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Evolution of a Surgeon: A 40-year Perspective

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I have looked forward to this day with anticipation and some trepidation for nearly 2 years. When I first joined the SSAT, about 30 years ago, I was then and am now in awe of the SSAT, and for me it has never lost its luster. When I was inducted, some of the founders were still active, providing me with special memories of those early days, and over the years, I have made many friends and acquaintances through my involvement in the SSAT. In a sense I grew up in this organization.

Several SSAT members have served as role models for me over my career and as such were my heroes: Wally Ritchie, Frank Moody, and Bill Silen come immediately to mind. Each in his own way was a help to me, willing to provide advice, support, or encouragement for a young surgeon trying to understand the traditions and pitfalls, as well as the opportunities of academic surgery. Other SSAT members who have contributed to my professional development include Ted Copeland, Joe Fischer, Stan Dudrick, Bernie Jaffe, Lou Flint, Jim Thompson, Isidore Cohn, Tom DeMeester, and the late Jim Thompson, to name just a few. And I can't fail to mention Larry Cheung and Bing Rikkers, Frank Moody's disciples in their Utah days, who were always good comrades.

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My last words of appreciation go first to Dr. Eugene Jacobson. Gene, when he was the first Professor and Chairman of Physiology at the new University of Texas Medical School in Houston, took me into his GI laboratory, despite the fact that I was a surgeon, and turned a neophyte into a fairly competent investigator. I understood that I would never win a Nobel Prize, but he managed to train me well enough for me to be awarded several NIH ROI grants, a good start to any young surgeon's career. For that I thank Gene and also Wally Ritchie who first whetted my appetite for bench research during my time at the Walter Reed Army Institute of Research.

Last, but not least, I want to recognize Dr. John Ochsner who recruited me to the Ochsner Clinic in 1976 and who has served ever since as my role model, mentor, and friend. John is truly one of the great surgeons of the twentieth century. I have been very lucky to follow behind a man of such extraordinary ability, character, and commitment to surgery and to his patients.

Speaking of research and great surgeons, I recently ran across these words: "...the achievement of the surgeon and his assistants becomes one of the greater glories of science.... in the operating room all results of the most improbable reaches of research, all the immense accumulation of medical knowledge are drawn upon in a determined drive towards ... preservation of one human life."[1]

Those words were written in an article that appeared in *Time Magazine* on May 3, 1963 entitled, "The Best Hope of All." A few months later I entered medical school and, perhaps naively, began a quixotic journey to become a doctor. The article in *Time* was written to extol the new "modern surgeon" who pursues knowledge to establish a scientific foundation for surgical treatment and who dares to perform procedures so radical that they were almost

unimaginable a few years before. In the same article Dr. Donald Effler, the Cleveland Clinic surgeon, was quoted as saying, "A great surgeon must have a fierce determination to be the leader in his field. He must have a driving ego, a hunger beyond money. He must have a passion for perfectionism." The surgeon luminaries of the twentieth century, including many of our predecessors in SSAT, possessed powerful personalities and fierce determination in order to achieve success and to further the development of surgery. Throughout most of the twentieth century, the image of a surgeon was that of a commanding presence, capable of controlling all facets of patient care, a leader the Germans called a *geheimrat*. Advances required strong personalities with great self-confidence, ego strength, and limitless perseverance.

Today the surgeon's image is changing as a result of many factors—social, organizational, legal, economic, and political. For the most part these forces are beyond the control of the surgeon. Today's surgeon, of necessity, must fit in with a team of healthcare professionals and interact collegially with them to be successful.

Becoming a part of a team with other specialists has made it impossible for a surgeon to fulfill the traditional role of "master of the ship." It's acknowledged that a surgeon should understand and be aware of every aspect of his patient's disease and care, but in fact, many others play important roles and make it impractical to retain complete control over the patient's care.

Nevertheless, it is the surgeon who stands before the patient and draws up the contract that permits the surgeon and the team to embark on a plan to correct a surgical problem. And it is the surgeon to whom the patient has entrusted his life and welfare.

Surgeons understand the human cost of failure better than any other professional group in our society. We know that the only thing that really counts is results, i.e., solving a problem with the least cost of human suffering and with optimal benefit to the patient. The commitment to surgery is the defining event for the patient and for the surgeon. Style and artifice are useless if not effective; and founts of knowledge and intellectual speculation are useless unless at the defining moment they provide clarity, thought, and direction to guide the surgeon's hand.

A new distraction is now foisted on a surgeon as a result of rapid communication. The nearly instantaneous spread of new ideas, not only to the medical community but also to the public, brings pressure on the contemporary surgeon to wade through a morass of information, released unedited and untested into the public awareness. The pressure to be *au courant*, to know the latest claims and counterclaims, and to be able to discuss them with the next patient who walks in your office can be a demanding exercise. So much of what is available to the public is, at best, half-baked, sometimes untrue, and often misunderstood to the point it can become a major impediment to winning a patient's confidence. Unfounded claims can create unrealistic expectations that do not account for the full range of possible outcomes and make obtaining informed consent difficult. The public is ill-equipped to evaluate medical information, prioritize its importance, and make rational decisions.

As surgeons, we cannot become deluded by claims of what could or should be, and as surgeons we face our own stern realities in which events may unfold unpredictably and absolute control is an illusion. This reality now blends into today's world where statistics, algorithms, and consensus opinions tell us what others say we should achieve. This places pressure on every surgeon to be risk averse. Unfortunately, many problems we face are complex, their solutions involve risk for both the patient and the surgeon, and statistical probabilities are not always achievable. We struggle to deal with outsiders from the secular world who want to control and quantify the unquantifiable, thus deterring performance and inhibiting innovation. How and if this tension can be resolved remains an open question.

Managers in today's world believe process and controls produce a better product. I suppose it was just a matter of time until the "organization man" that we derided in the 1960s and 1970s turned his managerial skills toward the unbowed world of medicine. This raises the question whether surgeons have to become subservient to the organization man to survive. Will "best practices" and treatment "guidelines" retard innovation and produce mediocrity or will they provide a constructive framework for producing better outcomes? Standardization of routine processes insures safety from technical and administrative errors, to be sure. Computer programs have already improved our ability to collate information and to track and coordinate patient care. However, fear of intrusive oversight and misuse of information can create a "gotcha" mentality that will produce a chilling effect on surgical decision making. Information that can be manipulated against anyone who dares to challenge orthodoxy confers unfettered power on the organization man. Fear breeds temerity, a surgeon's enemy when there is a need to make decisions, act with partial information, or use experiential judgment.

Where then will the surgeon leaders of the twenty-first century come from? Will they be as talented, imaginative, and determined as the personalities attracted to our profession in the past? Are these types needed or even wanted in the new world order? In her book *The Scalpel's Edge*,[2] Pearl Katz opines that the new surgical heroes may be those who admit doubt and uncertainty, communicate sensitively with patients in an effort to have patients participate in decision making, communicate openly with

their colleagues, and take risks not for their patients but with their patients. Katz's vision of the surgeon's role in the future, as seen through the eyes of a cultural anthropologist, bespeaks a humanistic adaptation that is already underway. It appears that the boldness and rugged individualism that characterized so many of our surgeon pioneers will have to be sublimated and further modified for the next generation of surgeons to be effective leaders.

The technological explosion in American surgery began in 1989 when the application of the laparoscope to cholecystectomy was proven to be not only doable but teachable to thousands of trained surgeons. Its advantages over standard surgery caused a stampede to learn the technique.

In my case, I saw two laparoscopic cholecystectomies performed in a small community hospital in early 1990. And within a few weeks, I had performed my first laparoscopic cholecystectomy, having cobbled together the rudimentary equipment. This was as close to see one, do one, teach one as it gets. From that experience, I developed renewed respect for our pioneering predecessors who performed much more risky procedures with even less guidance under even more primitive conditions. Because it could be performed by thousands of surgeons hundreds of times and because it is so perfectly amenable to minimally invasive techniques, laparoscopic cholecystectomy did more, in my opinion, to advance all of surgery, and especially gastrointestinal surgery, than any other surgical innovation in my professional life.

The parallel development of small, modular, digital computers was a fortuitous congruence that led visionaries to see the great potential created by combining minimally invasive surgery with the power of computerized control. The impact of these developments is so far reaching that they have truly created a new paradigm affecting every aspect of modern surgery. A partial list of impacted areas would include training, workforce requirements, facilities, economics, levels of specialization, certification and credentialing, litigation, reimbursement patterns, and not the least affected—patients' expectations.

Nevertheless, the technological developments of the past 20 years, while providing a thrust to the future of surgery that I never dreamed of, have produced a host of complex problems. Among those concerns is the future of general surgery. As early as 1991, in the title of his SSAT Presidential address, William Silen implored, "Where Have the General Surgeons Gone?".[3] He presciently predicted that as the number of specialists and consultants increase, costs would escalate, rapport with the patient and trust in the physician would erode, malpractice litigation would escalate, and college students' interests in medical careers would wane. Have not all of his predications come to pass?

The extent of the threat to general surgery as a specialty began to come into focus just as the new millennium began. The AMA Physician Database showed a decline of just over 2,600 general surgeons in 4 years, a fall in absolute numbers from 27,509 in 1998 to 24,902 in 2002. This occurred despite a population growth in the U.S. of approximately 25 million each decade since 1970. Concomitantly, the production of general surgeons in the U.S. over the past 25 years has been remarkably constant at an even 1,000 per year. This has continued through the match in 2007 when over 99% of 1,055 positions were filled.

There are two significant and relevant demographic factors that are noteworthy, although their impact on the future of general surgery is uncertain. The first is that in 2001 the percentage of positions filled by U.S. medical school graduates fell below 90% for the first time in history.[4] And in 2007 the percentage filled by U.S. graduates fell below 80%. This pattern is not universal for all specialties. For example, anesthesiology trends are the reverse, having filled only 30% of their slots in 1996 (their nadir) and increasing dramatically to 98% filled with 78% U.S. graduates in 2007. Likewise, diagnostic radiology filled only 50% in 1996 compared to 100% in 2007 with 89% U.S. graduates. Clearly there is a declining interest in general surgery and its related specialties among U.S. medical graduates.

The second demographic of note is that women now comprise over 50% of medical school graduates. And there has been a drop of over 50% in the total number of men applying to medical school since 1974. Bucking these trends, general surgery remains a white male dominated specialty with little more than 20% being females. The gender factor is widely assumed to have a negative impact on the surgical workforce by limiting the available candidates for residency because of lifestyle issues and by reducing the availability of practicing general surgeons due to a greater likelihood of women choosing to interrupt or shorten their careers.

These data augur for a further decline in the general surgery workforce that will limit available candidates for further specialty training. Because the number of federally funded entry positions in general surgery is capped by the Balanced Budget Act of 1997 at about 1,000 per year, competition for candidates to fill subspecialty slots will be fierce. And it is not surprising that several specialties have already successfully petitioned the American Board of Surgery to allow them to accept candidates after only 3 or 4 years of general surgery training.

But what explains the actual decline in the number of practicing general surgeons that is already occurring? Dr. David Cosman, a practicing vascular surgeon in Los Angeles, writes an opinion column in *General Surgery News* expressing his views on a wide range of subjects including medical economics, politics, practice, and the future of surgery. He recently opined that "there is a rising

tide of physician dissatisfaction in this country.... Demoralized by decreased reimbursements, endless regulatory rituals, useless compliance exercises, and a distrustful patient population, physicians are on the ledge, and it won't take much more to push them over the edge."[5]

This sentiment is shared by more and more practicing surgeons who don't see a way out of the quagmire they find themselves in. Reimbursement for surgical services in real dollars is approximately 30% of what it was 15 years ago, and yet practice overhead has more than doubled largely due to inflation, regulatory mandates, rising insurance premiums, and administrative cost increases. In a statement to a senate committee this year (Senate Committee on Health, Education, Labor and Pensions, February 12, 2008) the American College of Surgeons, addressing healthcare workforce issues for the future, concluded that "the single most important factor shaping the surgical workforce issue is declining reimbursement." These concerns beg the question of whether it is too much to ask that present and future surgeons have some hope of prosperity and security. Is it any wonder that more and more general surgeons are either retiring early or seeking another career?

One thing is certain; the workforce is declining as the American populace grows larger and older. These kinds of trends take decades to produce and decades to reverse. Unfortunately, there is no plausible evidence to suggest that the public or our elected officials perceive a physician shortage or, more specifically, a shortage of surgeons. The exceptions to this reality are limited to rural areas that have little or no service and lack the political influence to affect public policy. Surgeons need formidable public relations and formidable political advocacy to stabilize and hopefully improve reimbursement. So far, as a profession, we have not developed effective political representation, and, unfortunately, we have no natural allies to champion our cause. Alone we have little political leverage. This is not a condemnation of our surgical societies, all of which were founded for educational, not political purposes. Furthermore, traditional professional societies may not be the best means through which to achieve political influence. Yes, the American public does think there is a healthcare crisis, as the media and opinion polls remind us daily, but the concern of the American public is solely about their individual cost and their access to care, not surgeon's pay and lifestyle.

On the production end of the equation, general surgery residency numbers remain constant for now only because the number of international applicants remains robust. Basically, surgery positions fill with qualified U.S. applicants and then top off with qualified foreign graduates. The decline in U.S. seniors choosing careers in surgery augurs poorly for the future, and the increasing reliance by American training programs on foreign medical graduates to fill positions makes the continued supply of surgical specialists tenuous.

This concern, first brought to prominence by the 2001 general surgery match results, has been the subject of much discussion. After reviewing dozens of articles written about the disaffection of graduating seniors for general surgery, and after trying to digest reams of demographic data, it seems fairly transparent to me: Today's contemporary generation (or Generation X, defined as anyone born after 1965) is not as attracted to general surgery (or its subspecialties) because they see in them less relative value as compared to other specialties and other professions. The simplistic explanation has been to blame "lifestyle issues." This catch phrase implies that the younger generation is not as committed or as willing to work as previous generations. The notion that if surgeon educators could just make surgical training more attractive and user friendly, and things will get better, is frankly naive. Maybe some medical students have been scared off because they see how long and hard surgeons work or how stern and demanding they can be at times. Clearly some react negatively to the surgical ethos. Unfortunately, the cause of disaffection is much deeper and not so easily corrected.

One important influence on a career's attractiveness is financial. A former medical director at the Ochsner Clinic said, "when someone says it's not about the money, it's the principle of the thing, it's always about the money." Professor Michael Porter of the Harvard Business School, at this year's annual meeting of the American Surgical Association,[6] characterized healthcare as a "zero sum competition", meaning that all the participants in the healthcare community are pitted against each other to carve out more value at the expense of others. Therefore, is it any wonder that the next generation is questioning commitment to a specialty whose status has become financially compromised and whose services, especially in general surgery, have been, I think, intentionally devalued? Isn't fair compensation a reasonable expectation for years invested in a surgeon's education, for the stresses and interruptions in family life, and for a life of commitment to the frailties of others? How can anyone expect to have balance in their life if they are chronically overworked and financially strapped?

Fortunately, there are still highly motivated and talented candidates who are willing to pay the price necessary to be molded into what is one of the most personally rewarding professions that exists, that of a surgeon. The intangible rewards are still among the most satisfying of any profession I know. But the reality is that the life of a surgeon is not easy and it's not always possible to plan your practice around your personal life. It would be misleading to promise surgical candidates a rose garden. I would much prefer to train

young surgeons with realistic expectations, committed to a life of professional attainment and responsibility, than to do anything to weaken the fabric of our profession. And it is incumbent upon those of us in leadership roles to make certain that we stand steadfast against any attempts to compromise or minimize the requirements necessary to become a surgeon. If we overreact to a few poor years in the match and if we begin to undermine the basic tenets of surgical education that have been shown to be tried and true for over 100 years, we will do a lasting disservice to future generations.

We, in our professional capacity, can do very little to change the practice environment that is eating away at so many of our colleagues. The forces producing practice dissatisfaction are, for the most part, beyond our control and reflective of political and societal ills that will require a sea of change to rectify. But we can take seriously and responsibly our stewardship of the next generation of surgeons. To that end, we must protect the depth and breadth of surgical experience as the bedrock of training.

The science of experience teaches us that mastering most complex human endeavors requires a minimum of 10 years' experience. Surgeon educators have and will continue to develop new methods to teach complex subjects, but there is a limit to how fast the human mind can absorb large quantities of information, synthesize it, and apply it to an almost infinite number of circumstances. Furthermore, training parameters must be designed to adequately train the slowest, not just the quickest and most facile. When dealing with human life we are obligated to maintain training goals that aim, as in aviation, for zero defects. In medicine, in contrast to other professions such as civil engineering, solutions to urgent and complex problems must be acted on in real time, often with partial information. Surgeons must be trained to manage the worst scenarios and to confront the unexpected. The human condition comes in limitless variations, making it essential that each surgeon has the capacity to respond flexibly and reflexively. Professional discipline and technical skills are gained through long hours of repetition and through struggling under adverse circumstances. William Halsted and other great surgeon educators of the twentieth century understood and stated explicitly that it takes time and years of experience to train a surgeon.

It is popular today to appear flexible and understanding. But in my 40 years in surgical education, as a trainee or trainer, I can see no justification for being anything but demanding and rigorous in the design of the training process. In surgery, the only acceptable performance goal is the best that can be achieved for each and every patient. Nothing less is acceptable. This can only be accomplished if each surgeon is broadly and expertly trained and experienced.

While 10 years is probably a minimum required to achieve expertise in most complex fields, including surgery, more and more experience alone is not a guarantee of success. Gaining experience is only the starting point. Anders Ericsson, the editor of the *Cambridge Handbook of Expertise and Performance*,[7] states, "The number of years experience in a domain is a poor predictor of performance." This observation is particularly relevant to the experienced and mature surgeon. Ericsson holds that rather than through more and more experience, sustained performance is achieved through what he calls "dedicated exertion", i.e. repeatedly practicing the most difficult tasks that lead to excellence and consistent performance. If a task gets easy and the mind wanders, routine tasks may be executed mindlessly and mistakes occur.

A recent study from Harvard, for example, reported the causes of surgical technical errors that had resulted in malpractice claims.[8] The majority (or 73%) involved experienced surgeons, and 84% occurred in routine rather than advanced procedures requiring special training. Therefore, successful performance requires more than experience or "time in grade" in U.S. Army jargon, but continuing focus on decision-making and constant awareness in routine operations for the occurrence of complex circumstances.

The importance of experience in training leads me to a few thoughts on the design of surgical training in the future. You have already deduced that I am "old school." That I feel surgical training must be, of necessity, long enough and rigorous enough for the trainee to acquire not only practical experience but also to acquire intangibles like mental and emotional discipline. In my opinion, early specialization after only 3 years of general surgery, as has been proposed,[9] will produce a surgical workforce of narrowly trained specialists who lack the foundation, maturity, and breadth of experience to meet the challenges they will surely confront in their careers. If the perceived disaffection of senior medical students is used as a reason to reduce the rigor of general surgery training prior to specialization in an attempt to make surgery more alluring, it will severely diminish the effective workforce of qualified general surgeons. An unintended consequence will be to create several tiers of qualification and credentialing that will be a nightmare to administer and unravel. Credentialing committees will be forced to rely on formulas to determine competency, moving standards toward the lowest common denominator. Litigation over qualifications will ensue, producing a morass that the courts are ill prepared to adjudicate. Gaps in coverage of specific conditions will emerge, and hospitals, as they become increasingly reliant on fragmented specialists, will have to enlarge their staffs to maintain continuity of care.[10] Who will be empowered to convene the specialists to assign ultimate responsibility for

the whole patient? I fear that into this void will lead an opportunist, perhaps with little or no surgical experience, to seize the role of ringmaster. All of this will magnify the anticipated workforce shortages, and the redundancy of specialists will lead to rising costs. In the end, continuity of care will be sacrificed and patients will suffer.

Thirty-five years ago a Yale psychologist, Irving Janis, published an essay in the Yale Alumni Magazine to explain how a group of intelligent people working together to solve a problem can sometimes arrive at the worst possible answer.[11] He called his radical new theory "group think." The consequences of such an error can be devastating. A minor consequence would be that a proffered solution simply delays resolution of a problem. More serious consequences can lead to tragic outcomes such as the Bay of Pigs fiasco, the escalation of the Vietnam War, or now, the prosecution of the Iraqi War.

Today, group think is studied in military colleges, political science classes, business schools and academia. In response to criticism regarding decisions leading up to the Iraqi War, the CIA announced it has initiated new procedures to minimize the risk of "group think." John A. Kringan, head of the CIA's Directorate of Intelligence, has outlined new procedures setting up "alternative analysis" teams to guard against decisions going off in the wrong direction for the wrong reasons. This process provides for an external authority to test the assumptions and conclusions of the group before potentially damaging or irreversible action is taken.

My concern is that the future of surgical training, its basic premises and format, be examined and debated, and any proposed changes subjected to the equivalent of an alternative analysis, before anything is done that could permanently weaken the foundation of surgery in America. A minimum of 5 years of surgical training before specialization should be retained as a foundation until all the consequences of compressed general surgery training have been explored.

Tomorrow's surgeon is faced with mastering more knowledge, not less; more complexity, not less; and the hard earned lessons of the past must be passed on to the next generation. It is crucial that we shape the scope of knowledge and experience that will be required of future surgeons and that we not be unduly influenced by transitory exigencies. In the end we cannot control all the forces buffeting our society, but we can and should control the fundamental qualifications necessary to fulfill our responsibility to the future of our profession. And above all, we must instill in future surgeons, in Dr. Effler's words, "a passion for perfectionionism." Nothing less will do.

"Be not the first by whom the new are tried

Nor yet the last to lay the old aside"

Alexander Pope

Essay on Criticism, 1711

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Eradication of Barrett Esophagus with Early Neoplasia by Radiofrequency Ablation, with or without Endoscopic Resection

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Abstract
Background Radiofrequency ablation is safe and effective for complete eradication of nondysplastic Barrett esophagus (BE). The aim was to report the combined results of two published and two ongoing studies on radiofrequency ablation of BE with early neoplasia, as presented at SSAT presidential plenary session DDW 2008.
Methods Enrolled patients had BE ≤12 cm with early neoplasia. Visible lesions were endoscopically resected. A balloon-based catheter was used for circumferential ablation and an endoscope-based catheter for focal ablation. Ablation was repeated every 2 months until the entire Barrett epithelium was endoscopically and histologically eradicated.
Results Forty-four patients were included (35 men, median age 68 years, median BE 7 cm). Thirty-one patients first underwent endoscopic resection [early cancer (n=16), high-grade dysplasia (n=12), low-grade dysplasia (n=3)]. Worst histology remaining after resection was high-grade (n=32), low-grade (n=10), or no (n=2) dysplasia. After ablation, complete histological eradication of all dysplasia and intestinal metaplasia was achieved in 43 patients (98%). Complications following ablation were mucosal laceration at resection site (n=3) and transient dysphagia (n=4). After 21 months of follow-up (interquartile range 10–27), no dysplasia had recurred.
Conclusions Radiofrequency ablation, with or without prior endoscopic resection for visible abnormalities, is effective and safe in eradicating BE and associated neoplasia.

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Introduction

Barrett esophagus (BE) is a condition characterized by a change of the normal squamous esophageal lining into a columnar epithelium containing specialized intestinal metaplasia (IM), due to longstanding exposure to gastroesophageal refluxate.[1,2] BE is the best-recognized risk factor for the development of esophageal adenocarcinoma, and patients diagnosed with nondysplastic BE are, therefore, advised to undergo endoscopic surveillance with biopsies every 1 to 3 years.[3] By histological evaluation of these biopsies, malignant progression to low-grade dysplasia (LGD), high-grade dysplasia (HGD), or early cancer (EC) may be detected.[1,2] Early neoplasia (i.e., HGD and/or EC) can be treated by surgical esophagectomy. Given the morbidity and mortality that may be associated with esophagectomy, less invasive endoscopic alternatives have been considered. Endoscopic resection (ER) is the cornerstone of endoscopic therapy, since it provides a relatively large tissue specimen for histopathological evaluation, enabling proper selection of patients for subsequent endoscopic versus surgical therapy.[4–6] Selected patients with HGD or EC limited to the mucosal layer (T1m) have a minimal risk of lymphatic involvement, and ER in these patients has been reported to have a 5-year disease-specific survival of 95%.[5] Patients with submucosal invading lesions (T1sm), however, have a 15–30% risk of lymphatic involvement, warranting surgical esophagectomy with resection of surrounding lymph nodes.[7,8]

After focal ER of HGD/EC, the residual BE still holds the potential of malignant degeneration, and metachronous lesions occur in 30% of patients.[9] Additional treatment of the residual BE after focal ER is therefore advocated, and different treatment modalities have been proposed for this end. The residual BE may be completely removed with stepwise radical endoscopic resection (SRER).[10–12] This approach allows for histopathological evaluation of the entire BE segment and removes all oncogenetic alterations that are present in the pretreatment BE.[13] SRER, however, is technically demanding, only amendable for patients with a BE <5 cm, and has a significant stricture rate.[10–12] Ablating the residual BE with argon plasma coagulation (APC) or photodynamic therapy (PDT) has also been described, but these techniques do not always result in complete eradication of all Barrett epithelium, preexisting oncogenetic alterations may still be found in residual areas of BE, and both techniques are associated with issues of variable ablation depth and safety.[14–19] Furthermore, after APC and PDT, areas of IM may become hidden underneath the newly formed squamous epithelium after ablation (a.k.a., "buried Barrett"), and some fear that these buried glands may progress to dysplasia and adenocarcinoma without being detected endoscopically.[20,21] Stepwise circumferential and focal radiofrequency ablation (RFA) using the HALO system is a novel and promising ablative modality. Primary circumferential ablation is performed using a balloon-based bipolar electrode, while secondary treatment of residual BE is performed using an endoscope-mounted bipolar electrode on an articulated platform. Studies involving circumferential ablation were initially conducted in the porcine animal model and in humans prior to esophagectomy in order to determine dosing and technique parameters.[22–24] Subsequently, RFA has been proven safe and effective for the eradication of dysplasia and IM in a number of clinical trials involving patients without dysplasia, with LGD or HGD, and after ER of EC and visible lesions.[25–27] In addition, no buried Barrett glands have been found in over 4,000 neosquamous biopsies obtained during follow-up,[25–27] oncogenetic abnormalities as present in the pretreatment BE are absent in the regenerated neosquamous epithelium after RFA,[28] and the functional integrity of the esophagus is not affected by RFA.[29] In this paper, we will present the results reported in Abstract 215, which was selected for oral presentation during the SSAT presidential plenary A session, at the Digestive Disease Week 2008, San Diego, CA, USA.[30] We will review our results, as available up until November 30, 2007, of stepwise circumferential and focal ablation in 44 patients with BE and HGD/EC, who were consecutively treated in four different, IRB-approved, study protocols at the Academic Medical Center, Amsterdam, the Netherlands.

Materials and Methods

Patient Selection

Starting July 2005, patients between 18 and 85 years old were consecutively included in a series of IRB-approved clinical protocols evaluating the effect of RFA on BE with early neoplasia, and conducted at the Academic Medical Center, Amsterdam, the Netherlands. Patients were eligible if they had endoscopically visible BE (≤12 cm) with HGD or EC diagnosed at two separate endoscopies by an experienced gastrointestinal pathologist (FtK). Any visible endoscopic abnormalities, or EC without a clear lesion detected by biopsies, were removed with ER prior to ablation, as per the protocol. In case of prior ER, histological evaluation of the specimen could not show vertical resection margins positive for cancer (R+), deep submucosal invading cancer (>T1sm1), poorly or undiffer-

entiated cancer (G3, G4), or presence of lymphatic/vascular invasion (V+). Patients with esophageal stenosis at baseline and patients with invasive cancer in biopsies obtained after ER but prior to RF ablation were also excluded. Our four serial and unique study protocols were as follows:

1. The first prospective study on circumferential RFA of HGD/EC in patients with a median BE segment of 5 cm [interquartile range (IQR) 5–7] using the HALO^{360} ablation catheter, with prior en-bloc ER of visible lesions and EC. Halfway through this study, the focal HALO^{90} ablation device became available.[26]
2. The second prospective study on RFA for the treatment of HGD and EC in patients with a median BE length of 7 cm (IQR 6.5–8) had a study protocol similar to the first study. Based on the experiences from the first trial, however, the protocol for this second trial had been optimized by thorough cleaning of the ablation zone and electrode surface in between ablation cycles, and the focal HALO^{90} device was available from the start of the study. In addition, patients with prior piecemeal ER of visible lesions were also included.[27]
3. The first ongoing European multicenter trial to evaluate the safety and efficacy of RFA in patients with a Barrett segment up to 12 cm long, with early neoplasia, with or without prior ER.[31]
4. An ongoing prospective randomized multicenter trial comparing SRER and RFA for the eradication of dysplasia and IM in patients with a BE <5 cm containing early neoplasia.

Endoscopic Procedures and Medication

All endoscopic procedures were performed on an outpatient basis using intravenous conscious sedation comprised of midazolam and/or fentanyl. After the procedure, patients were clinically observed for 2–4 h before they were discharged. All patients were prescribed high-dose proton pump inhibitors (i.e., esomeprazole 40 mg bid) as a maintenance dosage during the entire study period. Sucralfate suspension 5 mL (200 mg/mL) qid and ranitidine 300 mg before bedtime were prescribed for 2 weeks after each therapeutic endoscopy. In case of postprocedural discomfort, patients were allowed to take acetaminophen 500 mg (max. 6/24 h), and if this did not suffice, diclofenac suppositories 100 mg bid were permitted.

Endoscopic Ablation Systems

Both ablation systems that were used (HALO Ablation Systems, BÂRRX Medical, Sunnyvale, CA, USA) have 510(k) clearance by the Food and Drug Administration in the USA and the CE Mark for Europe for the treatment of BE. The HALO ablation system comprises two distinct ablation systems: the HALO^{360} system for primary circumferential ablation and the HALO^{90} system for secondary focal ablation. The HALO^{360} system includes an energy generator, ablation catheters, and sizing catheters. The HALO^{360} energy generator delivers radiofrequency (RF) energy to the electrode and has an integrated pressure–volume system to inflate the sizing balloon and automatically measure the inner esophageal diameter. The sizing balloon catheter consists of a 4-cm noncompliant balloon that is used for measuring the inner esophageal diameter of the targeted portion of the esophagus, prior to circumferential ablation. The sizing catheter is introduced over a guide-wire and its balloon is inflated in an automated manner to 4 psi (0.28 atm). Based on the baseline balloon volume–geometry and the volume needed to inflate the balloon to 4 psi, the mean esophageal inner diameter is calculated. Measurement is repeated moving distally, for every centimeter of the targeted esophagus, until an increase in diameter indicates the transition to the stomach or hiatal hernia. The HALO^{360} ablation catheter has a balloon at its distal end that is completely encircled by 60 electrode rings that alternate in polarity, over a length of 3 cm. The HALO^{360} ablation balloon is available in five outer diameter sizes (22, 25, 28, 31, and 34 mm). Extensive dosimetry studies in the porcine esophagus and human esophagus prior to surgical esophagectomy have shown that, for circumferential ablation, two applications of RF energy at 10–12 J/cm^2 and 40 W/cm^2 is the most effective regimen to ablate the full thickness of the epithelial layer, without injuring the submucosa. Focal ablation of residual BE tissue was performed with the HALO^{90} system. The HALO^{90} system consists of the focal ablation catheter and an energy generator. The bipolar electrode array of the HALO^{90} catheter is 20 mm long and 13 mm wide and is mounted on an articulated platform that can be attached to the tip of an endoscope with a flexible strap. The electrode array geometry and spacing are identical to those of the balloon-based electrode (Fig. 1).

Endoscopic Work-Up

Prior to ablation, all patients underwent at least two high-resolution endoscopies with narrow band imaging (NBI) (GIF-Q240Z, Lucera 260 system, Olympus, Tokyo, Japan, or GIF-H180, Excera II-system and a high-definition monitor, Olympus Europe, Hamburg, Germany) to have the BE segment thoroughly inspected by an expert endoscopist. The maximum length of the circumferential and contiguous Barrett epithelium was recorded according to the Prague classification system.[32] The maximum proximal extent of the Barrett mucosa (i.e., isles) was additionally documented, as isolated islands are not categorized in the Prague system.

Figure 1 The HALO ablation system used for circumferential and focal RFA of BE. *Left*: The HALO^{360} generator and HALO^{360} ablation catheter used for primary circumferential ablation. *Right*: The HALO^{90} ablation catheter fitted on the tip of an endoscope, for secondary focal ablation.

Visible lesions were classified in concordance with the Paris classification; type 0-I being polypoid, type 0-IIa slightly elevated, type 0-IIb flat, type 0-IIc depressed, and type 0-III excavated.[33] Biopsies were obtained from all visible lesions detected upon white light endoscopy or by advanced imaging techniques (NBI, autofluorescent imaging), and random four-quadrant biopsies were taken every 1–2 cm of the whole BE segment. To assess infiltration depth of lesions and lymph node involvement, all patients underwent endoscopic ultrasound (EUS) using electronic radial endoscopes (GF-UE160, Olympus GmbH, Hamburg, Germany) in conjunction with an Aloka SSD-5000 ProSound processor (Aloka, Meerbusch, Germany). In addition, computed tomography scanning of thorax and upper one-third of the abdomen was performed in all patients with EC to detect any metastatic disease.

ER Procedures

All visible lesions and EC were removed with ER prior to ablation. The objective of the ER was twofold. Firstly, ER allowed for histological evaluation and staging, enabling optimal selection of patients eligible for endoscopic treatment. Secondly, ER of visible lesions ensured that the subsequent ablation could be performed on an endoscopically flat mucosa. ER was performed using the ER-cap technique (Olympus GmbH) after submucosal lifting, or the multiband mucosectomy (MBM) technique (Duette™, Cook Endoscopy, Limerick, Ireland). Lesions with a diameter <2 cm were resected en-bloc; larger lesions were resected in multiple pieces (piecemeal procedure). All resected specimens were retrieved, pinned down on paraffin, and fixed in formalin for histopathological evaluation.

Endoscopic Ablation Procedures

For primary circumferential ablation, the esophageal wall was sprayed with acetylcysteine (1%) and flushed with plain water to remove excessive mucous. After recording the esophageal landmarks (i.e., top gastric folds, maximum extent of BE), the endoscope was removed, leaving a guide-wire (Amplatz extra stiff 0.035 in., Cook, Denmark, Europe) behind. A sizing balloon was introduced and the inner esophageal diameter was measured for every centimeter of the targeted BE segment, moving proximally to distally. Based on the measurements, an ablation catheter with an appropriate outer diameter was selected. The ablation catheter was introduced over the guide-wire, followed by the endoscope to allow the ablation procedure to be performed under endoscopic guidance. The electrode was placed 1 cm above the maximum proximal extent of the BE, the balloon was inflated, and the electrode was activated (12 J/cm^2, 40 W/cm^2). This resulted in a 3-cm-long, circumferentially ablated segment. Depending on the length of the BE segment, the ablation catheter was advanced and, allowing an overlap of 5–10 mm, repositioned distal to the first ablation zone. Ablation was repeated until the entire length of the BE segment had received one application of energy. Then, the ablation zone and electrode surface were cleaned. In the first 11 patients,[26] cleaning was performed by advancing the ablation balloon into the stomach, where it was inflated and flushed with water through the endoscope to rinse off excessive coagulum. The ablation zone was also rinsed with water through the spraying channel of the endoscope. For the next 12 patients,[27] the ablation catheter was removed and the electrode surface was cleaned outside the patient. The ablation zone was more rigorously cleaned compared to the first trial by forcefully spraying water through a spraying catheter using a pressure pistol (Alliance™, Boston Scientific, Limerick, Ireland, UK). In the following patients, cleaning was optimized by the use of a soft distal attachment cap fitted on the tip of the endoscope that was used to slough off most of the coagulum from the ablation zone, prior to forceful rinsing with water through a spraying catheter. After the cleaning procedure, the entire ablation zone was ablated a second time, using the same energy settings.

For secondary focal ablation with the HALO^{90} system, the mucosa was sprayed with acetylcysteine (1%) and flushed with plain water. The HALO^{90} electrode was fitted on the tip of the endoscope, introduced, and used for targeted ablation

of residual Barrett epithelium. The squamocolumnar junction was routinely ablated when the HALO^{90} electrode was introduced to ablate residual isles or tongues. The HALO^{90} system only became available at the end of the first trial, and the energy settings were escalated from 2×12 to 2×2×12 J/cm^2 and, eventually, to 2×2×15 J/cm^2 at 40 W/cm^2. All areas were ablated with cleaning of the electrode and ablated area in between ablation cycles, as previously described for the circumferential ablation procedure.

Treatment Protocol

After a minimum of 6 weeks after any ER, patients were treated with primary circumferential ablation using the HALO^{360} system. After 6 to 8 weeks, patients were scheduled for endoscopy to assess the treatment effect. Depending on the extent of residual BE, patients underwent a second HALO^{360} procedure, or secondary focal ablation using the HALO^{90} system. In the first study protocol, all patients were treated with a second circumferential ablation using the HALO^{360} system, regardless of the extent of the residual BE, since the HALO^{90} system for focal ablation was only introduced halfway through the study.[26] Additional ablation was repeated every 6–8 weeks, and a maximum number of two circumferential and three focal ablation sessions were allowed to achieve complete eradication of all IM. Persisting IM after the maximum number of ablations could be endoscopically resected using the MBM technique. Two months after the last treatment session, the endoscopic eradication of IM was assessed during endoscopy using high-resolution endoscopes with Lugol's staining (2%) or narrow-band imaging. To assess the histological clearance of IM, biopsies were obtained from four quadrants just distal to the neosquamocolumnar junction and every 1–2 cm from the neosquamous epithelium over the full length of the initial BE segment.

Follow-up

Patients were scheduled for follow-up endoscopy 2, 6, and 12 months after the last treatment session and then annually. High-resolution endoscopes with narrow-band imaging facilities were used to thoroughly inspect the esophagus for recurrence of IM, and four-quadrant biopsies were obtained for every 1–2 cm of the neosquamous epithelium over the original BE length and immediately distal to the neosquamocolumnar junction. Patients initially treated for EC underwent EUS every 12 months to exclude the presence of lymph node metastases.

Histopathological Review

All biopsies and ER specimens were embedded in paraffin, mounted on glass slides, and routinely stained with hemotoxylin and eosin. For the purpose of the described studies, all slides were reviewed by an expert GI-pathologist (FtK). The ER specimens were evaluated for the presence of dysplasia according to the revised Vienna classification,[34] tumor infiltration depth, tumor differentiation grade, presence of lymphatic or vascular infiltration, and the radicality of the resection at the deep resection margins. Biopsies were evaluated for the presence of IM, LGD, HGD, or EC, and in case of neosquamous biopsies, the presence of glandular mucosa underneath the neosquamous epithelium was assessed.

Ethical Considerations and Statistical Analysis

The Medical Ethics Committee at our institute approved all aforementioned study protocols, and written informed consent was obtained from all included patients. Statistical analysis was performed with SPSS 12.0.1 Software for Windows. For descriptive statistics, mean (±SD) was used in case of a normal distribution of variables and median (IQR) was used for variables with a skewed distribution. Where appropriate, the student *t* test and the Mann–Whitney test were used.

Results

Patients

A total of 44 patients was enrolled in the different study protocols, and all had finished treatment by 30 November 2007: 35 men, median age 68 (IQR 57–75) years, median Barrett length C5M7 (IQR C2–7, M4–9). Eleven patients were included in the first published trial on RFA,[26] 12 patients in the second published trial,[27] nine patients in the ongoing European multicenter trial,[31] and 12 patients were randomized to RFA in the ongoing randomized trial comparing RFA with SRER. A total of 36 ER procedures were performed in 31 patients prior to ablation. Nineteen were performed with the ER-cap technique after submucosal lifting and 17 with the multiband mucosectomy technique. There were 16 en-bloc and 20 piecemeal resections, with a median of two pieces per resection (IQR 2–3). The worst histological grade per patient found in the ER specimens was EC in 16 patients, all radically resected at the deep resection margin, HGD in 12 patients, and LGD in three patients. The worst histological grade of the BE after any ER, but prior to the first ablation procedure, was HGD in 32 patients, LGD in 10 patients, and residual nondysplastic IM in two patients.

Eradication of Dysplasia and IM

Complete histological eradication of dysplasia and complete endoscopic and histological clearance of IM was achieved in

43 patients (98%), after a median of one (IQR 1–2) circumferential ablation, two (IQR 1–2) focal ablation sessions, and escape ER in three patients (Fig. 2). These three patients had small areas of residual columnar epithelium that persisted after the maximum number of allowed ablation sessions. These areas were resected using the MBM technique and showed LGD (n=2) and HGD (n=1) upon histological evaluation. In one patient, the proposed treatment protocol failed (2%). After two ER sessions, one circumferential and two focal ablations, a persisting area of suspicious-looking columnar epithelium was observed and resected en-bloc using the MBM technique. Histology showed a T1sm1 adenocarcinoma, radically resected at the deep resection margins (R0). Two months after the escape ER, however, a suspicious 5-mm isle was identified. Additional resection of this area failed due to scarring resulting from the prior ER sessions. Since the patient strongly opposed surgical treatment, the area was ablated with APC (forced coagulation 60 W, gas flow 1.6 L/min, ERBE Vio System, Erbe Elektromedizin GmbH, Tübingen, Germany). Two subsequent follow-up endoscopies with extensive biopsies and EUS showed no signs of recurrent dysplasia or IM.

Adverse Events

In five patients, a complication occurred during ER (16%): there were four mild bleedings that could be easily managed with endoscopic hemostatic techniques and there was one esophageal perforation. The perforation was treated conservatively by placement of clips (resolution clips, Boston Scientific), and a covered esophageal stent (Esophageal Choo Stent, Fujinon Medical Holland B.V., Veenendaal, the Netherlands). In addition, the patient received immediate intravenous administration of antibiotics and acid suppressant therapy, an esophageal tube for suction and nil per mouth. He remained asymptomatic and no signs of leakage were seen on contrast swallowing examination. After 2 months, the defect had completely healed and treatment could be resumed. After initial circumferential ablation, a nontransmural laceration was

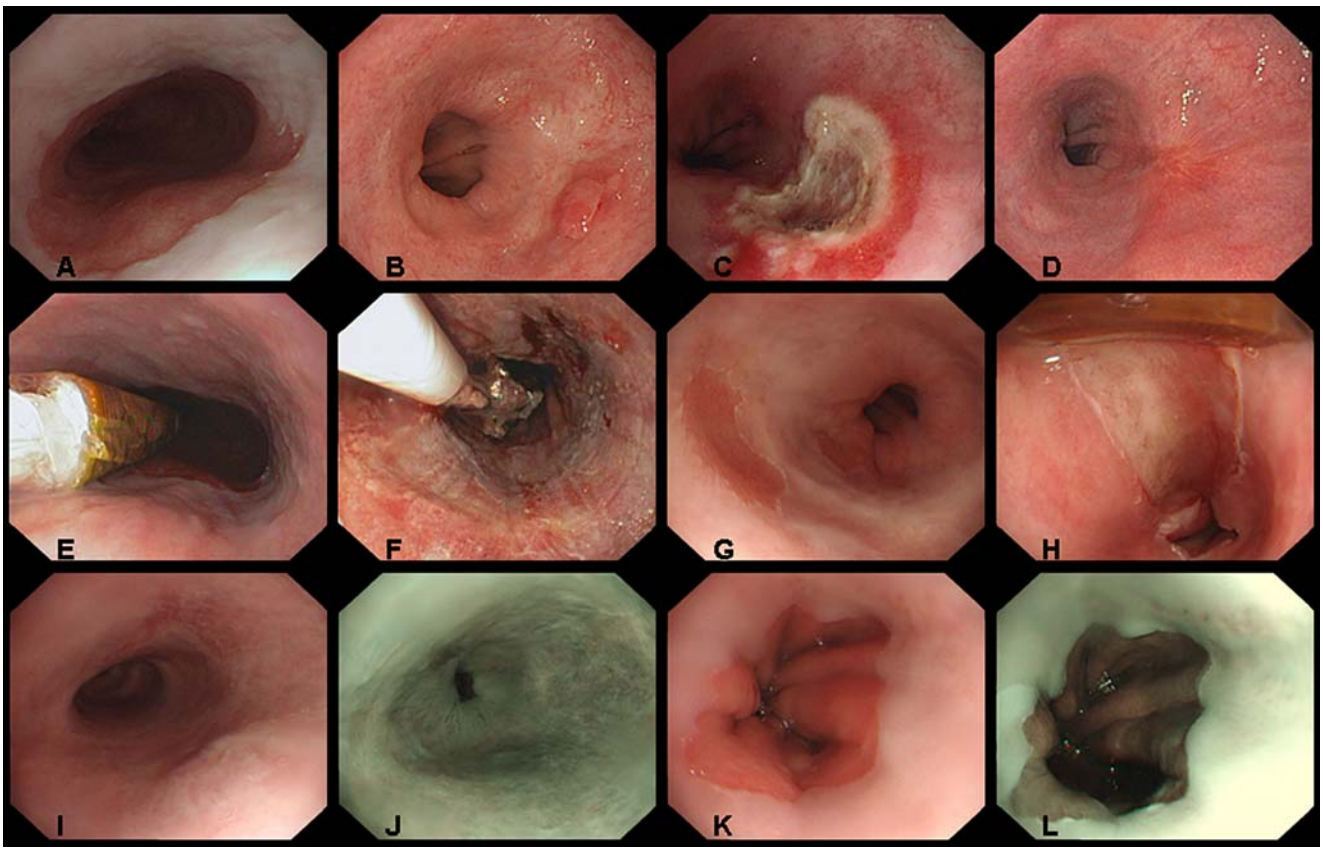

Figure 2 Endoscopic treatment of a C8M9 BE with HGD and a visible lesion treated with a combination of ER and RFA using the HALO system. **A** Antegrade view on a C8M9 BE. **B** View on a 0-I–IIa lesion at the 5 o'clock position. **C** View on the resection wound. The specimen showed a submucosal cancer with radical vertical and lateral resection margins. **D** Same area 6 weeks after the ER. The wound has healed completely with scarring. **E** HALO^{360} ablation balloon positioned 1 cm above the maximum extent of the BE. **F** Ablation effect after cleaning off the coagulum. **G** Residual isle of Barrett mucosa remaining 6 weeks after prior circumferential ablation. **H** Effect immediately after ablation with the focal ablation device. **I** Complete removal of he whole Barrett segment mucosa after one ER and two ablation sessions. **J** Corresponding image with NBI. **K** neosquamocolumnar junction after treatment. **L** Corresponding image with NBI.

observed in three patients (7%). All patients remained asymptomatic and no therapeutic interventions were required. The lacerations all occurred at the level of the ER scar in patients were an ablation catheter with a relatively large diameter was selected in relation to the esophageal inner diameter and who had undergone prior ER with a median extent of 33% of the circumference and 2.5 cm in length. Four patients (9%) developed dysphagia after ablation that could be resolved with a median of three (IQR 1–5) endoscopic dilatations. These patients all had prior widespread ER [median of three (IQR 1–5) pieces per procedure, 50% of the circumference and 2 cm in length], two had undergone two ER sessions, and one patient had a narrow esophagus at baseline. No lacerations or stenoses were observed in patients after ablative therapy if they had not had prior ER. Four patients (9%) were hospitalized after primary circumferential ablation for observation of fever (n=1), chest pain (n=2), and superficial mucosal laceration at a previous ER site followed by a negative contrast study (n=1). After conservative treatment and analgesics, all were discharged after 24–48 h.

Follow-up

During a median follow-up of 21 (10–27) months, no recurrence of dysplasia was observed. In one patient, a 1-mm BE island was identified 16 months after the last treatment, located at the upper end of the initial C9M10 Barrett segment; none of the other 43 patients showed endoscopic signs of BE during follow-up. Five patients had focal IM detected in biopsies obtained immediately distal to an endoscopically normal appearing neosquamocolumnar junction at a single follow-up endoscopy. In 1,475 biopsies obtained from neosquamous epithelium, only one (0.07%) showed buried glandular mucosa.

Discussion

This manuscript reviews our interim results of RFA for BE with early neoplasia from four different study protocols at the Academic Medical Center, Amsterdam, the Netherlands, and was written to accompany our oral presentation during the SSAT presidential plenary A session, at the Digestive Disease Week 2008.[30] A total of 44 consecutive patients with BE containing HGD and/or EC had finished treatment by November 30, 2007. Of these, 23 patients were treated in the first two pilot studies worldwide to evaluate the use of stepwise circumferential and focal ablation of BE with HGD/EC after prior ER of any visible abnormalities and EC.[26,27] The other 21 patients were included for the first European multicenter study on RFA of BE up to 12 cm containing HGD/EC,[31] or in an ongoing study comparing SRER with RFA in patients with early neoplasia in BE<5 cm. In all four studies, it was protocolized that any visible lesions and EC had to be removed with ER prior to ablation to enable histological evaluation for accurate staging of the infiltration depth and tumor differentiation and to ensure that subsequent RFA could be performed on an endoscopically flat mucosa. In the first study, six of the 11 patients had undergone an en-bloc resection of a visible lesion. No significant esophageal scarring was observed in these patients, and no complications such as mucosal injury or dysphagia occurred after ablation treatment. In the other three studies, patients with prior piecemeal ER or multiple ER sessions were included, and mucosal injuries (n=3) and dysphagia (n=4) were observed for the first time. The four patients presenting with dysphagia had all undergone widespread ER and/or were treated with a relatively large-diameter ablation catheter compared to the measured esophageal diameter. To prevent complications resulting from ER scarring, it is in our opinion that one should limit the extent of ER of visible lesions to 50% of the circumference and 2 cm in length. In addition, the HALO^{360} ablation catheter size should be selected conservatively in cases of prior ER, preferably one size smaller than the catheter that would be selected based on the esophageal inner diameter measurements. No esophageal stenoses were observed in patients without a prior ER who were exclusively treated with ablation therapy. These results are in concordance with the USA multicenter ablation of IM study, where no strictures were reported in 100 patients treated with RFA.[25] The absence of submucosal scarring as a result of RFA was also illustrated by our ability, in three patients, to remove focal areas of persistent Barrett mucosa after multiple ablation sessions using the multiband mucosectomy technique, without the need for submucosal lifting in three patients. This is a significant advantage compared to other endoscopic ablation techniques, after which escape treatment using ER is usually difficult as a result of submucosal scarring. In the 1,475 biopsies obtained from neosquamous epithelium during follow-up, only one biopsy showed focal IM hidden underneath the newly formed squamous epithelium. This biopsy was obtained at the upper end of an initial C9M10 Barrett segment, at the same level where, at a following endoscopy, a small 1-mm isle was identified with narrow-band imaging that may have been left untreated and unobserved at the preceding endoscopies. The fact that no buried glands were found in eight biopsies obtained at this level during other follow-up endoscopies, and the absence of any IM in an ER specimen to remove the 1 mm isle, suggests that the biopsy with buried IM may have sampled this minute isle tangentially, rather than sampling truly buried Barrett glands. Although this hypothesis cannot be confirmed, the 0.07% of subsquamous IM still compares

favorably to the 53% rate of buried glands reported after other ablation techniques.[14–21] Our findings were in concordance with the absence of buried glands in 3,007 neosquamous biopsies after RF ablation in the 100 patients described by Sharma et al.[25] Further studies on the adequacy of biopsies from the neosquamous epithelium after RFA should, however, clarify this issue further. Ablation at the GE-junction using the HALO^{360} catheter may be difficult, since the often tortuous course of the distal esophagus and widening into a hiatal hernia, present in most BE patients, may impede good circumferential contact of the electrode with the mucosa at this level. In addition, endoscopically differentiating cardia mucosa from Barrett mucosa at the top of the gastric folds after ablation treatment may be difficult. Therefore, all patients were treated with ablation of the GE-junction using the HALO^{90} catheter. The HALO^{90} device allows for targeted, focal ablation and was used to completely ablate the full circumference of the GE-junction to ensure that there was no small rim of residual Barrett mucosa left untreated at the transition of the columnar epithelium into the neosquamous epithelium. Despite this approach, focal IM was diagnosed in five patients (11%) in a single biopsy obtained just distal to a normal appearing neosquamocolumnar junction at a single follow-up endoscopy, not reproduced at following endoscopies. The clinical relevance of this finding may be debated. Since all patients had an initial diagnosis of HGD or EC, one may argue that finding residual IM in the cardia during follow-up means that the IM had not been completely eradicated and that the patients were not completely cured from their underlying disease. IM of the cardia, however, can be detected in up to 25% of patients with a normal appearing squamocolumnar junction and is not considered a premalignant condition in those cases.[35] In addition, we think that the patchy nature of this finding, and the fact that all patients will remain under endoscopic follow-up given their initial diagnosis of HGD/EC, does not justify additional treatment. As described in the "Materials and Methods" section, the treatment protocol for the second trial was improved based on the experiences from the first trial. These improvements were reflected in the median number of treatment sessions required to achieve complete eradication of IM. Although the median BE length was longer in the second trial [7 cm (IQR 6.5–8) vs. 5 cm (IQR 4–7)], the mean number of ablation sessions was lower (3.4 vs. 4.2 sessions). The three most significant changes in the protocol were as follows: firstly, the HALO^{90} catheter for secondary focal ablation only became available halfway through the first trial. Most patients had by then already undergone a second circumferential ablation session, regardless of the amount of residual BE, whereas in the second trial, the HALO^{90} device could be used to treat isles or tongues persisting after the first circumferential ablation. Secondly, the energy settings used for focal ablation were escalated from two ablations at 12 J/cm^2, to two times two ablations at 12 J/cm^2 ("double-double"), to double-double 15 J/cm^2 when the device became available during the first trial. In the second trial, the double-double 12 J/cm^2 dose was used initially, but in four patients, a step-up to double-double 15 J/cm^2 ablation was required to eradicate all IM. Since this "double-double 15 J/cm^2" approach proved effective without causing significant side effects, this dose is currently used in the ongoing studies. Thirdly, in the first study, the electrode surface of the HALO^{360} catheter was cleaned by inflating the balloon in the stomach and flushing it with water prior to the second ablation pass, without significant cleaning of the ablation zone. In the second trial, the electrode surface was cleaned with a wet gauze outside the patient, while the ablation zone was thoroughly cleaned by suctioning off the debris and high-pressure rinsing with water through a spraying catheter. The effect of this improved cleaning protocol was observed in the amount of surface regression after the primary circumferential ablation session; the median percentage of surface regression improved from 90% in the first trial to 99% in the second trial (p=0.035).[36] We think that, although it requires additional procedure minutes, meticulous cleaning of the electrode and ablation zone after the first pass improves the efficacy of RFA and should always be performed. The thorough cleaning protocol has, therefore, been incorporated in current trials.

Conclusion

Stepwise circumferential and focal RFA of Barrett epithelium with HGD or EC, with or without prior ER of focal lesions, is highly effective in achieving complete eradication of dysplasia and IM, without any serious adverse events. This novel treatment modality, therefore, appears to be a favorable alternative to esophagectomy, radical ER, APC, or PDT.

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Discussion

Eradication of Barrett Esophagus with Early Neoplasia by Radiofrequency Ablation, With or Without Endoscopic Resection

Jeffrey H. Peters, M.D. (Rochester, NY)

Members and guests, I believe we have just heard what I would call a paradigm changing paper. The search for a treatment for the epithelial changes of Barrett's, as opposed to the reflux related disease, has been ongoing for more than two decades. Drug therapy, surgery, a multitude of ablation technologies, thermal energy, laser, photodynamic therapy, all of these have fallen well short of the safety and effectiveness necessary for everyday clinical use. Radiofrequency ablation, however, seems poised to change this paradigm.

The authors have reported successful endoscopic mucosal resection followed by RF ablation in a relatively large cohort of patients with Barrett's and high grade dysplasia and/or early neoplasia. Successful eradication, as you heard, occurred in 43 of the 44 patients. There are a few caveats, however, that deserve highlighting. Firstly, remember, this is not a trial of RF ablation alone. Three quarters of the patients had mucosal resection prior to the RF; also, a very careful patient selection protocol was necessary to exclude those with submucosal cancer, a key issue; and finally, the longevity of the ablation is unknown at present. On the other hand, ablation was highly effective, successful, and was not associated with the development of strictures or buried submucosal glands.

This technology, this study, and others like presented at this meeting. Are changing the treatment paradigm of dysplastic Barrett's esophagus and early cancer. This is an excellent and well done study from one of the world's best units treating early esophageal neoplasia.

Jacques, I have a few brief questions for you. Firstly, visible lesions were key to the EMR technique. Most visible lesions are submucosal. You excluded all submucosal lesions. This suggests to me a very highly select patient population. Further, you didn't mention whether you had any patients with cancer on their biopsy that did not have visible lesions and what you would do under those circumstances. Surely you encountered such patients. Would you suggest how to approach these?

Second, ablation at the gastroesophageal junction may be an Achilles' heel of this technology. Reading the manuscript, Jacques has chosen to ignore biopsies right at the top of the stomach in the efficacy assessment. I would like for you to comment on this issue.

Finally, although your 98% success rate is spectacular, more widespread experiences suggest that it may not be quite this easy. Has your near uniform success continued as your experience has grown?

This is a wonderful contribution from the Amsterdam group and it is really a pleasure to see it presented here at the SSAT. Thank you for the honor of discussing it.

Jacques J. Bergman, M.D. (Amsterdam, Netherlands)

ell, thank you, Jeff, for those questions and those kind words. Coming back to your first question, are most visible lesions submucosal, the answer is no. The vast majority of visible lesions that we encounter are mucosal, but you bring up an important issue: we should simply teach our endoscopists how visual abnormalities look like so that they detect them at a stage that they are still mucosal. In our study, we excluded approximately 15% of our patients after EMR showed the resected lesion to be submucosal.

What do we do if we don't see visual abnormalities? I think that good endoscopic inspection is crucial before you decide that there is nothing there. We have a very low threshold of calling something a visual abnormality, and I would urge everybody who steps into this technique to do this. EMR is the crucial here: it is the final step in the diagnostic work-up and it is the first step in therapy. If you cannot do an EMR, you should not ablate. So if we didn't see any visual abnormalities, but as you said, it is only a quarter of our patient population, then that patient is eligible for immediate radiofrequency ablation.

The IM immediately distal to the neo Z line is a tough issue. We had that happen in five of our patients who during

follow up had IM detected in a single biopsy that was obtained immediately distal to the neo Z line. At subsequent follow up endoscopies, this was not reproduced. I think it is all reflecting sampling error. It will be very difficult to call an end point for absence of IM at the Z-line. What to do if you take biopsies at a certain point and you don't find it and you don't find it at the second or the third follow up and it the pops up at the fourth and the fifth and then again is absent at the sixth, how do we call this? This is one of the issues that I think we need to clarify if we define outcomes, and especially if we go into the long term follow up of these patients. You rightfully pointed out that we don't have long term follow up on these patients. We simply have to continue looking at these patients to prove that the complete removal of all Barrett's is indeed maintained. If we look at oncogenetic abnormalities, if we do brush cytology, if we do biopsies, then we see that the neo squamous epithelium really is "clean," in that sense.

Experience, how did it change during our consecutive studies? It maintained at the same level. We are presenting at this meeting the first results of the European multicenter study with three centers. The success rate for that was 96%. We are currently doing a multicenter study with 11 European centers, and we hope to present those data to you next year.

Stephen Attwood, M.D. (North Shields, UK)

Jacques, the real test of any new treatment is a randomized controlled trial. Can you randomize between EMR versus EMR and HALO, or can you randomize between surgical resection versus EMR and HALO, and what are the hurdles to setting up such a randomized trial?

Dr. Bergman: My presentation is competing with the presentation of the results of a randomized sham controlled study that is presented at the AGA plenary session. I think the main issue is if you want to do randomized studies, at least in the Netherlands, we have to do randomized studies comparing endoscopic treatment with another endoscopic treatment. Our Dutch guidelines state specifically that for the patient category that we included in this study, endoscopic treatment is the treatment of choice. So we could never do a randomized study with a surgical arm for this group. I know that in the U.K. there are thoughts about that, and of course, it will be the ultimate proof. In the Netherlands, we have moved beyond that. According to our guidelines, we don't need more proof to treat these patients endoscopically.

John G. Hunter, M.D. (Portland, OR)

As surgeons, we were very happy to be part of the randomized U.S. trial of radiofrequency ablation of HGD, which will be presented at the AGA. The proof of this therapy will be durability, and you have pointed out that 21 months is a little early to prove that the natural history of HGD has been changed by this therapy. The second proof is the elimination of sub-squamous glands, which have the potential to become malignant. In your salvage EMR cases, did you find sub-squamous glands beneath that beautiful neo-squamous epithelium?

Dr. Bergman: The first 23 patients that were in our studies, the first two studies that were published in Endoscopy this month, were all called back the last two months for an EMR of the neo squamous mucosa. So we EMR'd a part of their neo squamous mucosa out. Sixteen have been completed, and preliminary results don't show any submucosal or sub squamous IM in any of these. We took biopsies of the neo squamous mucosa and biopsies of untreated epithelium and, blinded, gave them to two expert pathologists asking them what is neo squamous and what is normal squamous. They cannot tell the difference.?

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Cervical Nodal Metastasis from Intrathoracic Esophageal Squamous Cell Carcinoma is not Necessarily an Incurable Disease

Daniel King-Hung Tong · Dora Lai Wan Kwong · Simon Law · Kam Ho Wong · John Wong

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Abstract
Background It remains controversial if metastatic cervical lymph nodes in patients with intrathoracic esophageal cancer signify distant metastases and are therefore incurable or if they should be regarded as regional spread with a potential for cure.
Material and Methods Patients with intrathoracic esophageal squamous cell carcinoma managed from 1995 to 2007, in whom metastatic cervical lymph node spread was confirmed by fine needle aspiration cytology, were studied. Treatment strategies and outcome were reviewed.
Results There were 109 patients, of whom 98 were men. Median age was 62 years (range, 34–88). Excluding those who underwent primarily palliative treatments, there were two main groups: 22 who had upfront chemoradiation therapy and subsequent esophagectomy ± cervical lymphadenectomy and 46 who had chemoradiation only. Significant downstaging occurred in 29 of the 68 patients (42.6%), of whom eight (11.8%) had complete pathological/clinical response. There was no mortality after esophagectomy. Median survival of patients with chemoradiation plus esophagectomy was 34.8 months compared to those with no surgery at 9.9 months, ($p<0.001$). Patients with stage IV disease at presentation by virtue of nodal disease survived longer than those with the same stage because of systemic organ metastases: 9.3 vs. 3 months, ($p<0.001$).
Conclusions Prognosis of patients with metastatic cervical nodes was not uniformly dismal. Up to 20% had reasonable survival after chemoradiation and surgical resection. Stage IV disease should be revised to segregate those with nodal and systemic metastases.

Keywords Esophageal carcinoma · Cervical lymph node metastases · Chemotherapy · Radiotherapy · Surgery

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Introduction

The most important prognostic factors in esophageal cancer patients are depth of tumor invasion (T), lymph node status (N), and presence of distant metastases (M). According to the American Joint Committee on Cancer (AJCC) staging system, the presence of cervical lymph node metastases from tumors located in the intrathoracic segment of the esophagus are regarded as M1 and stage IV disease.[1] The prognosis is poor, and the 5-year survival is as low as 3%.[2] Because of this presumed dismal prognosis, these patients are often treated with palliative intent.

There are existing data in the literature, however, that suggest that cervical lymph node metastases may not be uniformly fatal and could be regarded as regional spread instead of distant metastases.[3–5] Long-term survival might

be achievable in patients without systemic organ involvement. Multimodality treatment strategies including chemotherapy and radiotherapy have also gained popularity in recent years. In the neoadjuvant setting, prognosis in patients with resectable disease could be improved.[6,7]

It was hypothesized that, with multimodality treatments, prognosis of patients with cervical nodal metastases may be enhanced. The purpose of the present study was to evaluate our treatment methods and results in a cohort of patients with intrathoracic esophageal cancer who presented with cervical nodal metastases. The roles of chemoradiation therapy and surgical resection were assessed.

Material and Methods

Between 1995 and 2007, 767 patients with intrathoracic squamous cell esophageal carcinoma were managed by the Division of Esophageal Surgery, Department of Surgery, Li Ka Shing Faculty of Medicine, University of Hong Kong Medical Centre, Queen Mary Hospital. Patient data were collected prospectively in a database.

Staging investigations included endoscopy, barium contrast study, bronchoscopy, endoscopic ultrasound, neck ultrasound plus fine needle aspiration cytology, and computer tomography (CT) scans. Positron-emission tomography CT fusion scan (PET/CT) were only available since 2003. The staging system employed in this study was referred to the AJCC staging system.[1] Patients were recruited in this study if cervical nodal metastases were detected and confirmed by fine-needle aspiration cytological examination at presentation, thus all had stage IV disease. According to the AJCC classification, patients with upper third thoracic cancer and cervical nodal metastases were staged as IVa, while patients who had tumors of other levels and cervical nodes were staged as IVb. In addition, patients with systemic organ metastases, regardless of the location of the primary tumor, were classified as stage IVb. Those who had esophageal cancer located in the cervical segment or gastric cardia cancer were excluded. Tumor histologies other than squamous cell carcinoma were also not included.

The preferred treatment strategy would be to offer upfront chemoradiation therapy in patients who were judged suitable. For most patients, the regimen consisted of two cycles of chemotherapy given with concurrent external beam radiation. The chemotherapy regime comprised of two drugs: cisplatin at 100 mg/m^2 on day 1 and then day 22, and continuous infusion of 5-fluorouracil (5-FU) at 500 mg/m^2 per day for 5 days from days 1–5 and 22–26. Radiotherapy was given at a dosage of 40–46 at 2 Gy per fraction. It was delivered through anterior and posterior opposing fields to the primary esophageal tumor covering at least 1 cm lateral margins and 3 cm axial margins. For upper thoracic primaries, the radiation fields would extend to the neck to cover the cervical nodal metastases. Separated fields for cervical nodes were utilized in other cases where appropriate. Re-staging investigations were performed 4 weeks after completing the treatments.

In selected patients in whom curative resection could be anticipated and when cardiopulmonary evaluation was not prohibitive, surgical resection was offered. A transthoracic esophagectomy was the preferred approach. Cervical lymphadenectomy was performed in selected patients if there was evidence of residual tumor in the neck. If the cervical nodes were found to have been totally resolved, esophagectomy with two-field lymph node dissection was performed. The neck was closely monitored after surgery for recurrent disease.

For patients who had contraindications for chemoradiation, other palliative treatments were adopted. Palliative treatments included esophageal stenting, palliative surgery in selected patients, or supportive care only. Chemotherapy or radiotherapy alone was chosen as sole treatment in patients with contraindications for either modality or in patients who declined the full chemoradiation regimen but were keen to receive tumoricial therapy.

In patients who had surgical resection, complications were recorded prospectively. Medical complications were most cardiopulmonary. Surgical morbidities included anastomotic leakage, recurrent laryngeal nerve palsy, wound infection, neck seroma after neck dissection, hemorrhage, conduit ischemia, and any reason requiring surgical re-exploration. Any death within the same hospital stay as the esophagectomy was defined as hospital mortality. After discharge from hospital, patients had regular follow-up. Further radiological or endoscopic examinations were performed when there were symptoms or signs suggestive of recurrent disease.

Statistical Analysis

All data were collected prospectively. Calculations were performed by SPSS Software for Windows (version 11.5.2.1; SPSS, Chicago, IL, USA). Results are expressed as percentage or mean for continuous variables. Survival was calculated by Kaplan–Meier method. Log-rank test was used for testing significance, and $p<0.05$ was regarded as statistically significant. Chi-square test, Student's t test and Fisher's exact tests were used where appropriate.

Results

One hundred nine patients satisfied the inclusion criteria and were studied. Patient demographic data are shown in Table 1.

Table 1 Demographic Characteristics of the Studied Population

Characteristics	Number of patients, $n=109$
Median age in years (range)	62 (34–88)
Gender	
Male	98 (89.9)
Female	11 (10.1)
Level of tumor	
Upper third	25 (22.9)
Middle third	62 (56.9)
Lower third	15 (13.8)
Double tumor (both intrathoracic)	7 (6.4)
Pre-treatment T stage	
Tx	8 (7.3)
Tis	1 (0.9)
T1	3 (2.8)
T2	4 (3.7)
T3	73 (67)
T4	20 (18.3)
Pretreatment N stage	
N0	15 (13.8)
N1	94 (86.2)
Pretreatment M stage	
M1a	17 (15.6)
M1b	92 (84.4)
Overall stage	
Stage IVa	17 (15.6)
Stage IVb (non-regional LN)	68 (62.4)
Stage IVb (systemic organ metastases)	24 (22)

Unless otherwise stated, figures in parenthesis indicate percent of patients.
Stage IVa Thoracic upper third tumor with cervical lymph node spread, *Stage IVb(LN)* intrathoracic tumor with non-regional lymph node spread, *Stage IVb(organ)* systemic organ metastases

Treatments given for the 109 patients are shown in Fig. 1. Thirty patients were given only palliative treatments because of either unresectable, widespread metastases, inadequate physiologic reserve to tolerate aggressive treatment, or refusal of treatment. Primary surgical resection was performed on three patients; all had esophagectomy together with selective cervical lymphadenectomy. They did not receive upfront chemoradiation therapy primarily because of patients' choice. All three patients had bulky neck nodes, two of whom developed pneumonia postoperatively; one had unilateral recurrent laryngeal nerve injury while one other had bilateral vocal cord palsy. Another patient had a bypass procedure for unresectable tumor and palliation of severe dysphagia. There was no hospital mortality among these four patients.

There were 75 patients who had upfront chemoradiation treatment, five of whom deteriorated during treatment and were not restaged. Two of these five patients died of treatment-related gastrointestinal toxicity and pneumonia. The other three succumbed to malignant cachexia.

After chemoradiation, 70 patients were restaged, of whom 22 underwent tumor resection because potentially curative resection was deemed possible and risk assessment was satisfactory (Figs. 1 and 2a). Eight of these patients had selective unilateral neck dissection, and residual cervical nodal disease was proven in seven on histological examination of the resected specimens. In none of the other 14 patients was there evidence of cervical disease on preoperative imaging studies or intraoperative assessment. Pathological staging showed that 15 patients (68%) had significant downstaging of disease from stages IV to 0–III. Six patients (27%) had complete pathological response, and two (9%) had pT0N1M0 disease. R0 resection was possible in 18 (82%) patients, while in the remaining patients, they were R2 resections. Four out of the eight patients who had selective neck dissection subsequently developed systemic organ metastases, two of whom also had cervical nodal recurrences. In the 14 patients who did not have neck dissection, none developed cervical nodal recurrence but two had systemic organ metastases. Thus, none of the 22 patients had isolated cervical lymph node recurrence.

Two patients had bypass surgery after chemoradiation; in one, resection with curative intent had been planned but extensive lung adhesion and fibrosis precluded safe access to the mediastinum after thoracotomy was attempted. A Kirschner bypass was performed. The other patient had a bypass operation to relieve his total dysphagia.

Forty-six patients were not operated on after chemoradiation (Figs. 1 and 2b). In seven of these patients, significant downstaging occurred and curative esophagectomy was possible but was turned down. Esophagectomy was not offered to the other patients because of locally advanced or metastatic disease (including eight patients who had systemic metastases at initial presentation) and/or high risk on cardiopulmonary assessment. Some of these patients had further palliative treatments including chemotherapy, radiotherapy, or esophageal stenting.

Postoperative complications of the 22 patients who underwent esophagectomy after chemoradiation are shown in Table 2. There was no mortality in this group of patients.

Survival Analyses

Patients who received palliative treatments only and the few patients who had primary surgical resections all had very poor prognosis, with a median survival of less than 5 months. The three patients who had esophagectomy only survived for 6.2, 5.8, and 4.7 months, respectively. Of those who received upfront chemoradiation therapy, the 22 patients who had chemoradiation followed by esophagectomy had the longest survival. The median survival of this group of patient was 34.8 vs. 9.97 months for the 46

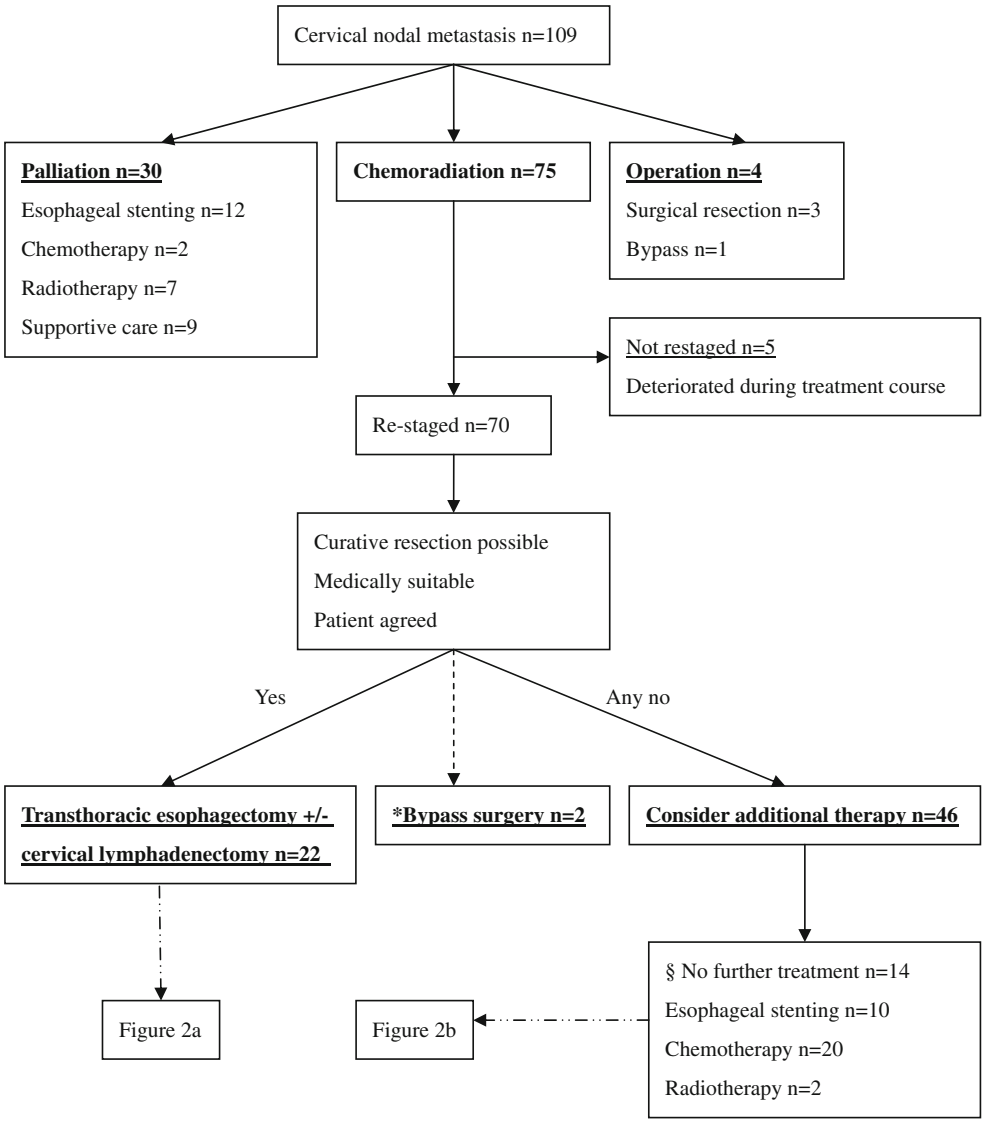

Figure 1 Management of patients with cervical nodal metastases.

* See text for description.

§ Primary treatment but each patient can have more than one form of treatment.

patients who had chemoradiation only but whose tumors were not resected, $p<0.001$ (Fig. 3). Two- and 3-year survival rates in the former group were 51% and 42%, respectively, compared with 11% and 5% in the latter group. In the seven patients who experienced good response to chemoradiation but no esophagectomy was performed because primarily of patients' choice even if curative surgery was anticipated, median survival was 11.2 months, still much less than the 34.8 months in those who had esophagectomy; the difference was however statistically insignificant, $p=0.38$.

Patient survival was also assessed stratified according to the pretreatment clinical staging (Fig. 4a,b). Patients who had stage IV disease by virtue of their nodal status had significantly better survival compared to those who had systemic organ metastases. Median survival was longer in those with stage IVa patients (upper third tumor with cervical nodal metastases—12.3 months) compared to those with stage IVb (by nodal status—8.52 months), although the difference was not statistically significant.

Discussions

We have shown in this study that prognosis in patients with cervical nodal metastases from intrathoracic squamous cell cancer of the esophagus is not uniformly dismal. In selected patients, downstaging with chemoradiation therapy with

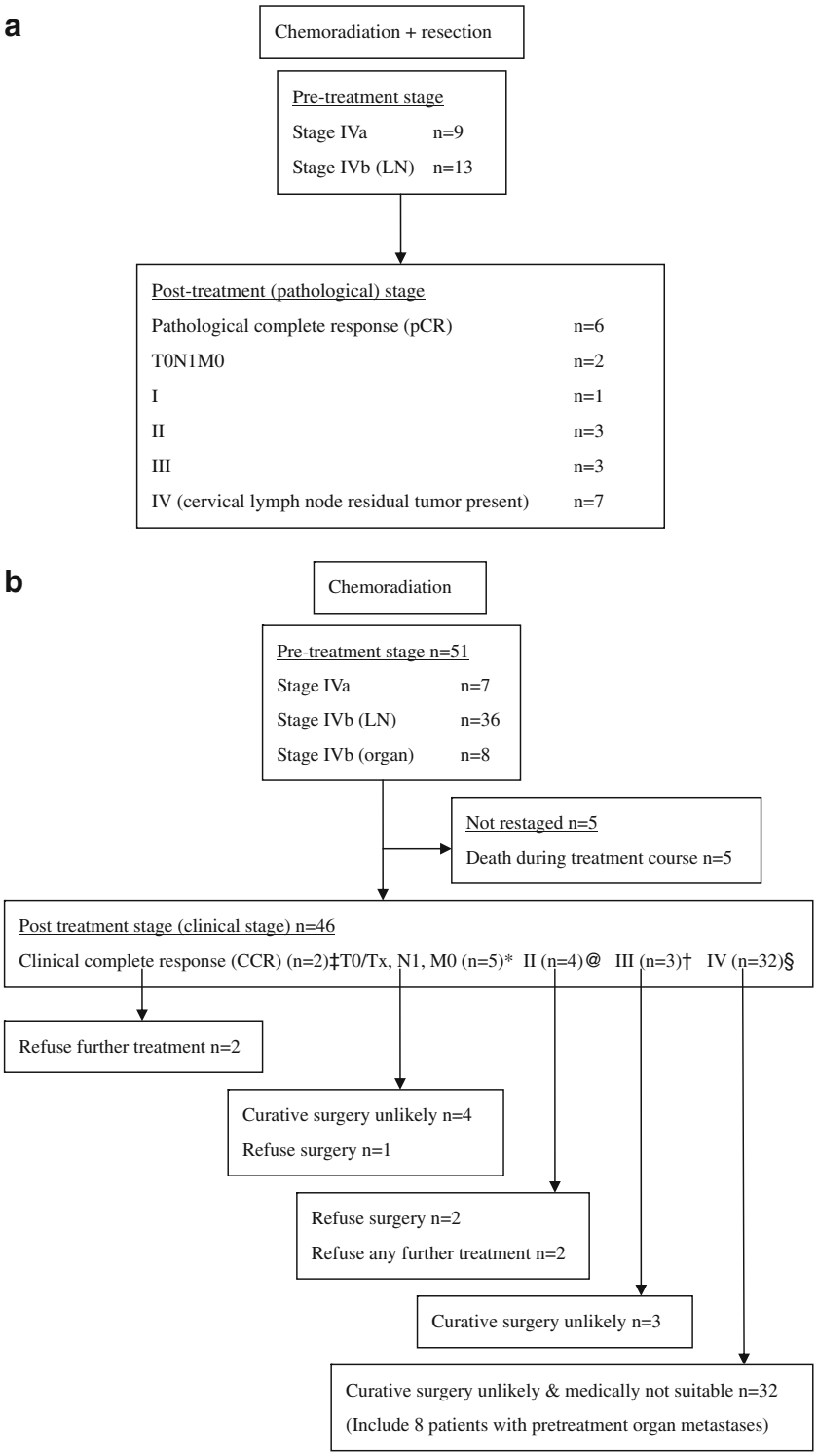

Figure 2 **a** Pathological staging of patients after chemoradiation followed by surgical resection. **b** Subsequent management of patients receiving chemoradiation as initial treatment but in whom no esophagectomy was performed.

.‡ 2 had no further treatment

* 4 had chemotherapy, 1 had esophageal stenting

@ 1 had chemotherapy, 1 had esophageal stenting

† 1 had chemotherapy, 2 had no further treatment

§ 14 had chemotherapy, 8 had esophageal stenting, 2 had radiotherapy, 8 unsuitable for further treatment

Table 2 Complications of Patients After Chemoradiation Followed by Esophagectomy

Complications	Number of patient, n=22 (%)
Medical complications	
Pneumonia	2 (9.1)
Myocardial infarction	1 (4.5)
Surgical complications	
Anastomotic leakage[a]	2 (9.1)
Hemorrhage[b]	1 (4.5)
Ischemic conduit[c]	1 (4.5)
Wound infection	1 (4.5)
Neck seroma	1 (4.5)
Vocal cord palsy (unilateral)	
Transient)	4 (18.1)
Permanent)	1 (4.5)
Hospital mortality	0 (0)

[a] One subclinical leak detected on radiological study only
[b] Bleeding from gastric stapled line requiring surgical re-exploration for control
[c] Ischemic gastric conduit requiring re-exploration and take-down of conduit, colonic reconstruction was subsequently performed

salvage esophagectomy could result in reasonable long-term survival. This strategy of upfront chemoradiation with or without salvage surgery in this group of patients has not been adequately studied.

Cervical metastases from intrathoracic esophageal cancer by definition signify stage IV disease with a poor outcome.[1,2] Treatment options in this group of patients are mainly directed toward palliation; the role of surgery is very limited. Operations on these patients with advanced disease are often risky, morbidity and mortality rates can be substantial, and survival is expectedly poor.[8,9]

Three-field lymphadenectomy entails nodal dissection along the recurrent laryngeal nerves in the thoracic cavity

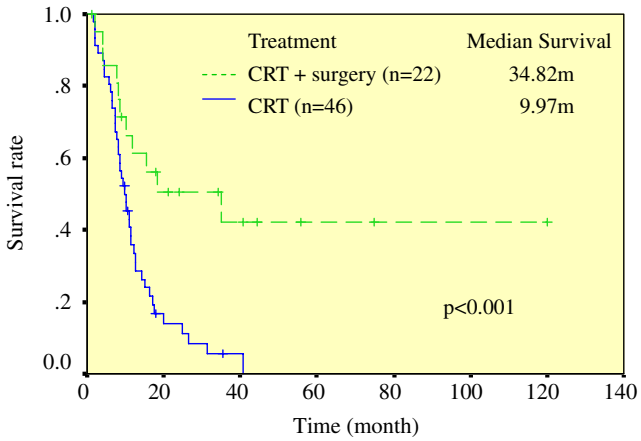

Figure 3 Survival of patients with chemoradiation and esophagectomy (CRT+surgery) vs. chemoradiation only (CRT).

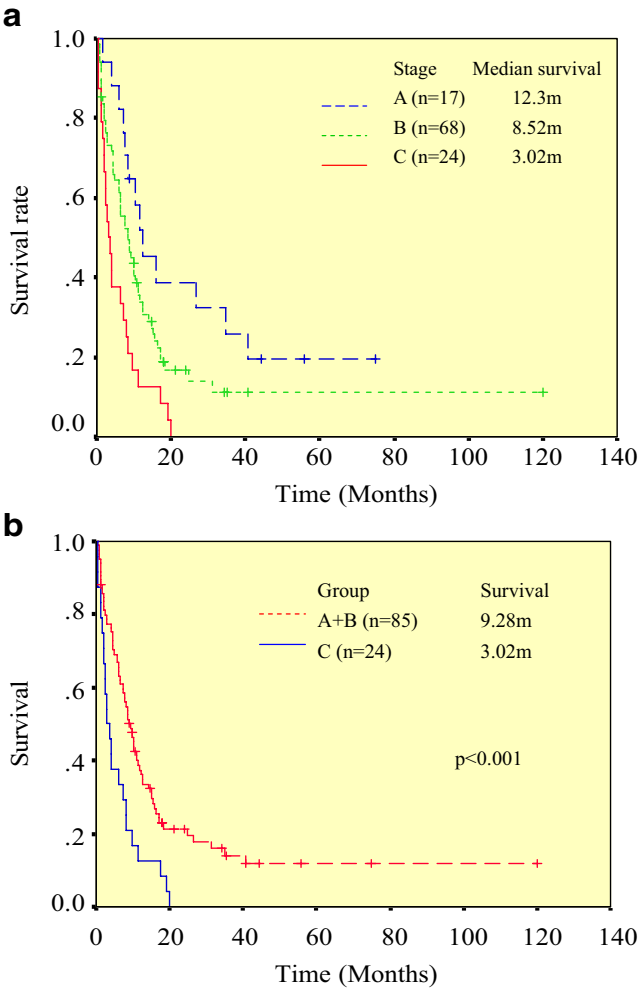

Figure 4 **a** Survival of patients with stage IVa (thoracic upper third + cervical lymph node) (*A*), stage IVb (non-regional lymph node metastases) (*B*) and stage IVb (systemic organ metastases) (*C*) disease. *A* vs. *B*, p=0.08, *A* vs. *C*, p=0.0002, *B* vs. *C*, p=0.0066. **b** Survival of patients with stage Iva and IVb (non-regional lymph node metastases) (*A*+*B*) compared to those with stage IVb (organ metastases) (*C*). p<0.001.

extending to the "third" field in the neck. The rationale of three-field lymphadenectomy is that cervical nodes are found to be involved in more than 30% of patients who undergo cervical lymphadenectomy.[10] In Japan, this is regarded as the standard procedure in treating patients with intrathoracic esophageal cancer, although its benefits remain controversial and unproved by the limited number of randomized trials.[11,12] Advocates of this extended procedure claim superior survival, even for patients with cervical nodal metastases.[10] A 26% overall 5-year survival rate was reported by Shimada and associates for patients with isolated cervical nodal metastasis from thoracic esophageal cancer. He also showed that the overall 5-year survival could be as high as 40% in selected patients when treated with three-field lymph node dissection.[5] The use of

this technique is not confined in Japan; Western centers have also published similar results.[3,13]

There are however differences between patients undergoing "routine" three-field lymphadenectomy and those in the present study. Three-field lymphadenectomy is most often carried out in patients with clinically unsuspected cervical nodal disease, and cervical nodal metastases are only found on microscopic examination of the neck dissection specimen. Lerut et al. reported that cervical lymph node involvement was unforeseen in 75.6% of patients who underwent resection.[3] In our patients, all had obvious nodal disease in the neck, whether clinically palpable or detected by preoperative imaging studies. The tumor burden is thus much more compared to those with subclinical metastases. In addition, patients selected for three-field lymphadenectomy would have a primary tumor that could be curatively resected and absence of other distant metastases. This explains in part the superior results from Japan even in patients with cervical nodal metastases. In our three patients who had esophagectomy without upfront chemoradiation, outcome was poor with very limited survival. All three had locally advanced cancer and bulky cervical nodal metastases. It is not fair to compare these patients who had primary surgical resection with those who had upfront chemoradiation and resection because of the small patient number and selection bias. However, the very encouraging survival experienced by the chemoradiation and surgery group suggests a survival advantage with the latter approach.

Our approach was to treat selected patients with cervical nodal metastases upfront with chemoradiation therapy, and only in those with proven or likely residual neck disease was additional neck dissection performed. The rationale was to avoid unnecessary morbidity associated with additional neck dissection. With these selection criteria, morbidity after esophagectomy in the 22 patients was satisfactory. Recurrent laryngeal nerve palsy rate was fairly high at 23% (five out of 22 patients); fortunately, in four patients, vocal cord palsy was transient with rapid full recovery. Patients with or without neck dissection were all monitored closely for possible cervical nodal recurrence. Indirect evidence from our own experience and also from others suggests that isolated cervical nodal recurrence may be uncommon after two-field lymphadenectomy.[14–16] And if they do recur, further neck dissection could then be carried out. In the present series, unilateral neck dissection was carried out in eight patients, and residual disease in the neck was proven in seven. In the other 14 patients, complete clinical response in the neck was attained post chemoradiation. Only two out of these 22 patients (both had had neck dissection) developed subsequent cervical nodal recurrence, but both also had systemic recurrence. No patient therefore had isolated recurrence in the neck to warrant further neck dissection.

If chemoradiation is effective in patients with cervical nodal spread, the role of additional surgical resection may be questioned. In our 22 patients who underwent esophagectomy, six showed no evidence of residual disease in the surgical specimen. In these patients, operation could have been avoided. However, available staging methods cannot tell certainly whether truly complete pathological response has been achieved. Surgical resection remains the only means to ensure disease clearance. In those whose responses were clinically short of complete, additional surgical resection was justified, provided a R0 resection can be anticipated. Overall R0 resection was possible in 82% of our patients. Admittedly, our patients were highly selected. It would have been ideal to be able to compare outcome with another similar group of patients who had good response to chemoradiation with similar pre- and post-chemoradiation stage distribution but who did not have additional surgery. In our seven patients who experienced good response to chemoradiation but no esophagectomy was performed because primarily of patients' choice even if curative surgery was anticipated, median survival was shorter than those who had surgery, although the difference was statistically insignificant. Direct comparison however may not be valid because of differences in stage distribution and selection bias. Randomized trial in this setting is anticipated to be difficult if not impossible.

The overall outcome of patients with systemic organ metastases was grave and was significantly worse than those who had stages IVa or IVb disease by virtue of nodal disease only. Median survival was only 3 months in this group of patients, and therefore, esophagectomy was not justified. Palliative treatment to maintain quality of life should be the primary goal. The current stage IV classification does not clearly distinguish nodal and systemic metastases; stage IVb disease encompasses patients with cervical nodes only and those with systemic organ metastases. Our data show that, in selected patients even with gross cervical nodal metastases, long-term cure could be achieved, while prognosis in those with systemic organ metastases was uniformly dismal. Our study offers additional evidence to support a revision of the present tumor staging system to take into account the differential survival between the two groups of patients.

In summary, we have shown in this study that, in selected patients with clinically overt cervical nodal metastases, upfront chemoradiation followed by esophagectomy ± cervical lymphadenectomy could result in satisfactory prognosis. It also provides additional evidence that the present tumor staging system that does not distinguish patients with nodal and systemic organ metastases is outdated and should be revised. Not all patients with cervical nodal metastases should be condemned; treatment strategies should be individualized.[17] Further

investigations to identify the best treatment for these patients are warranted.

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Discussion

Dr. Marco G. Patti (Chicago, Illinois): This is another great study that comes from one of the best centers in the world for the treatment of esophageal cancer, and over the years, data from the group of Professor Tong and Dr. Simon Law have had tremendous impact on the way this disease is treated.

Dr. Tong and his colleagues studied patients with intrathoracic squamous cell carcinoma and vert cervical node metastases considered a stage IVa. They hypothesize that, with multimodality treatment, chemoradiation therapy, followed by surgery, the prognosis of these patients could be improved. The patients had a high complication rate, but there were no deaths and the median survival was almost 35 months. In summary, I think that these results suggest that, in selected patients, the combination of chemoradiation therapy with salvage esophagectomy can result in a reasonable long-term survival.

I have the following questions for the authors.

Should we treat all patients with cervical lymph node metastases in this way or just the patients who respond to neoadjuvant therapy?

Second, considering that, in the Western world, most patients have an adenocarcinoma of the distal esophagus, should we extrapolate your data and treat in a similar way patients who have celiac or para-aortic nodule involvement?

And finally, how would you modify the staging system?

Again, congratulations on this very nice study and on your presentation.

Dr. Daniel K. Tong (Hong Kong, China): For your first question, patients with cervical nodal metastasis are a heterogeneous group, comprising those with or without systemic (organ) metastases and also those with locally resectable or unresectable disease. Patients with systemic metastases should be given palliative treatment only and surgical resection is not indicated. For those without systemic metastases, responders to neoadjuvant therapy will often convert locally unresectable or borderline resectable to potentially "curative" resections. In selected non-responders, potentially "curative" resections, i.e., gross tumor clearance achieved, can still be carried out. Our policy is therefore to resect whenever potentially "curative" resections can be performed. If residual disease is too advanced, such as invading to adjacent structures like the carotid artery, surgery is not indicated. Decision should be individualized.

For your second question: In our patient population of only squamous cell cancers, patients with lower third tumors and cervical nodal metastases were also included. There was no apparent difference in outcome between these patients and those with more proximally located cancers. We in Asia do not really have a significant number of patients with Barrett's adenocarcinoma of the distal esophagus. But for our possibility with distal squamous cell cancers and obvious celiac nodal metastases, our policy is also to treat upfront with chemoradiation and then consider surgical resection afterwards. There are two caveats to this: first, the diagnosis of celiac node is sometimes not certain; "celiac nodes" often turn out to be "left gastric or paragastric" in location, and the disease stage would be different. And second, similar to the situation in the neck, a celiac node can be a 1-cm node that can be easily removed vs. a massive celiac node that has enveloped the whole celiac axis where a curative resection is not possible. So again decision to operate has to be individualized.

For the modification of the staging system, we believe that cervical nodes should be classified as regional disease. The trend in other gastrointestinal cancers is to stage N disease according to the number of nodes involved; we think it would be the same for esophageal cancer.

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Medical or Surgical Management of GERD Patients with Barrett's Esophagus: The LOTUS Trial 3-Year Experience

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Abstract
Introduction The long-term management of gastroesophageal reflux in patients with Barrett's esophagus (BE) is not well supported by an evidence-based consensus. We compare treatment outcome in patients with and without BE submitted to standardized laparoscopic antireflux surgery (LARS) or esomeprazole treatment.
Methods In the Long-Term Usage of Acid Suppression Versus Antireflux Surgery trial (a European multicenter randomized study), LARS was compared with dose-adjusted esomeprazole (20–40 mg daily). Operative difficulty, complications, symptom outcomes [Gastrointestinal Symptom Rating Scale (GSRS) and Quality of Life in Reflux and Dyspepsia (QOLRAD)], and treatment failure at 3 years and pH testing (after 6 months) are reported.
Results Of 554 patients with gastroesophageal reflux disease, 60 had BE—28 randomized to esomeprazole and 32 to LARS. Very few BE patients on either treatment strategy (four of 60) experienced treatment failure during the 3-year follow-up. Esophageal pH in BE patients was significantly better controlled after surgical treatment than after esomeprazole (p=0.002), although mean GSRS and QOLRAD scores were similar for the two therapies at baseline and at 3 years. Although operative difficulty was slightly greater in patients with BE than those without, there was no difference in postoperative complications or level of symptomatic reflux control.
Conclusion In a well-controlled surgical environment, the success of LARS is similar in patients with or without BE and matches optimized medical therapy.

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Introduction

Patients with gastroesophageal reflux disease (GERD) who have Barrett's esophagus (BE) suffer a severe degree of acid and nonacid reflux.[1] Acid suppression therapy often results in incomplete reflux control in patients with Barrett's esophagus, both symptomatically and as measured by pH monitoring.[2,3] The outcomes of anti reflux surgery are considered by some to be suboptimal.[4–7] The situation is even more complex since until quite recently the long-term benefits of laparoscopic anti-reflux surgery have not been compared with optimal acid suppression in a prospective trial. A trial of similar design has previously reported the long-term outcomes of open surgery versus omeprazole for the management of GERD[8,9] and demonstrated an advantage of surgery, but both treatment arms were burdened by a significant number of treatment failures (35% at 3 years).

The Long-Term Usage of Acid Suppression Versus Antireflux Surgery (LOTUS) study is being undertaken to compare the long-term effects of standardized laparoscopic antireflux surgery (LARS)[10] with optimal proton-pump inhibitor (PPI) therapy [esomeprazole (ESO)].[11] Within the context of the LOTUS Trial, we had the opportunity to look specifically at the relative efficacy of these therapeutic options in control of reflux disease manifestations in BE patients, as reflected by symptom and esophageal pH control and improved quality of life.

Methods

Study Design and Objectives

The present study addressed two aspects relevant to the management of BE patients: firstly, whether long-term medical treatment with esomeprazole was comparable in efficacy with that of LARS and secondly, to analyze outcomes after LARS in BE compared to non-BE patients, focusing on subjective patient experiences and on measured objective criteria. The trial protocol was approved by local ethics committees, and written informed consent was obtained from all patients.

Patients

The target population consisted of adults aged 18–70 years with confirmed GERD, with or without BE. For the purposes of the trial, BE was defined as intestinal metaplasia on biopsy of endoscopically apparent columnarization. The patients had to have a history of chronic reflux esophagitis (>6 months) or chronic symptomatic GERD (>6 months) with pathological 24-h pH metry, according to local standards, and a requirement for long-term acid suppressive therapy. All patients were required to have had pH monitoring and manometry within 12 months prior to randomization and all had to be considered suitable for both surgical treatment and for long-term management with a PPI (esomeprazole). For this reason, any patient who had a primary need for surgery (e.g., for paraesophageal hernia or failure of medical therapy to control symptoms adequately) was not eligible to be recruited. Additionally, patients had to be capable of completing quality-of-life questionnaires. Patients who required PPI treatment for diseases other than GERD were excluded from the study, as were those who had a history of esophageal, gastric, or duodenal surgery or who had other diseases that might have a negative impact on their subsequent treatment within the study.

Study Schedule and Measurements

The study schedule and principal measurements have been described in detail elsewhere.[12] Before randomization, the protocol mandated a 12-week run-in period, which allowed baseline recordings to be made and medical treatment with esomeprazole 40 mg od to facilitate healing of the esophagitis. An investigational week was then scheduled without therapy to allow endoscopy, assessment of esophagitis according to the Los Angeles classification,[13] biopsy sampling, laboratory screening, and 24-h pH metry with manometry and symptom association probability (SAP).

At the end of the run-in period, eligible patients were randomized in blocks of four to two parallel study arms, receiving either surgery or maintenance medical treatment with esomeprazole 20 mg od for their disease. Medical treatment was started at 20 mg od but could be dose adjusted, not to exceed 20 mg bid. Surgery had to be performed within 3 months of randomization, but patients could be treated with esomeprazole up to 40 mg od while awaiting surgery. Follow-up clinic visits took place every 6 months (with surgical patients having extra visits for the operation and a 1-month postsurgical check-up).

Surgical Technique

A laparoscopic Nissen fundoplication was recommended to be performed in all patients, according to a standardized technique agreed upon by the surgeons at the beginning of the study. The pre-, per-, and postoperative outcomes and work-up programs are described in detail elsewhere.[10,12] The procedures were performed at 39 centers across Europe, and all participating surgeons were experienced

independent operators who had been performing >20 per annum before the start of the study.

Endoscopy, Symptom, and Safety Assessment

At endoscopy, the esophagus, cardiac region, stomach, and duodenum were examined, and biopsies were taken from the esophagus, Z-line, antrum, and corpus to assess for the presence of microscopic esophagitis, gastritis, and *Helicobacter pylori*. If there was any suspicion of Barrett's esophagus or malignancy, additional biopsies were taken and referred for pathological examination.[14] The gastroesophageal junction was defined as the junction between the proximal margin of the gastric mucosal folds and the tubular esophagus. In the case of an oral extension of the columnar lined esophagus of more than 2 cm, biopsies were taken from each quadrant of the circumference by 2 cm intervals in oral direction. An additional two biopsies were to be taken from tonguelike protrusions or islets of columnar epithelium.

Symptoms related to GERD as well as other gastrointestinal (GI) symptoms including epigastric pain, flatulence, bloating, diarrhea, ability to vomit, and ability to belch were scored by use of the Gastrointestinal Symptom Rating Scale (GSRS) questionnaire[15] and by investigator assessments (the latter for ability to vomit and ability to belch). Quality of Life was also assessed by the validated Quality of Life in Reflux and Dyspepsia (QOLRAD) questionnaire,[16] both questionnaires being administered to patients at randomization and annually thereafter.

Treatment Endpoints and Statistical Analyses

The main analyses were conducted using the intention to treat population that included all randomized patients. Time to treatment failure was defined as follows for the two study treatments:

> *In the medical arm:* The need for treatment other than esomeprazole for control of symptoms of reflux disease was assessed by asking the question "Do you have sufficient control of your heartburn and acid regurgitation?" If the answer was no and was backed up by the need for other regular therapy, the dose of esomeprazole was increased to 40 mg od for 8 weeks and could be adjusted to 20 mg bd for a further 8 weeks. If this proved insufficient to control symptoms, the patient was classified as a "treatment failure." *In the surgical arm*: The same questions were asked about symptom control as in the medical arm, and if the answer was no and again backed up by the need for regular drug treatment, the patient was classified as a "treatment failure." The patient was also classified as a treatment failure if there were postoperative complaints requiring medical action, per-operative death, post-operative death within 30 days after surgery, dysphagia requiring further treatment, or any other requirement to re-operate for symptom control. In the case of functional esophageal stenosis, one dilatation was allowed.

In a post hoc analysis, mean scores of GI symptoms (none=0, mild=1, moderate=2, and severe symptoms=3) from 6 months to 3 years were compared using a two-sample *t* test. Change from the randomization value to the average of the 1-, 2-, and 3-year values of the GSRS and QOLRAD scores were compared using an analysis of variance, with values from the randomization visit as covariate. Comparisons of all of the above variables were made between medical and surgical treatments in patients with BE versus those without BE.

Results

Out of 554 patients with chronic GERD, 60 patients were found to fulfil the given criteria for BE, of whom 28 were randomized to medical treatment and 32 to antireflux surgery (Table 1). As seen in this table, no major differences existed between chronic GERD patients with or without BE in relation to demographic and disease-specific characteristics. Patients allocated to LARS had slightly higher baseline total acid exposure time (ns) than those treated by ESO. When it came to the classification of the extent of the BE segment, this was estimated by the assessment of the circumferential extent, the number of tonguelike protrusions, and the estimation of the length of the longest tongue. The presence and number of columnar islands is also described. Valid baseline data on all of these variables were captured for 27 LARS patients and 24 of those randomized to ESO (Table 2). Again the groups were well-balanced regarding corresponding parameters.

In the patients with BE, 100% of operations were completed as a 360° Nissen fundoplication, compared to 98% in the patients who did not have BE. There were no conversions to open surgery—all procedures were completed laparoscopically. At the time of surgery, some differences between BE and non-BE patients emerged (Table 3). The parameters that showed differences included operative duration, the size of hiatus hernia, and the number of sutures used to repair the crurae. There was a high frequency of hiatus hernia (83% in BE vs 67% in non-BE) and when present, the hiatus hernia was often larger in BE patients (>5 cm in 37% patients with BE compared to 15% in non-BE patients). In BE patients, more than three crural sutures were required in 40% vs 28% in those without BE. Comments by the surgeons on any operative difficulty were recorded more commonly in patients with BE (23% vs 13%). However, we observed no difference in the median

Table 1 Demographic and GERD-Specific Characteristics of BE and Non-BE Patients by Therapeutic Strategy

	LARS		ESO	
	Non-BE	BE	Non-BE	BE
Female/Male (*n*)	85/171	4/28	60/178	7/21
Mean age (years)	45	47	45	50
Mean body mass index (kg/m^2)	27	28	27	27
GERD duration (*n*)				
<1 year	78	6	68	12
1–5 years	130	16	125	10
>5 years	46	10	44	6
Esophagitis grade				
None	120	14	109	20
LA grade A	72	7	54	1
LA grade B	55	9	68	4
LA grade C	9	1	7	3
LA grade D	0	1	0	0
% time pH <4				
Median (P10/P90)	7.9 (2.0–21.4)	13.2 (3.6–46.8)	8.8 (2.5–22.8)	7.4 (1.1–38.6)

operative time between patients with and without BE, although a trend emerged in that the BE group contained a larger proportion of operations lasting for more than 2 h (30% vs 23%, ns). There was no apparent difference between the groups in postoperative complication rates, although we observed a somewhat longer postoperative hospital stay in BE patients, with 63% staying 3 days or longer vs 47% for non-BE (p=0.11). Six months postoperatively, ambulatory 24-h pH metry was completed. From a baseline total acid exposure time of 13.2%, LARS reduced it to a median of 0.4% at 6 months. The corresponding data from ESO-treated patients were 7.4% and 4.9%, respectively, showing a significantly superior reduction in esophageal acid exposure after LARS (p=0.002).

When the symptomatic and overall therapeutic outcomes were evaluated at 3 years, there was one treatment failure in BE patients submitted to LARS and three in those treated medically (ns). The symptomatic outcomes, as reflected by the GSRS and QOLRAD scores, are detailed in Tables 4 and 5, showing normal values and no significant differences between BE and non-BE patients or between LARS and ESO strategies.

The side effects of a total fundoplication were compared between BE and non-BE patients in the LARS group. As seen in Fig. 1, BE as well as non-BE patients expressed similar profiles of obstructive and gas bloat-like complaints and, if anything, a trend was observed towards less complaints in the BE group.

Discussion

The LOTUS Trial constitutes a large and strictly defined chronic GERD population, in which a head-to-head comparison can be completed with respect to the pros and cons of management concepts based either on modern medical therapy or on standardized laparoscopic surgical treatment.[12] Within this clinical trial setting, we could study in more detail the patients with BE and we found that the clinical response to therapy over 3 years was essentially identical in BE compared to non-BE patients. When comparing surgery versus medical treatment for Barrett's esophagus, the symptom outcomes are similar at 3 years, but pH data indicate more complete control of reflux on LARS than modern medical therapy could accomplish.

In terms of symptom and disease history, we observed few if any important differences between our BE and non-BE patients at baseline. It could, therefore, be implied that

Table 2 Endoscopic Characteristics of the Columnar Lined Mucosa (CLE) at Baseline in BE Patients Allocated to Either LARS or ESO Therapy

	LARS	ESO
Circumferential CLE (*n*)	10	10
Extent ≤2 cm	1	4
3–5 cm	7	2
>5 cm	2	4
Tongues present (*n*)	16	13
Number of tongues: (Missing)	(1)	–
1	8	7
2	4	2
3	3	3
4	0	1
Longest tongue (*n*) (missing)		(1)
≤2 cm	11	9
3–5 cm	4	3
>5 cm	1	–
Isolated islands	1	1

Table 3 Peroperative Recordings During Laparoscopic Antireflux Repair in BE and Non-BE Patients

	Non-BE	BE
Hiatus hernia diameter (cm)		
1–2	29 (13%)	3 (10%)
3–4	73 (34%)	9 (30%)
5–6	30 (14%)	10 (33%)
>6	4 (2%)	1 (3%)
Number of crural sutures		
0	1	1
1–2	150 (69%)	17 (57%)
3–4	59 (27%)	12 (40%)
>4	4 (2%)	0
Esophagus mobilized to ≥3 cm intra-abdominally	212 (97%)	30 (100%)
Any operative difficulty	28 (13%)	7 (23%)
Any complication	16 (7%)	2 (7%)
Peroperative blood loss (ml)		
<100	5	–
100–300	8	–
301–1,000	2	–
>1,000	2	1
Pneumothorax	6 (3%)	1 (3%)
CO_2 retention	3 (1%)	–
Emphysema	7 (3%)	–
Total operating time (h)		
<1	18 (8%)	4 (13%)
1–2	148 (68%)	17 (57%)
2–3	45 (21%)	7 (23%)
3–4	3 (1%)	1 (3%)
>4	3 (1%)	1 (3%)
Postoperative hospital stay (n)		
1 day	62 (8%)	6 (20%)
2 days	53 (24%)	5 (17%)
3 days	38 (17%)	6 (20%)
4 days	26 (12%)	5 (17%)
5–6 days	27 (12%)	7 (23%)
7–15 days	10 (5%)	–

the pretreatment assessment of acid reflux variables did not separate the BE patients from the GERD patients without Barrett's esophagus. In terms of more general functional outcomes after surgery, we conclude that our BE patients responded in harmony with those GERD patients who did not have BE. It can be argued that the present study comprised relatively few patients with BE, opening up the possibility of type II errors. On the other hand, this large, multinational, European trial represents a chronic GERD population in which no selection criteria have been practised except for those defined in the protocol to regulate the enrolment. Therefore the ~10% of all our randomized patients who fulfilled the criteria for BE are representative of those patients presenting in clinical practice who seek medical long-term management advice.

Our per-operative data suggest that anti reflux surgery is more challenging in BE patients. The size of the hiatal hernia in BE mandates the surgeon to spend more time and effort in mobilizing the esophagus and restoring the anatomy of the gastroesophageal junction, as well as carefully repairing the hiatal defect by crural suturing. This course of action takes more time, even in the hands of experts. These steps are pivotal components in the surgical strategy to reconstruct the anatomy of the junction accurately, with the functional consequences that has on the reflux-preventing mechanisms.[17]

Reported outcomes for medical as well as surgical therapy of BE vary considerably in the literature. Few of these, if any, are multicenter randomized prospective trials of laparoscopic surgery with more than 3 years of follow-up. Many are cohort studies without medical comparisons,[4,7,18–24] often with mixed open and laparoscopic antireflux procedures and without standardization of the operation type. Some are randomized but mostly pre-date laparoscopy.[25–29] The only randomized trials of GERD with the current treatment modalities are single center and too small to assess outcomes in Barrett's esophagus.[30]

Table 4 Therapeutic Outcome, as Assessed by the Gastrointestinal Symptom Rating Scale at 1, 2, and 3 Years After LARS or ESO Therapy, in Non-BE and BE Patients—Mean Scores

	LARS				ESO			
	Baseline	1 year	2 years	3 years	Baseline	1 year	2 years	3 years
Non-BE								
Diarrhea	1.8	1.8	1.8	1.8	1.8	1.7	1.6	1.7
Indigestion	2.5	2.9	2.8	2.7	2.4	2.2	2.1	2.3
Constipation	1.7	1.7	1.7	1.6	1.6	1.6	1.5	1.6
Abdom pain	2.1	1.8	1.8	1.8	2.0	1.8	1.8	1.8
Reflux	1.9	1.2	1.2	1.2	1.7	1.7	1.7	1.7
BE								
Diarrhea	1.5	1.5	1.6	1.7	1.6	1.6	1.4	1.3
Indigestion	2.0	2.6	2.5	2.7	2.5	2.4	1.9	1.9
Constipation	1.6	1.4	1.7	1.8	1.6	1.6	1.6	1.4
Abdom pain	1.6	1.7	1.5	1.5	1.9	1.7	1.4	1.5
Reflux	1.5	1.1	1.2	1.3	1.7	1.5	1.4	1.3

Table 5 Health-Related Quality of Life, as Assessed by the QOLRAD Score at Baseline and 1, 2, and 3 Years after LARS or ESO Therapy, in BE and Non-BE Patients—Mean Scores

	LARS				ESO			
	Baseline	1 year	2 years	3 years	Baseline	1 year	2 years	3 years
Non-BE								
Emotional	6.4	6.9	6.9	6.9	6.4	6.5	6.7	6.6
Sleep	6.4	6.9	6.9	6.9	6.3	6.5	6.5	6.5
Food/drink	6.1	6.8	6.8	6.9	6.2	6.3	6.3	6.4
Physical/social	6.5	6.9	7.0	6.9	6.5	6.7	6.7	6.8
Vitality	6.2	6.8	6.9	6.9	6.2	6.4	6.4	6.5
BE								
Emotional	6.5	6.9	6.8	7.0	6.5	6.9	6.8	6.8
Sleep	6.5	7.0	6.9	7.0	6.5	6.7	6.8	6.8
Food/drink	6.5	6.9	6.8	6.9	6.2	6.5	6.4	6.5
Physical/social	6.7	7.0	7.0	6.9	6.5	6.8	6.8	6.7
Vitality	6.7	6.9	6.9	6.9	6.2	6.7	6.7	6.6

In the medical arena, it seems as though the better and more well-defined the therapeutic trial conditions are, the less impact presence of BE has on the level of reflux control and endoscopic healing rates.[31] Accordingly, the present data add to the view that the grading of the mucosal breaks according to the Los Angeles system[13] outweighs the influence of the presence of BE on clinical responsiveness to therapy.[32] In the surgical literature, the picture is

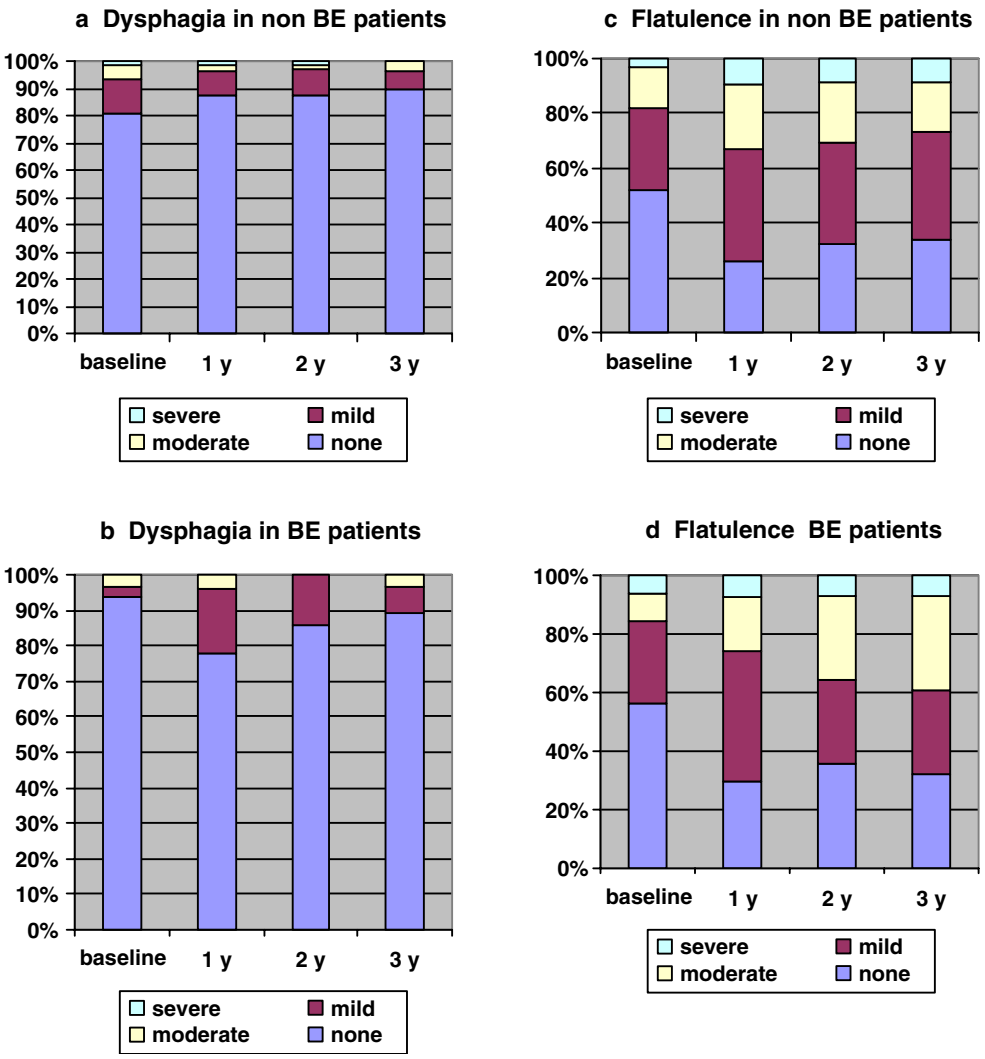

Figure 1 **a** Dysphagia in non-BE patients, **b** dysphagia in BE patients, **c** flatulence in non-BE patients, **d** flatulence BE patients.

even more diversified, usually without objective assessment of postsurgical outcomes by, e.g., the use of 24-h pH metry.[33–35] Our study has the obvious strength of combining subjective assessments (using standardized questionnaires) with the objective tool of pH monitoring. It was therefore gratifying that we could show a complete control of acid reflux postoperatively. Another factor of importance is the selection of BE patients for surgical repair. In our series, most of our patients had columnar-lined segments longer than 2 cm, indicating that our BE patients did not represent the "easy" cases of Barrett esophagus.[36] Our 3 year outcomes after LARS would not endorse the alleged concept of "tailored" surgical approaches in BE, incorporating even open transthoracic approaches and also more radical operative procedures.[7,21,37]

The results in the LOTUS Trial have shown symptom responses that are dramatically better than previous reports on prospective randomized trials.[9,25,26] There may be a number of explanations. Firstly, both the surgical and medical treatment arms were optimized.[10,11] Medically treated patients were given escalating doses of esomeprazole in a standardized manner, allowing much more effective acid suppression than that seen in previous studies. Surgical patients received a standardized laparoscopic Nissen fundoplication by a group of expert surgeons. The degree of standardization and the effect of this on the outcome in relation to low surgical complications have been published recently.[10] Only one previous study attempted to standardize the Nissen procedure, but it was by open surgery with relatively inexperienced surgeons.[27] In that study, standardization of the surgery was dictated by the senior author.[38] In our study, a group of relatively expert surgeons came to a consensus about how to standardize the Laparoscopic Nissen procedure and then were able to achieve this operation in all of the patients with BE. Having an operation standardized by a laparoscopic approach is very effective and can be policed by every member of the operating team because the view of the operative field is available to all team members. Efforts to achieve the wrap quality and fixation and the assurance of hiatal closure in all patients are easily monitored by the laparoscopic image. As a consequence, we believe the rate of complications seems significantly less than reported in the literature.[39–44] Debate about the value of laparoscopy is now superseded[45–47], and the arguments about the persistent need for PPI[48] are not supported by our low symptom failure rate after surgery (<5%).

Remaining crucial issues to be addressed in BE relate to the arrest of CLE progression, control of mucosal inflammation, attenuation of the proliferative drive on the columnar-lined epithelium with eventual mitigation of the risk of developing related dysplastic lesions.[49,50] The current human experimental model—the LOTUS Trial, comparing acid versus complete reflux control, offers a unique opportunity to address these pivotal questions. However, a longer follow-up period is then required with endoscopic surveillance and vigorous biopsy sampling. Subsequent analyses from the ongoing LOTUS Trial will cast further light on these important questions.

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Discussion

Thomas J. Watson, M.D. (Rochester, NY): Mr. Attwood and all of the participants in the Lotus trial are to be congratulated for their fine work in designing and executing this large international study. The good news for surgeons is the fact that the failure rate of laparoscopic fundoplication in patients with Barrett's esophagus was quite low, 3% at three years followup, much better than prior reports and demonstrating what a standardized operative technique performed in the hands of experienced surgeons can achieve.

Worthy of emphasis is what this report contains and what it does not. We have heard today about symptomatic and objective control of GERD. We have not heard about prevention of Barrett's progression to dysplasia or carcinoma or induction of histologic regression by medical or surgical therapy. Another important point is that patients not responding to attempts at symptom control which medical therapy were not eligible for enrollment in the study. So, if I interpret the results correctly, those patients who responded well to medical therapy prior to enrollment continued to respond well to medical therapy after enrollment, a most unstartling conclusion.

Mr. Attwood, my first question therefore is how the exclusion of patients deemed symptomatic failures to medical therapy should influence our interpretation of the trial results?

A final point of emphasis is that even though surgical and medical therapy resulted in similar symptomatic success rates, objective control of GERD was far superior in the surgical cohort, highlighting the time-proven principle that symptoms are an unreliable barometer of reflux severity. Given that pH scores remained, on average, in or near the abnormal range in patients on medical therapy, what are the implications regarding how we should monitor the effectiveness of reflux control in medically treated patients with Barrett's esophagus.

Again I congratulate you on a fine presentation and look forward to future reports as the data from this trial continue to mature. Thank you.

Stephen E. Attwood, M.D. (North Shields, UK): Thank you very much, Dr. Watson, for those comments.

I agree with all of the concerns that you have raised. It is very important to understand that you cannot translate this trial into routine medical practice for the decision of whether you should or should not operate. If a patient has failure to respond to medication, there are two ways that patient can be analyzed. One is that perhaps they are not suffering reflux. The other is that they have a positive pH monitoring with severe acid reflux and then they are very likely to improve with surgical therapy and they should be offered surgical therapy primarily.

The interpretation of this study does slightly bias in favor of the medical arm because we have excluded all those patients who did not respond, so I do agree with you. However, it is very important I think from the point of view of the literature to have a level playing field at the beginning of the study in order to identify comparative outcomes of the two treatment strategies and I think the long-term benefits of one treatment or another will be revealed by the 5 and 10-year followup. The question about whether this excess acid exposure that persists in the medically treated arm is that important – we do not know. It is not important at three years because the patients are well, but because we have a control group on no medication we will be able to see if there is a difference in histological changes at 5 and 10 years.

Donald E. Low, M.D. (Seattle (WA): Mr. Attwood, first of all, congratulations. I enjoyed your paper very much. I have two questions.

The first is, you are assessing surgical failure on the basis of a requirement for adding PPI therapy post-op. How is that decision made? We know from previous work there is a large group after antireflux surgery that will be on PPIs that have no demonstrable reflux disease. It would seem that you are setting yourself up for a situation that may be hard to interpret.

The second question is, we are increasingly told that patients with large hiatal hernias and Barrett's will be at increased risk for esophageal shortening and therefore should be considered for a Collis procedure at the same time. Is this part of the equation in how you are making your surgical decisions?

Dr. Attwood: Those two questions are very interesting. The second question about shortening – within the study no Collis Nissen operations were performed out of the 248

surgical procedures. So within European practice, the need for a Collis Nissen is extremely rare and also I point out we have a very low re-hernia rate, so we are not seeing people get a recurrent paraesophageal hernia.

In relation to assessment of failure in either group, we are asking our investigators to do pH monitoring to identify are these patients actually refluxing at the time they are either dose escalated with their pH with omeprazole or with addition of PPI after surgery. Of course they have not had very many who have needed a PPI after surgery but that is what we will do.

Giovanni Zaninotto, M.D. (Padova, Italy): Steve, do you have any detailed information on esophageal physiology in short- and long-Barrett segment patients before and after surgery?

Dr. Attwood: Unfortunately the group is relatively small. We have only 30 patients in the surgical group and when we divide those into those with short and long Barrett's at the moment we see no difference but in such a small group it is unlikely we would. We would probably need a larger group.

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Quality of Life and Symptomatic Response to Gastric Neurostimulation for Gastroparesis

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Abstract
Background Gastroparesis can be a difficult problem with patients suffering from nausea, vomiting, bloating, and pain intractable to medical management. Gastric neurostimulation has been developed as an adjunctive treatment for patients with diabetic and idiopathic gastroparesis unresponsive to pharmacologic and dietary treatment. The purpose of this study is to report symptomatic and quality-of-life response to gastric neurostimulation.
Methods This study was approved by the institutional review board, and patients had informed consent. The gastric neurostimulation device (Enterra therapy, Medtronic, Inc., Minneapolis, MN, USA) is approved by the Food and Drug Administration under the Humanitarian Device Exemption. Candidates for placement were patients with either idiopathic or diabetic gastroparesis who had symptomatic failure to dietary changes and to prokinetic and antiemetic drugs. Before placement, the patients' symptoms were recorded, and patients completed the Gastrointestinal Symptom Rating Scale (GSRS, three domains: dyspeptic syndrome, indigestion syndrome, and bowel dysfunction syndrome) and the Short Form-36 (SF-36, eight domains: physical functioning, role-physical, role-emotional, bodily pain, vitality, mental health, social functioning, general health, plus a health transition item). The device was surgically placed using a hybrid laparoscopic/open technique. Patients were followed and adjustments made on the device until satisfactory symptom control was achieved. At that time, patients completed both the GSRS and SF-36, and comparisons were made to preoperative values.
Results Forty-two patients had the device placed, 29 women, aged 41 (SD +14) years, 24 diabetic patients, 17 idiopathic patients, one postgastrectomy patient. Median follow-up was 12 months (range 1–42 months). There was a 2% immediate postoperative morbidity rate and 7% long-term morbidity rate (device extrusion). Thirty-one patients (74%) responded to gastric neurostimulation of variable degrees. Eleven patients had no response or had worsening symptoms. Of the patients who responded, there were statistically significant improvements in all three domains of the GSRS. Median scores (with interquartile ranges): dyspeptic syndrome, 9 (7–11.5) to 4 (2.5–6), $p=0.02$; indigestion syndrome, 5 (2–7) to 4 (0–5), $p=0.05$; bowel dysfunction syndrome, 3 (2–3) to 1 (0–1), $p=0.01$. In the SF-36, there were statistically significant improvement in the health transition item, 4 (4–5) to 1.5 (1–3), $p=0.01$; and social functioning domain, 25 (12.5–62.5) to 75 (50–87.5), $p=0.03$.
Conclusions Three quarters of gastroparesis patients responded to gastric neurostimulation to variable degrees. Gastrointestinal-specific symptoms are improved in responders. Patients felt that there health and social functioning (SF) improved, although there was no significant difference in the other domains. These results are encouraging considering that these patients had intractable symptoms with no other effective treatments available.

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Keywords Gastroparesis · Gastric neurostimulation · Nausea · Vomiting · Quality of life

Introduction

Gastroparesis has always been a problem difficult to manage. Patients may suffer from persistent nausea,

vomiting, abdominal or epigastric pain, and bloating. Often, these symptoms are intractable to medical management.[1] The effects of gastroparesis extend beyond symptoms, with employment opportunities and social interactions severely restricted. The most common causes of gastroparesis include diabetes mellitus, idiopathic causes, and post-gastrectomy/post-vagotomy syndromes. However, more rare causes include autonomic nervous system dysfunction, scleroderma, infiltrating diseases, such as amyloidosis, gastrointestinal pseudo-obstruction, central nervous system diseases, cyclical vomiting syndrome, and functional gastrointestinal disorders.[1,2] In addition, it is imperative that a mechanical cause of the delayed gastric emptying be sought, such as malignancy, adhesions, or herniation. The severity of the gastroparesis problem is significant, as the number of hospitalizations has increased dramatically.[3]

Traditionally, gastroparesis has been a medical problem. The standard treatments have included antiemetics, prokinetic agents, and pain management.[4] Gastrectomy has been reserved only for the most intractable of cases, although it has been found effective in patients who suffer from post-gastrectomy/post-vagotomy gastroparesis.[5,6] Work in understanding gastric neurophysiology has lead to the development of a device to provide gastric neurostimulation in hopes of improving gastroparesis.[7–9] This device (Enterra therapy, Medtronic, Inc., Minneapolis, MN, USA) has been approved for use under the Humanitarian Device Exemption by the US Food and Drug Administration for diabetic and idiopathic gastroparesis. The purpose of this study is to determine the effectiveness of Enterra therapy in improving the symptoms and quality of life of patients suffering from diabetic or idiopathic gastroparesis.

Methods

This study was approved by the Institutional Review Board of the Henry Ford Health System.

Patients As per Food and Drug Administration (FDA) requirements, only patients with diabetes or idiopathic gastroparesis were eligible for Enterra gastric neurostimulation. To be eligible, patients must also have had radiologic or endoscopic proof that their symptoms were not because of mechanical gastrointestinal obstruction. Patients must have been symptomatic. Initially, symptoms felt to be related to gastroparesis, including nausea, vomiting, postprandial abdominal or epigastric pain, or bloating. In addition, patients must have a failed medical therapy with antiemetic medications, prokinetic medications, and/or pain medications. Patients also must have a failed dietary therapy to try to limit their symptoms, yet have had adequate nutritional intake. Patients must have had gastric emptying scintigraphy demonstrating delayed gastric emptying. Patients were counseled extensively as to the device, its FDA status, operative complications, lifestyle restrictions after the device is placed, requirement for long-term follow-up, and possible replacement of the device because of complications or battery depletion. Patients who wished to proceed with placement, then received informed consent.

Disease-Specific and Generic Quality-of-Life Instruments Before having the gastric neurostimulator implanted, usually at the first visit, patients completed the Gastrointestinal Symptom Rating Scale (GSRS)[10] and the SF-36[11] as symptom severity and quality-of-life measures, respectively.

The GSRS is a 15-item scale measuring three domains of gastrointestinal symptoms: dyspeptic syndrome, indigestion syndrome, and bowel dysfunction syndrome. The GSRS is a valid and reliable instrument for the measurement of gastrointestinal-related symptoms and has been applied to a wide variety of gastrointestinal diseases. The items are scored on a Likert scale from 0 to 3, with 0.5 steps allowed between the whole numbers. There are three domains: dyspeptic syndrome (five items, best possible score 0, worst possible score 15), indigestion syndrome (four items, best score 0, worst score 12), and bowel dysfunction syndrome (six items, best score 0, worst score 16). In addition, there is a specific item, #5, which addresses "nausea and vomiting," the main symptom that gastric neurostimulation is suppose to treat. The GSRS was chosen for this study because it has been shown to be reliable and valid for a variety of gastrointestinal disorders. Specifically, for the purposes of this study, because it assesses pan-gastrointestinal symptoms, it can evaluate the effects of gastric neurostimulation on a variety of gastrointestinal symptoms.

The SF-36 is a generic 36-item quality-of-life instrument measuring eight domains of quality of life and a health transition item. The eight domains of the SF-36 are physical functioning (PF), role-physical (RP), role-emotional (RE), bodily pain (BP), vitality (VT), mental health (MH), SF, and general health (GH). The best possible score is 100, and the worst possible score is 0. The "health transition" item (question #2 in the instrument) addresses patient-perceived change in health: Compared to 1 year ago, how would you rate your health in general now? The best possible score is 1 (much better now than 1 year ago), and the worst possible score is 5 (much worse than 1 year ago). The SF-36 has been shown to be a valid and reliable for the measurement of quality of life for a variety of medical and surgical disorders. It has been previously used to assess gastroparesis and gastric neurostimulation. This instrument was chosen for this study to provide a global quantitative assessment of quality of life not only for the effect of

gastric neurostimulation, but also to be able to compare gastroparesis to other disease processes.

The Enterra Gastric Neurostimulator The Enterra gastric neurostimulator (Medtronic, Inc., Minneapolis, MN, USA) consists of an implantable device that generates electrical pulses through two leads that are implanted into the muscular layer of the stomach.[4] The electrical pulses are cycled at a specific voltage, pulse width, rate, on-time and off-time. The electrical dosing can be adjusted based on symptom response by manipulating any of these parameters.

Operative Technique The gastric neurostimulator was implanted either through a completely open[12] (in patients whose prior operations did not allow for laparoscopic placement) or a hybrid laparoscopic/open technique. Because of difficulty in a completely open laparoscopic technique, the hybrid technique was developed. Although many surgeons place the device entirely laparoscopically, the hybrid technique has reduced the operating time from over 2 h to approximately 30 min. In addition, as a larger incision is required to place the device in a subcutaneous pocket, no change in the number or length of the incisions is incurred by the hybrid technique. The patients were brought into the operating room and placed on the operating table in the supine position. After general, endotracheal anesthesia was induced, the abdomen was prepped and draped in the usual sterile fashion. Prophylactic preoperative antibiotics and deep venous prophylaxis were used in all cases. The abdomen is entered through an infraumbilical incision either with a Veress needle or Hasson cannula. The abdomen is insufflated with CO_2 gas to a pressure of 15 mmHg and kept at this pressure throughout the laparoscopic portion of the case. A 10-mm port is placed in the left upper quadrant, with care taken to place the port as close to the greater curvature as possible. A 5-mm port is placed in the right upper quadrant. The pylorus is identified. A ruler exactly 10 cm in length and 1 cm in width is passed into the abdomen and placed at the pylorus. The spot exactly 10 cm from the pylorus to the gastric greater curvature is measured. The ruler is left in place, and using the hook electrocautery, two spots at the corners of the ruler, exactly 1 cm apart, are scored on the gastric serosa. At this point, an endo-Babcock clamp is used to grasp the stomach at the scored area on the greater curvature. This portion of the stomach is brought to the left upper quadrant trocar site. The gas is evacuated from the abdomen. The skin and fascia are enlarged just enough to allow the scored portion of the stomach to be visualized.

The scored marks on the stomach are identified. The leads are placed into the muscular layer of the stomach so that the blue polypropylene suture is within the muscular layer. At this time, the surgeon scrubs out of the procedure and performs an upper endoscopy to determine that the suture has not penetrated the gastric mucosa. It is essential that the electrodes are within the gastric muscle. The electrodes must be placed exactly parallel, exactly 1 cm apart. However, whether the electrodes are placed in line with the long or short axis of the stomach is irrelevant. Once the electrodes are with the gastric muscular layer, they are sutured to the stomach with silk suture. A plastic disk is advanced over the needle and suture to the exit point of the polypropylene suture from the stomach and secured in place with ligaclips and suture. The stomach is returned to the abdomen and the fascial defect closed with running, absorbable suture.

The device is connected to the electrodes and interrogated. The impedance between the electrodes is tested and must be between 200 and 800 Ω. Once this is confirmed, a pocket is created in the left upper quadrant and the device placed within this pocket.

The abdomen is reinflated and the stomach inspected to confirm adequate placement of the electrodes with no redundant lead length within the peritoneal cavity. The trocars and gas are removed, and the skin closed with intradermal, absorbable suture. The device is interrogated once again and is turned on with the following settings: voltage 5 V, pulse width 330 μs, rate 14 Hz, time-on 0.1 s, time-off 5 s. These are considered the "minimal" settings.

Postoperatively, patients are kept in the hospital 1 or 2 days. They are started on a gastroparesis diet and discharged when tolerating this diet, with all of their preoperative gastroparesis-related medications.

Follow-Up Patients are initially seen in the outpatient clinic within 2 weeks to insure that there are no adverse postoperative events. At that time, they are asked about symptom relief. They are seen every 2 to 4 weeks, and during these visits, symptom relief is assessed. A "response" was defined as the patient reporting that the symptoms related to gastroparesis have improved. If the patient still has significant symptoms, adjustments are made on the device to increase the electrical dosing. These adjustments are made in small increments either until satisfactory symptom relief is achieved or, maximally, electrical dosing is achieved. At that time, outcome endpoint was achieved. Patients were asked to complete the GSRS and SF-36. Symptomatic improvement was the only endpoint assessed. Follow-up gastric emptying scintigraphy was not done. For patients with satisfactory relief, follow-up was lengthened to every 3 to 6 months. For patients with no relief of symptoms, patients were offered to have the device turned off to confirm that there were no changes in symptoms with the device off. If there was no

effect on symptoms with either the device on or off, patients were offered to have the device removed.

Statistical Analysis All statistical analysis was done using the Stata Statistical computer program.[13] The GSRS and SF-36 data were tested for normality using the Wilk–Shapiro test and found not to follow a normal distribution. Therefore, these data are presented as medians with interquartile ranges and analyzed using the Mann–Whitney *U*-test. In addition, the SF-36 data are also presented as a "top-box" analysis.[14] As many of the responses of the SF-36 by patients leads to a score of 100 (the top-box), the frequency of the number of these top-box scores will also be presented. As these are nominal data, they will be analyzed using the chi-squared test. A p value of <0.05 was considered significant.

Results

Demographics Between August 2004 and April 2008, 42 patients underwent placement of the Enterra gastric neurostimulation. Twenty-nine patients (69%) were female, with a mean age of 41 (SD +14 years, range 19–71 years). Twenty-four patients (57%) were diabetic, 17 (24%) idiopathic, and one (2%) with postgastrectomy. This postgastrectomy patient underwent an antrectomy and vagotomy by another surgeon for diabetic gastroparesis and, therefore, qualified for the device because of his original cause of gastroparesis. Of the idiopathic patients, four (24%) had small bowel pseudo-obstruction with or without colonic inertia, and two (12%) others had associated diffuse autonomic nervous system dysfunction.

Operative Complications and Long-Term Adverse Events There were no immediate postoperative deaths. One patient developed a hematoma in the device pocket for an overall morbidity rate of 2%. In the long term, three patients (7%) had erosion of the device through the incision, requiring revision, but not removal of the device. Two deaths occurred: one because of a pulmonary embolism after a laparoscopic cholecystectomy 4 months after placement of the neurostimulator; another because of uncontrollable hemorrhage from profound coagulopathy during a pancreas transplant 17 months after device placement.

Follow-Up Visits and Frequency of Dosing Adjustments The median follow-up was 12 months (range: 1–42 months). Initially, patients were seen in the outpatient clinic every 2 to 4 weeks and adjustments made on the electrical dosing in incremental fashion. Thirty-five patients (83%) required increased electrical dosing because of unsatisfactory symptomatic response to the minimal settings.

Response Rate Overall, 31 of 42 patients (74%) had a response to gastric neurostimulation. The degree of symptomatic relief as reported by the patients varied from a noticeable improvement, but with persistent symptoms, to complete symptomatic relief. Of the six patients who required feeding tubes, five patients (all responders) were able to eat enough to have the feeding tubes removed. One patient (a nonresponder) continued to require enteral feedings.

Time to Response Of the patients who responded to gastric neurostimulation, the median time to response was 1.5 weeks (range: 0 to 32 weeks). This includes eight patients (26%) who had immediate symptomatic response. Of the patients who did not respond, the median time in determining that there would be no response was 15 months (range 4 to 22 months).

Response by Gastroparesis Type Of the 24 diabetic patients, 19 (79%) responded to gastric neurostimulation, while 12 (71%) of the idiopathic patients responded (p= NS). The postgastrectomy patient did not respond to neurostimulation. Of the idiopathic patients, five of the nonresponders had diffuse autonomic nervous system dysfunction (two patients) or pan-gastrointestinal motility dysfunction as manifested by colonic inertia, small bowel pseudo-obstruction, and biliary dyskinesia (two patients). One patient with idiopathic gastroparesis and small bowel pseudo-obstruction had a symptomatic response.

Response by Primary Symptom Of the 31 patients whose primary symptom complex was nausea and vomiting, 27 (87%) responded to gastric neurostimulation, while four (36%) patients whose primary symptom complex was pain and bloating responded (p=0.02). Of the 31 patients who responded to gastric neurostimulation, nine (28%) regularly used narcotic pain medications before placement of the device, compared to nine of the 11 (82%) of the nonresponders (p=0.004). However, of the responders, three (33%) were able to be weaned off narcotics.

Gastrointestinal Symptoms and Quality-of-Life Response The median time from placement of the device to maximal symptomatic improvement and, therefore, patients completing their follow-up GSRS and SF-36 instruments was 4 months (range 0–9 months). Table 1 shows the change in the median GSRS scores (with interquartile ranges) for the responders. There were statistically significant improvements in all three domains. The most dramatic improvement was with item #5—Nausea and vomiting, which decreased from the worst score, 3, to 1.

Table 2 shows the change in median SF-36 scores (with interquartile ranges) for the responders. The most important

Table 1 Preoperative and Postoperative GSRS Domain Scores (with Interquartile Range) in Patients who Responded to Gastric Neurostimulation

	Dyspeptic syndrome	Indigestion syndrome	Bowel dysfunction syndrome	Nausea and vomiting item
Preoperative	9 (7–11.5)	5 (2–7)	4.5 (3.5–7.5)	3 (2–3)
Postoperative	4 (2.5–6)	4 (0–5)	3 (0–6.5)	1 (0–1)
p value	0.02	0.02	0.05	0.01

change was in the health transition item, where the median score improved from 4 ("somewhat worse that 1 year ago") to 1.5 (between "much better than 1 year ago" and "somewhat better than 1 year ago"). Although there appeared to be improvement in all domains, the only domain that was statistically significant was the SF domain. Table 3 shows the top-box analysis. This shows an even more dramatic improvement in health transition where there were no patients in the top-box before placement of the gastric neurostimulator, while 54% were in the top-box after. Interestingly, although the change in median RP scores was not statistically significant, the change in top-box score was.

The nonresponders showed no change in scores. Figure 1 shows the overall change in GSRS scores for the entire cohort of patients including the responders and nonresponders.

Device Removal Of the 11 nonresponders, five have had the device removed. Two at the time of total gastrectomy, one at the time of feeding tube placement, one patient with diffuse autonomic nervous system dysfunction who would require magnetic resonance imaging exams for assessment and management of her disease, and one because of pain at the site.

Discussion

This study demonstrates that, in some patients who had otherwise intractable symptoms related to diabetic or idiopathic gastroparesis, gastric neurostimulation provided some measure of relief. Clearly, gastric neurostimulation is no panacea. About one quarter of the patients had no symptomatic improvement whatsoever. Other investigators have also reported response rates in the 50% to 90% range.[4,15–21] Preoperatively, identifying this group of non-responders is important.[22] It is also interesting to note that, in those patients who did respond, not only were symptoms of nausea and vomiting improved, but also other gastrointestinal symptoms associated with indigestion syndrome and bowel dysfunction syndrome also improved (Table 1) They also had a sense that their general health improved and, interestingly, the SF domain of the SF-36 also improved (Tables 2 and 3), implying that the benefit of symptom control extended beyond symptomatic improvement.

This study suggests a group of gastroparesis patients who may not benefit from gastric neurostimulation. Diabetes patients appear to have a better response to gastric neurostimulation than idiopathic patients, although this was not statistically significant. Maranki et al.[22] also found this to be the case in their series. However, idiopathic patients who have other associated nervous system or motility disorders, such as autonomic nervous system dysfunction or small pseudo-obstruction and colonic inertia, are particularly at risk for lack of response. These associated conditions have not been greatly explored in the extant literature. These patients can be identified because they frequently will have histories of esophageal motility problems, biliary dyskinesia, colonic inertia, and small bowel "obstruction." In addition to the type of gastroparesis, this study also demonstrated that primary symptom has some predictive value. Patients whose primary symptoms are nausea and vomiting respond better than patients whose primary symptoms are bloating and pain. Patients who are chronic users of narcotic pain medications will frequently continue to have symptoms despite maximal electrical stimulation. Maranki et al.[22] also reported this finding. Another group, where not much information exists, is the postgastrectomy/postvagotomy patients. It is unclear how well this group responds to neurostimulation. The one postgastrectomy patient in this series did not respond to therapy. On the other hand, McCallum et al.[23] report statistically significant improvement in patient with post-surgical gastroparesis with gastric neurostimulation. The

Table 2 Median Preoperative and Postoperative SF-36 Scores (with Interquartile Range) in Patients Who Responded to Gastric Neurostimulation

	Health transition	Physical functioning	Role physical	Role emotional	Bodily pain	Vitality	Mental health	Social functioning	General health
Preop	4 (4–5)	30 (10–65)	0 (0–50)	33 (0–100)	22 (12–41)	30 (20–40)	56 (48–68	25 (12.5–62.5	25 (25–47)
Postop	1.5 (1–3)	60 (30–90)	75 (0–100)	67 (0–100)	62 (31–72)	35 (20–60)	60 (56–76)	75 (50–87.5)	47 (22–62)
p value	0.01	NS	NS	NS	NS	NS	NS	0.03	NS

Table 3 Top-Box Analysis in Patients Who Responded to Gastric Neurostimulation

	Health transition (%)	Physical functioning (%)	Role physical (%)	Role emotional (%)	Bodily pain (%)	Vitality (%)	Mental health (%)	Social function (%)	General health (%)
Preop	0	6	12	47	6	0	0	12	0
Postop	54	9	45	45	0	0	0	18	0

causes for this variation in response are not fully elucidated, although a loss of the interstitial cells of Cajal within the gastric muscle has been implicated.[24] Nevertheless, it is clear that diabetic patients whose primary symptoms are nausea and vomiting appear to have the best response to the device.

Opinion as to the management of the device is not uniform. There are two basic approaches. One is the "set it and forget it approach." In this approach, it is assumed that it may take several weeks, and possibly up to a year, for patients to have a response to the minimal electrical dosing. In this approach, many patients will require feeding jejunostomies to maintain nutritional support, as well as continued antiemetic medical therapy. The other approach, which was practiced in this series, is frequent, incremental increases in electrical dosing until satisfactory symptom relief is achieved. Whether the increase in electrical dosing versus "tincture of time" is the cause of the improvement cannot be determined without a randomized trial. However, in the experience reported here, those patients who have had good symptomatic relief at higher doses whose electrical dosing was reduced (to prolong battery life) had a worsening of their symptoms. In addition, the time to respond in those patients who did not have immediate response was weeks rather than months. Therefore, it appears that patients may require different dosing levels that can be achieved with frequent follow-up and device adjustments.

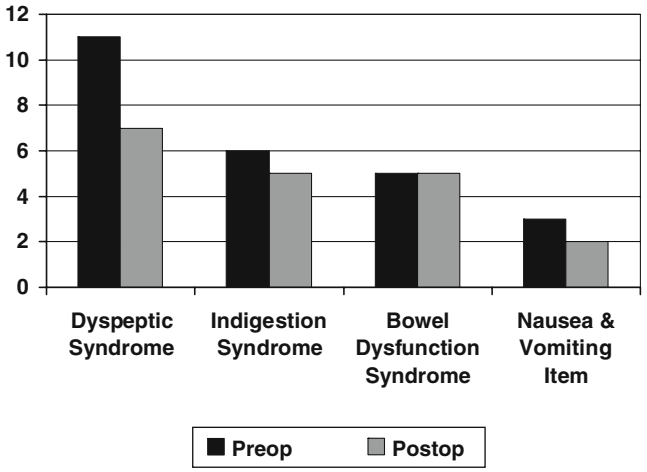

Figure 1 Comparison of preoperative and postoperative GSRS scores for the entire cohort of patients receiving gastric neurostimulation, both the responders and nonresponders.

There is a natural variability of the disease, and systemic illness can dampen the effect of gastric neurostimulation on gastroparesis-related symptoms. Data were previously presented that gastroparesis is a disease with a variable natural history.[25] Patients normally have their "good days and bad days." In addition, systemic illnesses affect the effectiveness of the device. Such issues as glucose control, infection, emotional and physical stress, thyroid function, adrenal function, menstrual cycles, conditional vomiting, and migraines all can lead to worsening symptoms of gastroparesis despite adequate function of the device. When patients return with recurrent symptoms after a period of good symptom relief, such problems need to be sought and addressed.

There is a halo effect of symptomatic improvement on other aspects of quality of life and gastrointestinal symptoms. What this study demonstrated is that gastrointestinal symptoms other than nausea and vomiting can be improved with gastric neurostimulation. The GSRS that measures in addition to dyspeptic syndrome (abdominal pain, heartburn, acid regurgitation, sucking sensations in the epigastrium, and nausea and vomiting) also measures indigestion syndrome (borborygmus, abdominal distention, eructation, and increase flatus) and bowel dysfunction syndrome (decreased passage of stools, increased passage of stools, loose stools, hard stools, urgent need for defecation, and feeling of incomplete evacuation) showed improvements in gastrointestinal function not generally considered related to gastroparesis. Similarly, although the largest effect of gastric neurostimulation was seen in the health transition item of the SF-36, the only domain which showed a statistically significant difference was in SF. Patients who suffer from gastroparesis are, in fact, very limited in their SF—they are unable to go out to restaurants, visit family and friends for meals, etc., because of their persistent nausea and vomiting. The control of these symptoms allows these patients to have more meaningful social interactions that were previously limited. The above two examples as point to why having validated, reliable quality-of-life instruments can identify areas of patient-centered outcomes that researchers may miss.

In conclusion, gastric neurostimulation for diabetic or idiopathic gastroparesis with the Enterra device leads to symptomatic and quality-of-life improvement in the majority of patients. Nausea and vomiting are the symptoms most

reliably controlled, while bloating and pain control have a much higher failure rate. This effect can extend beyond mere control of nausea and vomiting to include other gastrointestinal symptoms and quality of life. More research is needed in better patient selection and device management.

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Discussion

Quality of Life and Symptomatic Response to Gastric Neurostimulation for Gastroparesis

Richard C. Thirlby, M.D. (Seattle, WA): Dr. Velanovich, thank you for a concise objective review of the current status of gastric stimulation for patients with idiopathic and diabetic gastroparesis. Our results with over 150 of these procedures are nearly identical to yours.

I have several questions or comments.

First, our practice has been to begin the adjustment of the device after about six weeks if symptoms are not improving. You began adjustments as early two weeks. As a result you increased the settings in 83% of your patients. That number is much higher than in our experience. It would seem to me that you are likely increasing output and thereby decreasing battery life unnecessarily in many of your patients who would have improved on their own. Do you have any evidence that this early adjustment is a good idea and/or have you ever turned down the dosing after several months?

Second, your manuscript states that, “Adjustments are made in small increments either until satisfactory symptom relief is achieved or maximally electrical dosing is

achieved. At that time outcome end point was achieved." Patients frequently improve for weeks. Unless I am missing something your methodology is going to underestimate the benefit of the device. Did you ever increase the settings after your followup data collection? I think this study would be much cleaner and would have demonstrated more benefit if you had arbitrarily selected a 6 or 12-month followup date to collect your data.

Third, you reported that there are not statistically significant improvements in most of the domains of SF36. Given the fact that there was a dramatic doubling or tripling of the scores in several domains which were not significant, would you agree that your study is not significantly powered to make definitive conclusions about the effect of gastric stimulation on HRQL?

Fourth, you appropriately question the value of gastric stimulation in some patient cohorts, but you never really commit yourself. I would like you to give us your opinion about patients who are not appropriate. In my experience, patients with symptoms of small bowel or colonic disease do not do well. Would you now refuse to operate on patients with gastroparesis and documented colonic inertia or pseudo-obstruction? And what about post-viral or scleroderma patients?

Also, you mentioned narcotics in your manuscript. Our practice is to demand cessation of all narcotics prior to device placement.

And finally, a wise mentor once told me to regard single author publications with skepticism. I agree with all of your data and your conclusions; however, I would suggest getting the help of a resident or a gastroenterologist in dealing with these labor-intensive patients. Your life will improve and your data may become a little bit more objective.

Thank you.

Vic Velanovich, M.D. (Detroit, MI): Thank you, Dr. Thirlby. Let me get directly to your questions.

With respect to frequent adjustments of the device, this includes my early experience and so some of these adjustments may have been unnecessary. Although Dr. McCallum does not adjust the device very often, up to three-quarters of his patients require feeding tubes, whereas only 3 of my 42 patients did. This difference could be related to aggressive adjustments of the device.

With regard to the second question about whether to do the followup quality of life scoring at some arbitrary time point, that was the plan initially, was to pick a set time point. However, gastroparesis is a waxing and waning disease and in fact if you pick an arbitrary time point you could get, in my opinion, you could record a score which is lower than what the patient usually is if the patient comes in on a "bad day," so I think the opposite is true, that you underestimate the quality of life effect if you set a specific time point.

With regard to the question about statistical power, you are right. I think with more patients the difference in quality of life scores would reach statistical significant.

As far as patient selection goes, I now think that diabetic patients with nausea and vomiting do the best. Idiopathic patients with pain and bloating do the worst, particularly if they are on narcotics. I have to say I am very impressed that you can demand that patients discontinue narcotics, you must have more influence over your patients than I, because I cannot get them to stop taking narcotics. If you can, please tell me how.

Lastly, with regard to a gastroenterologist, I would love to have a gastroenterologist involved. Maybe you could send me one of yours so I could use him, because it is hard for me to engage them in followup of these difficult patients.

Thank you.

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Loss of Heterozygosity Predicts Poor Survival After Resection of Pancreatic Adenocarcinoma

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Abstract
Background American Joint Committee on Cancer (AJCC) staging for pancreatic adenocarcinoma is a validated predictor of prognosis but insufficiently discriminates postresection survival. We hypothesized that genetic analysis of resected cancers would correlate with tumor biology and postoperative survival.
Methods Resected pancreatic ductal and ampullary adenocarcinomas (n=50) were analyzed for loss of heterozygosity (LOH) at 15 markers including 5q(*APC*), 6q(*TBSP2*), 9p(*p16*), 10q(*PTEN*), 12q(*MDM2*), 17p(*TP53*), and 18q(*DCC/SMAD4*). *KRAS* exon 1 mutations were detected by sequencing. The primary endpoint of this interim data analysis was survival at 18 month median follow-up.
Results Negative margins were achieved in 43 (86%) cases. AJCC stage was: Ia/b (3), IIa (16), IIb (31). *KRAS* mutations were detected in 31 cases (62%) and LOH in 26 (52%) with mean fractional allelic loss score 23±16%. Median survival was significantly shorter with LOH (15.2 months versus not reached; p=0.021) and *KRAS* mutations (19.6 months versus not reached; p=0.038). Combining *KRAS* mutation with LOH was a powerful negative predictor in Cox regression (HR=10.6, p=0.006). Stage, nodal and margin status were not predictive of survival.
Conclusion LOH and *KRAS* mutations indicate aggressive tumor biology and correlate strongly with survival in resected pancreatic ductal and ampullary carcinomas. Genetic analysis may improve risk stratification in future clinical trials.

Keywords *KRAS* · Loss of heterozygosity · Pancreatic adenocarcinoma · Surgical resection

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Introduction

Surgical resection is the only therapy for pancreatic cancer offering some potential for long-term survival[1,2]. Pancreatic resection can be performed safely with mortality less than 1% overall[3,4] and 4% in the elderly[5]. Unfortunately, early disease progression[6,7] reduces actuarial 5-year survival to 18–23%[1,4]. Actual 5-year survival remains only 12%[8]. Extended lymphadenectomy[9] and regional pancreatectomy[10,11] provide no survival benefit despite potential clearance of microscopic tumor in regional lymph nodes and perivascular soft tissue.

These observations suggest that the future of pancreatic cancer treatment depends on improvements in systemic therapy. Reliable algorithms to stratify the risk of postoperative recurrence are essential for designing clinical trials as well as personalizing treatment decisions. At the present time, the marked heterogeneity of outcomes following surgical resection makes this objective difficult to achieve. Traditional AJCC staging[12] and novel prognostic scoring systems like the Memorial Sloan-Kettering Cancer Center (MSKCC) nomogram[13] and lymph node ratio[14] have been validated[15,16], but improvements in prognostic accuracy for individual patients are still needed.

We hypothesized that tumor genetics reflect the underlying biology of pancreatic and ampullary adenocarcinomas and may improve the prediction of postresection survival. Genetic testing has a major impact on the prognostic assessment of breast[17,18] and non-small cell lung cancer[19] and contributes to the rational selection of chemotherapy agents for breast[20,21] and colon cancer[22]. Previous genetic studies have identified KRAS mutations as an early event in carcinogenesis among patients with chronic pancreatitis as well as pancreatic intraductal neoplasia[23]. Published rates of *KRAS* mutation approach 90% among patients with advanced pancreatic carcinoma[24]. Consistent with the two-hit hypothesis[25], pancreatic cancer features multiple additional genetic abnormalities. Among them are known tumor suppressor genes like the cyclin-dependent kinases (*CDKN2A/p16*), *TP53*, and the *DCC/SMAD4* complex, as well as mutations in *Her-2/neu* and *BRCA2*[26,27] and modulators of angiogenesis like thrombospondin[28]. Interactions between these genetic factors may determine the phenotype of the resulting adenocarcinoma and adversely impact patient survival independent of descriptive findings such as tumor size and nodal status or surgical outcomes such as resection margin status.

The primary objective of this pilot study was to determine whether tumor loss of heterozygosity influences survival after potentially curative pancreatic resection for pancreatic and ampullary adenocarcinoma. We performed retrospective analysis of 50 pancreatic resections performed in 2006 for pancreatic and ampullary adenocarcinomas to determine the relationship between allelic losses at common tumor suppressor gene loci, the existence of concurrent *KRAS* mutations, and postoperative survival. We analyzed both pancreatic and ampullary carcinomas due to reported similarities in their genetic profiles[29].

Methods

Patient Population and Data Collection

All study procedures were approved by the University of Pittsburgh Institutional Review Board. Medical records were reviewed for fifty patients that underwent surgical resection for pancreatic ductal adenocarcinoma or ampullary adenocarcinoma during 2006. No patients had any evidence of metastatic disease at the time of surgery. Pathologic examination was performed according to a standard protocol to examine six surgical resection margins[30].

The primary endpoint of the study was overall survival measured from the date of surgery. Demographic variables, imaging results, operative findings, and tumor characteristics were recorded including detailed TNM data, histologic grade, and AJCC stage. Details of chemoradiotherapy were recorded. Patients with suspected locally-advanced pancreatic cancer were offered neoadjuvant therapy. Adjuvant therapy was offered to all resected patients, and gemcitabine was the drug of choice. Disease-specific survival probabilities at 12 and 24 months were calculated using Brennan's prognostic nomogram based on the following variables: age, gender, portal vein resection, splenectomy, status of the surgical margin, tumor grade, location of tumor, lymph node status, pain, T stage, weight loss, and pathologic axis[13].

Molecular Analysis

The pathologic diagnosis was confirmed in each case (AMK). Clusters of malignant cells were identified on hematoxylin-eosin stained slides, and manual microdissection of 4 μm unstained histologic sections was performed using a high-resolution stereomicroscope. Normal pancreatic or ampullary tissue served as an internal control to determine whether allelic imbalances were informative.

DNA was isolated from each target using the DNeasy tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. A panel of 15 polymorphic microsatellite markers (Table 1) was used to identify loss of heterozygosity (LOH) for tumor suppressor genes located at the following chromosomal loci: 5q (*APC*), 6q (*THBS2*), 9p (*CDKN2A/p16*), 10q (*PTEN*), 12q (*MDM2*), 17p (*TP53*), and 18q (*DCC/SMAD4*). PCR amplification was performed using fluorescently-labeled primers. The

Table 1 Microsatellite Markers, Their Corresponding Chromosomal Loci, and Potential Deleted Tumor Suppressor Genes of Interest

Microsatellite marker	Locus	Gene	Significance
D5S.1384	5q23.3	Adenoma polyposis coli; APC	Activation of c-myc proto-oncogene and cell proliferation; accumulation of β-catenin[55]
D5S.659	5q23.2		
D6S.297	6q27	Thrombospondin; THBS2	Tumor cell growth and angiogenesis[56]
D9S.251	9p21.3	Cyclin-dependent kinase; CDKN2A/p16	Release of cell cycle arrest mediated by cyclin D at the G1-S checkpoint[57]
D9S.1679	9p22.2		
D9S.1748	9p22.2		
D10S.520	10q23.31	PTEN	Activation of AKT; inhibition of apoptosis and increased cell proliferation[47]
D10S.1171	10q23.31		
D12S.375	12q21.1	Mouse double minute 2; MDM2	Deactivation of p53[58]; upregulation of angiogenesis via ERK1/2[59]
D12S.1036	12q21.1		
D17S.516	17p13.1	TP53	Release of cell cycle arrest and apoptosis in response to DNA damage; accumulation of genetic aberrations[60]
D17S.768	17p13.1		
D17S.1844	17p13.1		
D18S.364	18q21.2	DCC/SMAD4	Release of growth inhibition and increased cell proliferation mediated by TGF-β receptor[28]
D18S.1119	18q21.2		

products of amplification were detected by capillary gel electrophoresis (ABI3730; Applied Biosystems, Foster City, CA). The relative fluorescence values (peak heights) were obtained for individual alleles, and the ratio of peaks was calculated using GeneMapper software v.3.2 (Applied Biosystems, Foster City, CA). Detection of mutations in the KRAS gene (exon 1, codons 12 and 13) was performed by direct nucleotide sequencing using the BigDye Terminator Kit (ABI3130; Applied Biosystems, Foster City, CA). Both sense and antisense sequencing was performed to assure the detection of heterozygous mutation in the *KRAS* gene. Sequencing data were analyzed for the presence of mutations using Mutation Surveyor v.3.01 (SoftGenetics) software.

The LOH ratio was established by dividing the peak ratio of the normal sample to the peak ratio of the tumor sample. Individual microsatellites were considered uninformative if analysis of normal tissue demonstrated a single peak representing either hemizygous loss of one marker (true allelic loss) or the inheritance of identical microsatellites from each parents (false-positive allelic loss). For informative alleles, high-level allelic imbalance (ratios <0.5 or >2) was interpreted to indicate LOH. These criteria required at least 50% of cells in the dissected target to display a given mutation, stringent conditions which prevented misclassification of allelic losses[31]. The fractional allelic loss (FAL) rate for a given sample was defined as the number of loci with LOH divided by the total number of informative microsatellite loci.

Statistical Analysis

The relationship between genetic alterations and clinical outcome was studied. The cohort was dichotomized into an LOH positive group (at least one LOH documented at any locus) and an LOH negative group (no LOH at any informative loci). Data were analyzed using Statistical Package for Social Sciences, SPSS 15 (SPSS, Chicago, IL) and STATA 10 (StataCorp, College Station, TX). Chi square or Fisher test were used to compare categorical variables. The Kaplan-Meier method was used to determine estimates of survival and differences were determined using the log-rank test. Cox proportional hazard model was used for multivariable assessment of survival. Statistical significance was assumed at 0.05. Results are expressed as proportions and means ± standard deviations.

Results

Outcomes from fifty consecutive patients undergoing pancreatic resection for pancreatic ductal (n=42) or ampullary (n=8) adenocarcinoma during 2006 were analyzed. The age and gender distribution of the study population reflected the demographics of pancreatic cancer (mean age 68±12 years; 26 men and 24 women). 37 patients underwent pancreaticoduodenectomy (25 pylorus-preserving; 12 standard), with five distal pancreatectomies, four total pancreatectomies, and four undesignated pancre-

atic resections. Ten patients (20%) underwent resection and reconstruction of the portal or superior mesenteric veins reflecting aggressive surgical management of suspected venous invasion. Histologic confirmation of venous invasion was observed in 6/10 (60%). Negative resection margins were achieved in 43 patients (R0 86%), with microscopically-positive margins in 7 patients (R1 14%). There were no postoperative deaths.

Forty patients received multimodality treatment (80%). Four patients (8%) received preoperative chemoradiation prior to surgical resection for suspected locally-advanced disease, none of which had a subsequent positive surgical margin. 26 patients received adjuvant chemotherapy (26/50; 52%), and eleven received adjuvant chemoradiotherapy (11/50; 22%). All patients with a positive resection margin received adjuvant treatment except for 78 year old man who did not recover sufficiently within eight weeks following total pancreatectomy.

The results of detailed pathological examination are shown in Table 2. Tumors averaged 3.1±1.4 cm in diameter, and 20% (10/50) were larger than 4 cm. The majority (82%) of lesions invaded the peripancreatic soft tissue consistent with the following T stages: T3 (41/50, 82%), T2 (6/50, 12%), and T1 (3/50, 6%). 40% of lesions (20/50) were poorly-differentiated, and 86% (43/50) demonstrated either lymphovascular or perineural invasion. 62% of patients (31/50) had positive lymph nodes. Twelve patients (24%) had advanced nodal disease (lymph node ratio ≥ 0.4), while the average lymph node ratio for the entire cohort was 0.23±0.25. The distribution by AJCC stage was: Ia (1/50; 2%), Ib (2/50; 4%) IIa (16/50; 32%), and IIb (31/50; 62%).

Table 2 Pathological Characteristics of Resected Tumors

Parameter	Entire cohort	LOH(+)	p vs. LOH(−)
Number	50	26	
Histologic origin			
Pancreatic ductal	42 (84.0%)	21 (50.0%)	0.704
Ampullary	8 (16%)	5 (62.5%)	
Resection margin			
Positive	7 (14%)	4 (57.1%)	1.000
Negative	43 (86%)	22 (51.2%)	
Histologic grade			
Grade I	2 (4%)	1 (50.0%)	0.942
Grade II	28 (56.0%)	14 (50.0%)	
Grade III	20 (40.0%)	11 (55.0%)	
Nodal status			
Positive	31 (62.0%)	16 (51.6%)	0.944
Negative	19 (38.0%)	10 (52.6%)	
LVI or PNI			
Present	43 (86%)	22 (51.2%)	1.000
Absent	7 (14%)	4 (57.1%)	
Stage			
Ia/b	3 (6%)	2 (66.7%)	0.871
IIa/b	47 (94%)	24 (51.1%)	
KRAS mutation			
Present	31 (62%)	17 (54.8%)	0.608
Absent	19 (38%)	9 (47.4%)	

Data in the first column reflects the entire cohort (n=50), while the second column presents the LOH positive group (n=26). The last column shows the significance value for statistical comparisons between LOH-positive and LOH-negative tumors

LVI Lymphovascular invasion, *PNI* perineural invasion

Genetic aberrations were defined as the presence of a *KRAS* mutation or at least one allelic imbalance and were identified in 80% (40/50) of resected tumors. Loss of heterozygosity was detected in 26 patients (52%), while *KRAS* exon 1 mutations were found in 31 patients (62%). Seventeen patients (34%) exhibited both a *KRAS* mutation and at least one allelic loss. Among tumors with LOH, the average fractional allelic loss rate was 23.6±16.1% (median 18.2%). The incidence of specific allelic losses in order of frequency was: 18q (*SMAD4*, n=11), 12q (*MDM2*, n=10), 17p (*TP53*, n=9) and 9p (*p16*, n=9), 5q (*APC*, n=6), 10q (*PTEN*, n=4), and 6q (*THSP2*, n=2). No statistically-significant differences were observed in the following variables between LOH-negative and LOH-positive tumors: patient age (LOH-negative 69.6±11.8 years versus LOH-positive 66±12.5 years, p=0.348), tumor size (2.9±1.5 cm versus 3.2±1.3 cm, p=0.400), histologic grade, total number of recovered lymph nodes (11.8±5.8 versus 9.4±4.8, p=0.119), number of positive lymph nodes (2.0±2.1 versus 1.8±1.9, p=0.636), lymph node ratio (0.22±0.27 versus 0.23±0.24, p=0.865), resection margin status, or the frequency of *KRAS* mutations (58.3% versus 65.4, p=0.608).

Survival outcomes for a total of 20 deaths were determined at a median follow-up of 18 months and stratified by the presence of genetic aberrations. The frequency of multimodality treatment was independent of LOH status (LOH- 83% vs. LOH + 77%) and the presence of *KRAS* mutations ($KRAS^{wt}$ 74% vs. $KRAS^{mut}$ 84%). There were 6 deaths in the LOH-negative group and 14 deaths in the LOH-positive group during follow-up. Univariate analysis demonstrated significant differences in overall survival between LOH-positive (median 15.2 months) and LOH-negative patients (median survival not reached; p=0.021, Fig. 1). Similarly, the presence of a *KRAS* mutation also reduced median survival. Four deaths were observed in patients with tumors expressing wild-type *KRAS* (median survival not reached), while 16 deaths occurred in the presence of mutant *KRAS* (median survival 19.6 months; p=0.038, Fig. 1). Striking survival differences were observed among the 17 patients with both a *KRAS* mutation and at least one allelic loss (Fig. 2). Although 13/17 affected patients (76.5%) received multimodality treatment, median overall survival for this group was only 12.3 months (95% CI: 10.4–14.3 months) as compared to the *KRAS* only or

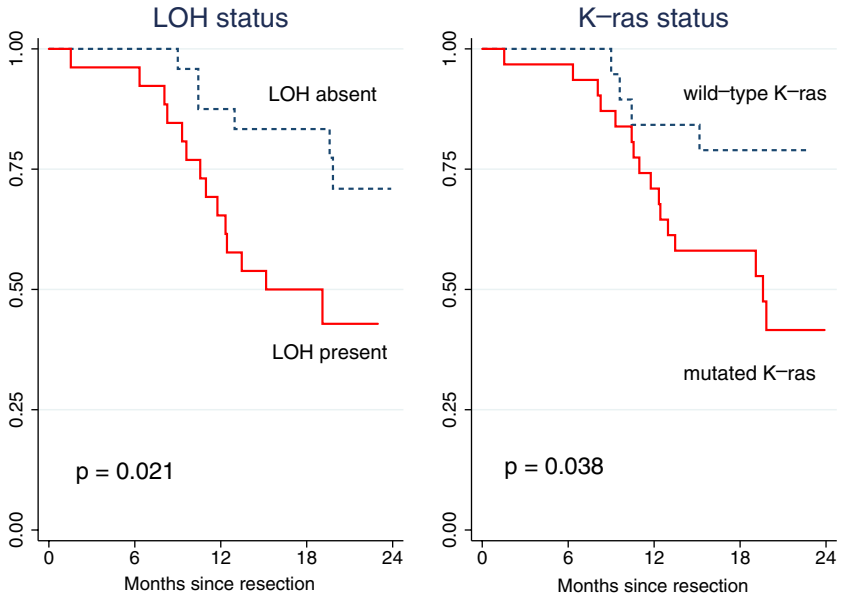

Fig. 1 Overall survival of patients with resected pancreatic ductal or ampullary adenocarcinoma cancer stratified by LOH status (*left panel*) and *KRAS* mutations (*right panel*).

LOH only groups (Fig. 2; p<0.001). By comparison with tumor genetic analysis, none of the traditional pathological factors were predictive of survival at 18 month median follow-up: patient age (p=0.536), tumor grade (p=0.633), tumor size (p=0.511) and T stage (p=0.730), resection margin status (p=0.536), histological origin of pancreatic ductal versus ampullary adenocarcinoma (p=0.403), nodal status (p=0.558), and lymphovascular or perineural invasion (p=0.490). Furthermore, no significant survival differences were observed between the mean MSKCC nomogram scores of LOH-negative and LOH-positive tumors (241±31 versus 238±21 points, p=0.731) or of tumors expressing wild-type *KRAS* and mutated *KRAS* (247±41 versus 240±22 points, p=0.635).

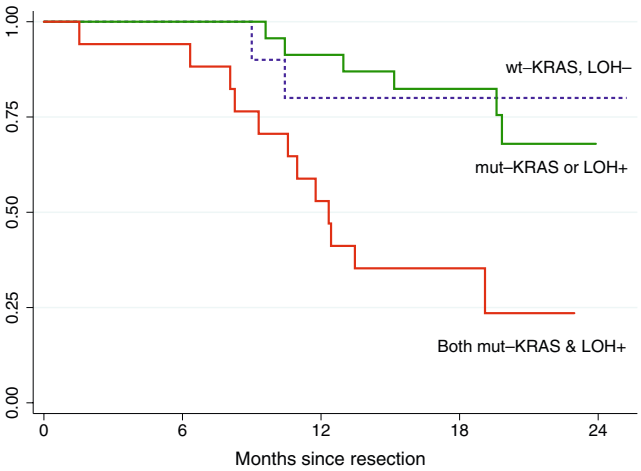

Fig. 2 Overall survival stratified by the presence of tumor genetic aberrations. Patients with either *KRAS* mutations or allelic imbalance had equivalent survival (median not reached). Patients exhibiting both *KRAS* and LOH (n=17) had significantly shorter overall survival (median 12.3 months, log rank test, p<0.001) after pancreatic resection.

We next developed Cox proportional hazard regression models using genetic variables as well as traditional parameters based on pathology (Table 3). The baseline model evaluated LOH status as a predictor of survival and demonstrated a hazard ratio of 2.9 (p=0.028) for LOH positive tumors. The second model evaluated the fractional allelic loss score (FA) as a predictor of survival, which is a continuous variable describing the percent of alleles lost at informative loci. The FAL score provides mathematically richer information as compared to the dichotomized variable of LOH status (present versus absent) and indicated a hazard ratio of 1.022 per percent increase in FAL score that was only borderline significant (p=0.062). The third model included both LOH status and the presence of a *KRAS* mutation, variables which were significant in univariable testing. The presence of LOH and mutated *KRAS* was predictive of survival with hazard ratios of 3.2 and 3.3.

The final Cox proportional hazards model evaluated the effect of genetic aberrations on survival in addition to traditional outcome parameters such as age, pancreatic ductal or ampullary origin, tumor size and grade, margin and nodal status, as well as the provision of multimodality therapy (Table 4). After adjustment for all variables included in this model, only LOH status and *KRAS* mutation were predictive of survival after resection of pancreatic and ampullary adenocarcinoma. The hazard ratio for survival in tumors with *KRAS* mutation and allelic imbalance was 10.6 (p=0.006), demonstrating a powerful correlation between tumor genetic aberrations and cancer-related death (Fig. 2).

Table 3 Baseline Cox Regression Models for Survival

	Sig	HR	95% CI for HR	
			Lower	Upper
Base model 1				
LOH status (referent: LOH-)	0.028	2.948	1.125	7.725
Base model 2				
LOH score (referent: LOH 0%)	0.062	1.022	0.999	1.045
Base model 3				
LOH status (referent: LOH-)	0.020	3.179	1.204	8.392
KRAS mutation (referent: wild type)	0.035	3.279	1.086	9.900

Model 1 uses a single dichotomized predictor (LOH- versus LOH+). Model 2 uses fractional allelic loss score expressed in percent. Model 3 uses a bivariable set of predictors based on predefined selection criteria (p value 0.2 or better in univariable analysis). A hazard ratio of more than 1 indicates an increased risk of death

Table 4 Combined Multivariable Cox Regression Model for Survival After Pancreatic Resection Adjusted for Known Predictors. The Risk of Death is Significantly Elevated Among Patients with LOH and Mutated *KRAS* (HR=10.6)

Variable	Sig.	HR	95.0% CI for HR	
			Lower	Upper
Age	0.218	1.036	0.979	1.097
Origin (referent: ductal)	0.969	1.034	0.196	5.445
Tumor size (cm)	0.887	0.978	0.717	1.333
Grade (I/II referent)	0.959	1.028	0.358	2.947
Margin status	0.525	1.696	0.333	8.644
Nodal status	0.378	1.612	0.557	4.666
Multimodality therapy	0.723	0.798	0.229	2.780
KRAS or LOH	0.526	1.740	0.314	9.652
Both KRAS and LOH	0.006	10.581	1.987	56.357

When specific allelic losses were analyzed in a Cox multivariable regression model, the strongest predictors of mortality were LOH at 6q (*THBS2*; HR=39.6, p=0.01), followed by 9p (*CDKN2A/p16*; HR=7.1, p=0.011) and 5q (*APC*; HR=6.0, p=0.012). *KRAS* mutations also had a strong effect (HR=5.1, p=0.013). On the other hand, loss of heterozygosity at 10q (*PTEN*; HR=0.014, p=0.025) had a protective effect. Associations between separate LOH events may have an additive effect on survival which is different from the effect of a single allelic loss[32]. We therefore performed multiple crosstabulations to identify concordant losses between two chromosomal loci (Table 5). Five associations were identified at frequencies significantly higher than predicted by random chance. The most frequent combined allelic losses were 18q with 17p and 18q with 12q.

Discussion

Survival after resection of pancreatic ductal or ampullary adenocarcinoma correlated with *KRAS* mutation and allelic imbalances. The study cohort was elderly with an equal gender distribution reflecting the typical demographics of pancreatic and ampullary carcinoma. Resected tumors exhibited a high rate of peripancreatic soft tissue invasion, poor differentiation, and nodal metastasis. The majority of patients underwent margin negative surgical resection (86%) and therefore had no measurable disease at the conclusion of treatment. 80% of patients received multimodality treatment in accordance with recent clinical trials[33,34]. The median fractional allelic loss score (18%) was identical to previous reports[35]. At 18 month median follow-up, survival outcomes were strongly correlated with the presence of LOH and *KRAS* mutations. The addition of any allelic imbalance to a *KRAS* mutation raised the hazard ratio for postoperative survival from 3.2 to 10.6. Traditional pathologic measures of outcome, including AJCC stage and margin status, were not significant at the time of this interim data analysis.

Genetic profiling has led to major advances in the understanding of cancer biology. Pancreatic adenocarcinoma exhibits multiple genetic events, including mutations of *Her-2/neu, KRAS, p16, TP53, DCC/SMAD4*, and *BRCA2*[24,26,27,36]. The impact of these genetic events on the prognosis and therapeutic options of patients with pancreatic cancer has not been established. Despite detailed pathological evaluation of resected tumors and the development of prognostic scoring systems to predict survival, the majority of clinical trials for pancreatic cancer remain 'negative' studies. This observation may be attributed to sub optimal stratification of recurrence risk among research subjects. Because a substantial improvement in survival cannot be expected from further advances in surgical therapy[9,11], the development of highly effective systemic agents should be the primary objective of future research. It is therefore exceedingly important to establish, validate, and utilize effective risk stratification models to conduct informative trials of systemic therapy.

Table 5 Concordant Loss of Heterozygosity Between Loci

Loci	Genes	No. of Concordant Pairs		
		LOH at both loci	No LOH	p value
6q and 17p	THBS2/TP53	2	41	0.029
9p and 10q	P16/PTEN	3	40	0.016
10q and 17p	PTEN/TP53	3	40	0.016
12q and 18q	MDM2/SMAD4	5	34	0.030
17p and 18q	TP53/SMAD4	5	35	0.017

The LOH technique has the ability to detect highly polymorphic changes in the tumor genome. Allelic loss is an important mechanism for gene inactivation by eliminating functional copies of a tumor suppressor gene. However, the relationship between loss of heterozygosity and loss of gene function is not direct. LOH indicates the deletion of a chromosomal locus adjacent to a gene of interest. Previous studies have used loss of heterozygosity analysis[35,37–39], gene knock-out models[40], and microarray technologies[41,42] to characterize the genetics of pancreatic cancer. The observed alterations are complex, and substantial heterogeneity has been detected within different regions of the same tumor[39], as well as between affected individuals[37].

Prior reports indicate that allelic losses are most common at the 9p, 17p, and 18q loci in patients with pancreatic carcinoma[35,37,38,43]. We identified LOH at these same loci in the current study in addition to 12q. Among Japanese patients with pancreatic cancer, poor prognosis has been associated with deletions at 12q, 17p, and 18q[35]. Such differences in chromosomal loci affected by LOH are a function of the study population as well as differences in tumor histologic grade. Loss of heterozygosity at 18q is associated with a malignant phenotype and correlates with poor prognosis in the current study as well as in prior reports[35,38,43]. Chromosome 18q carries several known tumor suppressor genes, including *DCC, SMAD2*, and *DPC4/SMAD4*. Inserting chromosome 18 into pancreatic carcinoma cells with a deletion of 18q suppresses metastatic potential and confers a dormant phenotype on rescued cells[44]. Loss of heterozygosity on chromosome 17p was evaluated by three markers for the *TP53* gene. *TP53* is a regulator of the cell cycle and a gatekeeper of apoptosis in response to genetic damage. Loss of *TP53* function immortalizes affected cells and is a key indicator of malignancy. LOH at 17p was identified in 18% of cases in the current study, a frequency much lower than 60% - 80% reported by Iacobuzio[37,43] but similar to the 23% rate identified with comparative genomic hybridization[36]. *TP53* associates with the *murine double minute* gene (*MDM2*, located at 12q13–14), a regulator of the *TP53* tumor suppressor gene product[32]. Loss of heterozygosity at 12q was identified in 20% of specimens in our study and may indicate additional defects in the cell cycle checkpoints downstream from *TP53*.

In the current study, loss of heterozygosity at 6q, 9p, and 5q carried the worst prognosis. These loci correspond to deletions of the *thrombospondin 2 (THBS2)*, *p16*, and the *APC* tumor suppressor genes. LOH at 6q has been reported previously[35,43]. Loss of heterozygosity at the *thrombospondin-2* locus on chromosome 6q was the strongest predictor of mortality but affected only two patients. Thrombospondin is a family of extracellular matrix glycoproteins consistent with their classification as landscaper genes[45]. *THBS2* modulates tumor growth through changes in the stromal microenvironment and is a potent inhibitor of angiogenesis[46]. Cyclin-dependent kinase *2/p16* is mapped to 9p21, a region known for frequent rearrangements in pancreatic cancer and certain other malignancies. It exerts negative control on cellular proliferation by inhibiting cyclin-dependent kinases and loss of its function leads to unopposed cellular growth[47].

KRAS and *PTEN* (10q) are strategic mediators of cellular development and transformation that are implicated in carcinogenesis[38,48,49]. We acknowledge the rather low prevalence (62%) of *KRAS* mutations in the current series. The presence of a *KRAS* mutation has been observed in the majority of pancreatic cancers[24]. The frequency of KRAS mutations is variable[26,27] and may range between 56%[38] and 69%[50]. KRAS mutations are an early event in carcinogenesis and are observed in smokers[51] as well as in patients with chronic pancreatitis. The protein products (p21, G-protein superfamily) of mutated *ras* genes are insensitive to negative inhibition and cause unopposed cellular growth as the result of Raf and Map kinase activation[49]. *PTEN* is a regulator of AKT activity and cellular proliferation encoded on chromosome 10q[52]. We observed an unanticipated protective effect of allelic imbalance at 10q23 region in the current study, which remains unexplained.

The current study has several limitations. A mechanistic evaluation of previously unreported concordant allelic imbalances at 9p/10q, 10q/17p, and 17p/18q is beyond the scope of the current study. The number of patients is relatively low and the median follow-up is short. This fact reduces statistical power and requires that dichotomous variables like the presence or absence of LOH be analyzed rather than the effects of allelic imbalances at individual loci. Finally, our decision to include both ampullary and ductal pancreatic cancer can be criticized, although the genetic fingerprints of these two adenocarcinomas are known to be very similar[29]. Our approach allowed the surgical outcomes of two histological variants of adenocarcinoma to be evaluated following identical surgical treatments.

We conclude that genetic aberrations increase the hazard ratio for postoperative survival in resected pancreatic and ampullary cancers. The combination of *KRAS* mutations and LOH was highly predictive of survival in both univariate testing as well as an adjusted Cox model. We did not observe any predictive value of other recognized pathologic variables at this interim data analysis, such as resection margin status[4,53], nodal disease[12,54], lymph node ratio[13,14,54] or nomogram score[13,16]. Longer follow-up and expansion of this cohort are underway to reproduce and validate the role of these genetic markers. We believe the future of diagnosis and stratification of recurrence risk

requires molecular analysis of tumor tissue. These methods may permit the rational selection of candidates for surgical resection and enable patient-specific adjuvant treatments to be developed.

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Discussion

Mark P. Callery, M.D. (Boston, MA): Dr. Franko, my congratulations to you and Dr. Jim Moser and your Pittsburgh colleagues on this excellent plenary paper. Custom tumor genotypic profiling will be, for sure, one wave of the future as we migrate to custom tumor therapies based on actual tumor biology.

My two questions are, and you actually stressed the first one a bit with your combined defects, how does your data

support the concept that tumor biology is worse when multiple cumulative genetic defects emerge? And my second question: Were you able to reconcile and segregate using your microdissection technique relative genetic defects between ductal cells and surrounding stromal cells? This could be a novel and worthwhile tactic for your research.

Congratulations.

Jan Franko, M.D., Ph.D. (Pittsburgh, PA): Thank you, Dr. Callery, for excellent questions. I will answer the second question first. I think it is very important to deal with the stroma. We did not do that in this study. I think it is a great idea for our future plans. And it is especially true when we look at the recent presentation on Sunday from the Pancreas Club where one of the groups presented the epithelial-to-mesenchymal transition results, and if that would be true, actually the whole issue regarding analysis of surgical margin comes into question. If one doesn't see malignant ductal cells at the margin, we call this margin histologically negative, when in fact there may be mesenchymal-like cells which represent cancer. So I think LOH analysis or any genetic technique to be used for the stroma is important, and I think it is something that we will get into, and hopefully we will be able to report soon.

If I am correct, the first question was related to differences between –

Dr. Callery: I wrote my question before I heard your updated slide. You answered it. The accumulated combined *KRAS*-LOH defects that support a multi-hit theory.

O. Joe Hines, M.D. (Los Angeles, CA): I like the concept, it is a great concept, and obviously it is the direction we are going to be going, but I want to ask you a specific question as to how you did the study. It seems that ampullary and pancreatic cancers were pooled in your analysis. I think that these are really two very different diseases. I know for many, many years pancreatic surgeons have reported their clinical data pooled and called this periampullary disease. Have you looked to separate the two groups, and what do you think about this issue of pooling ampullary and pancreatic together?

Dr. Franko: I think it is right to the point. Thank you for that question. Everything is relative. Many say that ampullary cancer has a substantially better prognosis, and it is mostly true because most patients are caught in early stage of disease. But when you go to stage by stage, even in a plenary session a couple of speeches before me, it was demonstrated they have pretty poor survival. I am not saying they are exactly the same. But if you go stage by stage, the survival is not that different.

At the time when I constructed the study, it came to the question of power to collect enough data, enough patients; I think only seven patients, about 14%, represent ampullary origin. If I do a sensitivity and subgroup analysis with the current follow-up as of today, all what I have said here holds true for pancreatic ductal carcinoma. It doesn't make too much sense to examine seven patients with ampullary carcinoma separatelly. In the future we will collect over 180 specimens, which is our current plan. We will separate pancreatic ductal and ampullary histologies.

I allowed to pool those together, because there is a nice review from Dr. Moore from I think 2004 where he described the current evidence and suggested that the genetic abnormalities in ampullary and pancreatic ductal actually are very similar. So that was the mechanistic reason why I pooled them together, and also I needed it for power of the study.

Andreas C. Hoffmann, M.D. (Los Angeles, CA): How did you do the PCR analysis? You said that you did PCR. I myself tried the k-ras as well. Did you use a probe method, such that you changed the probe in the design to detect the mutation, and did you correlate your PCR analysis with gene sequencing data?

Dr. Franko: The *KRAS* was done by gene sequencing. The LOH analysis is not necessarily done by PCR. It is amplified by PCR. But what we actually measure is the fluorescence of the markers which are tagged, and you compare normal tissue versus the malignant epithelium. So PRC is used just to get more signal, but it is not true PCR for LOH analysis, as opposed to *KRAS*. That is done through sequencing.

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High Expression of Heparanase is Significantly Associated with Dedifferentiation and Lymph Node Metastasis in Patients with Pancreatic Ductal Adenocarcinomas and Correlated to PDGFA and Via HIF1a to HB-EGF and bFGF

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Abstract
Background Pancreatic cancer still has one of the worst prognoses of all cancers with a 5-year survival rate of 5%, making it necessary to find markers or gene sets that would further classify patients into different risk categories and thus allow more individually adapted multimodality treatment regimens. Especially heparanase (HPSE) has recently been discussed as a key factor in pancreatic cancer.
Materials and Methods Paraffin-embedded tissue samples were obtained from 41 patients with pancreatic adenocarcinoma who were scheduled for primary surgical resection. Direct quantitative real-time reverse transcriptase polymerase chain reaction (TaqMan™) assays were performed in triplicates to determine HPSE, hypoxia inducible factor-1 alpha (HIF1a), platelet-derived growth factor alpha (PDGFA), heparin-binding EGF-like growth factor (HB-EGF), and basic fibroblast growth factor (bFGF) gene expression levels.
Results HPSE was significantly correlated to PDGFA ($p=0.04$) and HIF1a ($p=0.04$). The correlation of HIF1a to bFGF and HB-EGF was significant ($p=0.04$, $p=0.02$). Stepwise multiple linear regression models showed a significant independent association of HPSE with lymph node metastasis ($p=0.025$) and with dedifferentiation ($p=0.042$).
Conclusions Heparanase seems to be significantly associated with lymph node metastasis ($p=0.025$) as well as dedifferentiation ($p=0.042$). We assume that HPSE plays a crucial role for the aggressiveness of pancreatic cancer. Larger studies including more patients seem to be warranted.

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Keywords Heparanase · HIF1a · bFGF · PDGFA · HB-EGF · mRNA · FFPE tissue · qRT-PCR · Lymph node metastasis · Dedifferentiation · Aggressiveness

Introduction

From a histological perspective, the pancreas can be divided into an exocrine part, with ducts and acini and an endocrine component consisting of hormone secreting cells. The majority of pancreatic cancers derive from the exocrine section, with ductal adenocarcinoma representing 80–85% of all pancreatic cancers.[1] Even though in the last 10–15 years mortality rates of ductal adenocarcinoma have leveled off in Europe and have even decreased about 4% in the USA,[2,3] pancreatic cancer still has one of the worst prognoses of all cancers with a 5-year survival rate of 5%. Because symptoms appear in almost every case at an advanced stage of the cancer, only 10–20% of the patients with pancreatic malignancies are eligible for complete resection with curative intent. Thus, improvement in the treatment of this disease must be sought in approaches such as the use of markers or gene sets that would classify patients into various risk categories in order to allow more individually adapted multimodality treatment regimens.[4]

The presence of lymph node metastasis has a bad impact on the survival of pancreatic cancer patients, even though extended lymph node resection does not seem to provide the patients with a better postoperative survival.[5] Consequently, histopathological staging of lymph nodes is a standard procedure after every operation. However, overlooked metastases or disseminated tumor cells in lymph nodes examined by standard staining may result in some patients being falsely categorized as N0.[6] A gene set correlated to the presence of lymph node metastasis would supply useful prognostic guidance by providing auxiliary information on the likelihood of tumors developing lymph node spread. Additional information on the aggressiveness of the tumor might be gained by markers correlated to the stage of dedifferentiation, which is known to be an independent prognostic factor.[7]

Heparanase (HPSE) functions as an endoglycosidase that cleaves heparan sulfate chains of proteoglycans. The heparan sulfate chains are necessary to form a network containing among others type IV collagen, fibronectin, and laminin, which is an essential part of the basement membrane and the extracellular matrix.[8] Tumors expressing higher amounts of HPSE thus may have greater ability to break down the extracellular matrix barrier that normally would prevent them from spreading rapidly.[9] Indeed, Gao and others have stated that transfection of antisense HPSE results in a decreased invasive potential of pancreatic cancer cell lines.[10] Enclosed in the heparan sulfate glycosaminoglycans are growth factors and angiogenic factors, such as basic fibroblast growth factor (bFGF), which are released upon degradation with HPSE. The expression of bFGF is known to have a strong association with the expression of HPSE in many entities such as esophageal cancer,[11] but the connection between these factors is still poorly understood in pancreatic cancer and so far not evaluated. Heparin-binding EGF-like growth factor (HB-EGF) also binds to heparan sulfate proteoglycans (HSPG), which are cleaved in their heparin sulfate side chain from HPSE.[12] The importance of HB-EGF in pancreatic cancer is not fully understood yet.[13] Platelet-derived growth factor alpha (PDGFA), which interacts with heparin as well as HSPG,[14] has recently been discussed as a potential drug target in pancreatic cancer.[15] However, to date, the relationship between HPSE and PDGFA has not been evaluated in pancreatic cancer. Hypoxia inducible factor-1 alpha (HIF1a) has been shown to correlate with an unfavorable prognosis in many cancers and is known to regulate many genes linked to the angiogenesis pathway.[16]

In this pilot study, we investigated the interrelationships of the gene expressions of HPSE, PDGFA, HB-EGF, bFGF, and HIF1a using quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) of RNA extracted from formalin-fixed paraffin-embedded tissue (FFPE) of pancreatic carcinoma. We further analyzed the correlation of each of these gene expressions with clinical and histopathological variables such as tumor size (diameter/volume), primary tumor expansion (pT) regional lymph node metastasis (pN), grading/differentiation, and the survival time.

Patients and Methods

Study Population, Demographic Data, and Staging Procedures

FFPE samples were obtained from 41 patients with pancreatic adenocarcinoma with a median age of 65 years (range, 34–85 years) at time of operation who were scheduled for primary surgical resection. None of the patients received neoadjuvant or adjuvant radio-/chemotherapy. All patients were treated at the University Hospital of Cologne, North-Rhine-Westphalia, Germany, between December 1999 and July 2004. Demographic, clinical, and histopathological parameters are shown in Table 1. Informed consent was obtained from each patient in accordance with the requirements of the institution's board of ethics. TNM staging was performed according to the criteria of the International Union Against Cancer.[17]

Table 1 Patient Characteristics: Total Number of Patients, 41

Parameter (median age, 65 years; range, 34–85 years)	Number of patients (%)
Gender	
Male	23 (56.1%)
Female	18 (43.9%)
Histology	
Adenocarcinoma	41 (100%)
pT-category	
pT1	1 (2.4%)
pT2	6 (14.6%)
pT3	32 (78.0%)
pT4	2 (4.9%)
pN-category	
N–	7 (17.1%)
N+	33 (80.5%)
Not evaluated	1 (2.4%)
c/pM-category	
c/pM0	41 (100%)
Grading	
G2	22 (53.7%)
G3	19 (46.3%)
Residual tumor category	
R0	41 (100%)
Tumor size (diameter)	
Minimum	1 cm
Maximum	7 cm
Range	6 cm

UICC International Union Against Cancer; 1997 Tumor-Node-Metastasis (pTNM) Pathological Classification: *pT* primary tumor, *pN* regional lymph node metastasis, *c/pM* distant metastasis, *G* grade of differentiation, *R* residual tumor category

Microdissection

Representative H&E stained slides of each FFPE block were reviewed by a pathologist to estimate the tumor load per sample and to identify areas of tumor tissue. Section slides of 10-μm thickness were prepared for laser captured microdissection (PALM Microlaser Technologies AG, Munich, Germany) as described by Vallböhmer and others in 2005.[18]

Isolation of RNA and Complementary DNA Synthesis

The isolation of RNA from tissue samples was performed in accordance with a patented procedure at Response Genetics Inc. (Los Angeles, CA, USA, US patent no. 6,248,535). The complementary DNA (cDNA) preparation steps were accomplished as described previously.[19]

Quantitative Real-Time Polymerase Chain Reaction

Quantization of HPSE, PDGFA, HB-EGF, bFGF, and HIF1a messenger RNA (mRNA) expression levels was performed by qRT-PCR of amplified cDNA [ABI Prism 7900 Sequence Detection System (TaqMan) Perkin–Elmer Applied Biosystem, Foster City, CA, USA] as previously described by Kuramochi and others.[20] Beta-actin was used as an internal reference gene expression representing the total amount of RNA isolated. All genes were run on all samples in triplicates. Colon, liver, and Stratagene Universal Mix RNAs (Stratagene) were used as control calibrators on each plate. All Primers were selected using the Gene Express software (Applied Biosystems). All primers were validated before use, and gene expression data results are expressed as ratios between two absolute measurements (gene of interest/internal reference gene) to account for loading differences as described by Salonga and others in 2000.[21]

Statistical Analysis

All statistical tests were performed using the Software Packages SPSS® for Windows, Version 16.0, Chicago, IL, USA, and JMP 7.0 Software (SAS, Cary, NC, USA). The associations among the various gene expressions, and between each gene expression and clinicopathologic parameters were tested with Spearman's test for bivariate correlations. To evaluate whether gene expression levels can be used as independent variables to predict certain clinical factors, we used multiple linear regression analysis with stepwise selection on the gene set, tumor stage, tumor size (diameter/volume), and the dedifferentiation grade as covariates. A data mining technique provided by the SAS Institute was used to split gene expression in high- and low-level groups based on a platform that recursively partitions data according to a relationship between the X and Y values, creating a tree of partitions (recursive descent partition analysis). By searching all possible cutoff values, the software finds a set of cuts of X values (gene expression) that best predict the Y value (lymph node metastasis, grade). These data splits are done recursively, forming a tree of decision rules until the desired fit is reached; the most significant split is determined by the largest likelihood-ratio chi-square statistic. In either case, the split is chosen to maximize the difference in the responses between the two branches of the split. This method was previously used by Lu and others.[22] We used receiver operating characteristic (ROC) curve analysis to test the ability of the chosen cutoffs to discriminate low-grade (well-differentiated) from high-grade tumors. The level of significance was set to $p<0.05$. All p values reported were based on two-sided tests.

Results

The distribution of the log-transformed delta computed tomography (dCT) values is shown in Fig. 1.

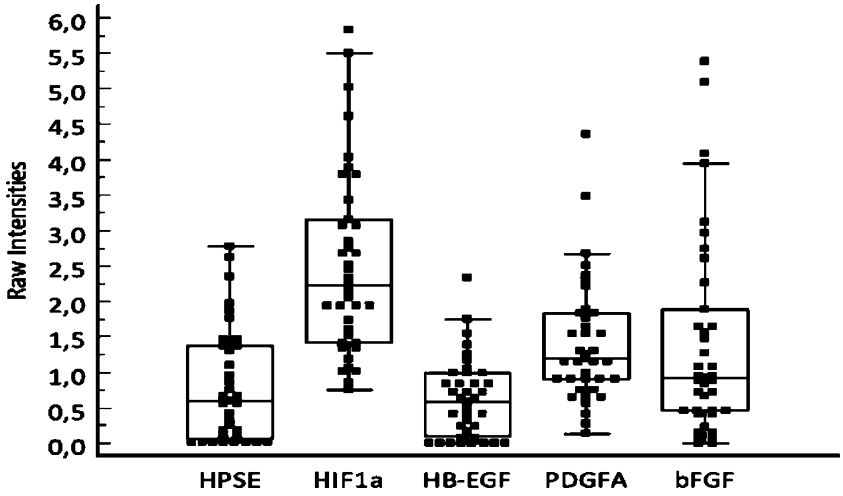

Figure 1 *Box-and-whisker plot* of the log-transformed dCT values for the studied genes.

Spearman's Test for Bivariate Correlations

Spearman's test on the log-transformed dCT values showed significant correlations among some of the genes. HPSE expression was significantly correlated with PDGFA (p=0.04) and HIF1 expressions (p=0.046). However, the expression of HPSE was not significantly correlated either with bFGF or HB-EGF expressions (p=0.39, p=0.15). The correlation of HIF1a with both bFGF and HB-EGF expressions was significant (p=0.04, p=0.02).

Partition Tree Analysis of Genes Based on Lymph Node Metastasis and Grade of Dedifferentiation

Patients were grouped by lymph node metastasis status according to whether they were pN0 (pN−) or >pN0 (pN+). Partition analysis showed HPSE to be the most significant gene for dividing the patients into the pN− or pN+ groups (with the highest log rank) at the cut point of the 70th percentile. The next in line were first HB-EGF and then bFGF (Fig. 2). Based on the partition analysis, we split every gene into a high- and a low-expression group and then performed a Spearman's test for correlation. High expression of HPSE was significantly correlated to lymph node metastasis at a significance level of p=0.025 in our study group. The correlation of HB-EGF after grouping in high and low gene expression with the lymph node stage was also significant at a level of p=0.01, whereas bFGF showed no correlation to the existence of lymph node metastasis.

Using the dedifferentiation grade as the predictable value to perform a recursive partition analysis, HPSE seemed to be the most significant divisor (Fig. 3). HPSE was also significantly correlated to the dedifferentiation grade of the samples with Spearman's test for correlation (p=0.02).

Multiple Linear Regression Analysis

We put pT, the grading, and HPSE as independent variables in a stepwise multiple linear regression model with lymph node metastasis as the dependent variable. The overall model fit had a significance level of p=0.025 with HPSE as the only factor significantly associated with lymph node metastasis (Tables 2, 3, and 4). Stepwise multiple regression analysis

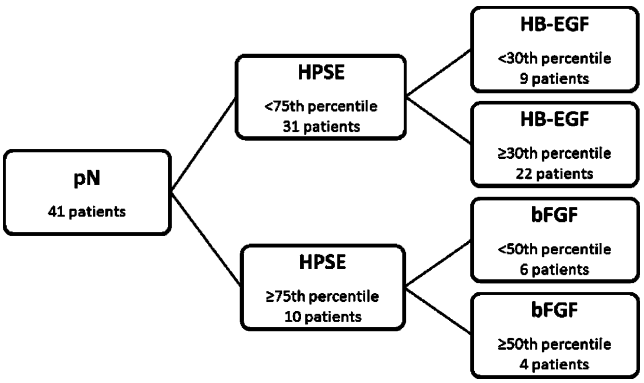

Figure 2 Recursive descent partition analysis: using lymph node metastasis as the factor to perform partition analysis on the chosen gene set.

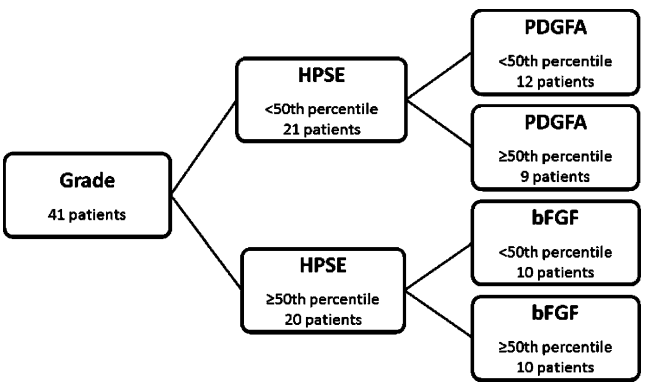

Figure 3 Recursive descent partition analysis: using grading as the factor to perform partition analysis on the chosen gene set.

Table 2 Multiple Linear Regression: Lymph Node Metastasis (ANOVA, Dependent Variable: Lymphknotenstatus)

Model		Sum of squares	*df*	Mean square	*F*	Significance
1	Regression	11,361	1	11,361	5,442	0.025[a]
	Residual	81,419	39	2,088		
	Total	92,780	40			

[a] Predictors: constant, HPSE75p

Table 3 Multiple Linear Regression: Lymph Node Metastasis (Coefficients, Dependent Variable: Lymphknotenstatus)

Model		Unstandardized coefficients		Standardized coefficients	*t* test	Significance
		B	SE	Beta		
1	Constant	1,774	0.260		6,837	0.000
	HPSE75p	1,226	0.525	0.350	2,333	0.025

Table 4 Multiple Linear Regression: Lymph Node Metastasis (ANOVA, Dependent Variable: Grading)

Model		Sum of squares	*df*	Mean square	*F*	Significance
1	Regression	1,043	1	1,043	4,443	0.042[a]
	Residual	9,152	39	0.235		
	Total	10,195	40			

[a] Predictors: Constant, HPSE50p

Table 5 Multiple Linear Regression: Grading (Excluded Variables, Dependent Variable: Lymphknotenstatus)

Model		Beta ln	*t* test	Significance	Collinearity statistics	
					Partial correlation	Tolerance
1	Grading	0.013[a]	0.083	0.934	0.013	0.995
	pT	0.215[a]	1,400	0.170	0.221	0.928

[a] Predictors in the model: constant, HPSE75p

Table 6 Multiple Linear Regression: Grading (Coefficients, Dependent Variable: Grading)

Model		Unstandardized coefficients		Standardized coefficients	*t* test	Significance
		B	SE	Beta		
1	Constant	2,300	0.108		21,233	0.000
	HPSE50p	0.319	0.151	0.320	2,108	0.042

Table 7 Multiple Linear Regression: Grading (Excluded Variables, Dependent Variable: Grading)

Model		Beta ln	*t* test	Significance	Collinearity statistics	
					Partial correlation	Tolerance
1	pT	−0.020[a]	−0.128	0.899	−0.021	0.917
	Lymphknotenstatus	−0.096[a]	−0.607	0.547	−0.098	0.941

[a] Predictors in the model: Constant, HPSE50p

to evaluate the most influential of the accessible factors on the dedifferentiation of the tumor showed a significant fit (p=0.042) of the overall model. The most significant independent factor associated with the grade of dedifferentiation was HPSE. Despite these results, HPSE expression showed no correlation to the overall survival of the patients (Tables 5, 6, and 7).

Receiver Operating Characteristic

The 50th percentile cutoff of the HPSE mRNA expression showed sensitivity (true positive rate) of 68.42% and a specificity (true negative rate) of 68.18% for the diagnosis low versus high grade. The area under the curve was 0.683 (CI 0.519 to 0.819) with a significance level of p=0.03. The positive likelihood ratio (true-positive rate/false-positive rate) was 2.15 and the negative likelihood ratio (false-negative rate/true-negative rate) 0.46 (Fig. 4).

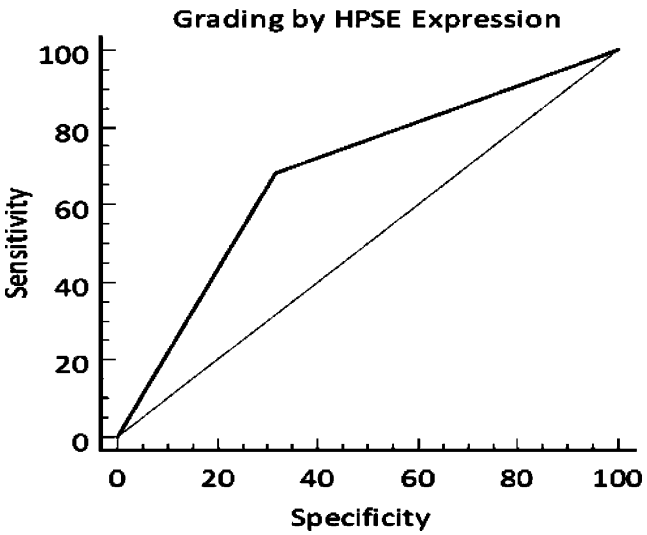

Figure 4 Receiver operating characteristic (*ROC*) curve analysis of the grade as the classification variable and dichotomized HPSE (50th percentile) as the discriminator.

Discussion

The goals of this study were to evaluate the extent to which HPSE contributes to the invasive potential and to the aggressiveness of pancreatic carcinomas and to examine its relationship to other genes involved in the various processes important for tumorigenesis. With regard to the first goal, our data showed that high HPSE expression is associated with the existence of lymph node metastasis. The implementation of Spearman's test and the multivariate analysis illustrated a significant association of HPSE expression with lymph node metastasis (p=0.025).

These data contradict the results of Kim and others, who found no correlation of HPSE expression with any staging factors of pancreatic cancer, including lymph node metastasis, primary tumor stage, or grading.[9] However, they did report correlation of HPSE expression with patient survival in early stage pancreatic carcinoma, a finding that we did not observe possibly because our set of samples included only a relatively small group of pT1 and pT2 patients. Our results are consistent with a recent study on 38 FFPE tissue samples from patients with gallbladder carcinoma that revealed a high correlation of HPSE protein expression with lymph node metastasis.[23]

Multivariate analysis revealed HPSE as a significantly independent factor associated with dedifferentiation (p=0.042). Patients in our study group with a higher grade of dedifferentiation showed a significantly higher expression of HPSE (Spearman's p=0.02). Koliopanos and others examined HPSE expression in 33 samples of pancreatic cancer, and in their patient cohort, there was a tendency toward significant correlation between dedifferentiation and higher levels of HPSE mRNA.[24] Doweck and others were able to show a significant correlation of HPSE expression to dedifferentiation in patients with head and neck cancer.[25]

HIF is a transcription factor that is activated under hypoxic conditions and then drives the induction of a large number of genes controlling functions such as angiogenesis, metabolism, invasion/metastasis, and apoptosis/survival.[26] It has been assumed previously that HPSE could promote the processes stimulated by HIF1 by inducing cox-2, which in turn induces HIF1 overexpression.[27] Our finding that the

mRNA levels of HPSE correlated significantly with HIF1a gene expression (p=0.046) lends support to this idea. A link between HPSE and HIF1-induced pathways suggests one possible mechanism by which HPSE might be involved in causing increased tumor aggressiveness.

There was a significant correlation of HPSE levels to the expression of PDGFA (p=0.04). As far as we know, the correlation between HPSE and PDGFA has not yet been evaluated in human cancer. The normal function of PDGF, through its receptors, is to promote a variety of events, including stimulation of cell growth, inhibition of gap junction communication, and inhibition of apoptosis; consequently, dysregulation of these cellular events may result in tumorigenesis.[28] Pancreatic tumors have been shown to overexpress PDGF receptors alpha and beta.[29] While the correlation of HPSE and PDGFA levels suggests a convergence of functions at some point, the question of whether HPSE is induced by increased activation of the PDGF pathways or whether HPSE plays a role in the induction of PDGF receptor levels remains to be elucidated.

Overexpression of bFGF is common in pancreatic cancer, and previous studies such as those of Han and others, who found a significant correlation of bFGF and HPSE in esophageal cancer, and El-Assal and colleagues, who reported that HPSE and bFGF appear to have a synergistic effect in hepatocellular carcinoma, led us to hypothesize that there also might be a strong connection between the two gene expressions in pancreatic cancer.[11,30] However, our results revealed no direct correlation between bFGF and HPSE, although bFGF was significantly correlated to HIF1a (p=0.04), the expression of which was, as mentioned before, significantly associated with HPSE expression.

Though heparan sulfate proteoglycans function as a co-receptor for, among others, HB-EGF, and therefore, HPSE expression might also influence HB-EGF mRNA levels,[12] we found no significant correlation between HB-EGF and HPSE expressions. A role for HB-EGF in metastasis of pancreatic cancer was suggested by a previous study in which gene chip analysis on pancreatic cancer cell lines was used to show that HB-EGF expression was significantly higher in metastatic cell lines.[31] However, immunohistochemical analysis of HB-EGF levels in 40 samples of pancreatic adenocarcinoma did not show a significant correlation between HB-EGF expression and lymph node metastasis.[13] These contradictory results might be partially explainable by the fact that, although Spearman's test on our patient cohort showed a significant correlation of lymph node metastasis with HB-EGF expression (p=0.01), multivariate analysis did not rate HB-EGF expression as independently associated with lymph node metastasis.

Conclusions

The results of this hypothesis-generating study seem to underline the importance of HPSE in pancreatic cancer. Though we could not verify HPSE as important for the overall survival of patients, we were able to fit HPSE in an angiogenesis model for pancreatic cancer and to illustrate co-expression of HPSE, HIF1a, and PDGFA. Considering the fact that HPSE seems to be a highly significant independent variable for lymph node metastasis (p=0.025) as well as for dedifferentiation (p=0.042), we assume that HPSE plays a role in determining the aggressiveness of pancreatic cancer. Because these results were obtained on a relatively small number of patients, larger studies including patients treated with actual chemotherapeutics seem to be warranted.

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Discussion

O. Joe Hines, M.D. (Los Angeles, CA): I enjoyed your presentation, and thank you for providing me the manuscript. You propose that heparanase can be used to characterize a more aggressive tumor phenotype in patients with pancreatic cancer, and in your abstract you state that you assume that based on your findings, heparanase plays a critical role in pancreatic cancer. In fact, it does, and there has been significant work already reported in this area. A few examples include work from Dr. Prinz and his group, who reported at this meeting in 2001 that serum heparanase levels are elevated in patients with pancreatic cancer, that the level correlates with survival and drops with gemcitabine treatment. In a very elegant study a group from UCSF recently found that heparanase expression progressively rises in a mouse model of multistage pancreatic carcinogenesis. So, Dr. Hoffmann, I have three questions for you.

First, I recognize that the study does not offer any functional data proving a relationship between these factors, and I understand the factors are only used here as potential markers for poor tumor biology, but the work does try to establish several links between these five factors, and these links may not actually be real. But since you tried to establish the links, let me ask one question related to this. There is no described relationship between heparanase, HIF, and EGF, no evidence that hypoxia drives EGF, yet you state in both the title and the text of the manuscript that there is a relationship. How do you speculate that these are related or are they just all elevated?

The second question is heparanase was found to be significantly associated with lymph nodes metastases.

Seven patients were node negative and 33 node positive. How many node negative patients had high heparanase expression and how many node positive patients had low expression?

And finally, assuming that this data set or even another panel of markers can be verified to identify a group of patients with especially aggressive tumors, how do you imagine that information would be utilized? This really is such an aggressive disease. Would this influence postoperative treatment? In my practice, almost every resected patient with a reasonable functional status receives adjuvant treatment regardless of nodal status. Or would you imagine that patients with a potentially resectable lesion but a poor panel of markers not be offered surgery?

I thank the moderators for inviting me to discuss the paper.

Andreas C. Hoffmann, M.D. (Los Angeles, CA): Thank you for your excellent discussion. To the first point, you are absolutely right; this is just a first step. The next step would be to get more patients, which is what we are doing at the moment and then we would have to use a biological model to substantiate the meaning of the used genes.

Secondly, though it is of course not possible to definitively answer this question from a correlative study, we did not just find these genes to be associated with higher aggressiveness by some random screening process. Rather, we had reasons at the outset as to how these genes might promote more aggressive tumors, and the fact that these gene expressions did then associate with various metrics of tumor aggressiveness strengthens the hypothesis of cause, not effect. Identifying genes that are associated with more aggressive tumors is useful to form a candidate oncogene pool that is available for further work to more definitively address the cause-or-effect question, such as in vitro experiments where the genes in question are transfected into cells.

For example, the insertion of mutated p53 into cells has been used to demonstrate that this gene directly causes many different effects, but it had to be identified first as being associated with more aggressive tumors.

As to the relationships of the different genes – I ran several genes out of the angiogenic pathway, HIF1 alpha is only one of them, also on different tumor entities, like gallbladder cancer, soft-tissue sarcoma, and I always found this correlation which in my opinion strengthens my hypothesis. I am validating these results at the moment on larger patients sets, so we will have to see whether the hypothesis of correlation can be scrutinized.

The third question, yes, in the medium there was a significant difference between lymph node positive and lymph node negative patients, and that leads to the last question, whether these results have any clinical meaning or what the potential benefit of using these genes could be.

Positive findings on genes associated to tumor aggressiveness and lymph node metastasis could aid in the preoperative staging process, meaning that a preoperative biopsy and analysis could provide essential information about potential treatment relevant facts earlier than it is currently available. It could eventually lead to an altered staging and treatment process with the possibility of neoadjuvant treatment for certain patient groups. By the time the pathologist examines the full extent of aggressiveness and local metastasis the first chance of a possible downstaging before performing the operation has already passed.

I think this is the first step to probably find markers which add something to a pathological staging.

Thank you very much.

Edward E. Whang, M.D. (Boston, MA): The major problem with current translational research is that we report data on biomarkers that have not been validated, and most of these biomarkers do not stand the test of time. In this study you used a relatively small data set to derive your cutoff points. Do you have the ability to validate your findings using an independent set of patients and data?

Dr. Hoffman: Yes. Actually I already added 100 patients more, also 20 noncancerous patients who had pancreatitis. I think if I use again the same cut points across, then I can validate the results with this bigger set. Of course, you are right; we have to see whether the results stand the test of time.

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Applying Proteomic-Based Biomarker Tools for the Accurate Diagnosis of Pancreatic Cancer

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Abstract
Background The proteome varies with physiologic and disease states. Few studies have been reported that differentiate the proteome of those with pancreatic cancer.
Aim To apply proteomic-based technologies to body fluids. To differentiate pancreatic neoplasia from nonneoplastic pancreatic disease.
Methods Samples from 50 patients (15 healthy (H), 24 cancer (Ca), 11 chronic pancreatitis (CP)) were prospectively collected and underwent analysis. A high-throughput method, using high-affinity solid lipophilic extraction resins, enriched low molecular weight proteins for extraction with a high-speed 200-Hz matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-MS; Bruker Ultraflex III). Samples underwent software processing with FlexAnalysis, Clinprot, MatLab, and Statistica (baseline, align, and normalize spectra). Nonparametric pairwise statistics, multidimensional scaling, hierarchical analysis, and leave-one-out cross validation completed the analysis. Sensitivity (sn) and specificity (sp) of group comparisons were determined. Two top-down-directed protein identification approaches were combined with MALDI-MS and tandem mass spectrometry to fully characterize the most significant protein biomarker.
Results Using eight serum features, we differentiated Ca from H (sn 88%, sp 93%), Ca from CP (sn 88%, sp 30%), and Ca from both H and CP combined (sn 88%, sp 66%). In addition, nine features obtained from urine differentiated Ca from both H and CP combined with high efficiency (sn 90%, sp 90%). Interestingly, the plasma samples (considered by the Human Proteome Organization to be the preferred biological fluid) did not show significant differences. Multidimensional scaling indicated that markers from both serum and urine led to a highly effective clinical indicator of each specific disease state.
Conclusions The proteomic analysis of noninvasively acquired biological fluids provided a high level of predictability for diagnosing pancreatic cancer. While the proteomic analysis of serum was capable of screening individuals for pancreatic disease (i.e., CP and Ca vs. H), specific urine biomarkers further distinguished malignancy (Ca) from chronic inflammation (CP).

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Keywords Pancreatic cancer · Proteomics · Mass spectrometry · MALDI · Serum

Introduction

While genomics has held a spotlight for some years now, proteins are the end product of these genes and modifications of these proteins are believed to hold the secret to many pathophysiologic changes. Recent advances in the field of proteomics are crucial to the discovery of biomarkers that may indicate the development of a particular disease. This technology has been applied to the identification of different proteins associated with various solid organ malignancies, such as prostate cancer,[1,2] ovarian cancer,[3] breast cancer,[4] and renal cell cancer,[5] but has yet to be utilized in a study of this size for the identification of biomarkers associated with lesions that give rise to pancreatic ductal adenocarcinoma, especially at an early stage. However, a number of smaller pooled and more focused studies have been completed in this realm, which complement the outcome of this study.

In contrast to the genetic background of an individual, the proteome varies not only from cell to cell but also differs from one physiologic or disease state to another. Proteins function as an integral piece in biological/molecular pathways within the cell, and, therefore, an in-depth study of the proteome, or protein signature of an individual as it relates to a specific disease state, is highly complementing and yet very complicated. This realization of the changing proteome between different disease states has led to the identification of protein biomarkers associated with specific diseases and has led to the development of the Human Proteome Organization (HUPO), which has begun to set standards for the characterization of human proteins. This relatively new global comprehension and categorization of the proteome and their interactions will undoubtedly lead to new diagnostic and treatment modalities.

Pancreatic cancer is now the fourth leading overall cause of cancer death in the United States, with over 34,000 estimated deaths in 2008.[6] The high mortality associated with pancreatic cancer is mostly attributed to advanced stage of disease at patient presentation. The overall 5-year survival for those with pancreatic cancer is about 5%, and only ~20% of patients are candidates for surgical resection and possible cure. For this small percentage of patients undergoing resection, even when followed by multimodal therapy, 5-year survival rates are still less than 25%.[7–9] To date, there are no modalities to aid in the early detection of pancreatic cancer and recent strategies to improve survival have focused solely on chemotherapy in the neoadjuvant setting or after resection.

Mass spectrometry (MS) is an analytical technique that measures the mass to charge (*m/z*) ratio of ionizing particles, which includes small molecules, proteins/peptides, and fatty acids. Various techniques are available to purify or enrich proteins in body fluid-derived samples prior to identification by MS. These techniques have been utilized on a limited basis to describe the proteome in those with pancreatic cancer, and studies have been mostly descriptive and the number of patients evaluated has been consistently low.[10–12]

There are currently no serologic markers identified to be specific to pancreatic cancer. Furthermore, few studies have attempted to differentiate those with pancreatic cancer from patients with chronic pancreatitis and patients without pancreatic disease. The unique aspect of our study is that we matched patient samples and used an individual, nonpooled analysis. The goal of this study is to combine a high-throughput (HTP) technique to enrich low molecular weight proteins from serum, plasma, and urine, with the use of a high-speed matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry to identify spectra associated with patients with and without pancreatic disease.

Materials and Methods

Sample Collection

Patients were enrolled into the Pancreatic SPORE (P20 CA101955) protocol at the University of Alabama at Birmingham from 2003 through 2005. Matched serum, urine, and plasma samples were prospectively collected from patients with or without pancreatic disease, and 100 μL aliquots were stored at −80°C. Radiographic imaging, operative details, and pathologic reports were used to determine healthy (H), cancer (Ca), or chronic pancreatitis (CP). Serum and plasma samples were first visually inspected for hemolysis and scored 0–3 (0—no hemolysis, 1—slightly pink, 2—darker pink, 3—red). Those samples that were classified as 2 and 3 were excluded from the study. Fifty samples per biological fluid underwent the final analyses.

C18 Cleanup

Ten microliters of serum/plasma sample was diluted 1:50 with distilled water and applied on an 800-μL 96-well Whatman® filter plate, which contained activated C18 resin. Proteins were let bound to the resin and extracted in 100 μL of 70% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA). One and a half microliters of extracted sample was applied on a MALDI target plate with 1.5 μL of 20 mg/mL sinapinic

acid in 50%ACN/0.1%TFA. The same C18 method was used for urine samples but without dilution. Samples were then analyzed with a MALDI-TOF instrument (Ultraflex III, Bruker Daltonics). Protein mass spectra were baseline-subtracted, normalized and calibrated with Flexanalysis (Bruker Daltonics), and further preprocessed and normalized with the scripts from the proteomic toolbox in Matlab 7.0 prior to statistical analyses.

Statistical Analysis

Whole spectral analysis was performed with weighted means average (WMA) cut-off of ±0.65 and fold difference cut-off of ±1.5. The *m/z* values found to be statistically significant with this analysis were further confirmed with additional single tailed (when applicable) nonparametric pairwise tests including Wilcoxon, WMA, and Student's *t* test. This initial filtering is followed by multidimensional scaling (MDS) and leave-one-out cross-validation (LOOCV) using a WMA, class validation, voted-weighed-scheme method previously reported[2] for use in various genomic studies.

Top-Down Directed Protein ID (Strategy 1; Nondepletion Method)

Protein identification was performed on serum samples as described previously[1] with modifications. Briefly, serum samples were pooled to give 900 μL per arm (H, CP, or CA). A solid core C18 extraction resin was utilized (Waters) in a large hand-packed column (Upchurch), followed by sequential strong cation exchange (SCX) fractionation using a hand-packed ToyoPearl column in a similar fashion with salt bumps carried out at 0.5, 0.7, 0.8, 0.9, and 1 M NaCl in 10 mM ammonium acetate/10% ACN. Five microliters of sample per salt fraction was desalted/concentrated with a C4 ziptip (Millipore) and analyzed with a MALDI-TOF instrument (Ultraflex III, Bruker Daltonics) to determine the fractions containing those proteins of interest. The fraction that contained the 9,713 *m/z* peak was further purified with a C18 macrotrap with increasing organic bumps. One dimensional polyacrylamide electrophoresis (PAGE) was then performed, and bands that migrated corresponding to 9.7 kDa were excised. Gel pieces were digested in-gel with trypsin, and digests were analyzed by tandem MS with a MALDI-TOF/TOF instrument. The Mascot search engine was used to identify each protein.

Top-Down Directed Protein ID (Strategy 2; Depletion Method)

Another 125 μL of "pooled" serum derived from the cancer patient group were depleted of the 12 most abundant serologic proteins using a Proteome Lab IgY-12 High Capacity Partitioning Kit (Beckman Coulter). The depleted sample was then desalted/concentrated with a C18 macrotrap (Upchurch). Further fractionation was performed using a C4 HPLC column (GRACE Vydac). Fractions were collected off-line in a 96-well plate and analyzed with the MALDI-TOF instrument using WARPLC to reconstruct the time-based fractions as they correspond to *m/z*. A fraction that contained the 9,713 *m/z* peak of interest was concentrated and run on a 1DE gel and analyzed as mentioned above.

Results

Fifty samples (15 H, 24 Ca, 11 CP; Table 1) per biologic fluid underwent MALDI-MS analysis as described. Mean ages (62.7, 59.6, 66.6 years) among H, Ca, and CP patients, respectively, were not different ($p=0.24$). There was a higher percentage of women in the H group (93%) when compared to the CP (27%) and Ca (33%) groups ($p<0.01$). In the Ca group, 79% of tumors were located in the pancreatic head, 80% had lymph node metastases, 40% were poorly differentiated, and the mean size was 3.1 cm. Using the top statistically significant eight serum features (4,470, 4,792, 8,668, 8,704, 8,838, 9,194, 9,713, and 15,958, the top scoring marker from this list 9,713* daltons with $z=1$), we were able to accurately differentiate Ca from H (sn 88%, sp 93%), Ca from CP (sn 88%, sp 30%), and Ca from both H and CP combined (sn 88%, sp 67%; Fig. 1; Table 2). When applying the top nine urine features (2,193, 2,463, 2,515, 2,834*, 5,268*, 5,412*, 5,662, 10,946, 12,117**m/z*), four of which were specific for differentiating Ca vs. CP (*denoted by an asterisk*), we accurately differentiated Ca from H (sn 90%, sp 92%), CA from CP (sn 90%, sp 90%), and Ca from both H and CP combined (sn 90%, sp 90%; Fig. 2; Table 2). Interestingly, the plasma samples (considered by the Human Proteome Organization to be the preferred biological fluid) did not show significant differences between patient groups. In addition, by combining features from both studies, we reanalyzed H vs. disease (Ca and CP) with a clear improved separation observed by MDS, along with a second analysis using the four top Ca specific urine features (2,834*, 5,268*, 5,412*, and

Table 1 Patient Information

Patient group	No. of patients	Median age (range)	No. of matched samples Serum/plasma/urine
Pancreatic cancer	24	66 (45–79)	24
Chronic pancreatitis	11	60 (47–72)	11[a]
Healthy	15	60 (32–78)	15

[a] Two urine samples from CP group were not available for this study

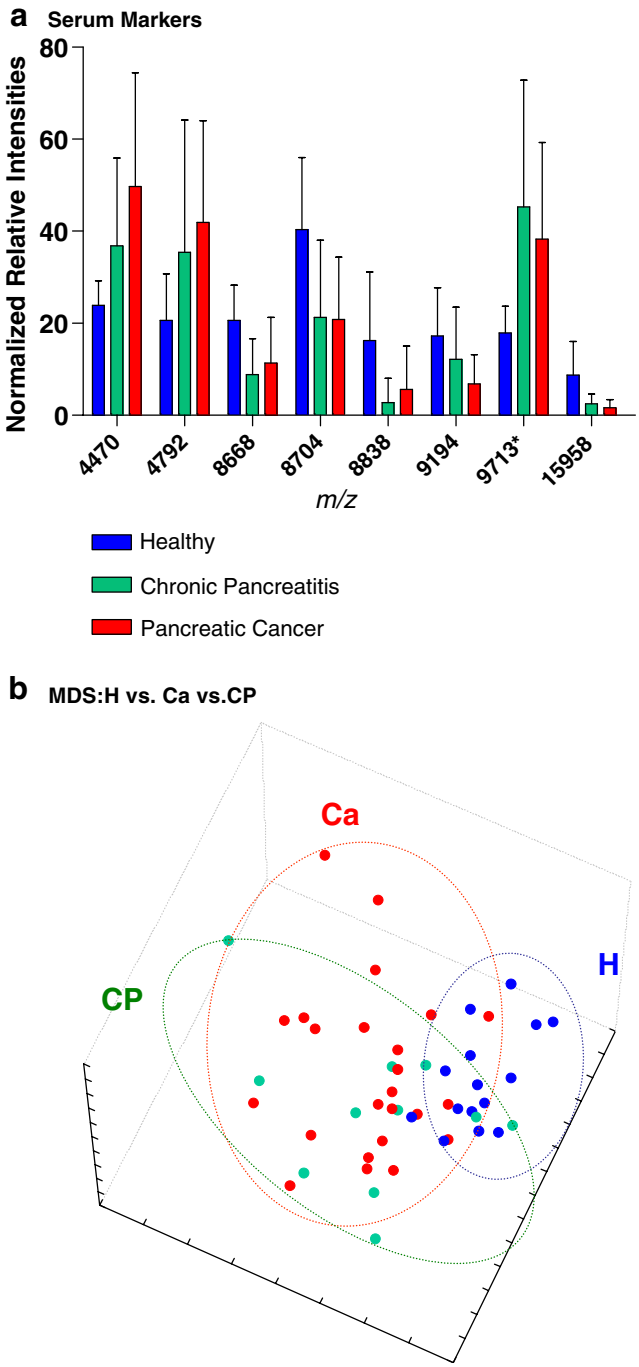

Figure 1 Serum markers; comparison of normalized peak intensities among healthy (*H*) vs. chronic pancreatitis (*CP*) vs. pancreatic cancer (*Ca*) patients: Statistically significant features (*m/z* values with $p < 0.05$) were identified between each patient group using two separate nonparametric pairwise analysis. **a** Eight *m/z* values were identified and are presented as the intensity mean±SD (4,470, 4,792, 8,668, 8,704, 8,838, 9,194, 9,713, and 15,958), the top scoring marker from this list 9,713* daltons ($z=1$) was characterized by tandem mass spectrometry (shown in Fig. 5). **b** multidimensional scaling (*MDS*) analysis was carried out for each patient based on the normalized peak intensities from all eight features. The resultant "qualitative" separation of each group is visualized in a three-dimensional plot.

12,117* *m/z*) which also indicated an improved separation between CP vs. Ca groups (Fig. 3).

For the MS characterization of the serologic protein at 9,713 *m/z*, two top-down-directed (whole protein detected by MS at every fraction) techniques were utilized (Fig. 4). In short, the native apolipoprotein CIII (ApoCIII) and modified forms of this protein were identified. This identification was carried out with a use of partial-trypsin search approach, since the current databases do not assume that the cleaved "active" protein is present. In the band(s) of interest, we detected the parent protein ApoCIII with no modifications (MH^+ 8,766 average mass), and the modified forms including $ApoCIII_0$ Gal/GalNAC (+365, MH^+ ~9,131 average mass), $ApoCIII_1$ plus one sialic acid (sia, +291, MH^+ ~9,422), $ApoCIII_2$ plus two sia (+582, MH^+ ~9,713) and loss of the C-term alanine from each modified form presumably by carboxypeptidase A.[13] The sequences (post-tryptic digestion, cleavage following K, R) of each modified form found in this study are as follows in addition to the posttranslational modifications on the last C-term peptide AA shown in bold T(x); SEAEDASLLSFMQGYM**K**/HATK/TAK/DALSSVQESQVAQQAR/GWVTDGFSSLK/DYWSTVK/DK/FSEFWDLDPEVR/P***T(x)***SAVAA. We were able to measure the active protein peptides to reconstruct the observed masses as shown in Fig. 5: 8,764.7 MW (average), with 91% coverage (PMF), and three peptides ID'd with tandem MS (i.e., MS^2).

Discussion

Proteomic technology has improved and is now allowing the high throughput identification of proteins and peptides in various tissues and body fluids on a patient specific (nonpooled) basis. The expanded study of peptides and proteins, known as proteomics, has led to the description of the proteome of individuals with various solid organ malignancies.[2–5,10] The accuracy of identifying those with cancer has been impressive, although there is limited data on the study of those with pancreatic cancer. Several groups have reported successes with surface-enhanced laser

Table 2 Predictive Values Based on Top Markers in Serum and Urine

		Ca vs. H (%)	Ca vs. CP (%)	Ca vs. H/CP (%)
Serum	Sensitivity	88	88	88
	Specificity	93	30	67
Urine	Sensitivity	90	90	90
	Specificity	92	90	90

All data was calculated based on LOOCV class validation using a voted weighted scheme weighted means average approach with the markers listed for each biological fluid respectively (i.e., serum or urine)

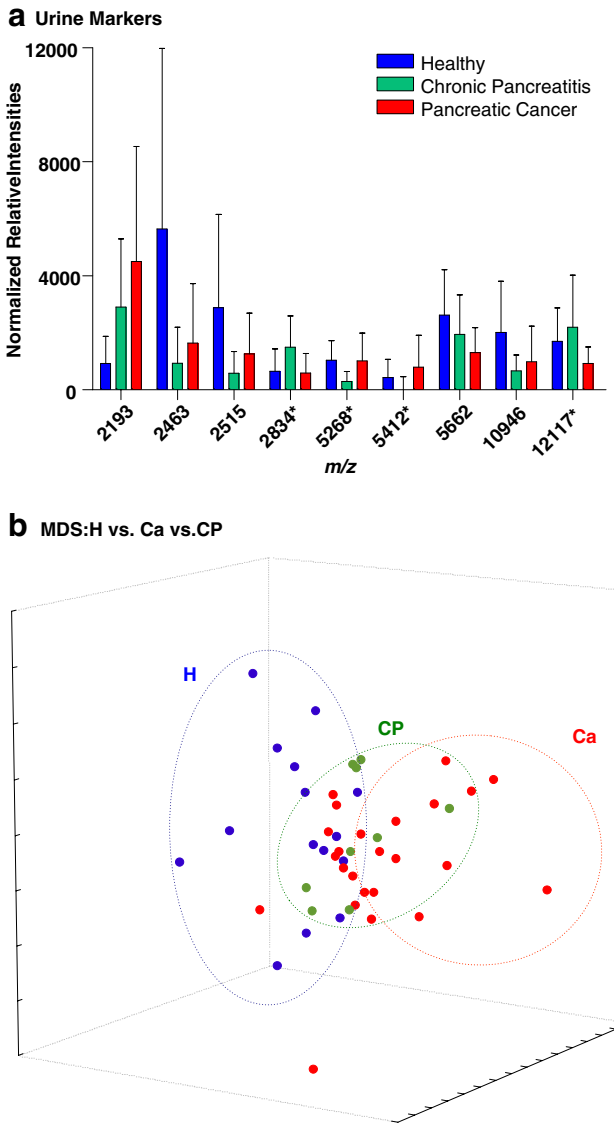

Figure 2 Urine markers; comparison of normalized peak intensities among healthy (*H*) vs. chronic pancreatitis (*CP*) vs. pancreatic cancer (*Ca*) patients: Statistically significant features (*m/z* values with $p < 0.05$) were identified between each patient group using two separate nonparametric pairwise analysis. **a** Nine *m/z* values were identified and are presented as the intensity mean±SD (2,193, 2,463, 2,515, 2,834*, 5,268*, 5,412*, 5,662, 10,946, 12,117**m/z*), four of which were specific for differentiating Ca vs. CP (*denoted by an asterisk*). **b** Multidimensional scaling (*MDS*) analysis was carried out for each patient based on the normalized peak intensities from all nine features. The resultant "qualitative" separation of each group is visualized in a three-dimensional plot.

desorption/ionization mass spectrometry[13]; however, the advantages of the latest in HTP MALDI-MS have yet to be investigated in those with pancreatic cancer. HTP MALDI instrumentation allows for higher resolution and identification of specific peaks within the protein spectra. During this study, we utilized MALDI-MS not only to analyze body fluids of patients with pancreatic cancer but also to compare the spectra to those with normal pancreatic architecture and chronic pancreatitis.

According to HUPO, plasma is the fluid of choice for biomarker discovery and identification. However, our plasma sample set did not produce data with acceptable specificity and sensitivity. When analyzing the serum samples in our set, the specificities and sensitivities were impressive, although the overall power is relatively low with eight features from the spectra studied. Interestingly, for the first time when exploring urine from this same perspective, the nine features identified differentiated cancer from all others with a high sensitivity.

Apolipoprotein CIII contains 79 amino acids and comprises about 50% of very low density lipoprotein in humans.[14] In humans, the ApoCIII gene has been mapped to the long arm of chromosome 11 and is expressed in the liver and intestine. Mutations have long been described in

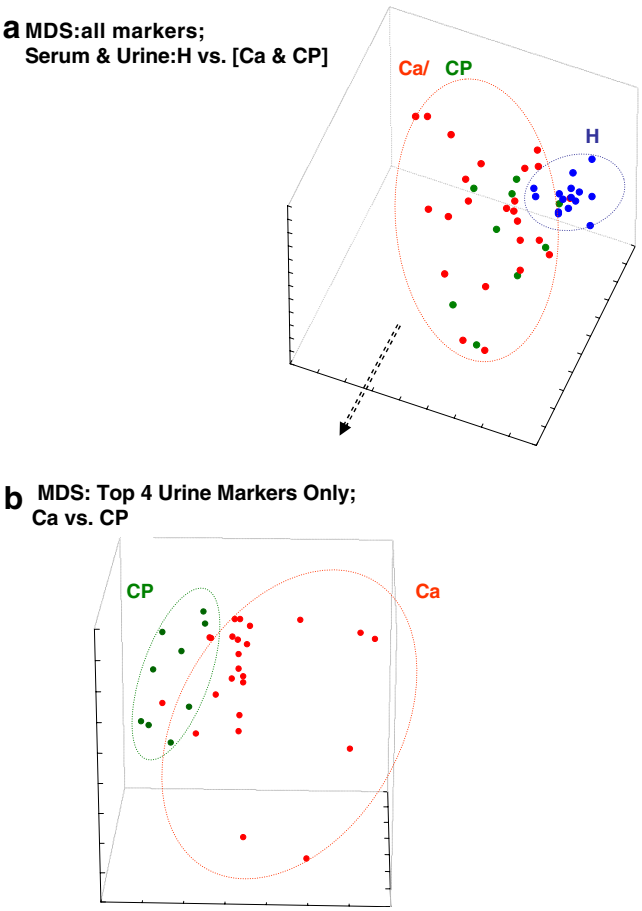

Figure 3 Improved separation among all groups with combined serum and urine markers: Statistically significant features from both studies were combined and reanalyzed for H vs. disease (Ca and CP). **a** A clear separation between H vs. disease groups (CP and Ca) is observed by multidimensional scaling. **b** Further analysis with four Ca specific urine features (2,834*, 5,268*, 5,412*, and 12,117**m/z*) indicates improved separation between CP vs. Ca groups.

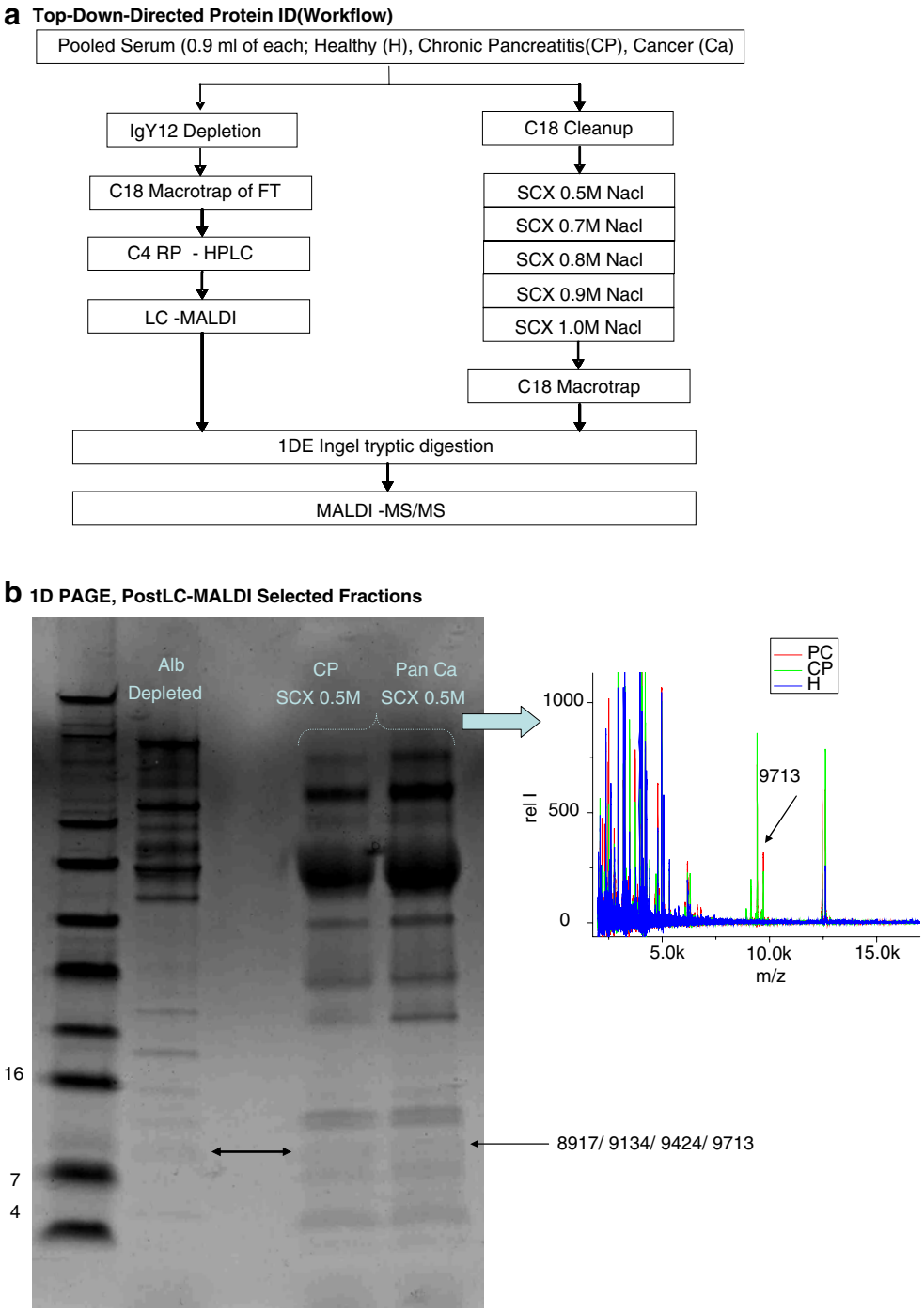

Figure 4 Protein identification (ID) strategies: Two experimental approaches were taken to fully characterize the serologic protein marker at 9,713 *m/z* found to be the most statistically significant of the eight identified. **a** Schematic diagram of protein ID experiments. **b** One-dimensional PAGE (*bottom-left*) following each separation approach visualized with coomassie stain and corresponding mass spectra (*bottom-right*) of semipurified protein illustrating the marker at 9,713 *m/z*.

ApoCIII regarding its role in lipid metabolism,[15] but very little have been learned about the role of ApoCIII in the native or mutated form as pertaining to pancreatic cancer. In a study from the University of Michigan,[16] $ApoCIII_1$ was found to be downregulated in pancreatic cancer sera. In contrast to this earlier study, our results of 24 separately analyzed patient sera show an upregulation of $ApoCIII_2$ that is reproducible throughout the analyses. We were also able to repeatedly identify differentially modified forms of ApoCIII along (see Fig. 5b).

The results presented herein from our pilot study of collected samples are very encouraging for distinguishing those with pancreatic cancer from those without disease or those with chronic pancreatitis. The advantage of this technology is that pancreatic cancer may be able to be distinguished from those with pancreatitis or diagnosed prior to radiologic or endoscopic examination. A larger prospectively collected sample set is necessary in order to validate and improve on our results. Standardization of sample preparation is crucial to prevent hemolysis and gain

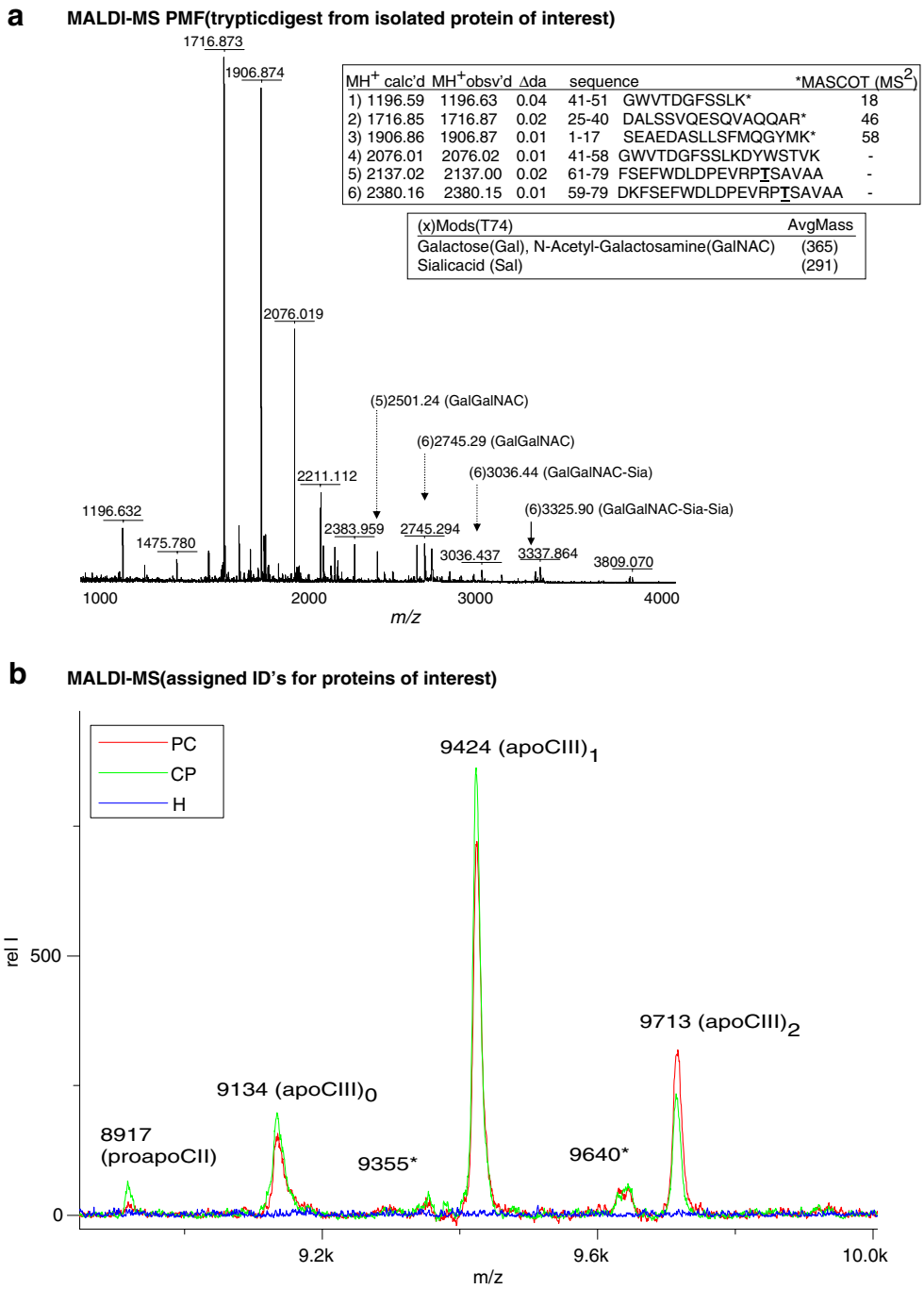

Figure 5 Apolipoprotein CIII (*ApoCIII*) identification with MALDI-TOF/TOF. In-gel tryptic digests of the 9,713 *m/z* bands were analyzed by tandem mass spectrometry. The peptide mass fingerprints and tandem mass spectra of each peptide led to identification of a protein corresponding to a multimodified form of apolipoprotein CIII. **a** The peptide mass spectrum of an enzyme digest matched well to the known human form(s) of ApoCIII. **b** Resultant protein mass spectra of SCX 0.5M NaCl isolated fractions from H (*blue*), CP (*green*), and Ca (*red*) pooled serum samples with the multiple peaks corresponding to differentially modified forms of ApoCIII based on tandem mass spectrometry.

of maximum yield from the spectra identified. Ultimately, multi-institutional studies will allow validation through a robust test set and possible creation of a protein profile specific to the diagnosis of pancreatic cancer.

Conclusion

Proteomic analysis of human serum and urine can provide a high level of predictability for diagnosing pancreatic cancer. This study is the largest in the literature to separately analyze nonpooled samples. Likewise, apolipoprotein CIII was found consistently overexpressed in the 24 cancer serum samples we analyzed, a finding that is unique to the literature. The eventual inexpensive proteomic analysis of noninvasively acquired biological fluids may be used to screen individuals for pancreatic cancer or differentiate benign from malignant disease. Further sample collection and standardized processing is necessary in order to verify and expand on the results presented in this manu-

script. Tertiary care centers and high volume pancreatic surgery centers should begin fluid and tissue collection from all cancer patients in order to expand our understanding of the genomic and proteomic changes involved in the development of cancer.

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Pancreatic Fistula Rates After 462 Distal Pancreatectomies: Staplers Do Not Decrease Fistula Rates

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Abstract
Introduction Pancreatic fistula is a major source of morbidity after distal pancreatectomy (DP). We reviewed 462 consecutive patients undergoing DP to determine if the method of stump closure impacted fistula rates.
Methods A retrospective review of clinicopatologic variables of patients who underwent DP between February 1994 and February 2008 was performed. The International Study Group classification for pancreatic fistula was utilized (Bassi et al., *Surgery*, 138(1):8–13, [2005]).
Results The overall pancreatic fistula rate was 29% (133/462). DP with splenectomy was performed in 321 (69%) patients. Additional organs were resected in 116 (25%) patients. The pancreatic stump was closed with a fish-mouth suture closure in 227, of whom 67 (30%) developed a fistula. Pancreatic duct ligation did not decrease the fistula rate (29% vs. 30%). A free falciform patch was used in 108 patients, with a fistula rate of 28% (30/108). Stapled compared to stapled with staple line reinforcement had a fistula rate of 24% (10/41) vs. 33% (15/45). There is no significant difference in the rate of fistula formation between the different stump closures ($p=0.73$). On multivariate analysis, BMI>30 kg/m^2, male gender, and an additional procedure were significant predictors of pancreatic fistula.
Conclusions The pancreatic fistula rate was 29%. Staplers with or without staple line reinforcement do not significantly reduce fistula rates after DP. Reduction of pancreatic fistulas after DP remains an unsolved challenge.

Keywords Distal pancreatectomy · Pancreatic fistula

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Introduction

Distal pancreatectomy (DP) is most often performed for primary benign or malignant lesions in the body or tail of the pancreas, for pancreatitis, or for trauma. The procedure usually involves resection of a portion of the pancreatic parenchyma to the left of the portal vein. The spleen can be resected or preserved depending on the nature of the lesion being removed.

The surgical mortality for pancreatic resection has been reduced significantly over the past 30 years. Mortality rates in high-volume centers are under 5%; however, morbidity rates continue to be as high as 47–64%.[1,2] Although distal pancreatectomy is a technically simpler operation than a pancreaticoduodenectomy, the morbidity remains substantial. Pancreatic fistula, the most frequent complication, results in varying degrees of morbidity for the patient. There are numerous definitions for pancreatic fistula; however, in 2005, an international working group proposed

a consensus definition and classification.[3] This standardized definition allows for comparisons between different surgical experiences and allows for more meaningful comparisons between series.

Pancreatic fistula is often associated with additional complications such as wound infections, intra-abdominal abscesses, fever, malabsorption, and delayed hemorrhage. These complications affect not only the patient's health but also significantly increase the cost of their healthcare.[4] This has lead to an extensive search for the best closure technique for the pancreatic stump. Techniques include hand-sewn approximation of the edges, ligation of the pancreatic duct, glues and patches, as well as staplers. However, none of these techniques have consistently affected the rates of pancreatic fistula.

The purpose of this study was to compare pancreatic fistula rates between different stump closure techniques at a high-volume tertiary care center. Secondly, we wanted to determine the incidence of different grades of pancreatic fistulas after distal pancreatectomy and, thirdly, to identify clinicopathologic factors that contribute to pancreatic fistula formation.

Materials and Methods

A retrospective review of clinical charts (January 1994 to December 2000) and a prospectively collected database (January 2001 to February 2008) identified 462 patients who underwent distal pancreatectomy, with or without splenectomy. Clinicopathologic variables were reviewed after obtaining approval by the institution's internal review board. Cardiac history was defined as patients with a history of myocardial infarction, coronary artery disease requiring bypass grafting or coronary stents, atrial fibrillation, or more than two medications to control their hypertension. Operative notes and postoperative hospital and outpatient records were reviewed for all patients. A Jackson–Pratt or Blake drain was routinely left at the time of operation. A drain amylase three times the upper limit of normal (>300 U/L) was considered amylase-rich fluid.

Definition of Pancreatic Fistula

Pancreatic fistula was defined as outlined by the international study group (ISGPF) classification.3 According to the ISGPF classification, a grade A fistula requires little change in management or deviation from the normal clinical pathway. Therefore, in our institution, a grade A fistula was defined as >30 cc per day of amylase-rich fluid (>300 U/L), which resulted in a delay in drain removal (>6 and <21 days). If a patient was discharged with a drain in place regardless of the character of the output, it was considered a grade A fistula. A grade B fistula included the surgically placed drain(s) >22 days, placement of a new drain by interventional radiology, or re-admission for the fistula. Any patient with a collection of amylase-rich fluid or abscess in the vicinity of the pancreatic stump was considered to have a pancreatic fistula. A grade C fistula includes the need/use of total parenteral nutrition (TPN) or reoperation for the pancreatic fistula.

Surgical Technique

Fish-Mouth With or Without Pancreatic Duct Ligation

The pancreas was transected with electrocautery or a ten-blade scalpel. The center was beveled in so as to be able to bring the anterior and posterior surfaces together with interrupted 3′0 silk U stitches. A single U stitch of 4′0 silk was used to ligate the pancreatic duct if the duct could be identified.

Falciform Patch

The mesothelial membrane of the falciform ligament was excised and applied to the cut margin of the pancreas with fibrin glue. The pancreatic transection margin was controlled with silk sutures after ligation of the pancreatic duct.

Fibrin Glue or Omental Patch

Pancreatic transection with pancreatic duct ligation, if the pancreatic duct was identified, and fish-mouth closure as described above were performed. Either fibrin glue or omentum was used to cover the pancreatic transection line.

Stapler

The pancreas was transected utilizing an endovascular stapler or a TIA stapler. More recently, a reinforcing bioabsorbable buttress mattress to the staple line was utilized (Seamguard™).

Statistics

Statistical analysis was performed utilizing SAS version 9. For the univariate analyses, we applied the chi-square test for binary and categorical outcomes and used the *t* test to

compare continuous variables. For the multivariate analyses, we applied a multivariate logistic regression model that included patient demographics and clinical variables of interest. *P* value of less than 0.05 was considered statistically significant.

Results

Patient Demographics and Pathologic Factors

During the 14 years of this study, 462 patients underwent a distal pancreatectomy. The annual distribution is depicted in Fig. 1. The patient demographics and clinicopathologic factors evaluated are outlined in Table 1. The median age of the patients was 58 years, and 60% were women. The three most common indications for distal pancreatectomy in our series were mucinous cystic tumors (19%), neuroendocrine lesions (18%), and pancreatic adenocarcinoma (15%).

Intraoperative Factors

Distal pancreatectomy with splenectomy were performed in 321 (70%) patients. Additional organs were resected in 116 (25%) patients, and a laparoscopic procedure was performed in 13 (3%) patients. Only 364 of 462 patients had an accurate blood loss recorded with a median estimated blood loss of 400 mL.

Postoperative Factors

The mortality was 0.8%. Four patients died postoperatively, two women and two men. One male patient died of other injuries resulting from vehicular trauma. One woman developed a retroperitoneal bleed and rupture of her transplanted kidney postoperatively. One woman died of a postoperative aspiration pneumonia, and one man died of a post-operative cardiac arrest related to his sarcoid cardiomyopathy.

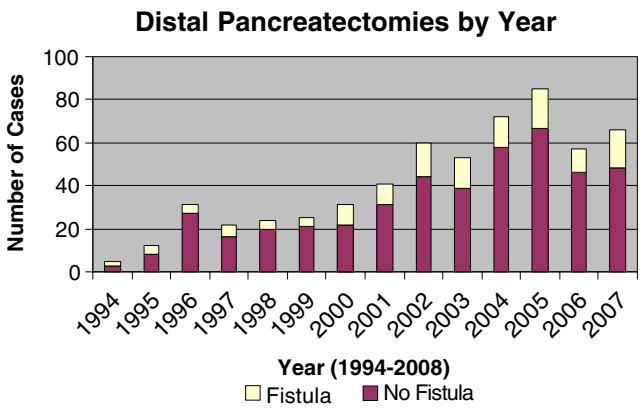

Figure 1 Number of distal pancreatectomies performed by year over time.

The overall pancreatic fistula rate was 29% (133/462). Almost half of the patients (227/462, 49%) had a fish-mouth suture closure, of whom 158 had a separate pancreatic duct ligation. Pancreatic duct ligation did not significantly reduce the rate of pancreatic fistula (29% vs. 30%). A stapled closure with or without staple line reinforcement was performed in 19% of patients (86/462). The type of stump closure or location of the pancreatic transection, based on length, width, and thickness of the pathologic specimen, did not affect the pancreatic fistula rate.

The most common type of fistula was a grade B fistula (52%, 69/133), requiring an operative drain for >22 days, an interventional drain placement or a re-admission. The most devastating fistulas, grade C fistulas, comprised only 4% (6/133) of all pancreatic fistulas and affected only 1% (6/462) of all patients undergoing a distal pancreatectomy (Table 2). The type of stump closure did not significantly affect the grade of fistula observed.

On univariate analysis, BMI>30 kg/m^2, a cardiac history, a prolonged operative time, and an increased length of stay were significant. On multivariate analysis, BMI>30 kg/m^2, male gender, and an additional procedure were significant predictor factors for a pancreatic fistula (Table 3).

Discussion

Despite significant improvements in the short-term outcome after pancreatic operations, pancreatic fistula following distal pancreatectomy continues to be a clinically relevant problem. In the current series, the mortality after distal pancreatectomy is 0.8%, similar to the mortality documented in recent reports (Table 4). The number of distal pancreatectomies performed per year has increased steadily at our institution. However, the yearly pancreatic fistula rate calculated has not deviated significantly from an annual rate of 29%, despite advances in perioperative care and the utilization of various stump closure techniques.

Our series represents the largest series of consecutive distal pancreatectomies reported from a single institution. Our pancreatic fistula rate of 29% is higher than the 5–26% cited in other series (see Table 4). This discrepancy may be due to our strict definition of pancreatic fistula, which used the ISGPF guidelines, whereas other series had variable definitions for pancreatic fistula. Specifically, no definition for pancreatic fistula was outlined in the series documenting a 5% pancreatic fistula rate.[5] Several series have quoted a low to non-existent pancreatic fistula rate when utilizing staplers with staple line reinforcement; however, they have included only a small numbers of

Table 1 Clinicopathologic Factors of the Entire Cohort

	All patients (n=462)	No fistula (n=229)	Fistula (n=133)	P value
Median age (range)	58 (11–92 years)	58 (11–92 years)	55 (18–82 years)	0.08
Gender (female)	276 (60%)	204 (74%)	72 (26%)	0.12
BMI>30 kg/m^2	101 (22%)	60 (59%)	41(41%)	0.003
Albumin<3.5 mg/dL	218 (47%)	153 (70%)	65 (30%)	0.64
Cardiac history	102 (22%)	81 (80%)	20 (20%)	0.02
History of DM	57 (12%)	43 (77%)	13 (23%)	0.33
Splenectomy	321 (69%)	226 (70%)	95 (30%)	0.56
Additional organ resection	116 (25%)	76 (23%)	40 (31%)	0.11
Cholecystectomy	28 (6%)	20 (71%)	8 (29%)	
Colon/SBR	21 (4.5%)	6 (29%)	15 (71%)	
Stomach	18 (4%)	13 (72%)	5 (28%)	
Adrenal	7 (1.5%)	3 (43%)	4 (57%)	
Other	42 (9%)	34 (81%)	8 (19%)	
Laparoscopic procedure	13 (3%)	8 (62%)	5 (38%)	0.31
Pancreatic stump closure				0.73
Suture (fish-mouth) with PD ligation	158 (34%)	112 (71%)	46 (29%)	
Suture (fish-mouth) no PD ligation	69 (15%)	48 (70%)	21 (30%)	
Falciform patch	108 (23%)	78 (72%)	30 (28%)	
Suture + fibrin glue	18 (4%)	11 (61%)	7 (39%)	
Suture + omental patch	21 (5%)	17 (81%)	4 (19%)	
Stapled	41 (9%)	31 (76%)	10 (24%)	
Stapled with Seamguard	45 (10%)	30 (66%)	15 (33%)	
Operative time (min)	189, 170	184, 167	202, 175	0.047
Mean, median (range)	(54–660)	(54–660 min)	(60–658 min)	
Pathology				0.34
Mucinous cystic tumor (cancer, borderline, adenoma)	88 (19%)	59 (67%)	29 (33%)	
Neuroendocrine	84 (18%)	59 (70%)	25 (30%)	
Pancreatic adenocarcinoma	68 (15%)	50 (74%)	18 (26%)	
Chronic pancreatitis + pseudocyst	57 (12%)	41 (72%)	16 (28%)	
IPMN	45 (10%)	36 (80%)	9 (20%)	
Serous cystadenoma	38 (8%)	28 (74%)	10 (26%)	
Normal pancreas as part of another operation	22 (5%)	16 (73%)	6 (27%)	
Metastases (renal and melanoma)	12 (3%)	5 (42%)	7 (58%)	
Solid pseudopapillary	9 (2%)	6 (66%)	3 (33%)	
Trauma	7 (1.5%)	5 (71%)	2 (29%)	
Other	32 (6.5%)	16 (66%)	8 (33%)	
Length of pancreas (cm), mean, median (range)	9.2, 9.0 (0.4–26 cm)	9.2, 9 (0.4–26 cm)	9.0, 9 (2–25 cm)	0.53
Width of pancreas (cm), mean, median (range)	4.6, 4.5 (1.8–15 cm)	4.6, 4.3 (0.8–15 cm)	4.7, 4.5 (1.6–11 cm)	0.51
Thickness of pancreas (cm), mean, median (range)	2.6, 2.5 (0.5–9.0 cm)	2.6, 2.5 (0.5–9 cm)	2.5, 2.4 (0.6–6.0 cm)	0.55
Overall mortality	4 (0.8%)			
LOS (days) mean, median (range)	7.5, 6 (0–60 days)	7.1 6 (0–42 days)	8.5, 7 (3–60 days)	0.036

patients.[6,7] Jimenez et al. claimed a 0% pancreatic fistula rate in 13 cases, while Hawkins et al. reported a pancreatic fistula rate of 3.5% in 29 cases.[6,7] We were unable to confirm these findings: Our pancreatic fistula rate was 33% in the 45 cases performed with staple-line reinforcement. Neither of those other two studies utilized the strict ISGPF pancreatic fistula definitions, possibly accounting for the discrepancy. Truty et al. utilized saline-coupled radiofrequency ablation in a swine model with a significant decrease in the pancreatic fistula rate. This method would need to be studied further in humans.[8] In short, we were unable to identify any superior techniques for closing the pancreatic stump.

The median age was 58 years old, and female patients comprised 60% of the patients, consistent with other large series in the literature.[5,9] Splenectomy was performed in 69% of the patients, which is slightly lower than the other large series where splenectomies were performed in 76–91% of the patients.[5,9–12] Median operative time was 189 min, shorter than the 245–258 min documented by Kleeff et al. and Lillemoe et al., which may reflect the smaller proportion of patients who underwent a splenectomy

Table 2 Characteristics of Patients with Different Types of Fistulas

	Type A (n=58)	Type B (n=69)	Type C (n=6)	P value
Age (mean, median, range)	53, 51 (23–78)	55, 56 (18–82)	58, 53 (49–74)	0.56
Female (%)	25 (41%)	34 (56%)	2 (3%)	0.64
BMI>30 kg/m^2	19 (46%)	20 (49%)	2 (5%)	0.89
Albumin <3.5	4 (28%)	10 (72%)	0 (0.00%)	0.53
Cardiac history	7 (35%)	12 (60%)	1 (5%)	0.70
History of DM	9 (69%)	4 (31%)	0 (%)	0.13
Splenectomy	37 (39%)	52 (55%)	6 (6%)	0.10
Additional organ resection	18 45(%)	21 (51%)	1 (2%)	0.75
Cholecystectomy	3	5	0	
Colon/SBR	6	8	1	
Stomach	2	3	0	
Adrenal	1	3	0	
Other	6	2	0	
Laparoscopic procedure	2 (40%)	3 (60%)	0 (0.00%)	0.87
Pancreatic stump closure				0.21
Suture (n=67)	34 (51%)	31 (46%)	2 (3%)	
Falciform patch (n=30)	15 (15%)	14 (47%)	1 (3%)	
Suture + fibrin glue (n=7)	1 (14%)	6 (86%)	0	
Suture + omental patch (n=4)	2 (50%)	2 (50%)	0	
Stapled (n=10)	1 (10%)	8 (53%)	2 (14%)	
Operative time (min)	203.2,169	206.2,185	146.5,154	0.32
Mean, median (range)	(100–658)	(85–434)	(60–200)	
Pathology				
Mucinout cystic tumor (cancer, borderline, adenoma)	16 (55%)	12 (41%)	1 (4%)	0.21
Neuroendocrine	9 (36%)	15 (60%)	1 (4%)	
Pancreatic adenocarcinoma	4 (22%)	13 (72%)	1 (6%)	
Chronic pancreatitis + psuedoscyst	7 (44%)	9 (66%)	0 (0.00%)	
IPMN	4 (44%)	3 (33%)	2 (23%)	
Serous cyst adenoma	5 (50%)	5 (50%)	0 (0.00%)	
Normal pancreas as part of another operation	2 (33%)	3 (50%)	1 (17%)	
Metastases (renal and melanoma)	3 (43%)	4 (57%)	0 (0.00%)	
Solid pseudopapillary	1 (33%)	2 (66%)	0 (0.00%)	
Trauma	0 (0.00%)	2 (100%)	0 (0.00%)	
Other	7 (88%)	1 (12%)	0 (0.00%)	
Length of pancreas (cm), mean, median (range)	8.5, 8.5 (3.2–14)	9.4, 9.15 (2–25)	9.6, 9 (7.3–13)	0.29
Width of pancreas (cm), mean, median (range)	4.6, 4.5 (2–9)	4.9, 4.5 (1.6–11)	4.8, 5 (2.1–7)	0.64
Thickness of pancreas (cm), mean, median (range)	2.5, 2.25 (0.8–6)	2.6, 2.5 (0.6–6)	2.6, 2 (1.5–5)	0.84
LOS (days)	9.0, 7 (3–60)	7.7, 6 (0–25)	12.2, 8 (4–26)	0.23

Table 3 Multivariate Analysis of Clinicopathologic Factors Predicting Pancreatic Fistula

	Multivariate
Age (continuous)	0.17
Male gender	0.05
BMI>30 kg/m^2	0.001
Splenectomy	0.86
Additional organ resection	0.04
Type of pancreatic stump closure	0.24
Pathology	0.52

and an additional organ resection in our series.[5,9] An additional organ was resected in 25% of our patients, as compared to 36% and 41% of patients in the Heidelberg and Hopkins groups, respectively.[5,9] Median estimated blood loss was 400 mL, which is consistent with the median EBL of 450 mL documented by Lillemoe et al., but significantly less than the 700 mL documented by Kleeff et al.[5,9] Median length of stay after distal pancreatectomy was 6 days, significantly shorter than the 10–12 days documented by the Hopkins and Heidelberg groups.[5,9] This is most likely due to our aggressive development and implementation of clinical pathways and the smaller number of additional procedures performed.

Table 4 Comparison to Other Clinical Series

Author (year)	Number of patients	Pancreatic fistula rate (%)	Mortality (%)	Prognostic factors
Lillemoe et al.[5]	235	5	<1	None identified
Fahy et al.[10]	51	26	4	Trauma Suture closure
Pannegeon et al.[11]	175	23	0	Transection at body No ligation of PD
Thaker et al.[6]	40	13	0	No staple line reinforcement
Lorenz et al.[13]	46	19		None identified
Ridolfini et al.[12]	64	22	1.5	Non-pancreatic malignancy Fibrotic pancreas Octreotide Splenectomy
Sierzega et al.[14]	132	13.6		Nutritional risk index<100
Kleef et al.[9]	302	12	2	OR time>480 min Stapler
Ferrone (2008)	462	29	<1	BMI>30 kg/m^2 Male gender Additional procedure

The three most common indications for distal pancreatectomy in our series were mucinous cystic tumors (19%), neuroendocrine lesions (18%), and pancreatic adenocarcinoma (15%), for which the pancreas is soft (normal) at the point of transection. In contrast, chronic pancreatitis and pancreatic pseudocyst, the most common indication for distal pancreatectomy in many other series, was the fourth most common indication (12%) in our series.[5,11] The transected pancreas in chronic pancreatitis is characteristically fibrotic and holds sutures more securely, a factor believed to be responsible for a lower leak rate (11).

On multivariate analysis, male gender, an additional procedure, and a BMI>30 kg/m^2 were the only significant predictors of a pancreatic fistula (Table 3). Increased technical difficulty with a male body habitus and heavier patients may explain the increased pancreatic fistula rate for this subset of patients. Prognostic factors documented by other published studies (Table 4) were not significant factors in our series. Pancreatic pathology, such as traumatic transection, non-pancreatic malignancy, or chronic pancreatitis, did not significantly impact the pancreatic fistula rate or demonstrate a significant difference in the type of pancreatic fistula. Surprisingly, patients undergoing a distal pancreatectomy for chronic pancreatitis with a firm pancreas did not have a lower fistula rate than patients with a "soft" pancreas (28% vs. 29%).

Prolonged operative time, prognostic in the Heidelberg series, was significant on univariate analysis but not on multivariate analysis. An additional procedure did significantly increase the rate of pancreatic fistula. When analyzing subsets of patients undergoing an additional procedure, patients undergoing a colonic or small-bowel resection had a pancreatic fistula rate of 71% (15/21) compared to 28% (5/18) for patients undergoing an additional gastric resection. This could be due to the paucity of bowel or omentum to seal the pancreatic stump with a living mesothelial patch. The site of pancreatic transection was predictive of a fistula in Belghiti's group,[11] but we were unable to document a length, width, or thickness cutoff predictive of pancreatic fistula formation.

Conclusion

In conclusion, this series has demonstrated that distal pancreatectomy can be performed safely for a variety of different conditions, with a low mortality of 0.8%. However, a postoperative pancreatic stump leak and resultant fistula continue to be a significant clinical problem for 29% of the patients in our experience. Grade A fistulas, requiring a prolonged period of drainage before spontaneous closure, occurred in 13% (58/462) of the patients. A more significant grade B fistula developed in 15% of the patients (52% of the patients who developed a pancreatic fistula). Only 1% (6/462) of the patients developed a grade C fistula, requiring a reoperation or a hospital admission and TPN treatment. No mode of pancreatic stump closure, including stapling with staple line reinforcement, was able to decrease the pancreatic fistula rate significantly from 29%. Pancreatic fistula and the method for stump closure continues to be a significant clinical challenge.

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Discussion

Pancreatic Fistula Rates After 462 Distal Pancreatectomies: Staplers Do Not Decrease Fistula Rates

Michael G. Sarr, M.D. (Rochester, MN): Dr. Ferrone, why aren't we smarter? We ought to be able to solve this problem of leaks, but it is consistent across the board, isn't it? So I have three questions.

There must be some up front bias in your study. For a thick gland, I doubt that some of your surgeons would be willing to put a stapler across. Why don't you comment on that. Second, there are some new techniques, such as Tissuelink®, and there is an experimental study that supports that. Is octreotide of any benefit? And third, why did you leave a drain?

Cristina R. Ferrone, M.D. (Boston, MA): To answer your first question, I think that there is definitely a bias, and we have actually only been using the Seamguard for the last two years. So there is some bias there, but most of those numbers are within the last two years. But it is true, when you put the stapler across and the pancreas is very thick and you feel the staples are just going to rip through, we tend to switch over and perform the fishmouth closure technique. We stratified by length, width, and thickness of the pancreas based on the pathologic specimen and weren't able to find any cutoff which predicted an increased risk of pancreatic fistula, which we were very surprised at. We were hoping to find a thickness cutoff that would indicate you should not be doing this or that, but unfortunately, we did not.

In terms of the second point, I think you are absolutely right. I think Dr. Trudy from your institution has a wonderful model within the pig model, and now has some patient data, suggesting that the tissue link actually will be a method which should be prospectively tried to see if that can actually decrease the fistula rate.

In terms of octreotide, I think you probably know better than anyone, having led the pancreatic study group which included a combination of distal pancreatectomies, middle pancreatectomies, and Whipples, and unfortunately octreotide wasn't able to significantly decrease the fistula rates. Based on the data from the trial you led we have not been that aggressive about using it at our institution, at least recently, because of that data.

And your third question?

Dr. Sarr: The drain.

Dr. Ferrone: I think the thought is that just because you don't know it (the pancreatic fistula) is there doesn't mean it is not there and therefore leaving a drain we at least consider somewhat safer. You can treat the patient maybe a little bit more aggressively and prevent them from coming back with fevers and abscesses and having to get an interventional radiology drain.

L. William Traverso, M.D. (Seattle, WA): Christina, very clearly presented.

You presented the A leak and B leak rates overall but you didn't present A and B for each of the types of techniques that you used. I wonder if you did that and found that that wasn't helpful? In our experience, by providing a wider

spectrum of leak severity grades, you can find that the majority of the leaks were the chemical leaks or A's and not the clinically significant B's. I wonder if you might have some important information here?

Finally, one comment is that the original International Study Group's definition of leak, according to Bassi in 2005 published in Surgery, was not the one that you used in your slide. It has been modified since by other groups in Boston but you did not use that one either. You have modified it even further. When you submit the manuscript please use the actual ISGPS definition the way it was written, or maybe you can't if you don't have a drain in place to measure drain amylase and volume.

Dr. Ferrone: In terms of that first question, we did actually do the subset analysis, and we compared A versus B and C, to compare clinically low impact versus the clinically high impact fistulas for all the different types of stump closure, and unfortunately we weren't able to find a difference.

I presented the ISGPF classification copied out of the text of the paper in 2005 by Dr. Bassi. Based on the ISGPF classification we utilized the grade A definition, to define a grade A fistula. And so we did not modify that in the sense that the clinical A classification was defined as no change in the managementand so for us the change in management would have been to leave a drain for more than five days.

Dr. Traverso: I like your modification, by the way, but, for the record: if we are all going to use the same definition, we should use it. The ISGPS definition is currently undergoing some clarification.

Roger G. Keith, M.D. (Saskatoon, SK, Canada): Do you have from your database any information on proximal duct status or proximal disease, which will likely contribute to your fistula rate?

Dr. Ferrone: We, unfortunately, do not. Not all of the patients had good MRCP's or CT scans that we could review to be able to evaluate that proximal duct.

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Surgical Management of Early-Stage Hepatocellular Carcinoma: Resection or Transplantation?

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Abstract
Background The surgical management of hepatocellular carcinoma in patients with well-compensated cirrhosis is controversial. The purpose of the current study was to compare the outcome of patients with well-compensated cirrhosis and early stage hepatocellular carcinoma treated with initial hepatic resection versus transplantation.
Methods Between 1985 and 2008, 245 patients underwent hepatic resection, and 134 patients underwent liver transplantation for early stage hepatocellular carcinoma. All patients had well-compensated cirrhosis. Prognostic factors were evaluated using univariate and multivariate analyses; survival was calculated using the Kaplan–Meier method.
Results Compared with transplantation, patients undergoing resection had larger tumors and a higher incidence of microscopic vascular invasion. Transplantation was associated with better 5-year disease-free and overall survival compared with resection. Hepatitis status, presence of microscopic vascular invasion, and tumor size were predictors for recurrence,

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while the presence of microscopic vascular invasion and tumor size conferred an increased risk of death. The disease-free survival advantage with transplantation was more pronounced in hepatitis C patients compared with non-hepatitis and hepatitis B patients. The overall survival advantage with transplantation persisted in cases of solitary lesions ≤3 cm, but was attenuated in patients with a MELD score ≤8.

Conclusion In well-compensated cirrhotic patients with early stage hepatocellular carcinoma, transplantation was associated with longer disease-free and overall survival. Patients undergoing resection did, however, have tumors with more advanced pathologic features. Patients best suited for initial resection as the treatment of hepatocellular carcinoma were those with a MELD score ≤8 without evidence of hepatitis.

Keywords Hepatocellular carcinoma · Early stage · Resection · Transplantation · Outcome

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common malignancy worldwide[1] and is the third largest cause of cancer-related deaths.[2] The incidence of HCC has been steadily rising with a near doubling of incident cases in the Western Hemisphere over the last two decades.[3] Although the majority of newly diagnosed cases are advanced,[4] through implementation of screening programs, more patients may be recognized with early stage disease.[4,5] Early stage HCC has generally been defined as the "Milan criteria:" a single tumor of 5 cm or less or no more than three tumor nodules, each of which is 3 cm or less in diameter.[6,7]

Whereas liver transplantation has been established as the optimal strategy for early stage HCC and poorly compensated cirrhosis/portal hypertension,[7–9] the preferred therapeutic approach for early stage HCC and well-compensated cirrhosis is not established. In many centers, the traditional approach has been hepatic resection,[10–12] since resection can be performed without delay and with low mortality.[13] However, as hepatic resection extirpates only the tumor and not the underlying predisposed cirrhotic liver, the rate of intrahepatic recurrence has been high.[14–16] Some investigators have therefore argued that liver transplantation, which treats both the tumor and the cirrhosis, may be a better therapeutic approach even in patients with early stage HCC.[17–20] Consensus treatment recommendations for patients with early stage HCC therefore remain poorly defined.

No prospective studies have been performed to address the question of resection versus transplantation for early stage HCC. In addition, such studies are unlikely to succeed given the difficulties in randomizing patients to resection versus transplantation.[21] While several retrospective studies[6,22–27] have attempted to compare outcome of hepatic resection versus liver transplantation, the results have been inconclusive. While some reports have suggested similar survival following resection versus transplantation,[6,18,25,26] other studies[23,24,27] have noted a trend toward improved survival with transplantation. These studies were limited, however, by small sample size and thus a potential lack of statistical power. In addition, most previous studies included all patients who were resected or transplanted for HCC and, in turn, failed to limit comparisons to only patients with early stage HCC who potentially might be eligible for both treatments. As such, the objective of the current study was to compare the results of initial hepatic resection versus primary transplantation for early stage HCC in a cohort of patients with well-compensated cirrhosis. Specifically, we sought to characterize long-term disease-free and overall survival after hepatic resection versus transplantation for early stage HCC. In addition, we attempted to identify potential predictors of outcome in each treatment group.

Patients and Methods

Between January 1985 and January 2008, 379 patients with early stage HCC who underwent resection (n=245) or transplantation (n=134) were identified from six major hepatobiliary/transplant centers in the United States [Johns Hopkins School of Medicine, Baltimore, MD (resection, n=36; transplantation, n=47); The University of Maryland Medical Center, Baltimore, MD (resection, n=2; transplantation, n=14)] and Europe [Department of Transplantation and Visceral Surgery, University Hospitals of Geneva, Geneva, Switzerland (resection, n=19; transplantation, n=25); Hepato-Biliary-Pancreatic and Transplantation Center, Curry Cabral Hospital, Lisbon, Portugal (resection, n=9; transplantation, n=48); Unit of Hepato-Biliary-Pancreatic Surgery, A.O. Ordine Mauriziano, Torino, Italy (resection, n=124; transplantation, n=0]; Department of Surgery, Liver Unit, Scientific Institute San Raffaele, Vita-Salute San Raffaele University, Milan, Italy (resection, n=55; transplantation, n=0)]. The study was approved by the Institutional Review Boards of the respective institutions. Only patients with early stage HCC treated with curative intent hepatic resection or liver transplantation were included in the current study. Early stage HCC was defined as a solitary HCC tumor of 5 cm or less or no more than three tumor nodules, each of which is 3 cm or less in diameter,[6,7] as well as absence of radiologic evidence of macroscopic portal vein or hepatic vein invasion.[28,29] Patients who did not fulfill these criteria were excluded. All patients were evaluated with a baseline history and

physical examination, serum laboratory tests, and a computed tomography or magnetic resonance imaging scan of the abdomen and pelvis. Following hepatic resection or transplantation, all patients were regularly followed and prospectively monitored for recurrence.

Data Collection

Data were collected using standardized data sheets that were subsequently synthesized and analyzed by the coordinating center (Johns Hopkins School of Medicine). The following data were collected for each patient: demographic; details of primary tumor treatment (e.g., hepatic resection versus transplantation); history of previous loco-regional therapy [e.g., transarterial chemoembolization (TACE); radiofrequency ablation (RFA), etc.]; primary tumor size, number, grade; presence of microscopic vascular invasion; hepatitis status; Child-Turcotte-Pugh score; serum laboratory exams [e.g., alpha-fetoprotein [AFP], international normalized ration (INR), bilirubin, creatinine, etc.]; postoperative complications; most recent follow-up date; vital status (e.g., alive versus dead); recurrent disease status (e.g., no evidence of disease versus recurrence); date of recurrence; date of death. Tumor grade was assessed using the scheme outlined by Edmondson and Steiner.[30] Microscopic vascular invasion was defined as the presence of tumor emboli within the central vein, the portal vein, or large capsular vessels or involvement of the segmental or sectoral branches of the portal vein or the hepatic veins.[28,31] The serologic presence of hepatitis B virus (HBV) antigen was considered evidence of HBV.[32,33] The serologic presence of hepatitis C virus (HCV) antibody was considered evidence of HCV infection. Complications were scored according to the Clavien grading system.[34] MELD score was calculated using the following formula: $\mathrm{MELD} = 9.57 \times \log_e(\text{creatinine mg/dL}) + 3.78 \times \log_e(\text{bilirubin mg/dL}) + 11.20 \times \log_e(\mathrm{INR}) + 6.43$.[35]

Statistical Analyses

Demographic variables of interest in transplant and resection patients were compared using Student's t test, Pearson's chi square test, or Fisher's exact test as appropriate. The outcome variables were recurrence (disease-free survival) and death (overall survival). Time to outcome was calculated using the date of diagnosis until the date of the event or the date of last follow-up time for patients who did not experience the event. Overall Kaplan–Meier survival curves were constructed for resection versus transplantation. The effect of demographic variables on disease-free and overall survival were initially examined using the Kaplan–Meier log-rank test. Univariate analyses were performed independently for resection and transplant. All variables significant to $P<0.20$ for the outcome were included in a Cox proportional hazards model using shared frailty to compensate for potential institutional effects.[36] Interaction terms were created for variables that were highly significant for only one surgical approach. Backward selection was performed to retain significant variables; age and MELD score were retained in the model regardless of significance. Separate stratified survival analyses were performed based on tumor size, tumor number, hepatitis status, and MELD score. Evaluation of perioperative (30 and 60 day) mortality was performed using logistic regression employing backward selection using variables significant to $P<0.20$ on bivariate analysis.

Results

Patient Demographics and Tumor Characteristics

Between 1985 and 2008, 379 patients with early stage HCC were identified who underwent hepatic resection ($n=245$) or liver transplantation ($n=134$). For those patients undergoing hepatic resection, the extent of the hepatic resection was wedge ($n=43$, 18%), segmentectomy ($n=35$, 14%), hemihepatectomy ($n=35$, 14%), or an extended hepatectomy ($n=9$, 4%). The clinicopathologic characteristics of the study patients are presented in Table 1. Patients who underwent hepatic resection were more likely to be older than transplantation patients (mean age, 65 years versus 55 years, respectively; $P<0.0001$). While more transplantation patients had alcohol abuse as the etiology of their underlying liver disease (resection: 27% versus transplantation: 54%; $P<0.001$), the incidence of hepatitis was comparable between the two groups ($P=0.19$). Patients in the two groups also had similar preoperative AFP levels ($P=0.48$); however, as expected, transplantation patients were more likely to have a higher preoperative MELD score ($P<0.001$).

The mean time from HCC diagnosis to surgical intervention was predictably shorter in the resection group (25 days) compared with the transplantation cohort (209 days; $P<0.001$). In turn, significantly more transplantation patients had received some type of preoperative loco-regional therapy (resection: 11% versus transplantation: 46%; $P<0.001$). Loco-regional liver-directed therapies included TACE ($n=64$, 17%), RFA ($n=18$, 5%), and ethanol injection ($n=4$, 1%). There was also a significant trend in the utilization of resection versus transplantation. Specifically, while the majority of hepatic resections (71%) were performed in the pre-MELD era (e.g., before February 27, 2002), most transplantations occurred in the post-MELD era (72%; $P<0.001$).

On final pathologic analysis, the majority of patients had solitary lesions ($n=331$, 87%). Of these solitary tumors,

Table 1 Demographic Data and Tumor Characteristics

Variable	Resection (n=245)	Transplantation (n=134)	P Value
Gender (M/F)	203/42	110/24	<0.001
Age (median, years)	65	55	<0.001
Child Pugh Class			0.003
A	233 (90%)	75 (77%)	
B	12 (10%)	59 (23%)	
Hepatitis			0.190
No hepatitis	40 (20%)	31 (23%)	
Hepatitis B only	36 (18%)	13 (10%)	
Hepatitis C only	113 (57%)	81 (60%)	
Hepatitis B and C	10 (5%)	9 (7%)	
Alcohol Use	67 (27%)	73 (54%)	<0.001
Treatment in post-MELD era	72 (29%)	96 (72%)	<0.001
MELD score	9.1±2.5	11.0±3.3	<0.001
Preoperative AFP level	276±1120	198±480	0.447
Treatment delay (days)	25±75	209±287	<0.001
Preoperative treatment	27 (11%)	62 (46%)	<0.001
Largest tumor size (>3 cm)	149 (61%)	36 (27%)	<0.001
Tumor number (median)	1.1	1.3	<0.001
Bilobar tumor location	3 (1%)	11 (15%)	<0.001
Histological grade			<0.001
Well differentiated	36 (15%)	54 (43%)	
Well moderately differentiated	8 (3%)	7 (6%)	
Moderately differentiated	118 (50%)	56 (45%)	
Moderately poorly differentiated	59 (25%)	2 (2%)	
Poorly differentiated	15 (6%)	6 (5%)	
Presence of microvascular invasion	71 (29%)	12 (9%)	<0.001
Satellite lesions	60 (25%)	11 (8%)	<0.001

most were >3 cm (n=183, 55%). Patients who underwent hepatic resection were more likely to have a tumor >3 cm in size (resection: 61% versus transplantation: 27%; P< 0.001), but were less likely to have bilateral disease (resection: 1% versus transplantation: 11%; P<0.001). Patients undergoing resection were also more likely to have evidence of satellitosis on final pathologic exam (resection: 25% versus transplantation: 8%; P<0.001).

Perioperative Morbidity and Mortality

The median length of stay was 12 days (range, 3 to 140 days) for the entire cohort of 379 patients with early stage HCC. Patients who underwent hepatic resection (median: 11 days, range 3 to 74 days) had a shorter hospital stay compared with patients who underwent transplantation (median: 19 days, range 4 to 140 days; P=0.007). Transplantation patients also had a higher rate of perioperative complications (65%) compared with hepatic resection patients (49%; P=0.001). The majority of complications in both groups were minor (Clavien grade 1–2; resection: 51% versus transplantation: 62%; P=0.34).

Of the 379 patients, six patients died within 30 days of surgery yielding a perioperative mortality rate of 1.6%. Thirty-day mortality was comparable in patients who underwent resection (n=4, 1.6%) versus transplantation (n=2, 1.5%; P>0.05). Because patients with underlying liver disease may suffer from delayed liver insufficiency and death, 60-day mortality was also assessed. An additional four patients died for an overall 60-day mortality rate of 2.6% (resection: n=6, 2.9% versus transplantation: n=6, 2.2%; P=0.13). The only factor associated with an increased risk of 60-day perioperative mortality was MELD score (OR=1.23, 95% CI 0.99–1.54, P=0.06).

Disease-Free Survival

Thirty-seven percent of patients (n=140) had documented recurrence (resection: 50% versus transplantation: 14%), with a median follow-up time of 2.5 years (2.3 years for resection; 3.3 years for transplantation). The overall 1-, 3-, and 5-year disease-free survival was 91%, 72%, and 54%, respectively. Disease-free survival was significantly better after liver transplantation (1-, 3-, and 5-year: 96%, 89%, and 82%, respectively) compared with hepatic resection (1-, 3-, 5-year: 88%, 62%, and 40%, respectively (P=<0.001; Fig. 1). The median disease-free survival time after hepatic resection was 45 months (95% CI, 40 to 54 months); in contrast, the median disease-free survival following transplantation had not been reached (P<0.001).

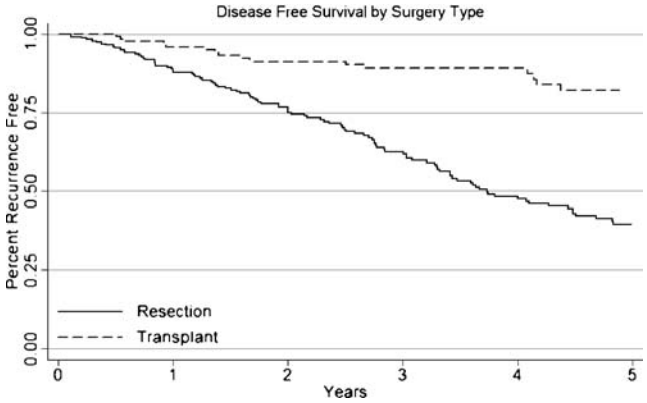

Fig. 1 Disease-free survival was significantly better after liver transplantation (1-, 3-, and 5-years: 96%, 89%, and 82%, respectively) compared with liver resection (1-, 3-, 5-years: 88%, 62%, and 40%, respectively ($P<0.001$).

On univariate analyses, several factors were associated with disease-free survival. Among all patients, those patients with hepatitis, tumor size, and microscopic vascular invasion (all $P<0.05$) had an increased risk of recurrence. On multivariate analysis, hepatitis status (HR= 2.50, 95% CI 1.47–4.24, P=0.001), microscopic vascular invasion (HR=3.90, 95% CI 2.20–6.90, $P<0.001$), tumor size (HR=2.6, 95% CI 1.44–4.72, $P=0.002$), and tumors >3 cm (HR=2.37, 95% CI 1.35–4.17, $P=0.003$) each remained independently associated with risk of recurrence (Table 2). Type of surgical approach also was a strong predictive of recurrence. Specifically, even after controlling for the aforementioned risk factors as well as age and MELD score, those patients treated with transplantation had a more than one-half reduction in the risk of recurrence compared with patients treated with hepatic resection (HR= 0.42, 95% CI 0.20–0.86, $P=0.018$).

In order to better evaluate the relative contribution of hepatic resection versus transplantation on the risk of recurrence, stratified analyses were performed. Transplantation was associated with an improvement in disease-free survival in patients with no hepatitis (5-year disease-free survival, resection: 60% versus transplantation: 86%; $P=0.03$) and HCV (5-year disease-free survival, resection: 35% versus transplantation: 84%; $P<0.001$; Fig. 2). However, the relative disease-free survival benefit of transplantation versus resection was attenuated in patients with no hepatitis (difference of 26%) versus those with HCV (difference of 49%). There was no statistically significant difference in disease-free survival in patients with HBV (5-year disease-free survival: resection: 27% versus transplantation: 58%; $P=0.10$).

Table 2 Prognostic Factors for Disease-Free Survival in Resection and Transplantation Patients

Variable	Risk Ratio of Recurrence (95% Confidence Interval)	*P* Value
Treatment with transplantation	0.42 (0.20–0.86)	0.0018
Tumor size>3 cm	2.37 (1.35–4.17)	0.003
Microvascular invasion	3.99 (2.20–6.90)	<0.0001
Tumor number	2.61 (1.44–4.72)	0.002
Hepatitis B positive	2.50 (1.47–4.25)	0.001

Overall Survival

The median overall survival for the entire cohort of 379 patients with early stage HCC was 62 months, and the 1-, 3-, and 5-year overall survival rates were 92%, 74%, and 52%, respectively. There was a difference in overall long-term survival between patients who were treated primarily with hepatic resection versus liver transplantation (Fig. 3). Specifically, median survival was 55 months in the resection group versus 120 months in the transplant group ($P=0.03$).

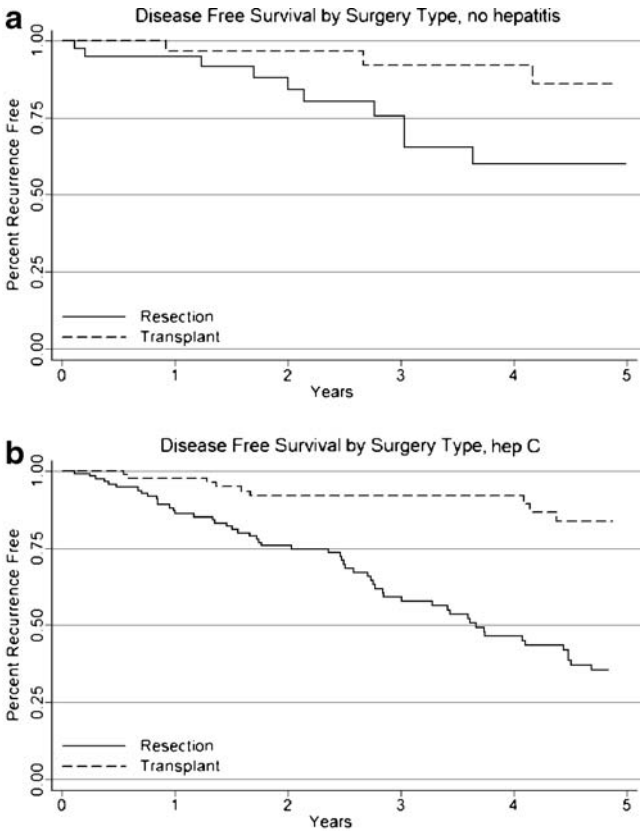

Fig. 2 Transplantation was associated with an improvement in disease-free survival in patients with (**a**) no hepatitis and (**b**) hepatitis C (both $P<0.05$). However, the relative disease-free survival benefit of transplantation versus resection was attenuated in patients with no hepatitis versus those with hepatitis C.

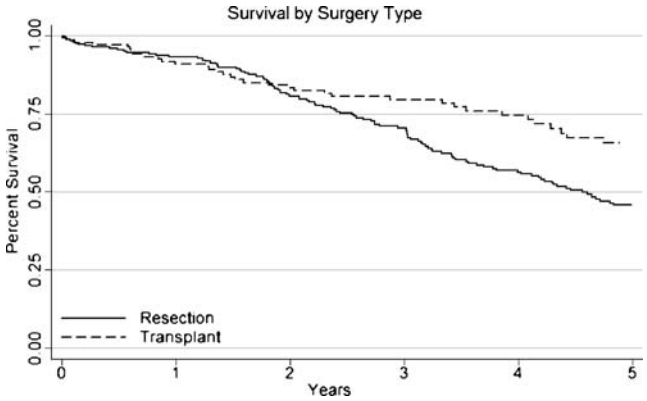

Fig. 3 Overall survival was significantly better after liver transplantation (1-, 3-, and 5-years: 91%, 79%, and 66%, respectively) compared with liver resection (1-, 3-, 5-years: 93%, 71%, and 46%, respectively (both $P<0.001$).

Factors associated with overall survival included tumor grade (HR=2.81, 95% CI 1.14–6.91, $P=0.02$) and presence of microscopic vascular invasion (HR=3.37, 95% CI 2.12–5.35, $P<0.001$; Table 3). Liver resection versus transplantation was also a strong predictor of worse overall survival. On multivariate analyses, patients who underwent hepatic resection were at a threefold increased risk of death compared with patients who were treated with transplantation (HR=3.16, 95% CI 1.51–6.52, $P=0.002$). Although recurrence was more frequent following hepatic resection, the implications of recurrent disease were more severe in the transplantation group. Recurrent disease in transplant patients was associated with an increased risk of death compared with recurrent disease in patients having undergone hepatic resection (HR=3.60, 95% CI 1.70–7.64, $P=0.001$). This finding may partly be explained by the fact that liver resection patients were likely to have recurred locally in the liver and thus were more likely to have undergone salvage transplantation (1%), re-resection (9%), or local liver-directed therapy (34%).

Stratified survival analyses were performed to identify the relative effect of hepatic resection versus transplantation on overall survival in specific subsets of patients. In patients with no history of hepatitis, 5-year overall survival following hepatic resection was 37% versus 74% for patients who underwent transplantation ($P=0.006$). Similarly, when analyses were limited to patients with a solitary lesion ≤3 cm, transplantation was associated with an improved 5-year survival (resection: 48% versus transplantation: 79%, $P<0.001$). While transplantation continued to be associated with an improved 5-year survival in patients with MELD score ≤8, the effect was not significant (resection: 41% versus transplantation: 69%, $P=0.21$; Fig. 4).

Table 3 Prognostic Factors for Overall Survival in Resection and Transplantation Patients

Variable	Risk Ratio of Recurrence (95% Confidence Interval)	P-Value
Treatment with transplantation	0.29 (0.14–0.62)	0.001
Microvascular invasion	3.37 (2.13–5.34)	<0.0001
Recurrence post transplantation	5.32 (2.12–13.4)	<0.0001
Tumor grade	2.81 (1.14–6.91)	0.024

Discussion

The management of patients with early stage HCC and well-compensated hepatic cirrhosis remains controversial. Many centers continue to recommend surgical resection; however, as experience with liver transplantation has improved and its indications refined,[7] some investigators have increasingly advocated transplantation. While a

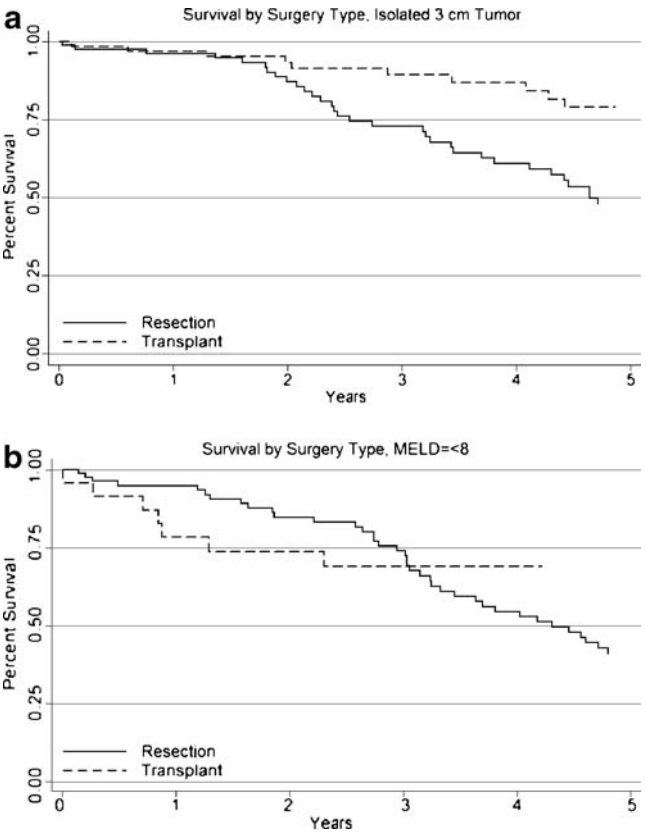

Fig. 4 (**a**) In stratified analyses limited to patients with a solitary lesion ≤3 cm, transplantation was still associated with an improved 5-year survival ($P<0.001$). (**b**) In patients with MELD score ≤8, the difference in overall survival comparing hepatic resection versus liver transplantation was not significant ($P=0.21$).

number of retrospective studies[6,22–27] have attempted to address this therapeutic dilemma, these reports have suffered from a number of shortcomings. Specifically, previous studies have included only a small number of total patients, thereby limiting the ability to detect any potential true difference in outcome between resection versus transplantation. In addition, most studies failed to restrict the study cohort to patients with only early stage HCC. Finally, virtually all previous studies have been based on single institution experiences and therefore may lack generalizability. The current study is unique in that we utilized a large, multi-institutional, multinational dataset to assess the relative benefit of resection versus transplantation in a cohort of patients specifically with early stage HCC. As such, findings from the current study provide important insight into the optimal treatment strategy for this group of HCC patients.

While perioperative morbidity and mortality rates are notoriously high in patients with decompensated cirrhosis,[37,38] the perioperative outcome for hepatic resection in the setting of well-compensated cirrhosis has been more favorable.[39,40] Several studies[39,41] have reported perioperative mortality rates of less than 5%, with some studies[13,37,40] even reporting no mortality for liver resection in well-selected patients with HCC. Aggregate data from the multiple centers included in the current study further demonstrate that hepatic resection can be associated with very low perioperative mortality (1.6%). This low mortality rate may reflect increasing expertise and sophistication in selecting appropriate patients with well-compensated cirrhosis for resection.[37,40,42,43] Of particular note was the finding that both 30- and 60-day perioperative mortality were comparable between the hepatic resection and liver transplantation groups. Even in relatively well-compensated patients, however, the risk of perioperative mortality may increase with the degree of hepatic functional impairment. Similar to previous studies,[37,42] we found that mortality following resection increased relative to the MELD score. Even in patients with well-compensated cirrhosis, MELD score may help to select the optimal candidates for hepatic resection versus those best served by immediate transplantation.

Following hepatic resection, recurrent HCC is not uncommon. In the current study, at the time of last follow-up, 50% of patients who had undergone hepatic resection had recurred. This translated into a 5-year disease-free survival of 40% following hepatic resection compared with 60% for liver transplantation. Better disease-free outcome following liver transplantation can be explained by the removal of the entire cirrhotic liver, which removes the chronic liver disease that otherwise acts as field of cancerization.[44] An alternative explanation for earlier recurrence following resection could be the presence of microscopic disease within the liver remnant that progresses postoperatively. A considerable proportion of patients may, however, survive without recurrence (40% to 50%) following hepatic resection.[14,45] The strategy of hepatic resection as a primary treatment of early stage HCC may therefore work best for patients with the lowest risk of recurrence.[46] In the current study, hepatitis status was one of the strongest predictors of disease-free survival. Whereas the relative disease-free survival benefit of transplantation versus resection was 49% in patients with HCV, the difference was only 26% in patients with no hepatitis, and there was no statistically significant difference in patients with HBV. Histologically, active hepatitis has been shown to increase the recurrence rate of HCC after hepatic resection.[47] Moreover, the natural history of patients with HCV is different from patients with no hepatitis or HBV—as HCV is more associated with the risk of multiple intrahepatic recurrent lesions.[48] Taken together, these data suggest that transplantation may be preferable in the setting of HCV with hepatic resection reserved for patients with early stage HCC and no hepatitis or HBV.

Although the incidence of microscopic vascular invasion increases with HCC tumor size,[49] microscopic vascular invasion can still be present with early stage small HCC. In the current study, 22% of patients who meet the criteria for early stage disease had evidence of microscopic vascular invasion. As reported by others,[50] microscopic vascular invasion was a strong predictor of worse disease-free and overall survival (Tables 2 and 3). As such, some investigators[12,51] have advocated using immediate salvage liver transplantation after hepatic resection in those patients discovered to have microscopic vascular invasion found on histopathologic study of the resected specimen, since these features have been related to higher early tumor recurrence. While the Barcelona Liver Cancer (BCLC) group has reported some promising preliminary results,[51] others[52] have argued that this approach may not be reasonable given that such unfavorable features also increase the probability of extrahepatic tumor recurrence.

Regarding overall survival following hepatic resection versus liver transplantation, several studies[6,18,23–27] have attempted to address this issue but have failed to provide definitive conclusions. The results of past studies have been difficult to interpret, as patients with all sizes of HCC and various stages of cirrhosis were included. In the current study, which was limited to only early stage HCC, we noted a clear superiority in 5-year overall survival following transplantation versus hepatic resection (Fig. 3). Further inspection of the survival curves demonstrated that, while overall survival was somewhat comparable over the initial 2 to 3 years, with longer follow-up, the benefits of transplantation became increasingly evident. This overall survival advantage with transplantation persisted even in cases of solitary lesions $\leq$ 3 cm, but was attenuated in

patients with a MELD score ≤8. These data were consistent with other studies that have shown MELD score to be a strong predictor of long-term survival in patients with cirrhosis undergoing hepatic resection for HCC.[37]

While liver transplantation may represent the better therapeutic option in a large number of patients with early stage HCC and well-compensated cirrhosis, there are several potential problems with such an approach. As transplantation is increasingly utilized, waiting lists get longer as demand exceeds organ availability.[5] The probability of patient drop out with a median waiting time of 6 months is 23%[22] and may increase to 30% to 50% when the waiting time exceeds 1 year.[53] In the current study, the median wait time between diagnosis and transplantation was 6.9 months. Given the general increase in wait times, more and more centers have adopted pretransplantation loco-regional therapy.[54–56] This fact was also true in our own experience, as nearly one-half (46%) of patients who underwent transplantation were treated with some type of loco-regional pretransplantation therapy. While existing data suggest that treatment before liver transplantation for HCC may reduce the risk of drop-out, its efficacy with regard to HCC recurrence and patient survival has yet to be determined.[54,57]

The current study had several limitations. Foremost was our inability to collect data on the number of patients who were listed for transplantation but subsequently had disease progression and were dropped off the waiting list. By failing to perform a true intention-to-treat analysis, the results may have been biased in favor of transplantation as a superior therapeutic approach. As such, our data showing a general superiority of transplantation over resection may only be applicable to patients who have a short waiting time (about 6 months). Another limitation involved the heterogeneity of the hepatic resection and liver transplantation cohorts (Table 1). We attempted to control for this heterogeneity by utilizing both multivariate as well as specific stratified subset analyses. However, as with all retrospective studies, the limitations of these techniques to control for both measured and unmeasured confounders is well established.[58]

In conclusion, patients with early stage HCC and well-compensated cirrhosis had better disease-free and overall survival following transplantation compared with hepatic resection. The wait time for patients in the current study, however was relatively short (6.9 months). Hepatic resection for early stage HCC was demonstrated to be safe, as the perioperatively morbidity and mortality in well-selected patients was low. Subset analyses revealed that patients best suited for initial resection for the treatment of hepatocellular carcinoma were those with a MELD score ≤8 and those without HCV. As such, the use of hepatic resection and liver transplantation should not be seen as opposing one another. Rather, different strategies should be employed depending on the degree of underlying liver function (e.g., MELD score), hepatitis status, as well as graft availability and expected waiting times in different centers.

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High Volume and Outcome After Liver Resection: Surgeon or Center?

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Abstract
Introduction In a case controlled analysis, we attempted to determine if the volume–survival benefit persists in liver resection (LR) after eliminating differences in background characteristics.
Methods Using the Nationwide Inpatient Sample (NIS), we identified all LR (n=2,949) with available surgeon/hospital identifiers performed from 1998–2005. Propensity scoring adjusted for background characteristics. Volume cut-points were selected to create equal groups. A logistic regression for mortality was then performed with these matched groups.
Results At high volume (HV) hospitals, patients (n=1423) were more often older, white, private insurance holders, elective admissions, carriers of a malignant diagnosis, and high income residents ($p<0.05$). Propensity matching eliminated differences in background characteristics. Adjusted in-hospital mortality was significantly lower in the HV group (2.6% vs. 4.8%, p=0.02). Logistic regression found that private insurance and elective admission type decreased mortality; preoperative comorbidity increased mortality. Only LR performed by HV surgeons at HV centers was independently associated with improved in-hospital mortality (HR, 0.43; 95% CI, 0.22–0.83).
Conclusions A socioeconomic bias may exist at HV centers. When these factors are accounted for and adjusted, center volume does not appear to influence in-hospital mortality unless LR is performed by HV surgeons at HV centers.

Keywords Liver resection · NIS · Propensity scores · Mortality · Volume

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Introduction

In advanced surgical procedures, improved outcomes may be directly related to volume for both hospitals and surgeons.[1–4] High volume (HV) surgeons and centers have improved outcomes with complex surgical procedures including major cancer surgery in population-based studies.[1,2,5] Examples of a volume based effect include carotid endarterectomy (CEA), coronary artery bypass grafting, aortic valve replacement, elective repair of abdominal aortic aneurysm (AAA), resection for lung cancer, esophagectomy, and pancreatic resection.[2,6,7] What is the relationship and significance of surgeon and center volume? In vascular surgery, HV surgeons have improved outcomes regardless of the volume of the hospital after CEA and AAA repair.[2] Harmon et al. found that low volume (LV) surgeons at HV centers have outcomes similar to HV surgeons at the same centers.[4]

There has been little investigation into whether differences in outcome between HV and LV surgeons could be

due to patient factors. Patient race, socioeconomic status, and geography are associated with an increased incidence of cardiovascular disease and increased mortality.[8,9] Furthermore, patients with low income, low level of education, or multiple comorbidities have worse outcomes in terms of cancer survival.[10] Patient payer status has also been associated with poor outcome, as uninsured patients have higher operative mortality for both elective and emergent AAA repair.[11] Liu et al. showed that in California, blacks and Hispanics are much less likely to be treated at a HV facility for complex surgery.[12]

The assumption for better patient outcomes by HV surgeons is that their surgical expertise is derived from repetition. However, it is possible that the mortality benefit of HV surgeons may be due in part to favorable patient characteristics. Few studies have examined the effect of surgeon volume on outcome while controlling for confounding factors within the patient population including race/ethnicity, socioeconomic status, insurance type, and comorbidity. We attempted to determine if improved outcomes by HV surgeons, specifically in the case of liver resection (LR), are reproducible when patient demographic factors are controlled at the population level.

Material and Methods

A retrospective analysis was performed using discharge records from the Nationwide Inpatient Sample (NIS) from 1998–2005 for all patients who underwent LR for which surgeon and hospital identifiers were available. The NIS is the largest US all-payer database for inpatient medical records. The Healthcare Cost and Utilization Project supports the database, which contains all patient discharge records from participating hospitals. The sample constitutes about 20% of hospital discharges in the United States. Seven million hospital discharges per year are compiled from one fifth of nonfederal community hospitals including both academic and specialty hospitals.

This study was reviewed by and received exemption from the University of Massachusetts Institutional Review Board as no personal identifiers are listed in the NIS data.

Study Population

The Clinical Modification of the International Classification of Diseases, 9th Revision (ICD-9-CM) diagnostic and procedural codes was used to identify diagnoses and procedures. Liver resection was defined as either a wedge resection or lobectomy (hemihepatectomy) for any cause. Patients who underwent LR (primary procedure code 50.22 or 50.3) were identified and included only if hospital and surgeon identifier data were available. This comprised 2,952 of 9,989 total LR (29.6%) performed in the NIS over the 8-year period. We excluded liver transplantation, total hepatectomy or other nonresectional liver procedures. We also excluded all cases with admission types of "newborn" or "trauma."

To evaluate volume, we categorized groups based on approximately equal sizes for purposes of comparison prior to any volume–outcome data analysis, as previously described.[6,13,14]

Provider Identifiers

Hospital and surgeon identifiers were used to determine the number of LR per individual hospital and surgeon. Since record sampling in the NIS does not correlate across years, a continuous single surgeon identifier was not possible. Each record or identifier is considered a unit assigned to a specific surgeon or hospital; therefore, the same surgeon may operate in each year recorded as a different individual surgeon. For example, due to the sampling in the NIS, it is possible for a surgeon's hospital to be included in 1 year and then not included the following year. Due to the variable for surgeon identifier changing twice during the study period and to concerns regarding the fidelity of surgeon and/or hospital identifiers from year to year, all volume calculations were done on a year to year basis. Extrapolation of the dataset using institutional weighting was not performed. In order to create equal cutoff groups, HV hospitals were defined as ≥20 LR/year and HV surgeons performed ≥10 LR/year.

Variables

Patient demographic characteristics compiled in the NIS were used. Age was incorporated as a continuous variable. Race was categorized by the following groups: white, black, Hispanic, or other (Asians, Pacific Islanders, and Native Americans). Race was missing in 9.5% of cases in this cohort. Income bracket, a categorical variable, was created by using the corresponding median household income from the respective residential zip code. For the years 1998–2002, quartiles were created based on demographics from 1999 where the maximum of the first quartile equaled 150% of the poverty level and the second and third quartiles were divided using the national median income as the upper limit of the second quartile. For the years 2003–2005, annual adjustments were made to separate patients equally with the same division between the second and third quartile. Due to changes in the variable during the years of study, a separate variable was created for highest income bracket. Four payer status groups were created including: Medicare, Medicaid, private insurance, or other.

To evaluate comorbidity the Elixhauser comorbidity index was used.[15] This previously validated index identifies 29

specific disease entities that are considered true preoperative comorbidities rather than complications of care.[16] Scores between zero and three were created based on how many comorbid diseases patients had.

Outcomes

Mortality was the primary endpoint examined in this study and was defined as death due to any cause prior to discharge. Secondary endpoints included cost of hospitalization and discharge location.

Case-controlled Analysis

Propensity scores were used to further investigate whether differences in outcomes at HV and LV centers were dependent on differences in patient population and disease characteristics as previously described. (Csikesz et al. in press) Candidate factors for the propensity model included available demographic and disease factors. The propensity groups markedly reduced demographic and hospital differences between patients who were treated at different volume centers.[17,18] A matched cohort was created in which all demographic/disease characteristic differences between HV and LV centers (n=767 in each group) were eliminated, allowing us to evaluate the effect of volume on mortality in a case-control fashion. Within each group, the association between each demographic or disease characteristic was determined by the χ^2 test.

Statistical Analysis

SAS 8.02 software (SAS Institute, Cary, NC, USA) was used to analyze data. Analyses were performed using SAS survey means command to account for the NIS' stratified 2-stage cluster design. The Shapiro–Wilk's test was used to evaluate continuous variables for normality. One-way analysis of variance was used to determine statistical significance. χ^2 analysis tested categorical variables. Statistical significance was defined as $p<0.05$. Continuous variables are presented as median and range.

Univariate predictor variables with a $p<0.10$ were included in the multivariate analysis. The effect of LR on the probability of in-hospital mortality, while controlling for confounding variables was accomplished using a logistical regression. A Hosmer–Lemeshow goodness-of-fit test confirmed the model. Variables assessed by logistical regression included: age (continuous), sex, race (white, black, Hispanic, or other), primary insurance (Medicare, Medicaid, private, or other), admission type (elective or emergent/urgent), Elixhauser comorbidity score, malignant diagnosis, and volume status. A model was first developed and included the comparisons of LV and HV surgeons and hospitals separately. Income level was not included in the logistic regression due to changes in coding and tracking over the years in the study. Then, separate models were used to test the effect of different surgeon/hospital volume combinations. Models were run with and without patients with missing variables. In order to account for variability in wedge resections, a separate model was run for only hemihepatectomy to ensure the findings are broadly applicable over varying degrees of LR.

Adjusted regression and mortality assessments were then performed on the case-controlled groups. The outcome variable was again in-hospital mortality. P values<0.05 were considered statistically significant. Logistical regression data were tabulated as hazard ratios and 95% confidence interval (CI).

Results

Demographics

Over the 8-year period, 2,949 LRs were included in this cohort from the NIS. The median number of states involved in the study was seven per year (range 4–9). The median number of surgeons per year involved in the study was 134 surgeons (range 98–163). Surgeons performed a median of one LR per year (range 1–83). A median of 64 hospitals performed a LR in a given year in this cohort (range 49–79). The median hospital volume of LR was two per year (range 1–196) (Fig. 1). Of these, 1,526 were performed at LV centers (60.1% wedge resections and 39.9% lobectomies) and 1,423 were performed at HV centers (50.9% wedge resections and 49.1% lobectomies). Low volume surgeons performed 1,767 LRs, 1,393 at LV hospitals and 374 at HV hospitals. High volume surgeons completed 1,182 LR, 133 at LV hospitals and 1,049 at HV hospitals (Fig. 2).

Table 1 demonstrates the demographics of the cohort. Patients treated at high volume centers were more likely to be older (57 years vs. 56 years), white race (76% vs. 69%), recipients of private insurance (56% vs. 48%), treated in an elective setting (93% vs. 79%), members of the highest income bracket (41% vs. 34%), and carriers of a malignant diagnosis (81% vs. 74%) ($p<0.05$). The median Elixhauser score was one and there was no difference in comorbidity between the two hospital groups.

Patient Outcomes

At HV centers patients were discharged home more frequently (78% vs. 75%), had higher mean total charges ($55,400 vs. $45,200), and a lower unadjusted mortality (3.2% vs. 6.5%)($p<0.05$). A multivariable logistic regres-

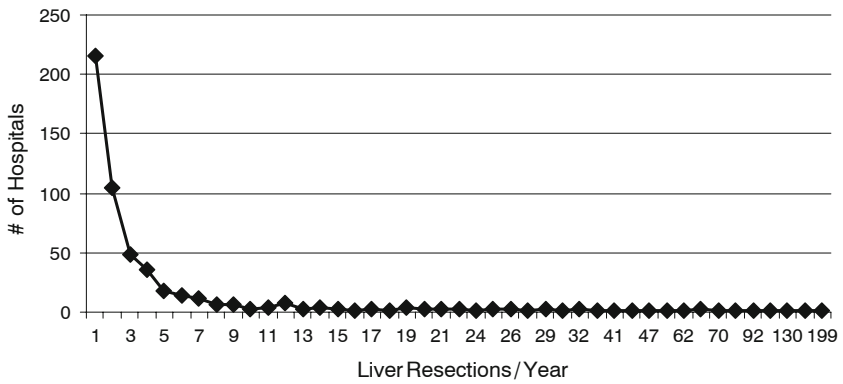

Figure 1 Liver resections performed per year by center.

sion was completed and independent variables predictive of in-hospital mortality were calculated (Table 2). Those associated with increased in-hospital mortality included: Medicare insurance (OR, 1.80; 95% CI, 1.16–2.77), increased medical comorbidity (HR, 1.25; 95% CI, 1.09–1.43), and emergent presentation (HR, 3.75; 95% CI, 2.45–5.74 (Table 3)). Female sex was protective (HR, 0.59; 95% CI, 0.41–0.87). Separately, neither treatment at a HV center (HR, 0.81; 95% CI, 0.48–1.38) or by a HV surgeon (HR, 0.68; 95% CI, 0.39–1.19) was protective.

In combination, only treatment by a HV surgeon at a HV center was associated with a 50% reduction in the risk of mortality (HR, 0.53; 95% CI, 0.32–0.89 (Table 3)). A separate analysis of only hemihepatectomy achieved similar results to ensure that this result is applicable over varying degrees of LR. Since LRs are commonly elective operations, a separate analysis omitting emergent LR was performed as well. There was no difference in results using only elective LRs in this cohort.

Adjusted Mortality Models

Distributions of measure variables were comparable between groups ensuring that presentation to high or low volume centers and surgeons was unrelated to patient traits. A total of 1,678 patients comprised this case-controlled cohort. Patients who underwent LR at HV hospitals had a lower adjusted mortality rate (2.6% vs. 4.8%; $p=0.02$)

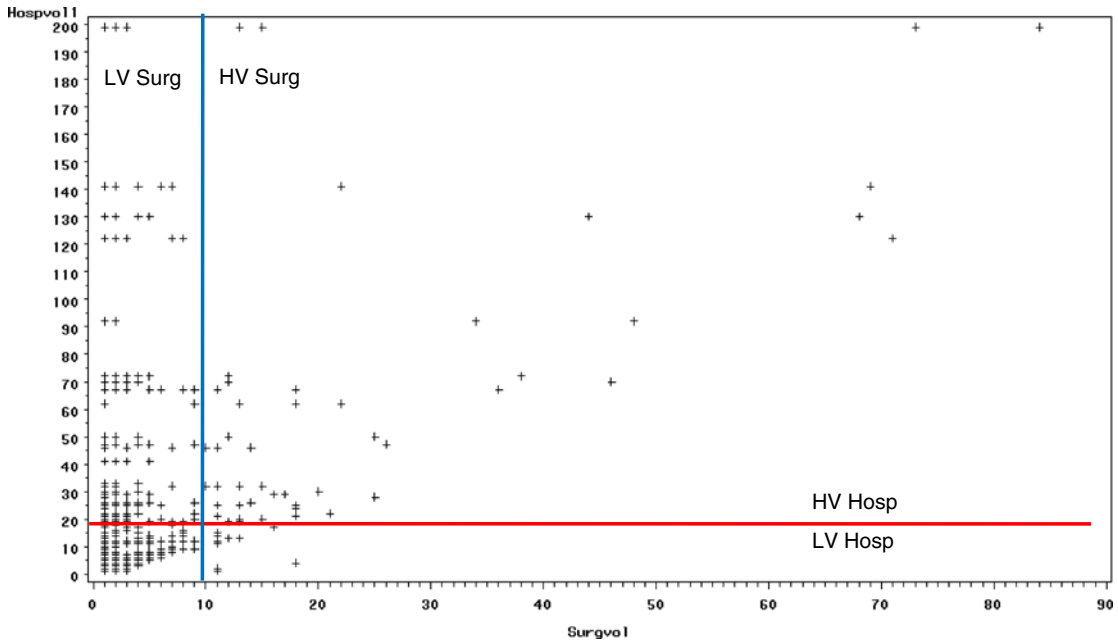

Figure 2 Surgeon and hospital volume scatter plot (*HV* high volume, *LV* low volume).

Table 1 Demographics by Hospital Volume

Demographic	Low volume (n=1,526)	High volume (n=1,423)	P value
Mean age (Median)	55.5 (59)	56.9 (59)	0.03
Female	52.5%	48.7%	0.04
Race			<0.0001
White	69.2%	75.6%	
Black	11.7%	6.8%	
Hispanic	11.3%	8.7%	
Other	7.7%	8.9%	
Primary insurance			0.001
Medicare	36.3%	31.7%	
Medicaid	7.4%	6.1%	
Private	48.4%	55.7%	
Other	8.0%	6.5%	
Admission type			<0.0001
Urgent	21.4%	7.0%	
Elective	78.6%	93.0%	
Highest income bracket	33.7%	41.4%	<0.0001
Malignant diagnosis	73.5%	80.56	<0.0001
Hepatocellular carcinoma	17.4%	21.7%	0.003
Metastatic cancer	50.3%	53.1%	0.13
Cirrhosis	7.9%	13.4%	<0.0001
Portal hypertension	3.8%	3.7%	0.91
Elixhauser score			0.68
0	25.0%	23.6%	
1	31.9%	33.2%	
2	23.1%	22.4%	
≥3	19.9%	20.9%	
Blood transfusion	24.4%	18.3%	<0.0001
Disp_4			<0.0001
Routine (home)	75.3%	78.2%	
Rehab	5.8%	3.4%	
Died	6.5%	3.2%	
Other	12.4%	15.2%	
Died	6.5%	3.2%	<0.0001
Mean LOS (Median)	8.4 (7)	8.0 (6)	0.12
Mean total charges (median)	$45,000 ($31,300)	$56,200 ($39,700)	<0.0001

compared to LV hospitals. A logistical regression of propensity matched groups was then performed to determine if independent risk factors for in-hospital mortality existed in this case-controlled cohort (Table 4). Medicare (HR, 2.29; 95% CI, 1.26–4.17), increasing Elixhauser comorbidity (HR, 1.42; 95% CI, 1.17–1.72) and emergent admission (HR, 4.43; 95% CI, 2.40–8.18) were independently associated with in-hospital mortality. Different combinations of surgeon and hospital volume such as HV hospital/LV surgeon (HR, 0.79; 95% CI, 0.37–1.72) or LV hospital/HV surgeon (HR, 1.08; 95% CI, 0.40–2.92) did not show a benefit with in-hospital mortality (Fig. 3). Only surgery by HV surgeons at HV hospitals remained beneficial (HR, 0.40; 95% CI, 0.21–0.80) for patients undergoing LR. All other combinations of volume groups of surgeons and hospitals did not prove to be protective.

Table 2 Logistic Regression of In-Hospital Mortality for All Patients Who Underwent Liver Resection (n=2,949)

Variable	Hazard ratio	95% CI	P value
Female	0.59	0.41–0.87	0.007
Primary insurance			
Private	1.00	–	–
Medicare	1.80	1.16–2.77	0.04
Medicaid	1.22	0.59–2.53	0.85
Other	1.97	1.09–3.58	0.05
Comorbidity count	1.25	1.09–1.43	0.001
Malignant diagnosis	0.73	0.46–1.16	0.42
Teaching hospital	1.06	0.66–1.68	0.91
Admission type			
Elective	1.00	–	–
Urgent	3.75	2.45–5.74	<0.0001
HV surgeon	0.68	0.39–1.19	0.18
HV hospital	0.81	0.48–1.38	0.44

CI Confidence interval, *HV* high volume

Discussion

Specialized procedures require surgical expertise, modern and up-to-date tertiary services, and patients who can tolerate surgery safely. Using the surgeon and hospital identifier in the NIS, we have found that a complex interplay between surgeon, center, and patient factors determines outcome after LR. Many surgical procedures have better outcomes when performed by HV surgeons or centers, but the volume–outcome relationship is not well-defined.[19,20] A volume-based outcome analysis is applicable to only a very small segment of surgical care and may apply only to a subgroup of operations.[21] Use of database research has elucidated some aspects of provider standards and outcomes after surgery. However, the reasons for

Table 3 Logistic Regression of In-Hospital Mortality of Different Volume Groups in All Patients who Underwent Liver Resection (n=2952)

Volume group	LV hospital	HV hospital
LV surgeon	1.0	0.93 (0.52–1.67)
HV surgeon	0.95 (0.40–2.28)	0.53 (0.32–0.89)

All results presented as hazard ratio (95% confidence interval)
LV Low volume, *HV* high volume

Table 4 Logistic Regression of In-Hospital Mortality for Case-Controlled Cohort (n=1,678)

Variable	Hazard ratio	95% CI	P value
Female	0.68	0.39–1.16	0.16
Primary insurance			
Private	1.00	–	–
Medicare	2.29	1.26–4.17	0.007
Medicaid	1.83	0.73–4.59	0.20
Other	1.00	0.28–3.54	0.99
Comorbidity count	1.42	1.17–1.72	0.0004
Malignant diagnosis	0.60	0.30–1.20	0.15
Teaching hospital	1.03	0.29–3.72	0.98
Admission type			
Elective	1.00	–	–
Urgent	4.43	2.40–8.18	<0.0001
HV surgeon	0.72	0.35–1.47	0.37
HV hospital	0.63	0.32–1.24	0.18

HV High volume

improved perioperative mortality have not been adequately studied.[20]

In large population-based studies, improved unadjusted results may appear to be better at HV hospitals.[5,13,22] This is due to inherent assumptions about the large cohort that are commonly accepted.[23] We have shown in this cohort of LR from the NIS database that a socioeconomic bias may exist at HV hospitals. Specifically, HV hospitals treated patients who were more often white, had private insurance, and were located in high income residencies. After eliminating these differences to create a case-controlled analysis between LV and HV hospitals, an adjusted mortality benefit was still evident at HV hospitals. Although these factors are controlled for in the propensity scoring, they still bear out an increased risk for mortality. When independently examined, within each subgroup, mortality rates are higher in LV hospital than HV hospitals (4.8% vs. 2.6%) despite controlling for the frequency of these demographic variables. This further emphasizes their effect in outcomes after LR in this cohort.

Surgeon volume was not protective independent of hospital volume. In this study the majority of liver resections completed by HV surgeons (89%) were done at HV hospitals. Similarly, LV surgeons tended to operate at LV centers (79%). Since improved mortality was not demonstrated independent of center volume, favorable patient characteristics may have led to the mortality benefit of other studies. Could part of the mortality benefit enjoyed by HV surgeons in other studies be due to beneficial patient characteristics? Although demographics may appear similar, causal effects and background characteristics from large data sets can be controlled by propensity scores. This method of case-controlled analysis makes the results more assessable and transparent.[23] Others have hypothesized that the volume parameters to determine "high" and "low" volume are misleading. We used equal groups to determine our volume threshold; this method is accepted but may not be the most accurate predictor of a volume–outcome effect.[19,24] Statistical analysis to determine a 'best-fit' for volume cutoffs using pseudo-r^2 resulted in unacceptably low cutoffs (data not shown).[24]

There are many factors which may lead to improved outcomes at HV centers including both those inherent to the hospital such as specialization, dedicated care teams and units, and clinical pathways. We have previously shown that centers that support transplantation achieve better perioperative mortality after liver and pancreas surgery regardless of surgeon specialization or volume (Csikesz et al. in press). Other processes of care that have been shown to independently improve patient outcomes after surgery include surgical specialization,[1,25] intensive care specialists running intensive care units,[26,27] multidisciplinary care,[28] and surgeon experience.[29] Furthermore, the effect of the interplay between hospital and surgeon volume may impact outcomes as well. This is a growing area of research that may help identify better parameters of "quality" or create more accurate risk adjustment to assess outcomes after surgery.

Several limitations to this study must be considered. This was a retrospective study and has the associated constraints due to the level of the NIS data. For example, we were unable to confirm the validity and accuracy of the diagnostic and procedure coding.[30] The main outcome measure of this study was in-hospital mortality. This may reflect a lower mortality rate compared with studies using 30-day mortality as most patients were likely discharged from the hospital prior to the potential death (if applicable). Our study used population-based data with only limited information on patient and treatment factors, thereby

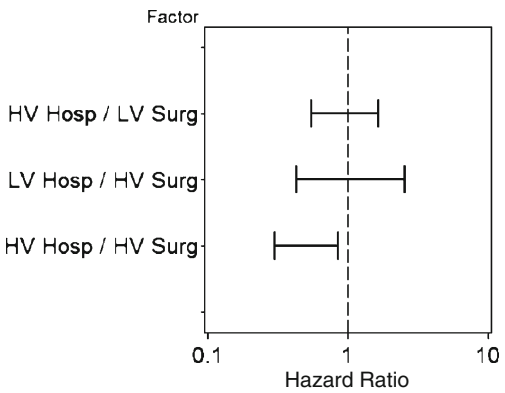

Figure 3 Logistic regression plot of hazard ratios for in-hospital mortality of volume groups in the case-controlled analysis. Referent value is LV surgeon/LV hospital (*HV* high volume, *LV* low volume, *Hosp* hospital, *Surg* surgeon).

limiting our evaluation of medical factors such as presence of cancer, cirrhosis, antibiotic use, mechanical ventilation, and prior surgery. Our data was comprised of only a median of seven states per year. This may not have been a representational cohort of a large population; demographic and practice patterns of surgeons performing liver procedures may vary from state to state. In order to account for this, we calculated the mortality rates for the whole NIS cohort for LR and did not find any difference in mortality among states with surgeon/hospital identifiers and those without. We also did not identify any significant regional differences in our cohort and states had uniform distribution of demographic and patient characteristics.

In conclusion, this large cohort of patients who underwent LR suggests that a socioeconomic bias may exist at HV centers. When these factors are accounted for and adjusted, center volume does not appear to influence in-hospital mortality unless LR is performed by HV surgeons at HV centers. Further processes of care that contribute to improved outcomes continue to be defined as volume may only be a surrogate for these other factors that improve patient care.

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Elijah Dixon, M.D. (Calgary, Alberta, CA): Dr. Shah, I would like to congratulate you on a nice presentation and a well written manuscript. You have taken a closer look at the volume outcome relationship as it applies to hepatic surgery and tried to drill down and see what effect the individual surgeon and hospital or institutional volume has on outcomes. The analysis that you used is a combination of standard logistic regression analysis and a case control matched analysis with propensity scores. You found that there were differences in the socioeconomic means of the patients that are treated at high and low volume hospitals, and that the individual effects of both surgeon and hospital volume on outcome are fairly weak, and that only the two of them together show us a statistically significant effect on postoperative mortality. So, I have three quick questions for you.

The first is, can you comment on how significantly you think your results or the lack of volume outcome effect can be explained by differences in case complexity at high and low volume centers and our inability to measure the differences in case complexity using administrative data? The second question. There is some evidence that volume may in fact be a surrogate for other aspects of care, and I wonder if you could hypothesize what some of the structural and process of care issues may be that can explain some of the volume outcome effect. And third, your analysis used two techniques. The case control matched analysis used a much smaller data set, a subset of your data, and I wonder if you could comment on what the value of that analysis is and its relative strength in comparison to the standard multivariate regression.

Thank you. I enjoyed your presentation.

Shimul A. Shah, M.D. (Worcester, MA): To answer your first question, the case complexity is something that we are not going to be able to account for in most administrative databases. Some of the cancer databases might allow us to look at tumor size, but even then, it is not going to give us the accurate assessment of how much work a high volume or low volume surgeon would have to do in a liver resection. And probably in that regard, as some centers have done already, we are going to need to collate some of our data and look at it prospectively and combine centers' experiences and try to understand this phenomenon. Maybe the high volume surgeons are doing more complex cases, so therefore the mortality benefit that you get is underscored in a study like this.

In terms of processes of care, I think with liver resection it is especially unique and isn't really accounted for in large population-based studies. For instance, high volume centers probably have a multidisciplinary tumor conference; if you work at a transplant center, that might account for improved outcomes after liver resection; whether you are at teaching hospital or a nonteaching hospital; or even something like having two high volume surgeons in a single center that work together during a liver resection probably improves outcomes. These kinds of factors are not accounted for in a large database. Our group has previously shown that liver resection at a transplant center significantly improves outcomes in terms of in-hospital mortality.

The use of propensity scores allows us to do a case controlled analysis. When you perform a logistic regression of thousands of patients, you are assuming that the cohorts are similar when they are not. So although you might find significant factors that are "independent", we really don't believe that they are truly independent unless you trim down the cohorts and make sure that the demographic and the hospital factors are similar.

Myrddin Rees, M.D. (Baskingstoke, UK): I have been a low volume surgeon in a low volume hospital, and I am currently a high volume surgeon in a high volume hospital, doing over 200 resections of the liver a year. I am also president of your sister organization, the Association of Upper Gastrointestinal Surgeons for Great Britain and Ireland. I would like to give you a U.K. perspective, which I think is relevant to this paper, which I enjoyed very much.

Over the past five years, two important things have occurred in England. We have centralized all major cancer resections for the esophagus, pancreas, and the liver. So we now only have high volume centers. However, we still had a spate of young surgeons being suspended in their first year as a consultant. As a result, AUGIS decreed and advised that all young surgeons be mentored for up to five years. So at my center we have three surgeons, and though I do the majority, my youngest surgeon always has me as first assistant on any difficult operation. As a consequence our results are the same and equal, and I recommend to you the team approach.

Thank you.

Nicholas J. Zyromski, M.D. (Indianapolis, IN): Congratulations on a beautiful presentation of a provocative paper. I understand that your outcome measure on this was specifically in-hospital mortality. My question is, does this database allow you to look at long-term survival and is that something that you are thinking about looking at in this situation?

Shimul A. Shah, M.D. (Worcester, MA): Unfortunately, this database doesn't allow us to look at long-term survival. So one thing that we are going to look into next is some of the cancer databases. Unfortunately, they don't always track surgeon volume, which is one of the limitations why it probably hasn't been done before. I think the key is getting a lot of people in this audience together and seeing what our own data is. Unfortunately, probably most of the people in this room are high volume surgeons, and in order to do some of these studies well, I think we need to get the low volume surgeons involved as well. Thank you.

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NOTES Rectosigmoid Resection Using Transanal Endoscopic Microsurgery (TEM) with Transgastric Endoscopic Assistance: A Pilot Study in Swine

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Abstract
Introduction Transanal endoscopic microsurgery (TEM) provides direct endoscopic access to the rectum and peritoneal cavity. The feasibility of natural orifice translumenal endoscopic surgery (NOTES) rectosigmoid resection using TEM was evaluated in swine. Transgastric endoscopic assistance to extend transanal colon mobilization was also investigated. Full-thickness circumferential rectal dissection was performed and extended proximally. After maximal sigmoid mobilization, the specimen was exteriorized and transected, and the proximal colon was stapled to the distal rectum. In a subset of animals, transgastric endoscopic access was used to mobilize the colon further.
Results Rectosigmoid resection using TEM was performed in two non-survival and seven swine cadavers (n=9). The mean procedure time was 3 h (2.5–4 h), and mean length of resected colon was 16.7 cm (10–25 cm). Transgastric endoscopic assistance was used in three cadavers and two non-survival swine (n=5) with a mean operative time of 3.5 h (3.5–3.75 h). The mean length of colon mobilized with transgastric and transanal endoscopic access was 24.4 cm (20–27 cm) vs. 16.7 cm which mobilized the transanal approach alone (p=0.016). A posterior anastomotic defect was noted in two animals.
Conclusion Transanal rectosigmoid resection with TEM is feasible in swine. Combined transgastric and TEM access is a promising new technique for NOTES colorectal resection.

Meeting presentations This study was accepted for presentation at the SSAT Plenary Session May 20, 2008 at Digestive Disease Week in San Diego, CA.

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Introduction

The optimal translumenal access route for natural orifice translumenal endoscopic surgery (NOTES) is an area of active investigation. Relative to transgastric and transvaginal access, transcolonic NOTES has been the least explored approach because of concerns related to fecal contamination and intra-abdominal infectious complications. Initial data on transcolonic peritoneoscopy[1,2] and cholecystectomy[3] in swine survival models have reported successful outcomes with a 9% cumulative incidence of septic complications, all related to technical failures to adequately close the colotomies.[1–3] With ongoing experience, transcolonic NOTES procedures of increasing complexity such as ventral hernia repair[4] and distal pancreatectomy[5,6] have been described

with good outcomes. The benefits of transcolonic access include in-line endoscopic visualization, the ability to create and close the colotomy with existing transanal endoscopic microsurgery (TEM) equipment, and suitability of both male and female patients. TEM utilizes a wide rigid proctoscope with a magnifying lens, specialized instruments, and pressure-controlled CO_2 insufflation. Wilhelm et al.[1] recently described flexible endoscopic transcolonic peritoneoscopy with colotomy closure using suturing and stapling devices passed through the TEM proctoscope. More recently, Whiteford et al.[7] described a novel technique for transanal sigmoid colectomy in human cadavers using TEM with complete circumferential resection of the colon and mesentery followed by stapled colorectal anastomosis. In addition to serving as a stable conduit to the peritoneal cavity, TEM permitted a single operator to complete the procedure transanally without the need for transabdominal assistance with the added benefit of superior visualization of pelvic structures provided by TEM optics and CO_2 insufflation. The authors found that the extent of resectable sigmoid colon was limited by the length of the proctoscope and anatomic factors including difficulties overcoming the acute angle at the sacral promontory with the TEM proctoscope.[7]

In an effort to evaluate transanal NOTES colorectal resection in a large animal model, our group investigated the feasibility of performing transanal rectosigmoid resection using TEM in swine. In addition, transgastric endoscopic access to assist transanal colonic mobilization and extend the length of resectable rectosigmoid was explored.

Materials and Methods

This study was approved by the Subcommittee for Research Animal Care of the Massachusetts General Hospital, Boston, MA. The objective of the study was to evaluate the feasibility of performing rectosigmoid resection transanally using TEM in a swine model. In a subset of animals, transgastric endoscopic access was also investigated to evaluate its ability to assist with colonic mobilization and extend the length of colon resected transanally. Intraoperative variables measured included length of rectosigmoid colon mobilized transanally, operative time, evidence of colonic wall injury and injury to adjacent organs after endoscopic colonic dissection, as well as anastomotic integrity. Healthy Yorkshire male swine or swine cadavers weighing 40–50 kg were used for all procedures. Animals were fed Ensure (Abbott Laboratories, North Chicago, IL, USA) and yogurt, transitioned to a clear liquid diet and fasted overnight before the procedures. In non-survival experiments, general anesthesia was induced with Telazol/xylazine 4.4+2.2 mg/kg IM, and animals were intubated. Anesthesia was maintained with isoflurane (1.5–3.0%) and oxygen (3.0 l/min). In cadaver experiments, animals were euthanized with pentobarbital 100 mg/kg IV after completion of unrelated procedures. NOTES procedures were performed within 15 min of euthanizing the animals.

Transanal Rectosigmoid Resection with TEM

Tap water enemas were administered through a rigid proctosigmoidoscope until all fecal material was cleared from the rectosigmoid colon. The TEM proctoscope (Storz, Culver City, CA, USA) was inserted rectally and sealed with the faceplate. Low-pressure CO_2 was insufflated, and the distal rectum was occluded circumferentially with a 2.0 silk pursestring suture placed 3–4 cm from the anal verge using the endostich device (US Surgical, Norwalk, CT, USA) to avoid fecal inflow from the proximal colon. Alternatively, the pursestring was placed transanally under direct vision using anal retractors. After luminal occlusion, the rectum was re-insufflated, and the mucosa was scored circumferentially using the Autosonix ultrashears (US Surgical) just distal to the pursestring. Circumferential rectal mobilization was started posteriorly by incising the posterior rectal wall full-thickness until the presacral plane was entered (Fig. 1). The posterior dissection was extended in the retro-rectal plane using the Autosonix ultrashears (US Surgical). The plane of dissection was extended medially, laterally, and anteriorly, staying close to the rectal wall to avoid injury to adjacent structures. *Posteriorly, every attempt was made at preserving the mesorectum*. Circumferential rectal and sigmoid mobilization was extended cephalad until limited exposure prevented further proximal dissection. The 7.5-cm proctoscope was exchanged for the 15-cm proctoscope, and further mobilization was completed by retracting the specimen maximally through the proctoscope. The peritoneal reflection, which is thick and redundant in swine, was partially divided anteriorly to enter the peritoneal cavity. When dissection could not be extended any further cephalad, the specimen was exteriorized transanally in preparation for transection and anastomosis (Fig. 2).

Transgastric Endoscopic Access

In experiments where transgastric endoscopic assistance was evaluated, transgastric peritoneal access was obtained when further cephalad dissection could not be completed transanally. A 12.8-mm gastroscope (Pentax Medical Inc., Montvale, NJ, USA) was introduced per-orally. An esophageal overtube was advanced, and the stomach was copiously irrigated with water. The gastroscope was replaced with a 12.8-mm therapeutic colonoscope (Pentax). A Huibregtse® single lumen needle knife (Cook Medical Inc., Winston-Salem, NC, USA) was used to incise the stomach transmurally on its anterior surface. The colonoscope was

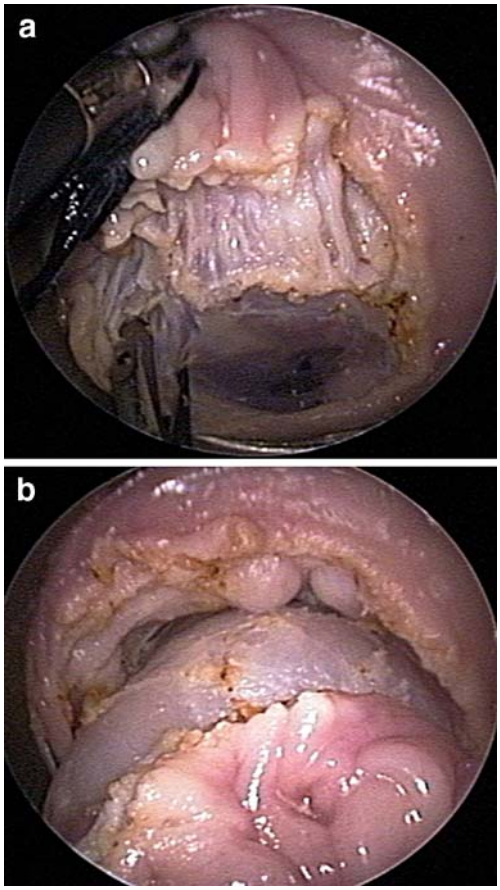

Figure 1 Transanal endoscopic rectal dissection using TEM. **a** The rectum is scored circumferentially followed by full-thickness dissection starting posteriorly where the presacral plane is entered. **b** Rectal mobilization is extended circumferentially and cephalad toward the sigmoid.

advanced through the gastrotomy into the peritoneal cavity. The lateral peritoneal attachments of the sigmoid colon were visualized and divided using needle knife cautery. *In non-survival animals, vascular structures, including the sigmoid mesentery, were preserved.* The rigid proctosigmoidoscope was used to manipulate the rectosigmoid and provide tension to facilitate transgastric dissection of residual peritoneal attachments. In combination with the endoscopic view and assistance with dissection provided through the TEM proctoscope, additional attachments were divided through the colonoscope. As these were cadavers and non-survival animals, the gastrotomy was not closed.

Rectosigmoid Resection and Colorectal Anastomosis

When sigmoid mobilization was completed, the proctoscope was removed, and the specimen was exteriorized, measured, and transected. The anvil of a 28 EEA stapler (US Surgical) was inserted into the proximal segment and secured using a pursestring. The proximal colon with the anvil was repositioned transanally, and the proctoscope was reinserted. CO_2 was re-insufflated, and a full-thickness pursestring was placed circumferentially through the open distal rectal cuff (Fig. 3a). The proctoscope was removed, the anvil on the proximal colon was pulled into the distal rectal ring, and the distal pursestring was tied around it. The stapler was introduced through the rectal stump, connected to the anvil, closed and fired (Fig. 3b). The anastomosis was inspected either through the proctoscope or with the colonoscope. The specimen was inspected for evidence of perforation. Non-survival animals were euthanized, and laparotomy was performed to evaluate the pelvic and abdominal cavities.

The Mann–Whitney *U* test (SPSS, Chicago, IL, USA) was used to evaluate differences between groups. A p value of less than 0.05 was considered statistically significant.

Results

Transanal rectosigmoid resection using TEM was performed in nine swine including seven fresh cadavers and two non-survival animals (Table 1).

Transanal Rectosigmoid Resection with TEM

Full-thickness and circumferential endoscopic rectal mobilization was completed transanally and extended cephalad toward the sigmoid (Fig. 1). In all animals, the peritoneal reflection was reached and partially divided anteriorly, and the peritoneal cavity entered with establishment of pneumoperitoneum. Proximal sigmoid dissection was

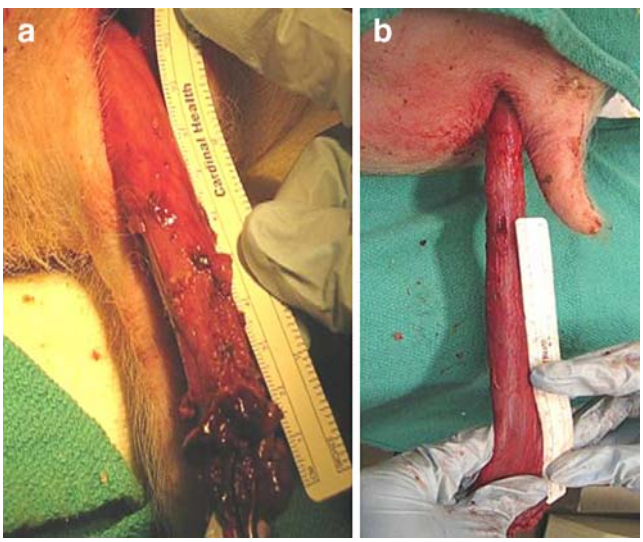

Figure 2 Transanal exteriorization of the mobilized rectosigmoid in a non-survival animal. **a** The length of colon dissected using TEM was measured at approximately 11 cm. **b** After additional sigmoid mobilization with transgastric endoscopic assistance, total specimen length of 25 cm is achieved.

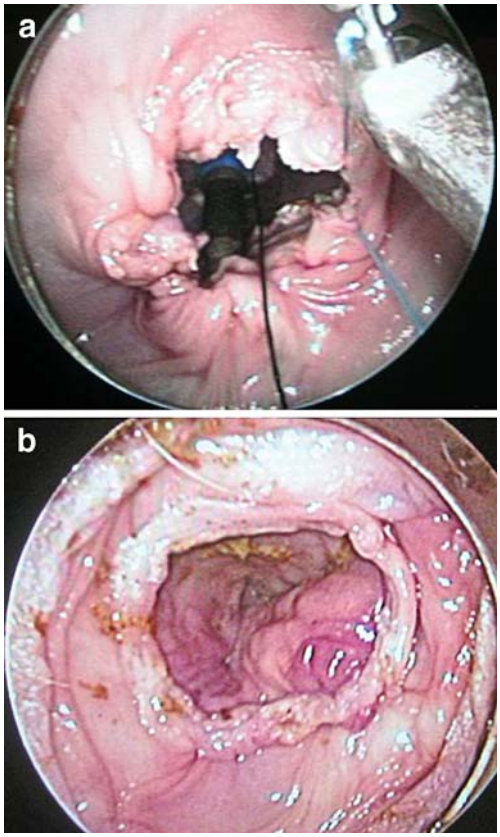

Figure 3 Stapled colorectal anastomosis. **a** A pursestring suture is placed around the distal open rectum. The anvil on the proximal colon is advanced through the distal pursestring, which is then tied around it. **b** Stapled anastomosis was completed and evaluated endoscopically.

limited by exposure because of the narrow size of the swine pelvis that could only accommodate 5 to 10 cm of the 4-cm wide and 15-cm long TEM proctoscope. The mean length of rectosigmoid colon exteriorized after TEM transanal dissection was 16.7 cm (range, 10–25 cm, Fig. 2a). The mean operative time was 3 h (range, 2.5–4 h) with progressive decrease in operative time over the study period (Table 1).

Combined Transgastric and Transanal Endoscopic Rectosigmoid Mobilization

Transanal dissection was combined with transgastric endoscopic assistance in five animals including three cadavers and two non-survival swine (Table 1). Transgastric peritoneal access with a colonoscope through an anterior gastrotomy was achieved without difficulty in all cases and was facilitated by the pneumoperitoneum previously established during TEM dissection. Addition of transgastric endoscopic dissection allowed an average gain of 5.8 cm of colon length (range, 2–14 cm, Fig. 2b), representing a 35% increase from the amount previously mobilized transanally using TEM. The additional length of colon mobilized ranged from 2 to 5 cm in cadavers and 5 to 14 cm in non-survival animals. Combining transgastric and transanal endoscopic dissection allowed a significantly longer segment of colon to be exteriorized relative to a purely transanal approach (Table 1). An average of 24.4 cm of rectosigmoid (range 20–27 cm) could be mobilized (p=0.016) with a mean operative time of 3.5 h (range 3.5–3.75 h).

Rectosigmoid Resection, Colorectal Anastomosis, and Laparotomy

On gross inspection, the resected specimens included the rectum and part of the sigmoid colon, its mesentery, and scattered lymph nodes. No colonic wall injury was noted except in one cadaver where a serosal tear was sustained anteriorly. The stapled anastomoses were intact in seven animals (Fig. 3b). A small posterior anastomotic defect was noted in two animals (one cadaver and one non-survival

Table 1 Operative Findings During Transanal Rectosigmoid Resection Using TEM With or Without Transgastric Endoscopic Assistance in Non-survival or Swine Cadavers

Animal number	Endoscopic access	Maximal length of exteriorized colon (cm)[a]	Specimen	Anastomosis	Duration (h)
Cadaver #1	Transanal	10	Intact	Intact	4
Cadaver #2	Transanal	12	Intact	Intact	3
Cadaver #3	Transanal	15	Intact	Small posterior defect	2.5
Cadaver #4	Transanal	20	Intact	Intact	2.5
Cadaver #5	Transanal+transgastric	27 (25+2)[a]	Intact	Intact	3.5
Cadaver #6	Transanal+transgastric	25 (22+3)[a]	Serosal tear	Intact	3.5
Cadaver #7	Transanal+transgastric	25 (20+5)[a]	Intact	Intact	3.5
Non-survival #8	Transanal+transgastric	20 (15+5)[a]	Intact	Intact	3.5
Non-survival #9	Transanal+transgastric	25 (11+14)[a]	Intact	Small posterior defect	3.75

[a] Total length in centimeters (TEM+transgastric). For combined transanal and transgastric procedures, the total length of exteriorized colon includes the length achieved transanally using TEM plus additional length achieved after transgastric endoscopic assistance.

animal) that likely resulted from an incomplete distal rectal pursestring suture. No injury to adjacent pelvic and abdominal organs was noted on necropsy.

Discussion

Several clinical reports have confirmed the feasibility and safety of translumenal peritoneoscopy,[8–10] appendectomy,[11] and cholecystectomy.[12,13] A wider range of NOTES procedures of varying complexity have been described in the experimental setting. No report on NOTES colorectal resection in a large animal model has been published to date. Transanal colorectal resections are routinely performed for rectal prolapse. Perineal proctosigmoidectomy of the redundant and prolapsing rectosigmoid is completed transanally. Recently, a novel endoscopic transanal approach for sigmoid resection was described in human cadavers using TEM.[7] Before this report, typical indications for TEM included local excision of high rectal lesions not amenable to endoscopic or transanal resection and early stage rectal cancers to avoid radical resection.[14] TEM is an attractive alternative to more invasive approaches with low morbidity and mortality.[14] While inadvertent peritoneal entry during full-thickness excision was historically managed with conversion to laparotomy to avoid peritoneal contamination, recent data has shown that peritoneal entry with closure is not associated with increased incidence of infectious complications.[15] This finding combined with the recent report on the feasibility of transanal radical sigmoidectomy using TEM in human cadavers suggests that this approach might provide relatively safe access to the peritoneal cavity to perform NOTES colorectal resection.

The results of this pilot study suggest that NOTES transanal rectosigmoid resection using TEM is also feasible in a porcine model. Relative to human cadavers, swine are a challenging model for TEM. In addition to difficulty overcoming the acute angle at the sacral promontory with the rigid proctoscope, the extent of sigmoid mobilization is limited by the narrow size of the swine pelvis that can only accommodate 5 to 10 cm of the 15-cm TEM proctoscope. Larger animals did not provide any benefit, as a negligibly wider pelvis was offset by a bulkier and more difficult to retract sigmoid colon. The thick and redundant peritoneal reflection in swine limited more proximal colon dissection and made access to the peritoneal cavity challenging. In addition, despite decompression with a needle, the swine bladder is flaccid and obscures the rectosigmoid. Swine also have a spiral colon configuration and lack a true splenic flexure, which, relative to humans, makes proximal colonic mobilization more challenging.

After TEM mobilization, an average length of 16.6 cm of rectosigmoid colon could be exteriorized compared to 24 cm of colon in human cadavers.[7] Combining transgastric endoscopic access compensated for some of the limitations encountered with transanal rectosigmoid mobilization. Transanal manipulation of the rectum using the rigid proctosigmoidoscope facilitated endoscopic division of the residual peritoneal reflection and attachments of the rectosigmoid. Exposure was also improved by placing animals in Trendelenburg and right lateral decubitus position, which shifted the bladder away from the sigmoid colon. One significant advantage of dual endoscopic access is the ability to synchronize both approaches to obtain additional colon length. The colonoscope can be used to visualize residual peritoneal attachments that are more easily divided with instruments inserted through the TEM proctoscope. Similarly, transanal manipulation of the rectum helps expose attachments that can be divided with the endoscopic needle knife. Overall, transgastric endoscopic assistance resulted in an average gain of 5.8 cm in colon length with up to 27 cm of total colon length mobilized using both transgastric and transanal endoscopic mobilization. In swine cadavers, this gain in length was minimal (2–5 cm) because of the fact that, relative to live animals, transgastric endoscopic dissection was more challenging. Distinguishing between peritoneal attachments and devascularized bowel wall was difficult in cadavers especially with the combination of cautery and cold pneumoperitoneum causing persistent fogging of the lens and a suboptimal endoscopic view. However, with the learning curve associated with transanal dissection, up to 22 cm of rectosigmoid could be mobilized transanally in swine cadavers, with a decrease in operative time from 4 to 2.5 h after the first pilot experiment. In contrast, transanal dissection was more challenging in non-survival animals. Up to 15 cm of rectosigmoid could be mobilized transanally, which is largely because of the need for meticulous dissection to achieve hemostasis during rectal wall dissection in non-survival animals. However, a longer segment of colon could be mobilized using transgastric assistance (5–14 cm), which is largely because of excellent endoscopic visualization relative to swine cadavers.

Regarding the integrity of the colorectal anastomosis and the accuracy of the colonic dissection that could be achieved after endoscopic colorectal dissection, the stapled line was incomplete in two of nine animals (22%). A small posterior anastomotic defect was identified in both cases that was most likely because of an incomplete pursestring on the distal open rectum. Placement of the distal pursestring suture through the proctoscope is challenging, especially posteriorly, because of limited space and the rigidity of the endostich device. We have modified our technique and now place the pursestring transanally under direct vision using anal retractors

with improved results. While not yet commercially available, novel devices may be better suited to place a pursestring suture on the distal rectal cuff such as a flexible suturing device inserted through the TEM proctoscope. With endoscopic rectosigmoid mobilization, injury to adjacent pelvic and abdominal organs was avoided, and with the exception of one cadaver, all resected specimens were intact.

While swine cadavers proved useful to begin this work, live swine are a more appropriate model for NOTES colorectal dissection. Suboptimal endoscopic visualization made differentiating between peritoneum and colonic wall difficult and, in one case, resulted in a serosal tear. Although bleeding complicates transanal rectal wall dissection, hemostasis can be achieved using the Autosonix Ultrashears (US Surgical). Bleeding occurred primarily during posterior rectal wall dissection, but once the presacral plane is entered, the rest of the dissection is relatively bloodless. With regard to transgastric sigmoid mobilization, no significant bleeding was encountered in live animals. Vascular structures were identified but not divided endoscopically to avoid profuse bleeding and devascularization of the proximal colon. We believe that additional colon length can be obtained when mesenteric division is performed. Options include placing endoscopic clips on vascular structures before division with the endoscopic needle knife or transanal division of the mesentery with a stapler under transgastric endoscopic visualization.

While some might question the logic and safety of creating both a colotomy and gastrotomy, our group and others have shown that secure gastrotomy closure is relatively easily attainable with a variety of closure devices *in survival animals*.[6,16–18] In addition, one of the major advantages of transanal colorectal resection using TEM is that, ultimately, the colotomy created is incorporated within the anastomosis.[7]

By providing access to the peritoneal cavity, TEM can also serve as a *safe* conduit to access the peritoneal cavity to perform various endoscopic abdominal and pelvic procedures. Extensive clinical experience over the last two decades has demonstrated that colotomy creation and closure during TEM procedures is associated with minimal morbidity.[15]

Conclusion

In this pilot study, transanal endoscopic rectosigmoid resection using TEM was found to be feasible in swine. The combination of TEM and transgastric endoscopic assistance allows additional colonic mobilization and is a promising technique for NOTES segmental colectomy. Given these findings, survival studies to investigate outcomes of combined transanal and transgastric endoscopic rectosigmoid resection using TEM are warranted.

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Who Should Do NOTES? Initial Endoscopic Performance of Laparoscopic Surgeons Compared to Gastroenterologists and Untrained Individuals

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Abstract
Introduction Natural orifice transluminal endoscopic surgery (NOTES) is a multidisciplinary surgical technique. If conventional endoscopic instrumentation can be easily mastered, surgeons with laparoscopic experience could head NOTES interventions.
Materials and Methods Thirty individuals were tested for endoscopic dexterity. Group 1 included seven gastroenterologists, group 2 included 12 laparoscopically experienced surgeons lacking endoscopic experience, and group 3 included 11 interns who had no hands-on endoscopic or surgical experience. Each individual repeated an easy (T1), medium (T2), and difficult (T3) task ten times with endoscopic equipment on a NOTES skills-box.
Results Group 3 had significantly poorer performances for all three tasks compared to the other groups. No significant differences were seen between groups 1 and 2 for T1 and T2. The initial T3 performance of group 1 was better than that of group 2, but their performance after repetition was not statistically different. Groups 2 and 3 improved significantly with repetition, and group 2 eventually performed as well as group 1.
Conclusions The data indicate that laparoscopic surgeons quickly learned to handle the endoscopic equipment. This suggests that a lack of endoscopic experience does not handicap laparoscopic surgeons when performing endoscopic tasks. Based on their knowledge of anatomy and the complication management acquired during surgical education, surgeons are well equipped to take the lead in interdisciplinary NOTES collaborations.

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Keywords NOTES · Skills-box · Dexterity · Spatial orientation · Surgeon · Gastroenterologist

Introduction

Natural orifice transluminal endoscopic surgery (NOTES) represents an entirely new surgical concept dating back to Kalloo's initial publication in 2004[1] and even earlier presentations at international conferences. Since that time, both surgeons and gastroenterologists have worked on the method of puncturing one of the visceral organs in order to perform intraabdominal surgical procedures.[2] American and European gastroenterological and surgical societies have formed collaborative organizations, such as the Natural Orifice Surgery Consortium for Assessment and Re-

search™ (NOSCAR™, USA)[3] and the EURO-NOTES Foundation (Europe),[4] in order to foster further developments in this surgical field. Though initially, gastroenterologists were more engaged and already reported in 2005 that "they have taken the lead,"[5] most human procedures have been performed by surgeons.[6–10] Still, the question of who should perform NOTES in the future, surgeons or gastroenterologists, remains actively discussed.

Evaluating the demands of NOTES may be helpful to answering the question. NOTES, in its current form, require expertise in advanced flexible endoscopy. In addition, procedure-specific surgical and anatomical knowledge is essential, and potential intra- and postoperative complications require surgical know-how and adequate and competent treatment. A NOTES physician has to have detailed knowledge to access the abdominal cavity translumenally, such as how to determine the correct access point and avoid injury to adjacent organs. Furthermore, the flexibility of the endoscope tip complicates the understanding of its distal orientation and requires detailed knowledge of tip placement relative to adjacent anatomic structures, especially when performing retrograde maneuvers.[11] Other, not yet discovered, challenges may be awaiting NOTES-performing physicians. Though gastroenterologists are experts at handling flexible endoscopes, surgeons have more knowledge of the procedure. Therefore, both gastroenterologists and surgeons could potentially qualify as the future NOTES physician; either specialist would have to learn and combine parts of the other's routine practice to be successful at NOTES. Important questions for the future may be to determine which part of the lacking knowledge is easier to acquire and whether gastroenterologists easily learn the procedure-related surgical knowledge or if it is easier to teach surgeons to handle the conventional flexible endoscopes.

We hypothesize that surgeons rapidly learn to handle the flexible endoscopes and that their initial performance is better than that of surgically untrained individuals. Furthermore, we assume that, for basic tasks and tasks of moderate difficulty, the performance is comparable between surgeons and gastroenterologists.

Materials and Methods

Participants

Thirty individuals were tested for endoscopic dexterity. Group 1 (G1) included seven gastroenterologists (GE) who had extensive experience in flexible endoscopy by having performed >200 endoscopies. Group 2 (G2) included 12 laparoscopically trained surgeons who lacked endoscopic experience but had performed at least 100 laparoscopic procedures. Group 3 (G3) included 11 interns who had no significant training for any surgical or laparoscopic device or endoscopes; they also never experienced any selection towards a manually oriented field of medicine (Table 1). Each participant executed each of three tasks exactly ten times after one initial warm-up attempt to verify a correct understanding of the task. No additional option for task training was given.

Equipment

All tasks were performed with flexible endoscopic equipment on a self-designed and constructed NOTES skills-box. A commonly used flexible endoscope (GIF-H180 Olympus Medical Systems Europe GmbH, Hamburg, Germany) and flexible endoscopic grasper (FD-410LR Olympus Medical Systems Europe GmbH, Hamburg, Germany) were utilized for all tasks.

Tasks

We considered task 1 (T1) to be a fairly basic task. It required the precise and single-handed maneuvering of the tip of the endoscope as well as the endoscopic grasper. Task 2 (T2) was of moderate difficulty and focused on hand–eye

Table 1 Distribution and Experience of Participants

Speciality	*N*	Gender	Median age (years)	Professional experience		Laparoscopic experience		Endoscopic experience	
				years	*n*	Number of procedures	*n*	Number of procedures	*n*
GE	7	1 ♀	37	≤6	2	0		>200	7
		6 ♂		>6	5				
Surgeon	12	2 ♀	38	≤6	3	100–200	3	0	
		10 ♂		>6	9	>200	9		
Trainee	11	6 ♀	24	0–1	8	0		0	
		5 ♂		<4	2				

GE Gastroenterologist

coordination and spatial orientation. Task 3 (T3) was assumed to be the most difficult task and combined the requirements of T1 and T2.

In T1, participants had to introduce the gastroscope, with retracted grasper, into the NOTES box, after 23 cm pass a 50-mm high barrier, advance the scope another 22 cm to the end of the box, approximate a target on the opposite wall, and ultimately touch a 5-mm diameter bull's eye with the endoscopic grasper (Fig. 1a).

In T2, participants had to introduce the gastroscope into the NOTES box, pick up a small fabric ball (swab, diameter 8 mm) from a height of 20 mm in the center of a 5-mm deep basket, and lay the swab down in a basket of the same size 35 mm lateral to the first one (Fig. 1b).

In T3, participants had to pick up a fabric ball with the grasper in a 90° flexion. The ball was on a small shelf approximately 20 cm from the box entrance at the right wall and at a height of 8 cm. The fabric ball was then placed in a basket approximately 8 cm to the left of the shelf (Fig. 1c).

Each participant assessed the difficulty of the tasks using a postperformance visual analog scale (VAS), from 1 for a very easy task to 10 for a very difficult task, in order to assess the individual appraisal of difficulty. The time needed to complete each task was measured and evaluated.

Statistical Analysis

A comparison of means between several groups was performed by one-way analysis of variance (ANOVA; Tukey–Kramer multiple-comparison test for statistical differences between groups). The Mann–Whitney *U* test was used to compare means between two means, and ANOVA for repeated measures was used to assess the differences in task performance through repeated executions. $P<0.05$ was considered significant. NCSS 2001 software (Number Cruncher Statistical Software, Kaysville UT, USA) was used for statistical calculations.

Results

Interns Executed All Tasks Slower than Gastroenterologists or Surgeons, Who Performed Similarly for T1 and T2

Groups 1 and 2 completed T1 [G1 median 23.3 s, 95% confidence interval (CI) 21.1–34.6; G2 median 30.6 s, 95% CI 19–38.8] and T2 in a comparable amount of time (G1 median 31.3 s, 95% CI 13.9–40.6; G2 median 29.8 s, 95% CI 25.9–36.7; Fig. 2a,b). Group 1 performed T3 faster than group 2 (G1 median 60.4 s, 95% CI 33.5–94.9; G2 median 68.4 s, 95% CI 57.4–93.3), but the difference was not significant (Mann–Whitney *U* test; Fig. 2c). Group 3 performed all tasks (T1 median 96.8 s, 95% CI 60.6–118.2; T2 median 57.7 s, 95% CI 42.7–67; T3 median 146 s, 95% CI 92.2–161.2) at a significantly slower pace than groups 1 and 2 ($P<0.0001$ for all tasks, ANOVA; Fig. 2a–c).

Subjective Difficulty of the Tasks was Perceived Equally Between the Groups and Independent of Task Performance

Individual appraisal of task difficulty on a VAS revealed no significant differences between the groups. The mean score for T1 was 4.7 (95% CI 3.8–5.6) for G1, 4.5 (95% CI 3.7–5.3) for G2, and 4.4 (95% CI 3.7–5.15) for G3 (Fig. 3a). For T2, the corresponding values were 4.7 (95% CI 3.4–5.3), 3.7 (95% CI 2.8–4.5), and 4 (95% CI 2.6–5.4) for G1, G2, and G3, respectively (Fig. 3b). Task 3 was rated as 7.1 (95% CI 5.9–8.3) by G1, 7.1 (95% CI 6.5–7.7) by G2, and 6.4 (95% CI 5.1–7.7) by G3 (Fig. 3c).

Improvement by Task Repetition was more Pronounced in the Surgical Group

Improvement in task performance through repetition, as measured by speed, can give an indication of how quickly a lack of experience in the handling of complex instruments

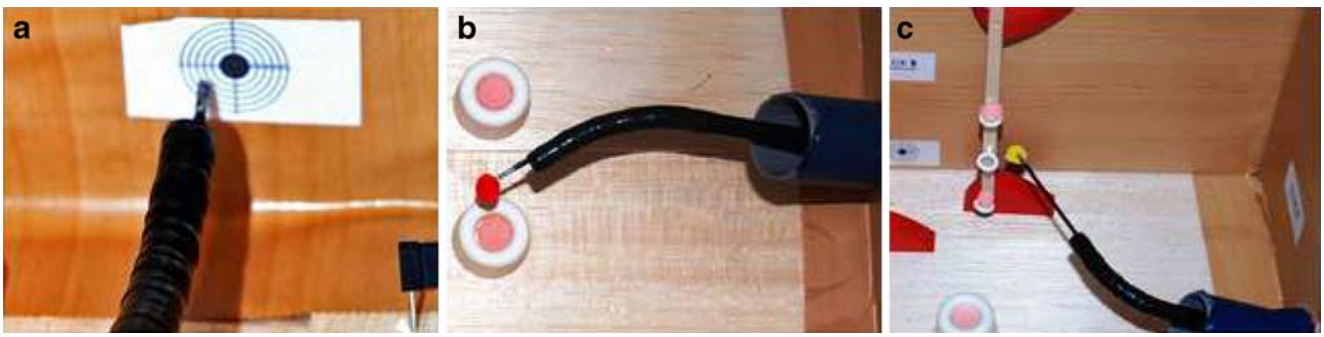

Figure 1 Tasks used to test for dexterity. **a** The approximation of the target in task 1, which involved touching the bull's eye with an endoscopic grasper. **b** Task 2 required grasping the fabric ball out of one basket and placing it into the second basket. **c** Task 3 required grasping the fabric ball from the middle ring on the shelf and laying it down into the right basket.

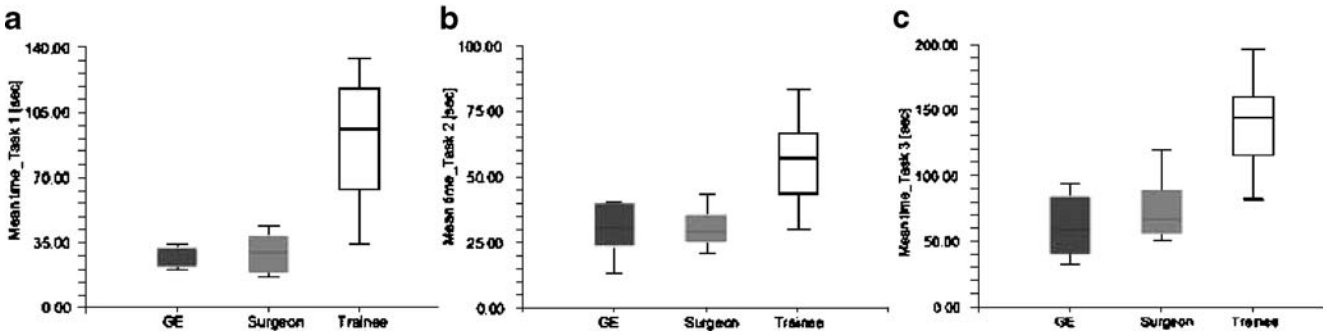

Figure 2 Boxplot comparisons of performance between groups for **a** task 1, **b** task 2, and **c** task 3. *Box lengths* represent the interquartile range (IQR) of 50% (from 25% to 75%), the *middle lines* represent the medians, and *T-bars* 75%/25% plus/minus 1.5 times the IQR. *GE* gastroenterologist.

and spatial adaptation can be overcome.[12,13] When considering who should perform novel tasks that require several degrees of knowledge, including basic surgical and anatomical knowledge, spatial orientation, manual dexterity, hand–eye coordination, instrument handling, and the management of potential complications, the steepness of the so-called learning curve in each category is essential knowledge.

Evaluating the improvement by repetition of the three groups showed that gastroenterologists, surgeons, and trainees improved similarly, if repeated task performance was compared to the initial task performance (time to complete task the first time; Fig. 4a,b). Gastroeneterologists showed no improvement by repetition in the difficult task (Fig. 4c). This indicates that this group has already an expertise in solving such difficult tasks and that further improvement cannot be gained by just ten repetitions.

Evaluating the time that surgeons or gastroenterologists needed to complete a task showed that there was no significant difference in improvement for the simpler tasks. (Fig. 5a,b). However, for the most difficult task, the initial performance by surgeons was significantly slower than that of gastroenterologists, but the surgeons improved significantly more over the course of the repetitions compared to gastroenterologists (ANOVA for repeated measures, $P=0.003$; Fig. 5c).

Laparoscopic or Endoscopic Experience Improved Task Performance Speed

Comparing test results between experts who had performed more than 200 laparoscopies or endoscopies and those without any endoscopic or laparoscopic experience found that the experienced groups performed significantly better for all three tasks (laparoscopic vs. inexperienced: T1 $P<0.019$, T2 $P<0.015$, T3 $P<0.055$; endoscopic vs. inexperienced: T1 $P<0.028$, T2 $P<0.088$, T3 $P<0.021$). These findings strongly correlate with the test results when comparing professional experience. Physicians with more than 6 years of experience had significantly better test results for all tasks compared to less experienced or inexperienced physicians ($P<0.001$).

Discussion

The data supports the hypothesis that endoscopically inexperienced laparoscopic surgeons are capable of quickly

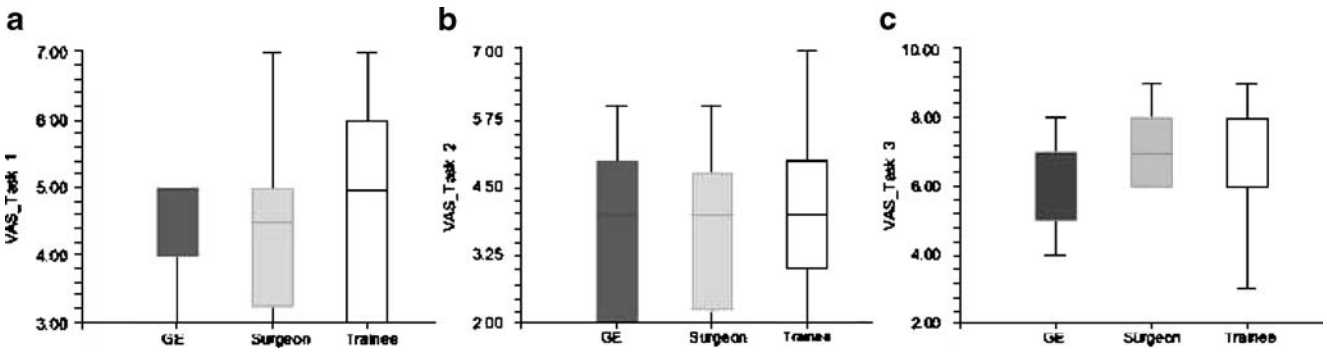

Figure 3 Individual appraisal of task difficulty on a visual analog scale. **a** Task 1, **b** task 2, and **c** task 3. *Box lengths* represent the IQR of 50% (from 25% to 75%), the *middle line*s represent the medians, and *T-bars* 75%/25% plus/minus 1.5 times the IQR. *GE* gastroenterologist.

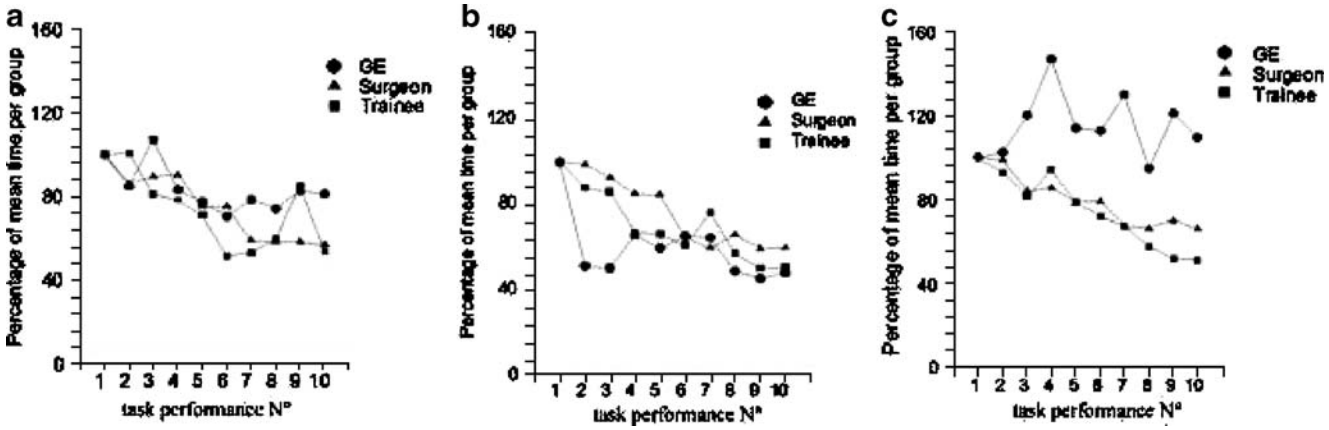

Figure 4 Learning curve adjusted for the mean time of initial execution by groups. **a** Task 1, **b** task 2, and **c** task 3. *GE* gastroenterologist.

(within ten repetitions) mastering the basic handling of a flexible endoscope. Their initial performance is superior compared to laparoscopically or endoscopically untrained persons. The results suggest that laparoscopic and/or surgical experience aids in learning to handle new instrumentation, such as flexible endoscopes. Admittedly, the better performance of experienced surgeons compared to interns may also be due to the selection of surgeon-specific traits and skills that facilitate a faster performance. In other words, young doctors who chose to be trained in the technically oriented field of surgery may be more dexterous than individuals who prefer less manually demanding fields of medicine. In addition, a laparoscopic surgeon who is already at an advanced level in his career has received years of manual training and has gone through a certain selection process by this training. Furthermore, laparoscopic surgeons are very familiar with the spatial orientation of the abdominal cavity and likewise the NOTES skills box.

While the gastroenterologists in our study were most familiar with the flexible endoscopes, they have never intentionally used them in an "open" space outside tubular structures. Overall, gastroenterologists performed the more difficult task better than the other test groups. These findings may be based on the fact that gastroenterologists do not depend on a "stable" visual horizon, which laparoscopically trained surgeons usually use as a benchmark. Furthermore, surgeons are accustomed to performing interventions where the image movements are not linked to the manipulated tools unlike in endoscopy. However, the present study demonstrates that surgeons can overcome these "new" hurdles after a short time of practice and quickly adapt to the required or sometimes helpful rotation of the horizon.

Hence, the limited experience in flexible endoscopy is unlikely to be a major handicap for surgeons for the upcoming NOTES era. The growing industrial interest in this new minimally invasive technique is currently resulting in the development of more NOTES-specific instruments and platforms. Presumably, this makes specific knowledge and the demanding ability to appropriately utilize conven-

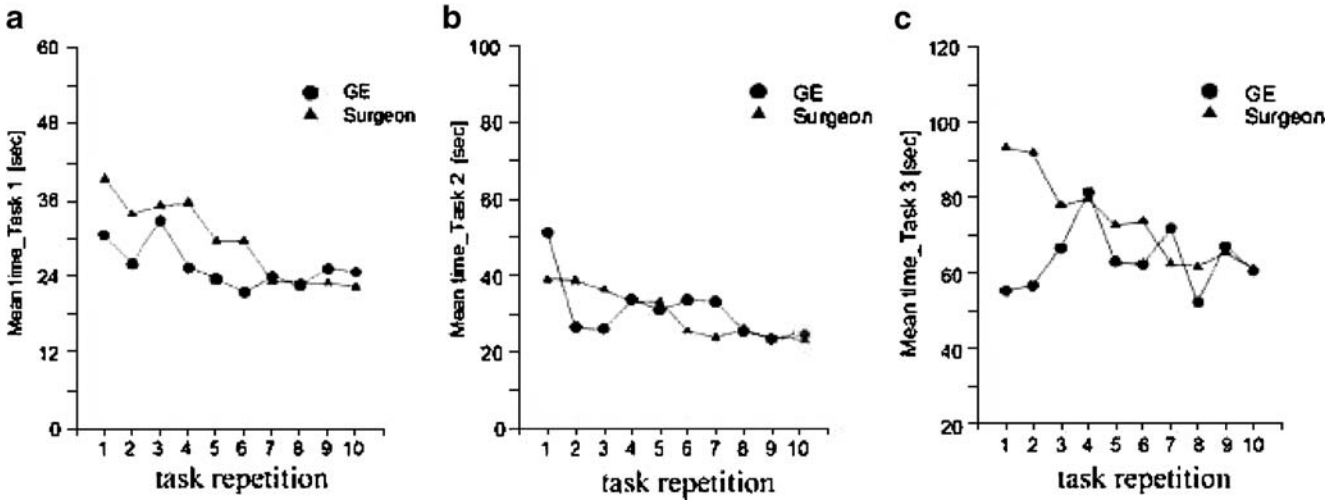

Figure 5 Improvement in task performance, as measured by speed, through repetition. **a** Task 1, **b** task 2, and **c** task 3. *GE* gastroenterologist.

tional endoscopes in NOTES procedures dispensable in the future.[14]

This study has certain restrictions and limitations. The reliability and validity of the chosen tasks for this study are not yet known. It remains unknown if results in our NOTES box correlate with NOTES performance in patients. Yet, we tried to choose realistic tasks, including testing for orientation, precision, and use of instrumentation in both a straight direction and in a 90° flexion. On the other hand, successful performance in NOTES is not only asking for the dexterous handling of a flexible endoscope. Intralumenal skills and experience, a characteristic of gastroenterologists, are crucial for this kind of surgery. Still, knowledge of intraabdominal anatomy and the procedure itself seems to be more important, and it is most certainly mastered by laparoscopic surgeons. Also, pre- and postoperative patient care and control of complications appears to be a surgical domain.

Conclusion

The present study supports the conclusion that surgeons will very quickly learn to handle flexible endoscopes. Because they have procedure-related knowledge and anatomical expertise, it appears logical that surgeons will assume control in NOTES procedures in the future. However, gastroenterologists most likely also have the means to acquire the surgical knowledge and skills and they also have the potential to conserve their role in the field of NOTES. At present, both surgeons and gastroenterologists do not have the complete skills set and applicable universal and intelligence platforms to successfully perform NOTES without each other. Therefore, it seems logical to create interdisciplinary teams to teach one another. In the short term, doctors, regardless of whether they are surgeons or gastroenterologists, with the best skills portfolio will be the NOTES physicians. In the long run, the training curricula for NOTES will be developed, and we may be able to identify who should perform NOTES: surgeons, gastroenterologists, or physicians trained in both fields.

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Perioperative Treatment with Infliximab in Patients with Crohn's Disease and Ulcerative Colitis is Not Associated with an Increased Rate of Postoperative Complications

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Abstract
Purpose The impact of infliximab (IFX) on postoperative complications in surgical patients with Crohn's disease (CD) and ulcerative colitis (UC) is unclear. We examined a large patient cohort to clarify whether a relationship exists between IFX and postoperative complications.
Methods A total of 413 consecutive patients—188 (45.5%) with suspected CD, 156 (37.8%) with UC, and 69 (16.7%) with indeterminate colitis—underwent abdominal surgery at the Massachusetts General Hospital between January 1993 and June 2007. One hundred one (24.5%) had received preoperative IFX≤12 weeks before surgery. These patients were compared to those who did not receive IFX with respect to demographics, comorbidities, presence of preoperative infections, steroid use, and nutritional status. We then compared the cumulative rate of complications for each group, which included deaths, anastomotic leak, infection, thrombotic complications, prolonged ileus/small bowel obstruction, cardiac, and hepatorenal complications. Potential risk factors for infectious complications including preexisting infection, pathological diagnosis, and steroid or IFX exposure were further evaluated using logistic regression analysis.
Results Patients were similar with respect to gender (IFX=40.6% men vs. non-IFX=51.9%, $p=0.06$), age (36.1 years vs.37.8, $p=0.43$), Charlson Comorbidity Index (5.3 vs. 5.7, $p=0.25$), concomitant steroids (75.3% vs. 76.9%, $p=0.79$), preoperative albumin level (3.3 vs. 3.2, $p=0.36$), and rate of emergent surgery (3.0% vs. 3.5%, $p=1.00$). IFX patients had higher rates of CD (56.4% vs. 41.9%, $p=0.02$), concomitant azathioprine/6-mercaptopurine use (34.6% vs. 16.6%, $p<0.0001$), and lower rates of intra-abdominal abscess (3.9% vs. 11%, $p<0.05$). After surgery, the two groups had similar rates of death (2% vs. 0.3% $p=0.09$), anastomotic leak (3.0% vs. 2.9%, $p=0.97$), cumulative infections (5.97% vs. 10.1%, $p=1$), thrombotic complications (3.6% vs. 3.0%, $p=0.06$), prolonged ileus/small bowel obstructions (3.9 vs. 2.8, $p=0.59$), cardiac complications (1% vs. 0.6%, $p=0.42$), and hepatic or renal complications (1.0 vs. 0.6% $p=0.72$). A logistic regression model was then created to assess the impact of IFX, as well as other potential risk factors, on the rates of cumulative

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postoperative infections. We found that steroids (odds ratio [OR]=1.2, p=0.74), IFX (OR 2.5, p=0.14), preoperative diagnosis of CD (OR=0.7, p=0.63) or UC (OR=0.6, p=0.48), and preoperative infection (OR=1.2, p=0.76) did not affect rates of clinically important postoperative infections.
Conclusions Preoperative IFX was not associated with an increased rate of cumulative postoperative complications.

Keywords Infliximab · Crohn's disease · Ulcerative colitis · Postoperative complications

Introduction

Infliximab (IFX) is a chimeric monoclonal antibody used in the treatment of patients with Crohn's disease (CD), ulcerative colitis (UC), rheumatoid arthritis, and a variety of other immune-mediated conditions. The drug targets tumor necrosis factor (TNF), a potent pro-inflammatory cytokine found in elevated concentrations in the inflamed tissues of such patients.[1] Although IFX has been demonstrated to improve the condition of CD and UC patients, it does not eliminate the eventual need for surgery in all such patients, and, for patients who do require surgery, questions have been raised regarding its safety in the perioperative period.[2–4] For example, some authors have reported that IFX use, before major abdominal surgery, may be associated with an increase in the rate of postoperative complications.[5,6]

This concern is not irrational. IFX is a potent immunosuppressor of cell-mediated cytotoxicity. Other immunomodulators of cell-mediated immunity, such as glucocorticoids and tacrolimus, have clearly been shown to increase postoperative infection rates.[7,8] Moreover, recent case reports have suggested an increased rate of tuberculosis, meningitis, pneumonia, and sepsis, arising from a variety of viruses and bacteria, in outpatients treated with IFX.[9,10] It might therefore be assumed that IFX would make postoperative complications more likely, especially in patients already weakened by chronic disease.

Interestingly, the literature relating to postoperative complications in IFX patients has not supported this commonly held assumption. Although one small study did suggest that inflammatory bowel disease (IBD) patients experienced more complications after surgery,[5] most other studies have found no statistically significant difference in surgical outcomes for these patients.[8,11,12]

Our study analyzed a large cohort of patients treated at the Massachusetts General Hospital (MGH) Crohn's and Colitis Center to compare the rates of postoperative complications between patients treated with IFX and not, before abdominal surgery for IBD. Our goal was to evaluate the impact of preoperative IFX treatment on postoperative complication rates in this cohort.

Methods

The MGH Institutional Review Board determined that this study was exempt from review.

Retrospective data were gathered on 413 consecutive patients who underwent abdominal surgery at MGH for complications of CD, UC, and indeterminate colitis (IC) between January 15, 1993 and June 27, 2007. These patients were identified from 1.8 million patients in the MGH medical records database, using the research patient database query tool (research patient data repository) to identify patients who underwent abdominal surgery for the diagnosis of UC (ICD-9:556), toxic gastroenteritis and colitis (ICD-9:558.2), CD (ICD-9:555), regional enteritis of the large intestine (ICD-9:555.1), regional enteritis of the small with large intestine (ICD-9:555.2), regional enteritis of the small intestine alone (ICD-9:555), and regional enteritis of an unspecified site (ICD-9:555.9; Fig. 1).

This initial search identified 455 patients, whose electronic medical records were individually reviewed to confirm that IBD had been recorded as the indication for surgery. As a result of this initial review, 42 patients were excluded because there was no history of IBD. These patients had been admitted for *Clostridium difficile* colitis, appendicitis, necrotizing enterocolitis, or other reasons.

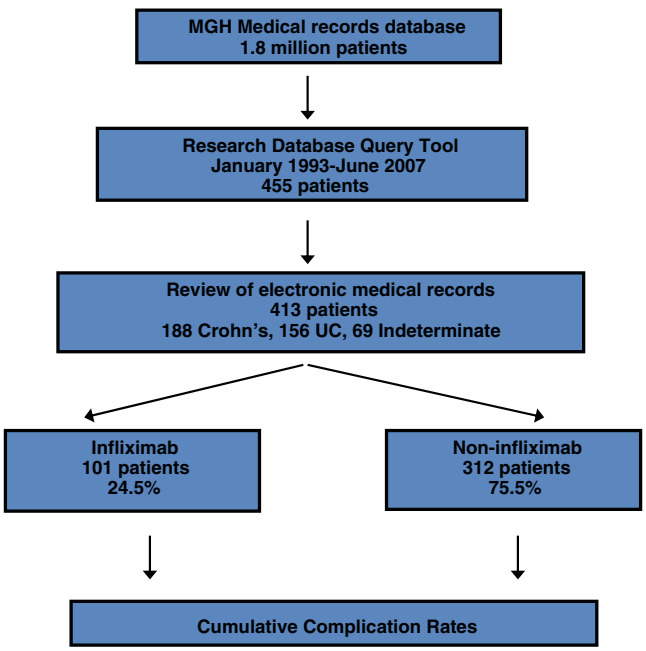

Figure 1 Cohort identification.

We next reviewed the medical records of the remaining 413 patients, including gastroenterology outpatient notes, surgical outpatient notes, hospital admission and discharge notes, operative reports, pathology reports, endoscopy reports, radiology reports, and admission and discharge medication lists. This search identified 101 patients who received IFX≤ 12 weeks before surgery. These 101 patients were then compared to the remaining 312 patients with respect to demographics, comorbidities, preoperative nutritional status, surgical indications, intraoperative findings, and rates of postoperative complications (defined as an occurrence within the first 30 days after the surgical procedure or during the index admission). In patients with more than one surgical procedure, we recorded the complications for the procedure that was performed after IFX treatment. If a patient did not receive IFX therapy at all, we recorded the complication rates after the first IBD-related procedure recorded in the medical record.

Controlling for Preexisting Medical Comorbidities

Given the wide range of preexisting comorbidities in both the IFX and non-IFX groups, we elected to calculate the age-adjusted Charlson Comorbidity Index for each patient to allow for a more standardized comparison. The Charlson Comorbidity Index, which was initially developed in 1987 (based on 1-year mortality data from internal medicine patients), has been extensively validated in the literature and has demonstrated excellent predictive validity for risk of mortality.[13,14]

Definitions of Postoperative Complications

Data were collected for the following postoperative complications: death, anastomotic leak (in those patients who had an anastomosis), sepsis, intra-abdominal abscess, wound infection, wound dehiscence, pneumonia, postoperative ileus>5 days, postoperative mechanical small bowel obstruction, portal vein thrombosis, deep vein thrombosis, pulmonary embolus, myocardial infarction, cardiac arrhythmia, liver failure, acute renal failure, and postoperative bleeding.

Because each of these individual complications occurred relatively infrequently, we calculated a total rate of postoperative complications. In addition, we grouped these complications into five general categories: cumulative infectious complications, hypomotility complications, thrombotic complications, cardiac complications, and hepato-renal complications (Table 1). The complications of anastomotic leak, bleeding and death were also evaluated separately in the subsequent statistical analysis.

Statistical Analysis

Categorical variables were presented as frequencies and percentages. Continuous variables were reported as mean and standard deviation (SD) or as medians and range, as appropriate. We performed comparisons between the cumulative rates of postoperative complications in IFX and non-IFX patients using Fisher's exact or chi-square tests, as appropriate, based on individual cell sizes. Continuous variables were evaluated using an independent *t*-test. We then explored the importance of the diagnosis of CD vs. UC, the impact of a preexisting intra-abdominal abscess at time of surgery, and the impact of steroid use on the rate of cumulative postoperative infectious complications with a logistic regression model.

A p value<0.05 was considered statistically significant on two-sided tests.

Results

Between 1993 and 2007, 413 patients underwent abdominal surgery at MGH for complications of IBD. The mean age was 37 years, and 49.2% were men. Of these, 188 (45.5%) had suspected CD, 156 (37.8%) had suspected UC, and 69 (16.7%) had IC. These patients underwent a variety of abdominal surgical procedures, and to our knowledge, IFX

Table 1 Definition of Cumulative Complications

Cumulative complication	
Infectious	Pneumonia, sepsis, anastomotic leak, enterocutaneous fistula, wound infection, dehiscence, intra-abdominal abscess
Hypomotility	Ileus> 5d, SBO
Thrombotic	Deep venous thrombus, portal vein thrombus, pulmonary embolus
Cardiac	Cardiac arrest, arrhythmia, myocardial infarction, congestive heart failure
Hepato-renal	Acute renal failure, acute liver failure
Bleeding	Bleeding requiring reoperation
Death	Death within 30 days after surgery
Anastomotic Leak	Anastomotic breakdown requiring reoperation and/or an abscess near anastomosis with a fistula to the anastomosis on a drain study

and non-IFX patients were not treated in a systematically different fashion by their surgeons (Table 2). One hundred sixteen (61.7%) of the patients with CD underwent ileocecal resection. The majority of patients with UC (N=146, 93.6%) underwent either a subtotal colectomy or total proctocolectomy. Overall, 304 patients (73.6%) had an anastomosis. One hundred eight patients had an ileoanal J pouch reconstruction.

Of the 413 surgical patients, 101 (24.5%) had been treated with IFX before surgery, and 312 (75.5%) had no prior IFX exposure. The IFX and non-IFX groups were similar demographically, although a somewhat larger proportion of CD patients had been treated with IFX. The IFX and non-IFX patients had similar rates of concomitant steroid use, but the IFX patients had a higher rate of preoperative azathioprine and 6-mercaptopurine exposure. Preoperative nutritional status, as indicated by preoperative albumin and hemoglobin levels, were similar. The non-IFX patients did tend to have higher preoperative white cell counts (Table 3).

Most patients within our cohort underwent planned (as opposed to emergent) surgery, and the rates of emergent surgery were similar for both groups (non-IFX 3.5% vs. IFX 3.0%, p=1.0). The indications for surgery were also similar in both patient populations. We observed no statistically significant difference between the groups in failure of medical management, rates of suspected perforation, and toxic colitis. However, IFX patients had a higher rate of suspected strictures (Table 4). IFX was generally avoided in patients with suspected malignancy or dysplasia or in patients with an intra-abdominal abscess (Table 4).

The IFX and non-IFX groups had similar cumulative rates of postoperative complications (IFX=16.8, non-IF=15.7, p=1). Isolated by categories of complications, rates of postoperative death, anastomotic leak, thrombotic complications, hypomotility, hepatorenal complications, and postoperative bleeding had a trend toward higher numbers in the IFX group, but this trend did not reach statistical significance (Fig. 2). Conversely, the rates of cumulative postoperative infections appeared higher in patients who did not receive IFX, but this was not statistically significant (Fig. 2). In the IFX group, two perioperative deaths were observed. One patient died on postoperative day no. (POD#) 70 after a prolonged hospital course complicated by postoperative intra-abdominal bleeding and cardiac arrest. The second died on POD#27 after a subtotal colectomy for perforated ileum secondary to chronic immunosuppression. Postoperatively, this patient had recurrent sepsis, renal failure, and atrial fibrillation with rapid ventricular response. In the non-IFX group, only one patient died. This non-IFX patient died on POD#58 with intra-abdominal fluid collections requiring interventional radiology drainage, renal failure, and atrial fibrillation with rapid ventricular response.

Table 2 Surgical Procedures Performed

Procedures	CD N=188*	UC N=156*	IC N=69*	Total N=413*
Small bowel resection/ strictureplasty	46	1	8	55
R colect/ileoocecectomy	116	3	20	139
Partial colectomy (transverse, left)	13	0	3	16
Subtotal colectomy	15	23	9	47
Total proctocolectomy	13	123	16	152
APR	6	2	7	15
Proctectomy	1	1	0	2
Anastomoses	158	116	30	304
IAPP	2	106	0	108

*These numbers are based on postoperative pathological diagnosis, not the preoperative clinical diagnosis.

Table 3 Comparison of Cohorts

Characteristic	IFX (n=101)	Non-IFX (n=312)	p value
Age (years) median (SD)	36.1 (17.6)	37.8 (19.1)	0.43
Gender			
Male	41 (40.6%)	162 (51.9%)	0.06
Female	60 (59.4%)	150 (48.1%)	
Ulcerative colitis	26 (25.7%)	100 (32.1%)	0.26
Crohn's disease	57 (56.4%)	131(41.9%)	0.02
Indeterminate colitis	16 (15.8%)	70 (22.4%)	0.20
Pre-op albumin mean (SD)	3.29 (0.76)	3.20 (0.79)	0.36
Pre-op hemoglobin mean (SD)	11.86 (1.99)	11.97 (3.0)	0.38
Pre-op WBC mean (SD)	8.7 (3.8)	10.2 (6.0)	0.02
Steroid exposure	76(75.3%)	240 (76.92%)	0.79
Concomitant azathioprine/ 6-mercaptopurine	37 (36.6%)	81 (26%)	0.04

Table 4 Indications for Surgery

	Infliximab (n=101)	Non-infliximab (n=312)	p value
Failure of med management	69 (68.3%)	199 (63.8%)	0.47
Suspected perforation	3 (3.0%)	18 (5.8%)	0.43
Toxic colitis	1 (1%)	2 (0.64%)	0.57
Malignancy or dysplasia	0 (0%)	12 (3.9%)	0.04
Symptomatic stricture	28 (27.7%)	49 (15.7%)	0.01
Intra-abdominal abscess	4 (4.0%)	34 (10.9%)	0.036

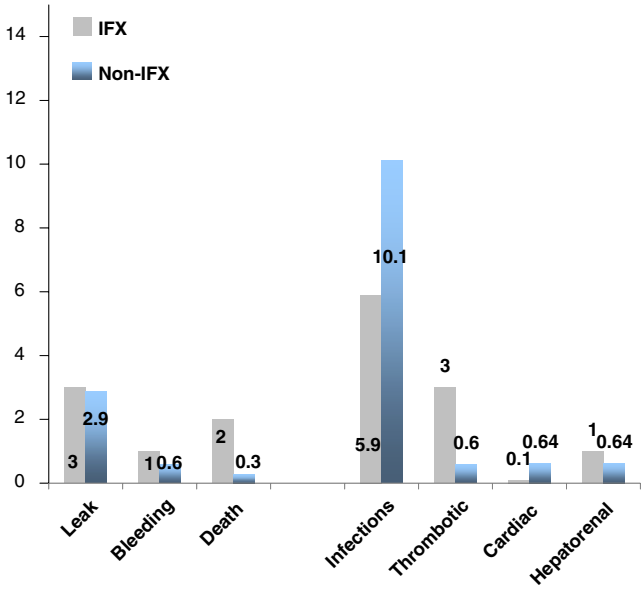

Figure 2 Comparison of cumulative postoperative complication rates (percent) based on IFX exposure. None of these was statistically significant.

Logistic regression analysis seeking to identify other factors that might have impacted the rates of postoperative infections was then performed. The model included the presence of a preexisting intra-abdominal abscess and steroid exposure and the diagnosis of CD or UC and IFX exposure. These variables were chosen either because the variable was statistically different between the IFX and non-IFX groups on our prior univariate comparison of cohorts or based on prior literature. None of these variables were found to be a reliable predictor of postoperative infections (steroids [OR=1.2, p=0.74], IFX [OR 2.5, p=0.14], preoperative diagnosis of CD [OR 0.7, p=0.63] or UC [OR 0.6, p=0.48], and preoperative infection [OR=1.2, p=0.76]).

The only statistically significant difference we observed was that the mean length of hospital stay was somewhat longer in patients treated with IFX (10.2 days [non-IFX] vs. 12.2 days [IFX], p<0.0001).

Discussion

Early case reports suggested that IFX, and other anti-TNF drugs, might be associated with an increased infection rate, both in the community setting and after surgery. However, larger studies that controlled for exposure to steroids, methotrexate, and other immunosupressants generally have produced more reassuring outcomes.[15] One prospective observational study, addressing 7,664 rheumatoid arthritis patients treated with anti-TNF medication in the community setting demonstrated that these drugs were not associated with any increased risk of serious infection, after adjusting for baseline risk (disease severity, comorbidity, extra-articular manifestations, baseline steroid use, and smoking).[16] Similarly, a large Medicare database study of 15,597 elderly patients with rheumatoid arthritis showed that those who used IFX had no greater rate of serious bacterial infections requiring hospitalizations, while glucocorticoid use did produce a dose-dependent increase in such infections.[17]

The data have also been varied in the surgical setting. For example, one retrospective study of 92 IFX patients who underwent various abdominal or orthopedic procedures showed that these patients experienced higher complication rates compared with historical controls.[6] On the other hand, a retrospective study of 768 rheumatoid arthritis patients (who underwent a total of 1,219 elective orthopedic procedures while being treated with IFX) showed no statistically significant difference between these patients and the control group in the rate of surgical site infections.[18] A smaller prospective study of 31 IFX patients showed no differences in infectious complications or healing rates, both short and long term, after orthopedic surgery.[19] In fact, these authors found that, when total complications (healing and infection) were analyzed, the IFX patients did better.[19]

Many of these earlier studies assessed surgical patients with rheumatoid arthritis. Some have argued that these studies of rheumatoid arthritis patients may not accurately predict outcomes in IBD patients, because IBD patients are generally more frail, have a worse preoperative nutritional status, a higher rate of high dose steroid use, and cannot avoid surgery. Thus, it has been argued that IBD patients are more likely to be affected by an additional immunosuppressant such as IFX.

Our study—which is, to our knowledge, the largest single institution study to date—did not support these arguments. To the contrary, we did not observe any statistically significant association between IFX use and rate of immediate postoperative complications in IBD patients. Although there may be many potential explanations for our observations, we believe that one important factor is the close collaboration between surgeons and gastroenterologists at our institution in presurgical decision making. IFX generally is prescribed on an outpatient basis, in an attempt to wean patients from steroids and treat active disease. Inpatients who are receiving intravenous corticosteroids, and who may be acutely ill, are taken directly to the operating room if they do not improve in a rapid fashion. While IFX is also used in the inpatient setting, both gastroenterologists and surgeons are mindful of the need to avoid delaying appropriate surgery in frail patients who are failing medical therapy.

Our findings are also consistent with several prior, smaller studies of patients with CD, which suggest that IFX may have little or no impact on postoperative outcomes in these

patients. One study evaluated 40 CD patients who had been treated with IFX before abdominal surgery compared to a control group of 39 patients without IFX exposure. They found no difference between the rate of early, late, minor, and major complications in the two groups.[12] A second study of 52 patients treated with IFX found no increase in the rate of septic complications associated with IFX use or with use of perioperative steroids or immunomodulator therapy.[11]

The data are more contradictory, however, for UC patients. One study compared a control group of 134 UC patients who had not been treated with IFX to 17 patients who had received IFX treatment. Some of the IFX patients had also been treated with tacrolimus. The authors found that preoperative treatment with IFX alone did not increase the incidence of postoperative complications. Interestingly, however, the patients who had been treated with a combination of IFX and tacrolimus did show a significantly higher rate of surgical morbidity.[8]

On the other hand, a study by Selvasekar et al.[5] analyzed 47 IFX patients who underwent an ileoanal pouch procedure for UC. These authors reported a significantly higher rate of pouch-related and infectious complications in the IFX patients. However, the IFX patients in this study had a higher (statistically significant) difference in their exposure to corticosteroids, which may have accounted for some part of the postoperative complication rate.[5] This may also explain some of the difference between Selvasekar's conclusions and the findings in our study. Our cohort had similar rates of exposure to corticosteroids regardless of preoperative IFX exposure. The IFX patients in our study generally were prescribed concomitant azathioprine or mercaptopurine, instead of steroids, to prevent development of antibodies to IFX.

Although the Selvasekar study demonstrated an increased rate of infections in UC patients who had received IFX, a prior study from the same institution did not show a similar effect in CD patients.[11] As a result, some authors have hypothesized that IFX may have different perioperative effects on patients with CD and UC.[5] Our study had a predominance of CD patients, and, therefore, if this hypothesis is correct, our prevalence of CD patients may have contributed to the lack of any statistically significant difference in postoperative complications. However, using logistic regression analysis and controlling for Crohn's and UC, we did not find a difference in the rate of IFX associated complications between patients with CD and those with UC. As a result, we have chosen to report these two cohorts together.

Our study demonstrates no major difference in surgical outcomes between the IFX and non-IFX patients. Nevertheless, our results need to be interpreted with some caution. First, this is a retrospective study. Although we reviewed medical records with extreme care, we could only isolate the data that were documented into the medical record by the treating physician at the time. In addition, despite the relatively large size of our cohort, the lack of statistical significance that we observed in complication rates may be because of insufficient statistical power. To identify a 5% difference in postoperative outcomes with a baseline complication rate of 15–25%, one would need a sample size of approximately 250 patients in each arm. Our study—while the largest to date in the literature—could only identify 101 patients who underwent IFX treatment within the 12 weeks preceding their abdominal surgery. While no statistical significance was observed between the groups, some of the trends seen in the IFX group such as the higher death rate are indeed worrisome, especially as the deaths in both groups were because of failure of multiple organ systems due to intra-abdominal sepsis. Furthermore, large-scale prospective studies are necessary before definite conclusions can be drawn.

Conclusions

Our study of 413 consecutive patients who underwent abdominal surgery for complications of IBD demonstrated no association between preoperative IFX use and an increased rate of postoperative surgical complications. Although reassuring, these results need to be interpreted with caution, as this is a retrospective study and the lack of difference could be because of insufficient power to detect the difference. While this is the largest study on this subject in the literature, a definitive answer on this important issue may need to await additional, large-scale prospective studies.

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Discussion

Andreas M. Kaiser, M.D. (Los Angeles, CA): Dr. Kunitake, thank you very much for your excellent presentation and certainly for the privilege to be the first to offer my comments.

The question that you raise in your paper is definitely a very important one, as surgeons are increasingly confronted with patients who are in need of surgery but are being treated with biological immunosuppressants. Your data – at first - seem to be reassuring. However, in light of the other studies that are out there (including e.g. the one following with the next presentation) that have suggested an increased risk with infliximab, one should remain very cautious and be careful with the interpretation. Most notably, it is crucial to look for causes of the observed variance. Even if the difference in your study was not statistically significant, the infliximab showed an odds ratio of 2.5 for infectious complications, which seems relevant given that there were more abscesses in the non-infliximab group to begin with. The other point is that the cohort size, even though it is very large compared to other studies, may still be too small and too little powered to detect such a significant difference between the different groups.

Another point that you should consider is that infliximab - depending on the circumstances - may be one of the confounding factors itself: e.g., there may on one hand be situations where the infliximab increases the risk if you compare it to a normal immune system. On the other hand, there may be different circumstances where the infliximab improves the overall morbidity or complication rate if a very bad disease is ameliorated prior to surgery and a sick patient is turned into a much better patient.

So I have a couple of questions that you may not yet have looked at but that you may want to look at in the future. First, did you look at the different subtypes of operations and try to find out whether there is a difference whether you did an ileoanal or whether you did a stricturoplasty? Second, did you look at the different diseases as such? And third, did you consider looking at the interval when the infliximab was actually delivered? You chose a cut-off time point of 12 weeks, but it may be that if you chose it closer to the infliximab administration between, let's say, zero to six weeks as compared to six to 12 weeks that there might have been a difference.

Thank you very much again and congratulations to your excellent work.

Hiroko Kunitake, M.D. (Boston, MA): These are excellent questions. Certainly I think your first point is a very valid one, which is that we have to look at this data with caution. Our study, although it is the largest study thus far, only has 101 patients in the infliximab group, and we calculated that we need at least 250 in the infliximab group in order to power our study well. So certainly this is preliminary data, but we are glad to present our results.

Now to your questions: You asked if we had looked at the influence of infliximab on the choice of operations

performed. We have begun to look at this and hope to soon be able to answer this question.

Regarding the effect of infliximab on patients with Crohn's compared to those with ulcerative colitis. Certainly in the literature thus far, most studies look separately at Crohn's patients and ulcerative colitis patients, and we initially did that as well. However, we found that there was no statistically significant difference in the rate of infection for each of these groups, and therefore in order to improve our numbers, we combined the two groups together, and we felt confident in doing that.

Finally, your question about the interval between infliximab treatment and surgery. I agree, many of these papers use two months or three months as a cutoff, but in fact when you look at their patient population, many of their patients were treated outside of that three-month period. For our study, we tried to include all patients with infliximab exposure within three months of surgery, because it is generally felt that infliximab is cleared in two months. So we included patients that were treated with infliximab within three months and we found results, which, quite frankly, we were a little bit surprised at. We were expecting to see a higher rate of infection with infliximab, but we did not.

Yoram Bouhnik, M.D. (Clichy, France): Thank you for this great presentation. Your data are very interesting. I would like to know if in infliximab-treated patients, some of them were treated not instead of surgery but as an "adjuvant therapy" before surgery, for example, to decrease the length of an ileal resection or to decrease the risk of temporary stoma, and if it was the case, if you have some results in this specific subgroup.

Dr. Kunitake: Thank you for your question. I hope I understand correctly that you are questioning whether or not infliximab is used as an alternative to surgery or not. On Monday we heard Dr. Bruce Sands and Dr. Richard Hodin speaking about the approach used at our institution which is that infliximab is used only when all other medical therapies have failed. Although many patients would prefer to postpone surgery as much as possible, there is very close collaboration between our gastroenterologists and surgeons and certainly when it is felt that the patient requires surgery, this is pursued.

Rosamaria Bozzi, M.D. (Naples, Italy): I wish to ask you in the patients that receive infliximab, what is the dose? They give infusions at zero to six weeks and then maintenance at eight weeks, and how many patients did make the maintenance of eight weeks? In Italy we give infliximab regularly, but when we have sub-obstructive symptoms or like sub-obstructive symptoms, we go directly to surgery, and we don't see other characteristic postoperative complications after the infliximab infusion. We prefer to perform surgery in patients with Crohn's or UC in high grade.

Dr. Kunitake: Thank you for your question. Our infliximab dosing is usually 5 mg/kg every eight weeks. There are some occasions where it has actually been increased to 10 mg/kg in a few patients to see if their response is better.

As far as the use of infliximab, I think what you are suggesting is that once they get to the point where there is definite stricture, infliximab should not be used because it will not be effective. I would like to say again that there is very close collaboration between gastroenterology and surgery at our institution such that if it is felt that these strictures are not resolvable with further medical management, our patients are taken to surgery.

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Use of Infliximab within 3 Months of Ileocolonic Resection is Associated with Adverse Postoperative Outcomes in Crohn's Patients

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Abstract
Background Few studies have evaluated preoperative infliximab use and postoperative outcomes in Crohn's patients. Our aim was to evaluate 30-day postoperative outcomes for Crohn's patients treated with infliximab within 3 months prior to ileocolonic resection.
Methods The study is a retrospective evaluation of data for patients undergoing ileocolonic resection after 1998 from a prospective Crohn's disease database. Patient characteristics and 30-day complications were compared for patients treated with infliximab within 3 months before surgery and an infliximab naïve group. The infliximab group was also compared with non-infliximab patients undergoing ileocolonic surgery before 1998.
Results Sixty of 389 Crohn's patients undergoing ileocolonic resection received infliximab. The infliximab and non-infliximab groups had similar characteristics, preoperative risk factors, and surgical procedure. However, steroid use was higher ($p<0.05$) in the non-infliximab group while concurrent immunosuppressive use was higher ($p<0.001$) in the infliximab group. Multivariate analysis showed infliximab use to be associated with 30-day postoperative readmission ($p=0.045$), sepsis ($p=0.027$), and intraabdominal abscess ($p=0.005$). The presence of diverting stoma ($n=17$) in the infliximab group was associated with lower risk of sepsis (0% vs. 27.9%, $p=0.013$). Similar results were noted when the infliximab group was compared to the pre-infliximab patients.
Conclusions Infliximab use within 3 months before surgery is associated with increased postoperative sepsis, abscess, and readmissions in Crohn's patients. Diverting stoma may protect against these complications.

Keywords Infliximab · Crohn's disease · Ileocolonic resection · Postoperative complications · Abscess · Sepsis · Anastomotic leak · Readmissions

Introduction

Various strategies have been adopted in an effort to treat exacerbations, maintain remission, and prevent or postpone surgery in Crohn's disease (CD) patients. Before 1998, this consisted of 5-ASA compounds,[1,2] steroids,[3,4] and immunosuppressants.[3–5] Failure of medical treatment, toxicity of medication, or steroid dependence prompted surgical intervention,[3–5] although surgery is associated with multiple advantages including relief of symptoms, improvement in quality of life, and withdrawal of potentially toxic medication.[6,7] Potential disadvantages of surgery also exist,[6–9] which have spurred the ongoing search for agents that could avoid surgery and maintain remission.

The demonstration of significant clinical response of CD patients to infliximab (IFX)[10,11] has changed clinical practice since 1998 with its use in patients unresponsive to other medications. IFX use has been shown to improve quality of life, maintain disease remission, facilitate discontinuation of

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steroids and immunosuppressive drugs, and avoid surgery.[12,13] Concerns regarding the potential harm of the medication in terms of septic perioperative outcomes for patients on IFX requiring surgery remain. Some studies report that IFX is associated with significant adverse outcomes such as severe infections, sepsis, abscess, cancer, infusion reactions, neurological complications, and death.[14,15] There is a paucity of data on perioperative outcomes for CD patients on IFX undergoing surgery. Two previous studies[22,23] reported outcomes in a small heterogeneous group of patients on the medication at variable time intervals before and after surgery. Since the complexity of surgery in CD patients may be variable and may, in itself, influence outcomes, we evaluated 30-day perioperative outcomes for CD patients who received IFX at any time within 3 months prior to undergoing ileocolonic resection. We hypothesized that by using a larger sample size and standardizing the timing of medication use and surgical procedure, any difference in postoperative outcomes for CD patients treated with IFX and an IFX naive group could be better determined. The aim of this study was to investigate outcomes for contemporary and historical cohorts of CD patients who underwent ileocolonic resection to see if use of IFX 3 months before ileocolonic resection may be associated with increased adverse postsurgical outcomes.

Methods

Patients

All patients undergoing surgery in the department of colorectal surgery at the Cleveland Clinic are currently accrued into an institution review board approved Crohn's disease database. Patient-related data pertaining to demographics, smoking history, ASA class, and indication of surgery; disease-related factors such as severity of disease, perioperative type, and dose of medication; and operative data such as type and extent of procedure performed and postoperative complications are prospectively maintained. From this database, data of all contemporary and historical cohort patients undergoing ileocolonic resection before and after 1998 were identified. One hundred and thirty-three patients who underwent ileocolonic resection from 1998 to 2007 had taken IFX. Of these, 24 had been administered IFX more than 3 months before surgery, 49 at some point after surgery, and 60 within 3 months before undergoing ileocolonic resection (IFX group). None of the patients who had taken IFX within 3 months of ileocolonic resection were treated with other types of antitumor necrosis factor. Medication use was verified with the pharmacy department at the Cleveland Clinic Foundation. Charts were reviewed, and all patients who took IFX contacted over the telephone to confirm the last dose of IFX infusion before ileocolonic resection. When patients could not recall the last date of their IFX infusion, the facilities where they received the medication were contacted for this information.

The 60 IFX patients were compared with 329 contemporary cohort patients undergoing ileocolonic resection who had never received IFX (non-IFX group). Differences between groups in relation to 30-day complications were evaluated. Since IFX has been used sometimes successfully for some patients in whom surgery was otherwise felt to be inevitable, patients treated with IFX may be expected to be sicker than those not on IFX. Thus, any potential adverse effects detected in the IFX patients undergoing surgery may be related to the fact that they are sicker rather than due to IFX. We, hence, chose to include a comparative group of patients in the pre-IFX era who underwent surgery since such patients may be expected to more accurately represent a comparative group with similar patients characteristics as might be expected if IFX were unavailable and, hence, not used. This group of 69 patients, who constituted a historical cohort (pre-IFX group), had undergone ileocolonic resection before IFX was approved in 1998.

Inclusion and Exclusion Criteria

Patients who had their first ileocolonic resection performed at outside institutions were excluded. Other exclusions included those with ulcerative colitis, indeterminate colitis, and other underlying immunodeficiency unrelated to their CD; when the last dose of IFX was longer than 3 months before surgery; if patients never took IFX until after surgery; and if they had perianal CD. Patients with a prior stoma for other reasons before their first ileocolonic resection for CD were also excluded. All patients included had ileocolonic CD confirmed endoscopically and/or radiographically.

Diagnostic Criteria

Demographics, comorbidity, and other patient characteristics were reviewed (Table 1). Diagnosis of CD was made clinically, endoscopically, and, where appropriate, radiographically. Failure of medical therapy was the most common indication for surgery—this consisted of persistent symptoms despite being on appropriate therapy for an appropriate length of time.

Surgical Procedure

The surgical procedure involved resecting part of the distal ileum and part of the proximal colon for ileocolonic CD and then anastomosing the ileum to the proximal colon to create an ileocolonic anastomosis. Loop stomas involved

Table 1 Patient Characteristics

	Non-IFX group (998–2007) n=329		IFX group (1998–2007) N=60	Pre-IFX group (1991 to 1997) N=69	p-Value
Gender (F)	178 (54.1%)		31 (51.7%)	33 (47.8%)	0.73[a] 0.66[b]
Age	36.84±14.37		35.83±11.90	37.96±12.49	0.92[a] 0.38[b]
Comorbidity	DM	5 (1.5%)	0 (0%)	0 (0%)	0.99[a]
	Cardiac	4 (1.2%)	3 (5.0%)	0 (0%)	0.8[a] 0.1[b]
	Renal	0%	0%	1 (1.4%)	0.99[b]
	HTN	18 (5.5%)	6 (10.0%)	9 (13.0%)	0.24[a] 0.59[b]
	Lung	5 (1.5%)	2 (3.3%)	2 (2.9%)	0.30[a] 0.99[b]
ASA Class	2	2	2		
Never smoked	141 (49.5%)	26 (48.1%)	33 (50.8%)	0.80* 0.78†	
Smoked	143 (50.2%)	28 (51.9%)	32 (49.2%)	0.80* 0.78†	

[a] p: Non-IFX vs. IFX
[b] p: Pre-IFX vs. IFX

the creation of a diverting stoma above the ileocolonic anastomosis with intention to close the stoma in the near future.

Definition of Variables

Intraabdominal sepsis was defined as the presence of abdominal complaints, fever, elevated white blood cell count, with a finding on imaging studies of an intra-abdominal fluid collection with or without anastomotic leak. Anastomotic leak was defined as patients with similar clinical presentations as those with intraabdominal sepsis which were found to have intraabdominal fluid collection and a true anastomotic leak that resulted in a surgical management of the leak. Patients with intraabdominal abscess clinically presented similarly and were found to have intraabdominal abscess that resulted in surgical or computed tomography-guided drainage of the abscess. Patients receiving 5-ASA derivatives, steroids, and immunosuppressives within 3 months of ileocolonic resection were considered to be on this therapy.

Outcome Measurement

Outcomes evaluated included 30-day mortality, wound infection, wound complications, anastomotic leak, sepsis, intraabdominal abscess, and readmissions rate.

Statistical Analysis

Fisher's exact test and Kaplan–Meier estimation with log rank tests were performed to assess differences in proportions between groups. Multivariable Cox models were used to assess the association between IFX use and each of 30-day outcomes (readmission, sepsis, and intra-abdominal abscess), adjusting for age, gender, comorbidities, penetrating abscess before surgery, diverting stoma, disease phenotypes, narcotics use, 6-mercaptopurine, azathioprine, and methotrexate. Odds ratios of the outcome with 95% confidence intervals were estimated for each variable in a multivariable model using R version 2.3.1 statistical program.

Results

Sixty of 389 CD patients undergoing ileocolonic resection received IXF (non-IFX—329). IFX and non-IFX groups had comparable patient characteristics (Table 1), disease behavior (Table 2), and operative procedure performed (Table 3). Table 4 gives the comparison of the perioperative medications used in the group.

Differences in Medication Use

As noted in Table 4, immunosuppressive use was higher in the IFX group (61.7%) compared with the non-IFX group (16.7%; p=0.001). However, steroid use was higher in the non-IFX group (76.9%) than the IFX group (65.0%; p= 0.05). When the IFX was compared with the pre-IFX group, immunosuppressive use was again higher in the IFX group (61.7%) compared with the pre-IFX 7.2% (p=0.001), while steroid use was higher in the pre-IFX group (80%; p=0.06). The 5-ASA use was similar between the groups.

Intraoperative and Postoperative Outcomes in IFX and non-IFX

Intraoperative complications, intraoperative, and postoperative transfusion use was similar between the groups.

Table 2 Disease Characteristics

	Non-IFX group (1998–2007) n=329	IFX group n=60	Pre-IFX group (1991 to 1997) n=69	p-Value
Nonstricturing/nonpenetrating Crohns	115 (48.7%)	16 (43.2%)	22 (44.9%)	0.68[a] 0.17[b]
Stricturing Crohns	66 (28.0%)	10 (27.0%)	20 (40.8%)	0.68[a] 0.17[b]
Penetrating Crohns	55 (23.3%)	11 (29.7%)	7 (14.3%)	0.68[a] 0.17[b]
Fibrostenosing Crohns	214 (65.0%)	36 (60.0%)	44 (63.8%)	0.45[a] 0.66[b]
Inflammatory	8 (2.4%)	0 (0%)	0 (0%)	0.61[a]
Abscess before or at surgery	144 (43.8%)	23 (38.3%)	22 (31.9%)	0.43[a] 0.44[b]

[a] p: Non-IFX vs. IFX
[b] p: Pre-IFX vs. IFX

Postoperative ileus, cardiopulmonary, neurological, and renal complications were also similar. Outcomes that were different on univariate analysis are as in Table 5. Although the non-IFX group had increased use of preoperative steroids, adverse postsurgical outcomes appeared to be lower in this group when compared with the IFX group. Using Cox multivariate analysis to adjust for differences in medication use, age, gender, comorbidity, disease phenotypes, and the presence of an abscess before or at surgery, the IFX group still appeared to have an increased risk of 30-day postoperative readmission (OR—2.33 [1.02–5.33], p=0.045, Table 6), sepsis(OR—2.62 [1.12–6.13], p=0.027, Table 7), and intraabdominal abscess (OR—5.78 [1.69–19.7], p=0.005, Table 8).

Presence of Diverting Stoma and Differences in Postoperative Adverse Outcome

IFX patients who had stoma (n=17) above their anastomosis had a lower incidence of sepsis when compared with those without a stoma (sepsis 0% vs. 27.9%, p=0.013). A slightly decreased rate of postoperative sepsis was also noted in the non-IFX group who had a stoma above their anastomosis (10.4% vs. 6.8%) though this was not statistically significant (p=0.40).

Comparison of Postoperative Outcomes between IFX and Pre-IFX Groups

When comparing the IFX group to the non-IFX group before 1998 (pre-IFX era), despite similar preoperative and perioperative factors, the IFX group still appeared to have higher postoperative sepsis (20 vs. 5.8%, p=0.021), anastomotic leak (10% vs. 1.4%, p=0.049), and readmission rate (20% vs. 2.9%, p=0.007). Because there were only five patients who had diverting stoma in the pre-IFX group, statistical analysis could not be performed to determine whether or not a stoma above anastomosis made any difference in adverse outcomes among this group.

Timing of IFX Use

Evaluation of postoperative outcomes for a subset of patients who received IFX within 2 months of surgery did not reveal any difference when compared with those who

Table 3 Characteristics at Operation

	Non-IFX group (1 998–2007) n=329	IFX group n=60	Pre-IFX group (1991 to 1997) n=69	p-Value
Laparoscopic-assisted	95 (28.9%)	18 (30.0%)	13 (18.8%)	0.91[a] 0.35[b]
Open	228 (69.3%)	41 (68.3%)	54 (78.3%)	0.91[a] 0.35[b]
Diverting stoma	60	17	5	
hand sewn	50 (20.9%)	8 (18.6%)	3 (6.2%)	0.69[a] 0.08[b]
Stapled	183 (76.6%)	35 (81.4%)	45 (93.8%)	0.69 0.08[b]

[a] p: Non-IFX VS. IFX
[b] p: Pre-IFX VS. IFX

Table 4 Medication Use before Surgery

	Non-IFX group (1998–2007) n=329	IFX group n=60	Pre-IFX group (1991 to 1997) n=69	p-Value
5-ASA-	196 (59.6%)	36 (60.0%)	35 (50.7%)	0.95[a] 0.29[b]
6MP/AZA/MTX	55 (16.7%)	37 (61.7%)	5 (7.2%)	<0.001[a] <0.001[b]
IFX	0 (0%)	60 (100%)	0 (0%)	<0.001[a] <0.001[b]
Steroids	253 (76.9%)	39 (65.0%)	55 (79.7%)	<0.052[a] 0.06[b]

[a] p: No IFX vs. IFX
[b] p: Pre-IFX vs. IFX

received the medication within 3 months of ileocolonic resection.

Discussion

The decision as to when to proceed with surgery or to persist with medical treatment in patients with CD is often difficult.[16] The need for surgery in patients who develop complications of the disease whilst on medical treatment is self-evident. Traditional strategies revolved around progressing to surgery when medical treatment with 5-ASA derivatives, steroids, and immunosuppression failed.[1,2] The availability of IFX in 1998 has been associated with its use and a decreased need for surgery[10,11] in addition to the long term side effects of IFX,[17] whether its use in patients undergoing surgery leads to adverse outcomes needs further investigation.

A study from the Mayo Clinic reported significant adverse outcomes associated with the use of IFX in ulcerative colitis (UC) patients undergoing ileal pouch-anal anastomosis (IPAA) procedures.[18] We found similar results in UC patients on IFX after IPAA.[19] The few studies investigating postsurgical outcomes in CD patients treated with IFX have not revealed any significant adverse outcomes in the IFX-treated and IFX-naïve groups[20,21]. These studies, however, included mixed groups of patients undergoing various procedures who received IFX at various periods before and after surgery. Colombel et al.[20] reported post operative outcomes for 52 CD patients treated with IFX who underwent abdominal operations. Patients who underwent a variety of procedures and some who received IFX 8 weeks before and 4 weeks after surgery were included. Marchal et al.[21] evaluated outcomes in 40 CD patients who received treatment with IFX within 12 weeks before surgery. This study was limited by small sample size, lack of standardi-

Table 5 Post Operative Outcomes

	Complication	Non IFX group (1998–2007) n=329 (%)	IFX group n=60 (%)	Pre-IFX group (1991 to 1997) n=69 (%)	Odd's ratio (95%CI)	p-Value
30-Day complications	Urinary complications	0	1.7	0.0		0.15[a] 0.47[b]
	Wound dehiscence	0.30	0.0	1.4		1.0[a] 1.0[b]
	30-Day mortality	0	1.7	0.0		1.0[a] 1.0[b]
30-Day complications	Readmission rate	9.4	20.0	2.9	2.40(1.15,5)* 8.37(1.79,39.15)†	0.019[a] 0.007[b]
	Sepsis	9.7	20.0	5.8	2.32(1.12, 4.82)* 4.06(1.23,13.37)†	0.024[a] 0.021[b]
	Intraabdominal abscess	4.3	10.0	4.3	2.50(0.92, 6.79)* 2.44(0.58,10.23)†	0.10[a] 0.30[b]
	Anastomotic leak	4.3	10.0	1.4		0.09[a] 0.049[b]
	Reoperation	3.0	8.3	0.0	2.9(0.95,8.81)*	0.06[a] 0.02[b]

[a] p: No IFX vs. IFX
[b] p: Pre-IFX vs. IFX

zation of surgical procedures, and the potential confounding effect of multiple operations.

Since patients with CD may have varying complexity of surgery, we chose to standardize the surgical procedure performed. Only patients undergoing ileocolonic resection with anastomosis (ICRA) were selected. In particular, those requiring additional procedures such as stricturoplasty, small bowel, or colonic resections were excluded. Since patients who underwent previous surgery may need more complex surgery, we excluded patients who had previously undergone surgery prior to ICRA. Although the half life of IFX is 10 days,[22] a previous study suggested that the use of IFX within 2 months prior to surgery may influence outcomes.[18] Since it is not clearly known whether the effect of IFX persists for a longer period, we chose to look at outcomes for CD patients treated with IFX within 3 months before surgery. We found that the use of IFX within 3 months before ileocolonic resection in CD patients appears to be associated with adverse outcomes such as 30-day postoperative intraabdominal sepsis, intraabdominal abscess, anastomotic leak, and readmission. Considering the function of TNF-alpha as a potent inflammatory mediator, one would expect that if this compound is blocked, there could be a potential risk for increased infection as shown in multiple studies.[23,24] Therefore, our finding of an increased incidence of sepsis and abscess after surgery is not surprising. It is also conceivable that the immunosuppressive effects of IFX may last well beyond the time when IFX is cleared from the body. A subgroup analysis of our data showed that there was no difference in the rate of complications for patients receiving IFX 2 and 3 months prior to ileocolonic resection.

In this study, we also found that having a stoma above an anastomosis appears to be associated with less postoperative infectious adverse outcomes. The presence of a defunctioning stoma has previously been demonstrated to reduce septic complications from anastomotic leak in other studies.[25] For those who did not have stoma above their anastomosis, perhaps some of these patients could not mount inflammation strong enough to control the infection due to blunted TNF alpha effect by IFX,[26–28] and ultimately, some of these patients proceeded to develop intraabdominal abscess, sepsis, and anastomosis leak.

Our study has some limitations. Firstly, the study is a retrospective review of data of a historical cohort. Subsequently, the results obtained may be overestimated or underestimated. Secondly, while our sample size for the IFX group is larger than published data, the sample size of 60 patients is still low; thus, differences in postsurgical outcomes that we found in this study might be further underestimated. Patients who were administered IFX more than 3 months before surgery and those who took IFX after surgery were also excluded; thus, the effect of IFX in these subsets of patients could not be ascertained. Furthermore, there was not enough sample size for the pre-IFX group to see if having stoma made a difference in postoperative outcome among this group.

In conclusion, use of IFX 3 months before ileocolonic resection appears to be associated with an increased risk of 30-day postoperative intraabdominal abscess, sepsis, anastomotic leak, and readmission rate. However, presence of stoma above the anastomosis appears to be associated with a decrease in these risks. A prospective study investigating IFX use 3 months before ileocolonic resection and anastomosis (with and without stoma) and postoperative outcome may help provide further crucial data in CD patients undergoing surgical procedures.

Table 7 Multivariable Logistic Regression Model Results for 30-day Sepsis

Variable	Odds ratio(95% CI)	*p*-Value
IFX	2.62 (1.12–6.13)	0.027
6MP/AZA/MTX	1.40 (0.66–2.98)	0.38
Steroids	1.10 (0.50–2.42)	0.81
Comorbidity	0.37 (0.08–1.67)	0.20
Penetrating abscess	1.71 (0.89–3.30)	0.11
Diverting stoma	0.28 (0.09–0.83)	0.021

Parameter estimate and odds ratio relative to a 5-year difference.

Table 6 Multivariable Logistic Regression Model Results for 30-day Readmission

Variable	Odds ratio(95% CI)	*p*-Value
IFX	2.33 (1.02–5.33)	0.045
6MP/AZA/MTX	1.14 (0.53–2.46)	0.74
Steroids	0.95 (0.45–2.03)	0.90
Comorbidity	0.98 (0.32–3.01)	0.97
Penetrating abscess	1.22 (0.63–2.35)	0.55
Diverting stoma	0.82 (0.35–1.92)	0.66

Parameter estimate and odds ratio relative to a 5-year difference.

Table 8 Multivariable Logistic Regression Model Results for 30-day Intraabdominal Abscess

Variable	Odds ratio(95% CI)	*p*-Value
IFX	5.78 (1.69–19.7)	0.005
6MP/AZA/MTX	0.41 (0.11–1.52)	0.18
Steroids	2.94 (0.63–13.6)	0.17
Comorbidity	0.30 (0.03–2.73)	0.29
Penetrating abscess	1.40 (0.55–3.57)	0.48
Diverting stoma	0.16 (0.02–1.25)	0.08

Parameter estimate and odds ratio relative to a 5-year difference.

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Epigenetic Regulation of WNT Signaling Pathway Genes in Inflammatory Bowel Disease (IBD) Associated Neoplasia

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Abstract
Introduction WNT signaling pathway dysregulation is an important event in the pathogenesis of colorectal cancer (CRC) with *APC* mutations seen in more than 80% of sporadic CRC. However, such mutations in the WNT signaling pathway genes are rare in inflammatory bowel disease (IBD) associated neoplasia (dysplasia and cancer). This study examined the role of epigenetic silencing of WNT signaling pathway genes in the pathogenesis of IBD-associated neoplasia.
Methods Paraffin-embedded tissue samples were obtained and methylation of ten WNT signaling pathway genes, including *APC1A*, *APC2*, *SFRP1*, *SFRP2*, *SFRP4*, *SFRP5*, *DKK1*, *DKK3*, *WIF1* and *LKB1*, was analyzed. Methylation analysis was performed on 41 IBD samples, 27 normal colon samples (NCs), and 24 sporadic CRC samples.
Results Methylation of WNT signaling pathway genes is a frequent and early event in IBD and IBD-associated neoplasia. A progressive increase in the percentage of methylated genes in the WNT signaling pathway from NCs (4.2%) to IBD colitis (39.7%) to IBD-associated neoplasia (63.4%) was seen (NCs vs. IBD colitis, $p<0.01$; IBD colitis vs. IBD-associated neoplasia, $p=0.01$). In the univariate logistic regression model, methylation of *APC2* (OR 4.7, 95% CI: 1.1–20.63, $p=0.04$), *SFRP1* (OR 5.1, 95% CI: 1.1–31.9, $p=0.04$), and *SFRP2* (OR 5.1, 95% CI: 1.1–32.3, $p=0.04$) was associated with progression from IBD colitis to IBD-associated neoplasia, while *APC1A* methylation was borderline significant (OR 4.1, 95% CI: 0.95–17.5, $p=0.06$). In the multivariate logistic regression model, methylation of *APC1A* and *APC2* was more likely to be associated with IBD-associated neoplasia than IBD colitis. (OR *APC1A*: 6.4, 95% CI: 1.1–37.7 p=0.04; OR *APC2* 9.1, 95% CI: 1.3–61.7, $p=0.02$).
Summary Methylation of the WNT signaling genes is an early event seen in patients with IBD colitis and there is a progressive increase in methylation of the WNT signaling genes during development of IBD-associated neoplasia. Moreover, methylation of *APC1A*, *APC2*, *SFRP1*, and *SFRP2* appears to mark progression from IBD colitis to IBD-associated neoplasia, and these genes may serve as biomarkers for IBD-associated neoplasia.

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Keywords IBD · Methylation · WNT signaling · Colorectal cancer

Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the intestines afflicting about one million individuals in the US, with 30,000 new cases each year.[1] IBD includes two distinct disease categories, Crohn's disease (CD) and ulcerative colitis (UC), both of which are associated with an increased risk of CRC.[2,3] Additionally, CRC is a major cause of increased mortality in IBD patients.[4] The risk of CRC in IBD increases with prolonged disease duration and is greater in those with extensive colitis.[5] This risk has been reported as ten- to 20-fold higher in UC patients with disease duration of 20 years or more, although current treatments may have modulated this risk.[6–8] As a result of the recognition of this increased risk, regular surveillance colonoscopy at 1–3 years interval is recommended for patients with long-standing disease.[9–12]

IBD-associated carcinomas arise in areas of dysplasia which are flat and difficult to recognize at colonoscopy.[13,14] Consequently, one may have to take 33 or more colonic biopsies to have a 90% confidence of finding dysplasia. To increase the accuracy of colonoscopic surveillance to 95%, nearly twice the number of biopsy specimens are required.[15] This makes surveillance labor intensive and expensive. Moreover, some of the recent studies have questioned the effectiveness of surveillance.[16–18] In order to improve the effectiveness of surveillance, there is a great need for objective and reliable molecular markers for early detection of IBD-associated neoplastic lesions especially among patients with long-standing disease.

DNA methylation-dependent silencing of cancer related genes is an important and early event in CRC. Methylation of promoter-associated CpG islands leads to binding of various proteins having methyl binding domains like MeCP2, MBD2, and MBD3; these proteins may then initiate a cascade of events, which eventually lead to transcriptional silencing.[19] Transcriptional silencing also involves changes in histone tails such as histone H3 lysine 9 methylation and histone H3 lysine 27 methylation as well as recruitment of histone deacetylases.[20,21] All of these modifications result in changes to the local chromatin structure, which in turn result in a tightly compacted chromatin, which in turn restricts the access to transcription factors facilitating transcriptional silencing. Aberrant age-related as well as cancer-specific methylation of genes like *p16*, *E-cadherin*, *hMLH1*, *p14*, *HPP1*, *ER*, etc., has already been reported in the setting of IBD-associated neoplasia (dysplasia and cancers).[22–26] It is believed that chronic inflammatory states like IBD may predispose to accelerated aberrant methylation and inactivation of tumor suppressor genes, which in turn may accelerate the development of cancer.

The WNT signaling pathway is crucial to the development of sporadic CRC.[27,28] Activating mutations in the WNT signaling pathway are seen in more than 80% of sporadic CRC.[29,30] Signaling in the WNT signaling pathway begins when the WNT ligand, a secreted factor binds to the Frizzled (Fz) receptor and LRP 5/6 co-receptor to initiate the signaling cascade.[31] Proteins of the disheveled (Dsh or dvl) family then interact with Fz receptors.[32] These interactions subsequently lead to inhibition of complex formed by *APC*, *AXIN*, and Glycogen synthase kinase-3β (*GSK-3β*). The *APC/AXIN/GSK-3β* complex normally plays a role in phosphorylation and degradation of β-catenin. As a result of the inhibition of the *APC/AXIN/GSK-3β* complex, β-catenin accumulates and is translocated to the nucleus[33] where it upregulates the transcription of many cancer-related genes including *MYC*,[34] *cyclooxygenase-2* (*COX-2*),[35] and *cyclin D1*(Fig. 1).[36] Powerful developmental regulatory pathways, like the WNT signaling pathway, are controlled by stringent negative regulation, which, if disrupted, can lead to their aberrant activation and tumorogenesis. WNT signaling pathway is normally inhibited by soluble Fz-related proteins (SFRPs) and WNT inhibitory factor (WIF1), which bind to WNT ligands extracellularly[37] and by proteins of the dickkopf (DKK) family, which bind to the LRP surface molecule.[38] Hence, inactivation of *SFRPs*, *WIF1*, and *DKKs* by mechanisms such as promoter methylation can lead to activation of WNT signaling in cancer cells. Similarly, inactivation of *APC* by mutation or promoter methylation or both can lead to prevention of degradation of β-catenin as *APC* normally plays a role in β-catenin degradation by complexing with other proteins as described previously. Although mutations of WNT signaling pathway genes including *APC* have been described in sporadic CRC, mutations of these genes are rare (0% to 6%) in IBD-associated neoplasia.[39]

Recent reports have explored the role of WNT signaling in IBD-associated neoplasia, but as yet little is known about the epigenetic regulation of this pathway in IBD-associated neoplasia.[40–42] Increased nuclear β-catenin and reduced cytoplasmic APC1A expression has recently been reported in the setting of UC-associated cancers.[43,44] Since mutation of WNT signaling genes is a rare event in IBD-associated neoplasia, we hypothesized that methylation of genes in the WNT pathway may be responsible for dysregulation of WNT signaling in these lesions. In the current study, we investigated if promoter methylation of WNT pathway genes occurs during the course of IBD-associated carcinogenesis and furthermore characterize the progression of the methylation events during the course of IBD associated neoplasia. This may help to find potential candidates for

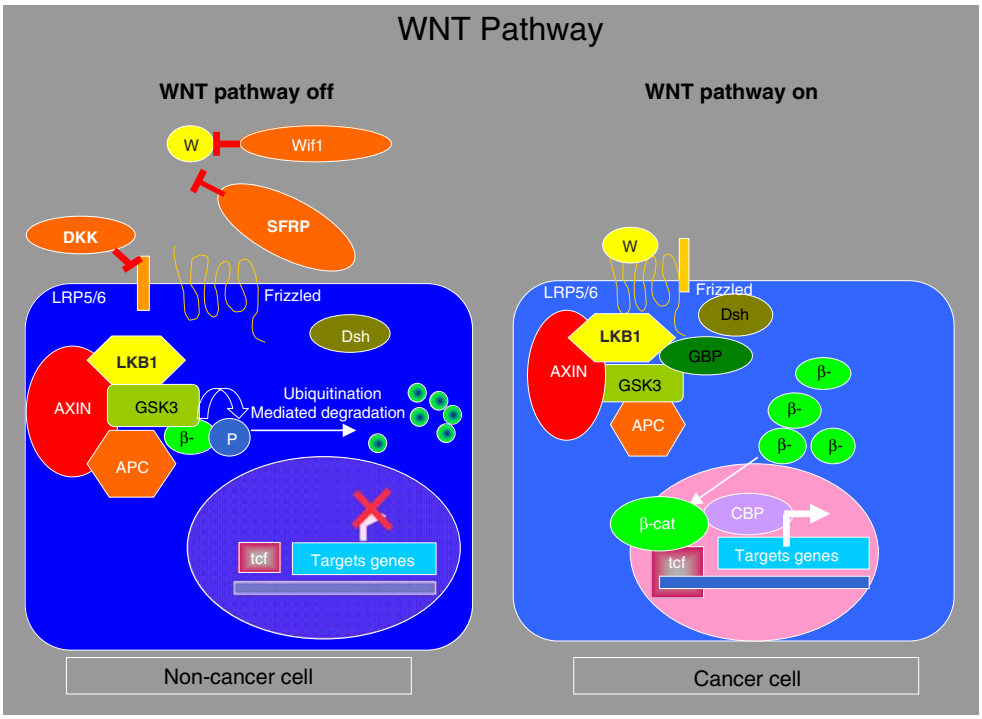

Figure 1 Overview of the genes involved in the WNT signaling pathway. When the pathway is turned on by binding of WNT, a cascade of events leads to translocation of β-catenin to the nucleus, which in turn upregulates the expression of many cancer-related genes.

investigation as biomarkers for early detection of IBD-associated neoplasia.

We now show that methylation of WNT signaling pathway genes is an early event that can be seen in long-standing IBD colitis and there is a progressive increase in methylation of the WNT signaling pathway genes during the development of IBD-associated neoplasia.

Materials and Methods

Patient Samples

Paraffin-embedded tissue samples were obtained from Johns Hopkins Hospital (JHH) pathology archives in accordance with regulations of the Institutional Review Board and HIPAA compliance. Tissue samples were obtained from 18 IBD patients who underwent colectomy from 1997 to 2006. Eleven of out 18 patients had IBD-associated neoplasia (either dysplasia or cancer) while seven were non-cancer IBD controls. A total of 41 IBD tissue samples were obtained including six IBD cancers, two high-grade dysplasias (HGDs), eight low-grade dysplasias (LGDs), and 25 noncancerous IBD colitis samples. Slides were first reviewed by an expert gastrointestinal pathologist at the JHH and then 5-um-thick sections of the desired paraffin-embedded tissue blocks were procured. In the case of IBD patients with dysplasia/cancer, we studied dysplasia/cancer samples as well as nondysplastic samples from other non-neoplastic areas of the colon. We also investigated methylation of the WNT signaling pathway genes in 27 NCs from 27 patients as well as 24 samples from 24 sporadic CRC patients. Sporadic CRC patients were stage matched to the IBD-associated cancer patients to determine if there were any differences between methylation of these genes between sporadic and IBD-associated cancers.

Methylation Analysis

Methylation analysis was performed using the methylation-specific polymerase chain reaction (MSP) strategy, as previously described.[45] DNA was extracted following a standard phenol-chloroform extraction protocol. Bisulfite modification of DNA was done using the EZ DNA methylation Kit™ (Zymo Research) as per manufacturer's instructions. Methylation-specific PCR was carried out in a 25-μl reaction containing 10× MSP buffer, 10 mM dNTPs, 33 pmol of each of the methylated or unmethylated primers, 0.5 unit of JumpStart™ REDTaq® DNA polymerase and 4 μl of bisulfite-treated DNA. Amplification cycles were as follows: one cycle of 95°C for 5 min followed by 35 cycles of 95°C for 30 s, annealing temp for 30 s, 72°C for 30 s, and a final extension step of 72°C for 5 min. In vitro methylated DNA (IVD) was used as a positive control for MSP. IVD was created by treating cell line DNA with Sassy methylated (New England Bolas) as directed. DKO, which is a double knockout derivative of the CRC cell line Hct116 with knockout of the major DNA methyltransferases (DNMT1−/− and DNMT3b−/−) was used as an additional

negative control. DKO lacks methylation at 95% of the known CpG sites.[46] Seven and a half microliters of each amplification reaction was loaded and run on 2% agarose gel containing GelStar™ Nucleic Acid Gel Stain (Cambrex Bio Science) and visualized by ultraviolet illumination.

We tested the promoter methylation of 10 WNT signaling pathway genes including *APC1A* (adenomatous polyposis coli1a), *APC2* (adenomatous polyposis coli2), *SFRP1* (secreted frizzled related protein1), *SFRP2* (secreted frizzled related protein2), *SFRP4* (secreted frizzled related protein4), *SFRP5* (secreted frizzled related protein5), *DKK1* (dickkopf1), *DKK3* (dickkopf3), *WIF1* (WNT inhibitory factor1), and *LKB1* (serine threonine kinase). All of these genes, except *APC2*, have been previously described to be hypermethylated and silenced in CRC by candidate gene approaches.[47–50] Hypermethylation of *APC2* was recently described by Schuebel et al. in their transcriptome-wide approach to find genes hypermethylated in colon cancer.[51] Table 1 summarizes the primer sequences used for the MSP reaction and the annealing temperatures used for the respective PCR reactions. Percentage of methylated genes for each tissue type sample was calculated using the following formula: (number of genes methylated)/(number of genes tested)×100. The means of samples belonging to each tissue type were then compared.

Statistical Analysis

Categorical variables were analyzed using Chi-square tests, while continuous variables were analyzed using Mann–Whitney *U* test. *P* values of less than 0.05 were considered significant. Logistic regression was used to calculate the odds ratios (ORs) and 95% confidence interval. All statistical analysis was performed using the STATA 9.2 software package (College Station, TX).

Results

All IBD patients had long-standing disease with median disease duration of 12 years (12.5 years for those with IBD-associated neoplasia vs. 12 years for controls; $p=0.2$). The median age of IBD colitis samples was 52 years, while the median age of patients with IBD-associated neoplasia was 54 years ($p=0.4$). The cancers from IBD patients were Stages I and II and patients with sporadic CRC were stage-matched to IBD-associated cancers. Family history of cancer was present in 36% of the patients with IBD-associated dysplasia or cancer, while 42% of the patients with IBD colitis without neoplasia had a positive family history of cancer.

DNA was extracted and successful methylation analysis was performed in 99.2% of samples. Methylation of the WNT signaling pathway genes was seen for all genes in our samples, except for *LKB1*, which was uniformly unmethylated. *LKB1* was therefore excluded from further analyses. Table 2 summarizes the methylation frequencies of WNT signaling pathway genes according to the tissue type analyzed. Methylation of the WNT signaling pathway genes varied according to the tissue type analyzed. NCs

Table 1 Primer Sequences, Annealing Temperatures, and Cycle Number for the Tested Genes

Genes	Forward	Reverse	Annealing Temp.	Cycles
APC1A-U	GTGTTTTATTGTGGAGTGTGGGTT	CCAATCAACAAACTCCCAACAA	60	35
APC1A-M	TATTGCGGAGTGCGGGTC	TCGACGAACTCCCGACGA	60	35
APC2-U	TGGTAGTGTTGTTTGTTTAGGTTTGGATTG	ACCAAAAATCCCAACCCAAAATAACCTCAAAACA	56	35
APC2-M	GTCGTTTGTTTAGGTTCGGATC	GACCCGAAATAACCTCGAAACG	56	35
SFRP1-U	GTTTTGTAGTTTTTGGAGTTAGTGTTGTGT	CTCAACCTACAATCAAAAACAACACAAACA	60	35
SFRP1-M	TGTAGTTTTCGGAGTTAGTGTCGCGC	CCTACGATCGAAAACGACGCGAACG	60	35
SFRP2-U	TTTTGGGTTGGAGTTTTTTGGAGTTGTGT	AACCCACTCTCTTCACTAAATACAACTCA	60	35
SFRP2-M	GGGTCGGAGTTTTTCGGAGTTGCGC	CCGCTCTCTTCGCTAAATACGACTCG	60	35
SFRP4-U	GGGGGTGATGTTATTGTTTTTGTATTGAT	CACCTCCCCTAACATAAACTCAAAACA	60	35
SFRP4-M	GGGTGATGTTATCGTTTTTGTATCGAC	CCTCCCCTAACGTAAACTCGAAACG	60	35
SFRP5-U	GTAAGATTTGGTGTTGGGTGGGATGTTT	AAAACTCCAACCCAAACCTCACCATACA	60	35
SFRP5-M	AAGATTTGGCGTTGGGCGGGACGTTC	ACTCCAACCCGAACCTCGCCGTACG	60	35
DKK1-U	GGGGTTGGAATGTTTTGGGTTTGT	ACCTAAATCCCCACAAAACCATACCA	60	35
DKK1-M	GTCGGAATGTTTCGGTTCGC	CTAAATCCCCACGAAACCGTACCG	60	35
DKK3-U	GGGGTTTTGGTTTTTTTTTGTTTTTGGGT	AACCACCACCTATATATCCCAAAACACA	60	35
DKK3-M	CGGTTTTTTTTCGTTTTCGGGC	CGCCTATATATCCCGAAACGCG	60	35
WIF-1-U	GGTTTTTGAGTGTTTTTTTTTGGGTTT	AATACAATACACCCAATAAAACACCCA	60	35
WIF-1-M	GTTTTTGAGTGTTTTTTTTCGGGTTC	AATACGATACGCCCAATAAAACG	60	35
LKB1-U	GGATGAAGTTGATTTTGATTGGGTT	ACCCAATACAAAATCTACAAACCAACA	60	35
LKB1-M	ACGAAGTTGATTTTGATCGGGTC	CGATACAAAATCTACGAACCGACG	60	35

Table 2 Summary of Methylation of WNT Signaling Genes in Different Tissue Types

Genes	NC (*N*=27)	IBD colitis (*N*=25)	IBD-associated neoplasia (dysplasia and cancer) (*N*=16)	NC vs. IBD colitis	IBD colitis vs. IBD-associated neoplasia
	Percentage Methylation			*p* value	
oo	0	16.0	43.8	0.04[a]	0.05[b]
APC2	0	48.0	81.3	<0.01[a]	0.03[a]
SFRP1	0	54.1	87.5	<0.01[a]	0.03[a]
SFRP2	29.6	52.0	86.7	0.10	0.03[a]
SFRP4	0	68.0	81.3	<0.01[a]	0.35
SFRP5	0	44.0	62.5	<0.01[a]	0.25
DKK1	0	28.0	50.0	<0.01[a]	0.15
DKK3	0	8.3	12.5	0.13	0.67
WIF1	7.4	41.7	68.8	<0.01[a]	0.09
Mean of percentage methylation	4.2	39.7	63.4	<0.01[a]	0.01[a]

p values were calculated using chi-square test for genes and Mann–Whitney *U* test for mean of percentage methylation
[a] Significant *p* value
[b] Borderline significant p value

were uniformly unmethylated for all the genes, except *WIF1*, which was methylated in 7%, and *SFRP2*, which was methylated in 29%. Methylation of some genes can occur in NCs as a process of aging, a process termed age-related methylation. There was no age-related methylation pattern seen for either *SFRP2* or *WIF1* among the NCs. Methylation of all the WNT signaling pathway genes (except *LKB1*) was seen frequently in samples with IBD colitis and IBD-associated neoplasia. The frequency of methylation of the WNT signaling pathway genes seen in samples with IBD colitis was significantly higher than those seen in the NCs (see *p* values in Table 2), except for *SFRP2* where the difference was not statistically significant (p=0.10). Furthermore, the frequency of methylation of the WNT signaling pathway genes (except *LKB1*, which was unmethylated in all IBD-associated neoplasias) increased in samples with IBD-associated neoplasia compared to samples with IBD colitis. Methylation of *APC1A, APC2, SFRP1*, and *SFRP2* was significantly higher in IBD-associated neoplasia compared to IBD colitis (APC1A 43.8% vs. 16.0%, p=0.05; *APC2* 81.3% vs. 48.0%, p=0.03; *SFRP1* 87.5% vs. 54.1%, p=0.03 and *SFRP2* 86.7% vs. 52.0%, p=0.03, respectively). Figure 2 shows the methylation profile of WNT signaling pathway genes in one of the IBD patients with cancer as a representative sample. Methylation analysis of the genes in WNT signaling pathway is shown from the cancer and areas of non-cancerous surrounding colitis.

Furthermore, the methylation of all the WNT signaling pathway genes was tested in 24 sporadic CRC who were matched in stage (stages 1 and 2) to the corresponding IBD-associated cancers. The methylation frequencies of the WNT signaling pathway genes seen in the sporadic CRC was similar to those seen in IBD-associated neoplasias (including dysplasias and cancers) except for *SFRP4* and *WIF1*. *SFRP4* was significantly more methylated in IBD-associated neoplasia (81.3%) compared to sporadic CRC (37.5%) (p<0.01). On the contrary, *WIF1* was significantly more methylated in sporadic CRC(100%) compared to IBD-associated neoplasia (68.8%), p<0.01. The remainder of the frequencies were comparable (p = ns; data not shown).

We then calculated the percentage of methylated genes in the WNT signaling pathway for each sample and compared the means for samples belonging to each tissue type. A progressive increase in the percentage of methylated genes was seen from NCs (mean=4.2%) to IBD colitis (mean=39.7%) to IBD-associated neoplasia (mean=63.4%) was seen (NC vs. IBD colitis, p<0.01, IBD colitis vs. IBD-associated neoplasia, p=0.01) (Fig. 3). There was no significant difference in the percentage of methylated genes

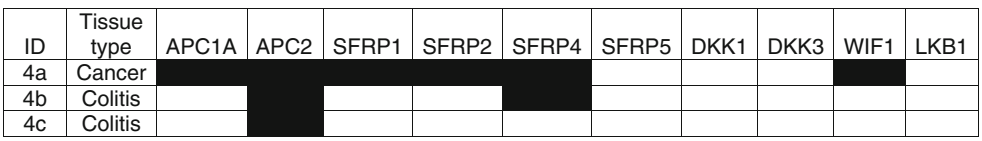

Figure 2 Methylation profile of individual WNT signaling pathway genes in paired samples from one of the IBD-associated cancers showing the increase in methylation from colitis to cancer. *Black bars* represent methylation, whereas *white bars* represent unmethylated sample. Note that the patient has acquired methylation of *APC1A*, *SFRP1*, *SFRP2*, and *WIF1* during progression to cancer.

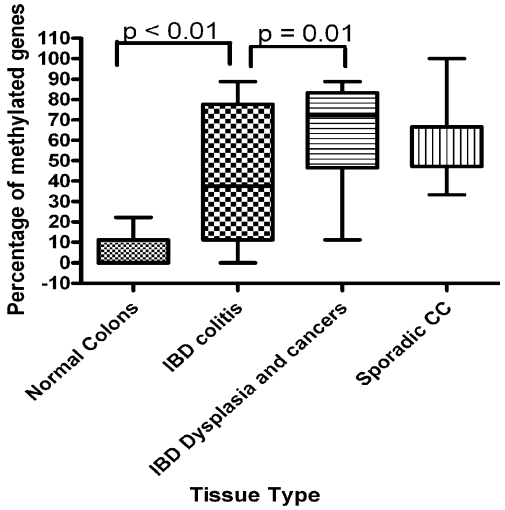

Figure 3 Percentage of methylated genes in different tissue types. Percentage of methylated genes increased from normal colons (n=24, mean=4.2%) to IBD colitis (n=25, mean=39.7%) to IBD-associated neoplasia (n=16, mean=63.4%) was seen (NC vs. IBD colitis, $p<$ 0.01, IBD colitis vs. IBD-associated neoplasia, p=0.01). There was no significant difference between IBD neoplasia (mean=63.4%) and sporadic CRC (mean=61.3%) p=0.42.

between sporadic CRC and IBD-associated neoplasia (61.3% vs. 63.4% respectively; p=0.42). However, methylation is an early event in IBD-associated neoplasia as relatively early lesions like LGDs show a high level of percentage of methylated genes (mean=75%).

We next used logistic regression model to find out whether methylation of these genes can be used to predict the presence of IBD-associated neoplasia, i.e., dysplasia or cancer. In the univariate analysis methylation of *APC2* (odds ratio [OR] 4.7 [95% CI 1.1–20.63], p=0.04), *SFRP1* (OR 5.1 [95% CI 1.1–31.9], p=0.04), and *SFRP2* (OR 5.1 [95% CI 1.1–32.3], p=0.04) was significantly associated with higher risk for IBD-associated neoplasia compared to IBD colitis. Methylation of *APC1A* was found to be borderline significant (OR 4.1 [95% CI 0.95–17.5], p=0.06). Moreover, in the multivariate model using these four genes, including *APC1A*, *APC2*, *SFRP1*, and *SFRP2*, methylation of *APC2* and *APC1A* was found to be significantly associated with higher risk for IBD-associated neoplasia [OR for methylated *APC2* 9.1 [95% CI 1.3–61.7], p=0.02 and OR for methylated *APC1A* 6.4 [96% CI 1.1–37.7], p=0.04) with an ROC value of 77.36%.

Discussion

Our study demonstrates for the first time epigenetic involvement of the WNT signaling pathway in IBD-associated neoplasia. We have shown that methylation of the WNT signaling pathway genes first begins in patients with long-standing IBD colitis and that the frequency of methylation of these genes increases progressively during the development of IBD-associated neoplasia. The percentage of methylated genes increases significantly from NCs to IBD colitis to IBD-associated neoplasia. A limitation of our current study is the relatively small sample size and the lack of expression and immunohistochemical data to fully confirm the silencing of the methylated genes.

However, the frequent methylation of the WNT signaling pathway genes in patients with IBD colitis suggests that chronic inflammation may play an important role in the methylation of WNT signaling genes. Further increase in the methylation of these genes in neoplastic lesions (dysplasia and cancer) suggests a progressive role of methylation of WNT signaling pathway genes in the pathogenesis of IBD-associated neoplasia. This is further supported by the fact that relatively early lesions like LGDs in IBD patients show high levels of methylation of the WNT signaling pathway genes. In the current study, we did not analyze non-inflamed normal colons from patients with IBD. Recent data suggest that some degree of methylation can occur in the non-inflamed terminal ileum, but it is still significantly lower than that seen in inflamed mucosa.[52]

Chronic inflammation can lead to chronic injury, which may predispose tumor-related genes to methylation and silencing. This process has also been described in other chronic inflammatory states like Barrett's esophagus, which predisposes to esophageal cancer,[53] chronic gastritis, which predisposes to gastric cancer,[54] and cirrhosis, which predisposes to hepatocellular cancer.[55] WNT signaling pathway genes are some of the genes affected by methylation in patients with IBD. Methylation of *p16*, *E-cadherin*, *hMLH1*, *p14*, *HPP1*, *ER*, etc. has already been reported by us and others in the setting of IBD-associated neoplasia (dysplasia and cancers).[22–26] We studied the current panel of genes because of their importance to the biology of colorectal cancers.[27,28]

Dysplasia is regarded not only as the precursor, but also a marker, of coexisting malignancy in IBD patients. However, there is a great deal of controversy in the diagnosis of dysplasia. Inflammatory epithelial changes can mimic dysplasia, and there is a significant degree of intra- and inter-observer variability in the pathological diagnosis.[56] This is not limited to less experienced pathologists, since even expert pathologists can differ in the opinion too.[57,58] This has led to the recommendation that two pathologists, one of whom is an experienced gastrointestinal pathologist, should independently review biopsy specimens from cases of IBD-associated dysplasia.[59–61] This criterion was also used when we procured samples for the current study. Our findings of early methylation of *APC2*, *SFRP1*, *SFRP2*, and *APC1A* in IBD-associated dysplasia may provide a more objective marker for the diagnosis of these lesions. In fact,

all patients with LGD and HGD showed 100% methylation of the *APC2*, which suggests that this could be used as a marker for dysplasia.

APC1A (classical *APC*) is a classical tumor suppressor gene. Its gene product forms a complex with GSK-3β, axin/conductin, and β-catenin. Subsequently, β-catenin is phosphorylated and degraded. Mutation of *APC1A* prevents this degradation and causes accumulation of β-catenin in the cell. The β-catenin then translocates to the nucleus where it upregulates the transcription of many cancer-related genes. *APC1A* is mutated in 80% of sporadic CRC.[29] Additionally, *APC1A* mutations usually occur early in sporadic colorectal carcinogenesis since lesions like tubular adenomas have been found to harbor *APC1A* mutations. Earlier studies, which looked into the mutation of *APC1A* in IBD, found a 0–6% mutation rate, and that too in advanced lesion like HGD and cancers.[39,62] In contrast to its low mutation frequency, APC1A protein expression was found to be abnormal in 67% of the UC-associated neoplasia, which cannot be explained by low mutation frequency.[44] Our results suggest that methylation of *APC1A* is an early and frequent event in IBD-associated neoplasia, and our results now suggest that methylation-associated silencing may account for the decreased protein expression of *APC1A* in these lesions. Since methylation of *APC1A* is more common than mutation, epigenetic inactivation may be a predominant mechanism for inactivation of *APC1A* and subsequent development of cancer in IBD patients.

APC2, identified more recently, has a high degree of homology with *APC1A*.[63] *APC2* can also modulate WNT signaling like *APC1A*.[63,64] Apart from mutations in *APC1A*, IBD-associated neoplasias also differ in the timing and frequency of mutation of other tumor suppressor gene *P53*. *P53* mutations occur late in sporadic CRC, being more frequent in cancers compared to adenomas, whereas *P53* mutations occur early and are more frequent in IBD-associated neoplasia. Early lesions like LGDs and sometimes even non-dysplastic mucosa in ulcerative colitis can have *P53* mutations.[65,66] Interestingly, *APC2* has been shown to interact with a protein, 53BP2, which in turn interacts with *P53* and an anti-apoptotic gene *Bcl2*. This suggests a mechanistic role of *APC2* in the *P53/Bcl2*-linked pathway of cell cycle progression and cell death.[67] Interaction of *APC2* with *P53* pathway is very interesting if viewed in the context of IBD-associated neoplasia. Early dysplastic lesions show a high level of *P53* mutation and *Bcl2* overexpression.[65,68,69] Similarly, we have found *APC2* to be methylated to high levels in early dysplastic lesions. Therefore, *APC2* methylation, *P53* mutation, and *Bcl2* overexpression might play a synergistic role in the pathogenesis of these lesions. This will require further investigation.

The frequent inactivation of *SFRP1* and *SFRP2* in IBD-associated neoplasias further highlights the importance of canonical WNT pathway in the pathogenesis of IBD-associated neoplasia. *SFRPs* can block the WNT signaling pathway either by interacting with WNT proteins or by forming nonfunctional complexes with frizzled receptors Fz.[70] Methylation of *SFRPs* can thus lead to activation of WNT signaling pathway even in the absence of mutations in *APC* and β-catenin.[48]

Recently, there has been increasing interest in developing stool DNA based biomarkers. In fact, the current ACS guidelines concluded that there is now sufficient data to include stool DNA as an acceptable option for CRC screening.[71] DNA shed into stool theoretically provides a more comprehensive sampling of abnormal cells than random punch biopsies for cancer surveillance among IBD patients. Stool DNA testing for hypermethylation of the *SFRP-1* promoter has already been shown to be a sensitive and specific screening tool for sporadic CRC.[72] Our findings of methylation of *APC1A*, *APC2*, *SFRP1*, and *SFRP2*, if validated in prospective studies, could be used to devise stool-based DNA methylation tools for early detection of dysplasia and cancer in IBD patients.

Conclusions

Methylation of the WNT signaling pathway genes is seen in patients with IBD colitis and the frequency of methylation of the WNT signaling pathway genes increases progressively during development of IBD-associated neoplasia. Moreover, the findings of early methylation of *APC1A*, *APC2*, *SFRP1*, and *SFRP2* in IBD-associated neoplasia may provide objective markers and a method for early detection of IBD-associated neoplasia. In IBD surveillance, strategies for CRC prevention would be strengthened by the addition of objective markers for dysplasia and cancer, which will be independent of the expertise of the pathologist. Further studies will be required to validate the findings of the current study.

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An FDA Approved Neurokinin-1 Receptor Antagonist is Effective in Reducing Intraabdominal Adhesions when Administered Intraperitoneally, But Not Orally

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Abstract
Introduction Postoperative adhesions pose a continued healthcare problem. We previously demonstrated that intraperitoneal (IP) administration of a neurokinin-1 receptor antagonist (NK-1RA) at surgery reduces intraabdominal adhesions in rats. The NK-1RA aprepitant (Emend™, Merck) is clinically approved for preventing postoperative nausea and vomiting; however, its effects on adhesion formation are unknown. Thus, we determined the effects of IP and oral administration of aprepitant on adhesion formation in a rat model.
Methods Adhesions were surgically induced in rats that were randomized to receive either one or five oral preoperative doses or a single intraoperative IP dose of aprepitant (50 mg/kg). Adhesions were scored at 7 days. In similar experiments using IP dosing, animals were sacrificed at 24 h and peritoneal fluid, and tissue were collected to assess fibrinolytic activity and tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) mRNA levels, respectively.
Results IP aprepitant reduced adhesion formation by 33% ($p<0.05$) compared with controls while oral aprepitant had no effect. Compared to controls IP aprepitant reduced tPA activity by 55% ($p<0.05$), increased PAI-1 mRNA levels by 140% ($p<0.05$), and had no affect on tPA mRNA levels.
Conclusion These data suggest that aprepitant maybe a useful pharmacologic agent for reducing adhesion formation clinically.

Keywords Intraabdominal adhesion · Neurokinin-1 receptor antagonist · Substance P · Aprepitant · Emend

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Introduction

Postoperative adhesions comprise a source of morbidity for patients undergoing intraabdominal surgery. Some of the complications caused by intraabdominal adhesions include pain, infertility in women,[1] small bowel obstruction,[2,3] bowel ischemia, and possibly death. The end result is increased hospital visits and increased cost of care for the postsurgical patient.[4–6]

Postoperative adhesions result from the injury and inflammation to the peritoneal lining of the abdominal cavity. Examples of peritoneal injury caused by surgery include cutting, crush, cautery, infection, ischemia, and rough handling of the peritoneal surfaces.[7] Areas of subperitoneal extracellular matrix are denuded followed by platelet adhesion and degranulation.[8] Inflammatory mediators such as transforming growth factor-β-1, prostaglandins, leukocyte chemotaxins, and fibrinogen are released and aggregate in

the wound,[8] leading to the formation of fibrin bands. When these fibrin bands form between two opposing surfaces, an adhesion may form. The fibrin bands can act as scaffolding for inflammatory cell in growth.[7] The wound is first populated by neutrophils, followed by macrophages, angioblasts, and fibroblasts. Fibroblasts deposit collagen into the fibrin band leading to permanent adhesion formation by 7 days following surgery.[7,8]

Under normal circumstances, the fibrin bands are degraded by plasmin prior to recruitment of inflammatory cells. However, under postoperative conditions, plasminogen activator activity is decreased and the fibrin bands persist and progress to adhesion maturation.[7] Peritoneal fibrinolytic activity is closely associated with levels of tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1).[9] tPA functions by activating plasmin while PAI-1 acts by directly inhibiting tPA. Previous studies have demonstrated that modulation of tPA activity and PAI-1 levels can affect adhesion formation; downregulation of the fibrinolytic system results in increased adhesions; while upregulation results in decreased adhesions.[10–12]

Many methods for preventing postoperative intraabdominal adhesions have been investigated in the past, such as antibiotics, anti-inflammatory medications, and physical barriers.[13–16] To date, no pharmacologic compounds have been approved for use in preventing adhesions. Currently approved anti-adhesion methods are limited to physical barriers only. By physically separating two inflamed surfaces, adhesion formation can be reduced. However, the anti-adhesion activity of barrier-based methods is likely limited to where they are placed, potentially leaving adhesiogenic surfaces. Placement of a solid barrier is also limited to open abdominal procedures. A pharmacologic method may allow for treatment of all adhesiogenic surfaces as well as allow adhesion prevention during laparoscopic operations.

One class of pharmacologic compounds previously investigated by this laboratory is the neurokinin-1 receptor antagonists (NK-1RA). The neurokinin-1 receptor (NK-1R) is a high affinity receptor that is activated by binding tachykinins of which the proinflammatory neuropeptide substance-P is the best studied. Our laboratory has demonstrated that both substance-P and NK-1R are upregulated during peritoneal injury.[17] We also showed that intraperitoneal administration of an NK-1RA increases peritoneal tPA activity and decreases adhesion formation.[18] This drug family shows promise for decreasing intraabdominal adhesions.

Currently, the only FDA-approved NK-1RA is aprepitant (Emend™, Merck, Rahway, NJ, USA), which is in use for postoperative and chemotherapy-related nausea and vomiting. The perioperative usage of this drug and its pharmacologic activity create a unique opportunity for study of its potential anti-adhesion effects. Our goal was to evaluate the effect of orally or intraperitoneally administered aprepitant on the reduction of abdominal adhesion formation in a rat model.

Methods

Materials All chemicals were obtained from Sigma (St. Louis, MO, USA) unless otherwise noted. The highly specific, nonpeptide NK-1RA 5-[[(2R,3S)-2-[(1R)-1-[3,5-bis(trifluoromethyl)phenyl]ethoxy]-3-(4-fluorophenyl)-4-morpholinyl] methyl]-1,2-dihydro-3H-1,2,4-triazol-3-one (aprepitant) was used in this study (Merck, Rahway, NJ, USA). This antagonist is highly specific for the NK-1R and has no affinity for the NK-2 or NK-3 receptors.[19] Sterile 100% dimethyl sulfoxide (DMSO) was used to dissolve the pure aprepitant for intraperitoneal delivery while a solution of 2% carboxymethylcellulose (CMC) was used for delivery of the oral formulation (Emend™) as slurry.

Animals Male Wistar rats weighing 175–200 g (Charles River Labs, Wilmington, MA, USA) were used for all experiments. Rats were housed at a constant room temperature of 25°C, with 12-h light and dark cycles, and were provided standard rodent chow (Purina, No. 5001) and water ad libitum. The Institutional Animal Care and Use Committee at Boston University School of Medicine approved these studies, and all procedures and animal care were performed in accordance with recommendations outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Sialogogic assay A sialogogic assay was performed as described by Leeman et al. to determine whether oral and intraperitoneal administration of aprepitant effectively blocks NK-1R activity in rats.[20] Rats were gavaged or intraperitoneally injected with aprepitant (50 mg/kg) or vehicle alone (N=3 per group). Animals were anesthetized and intravenously injected with SP (0.1 μg/100g) 3 h after oral gavage or 30 min after intraperitoneal injection. Saliva from the sublingual salivary gland was collected immediately with a Pasteur pipette and weighed to determine volume.

Induction of intraabdominal adhesions Intraabdominal adhesions were induced in rats using our previously described model.[18] Briefly, animals were anesthetized using continuous isoflurane 2–4% in 100% oxygen. A midline laparotomy was performed, and three ischemic buttons were created along the paracolic gutters bilaterally, spaced 1 cm apart. The abdomen and skin were closed using braided absorbable sutures and clips, respectively. Animals received a subcutaneous dose of buprenorphine (0.1 mg/kg

body weight) at the time of operation, and then every 12 h as needed for up to 72 h postoperation.

Experimental design The first group of rats received an intraoperative 1 ml peritoneal lavage with either aprepitant (50 mg/kg, $N=14$), or vehicle control (100% DMSO, $N=12$), followed by gentle abdominal massage to distribute the solutions throughout the peritoneal cavity. A second group received oral gavages of 1 ml (50 mg/kg) of aprepitant suspended in 2% CMC or the vehicle alone. Two dosing schedules were used: one dose given 3 h preoperatively (aprepitant $N=8$, vehicle $N=7$), or five doses given preoperatively every 12 h over 60 h (aprepitant $N=13$, vehicle $N=14$). Since a single oral dose of aprepitant effectively blocked the sialogogic response suggesting that this dose may be globally effective in the rat, we chose to determine if one oral dose of aprepitant was effective in reducing adhesion formation. However, given the fact that the oral formulation of aprepitant is less than 60% bioavailable, we decided to significantly increase the number of doses of aprepitant the rats received to five preoperative oral doses. To determine the effect of aprepitant on the intraperitoneal fibrinolytic system and gene expression, the experimental design described above for intraperitoneal administration was used with the addition of a nonoperative group receiving only an intraperitoneal injection of aprepitant or DMSO. For this second set of experiments, the rats were anesthetized with a ketamine–xylazine solution (75 and 5 mg/ml) at 24 h after surgery at which time a second laparotomy was performed to collect peritoneal fluid and ischemic button tissue for analysis of tPA activity and mRNA expression levels, respectively. Rats were then euthanized by a combination of pneumothorax and cardiac puncture. The peritoneal fluid was collected by rinsing the peritoneal cavity with 2 ml of 37°C phosphate buffer containing heparin (1 IU/ml). Approximately 1 ml of the fluid was recovered and added to an equal volume of 0.2 M sodium acetate, pH 3.9, and cellular debris was removed by microcentrifugation (2,000 rpm × 1 min). The resulting supernatants were immediately frozen in liquid nitrogen and stored at −80°C until assayed for tPA activity. Ischemic button tissue was removed within a 1-mm rim of surrounding tissue and frozen in liquid nitrogen and stored at –80°C until ready for reverse transcription polymerase chain reaction (RT-PCR) analysis.

Evaluation of fibrinolytic activity in peritoneal fluid Acetate-treated peritoneal fluid samples were acidified with 0.2 volumes 0.375 N HCl and then diluted tenfold with distilled water. The fibrinolytic activity due to tPA in each sample was assayed in duplicate by adding 50 μl of the diluted sample to wells of a 96-well microtiter plate containing 50 μl of tPA stimulator (0.6 mg/ml cyanogen bromide digested fibrinogen, American Diagnostica, Stamford, CT, USA). Next, 150 μl of assay buffer [16.7 μg/ml human plasminogen (Athens Research and Technologies, Athens, GA, USA), 667 μM S-2251 substrate (American Diagnostica, Inc.), and 20 mM Tris, pH 8.3] was added to each well and gently mixed. Cleavage of the S-2251 substrate by tPA-activated plasmin produces a yellow color that absorbs at 405 and 490 nm (calibration blank). The change in absorbance was measured at 37°C over a 6-h period with a Spectra Max 250 spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The activity of tPA in each sample was determined by extrapolation from a tPA (human; Calbiochem, San Diego, CA, USA) standard curve. In a separate control experiment designed to determine if the aprepitant or DMSO interfered with tPA activity assays, the above protocol was followed with the addition of a known concentration of pure tPA (2.5 U/ml) to peritoneal fluid samples before the addition of the S-2251 substrate.

RNA isolation and quantitative RT-PCR Ischemic buttons were homogenized by pulverization at –70°C on a liquid nitrogen-cooled plate. Total RNA was isolated from peritoneal ischemic button tissue (50 mg) with the SV Total RNA Isolation System (Promega), and RT-PCR was conducted with the Gene-AMP RNA PCR system (Applied Biosystems), as described by Reed.[17] The following primer sets were used to amplify tPA and PAI-1 (28 cycles of 95°C, 60°C, and 70°C for 30 s each): tPA, 5′TCTGACTTCGTCTGCCAGTG-3′ (sense) and 5′-GAGGCCTTGGAT-GTGGTAAA-3′ (antisense); PAI-1, 5′-ATCAACGACTGGGTGGAGAG-3′ (sense) and 5′-AGCCTGGTCATGTTGCTCTT-3′ (antisense). Real time PCR was performed on an Applied Biosystems 7000 Sequence Detection System machine using SYBR green. Levels of mRNA were normalized to glyceraldehyde 3-phosphate dehydrogenase, a constitutively expressed gene that did not vary among treatment groups.

Statistical analysis Data were analyzed with the Sigma Stat program (SPSS, Inc., Chicago, IL, USA) with one-way analysis of variance (ANOVA). When significant effects were detected ($p<0.05$), the difference between specific means was determined by the Student–Newman–Keuls test. If a test of normality failed, Dunn's test of ANOVA by ranks was used. Differences were considered to be statistically significant if $p<0.05$. All data are expressed as mean ± SEM.

Results

Intraperitoneal administration of aprepitant during surgery reduced intraabdominal adhesion formation The sialogogic assay demonstrated that aprepitant effectively blocked the

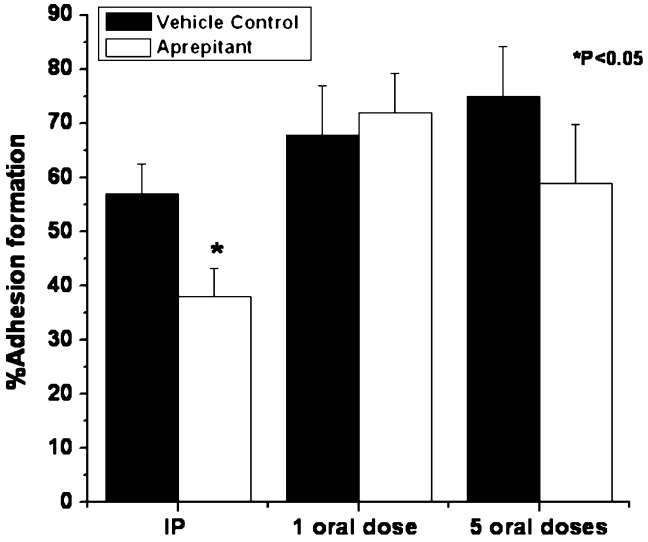

Figure 1 Intraperitoneal (*IP*) administration of aprepitant (50 mg/kg, $N=14$) at the time of operation shows decreased adhesion formation 7 days later compared with controls ($N=12$). Oral dosing with a single 3-h preoperative dose of aprepitant (50mg/kg; $N=8$) shows no significant difference in adhesion formation at 7 days postoperation when compared to vehicle control (2% CMC; $N=7$). Five oral doses of aprepitant (50 mg/kg; $N=13$), starting 2 days preoperation given every 12 h, shows no significant difference at postoperative day 7 when compared to vehicle control (2% CMC; $N=14$). Data are shown as mean ± SEM.

NK-1R by both enteral and intraperitoneal routes. Rats administered oral aprepitant showed a significant decrease in saliva production ($p<0.05$) compared to the vehicle (0.06±0.01 vs. 0.0003±0.0002 ml/200 gm body weight, respectively). Rats that received intraperitoneal aprepitant also showed a significant decrease in saliva production ($p<0.05$) compared to vehicle controls (0.11±0.03 vs. 0.02±0.001 ml/200 gm body weight, respectively).

To test the anti-adhesion effects of aprepitant, the drug was first delivered locally (IP) at the time of surgery. Intraperitoneal administration of aprepitant decreased intra-abdominal adhesion formation significantly ($p<0.05$) at postoperative day 7. Rats given the NK-1RA had a mean intraabdominal adhesion formation score of 38.1±5.1% compared to 56.9±5.6% in the vehicle control group (Fig. 1). Neither schedule of oral aprepitant showed a significant effect on adhesion formation at postoperative day 7. Rats receiving a single dose 3 h prior to surgery had mean adhesion scores of 72±7% (aprepitant) and 68±9% (vehicle control), while rats receiving five doses preoperatively, once every 12 h over 60 h, had mean adhesion scores of 59%±11 (aprepitant) and 75±9% (vehicle control; Fig. 1).

Intraperitoneal administration of aprepitant during surgery decreased tPA activity Total tPA activity at 24 h post-operation was significantly decreased ($p<0.05$) in peritoneal fluid from rats treated with a single intraoperative dose of aprepitant compared to vehicle controls. There was no effect on tPA activity when aprepitant was injected into non-operated animals 24 h prior to sacrifice (Fig. 2). In order to evaluate for potential interference of the aprepitant or DMSO with the tPA activity assay, 2.5 U/ml of tPA was added to peritoneal fluid from non-operated, aprepitant, and DMSO-treated animals. The assays were performed as described above. The addition of 2.5 U/ml increased tPA activity as expected, suggesting that aprepitant and DMSO do not inhibit the tPA assay (Table 1).

Intraperitoneal administration of aprepitant increased PAI-1 mRNA expression but and had no effect on tPA PAI-1 mRNA expression levels were 133% greater ($p<0.05$) in peritoneal adhesion tissue from rats treated with a single intraoperative dose of aprepitant compared to vehicle controls at 24 h postoperation. There was no difference in tPA mRNA expression levels in these same tissue samples (Fig. 3).

Discussion

This study demonstrates that intraperitoneal administration of aprepitant is effective in reducing adhesion formation while oral administration is not. One possible explanation for this observation is that despite the fact that orally administered aprepitant antagonizes the NK-1R, as demon-

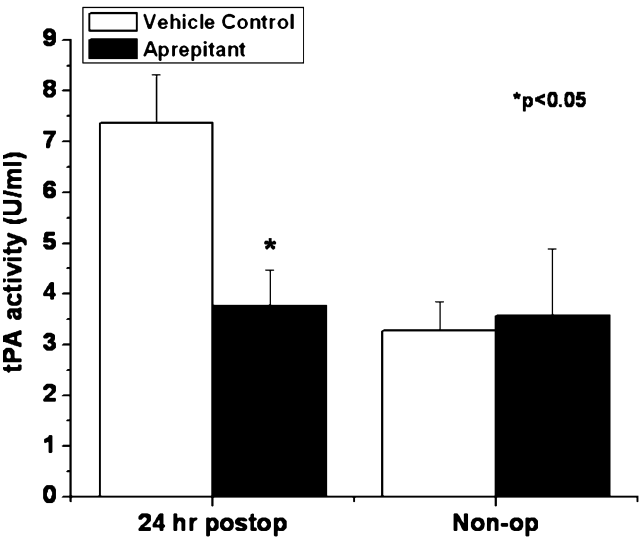

Figure 2 Twenty-four-hour postoperative (*postop*) tPA activity decreases in rats receiving IP aprepitant compared with vehicle controls (DMSO; $N=6$ per group). Non-operative (*nonop*) rats receiving IP injections of aprepitant or vehicle DMSO ($N=4$ per group) showed no difference between the two groups in tPA activity at 24 h post injection. Data are shown as mean ± SEM.

Table 1 tPA Activity Following the Addition of tPA to Peritoneal Fluid Samples

tPA Added (U/ml)	tPA Activity (U/ml)		
	Non-op	DMSO	Aprepitant
0	0±0.4	8.0±1.5	4.5±0.0
2.5	1.3±0.5	10.7±1.4	7.6±0.1

Data are shown as mean ± SEM
Non-op non-operated, *DMSO* dimethylsulfoxide vehicle

strated by the sialogogic assay, adequate peritoneal concentrations of aprepitant may not have been achieved at a critical time to prevent adhesion formation. Thus, the continuation of oral aprepitant administration after surgery would be of no further benefit to adhesion reduction. Our previous data suggest that there is a temporal window in which the NK-1RA is effective in reducing adhesion formation. We determined that the NK-1RA, CJ-12,255, must be available in the peritoneum no later than 5–6 h postoperation in order to reduce adhesion formation.[21] Other studies have also failed to show anti-adhesion effects of orally administered drugs that were effective by intraperitoneal administration. Oral doses of atorvastatin did not reduce adhesion formation in rats while intraoperative doses did.[22] Data presented here further support that the intraperitoneal administration of a pharmacologic agent during surgery may be the best method for adhesion prevention due to direct delivery to target tissues. This may be beneficial since a single intraperitoneal dose of a drug may limit the potential side effects associated with longer oral dosing regimens.

The tPA activity assays yielded unexpected results. Our previous work with the NK-1RA, CJ-12,255, showed increased postoperative tPA activity and increased *tPA* gene transcription compared to saline controls at 24 h.[18] However, intraperitoneally administered aprepitant decreased tPA activity compared to the DMSO vehicle. This correlated with the PCR results that showed tPA mRNA expression remained unchanged while PAI-1 mRNA expression increased, resulting in an overall downregulation of the fibrinolytic system. The tPA assays performed with an added known concentration of tPA demonstrate that neither aprepitant nor DMSO interfere with the tPA activity assay, eliminating this as a possible reason for the decreased measurement. Thus, the observed decrease in tPA activity is likely due to increased expression of PAI-1, not to changes in tPA expression.

In contrast to our current observations, several studies suggest that agents which elevate postoperative peritoneal fibrinolytic activity have the ability to reduce adhesion formation. In animal models, pharmacologic upregulation of peritoneal fibrinolysis, across a broad range of drug types, is associated with decreased adhesion formation. Such drugs include the NK-1RA CJ-12,255, methylene blue, atorvastatin, pentoxifylline, and octreotide.[18,21–30] Studies in both humans and animals demonstrate the ability of the peritoneal fibrinolytic system to regulate adhesion formation. Patients with the most severe adhesions have decreased postoperative tPA activity as well as increased levels of PAI-1.[31] In animals, increasing postoperative peritoneal fibrinolytic activity by either inhibition of PAI-1 or IP administration of tPA reduces adhesion formation.[32,33] Furthermore, Sulaiman et al. demonstrated that tPA-null mice have increased postoperative adhesion formation compared to their wild-type counterparts.[12]

While the fibrinolytic system has been demonstrated to have a strong relationship with adhesion formation, the current data suggest that aprepitant may reduce adhesion formation via other mechanisms. Possible alternative mechanisms include modulation of proinflammatory mediators such as cyclooxygenase-2 (COX-2) and transforming growth factor (TGF)-β. The COX-2 enzyme has been demonstrated to have proinflammatory, proangiogenic, and pro-fibroblastic effects, all of which may promote adhesion formation, and inhibition of the COX-2 enzyme, which is expressed by adhesion fibroblasts,[34] has been shown to reduce adhesion formation in rodents.[14,35,36] Overproduction of TGF-β, a multifunctional growth factor, correlates with adhesion formation in both humans and animals,[37–39] and in animal models, blocking TGF-β activity has been shown to reduce adhesion formation.[40,41] In support of a possible link between aprepitant and COX-2 and/or TGF-β, the NK-1R ligand substance P has been shown to upregulate COX-2 expression in cultured human umbilical vein endothelial cells and colonic epithelial cells,[42,43] and

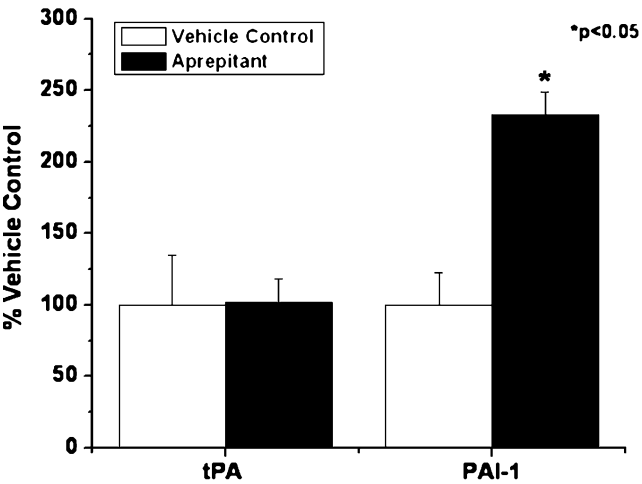

Figure 3 Twenty-four-hour postoperative rats showed a 233% greater PAI-1 mRNA level than vehicle controls (DMSO; $N=3$ per group). tPA mRNA levels were not statistically changed by aprepitant administration. Data are shown as mean ± SEM.

substance P has been shown to increase TGF-β expression in rat fibroblasts.[44] IP administration of aprepitant at surgery may also directly affect wound healing processes. Substance P released at sites of tissue injury, in addition to promoting inflammation, is thought to stimulate proliferation of epithelial, vascular, and connective tissue cells as part of the wound healing process.[45,46] Substance P may induce tissue fibrosis via augmentation of cytokine-induced fibroblast proliferation, effects on collagen organization, and regulation of matrix metalloproteinase expression.[47–50] We have previously reported that administration of the NK-1RA CJ-12,255, in addition to reducing adhesion formation, increases matrix metalloproteinase expression and activity in the postoperative peritoneum.[51] In the current study, aprepitant may reduce adhesion formation by modulating the inflammatory and wound-healing processes that begin with the onset of surgery well before the changes in peritoneal fibrinolytic activity occur. A possible scenario is that administration of aprepitant reduces postoperative fibrin band formation, thereby diminishing the need for fibrinolysis. Further investigation is needed to delineate the mechanisms by which aprepitant, as well as other NK-1RAs, reduce adhesion formation.

Conclusion

Intraperitoneal administration of the FDA approved NK-1RA, aprepitant, is effective in reducing intraabdominal adhesions while oral administration is not. The peritoneum may act as a selective barrier preventing orally administered or systemic drugs from significantly affecting processes in the peritoneum including adhesion formation. Thus, intraoperative peritoneal administration of a pharmacologic agent appears to be the most effective method for drug delivery to target adhesiogenesis. The results also suggest that the anti-adhesion effects of aprepitant may be via an alternative pathway other than the peritoneal fibrinolytic system. Although further investigation into the mechanism (s) of N K-1RA effects on adhesion formation is warranted, these studies support the use of an NK-1RA in adhesion prevention.

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Discussion

Margo Shoup, M.D. (Maywood, IL): I would like to congratulate the authors for trying to tackle a difficult problem that we all face with our patients with adhesion formation in a small bowel obstruction, and we really haven't made much headway in this in the last couple of decades. The authors in this paper attempt to study the effects of HA/CMC, or seprafilm, and neurokinin-1 receptor antagonist. The adhesions were measured at seven days postoperatively and placement of the buttons, and the Tpa was measured 24 hours after laparotomy. And we know that the synergistic effects of NK1RA and seprafilm is evident, but we are not really sure what is going on with the Tpa during all that. So I have a few questions for you.

Have you first looked at dose escalation studies with increasing NK1 receptor antagonists to evaluate the effects on Tpa, because this would clarify whether this is truly the mechanisms through which this is working. Also, you checked the Tpa levels 24 hours after surgery and, like I said, the adhesions at seven days. Have you looked at different time frames for both of these to see if there is more of a correlation? And at this point do you have any information on the status of the soluble seprafilm that is available in Europe, and if so, where do you think this may impact your study?

Thank you.

Rizal Lim, M.D. (Boston, MA): In terms of dose escalation of our antagonist, going back to the original parent compound, it is actually based off of a drug called ezlopitant. When we received this compound as a gift, the doses were actually based on the then maximum recommended dose of 25 mg/kg, which we used. But in earlier studies, as we started off with 5 mg/kg and then went to 10 mg/kg, we saw a progressive increase in adhesion prevention from those two doses.

In terms of the different time frames, looking at adhesions with this model specifically, our personal experience and data we have collected in the past have shown that when we look at adhesion formation beyond 7 days, we haven't really seen much of a difference in terms of severity. The same is true for tPA. In fact, what we have seen in previous studies is that tPA immediately post-op, at least within the rat, drops significantly and hits its nadir at approximately 24 hours and following that period of time begins to slowly rise back towards normal levels. So we chose that simply because it gives us a general idea of what the fibrinolytic activity within the abdomen is doing at its worst case scenario. We have also shown that giving the drug at 24 hours, we can alter that fibrinolytic activity.

And the final question, in terms of the soluble and gel forms of various barrier compounds, I am not firmly sure as to how far the various companies have progressed in terms of getting that approved within the U.S. But some of the implications which it may convey are that currently some of the biggest limitations of using HA/CMC barriers involve its actual application. It is a brittle, stiff material. It is difficult to use in certain cases such as laparoscopy, and I think that progressing to more of a gel type of device would improve its utility.

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VEGF Gene Therapy Augments Localized Angiogenesis and Promotes Anastomotic Wound Healing: A Pilot Study in a Clinically Relevant Animal Model

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Abstract
Background Anastomotic leak related to ischemia is a source of significant morbidity and mortality in gastrointestinal surgery. The aim of this study was to apply growth factor gene transfection for the purpose of up-regulating angiogenesis, increasing anastomotic strength, and ultimately preventing dehiscence.
Methods An opossum esophagogastrostomy model was employed. The human vascular endothelial growth factor ($VEGF_{165}$) gene was incorporated into a recombinant plasmid. The VEGF plasmid vector was then complexed with a cationic synthetic carrier, polyethyleneimine. Control animals received plasmid devoid of $VEGF_{165}$ (n=6). The experimental group received $VEGF_{165}$ plasmid (n=5). After esophagogastrectomy and gastric tubularization, plasmid was injected into the submucosa of the neoesophagus at the anastomotic site. Conduit arteriography was performed before and 10 days after injection. Euthanasia occurred on post-injection day 10 and the anastomosis was removed en bloc. A second group of animals treated with $VEGF_{165}$ were euthanized 30 and 37 days post injection. Blood flow was measured with laser-Doppler prior to euthanasia. Ex vivo anastomotic bursting pressure was performed. Tissue samples were procured for RNA extraction and von Willebrand Factor staining. Microvessel counts were obtained by two blinded observers. Tissue VEGF transcript levels were measured with reverse transcriptase polymerase chain reaction (RT-PCR).
Results There was one anastomotic leak in the control group. Experimental animals demonstrated significantly increased bursting pressure (104.25±6.2 vs 86.73±9.4 mmHg, p=0.021) and neovascularization (33.87±9.6 vs 20.33±8.1 vessels/hpf, p=0.032) compared to controls. In addition, there was a strongly positive correlation between the number of microvessels and bursting pressure (r=0.808, p=0.015, Pearson's). On angiographic examination, treated animals demonstrated more neovascularization compared to controls. RT-PCR demonstrated up to a 5.6-fold increase in VEGF mRNA in treated compared to controls.
Discussion This description of gene therapy in gastrointestinal surgery using $VEGF_{165}$ transfection demonstrates increased angiogenesis with subsequently improved anastomotic healing in a clinically relevant model.

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Keywords Angiogenesis · Healing · Vascular endothelial growth factor · Anastomosis

Introduction

Anastomotic leak is a primary cause of serious morbidity and mortality after gastrointestinal surgery. Anastomotic dehiscence is particularly vexing after esophagectomy and neoesophagus formation using the tubularized stomach—the most common conduit for esophageal replacement. Following esophago-gastric anastomoses, leak rates historically ranged from 10% to 25%.[1] Contemporary series still report leak rates of approximately 10% for both open esophagectomy and minimally invasive approaches.[2,3] The principle contributor to anastomotic failure is tissue ischemia.[2,4] Because the gastric conduit relies on a vascular pedicle representing approximately one quarter of its original blood supply, the devascularized stomach is particularly prone to ischemia and subsequent leak.

Angiogenesis is the natural response to tissue ischemia and plays an important role in anastomotic healing.[5] Vascular endothelial growth factor (VEGF) is one of the principal angiogenesis promoters.[6–8] Of the four known human isomers, $VEGF_{165}$ is the most abundant and bioactive form.[9,10] $VEGF_{165}$ plays a crucial role in wound healing by increasing microvessel permeability, promoting endothelial cell growth, and facilitating endothelial cell migration through the extra-cellular matrix.[11,12] Improved wound healing with the application of exogenous VEGF has been shown in multiple myocutaneous flap models.[13–17] To date, VEGF gene therapy has not been employed within the gastrointestinal (GI) tract to promote anastomotic healing.

Because most leaks occur 7–10 days after esophageal surgery, a sustained pro-angiogenic effect is required. An alternate approach to local growth factor protein delivery is to transfect target cells using a VEGF-encoding vector. Gene therapy can provide a stable, local source of VEGF for the ischemic anastomosis during the most vulnerable period. We hypothesized that delivery of the recombinant human $VEGF_{165}$ gene to the GI tract would up-regulate angiogenesis and subsequently improve anastomotic healing. The aims of this study were (1) to develop a method of VEGF gene delivery to cells in the GI tract, (2) to demonstrate up-regulation of angiogenesis at the anastomotic site, and (3) to demonstrate improved anastomotic healing through clinical outcomes and higher bursting pressures.

Methods

We compared angiogenesis and anastomotic healing in animals treated with recombinant human $VEGF_{165}$ plasmid to controls receiving injections of plasmid devoid of the VEGF coding sequence. The North American opossum (*Didelphis virginiana*) was chosen for its long intra-abdominal esophagus and its physiologic and anatomic similarities to the human foregut.[18] All animals were managed under the regulations of the Animal Care and Use Committee at the Portland VA Medical Center.

Plasmid

Recombinant polymerase chain reaction (PCR) methodology was utilized to generate a 649-base pair IgSP- $rhVEGF_{165}$ insert within the pCEF1α-DNT-IgSP expression vector (10.051 Kb, Fig. 1A). The purified 1 μg/μl VEGF plasmid DNA was condensed within a polyethyleneimine synthetic cation complex (jetPEI, MP Biomedicals, Inc., Irvine, CA), at a ratio of 10:1. Next, a 220-μl plasmid/PEI solution was mixed with 580 μl of the human fibrin sealant, Evicel® (Johnson & Johnson, Somerville, NJ). The Evicel was prepared at a 1:100 dilution from the manufacturer's recommended concentration. Control animals received the same formulation with the exception that the vector consisted of plasmid DNA without the $rhVEGF_{165}$ gene insert.

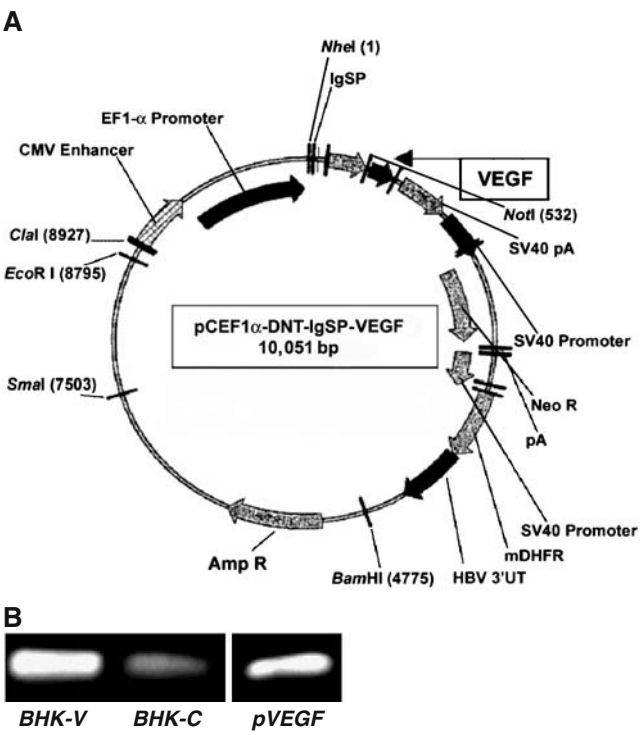

Figure 1 **A** Plasmid construct with rhVEGF165 fusion gene insert. **B** End-point PCR for VEGF amplification following *in vitro* gene transfection demonstrates bands of similar intensity in the cells transfected with rhVEGF165 and in the VEGF plasmid control, with a faint band in BHK control (BHK-V BHK cells + VEGF transfection, BHK-C BHK controls, pVEGF plasmid positive control).

Plasmid Validation

Plasmid gene transfer was validated by *in vitro* transfection of a cultured cell line. Baby hamster kidney (BHK) cells were incubated for 48 h after transfection with the PEI: rhVEGF plasmid complex. Messenger RNA was extracted from both control and treated cells using Trizol reagent (Invitrogen, Carlsbad, CA). End-point PCR was performed to confirm $rhVEGF_{165}$ mRNA expression by transfected cells (Fig. 1B). This *in vitro* model was also used to establish a preferred ratio of PEI to plasmid DNA for later use in the animal model.

Animal Model

The opossum model for esophago-gastric anastomosis previously developed by our laboratory closely mimics the gastric tubularization process used routinely for esophagectomy in humans.[19] All procedures were performed under general endotracheal anesthesia with inhaled isoflurane and supplemental oxygen; homoeostasis was maintained with heat lamps, warming blankets, fluid and oxygen supplementation as needed. The esophagus was divided just proximal to the gastroesophageal (GE) junction, and the stomach transected immediately distal to the GE junction. The stomach was prepared in the same fashion as for esophageal replacement—with ischemia produced in the stomach by ligating the left, right, and short gastric arteries, leaving the right gastroepiploic artery as the sole blood supply to the stomach. The stomach was tubularized with a resection line along the lesser curve, which was closed with a single layer monofilament suture. A gasrotomy was made at the tip of the fundus. One hundred microliters of either the VEGF plasmid or control plasmid/PEI solution was injected in four locations in the submucosa of the anterior fundus, and in four locations posteriorly with a 25-gauge needle at the future anastomosis site. Finally, an esophagogastrostomy was created with interrupted monofilament suture.

The first group of 11 animals was subdivided into two groups. The control group ($n=6$) received the empty plasmid, while the experimental group ($n=5$) received the VEGF plasmid. Animals were given liquids on postoperative day (POD) 1 followed by a full liquid diet on POD 2 and full chow on POD 3. Animals were euthanized on POD 10 at the time of anastomosis harvest.

A second group of six animals underwent VEGF delivery similar to the experimental group above. Three animals in this group were harvested 30 days after injection and anastomosis creation. The remaining three animals were harvested 37 days following injection. These animals served as subjects for temporal assessment of gene transfection.

Angiography

Visceral angiograms were performed in select animals from experimental and control groups at baseline and just prior to harvest. A femoral artery cutdown was performed, the femoral artery was cannulated using the Seldinger technique, and a contrast aortogram was performed to identify the celiac access. Next, the celiac artery was selectively catheterized with customized three French angiocatheters. Finally, selective gastric arterial angiograms were performed.

Blood Flow

Tissue blood flow analysis was performed using a laser Doppler probe as per previously reported methods using the Laserflo BPM^2 laser Doppler flowmeter (Vasamedics, Eden Prairie, MN).[19,20] A marking stitch was placed in the distal fundus for baseline and all subsequent blood flow measures. Prior to vascular ligation, blood flow velocity measurements (milliliter per minute per 100 g) were recorded for baseline. Nadir measurements were taken after gastric tubularization. Finally, measurements were obtained prior to tissue harvest on POD 10. Three consecutive *in situ* measurements were obtained for every animal at each time point and averaged.

Bursting Pressure

Following euthanasia, animals were subjected to bursting pressure measurements. The distal esophagus, stomach, and proximal duodenum were removed *en bloc*. Chilled normal saline mixed with methylene blue dye (1:180 mL concentration) was infused into the conduit at a constant rate with a power injector. Infusion occurred through the esophagus, and pressure (millimeter mercury) was continuously measured with a digital manometer attached to the duodenum. Anastomotic failure (bursting pressure) was recorded as the highest pressure obtained prior to frank leakage of blue saline. This value corresponded to the highest pressure obtained during infusion.

Tissue Preparation/Handling

Immediately following completion of bursting measurements, longitudinal tissue sections were taken through the anastomosis. Samples were collected for permanent section and for messenger RNA (mRNA) extraction. The former were preserved in formalin and the latter were incubated in RNA*later* solution (Ambion Inc, Austin, TX) at 4°C for 48 h and then stored at −80°C. Additional samples for mRNA extraction were taken from the esophagus 1 cm proximal to the anastomosis and from the liver.

Immunohistochemistry and Microvessel Counts

Angiogenesis quantification with endothelial staining and microvessel counts were obtained using previously validated methods.[21,22] Permanent sections were imbedded in paraffin and sectioned longitudinally. After slide fixation, the specimens were incubated with von Willebrand Factor (vWF) antibodies (A0062, Dako Inc, Dako, Denmark) for 60 min at a 1:4000 dilution followed by a secondary antibody for 30 min (rabbit IgG, 1:7500). Endothelial cells were then stained with NovaRed to detect vWF binding with methyl green for background staining. Negative staining controls were performed with rabbit IgG antibodies using the same protocol. Specimens were examined first under low power (×40 and ×100), and three areas corresponding to vascular "hot spots" were identified. These areas were then examined under high power (×200) and digital images were captured with a Leica DC 300F system (Leica Microsystems, Wetzlar, Germany). Microvessels in each hotspot were counted by two blinded observers using Image Pro-Plus version 6.1 software (Media Cybernetics, Inc, Bethesda, MD) following established profiles.[21]

Gene Expression

Extraction of mRNA from stored tissue sections was performed using the RNeasy MiniKit (Qiagen Inc, Valencia, CA) according to the manufacturer's recommended protocol. cDNA was prepared with the high-capacity cDNA reverse transcriptase kit (Applied Biosystems, Foster City, CA). Relative VEGF mRNA transcript copy number was assayed with quantitative RT-PCR. $VEGF_{165}$ and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers were custom-designed with the following sequences using the Primer3 program: $VEGF_{165}$ left primer 5'-CTACCTCCACCATGC CAAGT, right primer 5'-GCAGTAGCTGCGCTGATAGA, amplicon length 109 bp; GAPDH left primer 5'-AAGGG CACTGTAAAGGCAGA, right primer 5'-GTACTCG GCTCCAGCATCTC, amplicon length 114 bp (Operon Biotechnologies, Inc, Huntsville, AL).[23] Real-time RT-PCR was performed with the SyberGreen detection system (Applied Biosystems, Foster City, CA) on the Applied Biosystems 7900HT RT-PCR thermocycler. Cycle parameters were 95°C×10 min, (95°C×15 s, 55°C×1 min) × 40 cycles. GAPDH transcript was used as an endogenous control. After mRNA quantification, the $2^{-\Delta\Delta C}{}_{T}$ method was used for fold change derivation.[24,25]

End-point PCR was performed on in vitro transfected baby hamster kidney (BHK) cells using an Eppendorf Mastercycler (Eppendorf North America, Westbury, NY) with the following cycle parameters: 95°C×10 min, [95°C×15 s, 66°C×1 min] ×40 cycles. PCR products were analyzed using agarose gel electrophoresis.

Statistical Analysis

Data were analyzed with the SPSS program for Windows version 15.0 (SPSS, Chicago, IL). All data were tested for normality and parametric or non-parametric statistical tests were used as appropriate. A p value <0.05 defined significance. One-way analysis of variance (ANOVA) and Student's t test were used for comparison of means. The paired samples t test was used to compare the changes in blood flow after various interventions. Correlation between microvessel counts and bursting pressure was determined by the Pearson correlation coefficient.

Results

One animal in the control group developed an anastomotic leak. The animal was lethargic, anorexic, and had purulent drainage from its abdominal wound. After euthanasia it was discovered to have a wound infection with disruption of the abdominal closure and peritonitis subsequent to an anastomotic dehiscence. Dense adhesions between the left lobe of the liver and the anastomosis precluded bursting measures in one animal in each group. Animals in the 30- and 37-day survival groups demonstrated no untoward effects of treatment with postoperative courses as expected.

In vitro gene transfection was verified by end-point PCR as depicted in Fig. 1B. Relative gene expression in BHK cells transfected with $VEGF_{165}$ compared to control BHK cells transfected with the naked plasmid is shown by the prominent band for the former group. The faint band in the control well indicates a low background of VEGF expression expected with kidney cells. VEGF-treated BHK cells show a band of similar intensity to the positive control, represented by the VEGF plasmid.

Real-time quantitative RT-PCR analysis demonstrated a trend toward higher VEGF transcript levels in anastomotic tissues from experimental groups treated with $VEGF_{165}$, although these differences did not achieve statistical significance (Fig. 2). The fold increase was 4.34 (standard error range 1.43–13.13) for VEGF-treated animals for all anastomotic tissue and 5.61 (1.46–21.54) for those animals that underwent bursting measurements (p=0.222 and p=0.247, respectively). At 30 and 37 days post injection, the fold change had decreased by approximately one half (30-day FC=2.48 [1.03–5.92], 37-day FC=2.24 [0.85–5.90]). There was no difference in the relative amounts of VEGF mRNA between experimental and control animals in esophageal (FC=0.57 (0.27–1.18), p=0.46) and liver (FC=0.37 (0.20–0.71), p=0.161) specimens.

Blood flow results are depicted in Fig. 3. There was a significant drop in mean blood flow from baseline to nadir (p<0.001) and a significant increase from nadir to harvest

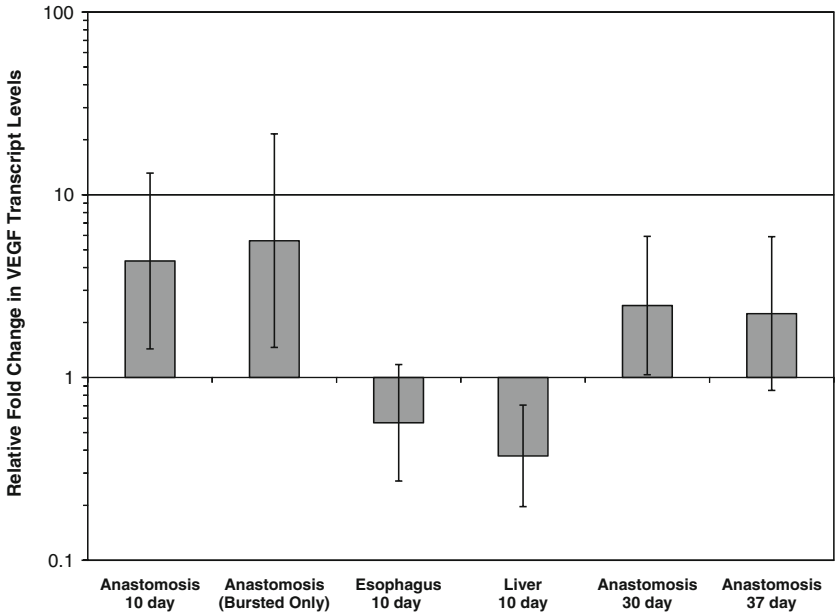

Figure 2 Relative fold change in VEGF mRNA levels for treated versus control animals (logarithmic scale, *error bars*= standard error ranges; n=10 for *Anastomosis*, *Liver*, and *Esophagus*; n=8 for *Anastomosis (Bursted Only)*; and n=3 for both *Anastomosis* 30 and 37 days; p=NS, Student's t test).

(p=0.001) for both groups. There was a trend toward increased blood flow at harvest in the experimental group compared to controls (6.68±2.88 vs 4.18±2.64 ml per minute per 100 g, p=0.191). No difference was observed between groups at the other two time points.

Angiography demonstrated a qualitative improvement in blood flow to the distal fundus in the animals treated with VEGF. Fig. 4 depicts standard and subtraction angiograms for two animals performed on POD 10. The stomach of both animals was insufflated with air, and the right gastroepiploic artery is prominent on the inferior border of the stomach along the greater curvature. In the control animal, there is an obvious paucity of vessels filling the fundus (Fig. 4A,B), while the experimental animal has filling of collaterals from the right gastroepiploic artery and neovascularization evident well out into the distal fundus (Fig. 4C,D). Filling of hepatic arteries is similar in both animals as seen at the top of the images, indicating an equivalent phase of contrast injection.

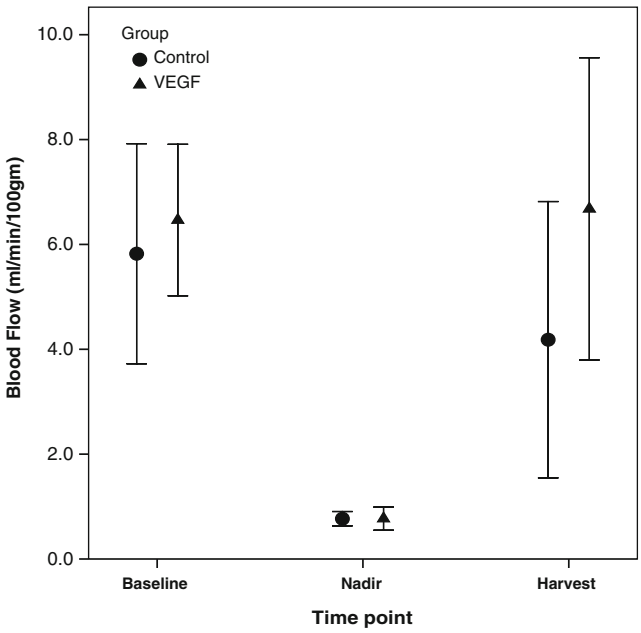

Figure 3 Mean tissue blood flow for control and VEGF-treated animals before and after each intervention. Groups are not significantly different at each time point (Student's t test). For both groups, nadir is significantly different than either baseline or harvest (p<0.05, paired t test; *error bars*=1 standard deviation).

Histology with immunostaining for microvessels showed a relative paucity of microvessels in the control group compared to the experimental (Fig. 5). Endothelium was stained brown in the presence of vWF antibodies and there is no endothelial staining in the negative controls. The mean number of microvessels was significantly higher in the treatment group, with 33.87±9.6 vessels/high-powered field in experimental versus 20.33±8.1 in controls (p=0.032).

Bursting pressures were obtained in four animals from each group. Mean bursting pressures were significantly higher in the experimental group compared to controls (104.25±6.2 vs 86.73±9.4 mmHg, p=0.027, Fig. 6). A strong correlation was observed between the number of microvessels and the bursting pressure (R=0.808, p=0.015, Fig. 7).

Discussion

In this study, we have successfully developed a vehicle for VEGF gene transfer to the gastrointestinal tract using a

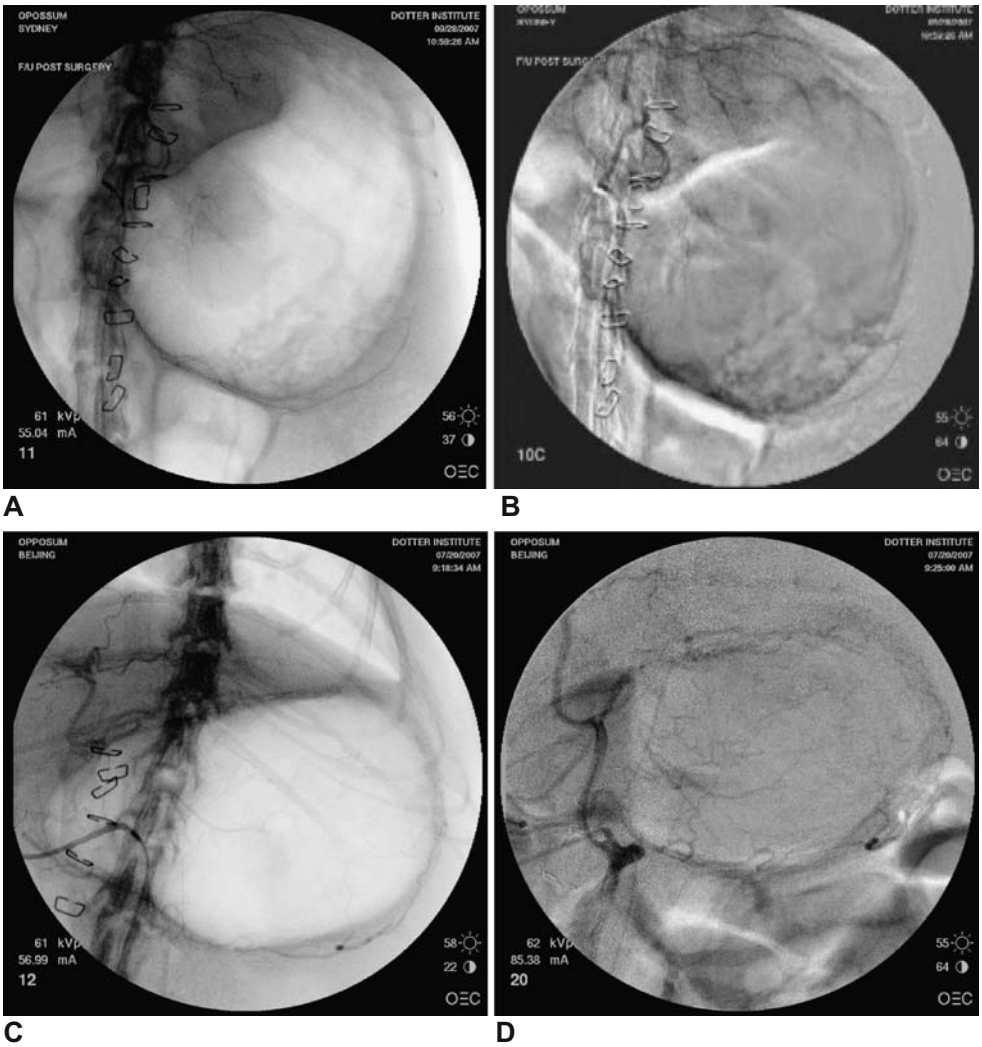

Figure 4 Representative angiograms for control (**A**, **B**) and treated (**C**, **D**) animals on POD 10. In images **A** and **C**, the stomach has been filled with air, and the right gastroepiploic artery fills along the greater curvature. Images **B** and **D** are digital subtraction images showing substantial filling of the fundus vessels only in the VEGF-treated animal (**D**).

non-viral vector system. We have demonstrated amplified angiogenesis following delivery of the recombinant $VEGF_{165}$ gene in an ischemic model of esophageal surgery. The subsequent improvements we observed in anastomotic healing measured by clinical outcome and bursting strength validate the potential for applying therapeutic angiogenesis in gastrointestinal surgery.

Despite a four- to fivefold increase in VEGF transcript levels in the treated arms, differences in VEGF transcript levels between control and experimental groups did not achieve statistical significance. The $VEGF_{165}$ coding region is highly conserved across species and the *Didelphis* VEGF gene has not been sequenced.[26] All $VEGF_{165}$ primers tested cross-reacted with opossum VEGF. We were, therefore, unable to distinguish between vector-derived and endogenous (opossum) VEGF transcript. Nonetheless, despite the lack of statistical significance, there was a reproducible trend of higher transcript levels in anastomotic tissues from experimental animals compared with controls. These differences were not observed in untransfected liver or proximal esophageal tissues, suggesting the presence of transgene product within transfected anastomotic tissues.

A lack of systemic effects and effects on distant tissues are important requirements of tissue-directed gene therapy. The lower transcript levels we observed in the liver specimens of VEGF treated animals suggest a low likelihood of systemic plasmid release and unlikely transfection of distant tissue. The esophageal specimen was sampled from within a centimeter of the anastomosis, and the results indicate that transfection is not occurring across the anastomosis. This finding indicates that gene delivery is limited to the site of plasmid injection in this model. No animals manifested signs or symptoms of systemic illness other than those associated with routine surgical recovery. Furthermore, there was no evidence of hemangiomas or other vascular lesions as has been reported in other studies.[27] Regulated transgene expression following limited spatial and temporal gene delivery will be an important component of utilizing this therapy in humans.

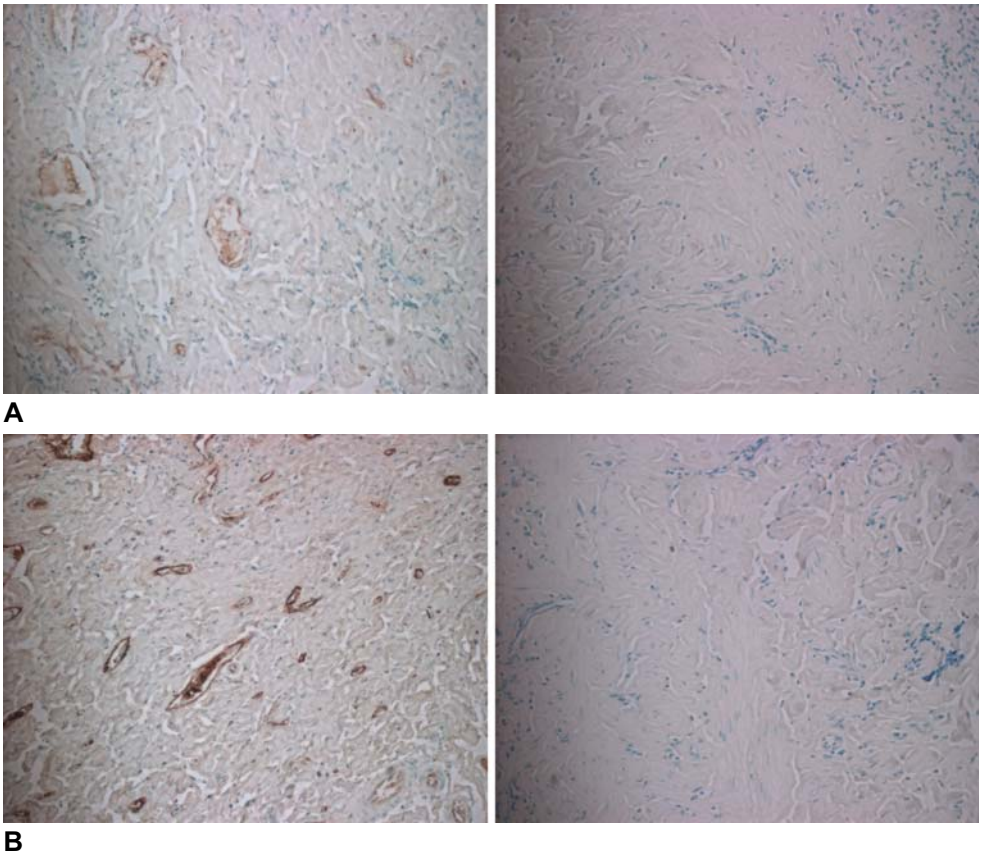

Figure 5 Representative histologic images showing von Willebrand Factor staining for endothelium in control (**A**) and treated (**B**) animals with corresponding negative controls for each group on the right. Microvessel counts demonstrated a significantly higher mean number of microvessels/high-powered field in the VEGF treated group (p=0.032, Student's t test; ×200).

Temporal changes in VEGF mRNA expression were observed between treated animals harvested on POD 10 compared to those harvested POD 30 and 37 and controls. While we anticipated a degree of sustained release of the plasmid DNA/PEI complex, we expected attenuation of recombinant human VEGF from the somatic opossum genome by 30 days. The fibrin suspension may have affected the transfection capacity of the system. The rationale for using a dilute fibrin suspension is that it preserves the plasmid at the anastomotic site. Instead of the vehicle incorporating into target cells (native endothelium and recruited macrophages), some of the plasmid DNA may have remained unavailable for immediate uptake. This sustained release phenomenon may explain the approxi-

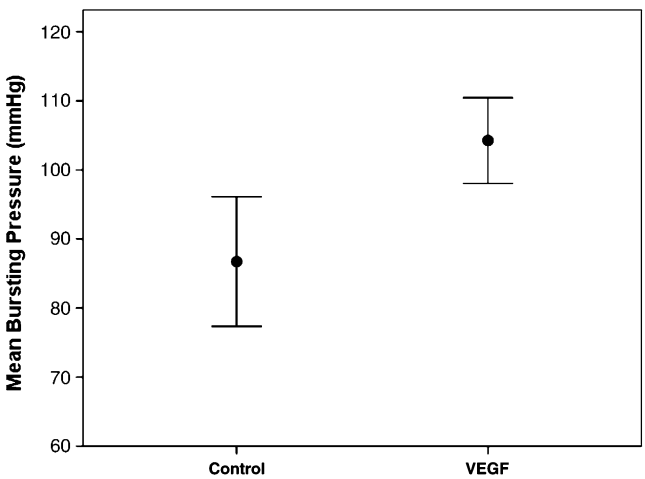

Figure 6 Bursting pressure was significantly higher in the VEGF treated group (*circle* represents mean value; *error bars*=1 standard deviation; p=0.021, Student's t test).

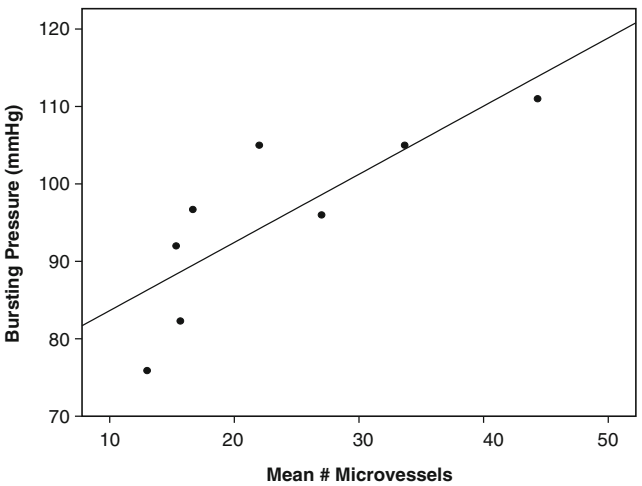

Figure 7 A strong correlation was observed between mean number of microvessels and bursting pressure (Pearson's correlation coefficient=0.808, p=0.015).

mately twofold increase in VEGF transcripts for the 30- and 37-day survival groups. Future experiments are needed to investigate alternate gene transfection systems.

Because we anticipated that few animals would develop frank anastomotic dehiscence, bursting pressure served as a proxy for anastomotic healing and strength. The higher bursting pressure measurements in VEGF treated animals corresponds with studies that have shown the opposite trend in animals treated with anti-angiogenic agents. For example, te Velde et al.[28] treated mice with angiostatin, a potent agiogenesis inhibitor, after colon transection and anastomosis. Mice that received angiostatin developed significantly more leaks, adhesions, and peritonitis, and anastomotic bursting strength was impaired and neovascularization was significantly reduced. Our data verified that the pro-angiogenic effects of VEGF produced converse results—no clinically significant leaks and a significantly higher bursting pressure in treated animals. While bursting pressure is a reasonable proxy for the rapidity and completeness of anastomotic healing, it is not a perfect test. The two animals—one in each group—which presented at harvest with dense adhesions between the liver and stomach may have both harbored small leaks, which were contained by the liver. Neither animal showed clinical signs of systemic illness, both had normal postoperative courses with respect to feeding and without signs of peritonitis, and there was no evidence of abscess, turbid peritoneal fluid or other signs of leak at harvest.

Our results demonstrate a strong correlation between the number of microvessels and bursting pressure. Several authors have suggested that VEGF-induced neovasculature is functionally impaired.[29,30] Increased vessel porosity resulting in increased leakage of plasma into the extravascular space may contribute to tissue edema and actually impair the healing process. However, based on our results it appears that a critical threshold has not yet been reached wherein the dysfunction of the new vessels adversely affects delivery of vital substrates to the already ischemic tissue. Furthermore, this strong correlation provides an important link between neovascularization at the histologic level and clinical and bursting pressure outcomes. Whether increasing the dose of VEGF delivered to these tissues will lead to progressive increases in vessel counts is unknown but may be a productive area for further research.

While this is the first study to harness therapeutic angiogenesis in GI surgery, previous investigators have demonstrated successful VEGF gene therapy in the intestinal tract. In their rat model for duodenal ulcer, Deng et al.[31] demonstrated improved healing of ulcers in animals treated with both naked VEGF plasmid DNA and those transfected with VEGF using an adenoviral vector. The authors showed that VEGF gene delivery using both methods enhanced VEGF production. They also observed up-regulation of other pro-angiogenic factors such as PDGF. The implication is that multiple factors are important in the tissue response to ischemic injury and healing, which may be initiated with VEGF gene therapy. While these authors have demonstrated higher gene transfer efficiencies with adeno-associated viral vectors, we have shown in this study and in previous work that non-viral vectors can achieve adequate up-regulation of target epitopes and a corresponding clinical response.[32] Although non-viral vectors potentially sacrifice higher transfection efficiencies, there are many benefits to non-viral gene transfer. These advantages include much lower immunogenic potential and lower toxicity—important considerations for future trials in humans.[33,34]

Multiple genes are responsible for controlling the complex process of vascular remodeling. Potential therapeutics combining VEGF transfection with delivery of other coding sequences such as those for the angiopoetins may be critical for limiting the malignant potential associated with angiogenesis and for improving the functionality of new vessels. For example, it has been shown that Angiopoetin-1 (Ang-1) stabilizes and matures the vessel growth induced by VEGF. It has also been shown that vessels regulated by Ang-1 have a lower propensity to leak than those in tissue treated only with VEGF.[29,35] Tight regulation of angiogenesis will be necessary for incorporation of similar methods in the setting of neoplasia. Future studies that explore the use of multiple growth factors or the transfection of multiple genes, various delivery methods, stem cells, and the application of the delay phenomenon will provide invaluable information in the field of gastrointestinal healing.

Conclusions

This pilot study employing gene therapy to up-regulate angiogenesis demonstrates that significant clinical benefit can be derived from VEGF-induced neovasculature at a healing gastrointestinal anastomosis. While future investigations examining the regulation of angiogenesis are required for the safe introduction of this therapy in humans, VEGF gene transfection has the potential for broad applications to improve healing throughout gastrointestinal surgery.

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Discussion

John W. Harmon, M.D. (Baltimore, MD): First, I would like to thank Dr. Enestvedt and Dr. Jobe for providing me with a copy of the manuscript. I congratulate you on nice work and a nice presentation.

As many of you in the audience are aware, the dream of gene therapy has not been fulfilled. As people say, the success of gene therapy is just around the corner, just over the horizon, and it always will be. The problem is how to deliver the gene therapy. You have viral and nonviral approaches. Dr. Enestvedt and his group are using a DNA plasmid vector which is nonviral. This avoids the problem of the systemic inflammatory response syndrome, which has notoriously appeared in certain viral trials, not often, but enough to cause a big problem. So it is very attractive that they are using a nonviral approach. So what about nonviral delivery of DNA plasmids?

The simple injection of DNA plasmidsinto tissue without anything else basically gives you a little tiny bit of transfection. We have used it, but it is not satisfactory. There is a gene gun approach in which small pellets, gold pellets, are coated with DNA, and shot into the tissue. This improves transfection efficiency, but it is highly variable and has few advocates.

Dr. Enestved's group is using a cationic lipid carrier to bring the DNA into the cells. The DNA, of course, has to get not only across the cell membrane but also across the nuclear membrane, double transport. I am impressed with this PEI transfection cationic lipid; I am impressed that it worked at all. In our hands, these cationic lipid carriers actually reduced transfection, as we have published. I looked into the literature regarding this jet PEI. It seems to be an advance, but there are conflicting reports; some people using it finding that it didn't work, others that it did.

We found that these kinds of carriers worked very well in tissue culture, especially without serum. We found that by adding serum, we progressively reduced the effectiveness of the approach, presumably as the cationic carrier with the DNA bound to the peptides, and we found they actually reduced transfection.

So, Dr. Enestvedt, my question for you is how certain are you that you are getting satisfactory transfection? You did get a nice biological response for some of your parameters. I was particularly impressed with the figure where you found a positive correlation between the bursting strength and the microvessels. However, this could possibly be a nonspecific effect.

It is of concern that real-time PCR didn't show an increase. There was an increase, but it was not statistically significant. In our lab using electroporation, instead of using the cationic lipid, we used the same kind of plasmid and the same PCR technique, we saw a 700-fold increase that lasted for a month.

Finally, I applaud you for tackling what is now becoming a classically difficult, but terribly important problem. Certainly if we could improve the healing of wounds, particularly bowel anastomoses, but many other wounds as well, using a gene therapy approach, we would be doing a very good thing for mankind, and you are part of the effort, and I applaud you.

Kristian Enestvedt, M.D. (Portland, OR): Dr. Harmon, thank you for your insightful analysis. The PCR results are definitely a concern for us. I think there are several explanations for our findings. The first is something I mentioned previously, which is the sample size. I think this does play a role. The vehicle that we chose for transfection, the PEI vehicle, I think has demonstrated a pretty reasonable effect. I think the problem with our study is the combination of the PEI with the fibrin suspension. The PEI, as you mentioned, is a strong cation, and the fibrin has a strong negative charge. This serves to tightly bind the PEI vector to the fibrin. So that when you inject it into the tissue, there is very limited lateral diffusion. If we don't sample exactly that needle tract and extract RNA from it, I think that we see less transgene in those tissue samples. The range for the fold change was on the order of 1.3 to 200. So we do see a reasonable transfection efficiency using this carrier system. I think a bigger problem is the PEI and fibrin combination sequestering the transgene in a very localized area, because, as you pointed out, we do see the appropriate downstream effect. This indicates that the VEGF growth factor is actually being disseminated to the tissue, and that is important, because that is what we need for the clinical response.

I am confident that we have addressed these issues because we have our PCR results from a second, larger study that I described, and with a doubling in the number of animals, we see a statistically significant difference in the transcript levels. In addition to the larger sample, we have removed the fibrin suspension. We are complexing only the PEI with the vector, adding PBS, injecting that solution, and now we see a significant difference in the transcript levels. Thank you, again, Dr. Harmon, for your questions.

Joerg Haier, M.D. (Muenster, Germany): First, congratulations on this very interesting approach. I have two questions, actually. Do you have an idea which type of cells is taking up your plasmid construct and which cells are actually responding to this, which means where is the VEGF coming from? And the second is, what is the advantage instead of using a slower-release, direct-protein construct?

Dr. Enestvedt: Those are excellent questions. There is a simple answer to the first:the cell types are infiltrating macrophages that are a normal response of wounding, that are abundant in the wound by postoperative day two. Those are the target cells, they take up the vectoras along with some of the native endothelial cells, and those are the cells that ultimately produce VEGF. The endothelial cells respond by sprouting new vessels.

Dr. Haier: Why are you using a genetic construct instead of direct protein release?

Dr. Enestvedt: Protein injected with a Matrigel would probably work with similar success. The problem with direct protein delivery is that the half-life is very short, and we need a sustained effect to protect the healing anastomosis. Our lab has shown in previous studies that using PEI, we have the optimal time of peak transgene expression by postoperative day four, which is when we want high VEGF levels to protect the healing anastomosis. There certainly are other methods for the delivery of VEGF but we favor this approach because of its time-course effects.

Dr. Haier: But there are some glycocalix coatings for suture materials available that release the proteins in similar kinetics. It might be an option too.

Dr. Enestvedt: Thank you, that is an excellent suggestion.

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Sodium-Coupled Transport of the Short Chain Fatty Acid Butyrate by SLC5A8 and Its Relevance to Colon Cancer

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Abstract
Introduction SLC5A8, expressed predominantly in the colon, is a Na^+-coupled transporter for short-chain fatty acids. In this paper, we report on the characterization of butyrate transport by SLC5A8 and the relevance of SLC5A8-mediated butyrate transport to colon cancer.
Results SLC5A8 transports butyrate via a Na^+-dependent electrogenic process. Na^+ activation of the transport process exhibits sigmoidal kinetics, indicating involvement of more than one Na^+ in the activation process. *SLC5A8* is silenced in colon cancer in humans, in a mouse model of intestinal/colon cancer, and in colon cancer cell lines. The tumor-associated silencing of *SLC5A8* involves DNA methylation by DNA methyltransferase 1. Reexpression of SLC5A8 in colon cancer cells leads to apoptosis but only in the presence of butyrate. SLC5A8-mediated entry of butyrate into cancer cells is associated with inhibition of histone deacetylation. The changes in gene expression in SLC5A8/butyrate-induced apoptosis include upregulation of pro-apoptotic genes and downregulation of anti-apoptotic genes. In addition, the expression of phosphatidylinositol-3-kinase subunits is affected differentially, with downregulation of p85α and upregulation of p55α and p50α.
Conclusion These studies show that SLC5A8 mediates the tumor-suppressive effects of the bacterial fermentation product butyrate in the colon.

Keywords Colon cancer · Tumor suppression · Histone deacetylation · DNA methylation · Dietary fiber · Commensal bacteria

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Introduction

Short-chain fatty acids such as acetate, propionate, and butyrate are produced in the colonic lumen by bacterial fermentation of dietary fiber.[1,2] One of the mechanisms by which high fiber intake promotes colonic health is by providing substrates for bacterial fermentation in the colonic lumen to generate short-chain fatty acids. These bacterial metabolites serve as the primary nutrients for colonocytes and promoters of cell differentiation.[1,2] The short-chain fatty acid butyrate induces differentiation of colonocytes and promotes apoptosis in colonic tumor cells. The ability of butyrate to induce apoptosis in tumor cells is related to its function as an inhibitor of histone deacetylases (HDACs).[3,4] High intake of dietary fiber is known to be protective against colon cancer. To produce these aforementioned effects, butyrate has to first enter the colonocytes. We and others have recently identified a Na^+-coupled transporter for short-chain fatty acids.[5–8] This transporter is known as SMCT1 (i.e., sodium-coupled monocarboxylate transporter

1) or SLC5A8 according to Human Genome Organization nomenclature and is expressed abundantly in the colon. We hypothesize that the primary function of this transporter is to mediate active entry of butyrate and other short-chain fatty acids from the colonic lumen into colonocytes to facilitate the biologic effects of these bacterial metabolites inside the cells.[9,10]

Butyrate also stimulates the absorption of salt and water in the colon.[11,12] The current prevailing theory on how butyrate exerts its stimulatory effects on Na^+ absorption implicates a functional coupling between a butyrate/HCO_3^- exchanger and a H^+-coupled monocarboxylate transporter (MCT1) in the lumen-facing apical membrane. This proposed mechanism of butyrate action does not incorporate the potential role of SLC5A8 in the process. Furthermore, the expression of MCT1 in the apical membrane of colonic epithelium is controversial.[13–16] Therefore, the relevance of MCT1-mediated transport of short-chain fatty acids to Na^+ absorption remains unclear. In contrast to MCT1, there is unequivocal evidence for the expression of SLC5A8 in the apical membrane of colonocytes.[8,16–18] Therefore, the Na^+-coupled electrogenic nature of this transporter has profound implications in the role of butyrate as a stimulator of Na^+ absorption in the colon.

Previous studies from other laboratories have shown that *SLC5A8* is silenced in colon cancer[8,19] and that reexpression of the transporter in colon cancer cell lines leads to growth arrest.[19] However, the underlying mechanism of tumor suppression has not been addressed. We hypothesize that SLC5A8-mediated concentrative entry of butyrate into colon cancer cells is responsible for tumor cell-specific induction of growth arrest. The present investigation was undertaken to delineate the kinetic features of butyrate transport via SLC5A8 and to examine the relevance of SLC5A8-mediated transport of butyrate to colon cancer.

Materials and Methods

Generation of Wild-Type (V251F) Human SLC5A8 complementary DNA

The SLC5A8 complementary DNA (cDNA) that we cloned originally has valine at position 251[5] whereas the clones reported in the GenBank™ by other investigators have phenylalanine at this position. Therefore, we generated a V251F variant of our human SLC5A8 cDNA to compare the kinetics of butyrate transport via wild-type SLC5A8 (F251) with that of our original clone (V251). The mutagenic primers were 5′-ATTATAGGAGGGACCTTCACATGGACCAGC-3′ (sense) and 5′-GCTGGTCCATGTGAAGGTCCCTCCTA TAAT-3′ (antisense). The codon subjected to mutation is underlined. Generation of the variant was accomplished using the QuikChange™ Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). The wild-type (F251) and variant (V251) cDNAs were subcloned in pGH19, a *Xenopus laevis* ocyte expression vector.

Functional Expression of Human SLC5A8 in *X. laevis* Oocytes

Capped complementary RNAs (cRNAs) from human SLC5A8 cDNA clones were synthesized using the mMessage-mMachine kit (Ambion, Austin, TX, USA). Mature oocytes from *X. laevis* were injected with 50 ng of cRNA. Water-injected oocytes served as controls. The oocytes were used for electrophysiological studies 3–7 days after cRNA injection using the two-microelectrode voltage-clamp method. Oocytes were superfused with a NaCl-containing buffer (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid, pH 7.5) followed by the same buffer containing butyrate. The membrane potential was clamped at −50 mV. The differences between the steady-state currents measured in the presence and absence of 2.5 mM butyrate were considered as the substrate-induced currents. In the saturation kinetics, $K_{0.5}$ (i.e., the concentration of butyrate inducing half-maximal current) was calculated by fitting the values for the butyrate-induced currents to the Michaelis–Menten equation. The Na^+-activation kinetics was analyzed by measuring the butyrate-specific currents in the presence of increasing concentrations of Na^+. The concentration of Na^+ was varied by adjusting the concentration of NaCl in the superfusion buffer with equimolar concentrations of *N*-methyl-D-glucamine chloride. The data were analyzed according to the Hill equation to determine the Hill coefficient (h, the number of Na^+ ions involved in the activation process) and $K_{0.5}$ for Na^+ (i.e., concentration of Na^+ necessary for half-maximal activation). Since the expression levels varied significantly among different oocytes, kinetic analyses were done by normalizing the expression levels (the maximally induced SLC5A8-specific currents in each kinetic experiment in individual oocytes was taken as 1).

Data Analysis

Electrophysiologic measurements were made with four different oocytes, and the data are presented as means ± SE. The kinetic parameters were determined using the computer program Sigma Plot, version 6.0 (SPSS, Inc., Chicago, IL, USA).

Immunofluorescent Localization of SLC5A8 in Cultured Cells

Cultured cells, grown on glass slides, were fixed in methanol, washed with 0.01 M phosphate-buffered saline (PBS; pH 7.4), and blocked with 1× Power Block (Biogenex, San Ramon, CA, USA) for 60 min. Slides were then incubated at 4°C overnight with the primary rabbit polyclonal antibody against SLC5A8 (1:1,000). Negative control slides were treated identically but in the absence of the primary antibody. Slides were rinsed and then incubated for 30 min at room temperature with goat anti-rabbit IgG coupled to Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA) at a dilution of 1:1,500. Coverslips were mounted with Vectashield Hardset mounting medium with 4′,6-diamidino-2-phenylindole (a nuclear stain), and slides were examined by epifluorescence using the Zeiss Axioplan 2 microscope (Carl Zeiss Inc., Oberkochen, Germany).

Collection of Primary Tumor Tissues

Human normal colon and colon tumor specimens were collected from 18 adult patients with colorectal cancer, with patients' informed consent and approval from the Medical College of Georgia institutional review board. Details of most of these patients have been described previously.[20]

Collection of Tissues from $Apc^{Min/+}$ Mouse

Apc$^{Min/+}$ male mice on a C57BL/6 background (Jackson Laboratories, Bar Harbor, ME, USA) were bred with female C57BL/6 mice. Genotyping was done by PCR. Mice were euthanized by CO_2, and the colon was collected. Tissue sections were fixed in 10% buffered formalin.

Ectopic Expression of SLC5A8 in Colon Cell Lines

Cells were seeded either in 10-cm (for protein and RNA) or in 35-mm (for florescence-activated cell sorting, FACS) culture dishes and cultured in the absence of pyruvate. Cells were transfected with pcDNA or SLC5A8 cDNA using Fugene 6 and Opti-MEM. pEGFP-N1 was used for co-transfection to determine transfection efficiency. After 24 h, cells were treated with or without butyrate (0.5 mM) for 24 h. Cells were collected, centrifuged and washed twice with PBS. Preparation of RNA and protein lysates, and FACS analysis were done as described previously.[21]

Reverse Transcriptase Polymerase Chain Reaction

This was done as previously described.[21] The PCR primers for specific gene products were designed based on the nucleotide sequences available in GenBank.

Western Blot Analysis

Fifty micrograms of protein was fractionated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the fractionated proteins were transferred to a nitrocellulose membrane (Schleicher & Schull). Membranes were blocked with bovine serum albumin and then exposed to respective primary antibody at 4°C overnight, followed by treatment with appropriate secondary antibody at room temperature for 1 h. Proteins were visualized by ECL Super Signal Western System (GE Healthcare).

Measurement of HDAC Activity

A commercially available kit (BioVision) was used to determine HDAC activity. CCD841 and SW480 cells were transfected with pcDNA or SLC5A8 cDNA followed by treatment with or without butyrate (0.5 mM) for 24 h. Cells were lysed, and 100 μg of lysate was used for the assay according to the manufacturer's instructions.

Results

Comparison of Butyrate Transport Between Wild-Type Human SLC5A8 and Its F251V Variant

We have reported the transport function of human SLC5A8 that we cloned from human intestine.[5] There are four entries in the GenBank™ database for the amino acid sequence of human SLC5A8 (NP_666018, AAI10493, AAP46193, and AAP46194). Comparison of amino acid sequences among these four clones and our clone shows four regions where the sequences differ: Ile or Val at position 193, Val or Phe at position 251, Val or Ile at position 440, and Ile or Met at position 490. Only our clone has valine at position 251, whereas all the four clones reported in the GenBank™ have phenylalanine at this position. The substitutions at positions 193, 440, and 490 are conservative and therefore are unlikely to produce dramatic effects on transport function. In contrast, Val-to-Phe substitution at position 251 may exert significant influence on transport function because of the differences in the chemical structure (aliphatic versus aromatic) and bulkiness of the amino acids involved in the substitution. Therefore, we mutated valine at position 251 in our clone to phenylalanine found in wild-type transporter and then compared the characteristics of butyrate transport between our clone (V251) and the wild-type clone (F251). Both clones, when expressed in *X. laevis* oocytes, induced inward currents in the presence of butyrate, but the magnitude of the currents was significantly higher for the wild-type transporter than for the F251V variant (Fig. 1a).

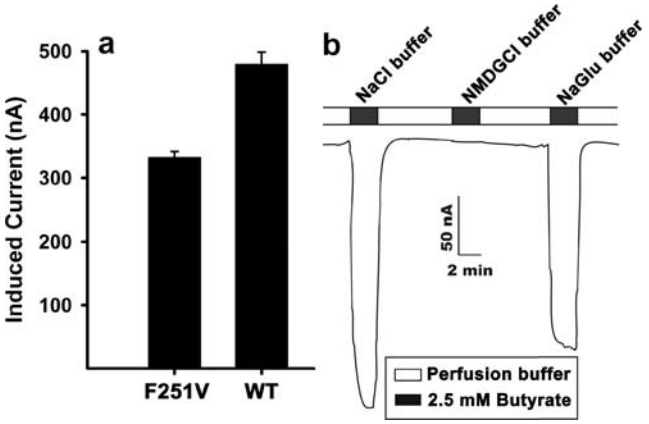

Figure 1 Butyrate-induced currents in oocytes expressing the wild-type SLC5A8 or the F251V variant. **a** Comparison of the magnitude of inward currents induced by 2.5 mM butyrate in oocytes expressing either the wild-type SLC5A8 or the F251V variant. Data are from three different oocytes (5 days following cRNA injection). **b** Na^+ dependence of butyrate-induced currents in oocytes expressing the wild-type SLC5A8. Oocytes expressing the transporter were perifused with 2.5 mM butyrate in three different buffers containing NaCl (presence of Na^+ and Cl^-), *N*-methyl-D-glucamine chloride (absence of Na^+ but presence of Cl^-), or Na gluconate (presence of Na^+ but absence of Cl^-). Data are from a representative oocyte (3 days following cRNA injection); similar results were obtained in four different oocytes.

The current induced by 1 mM butyrate was 480±19 nA for the wild-type transporter; the corresponding value was 332±10 nA for the F251V variant ($p<0.001$). With both clones, the butyrate-dependent currents were seen only in the presence of Na^+. Figure 1b describes the data for the wild-type transporter. Similar results were obtained with the F251V variant (data not shown).

We then compared the saturation kinetics for butyrate between the wild-type SLC5A8 and the F251V variant (Fig. 2a). The kinetics was identical for both clones. The Michaelis constant for butyrate was 51±3 μM for the wild-type transporter and 55±2 μM for the F251V variant ($p>0.05$). The Na^+-activation kinetics showed significant difference between the wild-type SLC5A8 and the F251V variant (Fig. 2b). The relationship between Na^+ concentration and butyrate-induced currents was sigmoidal for both clones with an identical value for the Hill coefficient (1.9±0.1), but the $K_{0.5}$ value for Na^+ showed significant difference (13±1 and 23±1 mM for the wild-type transporter and the F251V variant, respectively; $p<0.001$), showing that the wild-type SLC5A8 has greater affinity for Na^+ than the F251V variant.

Expression of SLC5A8 in Colon Cancer

SLC5A8 functions as a tumor suppressor in colon, but the underlying mechanism has not been addressed.[8,19] We hypothesized that SLC5A8-mediated concentrative entry of butyrate into colon cancer cells induces tumor cell-specific apoptosis. To test this, we first examined the expression of *SLC5A8* in paired samples of normal and cancer colon tissues from 18 patients to confirm the silencing of this gene in colon cancer. We found greater than 90% reduction in SLC5A8 mRNA levels in tumor tissue than in normal tissue (Fig. 3a and b). In parallel, we

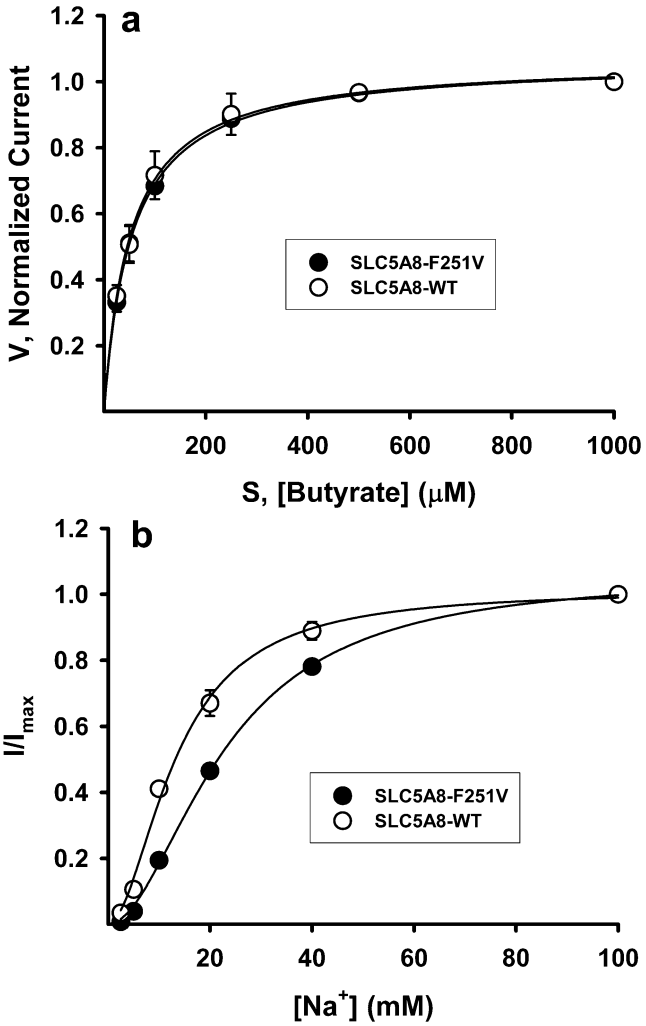

Figure 2 Kinetics of butyrate transport via SLC5A8. **a** Saturation kinetics for butyrate transport via the wild-type SLC5A8 and the F251V variant. Oocytes expressing either the wild-type transporter or the variant were perifused with increasing concentrations of butyrate in the presence of NaCl, and the induced inward currents were monitored. The experiment was repeated in four different oocytes for each clone. The currents in each oocyte were normalized by taking the maximal current induced by 1 mM butyrate as 1. **b** Na^+-activation kinetics for butyrate transport via the wild-type SLC5A8 and the F251V variant. Oocytes expressing either the wild-type transporter or the variant were perifused with 1 mM butyrate in the presence of increasing concentrations of Na^+, and the induced inward currents were monitored. The experiment was repeated in four different oocytes for each clone. The currents in each oocyte were normalized by taking the maximal current induced in the presence of 100 mM Na^+ as 1.

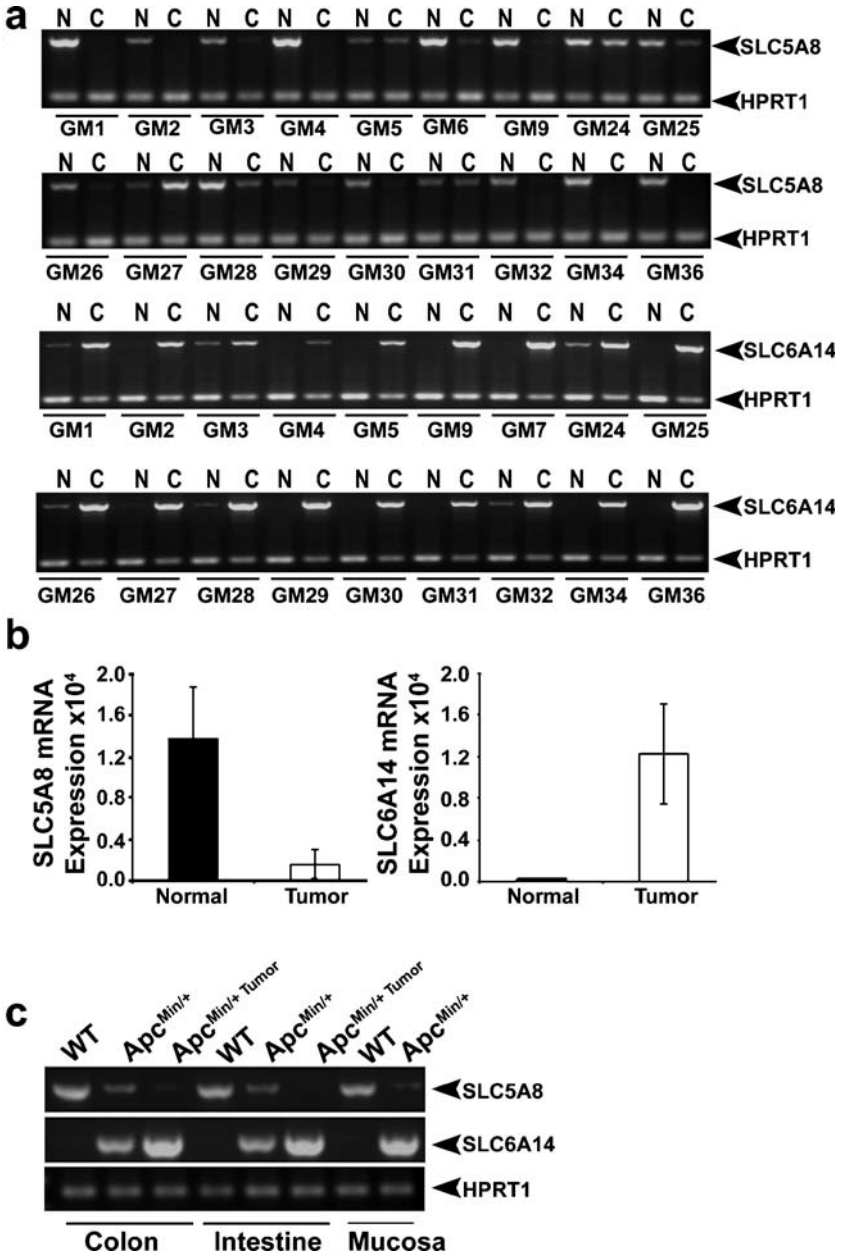

Figure 3 Silencing of *SLC5A8* in colon cancer. **a** Silencing of *SLC5A8* and upregulation of *SLC6A14* ($ATB^{0,+}$) in primary tumors derived from human colon. Reverse transcriptase polymerase chain reaction (RT-PCR) was used to monitor the steady-state levels of mRNAs specific for SLC5A8 and SLC6A14 in paired normal (N) and cancer (C) specimens. Representative gels with samples from 18 patients are shown. The patients' serial numbers are given as GM1, GM2, etc. HPRT1 mRNA was used as an internal control. **b** Quantification of SLC5A8 mRNA and SLC6A14 mRNA in normal and tumor tissues after normalization with HPRT1 mRNA levels. **c** Steady-state levels of mRNAs for SLC5A8, SLC6A14, and HPRT1 in intestinal and colonic tissues from wild-type mice and $Apc^{Min/+}$ mice.

showed that the expression of *SLC6A14*, the gene coding for the amino acid transporter $ATB^{0,+}$, is upregulated in the tumor tissue compared to the normal tissue, confirming our earlier findings.[20] The data from the $Apc^{Min/+}$ mouse model of intestinal/colon cancer corroborated these findings (Fig. 3c).

Cancer-Associated Silencing of SLC5A8 Involves DNA Methylation

The silencing of *SLC5A8* was also evident in a majority of colon cancer cell lines (Fig. 4a). The silencing of *SLC5A8* in these tumor cells occurs via DNA methylation because treatment of these cells with the DNA methylation inhibitor 5′-azacytidine induced the expression of the transporter (Fig. 4b). To determine which DNA methyltransferase is responsible for this process, we compared the expression of *SLC5A8* in parent HCT116 cells that express *DNMT1* and *DNMT3b*, with that in isogenic cells with selective deletion of *DNMT1*, *DNMT3b*, or both (DKO; Fig. 4c and d). *DNMT1*-null cells expressed the transporter mRNA and protein whereas *DNMT3b*-null cells did not, suggesting that DNMT1 is responsible for DNA methylation associated with the silencing of *SLC5A8*. This was confirmed by the findings that treatment of colon cancer cells with procainamide, a specific inhibitor of DNMT1,[22] induced *SLC5A8* expression (Fig. 4e).

The data from the $Apc^{Min/+}$ mouse model of intestinal/colon cancer indicate that the adenomatous polyposis coli

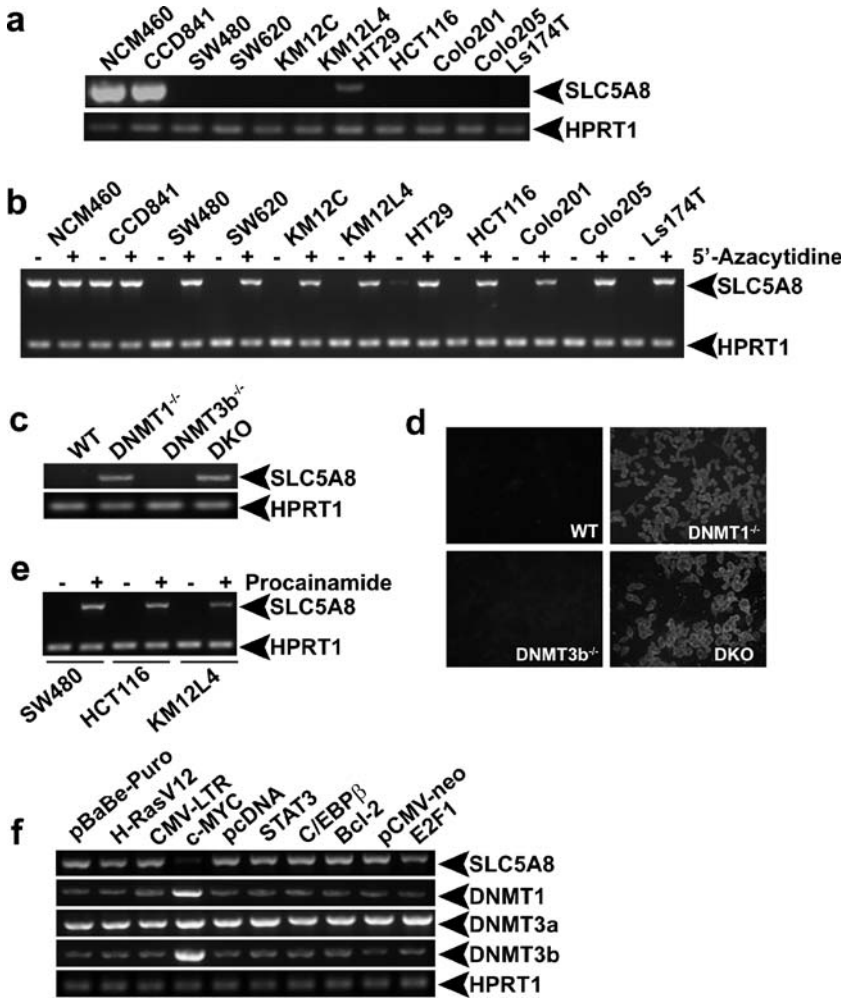

Figure 4 Involvement of DNMT1 in the silencing of *SLC5A8* in colon cancer cells. **a** Steady-state levels of mRNAs for SLC5A8 in two non-malignant human colon cell lines (NCM460 and CCD841) and nine human colon cancer cell lines (SW480, SW620, KM12C, KM12L4, HT29, HCT116, Colo201, Colo205, and Ls174T). **b** RT-PCR for SLC5A8 mRNA in non-malignant and cancer colon cell lines after treatment with or without 5′-azacytidine (2 μg/ml; 72 h). HPRT1 mRNA was used as an internal control. **c** Expression of SLC5A8 mRNA in HCT116 cell line (a human colon cancer cell line) and in isogenic cell lines with the deletion of *DNMT1*, *DNMT3b*, or both (DKO). **d** Expression of SLC5A8 protein, as assessed by immunofluorescence, in HCT116 cell line (a human colon cancer cell line) and in isogenic cell lines with the deletion of *DNMT1*, *DNMT3b*, or both (DKO). **e** Induction of *SLC5A8* expression by procainamide (10 μM, 48 h treatment), a specific inhibitor of DNMT1, in colon cancer cells. **f** Induction of *DNMT1* and silencing of *SLC5A8* by c-MYC in the non-malignant colon cell line CCD841. Cells were transiently transfected with cDNAs for c-MYC and other oncogenes, and the expression levels of mRNAs for SLC5A8 and various DNMTs were examined by RT-PCR.

gene (*APC*) is involved in the control of *SLC5A8* expression. Inactivation of *APC* leads to expression of the oncogene c-*MYC*.[23,24] To determine whether *c-MYC* is responsible for the tumor-associated upregulation of *DNMT1* and consequent silencing of *SLC5A8*, we expressed c-MYC ectopically in CCD841 cells (a non-malignant colon cell line) and analyzed the effects on the expression of *DNMT*s (Fig. 4f). *DNMT1* was induced by c-MYC. Ectopic expression of several other oncogenes did not have any effect. The c-MYC-induced upregulation of *DNMT1* was associated with downregulation of *SLC5A8*. The expression of DNMT3b was also induced by ectopic expression of c-MYC, but our earlier studies with HCT116 cells have shown that this isoform is not responsible for the silencing of *SLC5A8*. These findings suggest that inactivation of *APC* in colon cells leads to *c-MYC* expression, with consequent induction of *DNMT1* and silencing of *SLC5A8*.

Involvement of SLC5A8-Mediated Transport of Butyrate in Tumor Cell-Specific Induction of Apoptosis in Colon Cells

We then examined the relationship of butyrate to the tumor-suppressive function of SLC5A8 in the colon by assessing the influence of reexpression of the transporter in SW480 cells (a human colon cancer cell line in which SLC5A8 is

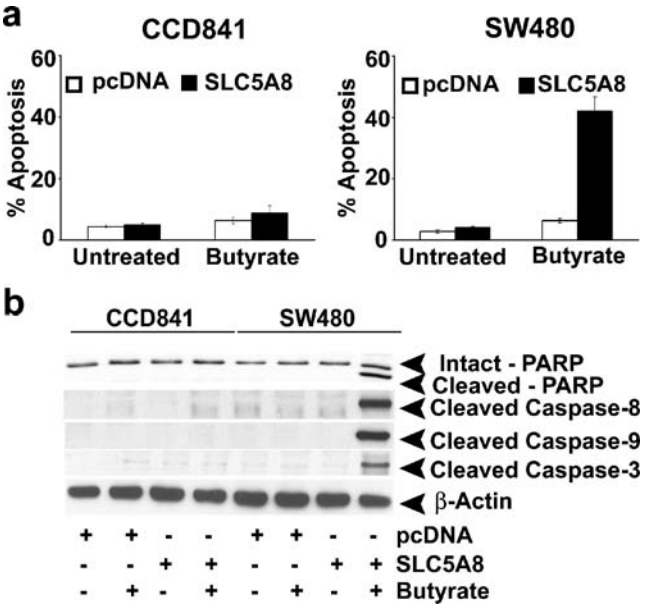

Figure 5 Tumor cell-specific induction of apoptosis by SLC5A8/butyrate. **a** CCD841 (a non-malignant human colon cell line) and SW480 (a human colon cancer cell line) cells were transfected with pcDNA or SLC5A8 cDNA. Twenty-four hours following transfection, cells were treated with butyrate (0.5 mM) for 24 h, and apoptosis was analyzed by FACS. **b** Cell lysates, prepared from CCD841 and SW480 cells, which were transfected with pcDNA or SLC5A8 cDNA and then treated with or without butyrate, were resolved by SDS-PAGE and the membranes analyzed for PARP cleavage and activation of various caspases using appropriate antibodies. β-Actin was used as a loading control.

completely silenced) on apoptosis. To investigate the tumor cell-specific selectivity of this process, we used CCD841 cells (a non-malignant human colon cell line, which expresses SLC5A8 abundantly) in parallel. With transfection of vector alone, neither CCD841 cells nor SW480 cells underwent apoptosis upon exposure to butyrate (Fig. 5a). In contrast, when transfected with SLC5A8 cDNA, butyrate induced apoptosis in SW480 cells but not in CCD841 cells. The apoptosis induced by SLC5A8/butyrate in SW480 cells was associated with poly-ADPribose polymerase (PARP) cleavage and activation of caspases 3, 8, and 9 (Fig. 5b).

Relevance of HDAC Inhibition to SLC5A8/Butyrate-Induced Apoptosis in Colon Cancer Cells

It is likely that SLC5A8/butyrate-induced apoptosis in tumor cells involves entry of butyrate into cells via SLC5A8 and subsequent inhibition of HDACs. We examined this by analyzing HDAC activity in CCD841 cells and SW480 cells with and without ectopic expression of SLC5A8 and with and without exposure to butyrate. HDAC activity was higher in tumor cells than in normal cells as evident from HDAC activity (Fig. 6a) and from the acetylation status of histone-H4-lys[16] (Fig. 6b). Butyrate did not influence HDAC activity in normal cells with or without the ectopic expression of SLC5A8. In contrast, exposure of cancer cells to butyrate inhibited HDAC activity but only following ectopic expression of SLC5A8. These data show that SLC5A8/butyrate-induced apoptosis in tumor cells involves HDAC inhibition.

Effects of SLC5A8/Butyrate on Expression Pattern of Genes Associated with Apoptosis and Cell Cycle in Colon Cancer Cells

We examined the effects of SLC5A8-mediated entry of butyrate into colon cancer cells on the expression pattern of several genes involved in apoptosis and cell cycle control (data not shown). The induction of apoptosis in SW480 colon cancer cells by SLC5A8/butyrate was associated with upregulation of pro-apoptotic genes (p53, Bax, Bad, Bak, FAS ligand, FAS receptor, TRAIL, and TRAIL receptors) and downregulation of anti-apoptotic genes (Bcl-2, Bcl-W, Bcl-xL, Bfl-1, and survivin). We also found differential effects on the expression of phosphatidylinositol-3-kinase subunits, with downregulation of p85α and upregulation of p55α and p50α.

Discussion

In this paper, we report findings that have potential physiologic, clinical, and therapeutic relevance to the role

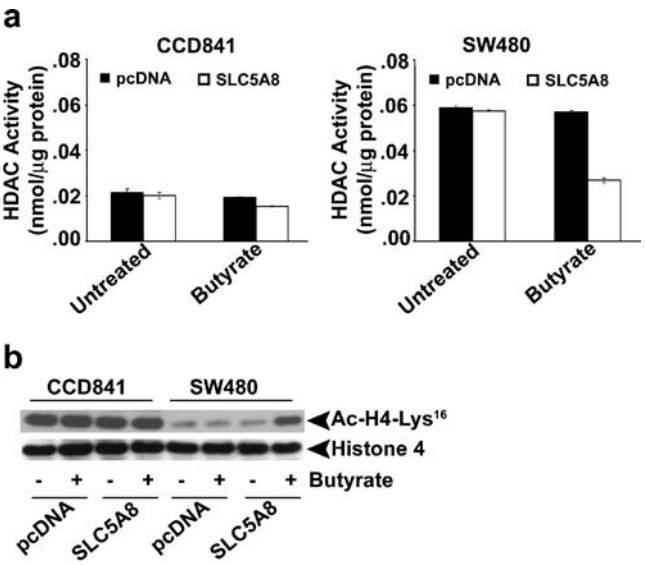

Figure 6 Relevance of HDAC inhibition to SLC5A8/butyrate-induced apoptosis in colon cancer cells. **a** CCD841 and SW480 cells were transfected with either pcDNA or SLC5A8 cDNA, and then treated with or without butyrate (0.5 mM). Cell lysates were used for measurement of HDAC activity with a commercially available kit. **b** Western blot analysis was carried out with these protein samples using antibodies against histone H4 and acetylated histone H4 (Lys16).

of butyrate in colon. We describe the features of butyrate transport via SLC5A8 and also provide evidence for SLC5A8/butyrate-mediated inhibition of HDACs as the underlying mechanism of the tumor-suppressive function of SLC5A8. The single-nucleotide polymorphism database reports the occurrence of Phe-to-Val mutation in human population. With both the wild-type transporter and the F251V variant, we found that butyrate is transported in a Na^+-coupled manner and that both clones exhibit similar substrate affinity ($K_{0.5}$, ~50 μM). The activity of the wild-type transporter is greater than that of the F251V variant. Furthermore, the wild-type transporter has higher affinity for Na^+ than the variant. The Na^+-coupled, high-affinity, electrogenic transport of butyrate via SLC5A8 provides a molecular basis for the ability of this short-chain fatty acid to promote Na^+ absorption in the colon.

Butyrate may have protective effect against diarrheal diseases due to its ability to enhance Na^+ and water absorption.[25,26] In addition, this short-chain fatty acid decreases intracellular levels of cyclic AMP (cAMP) and consequently interferes with Cl^- secretion.[27,28] Diarrheal diseases such as cholera and traveler's diarrhea are caused by bacterial toxins that elevate cAMP levels in intestinal and colonic epithelial cells.[29,30] Oral rehydration solution, which contains glucose, Na^+, Cl^-, and HCO_3^-, is used effectively for the treatment of cholera and other diarrheal diseases.[31] Since the activity of Na^+-coupled glucose transporter in the small intestine is intact in these disease conditions, administration of glucose into intestinal lumen enhances Na^+ absorption via this transporter, with consequent increase in Cl^- and water absorption. This prevents dehydration. Even though the oral rehydration solution is effective, improvements in efficacy of this solution are definitively desirable.[31] Since SLC5A8 is expressed in the apical membrane of not only the colon but also the ileum,[8,16–18] presence of butyrate in the intestinal lumen would facilitate Na^+ absorption markedly via the transporter. Such an effect would be followed by Cl^- absorption and water absorption. Our studies thus provide a strong rationale for the addition of butyrate to oral rehydration solutions as a means to improve their therapeutic efficacy against diarrheal diseases.

Our studies with $Apc^{Min/+}$ mice demonstrate that *Apc* controls the expression of *SLC5A8* with c-MYC and DNMT1 as the mediators. Inactivation of *APC* leads to upregulation of WNT/β-catenin signaling with consequent overexpression of *c-MYC* and *DNMT1* (http://www.stanford.edu/~rnusse/pathways/targets.html).[23,24] Our studies with the colon cancer cell line HCT116 and with $Apc^{Min/+}$ mouse intestinal tissue show that *DNMT1* is upregulated in response to c-MYC and that DNMT1 alone is sufficient to silence *SLC5A8*. These data link dietary fiber/bacterial flora to colonic health via SLC5A8. In normal colon with functional APC, WNT signaling is silenced with consequent suppression of *c-MYC* and its downstream target *DNMT1*. This results in robust expression of SLC5A8 in the apical membrane of the polarized colonocytes to mediate the beneficial effects of butyrate. When *APC* is mutated, WNT signaling is activated, resulting in the silencing of *SLC5A8*. This prevents the biologic actions of butyrate in colonocytes. Thus, the bacterial metabolite butyrate, arising from fermentation of dietary fiber, is an effective tumor suppressor in colon, and the process involves SLC5A8-mediated entry of butyrate into cells. Tumor cells silence the expression of SLC5A8 to evade butyrate-induced cell death. Therefore, pharmacologic means to reactivate *SLC5A8* in tumor cells may represent a novel therapeutic approach in the treatment of colon cancer.

The Michaelis constant for butyrate transport via SLC5A8 is ~50 μM. The luminal concentration of butyrate in normal colon reaches millimolar levels. At these high concentrations, non-SLC5A8-mediated transfer processes such as non-ionic diffusion and anion exchangers become quantitatively more important than SLC5A8 in butyrate entry into colonic epithelial cells. Why would tumor cells silence *SLC5A8* if non-SLC5A8-mediated entry of butyrate is predominant under physiologic conditions? It has to be borne in mind that tumor cells lose their polarity. Therefore, transformed cells in the colon do not have direct access to luminal butyrate, and the transporter may be expected to be expressed throughout the plasma membrane of these transformed cells. This becomes relevant to our recent findings that SLC5A8-mediated pyruvate entry into tumor cells induces apoptosis.[21] Because of the loss of polarity upon transformation, tumor cells in the colon may silence *SLC5A8* to prevent the entry of pyruvate from the blood. Thus, the tumor-associated silencing of the transporter may prevent the entry of butyrate from the lumen and the entry of pyruvate from the blood, thus preventing cell death inducible by these histone deacetylase inhibitors.

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Discussion

Stephen A. McClave, M.D. (Louisville, KY): We had this collective knowledge that butyrate is the ultimate food for the colonic epithelium. It has the ultimate trophic effect on cell proliferation and health of the colon. It is interesting to me that the work of Cresci, Ganapathy and others, as they have teased apart the mechanism of this butyrate transport, have led to four clinical applications of this butyrate transporter.

In critical illness, we are adding inulin and fructooligosaccharides and prebiotic fiber to enteral formulas. By stimulating the butyrate transporter, we are downregulating or inhibiting NFkB expression, which downregulates inflammation and reduces oxidative stress. In the ICU that means fewer complications. For patients with severe short bowel syndrome on TPN, dietary fiber facilitates colonic salvage by transporting butyrate across the colonic epithelium. Patients can thus salvage up to 500 calories a day, which can make the difference in gut autonomy and whether or not they get

off TPN. In chronic diarrheal diseases in third world countries, adding rice to oral rehydration solutions takes advantage of the sodium transport that is tacked on to the butyrate absorption in the colonic epithelium. In other words, the oral rehydration solution targets small bowel, glucose, and sodium mediated pumps. By adding the rice, we bring in the butyrate transporters and the colon gets further sodium absorption and the diarrhea management gets even easier. And then the fourth and probably most exciting application is what Dr. Cresci is talking about in colonic adenocarcinoma, that the tumor has this uncanny ability to protect itself by turning off the appropriate immune mechanisms that otherwise get rid of the cancer. Furthermore, as we understand the intricacies of this butyrate transporter, that may give us the opportunity in the future to turn this transporter back on and eradicate the cancer.

These investigators are to be applauded for the sophistication of this work, and I anticipate that the results of your efforts are going to go directly to the bedside.

I have one question for you. Listening to your talk, inhibiting or allowing methylation to occur, expressing or not expressing this transporter, I get the impression we have got a light switch on the wall: we can either turn the transporter on or turn it off to protect against cancer. With that in mind, how do we explain the difference between a lifelong history of high fiber in the diet that seems to protect against cancer compared to a 50 year old that gets a big polyp taken out and then goes on four years of fiber, yet sees no benefit?

Gail Cresci, M.S., R.D. (Augusta, GA): Thank you, Dr. McClave, for your great comments, and that is a great question. We have actually done some further studies in the lab where we are looking at normal versus germ free mice, and we have looked for expression of the transporter there, and we see silencing of the transporter in the germ free mice. We are in the process of re colonizing those mice to see if the transporter expression returns, and early stage results have shown that it does return. So I am not sure if it takes a little bit more time than just a quick turn on and turn off switch.

We also know that tumor cells lose their polarity, and so if they lose their polarity, then these transformed cells in the colon don't have access to the luminal butyrate, and it may be that in that case, the dietary fiber in the lumen may not be effective and perhaps other substrates for the transporter, such as pyruvate, which would be more involved in the plasma, may be a better means to affect these tumors.

Thank you very much.

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Evolving Management of Colonoscopic Perforations

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Abstract
Background Perforations of the large bowel during diagnostic or therapeutic colonoscopy are a rare but significant complication. Their treatment has evolved over the last decade, but there are still no specific guidelines for their optimal management.
Materials and Methods Retrospective review of 105,786 consecutive colonoscopies performed in a 21-year period allowed assessment of the medical records in all patients treated at our institution for colonoscopic perforation.
Results Thirty-five patients suffered perforation (perforation rate 0.033%) during colonoscopy from January 1986 to October 2007 (14 men, 21 women; mean age 69.4 years). Twenty-four of the perforations occurred during diagnostic colonoscopy, whereas 11 during therapeutic colonoscopy. Twenty-three (66%) of the patients underwent operative treatment and 12 (34%) were managed nonoperatively. The average length of stay was 15.2 days, and there was one death (2.9% 30-day mortality rate) among the patients.
Conclusions Perforations from diagnostic colonoscopy usually are large enough to warrant surgical management, whereas perforations from therapeutic colonoscopy usually are small, leading to successful nonoperative treatment. Over the last decade, the surgical treatment of colonoscopic perforations has evolved, as there has been a trend that favors primary repair versus bowel resection with successful outcome. Careful observation and clinical care adherent to strict guidelines for patients treated nonoperatively is appropriate in order to minimize morbidity and mortality and identify early those who may benefit from operation. Each treatment, however, has to be individualized according to the patients' comorbidities and clinical status, as well as the specific conditions during the colonoscopy that lead to the perforation.

Keywords Colonoscopy · Colon perforation · Operative management · Nonoperative management

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Introduction

Since the introduction of flexible fiber-optic colonoscopy in 1969 at the Department of Surgery at Beth Israel Medical Center in New York City by Drs. Wolff and Shinya,[1] there have been numerous reports on the safety, cost-effectiveness, and low morbidity and mortality rates of diagnostic and therapeutic colonoscopy.[2] Perforation is a significant and well-recognized, although rare, complication of fiber-optic colonoscopy. Its frequency is estimated to be between 0.01% and 0.6% in the various published series.[3–6]

However, the management of colonoscopic perforations remains controversial, since there are no specific guidelines, and has evolved during the last decade. Most authors emphasize the need for operative treatment of these patients, but, more recently, there have been reports of suc-

Table 1 Indications for Colonoscopy by Colonoscopy Type

Diagnostic	Therapeutic
Tumor	Polypectomy
Low GI bleeding	Laser application
Change in bowel habits	Colonic decompression, e.g., volvulus
Inflammatory bowel disease	Coagulation for bleeding
Abdominal pain	A–V malformation
Anemia	
Obstruction	
Diverticulosis	
Routine examination (screening)	

cessful nonoperative management in selected patients.[7–10] The purpose of this study is to determine the incidence of perforation after colonoscopy of our institution, the clinical presentation and diagnosis workup of these patients, the evolving optimal management, and, finally, the impact of the mechanism of injury in the selection of the ideal treatment modality.

Materials and Methods

After approval by the Institutional Review Board of our hospital, a retrospective review was carried out of colonoscopic perforations of the large bowel during a 21-year period, in an attempt to identify their incidence, optimal management, and clinical outcome. The electronic database of the endoscopic suite of our medical center was analyzed to identify the patients that had "perforation" or "abdominal pain" as an acute or late complication after or during the colonoscopy. Medical records of the patients that suffered a colonoscopic perforation were reviewed for the following data: patient demographics, past medical and surgical history, type and indication for colonoscopy, clinical presentation after the suspected perforation, diagnostic laboratory and radiological studies, time between colonoscopy and diagnosis and treatment, type of management, intraoperative findings, final pathology, length of hospital stay, clinical course, and final outcome. The general indications for colonoscopy are given in Table 1, based on the type of colonoscopy either as diagnostic or therapeutic.

Results

Demographics and Endoscopy

From January 1986 to October 2007, 105,786 colonoscopies were performed in the endoscopy suite of our institution that resulted in 35 perforations, meaning a perforation rate of 0.033%. The study group consisted of 14 (40%) men and 21 (60%) women, with a mean age of 69.4 years (range 43–88 years). All colonoscopies were performed or supervised by either attending gastroenterologists or attending general surgeons. Mild analgesia and anesthesia, administered by attending anesthesiologists in most of the cases, were used for all the patients in order to achieve their comfort.

Of the 105,786 colonoscopies performed in the 21-year period, 68,082 (64%) were diagnostic and 37,704 (36%) were therapeutic. Twenty-four out of the 35 perforations (69%) occurred during diagnostic colonoscopy (0.035% perforation rate), whereas 11 perforations (31%) occurred during therapeutic colonoscopy (0.029% perforation rate). The average age of patients with perforation from diagnostic procedures was 72.6 versus 65.9 years in the therapeutic procedure group. The indications for diagnostic and therapeutic colonoscopy of the 35 patients that suffered perforation are described in Tables 2 and 3.

Presentation and Diagnosis

After colonoscopy, 33 (94%) patients developed abdominal pain, which was the most consistent symptom. The most frequent occurring sign was tachycardia (19 patients or 54%), followed by guarding and rebound tenderness, abdominal distention, leukocytosis, fever, hypotension, whereas only two (6%) patients remained asymptomatic (Table 4).

Seven patients (20%) were diagnosed at the time of colonoscopy, whereas the majority of patients were diagnosed within 12 h (25 patients or 71%). Two patients (6%) were diagnosed after 12 h but before 24 h, and one patient (3%) had delayed diagnosis after 24 h (Table 5). All seven patients in whom the perforation was seen during the colonoscopic procedure were immediately taken to the operating room without any radiologic studies. For the rest of the patients in whom the perforation was not directly seen during the endoscopy but was suspected based on the signs and symptoms, either an upright chest or abdominal radiograph was obtained looking for free intraperitoneal air. In addition to plain films, stable patients with no peritoneal

Table 2 Indication and Number of Perforations for Diagnostic Colonoscopies

Indication for colonoscopy	Number of perforations (%)
Routine examination (surveillance)	10 (42)
Low GI bleeding	8 (34)
Change in bowel habits	2 (8)
Inflammatory bowel disease	2 (8)
Abdominal pain	1 (4)
Tumor	1 (4)
Total	24 (100)

Table 3 Indication and Number of Perforations for Therapeutic Colonoscopies

Indication for colonoscopy	Number of perforations (%)
Polypectomy	9 (82)
A–V malformation	2 (8)
Total	11 (100)

signs on physical examination would also undergo either a gastrografin enema or computed tomography (CT) of the abdomen and pelvis with rectal water-soluble contrast in order to try to identify the exact location and extent of the perforation or other pathology, like formation of abscess or intra-abdominal fluid.

Regarding the site of the perforation, the majority (18 out of 35 or 51%) were found at the sigmoid and rectosigmoid, followed by the descending and transverse colon (four out of 35 or 11%), the cecum (three out of 35 or 9%), and the ascending colon (two out of 35 or 6%), whereas in two cases no perforation was found during the operation and in one case the site was unknown (Fig. 1). The location of the perforation did not vary with therapeutic versus diagnostic colonoscopies, with the sigmoid–rectosigmoid region being the predominant site for both types of colonoscopy.

Treatment

Twenty-three or 66% of the 35 patients that suffered perforation underwent operative treatment (21 after diagnostic colonoscopy vs. one after therapeutic colonoscopy), whereas 12 or 34% of the patients were managed nonoperatively (three after diagnostic colonoscopy vs. ten after therapeutic colonoscopy). In total, 88% (21 out of 24) of the patients that suffered perforation after diagnostic colonoscopy underwent exploratory laparotomy and the 12% (three out of 24) were managed nonoperatively. On the other hand, 9% (one out of 11) of the patients that suffered perforation after therapeutic colonoscopy underwent exploratory laparotomy and the 91% (ten out of 11) were managed nonoperatively. Table 6 shows the patients who received Hartmann's procedure or other kind of bowel resection versus those who underwent primary repair of the bowel wall defect with or without protective ostomy.

Nonoperative treatment consisted of placing the patients in a monitored bed, keeping them nil per os for bowel rest, with a nasogastric tube for drainage of gastric contents, and on broad-spectrum intravenous antibiotics for coverage against the colonic flora. Serial abdominal exams were performed in order to monitor for development of peritoneal signs. This was the case with one patient who initially was treated nonoperatively but 8 h later developed signs of peritoneal irritation and therefore was taken for operation. If during the course of conservative management the patient's condition changed to the worse, either a CT scan or a gastrografin enema would be obtained to evaluate possible further intra-abdominal pathology and guide further treatment.

Table 4 Clinical Presentation and Frequency

Symptoms and signs	Number of patients (%)
Abdominal pain	33 (94)
Tachycardia	19 (54)
Guarding and/or rebound tenderness	14 (40)
Abdominal distention	12 (34)
Leukocytosis	7 (20)
Perforation seen during colonoscopy	7 (20)
Fever (>38°C)	5 (14)
Hypotension	2 (6)
Asymptomatic	2 (6)

Table 5 Time Interval Between Perforation and Diagnosis

Time between perforation and diagnosis	Number of patients (%)
During colonoscopy	7 (20)
<12 h	25 (71)
12–24 h	2 (6)
>24 h	1 (3)
Total	35 (100)

Length of Stay and Final Clinical Outcome

The average length of hospitalization for all patients was 15.2 days (range 3–42 days). The patients that suffered a

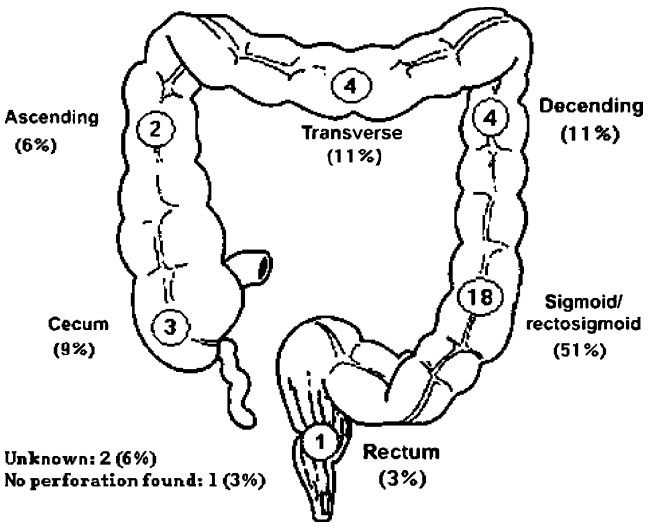

Figure 1 Site of perforation for all 35 cases.

Table 6 Type of Operative Treatment

Type of operation	Number of cases (%)
Hartmann's or other resection	15 (68)
Primary repair with or without protective ostomy	7 (32)
Total	22 (100)

perforation during diagnostic colonoscopy had an average length of stay of 17.1 days (range 4–42 days; 19.4 days in the operative group vs. 5.3 days in the nonoperative group), whereas the patients that suffered a perforation during therapeutic colonoscopy had an average length of stay of 6.1 days (range 3–12 days; 5 days in the operative group vs. 6.3 days in the nonoperative group; Table 7).

We had only one fatal outcome (one out of 35 patients, or 2.9% 30-day mortality; Table 8), which occurred in an 81-year-old male patient that underwent diagnostic colonoscopy for evaluation of low gastrointestinal bleeding. The patient had multiple comorbid conditions including severe coronary artery disease, aortic stenosis, and diabetes mellitus and expired in the surgical intensive care unit 2 days postoperatively due to extensive myocardial infarction.

Discussion

With technological advancements, colonoscopy has enjoyed a large number of broad diagnostic and therapeutic applications since its introduction at the Beth Israel Medical Center by Wolff and Shinya in June of 1969.[1] A main reason for the lack of guidelines for management of colonoscopic perforations is the presence of a large number of variables that need to be considered in order to make such guidelines practicable. Bowel preparation, diagnostic versus therapeutic colonoscopy, interventions performed, underlying disease process, clinical patient history, clinical status after the perforation, radiologic studies and laboratory data, and timing of recognition of the perforation are some of the variables that have to be taken into account when selecting the optimal treatment modality. The incidence of perforation in the high-volume centers is estimated between 0.01%

Table 7 Average Length of Stay Divided by Type of Colonoscopy

Type of treatment	Diagnostic colonoscopies, days	Therapeutic colonoscopies, days
Operative	19.4 (11–42)	5
Nonoperative	5.3 (4–7)	6.3 (3–12)
Average	17.1 (4–42)	6.1 (3–12)

Table 8 Final Clinical Outcome Divided by Type of Colonoscopy

	Type of treatment	Survived, *n* (%)	Died, *n* (%)	Total, *n* (%)
Diagnostic colonoscopies	Operative	20	1	21 (88)
	Nonoperative	3	0	3 (12)
	Total	23 (96)	1 (4)	24 (100)
Therapeutic colonoscopies	Operative	1	0	1 (82)
	Nonoperative	10	0	10 (8)
	Total	11 (100)	0 (0)	11 (100)

and 0.6% in the various reported case series.[3–6] The incidence of perforation from diagnostic colonoscopy ranges between 0.14% and 0.65%, while the same incidence from therapeutic colonoscopy ranges from 0.15% to 2.14%.[11,12] This wide variation in the incidence of perforation is best explained, most probably, by the expertise of the individual endoscopist and by how meticulously medical centers search for and report postcolonoscopy perforations.[2] Our overall perforation rate of 0.033% or one perforation per 3,030 colonoscopies is in accordance with the above range reported in the literature. The perforation rate in the current study regarding diagnostic and therapeutic colonoscopy was 0.035% and 0.029%, respectively, during a 21-year period with 105,786 consecutive procedures. However, it has to be mentioned that there may be patients with perforations who presented late in a different hospital and, thus, were missed in the follow-up and not included in the above rate calculations.

Various mechanisms may result in perforation of the large bowel during colonoscopy as illustrated in Fig. 2. Five main mechanisms have been identified. First, the perforation may result from direct mechanical penetration of the tip of the colonoscope in the bowel wall, especially when visualization is poor (Fig. 2a). Second, bowing of a loop of the scope may cause sufficient lateral pressure to perforate the colonic wall, making the perforation invisible from the tip of the instrument (Fig. 2b). Third, perforation may occur along a pathologic area of the colon, such as stricture, diverticulum, or tumor (Fig. 2c). Fourth, aggressive air insufflation may cause colon overdistention and rupture (Fig. 2d).[13] Fifth, perforation may occur during a snare polypectomy or with direct thermal injury to the bowel wall (Fig. 2e).

Because colonoscopic perforation is regarded as surgical emergency, most authors in the past believed that the appropriate treatment is operative, suggestive that possible failed conservative management will result in further intra-abdominal contamination and inflammation of the bowel wall, diminishing the chances for primary closure of the defect and increasing mortality.[2,14] However, the results of the present study show that management of such perforations should differ from traumatic injury of unprepped

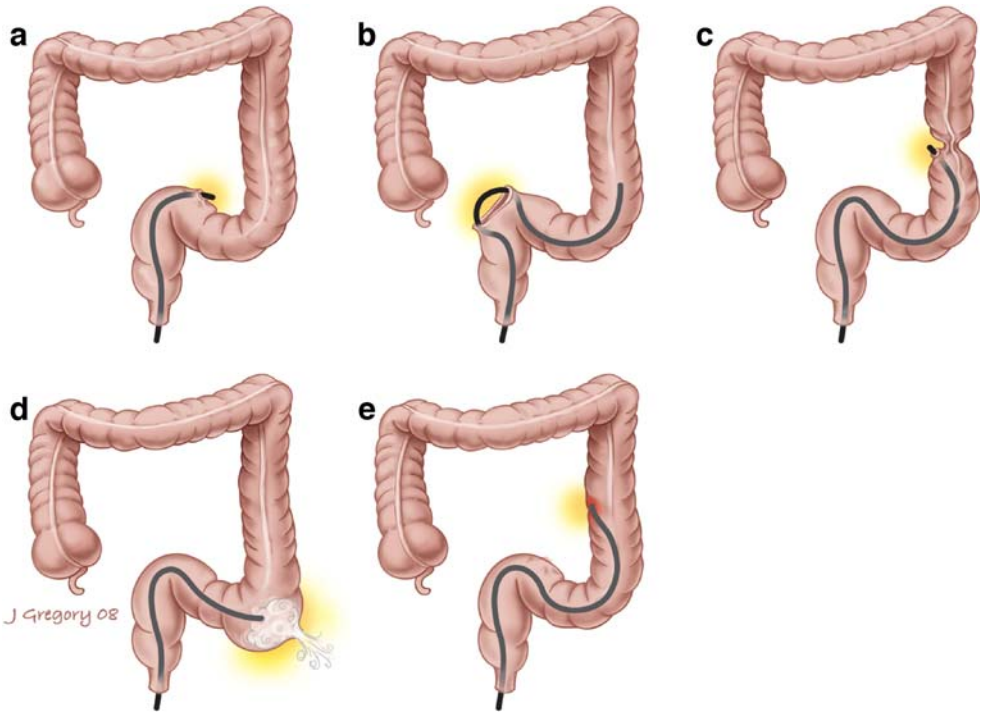

Figure 2 Mechanisms of perforation during colonoscopy. **a** Direct mechanical penetration of the tip of the colonoscope in the bowel wall. **b** Bowing of a loop of the scope may cause sufficient lateral pressure to perforate the colonic wall, making. **c** Perforation may occur along a pathologic area of the colon, such as stricture, diverticulum, or tumor. **d** Aggressive air insufflation may cause colon overdistention and rupture. **e** Perforation may occur during a snare polypectomy or with direct thermal injury to the bowel wall.

bowel, making the nonoperative approach in carefully selected patients a feasible approach with zero mortality. This is mainly first due to the fact that endoscopic perforations are routinely discovered early with prompt initiation of treatment and second due to the vigorous mechanical intestinal preparation before the colonoscopy which evacuates most of the fecal material and markedly decreases the intracolonic bacterial load.

As the current study shows, 88% of perforations during diagnostic colonoscopy required operative treatment. These perforations tend to be generally large enough in order not to seal by themselves. During the exploratory laparotomy, the abdominal cavity needs to be copiously irrigated and every attempt should be made to identify the exact site of the perforation, which in the majority of cases is the sigmoid colon. If there is no specific pathology and extensive wall inflammation at the site of the perforation, which is usually the case with patients that are diagnosed in the first 12 h after the colonoscopy, then a primary repair of the defect may be performed with or without creation of protective ostomy. If, however, the segment of the perforated bowel contains tumor, stricture, or a large injury with very inflamed wall, then colon resection should be the selected surgical option. Our data show that, over the last decade, surgeons at our institution have been favoring primary repair versus colon resection for the surgical treatment of colonoscopic perforations with excellent results (Fig. 3), a trend which has also been observed in trauma surgery.[15] We are also aware of successful methods of either laparoscopic or endoscopic repair in selected patients with colonoscopic perforations,[16] but none of our patients received such a treatment.

Ninety-one percent of the patients that suffered perforation during therapeutic colonoscopy were successfully treated nonoperatively. The main reason behind this is the fact that therapeutic endoscopic perforation is the result of an entirely different mechanism. During polypectomy, electrical current is applied to the base of the polyp. Prolonged application of this current may cause coagulation into the muscularis mucosa, resulting in a transmural burn and perforation. Also, if the pedicle of the polyp is long and the polyp touches the adjacent colonic wall, the transmitted current may cause the perforation in the wall opposite the

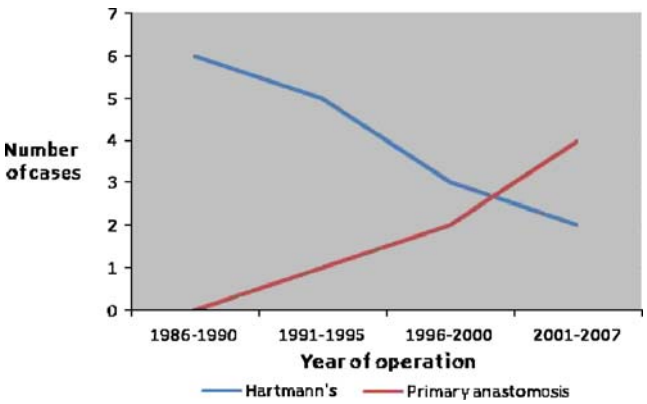

Figure 3 Change in surgical practice. Most colonoscopic perforations are treated with primary repair than with bowel resection over the last decade.

polyp.[2] In general, such perforations are quite small. The sudden opening in the distended colon will allow rapid egress of intracolonic air into the peritoneal cavity, resulting in pneumoperitoneum. Fortunately, these perforations seem to be rapidly sealed by the pericolonic fat or omentum leading to minimal contamination of the peritoneal cavity, warranting successful nonoperative management. The final outcome of such conservative management depends on careful clinical observation. However, clinical signs of peritonitis or deterioration of the patient should make the surgeon consider immediate operation since it indicates that the perforation has not sealed and there is ongoing contamination of the abdominal cavity with intracolonic material. Iqbal et al. agree that the presence of pneumoperitoneum alone is not an indication for operative management. In their study from the Mayo clinic, they reported on 72 patients with colonoscopic perforations, ten of which were treated nonoperatively, with low morbidity and length of stay. The authors also believe that timing of diagnosis is of great essence since patients diagnosed more than 24 h following colonoscopy have higher rate of fecal contamination.[17]

A management algorithm of colonoscopic perforations is suggested in Fig. 4, based on our experience. According to the illustration, if during the colonoscopy the scope is identified to have penetrated the bowel wall, the patient should be immediately taken to the operating room since the defect would be large enough (at least as wide as the diameter of the scope tip) to seal primarily. If, however, the perforation is suspected during or after the colonoscopy, based on the patient's symptoms, prompt physical exam will guide further actions. If the patient's vital signs are unstable or the physical exam reveals peritoneal signs, again, the best logical next step is operation. If the patient is stable with no signs of peritonitis, diagnostic radiologic workup should begin with an upright chest or abdominal film. Lack of free air in the X-ray warrants nonoperative treatment. The same conservative management should also be followed for the cases where the patient develops post-polypectomy syndrome, which can behave as frank perforation. On the other hand, presence of pneumoperitoneum should lead to further investigational studies, with either gastrografin enema or CT scan of the abdomen and pelvis with rectal water-soluble contrast. Studies that are negative for free fluid in the abdominal cavity, contrast extravasation, or clear perforation with extensive pericolonic inflammation warrant nonoperative management, whereas studies positive for the above findings should lead the surgeon towards operative treatment. Again, if during the course of conservative management the patient's clinical image worsens, consideration should be made for surgical treatment. The abovementioned algorithm, however, is only indicative and suggestive of the treatment that can be followed in a patient with colonoscopic perforation based on the clinical exam and radiologic findings. It has to be stated clearly that each case needs to be managed individually, taking into account the comorbidities of the patient and the exact interventions and mechanisms during the colonoscopy that lead to the perforation.

Conclusion

Perforation remains a serious complication of colonoscopy leading to significant morbidity if not diagnosed early. Perforations from diagnostic colonoscopy usually are large enough to warrant surgical management, whereas perforations from therapeutic colonoscopy usually are small,

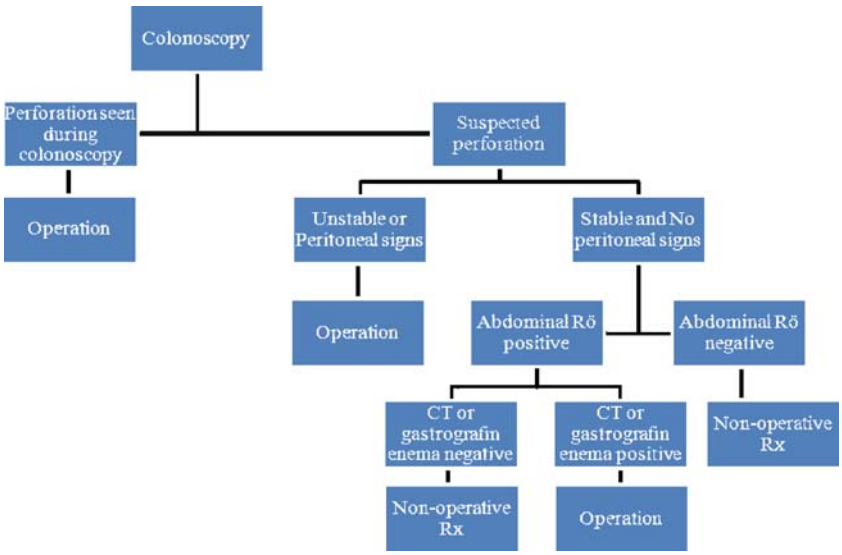

Figure 4 Suggested management algorithm for colonoscopic perforations.

leading to successful nonoperative treatment. Over the last decade, the surgical treatment of colonoscopic perforations has evolved, as there has been a trend that favors primary repair versus bowel resection with successful outcome. Careful observation and clinical care adherent to strict guidelines for patients treated nonoperatively is appropriate in order to minimize morbidity and mortality and identify early those who may benefit from operation.

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The Prognostic Superiority of Log Odds of Positive Lymph Nodes in Stage III Colon Cancer

Jiping Wang · James M. Hassett · Merril T. Dayton · Mahmoud N. Kulaylat

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Abstract
Background Literature showed that lymph node ratio (LNR) and total number of lymph nodes (TNODS) are independent prognostic factors in node-positive colon cancer. Our study assesses the prognostic superiority of the log odds of positive lymph nodes (LODDS) in the same patient population.
Material and Methods A total of 24,477 stage III colon cancer cases from the SEER registry were reviewed. Patients were categorized based on LNR into LNR1 to LNR4, according to cutoff points 0.07, 0.25, and 0.50, and based on LODDS into LODDS1 to LODDS5, according to cutoff points −2.2, −1.1, 0, and 1.1. The relative risk (RR), and 95% confidence interval (CI) were evaluated using the method of Kaplan–Meier and Cox model.
Results Patients with LNR4 could be classified into LODDS4 (61.4%) and LODDS5 (38.4%). The survival in these two groups was significantly different (5-year survival, 33.5% vs. 23.3%, $p<0.0001$). Univariate analysis showed that the higher LNR (RR=3.45, 95% CI=3.26–3.66) or low TNODS (RR=0.99, 95% CI=0.986–0.99) was significantly associated with poor survival. However, after adjusting for LODDS status, the association did not appear to be significant (LNR, RR=0.90, 95% CI=0.65–1.24, $p=0.52$; TNODS, RR=1.001, 95% CI=0.997–1.005, $p=0.54$).
Conclusion Colon cancer patients with LNR4 disease represent a heterogeneous group. The previously reported prognostic association of TNODS and LNR and outcome of stage III disease were confounded by LODDS.

Keywords Log odds · Lymph node ratio · Staging · Colon cancer

Introduction

The relationship between the number lymph nodes of involved with cancer, total number of lymph nodes (TNODS) examined, and the outcome of various malignancies is the subject of several studies.[1–10] Recent studies have also established the prognostic significance of TNODS harvested and lymph node ratio (LNR) defined as the quotient of the number of positive lymph nodes and TNODS.[11–20] Log odds of positive lymph nodes (LODDS), defined as the log of the quotient of the number of positive lymph nodes and the number of negative lymph nodes, has been introduced as a new prognostic factor in breast cancer prognostic research. Although the unique statistical features of LODDS are well documented, some studies have failed to show any significant advantage over LNR.[14,21] Johnson et al.[22] recently showed that "increasing the negative lymph node count is independently associated with improved long-term survival in patients with stage IIIB and IIIC colon cancer". This

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finding suggests that the LODDS (a function of the number of positive and negative lymph nodes) may have a potential prognostic role in patients with stage III colon cancer. We used the Surveillance, Epidemiology, and End Results (SEER) cancer registry database to evaluate the significance of LODDS in the prognosis of stage III colon cancer.

Material and Methods

Study Design

This is a retrospective, exploratory study based on SEER, a population-based registry sponsored by the National Cancer Institute that collects information on cancer incidence and survival from 12 population-based cancer registries including approximately 14% of the US population. Of the 12 registries, two were added in 1992. The information available for our analysis includes patient's age at diagnosis, gender, race, marriage status, location of the residence, primary tumor site, pathological grade, size, extension and metastasis, TNODS evaluated, number of tumor positive lymph nodes (TNM information available since 1988), and follow-up for vital status. Each tumor stage is coded as described by the AJCC Sixth Edition according to the TNM stage organization. The TNM stage is determined by the extent of primary tumor (T stage), the status and of regional lymph nodes (N stage), and the presence of distant metastasis (M stage). The numbers of positive lymph nodes are categorized into 1–3 (N1) and >4 (N2). Tumor grade is also categorized as low-grade (well or moderately differentiated) and high-grade (poorly differentiated, anaplastic, or undifferentiated).

Patient selection was described in detail elsewhere.[20] Excluded from the study are patients with in situ, stage I, II, or metastatic disease and those missing information on TNODS examined and patients with prior malignancies, malignancies other than adenocarcinoma, cancer NOS, or in appendix, rectum, rectosigmoid junction, anus, anal canal, and anorectum. Patients who had not undergone radical surgical resection and those who had received preoperative radiation were also excluded. Because this study used a preexisting publicly accessible database with no personal identifiers, exemption was obtained from the Institutional Review Board of the University at Buffalo.

Statistical Analysis

Patients' demographic characteristics were reported elsewhere.[20] LNR was defined as the quotient between the number of positive lymph nodes, i.e., involved with cancer, and TNODS harvested. Patients were grouped into four groups, LNR1 to LNR4, based on LNR value: $LNR<0.07$, $0.07\leq LNR<0.25$, $0.25\leq LNR<0.50$, and $0.50\leq LNR\leq 1.0$. The logit of the positive lymph nodes, i.e., the LODDS, was defined as the log of the ratio between the probability of being a positive lymph nodes and the probability of being a negative lymph nodes when one lymph node is harvested. It was estimated by: $\log\frac{(pnod+0.5)}{(tnod-pnod+0.5)}$, where the pnod is the number of positive lymph nodes and tnod is the total number of lymph nodes harvested, and 0.5 is added to both numerator and denomination to avoid singularity. Patients were sub-classified into five groups, LODDS 1 to LODDS5, according to the value of the ratio: LODDS1 ($LODDS<-2.2$); LODDS2 ($-2.2\leq LODDS<-1.1$); LODDS3 ($-1.1\leq LODDS<0$); LODDS4 ($0\leq LODDS<1.1$), and LODDS5 ($LODDS\geq 1.1$). The prognostic effects of LNR and LODDS were evaluated by using logrank test. Kaplan–Meier's curve was used to compare the performances of LNR, LODDS, and AJCC stage; univariate Cox proportional hazard model was used to identify potential prognostic factors such as patients' age at diagnosis, tumor size, tumor grade, ethnic group, number of positive lymph nodes, number of negative lymph nodes, TNODS harvested, LNR, and LODDS.[23,24] The multivariate Cox proportional model was used to evaluate the independent prognostic effect of the significant variables identified from the univariate Cox model. The LNR and LODDS were utilized as continuous variables in the Cox proportional hazard model. Data were analyzed using SAS version 9e (2002–2003, SAS Institute, Cary, NC, USA), and all statistical tests were two-sided with alpha equals to 0.05.

Results

The number of patients with stage III colon cancer identified was 24,477. Details of patient and tumor characteristics were reported elsewhere.[20] Based on LNR value, 2,860 patients (11.7%) were classified into LNR1, 9,729 patients (39.8%) into LNR2, 6,085 patients (24.8%) into LN3, and 5,830 patients into LNR4 (23.8%). The observed 5-year survival for patients with LNR1, LNR2, LNR3, and LNR4 groups is 64.8%, 56.2%, 45.1%, and 29.6%, respectively. The survival difference among these groups was statistically significant ($p<0.0001$; Table 1). Based on LODDS value, 3,089 patients (12.6%) were classified into LODDS1, 9,500 patients (38.8%) into LODDS2, 6,058 patients (24.8%) into LODDS3, 3,578 patients (14.6%) into LODDS4, and 2,252 patients (9.2%) into LODDS5. The observed 5-year survival for patient in LODDS1, LODDS2, LODDS3, LODDS4, and LODDS5 groups was 64.8%, 56.0%, 45.1%, 33.5%, and 23.3%, respectively. The survival differences were statistically significant ($p<0.0001$; Table 1).

Table 1 The Agreement Between LNR and LODDS

Name (5-year Survival)	LNR1 (64.8%)	LNR2 (56.2%)	LNR3 (45.1%)	LNR4 (29.6%)	Total (%)
LODDS1 (64.8%)	2,860	229	0	0	3,089 (12.6)
LODDS2 (56.0%)	0	9,500	0	0	9,500 (38.8)
LODDS3 (45.1%)	0	0	6,058	0	6,058 (24.8)
LODDS4 (33.5%)	0	0	0	3,578	3,578 (14.6)
LODDS5 (23.3%)	0	0	0	2,252	2,252 (9.2)
Total (%)	2,860 (11.7)	9,729 (39.8)	6,058 (24.8)	5,830 (23.8)	24,477 (100)

Our analysis showed good agreement between LNR and LODDS (Pearson correlation=0.97, $p<0.0001$; Table 1). However, based on LODDS, patients with LNR4 represent a heterogeneous group where 61.4% of the patients were classified as LODDS4 and 38.6% as LODDS5. The 5-year survival between these two groups was significantly different ($p<0.0001$).

Figure 1a shows the relationship between LNR and LODDS. When patients have a different LNR, LODDS has a one-to-one mapped value to each LNR, and the relative ranks between these two variables are the same, i.e., they both contain the same prognostic information. However, when patients have the same LNR value, LODDS has a smaller value as the TNODS increases for patients with LNR<0.5. In other words, when patients have the same small LNR value (LNR<0.5), LODDS penalizes retrieval of fewer lymph nodes. On the other hand, when patients have LNR value>0.5, LODDS has a larger value, as TNODS increases for patients with the same LNR value. Figure 1b shows the magnified result in Fig. 1a with LNR between 0.75 and 1.

The univariate Cox proportional hazards model identified nine variables that are significantly associated with survival: age at diagnosis, race, tumor size and grade, TNODS examined, number of positive and negative lymph nodes, LNR, and LODDS (Table 2). Both LNR and LODDS were included in the Cox model as continuous variables to avoid the potential influence of the empirical selection of cutoff points. For each additional lymph node removed, patients showed a 1% decreased risk of death (RR=0.99, 95% CI=0.986–0.990, $p<0.0001$). The number of negative lymph nodes had a similar behavior (RR=0.97, 95% CI=0.969–0.974, $p<0.0001$). LNR was also significantly associated with patient survival (RR=3.45, 95% CI=3.26–3.66, $p<0.0001$).

Multivariate Cox proportional model was used to evaluate the independence of these prognostic effects after controlling possible confounders. Since the TNODS examined and the number of negative lymph nodes are highly correlated, only one of them was included in the multivariate Cox proportional model. Model 1, which includes all the significant variables with the exception of LODDS, shows that all of the seven variables remain significantly associated with patient survival (Table 3). However, when LODDS was included in the model (model 2), the TNODS examined (RR=1.001, 95% CI=0.997–1.005, $p=0.54$) and LNR (RR=0.90, 95% CI=0.65–1.24) were no longer significantly associated with patient survival. A similar finding was observed when the

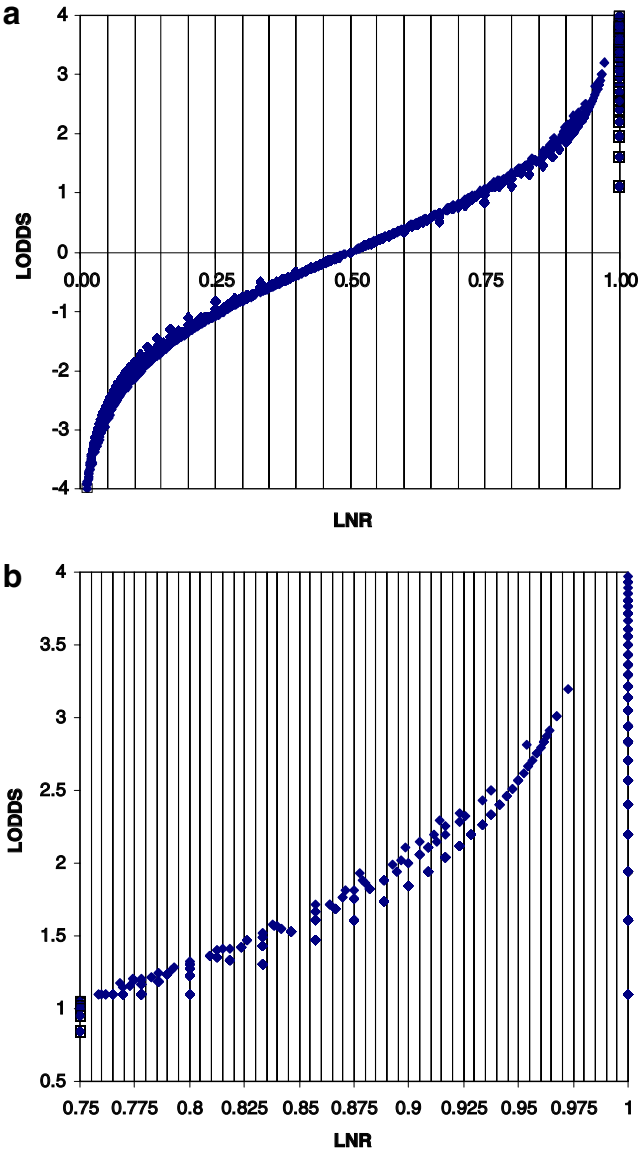

Figure 1 **a** The distribution of LNR and LODDS. **b** The magnified view of 1a for LNR between 0.75 and 1.0.

Table 2 Univariate Cox Proportional Model

Variable	*P* value	RR	95% CI
Age	<0.0001	1.04	(1.036, 1.039)
LNR	<0.0001	3.45	(3.255, 3.660)
LODDS	<0.0001	1.32	(1.307, 1.341)
Number of Negative lymph nodes	<0.0001	0.97	(0.969, 0.974)
Number of positive lymph nodes (PNOD)	<0.0001	1.07	(1.067, 1.074)
Total Number of lymph nodes (TNODS)	<0.0001	0.99	(0.986, 0.990)
Grade	<0.0001	1.40	(1.344, 1.451)
Tumor Size	<0.0001	1.003	(1.002, 1.003)
Race	<0.0001	1.13	(1.082, 1.170)

number of negative lymph nodes instead of TNODS was included in model 2 (data not shown).

Patients with stage IIIA colon cancer accounted for 8.9% (n=2,175) of all patients, stage IIIB for 59.8% (n=14,644), and stage IIIC for 31.3% (n=7,658). The 5-year survival for patients with stages IIIA, IIIB, and IIIC disease was 71.3%, 51.7%, and 34.0%, respectively, with $p<0.0001$ (Fig. 2). The LODDS status was used to test the homogeneity of these patients within various stages. The observed 5-year survival of patients with stage IIIA disease with LODDS1 to LODDS5 was 74.1%, 72.5%, 70.4%, 67.4%, and 61.2%, respectively (p=0.11; Fig. 3b). On the other hand, the 5-year survival of patients with stage IIIB disease with LODDS1 to LODDS5 was 63.7%, 54.4%, 44.4%, 35.8%, and 30.6%, respectively ($p<0.0001$; Fig. 3c). The observed 5-year survival for patients with LODDS2 to LODDS5 in stage IIIC was 49.7%, 41.7%, 29.8%, and 18.8%, respectively ($p<0.0001$; Fig. 3d). In other words, the majority of the stages IIIB and IIIC patients was either over or under staged according to the current AJCC TNM staging algorithm. The 5-year survival was not estimated for LODDS1 group since there were only 23 patients with stage IIIC classified as LODDS1.

Discussion

For years, the involvement of regional lymph nodes with cancer in malignant disease has been considered one of the most important prognostic factors. Other information pertaining to these regional lymph nodes such as the TNODS removed and the total number of negative lymph nodes identified has become the focus of several studies only in recent years.[1–10,22] This led to the development and adoption of new prognostic indices that incorporate all the lymph nodes information in a single identifiable parameter. Among the indices that have proven important and promising are the LNR and LODDS. The superiority of LNR as a prognostic method in various malignancies, including colon cancer, compared to the number of positive lymph nodes and TNODS retrieved alone has been confirmed in several studies.[11–20] There is however little information on the advantages of LODDS. Given its unique statistical features, LODDS has the potential to be a superior prognostic index.

In an analysis of the prognostic factors related to lymph nodes in 83, 686 breast cancer patients extracted from the SEER database, Vinh-Hung et al.[14] concluded that "the estimated LODDS provides results very similar to those with LNR". Yildirm et al.[21] reached a similar conclusion based on their analysis of 704 node positive breast cancer patients. To our knowledge, our study is the first study to show that LODDS is a better prognostic factor than LNR. Data depicted in Table 1 show that 61.4% of the patients in LNR4 are classified as LODDS4 and 38.6% as LODDS5, and the corresponding 5-year survival is 33.5% and 23.5%, respectively. In other words, about 40% of the patients in LNR4 are over-staged [5-year survival 29.6% (LNR4) vs.

Table 3 Multivariate Cox Proportional Hazard Model

Variables	Model 1				Model 2			
	P value	RR	95% CI		P value	RR	95% CI	
Age	<0.0001	1.04	1.037	1.040	<0.0001	1.04	1.037	1.040
Grade	<0.0001	1.17	1.120	1.216	<0.0001	1.16	1.116	1.212
Tumor size	<0.0001	1.003	1.002	1.003	<0.0001	1.003	1.002	1.003
PNOD	<0.0001	1.06	1.048	1.062	<0.0001	1.04	1.028	1.047
LNR	<0.0001	2.30	2.086	2.548	0.52	0.90	0.651	1.240
Race	<0.0001	1.24	1.190	1.301	<0.0001	1.24	1.188	1.299
TNODS	<0.0001	0.993	0.990	0.997	0.54	1.001	0.997	1.005
LODDS	–	–	–	–	<0.0001	1.28	1.185	1.393

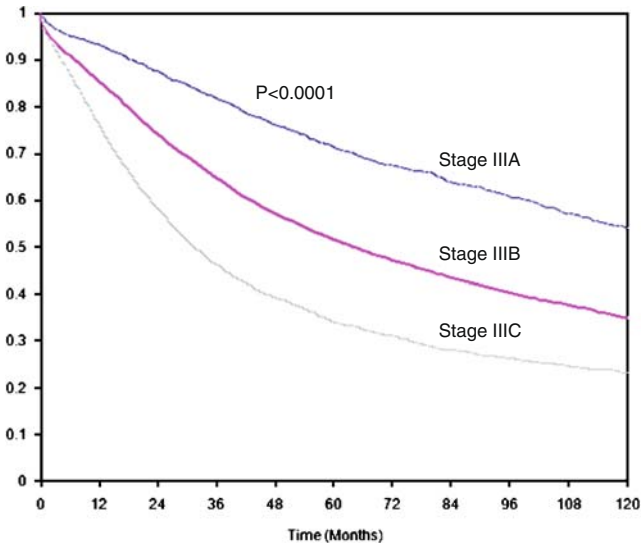

Figure 2 The survival curve for the node positive colon cancer patients stratified by AJCC stage.

23.3% (LODDS5)]. In addition, data reported in Table 3 further support the notion that LODDS is superior to LNR, TNODS, and number of negative lymph nodes. When LODDS are included in the multivariate Cox proportional hazard model (model 2), TNODS examined and LNR are no longer significant. Similarly, when LODDS and LNR are included in the multivariate Cox proportional hazard model along with number of negative lymph nodes, the number of negative lymph nodes and LNR are no longer significant (data not shown).

There are several reasons that make LODDS a superior prognostic factor to LNR, TNODS, and negative lymph nodes. Log of odds of positive lymph nodes is a function of the number of negative lymph nodes, whereas LNR is a function of total number of lymph nodes. In addition, with LNR, there is the assumption that patients with the same LNR have the same prognosis regardless of the TNODS harvested. This poses an interesting question: Does patient A

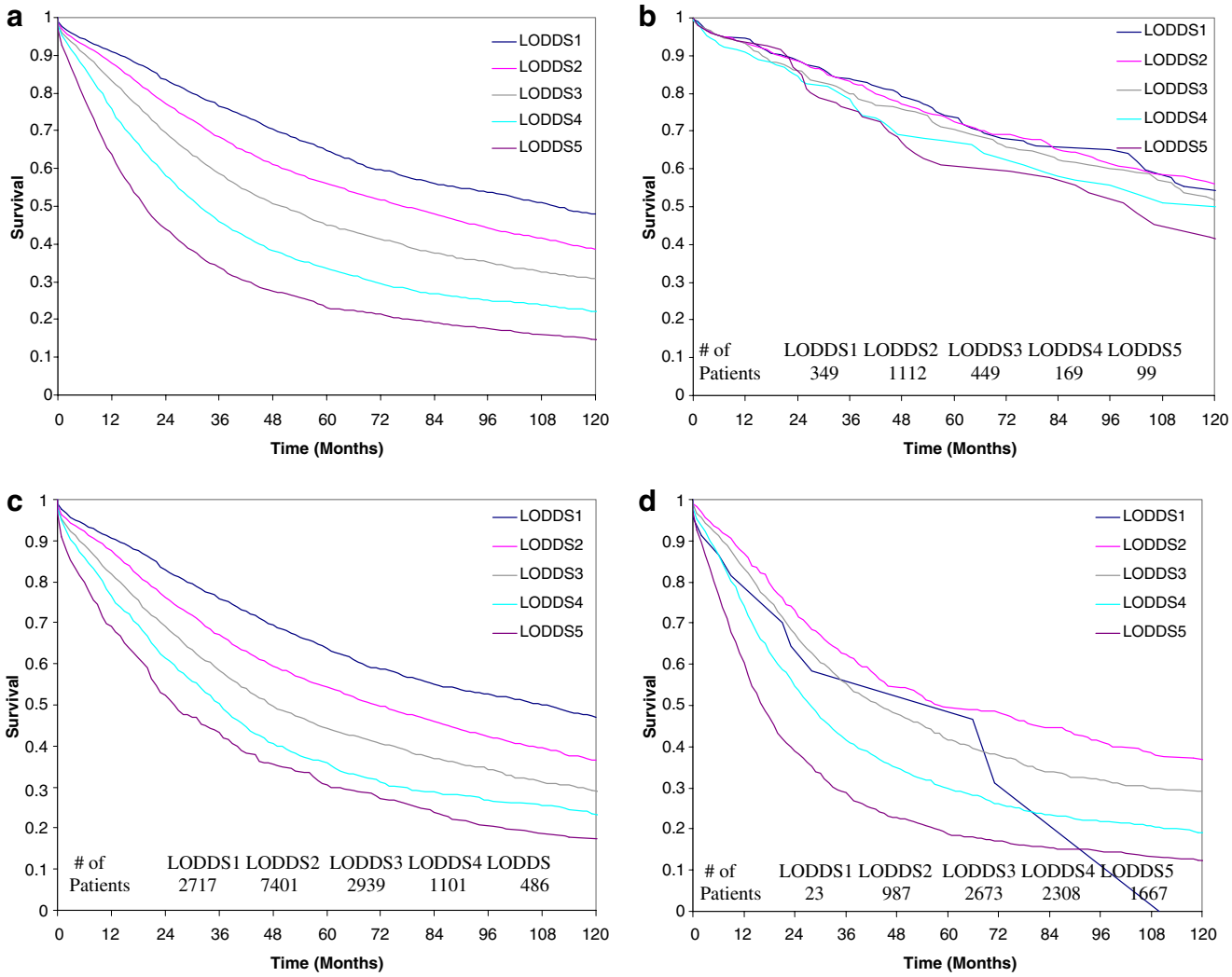

Figure 3 **a** The survival for stage III patients stratified by LODDS; **b** the survival for stage IIIA stratify by LODDS; **c** The survival for stage IIIB patient stratify by LODDS; **d** The survival for stage IIIC patient stratified by LODDS.

with four positive lymph nodes out of four lymph nodes harvested have the same prognosis as patient B with 20 positive lymph nodes out of 20 lymph nodes harvested? Intuitively, patient A has a better prognosis than patient B. Lymph node ratio however does not provide a good answer since the LNR for both patients is equal to 1. LODDS, on the other hand, can easily make the distinction between the prognosis of the two patients. Figure 1a shows that LODDS separates patients with the same LNR. When patients have a different LNR, LODDS has the same relative rank mapping to LNR. Whereas when patients have the same LNR, LODDS absorbed the information provided by TNODS.

Although this study is based on the SEER database and the results are limited by the nature of the data, our findings are congruent with those presented in current literature. More importantly, our results add more credence to the data already available in the literature that current AJCC stage III colon cancer patients represent a heterogeneous group[20] and that neither total number of lymph nodes harvested nor number of negative lymph nodes is an independent prognostic factor. The 5-year survival difference between LODDS1 and LODDS5 for patients with stage IIIB disease was 33%. For patients with stage IIIC disease, the 5-year survival difference between LODDS2 and LODDS 5 was 31%. Those differences were almost as large as the 5-year survival difference between stages IIIA and IIIC patients. This makes the current AJCC stage III an unacceptable staging tool in term of prognosis accuracy.

In summary, rapidly accumulating evidence shows the superiority of LNR over the AJCC N stage in node positive colon cancer.[20,25–28] This study further confirms our previous observation that current stage III colon cancer patients determined by AJCC TNM staging system represent a heterogeneous group.[20] Our results pertaining to LODDS are also consistent with those reported in the breast cancer research, i.e., LODDS have a better prognostic effect than N stage.[29] Therefore, it is scientifically sound and practical to consider ratio-based prognostic factors such as LNR or LODDS to be part of the staging system. Incorporating LNR or LODDS into the staging system of colon cancer will enable clinicians to more accurately assess the prognosis of patients. It also serves as a common platform when comparing inter-institutional and international treatment results. Used as a finer stratification tool for clinical trial design, it may help to find more specific chemotherapy for homogenous group patients. Unfortunately, since SEER data does not have treatment information, we are not able to evaluate the treatment plan for different groups of patients. In addition, external validation by using other large database for evaluating the prognostic effect of LODDS must be taken prior to the recommendation for its practical usage.

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Long-Term Results of Transanal Excision After Neoadjuvant Chemoradiation for T2 and T3 Adenocarcinomas of the Rectum

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Abstract
Introduction Traditionally, selected early distal rectal cancers have been considered for treatment by transanal excision (TAE) with acceptable oncologic results. With the frequent use of neoadjuvant chemoradiation (NCR) for the treatment of locally advanced rectal cancer, there is growing interest in the application of TAE for such lesions. We report our experience of TAE for T2 and T3 rectal cancers following NCR.
Material and Methods Between July 1994 and August 2006, 44 patients were identified as having undergone full-thickness TAE of pretreatment ultrasound-staged T2 and T3 rectal cancers that were treated with NCR. Fifteen patients were deemed medically unfit for radical resection, and 29 would have required abdominoperineal resection but were opposed to colostomy.
Results Our patient population consisted of 26 men and 18 women, with a median age of 69 (range, 43–89) and a median follow up of 64 months (6–153). Thirty-one patients had a clinical complete response (cCR) to NCR of which 19 (61%) had a pathologic CR (pCR). Seven (16%) of 44 patients sustained disease recurrence of which two were local only, two local and systemic, and three systemic only. Only four (9%) patients had died of disease at current follow up. Overall 5-year survival rates for T2/T3N0 and T2/T3N1 patients were 84% and 81%, respectively. Five patients underwent radical resection immediately following TAE for either positive margins or residual cancer. There was minimal morbidity with no perioperative mortality associated with TAE.
Conclusions TAE of T2 and T3 rectal cancers following NCR is a safe alternative to radical resection in a highly select group of patients for which recurrence and survival rates comparable to radical resection can be achieved. This study supports ongoing efforts to assess this approach in prospective, multi-center trials.

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Keywords Rectal cancer · Local excision · Neoadjuvant chemoradiation

Introduction

The treatment of early rectal cancer (T1) by local excision is generally considered an acceptable option. Despite several retrospective reports voicing concern of increased local recurrence rates,[1–8] a large number of studies including a multi-institutional prospective trial have reported good oncologic and quality of life outcomes.[9,10] However, the results of local excision for more advanced stages of rectal cancer such as T2 and T3 lesions, even with the use of adjuvant therapy, have been disappointing.[3,5–8]

Encouraged by a number of studies, most notably, the German Rectal Cancer Trial, a trend has developed toward the preferred use of neoadjuvant chemoradiation (NCR) for the treatment of locally advanced rectal cancers.[11–15] With a substantial number of rectal cancer patients experiencing significant responses to neoadjuvant treatment, there has been renewed interest in the application of local excision for such lesions. The few studies that address this approach, including a previous report from our own institution,[16] have been promising but have been limited by relatively small numbers and short follow-up intervals. We have sought to update our experience and evaluate long-term outcomes for patients with T2 and T3 rectal cancers treated by local, transanal excision following NCR.

Material and Methods

Patients

Institutional review board approval was obtained for this retrospective study from the H. Lee Moffitt Cancer Center and Research Institute Scientific Review Committee and University of South Florida Institutional Review Board. From July 1, 1994 to August 1, 2006, a total of 44 patients who underwent local excision for rectal adenocarcinoma by a transanal approach following NCR were identified. TAE was only offered to patients who either refused recommended radical surgery (most often secondary to the requirement of a colostomy; $n=29$) or who were deemed medically unfit to undergo radical resection by their treating surgeons ($n=15$).

Data Collection

Comprehensive chart reviews were performed for clinicopathologic information, and follow-up data were obtained from medical records and the Moffitt Cancer Center Tumor Registry. Collected clinicopathologic data included patient demographics, preoperative symptomatology, Charlson Index comorbidities,[17] risk factor history, family history, tumor stage, histopathologic variables, and recurrence data.

Pretreatment Evaluation

Following diagnostic confirmation of rectal cancer, patients were evaluated by endoscopic ultrasound (EUS) and computed tomography scan of the abdomen and pelvis. At our institution, NCR is routinely offered to patients with locally advanced (≥T3 and/or node positive) cancers at the time of diagnosis or to those who have very distal T2N0 cancers close to or involving the sphincter.

Neoadjuvant Regimen

Delivery of preoperative chemotherapy and radiation was performed in a standardized fashion. All patients received 4,500 cGy in 25 fractions to the pelvis utilizing a three-field technique (two lateral and one posterior). The top of the radiation field consisted of the midpoint of the sacroiliac joint, and the bottom of the field was marked at least 4 cm below the tumor. An additional 540 cGy boost was focused at the primary tumor site. Radiation was delivered concomitantly with 5-fluoruracil (5-FU), which was utilized as a radiosensitizing agent. The 5-FU was administered as a continuous infusion at a dose of 300 mg m^{-2} day^{-1}, 5 days/week on days of radiation. Therapy was delivered over the course of 5 weeks.

Post-neoadjuvant Chemoradiation Evaluation

Approximately 3–6 weeks following the completion of NCR, repeat examination was performed. Proctoscopy and digital rectal exam were supplemented by biopsy of any residual mass at the surgeon's discretion. Sixteen patients underwent repeat EUS. There were no patients that showed clinical evidence of tumor progression after NCR. Patients were classified as follows:

Clinical partial response (cPR): Patients found to have a residual mass post-NCR or significant rectal wall abnormality and, if biopsied, revealed the presence of residual cancer

Clinical complete response (cCR): Patients who had either no palpable/visualized mass or evidence of a scar at the site of the pre-NCR mass and, if biopsied, no pathologic evidence of cancer

Operative Technique

All patients in this study underwent local excision of their tumors by a standard transanal approach. Full-thickness excision was performed with the goal of achieving 1-cm gross circumferential margins around the tumor or residual scar. Specimens were oriented and secured on a rigid platform with a series of pins. Intraoperative frozen section assessment of peripheral and deep margins was routinely performed. The remaining defect in the rectal wall was then reapproximated in a single layer using absorbable suture material.

Pathologic Evaluation

Resection specimens were evaluated by a dedicated gastrointestinal pathologist. After gross inspection, samples were submitted in entirety for microscopic examination.

Hematoxylin and eosin staining was performed using standard techniques. Assessments of margin status were made when residual cancer was present. Classification of pathologic response was as follows:

Pathologic partial response (pPR): Tumors that displayed any evidence of residual cancer cells in the resection specimen

Pathologic complete response (pCR): No evidence of residual cancer in the resection specimen

Statistical Analysis

Comparisons of clinicopathologic data were performed using the chi-squared or Fisher's exact tests, as appropriate. Mean differences were examined for continuous data using analysis of variance. Survival endpoints considered in this study were (1) overall survival, defined as time from surgery to death from any cause, and (2) disease-free survival (DFS), defined as time from surgery to either rectal cancer recurrence or death with evidence of rectal cancer. Kaplan–Meier methods with the log-rank test were used to calculate the overall survival rate or DFS and differences in the survival curves.[18] All statistical tests performed were two-sided and declared at the 5% significance level. Statistical analyses were performed with Intercooled Stata (Stata Statistical Software, Release 9.0; Stata, College Station, TX, USA).

Results

Patient Demographics and Clinicopathologic Data

Our study population consisted of 26 men and 18 women with a median age of 69 years (range, 43–89) and a median follow up of 64 months (range, 6–153 months). The most common symptom at presentation was hematochezia, which was seen in 31 (70%) of the patients followed by constipation in eight patients (18%) and diarrhea in 7 patients (16%). Eleven (25%) of patients were asymptomatic at the time of diagnosis. Mean distance of the tumor was 5.2 cm from the anal verge with a mean pre-treatment diameter of 3.3 cm. The majority of tumors were classified as uT3N0 and were frequently moderately differentiated (Table 1).

Table 1 Clinicopathologic Data

Characteristics	Values
Number of patients	44
Pretreatment tumor diameter	
Mean (cm)	3.3±0.98 cm
Distance from anal verge	
Mean (cm)	5.2±2.1 cm
Differentiation	
Well	4 (9%)
Moderate	35 (80%)
Poor	2 (4%)
Not specified	3 (7%)
Pretreatment stage	
T2N0	10 (23%)
T3N0	22 (50%)
T2/3N1	11 (25%)
Unknown	1 (2%)
Clinical response	
Complete	31 (70%)
Partial	13 (30%)
Pathologic response	
Complete	25 (57%)
Partial	19 (43%)

Surgical Morbidity and Mortality

Eleven percent of patients experienced a treatment-related complication: Three patients (7%) had prolonged transient incontinence with resolution of their symptoms by 2, 6, and 12 months from surgery. One patient had a breakdown of their suture line and developed anorectal stenosis requiring dilatation. An additional patient sustained an in-hospital cerebrovascular accident but had no residual long-term sequelae. There were no perioperative deaths.

Response to Treatment

Response data are summarized in Fig. 1. Thirty-one (70%) patients had cCR, and 13 (30%) were noted to have had an incomplete response by clinical criteria (cPR). Of the 31 patients with cCR, 19 (61%) had a corresponding pCR, and 12 (39%) proved to have had only a pPR.

Outcomes

Outcome data are also summarized in Fig. 1. Of the 19 patients with cCR and pCR, 17 (89%) remained disease-free, one (5%) patient developed a local recurrence at 27 months, and one (5%) patient developed a systemic recurrence at 13 months. Of the 12 cCR patients that were subsequently found to have had a pPR, five underwent immediate radical surgery. Of these, three remained disease-free, one developed a local recurrence at 35 months, and one developed systemic recurrence at 48 months. Of the seven (cCR/pPR patients) that did not undergo immediate radical excision, five remained free of recurrence during follow up. One patient developed both local and systemic recurrence, and another suffered a

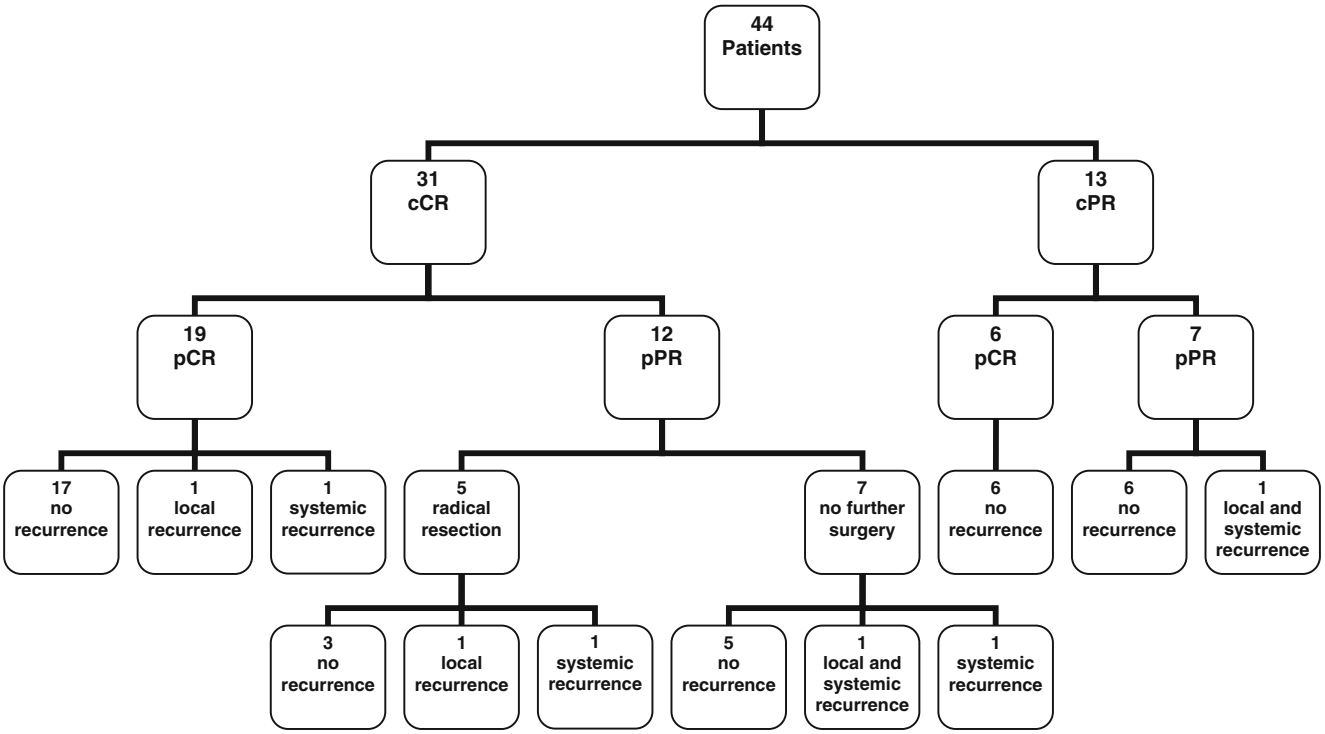

Figure 1 Flowchart of clinical and pathologic responses and corresponding outcomes.

systemic-only recurrence. Of the 13 patients with a cPR, the six patients with pCR remained free of recurrence. Of the seven patients with cPR/pPR, six were free from recurrence while one patient presented with both local and systemic recurrence at 12 months. Details of all recurrent cases are summarized in Table 2. Three- and 5-year overall survival rates were both 81% (Fig. 2). Comparisons were made to evaluate for differences in overall and DFS by stage (Fig. 3). Though DFS was lower at both 3 and 5 years in patients with node-negative disease compared to those with node-positive disease (92% vs. 79% and 88% vs. 79%, respectively), this did not reach statistical significance (p=0.6).

Discussion

Local excision is generally accepted as an option for the treatment of T1 adenocarcinomas of the rectum with favorable morphologic and histologic features and is associated with low rates of recurrence and surgical morbidity.[2,19–22] The use of local excision for more

Table 2 Summary of Recurrences

Pre-treatment stage	Clinical response to NCR	Pathologic response to NCR	Additional radical surgery performed	Type of recurrence	Time interval to recurrence (months)	Treatment of recurrence	Disease status
T2N0	cCR	pPR	APR	Local	35	Chemotherapy	DOD, 64 months
T2NO	cCR	pPR	LAR	Lung	48	Surgery, Chemotherapy	NED, 65 months
T3N0	cCR	pPR	No	Local and systemic	Unknown	Chemotherapy	DOD, 64 months
T3NO	cCR	pPR	LAR	Liver followed by Lung and Liver	62 and 87	Surgery, Chemotherapy	AWD, 102 months
T3NO	cCR	pCR	N/A	Liver	13	Chemotherapy	DOD, 36 months
T2N1	pCR	pPR	No	Local and Left groin followed by Liver, Lung and Bladder	12 and 22	Surgery, Chemotherapy	DOD, 29 months
T3N1	cCR	pCR	N/A	Local	27	Surgery	NED, 34 months

DOD Died of disease, *NED* No evidence of disease, *AWD* Alive with disease

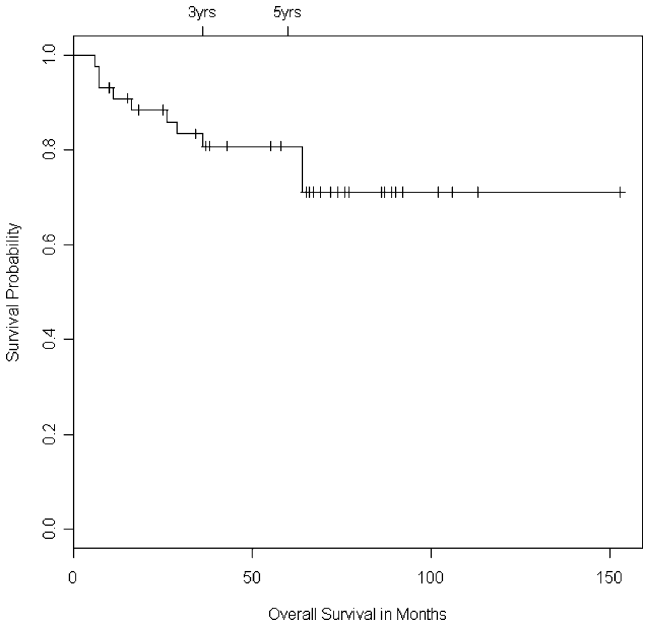

Figure 2 Kaplan–Meier analysis of overall survival for entire study population. Three- and 5-year overall survival rates were both 81%.

advanced lesions (T2 and T3) has been reported to have unacceptably high rates of recurrence (17–62%) even with the use of adjuvant chemoradiation strategies.[3,5–8] As such, the enthusiasm for local excision for T2 and T3 lesions has waned dramatically. However, with the increasing acceptance of NCR as the preferred approach for locally advanced rectal cancers, there is a renewed interest in the potential application of local excision for select circumstances. The neoadjuvant approach for locally advanced rectal cancer has been strongly supported by the results of the German Rectal Cancer Trial, which demonstrated a reduction in local recurrence, lower rates of short-term and chronic toxicities, and a higher rate of sphincter preservation.[11]

Associated with this increased use of neoadjuvant treatment is the observation that a complete pathologic response may be achieved in up to 30% of patients.[23] Radical surgery is still considered the standard of care for such patients but is associated with significant morbidity, including infection, anastomotic leak, need for ostomy, and genitourinary complications.[24–31] Consequently, the question has been raised as to whether radical surgery can be avoided in patients with a significant response to NCR. Habr-Gama et al.[32] have taken the very novel and controversial approach of observing patients with a cCR following the use of NCR for rectal cancer. They developed a treatment strategy based on the clinical response to NCR as follows: (1) patients with cCR diagnosed after NCR were offered surveillance and (2) patients with cPR were treated by radical resection. Among 265 patients undergoing NCR for resectable distal rectal cancer,[32] 71 patients with cCR underwent surveillance while 194 patients with a cPR underwent radical resection. Of these 194 patients, 22 had no residual disease (pathologic T0N0M0) and served as the comparison group for the patients with cCR. With a mean follow-up of 57 months in the observation group and 48 months in the resection group, 5-year overall survival was 100% vs. 88%, respectively, and 5-year DFS was 92% vs. 83%. With additional follow-up (mean, 59.9 months), there was an increase in the absolute number of recurrences in the observation group (13 vs. 5), leading to a reduction in 5-year overall survival to 93% and 5 year DFS to 85%.[33]

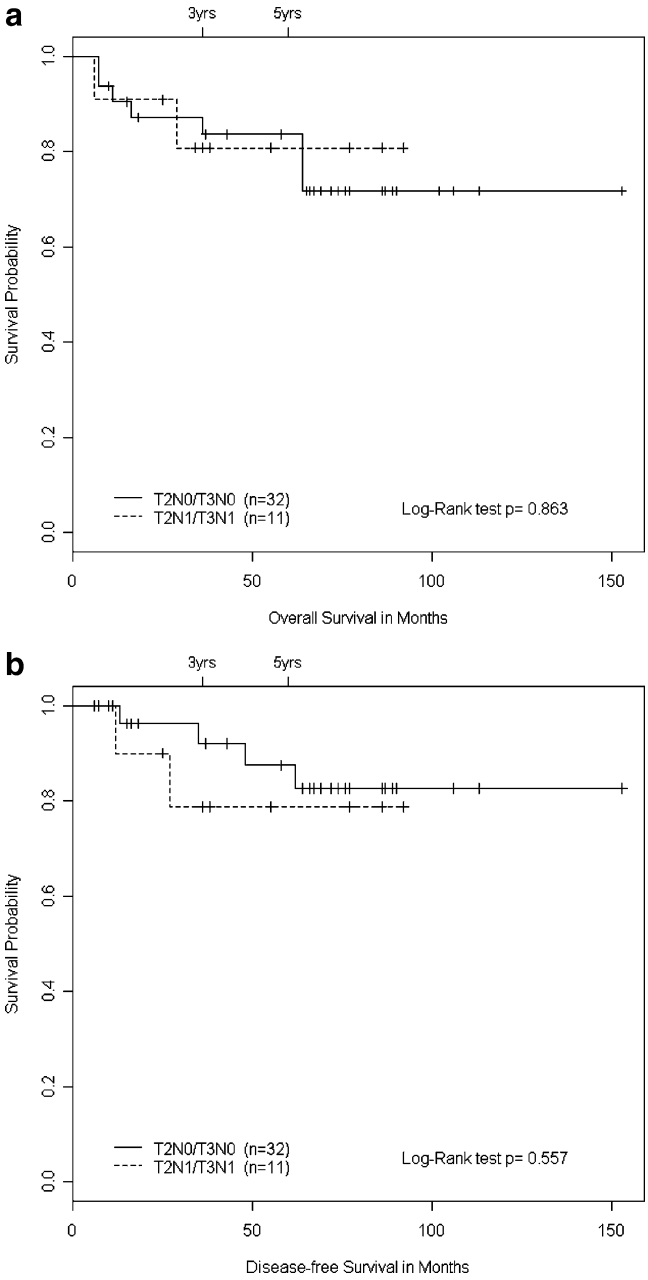

Figure 3 **a** Kaplan–Meier analysis of overall survival between patients with node-positive and node-negative disease. **b** Kaplan–Meier analysis of disease-free survival between patients with node-positive and node-negative disease.

Table 3 Incidence of Residual Mesorectal Disease in Patients with ypT0 Following NCR and Radical Resection

Author	Institution	Year	pT0 (*n*)	NODE+	Percent
Onaitis[44]	Duke	2001	34	4	12
Medich[43]	West Penn	2001	5	0	0
Hiotis[42]	MSKCC	2002	27	4	15
Habr-Gama[32]	Sao Paulo	2004	22	0	0
Stipa[46]	MSKCC	2004	31	2	7
Bedrosian[41]	MD Anderson	2004	22	2	9
Pucciarelli[45]	Padova	2005	56	1	2
Tulchinsky[40]	Tel Aviv	2006	17	1	6
		Totals	214	14	7

One of the significant concerns related to an observation-based approach is that a cCR is highly inaccurate in predicting a pCR.

Guillem et al.[34] have reported that digital rectal exam underestimated response in 78% of patients undergoing NCR for rectal cancer. Furthermore, digital rectal examination correctly identified only three of 14 pCRs. Similarly, radiologic imaging such as transrectal ultrasound, magnetic resonance imaging and positron emission tomography scanning have not been reliable in predicting response.[3,32,35–39] Consequently, observation alone following NCR for rectal cancer likely should not be routinely advocated.

Reluctance to adopt local excision for more advanced rectal cancer following NCR is primarily related to the concern of inadequate treatment of the locoregional lymph nodes. However, Tulchinsky et al.[40] have shown that, of patients with a pathologic T0 response at the primary tumor, only 6% had positive lymph nodes. As such, they have suggested that a T0 response may serve as a surrogate marker for mesorectal lymph node response. A review of eight studies in the recent literature (Table 3) demonstrates rates of lymph node positivity ranging from 0 to 15% in the setting of a T0 response to NCR.[32,40–46] These findings lend support to the notion that a select group of such patients may be adequately treated by local excision.

There are only a few studies in the literature that address the issue of local excision for rectal cancer following NCR. However, these have been limited by relatively low numbers of patients and short follow-up. These studies, which contain heterogeneous groups of T2, T3, and T4 tumors, have reported local and distant recurrence rates ranging from 0 to 12.5% and 0 to 20%, respectively.[47–51] With a median follow-up of greater than 5 years, we report comparably favorable outcomes. In our study of 44 patients, we have demonstrated 5-year DFS rates for stages II and III cancers of 88% and 79%, respectively. Similarly, 5-year overall survival rates for stages II and III disease were 83% and 81%, respectively. Both recurrence-free and overall survival rates compare favorably with results achieved by radical surgery.[24,52,53] Local and distant recurrence rates for our population were 9% and 11%, respectively.

Lezoche et al.[54] have recently reported the results of a prospective randomized study comparing transanal endoscopic microsurgery (TEM) with laparoscopic radical resection with total mesorectal excision after NCR for pretreatment T2N0 low or moderate grade rectal cancers. Thirty-five patients were randomized to each group, and there was no difference in response to NCR with 49% being downstaged in both groups. With a median follow-up of 84 months, overall survival was identical for the two groups (94%). The rate of local recurrence was 5.7% in the TEM group and 2.8% in the group treated with radical surgery, while the rates of distant recurrence were the same (2.8%). Results of this and previous retrospective studies evaluating local excision following NCR for locally advanced rectal cancer are summarized in Table 4.

A recent review of the aforementioned literature by Borschitz et al.[55] concluded that local excision for selected T2 and T3 rectal cancers treated by NCR was an acceptable option. They determined that the strongest prognostic

Table 4 Analyses of Local Excision Following NCR for Locally Advanced Rectal Cancer

Author	Institution	Year	T Stage	*N*	Median F/U (months)	Local recurrence	Percent	Distant recurrence	Percent
Schell[51]	U. of Florida	2002	T3	11	48	0	0	1	9
Ruo[50]	MSKCC	2002	T2/T3	10	28	1	10	2	20
Bonnen[48]	MDA	2004	T3	26	46	2	8	3	12
Caricato[49]	Rome	2006	T2/T3/T4	8	37	1	12.5	0	0
Lezoche[54]	U. of Rome	2008	T2	35	84	1	3	1	3
Nair	Moffitt	2008	T2/T3	44	64	4	9	5	11

factors were complete pathologic response (ypT0) or residual tumor isolated only to the submucosa (ypT1). No patients with a pCR (ypT0) developed local recurrence, while the rates in ypT1, ypT2, and ypT3 patients were 0–6%, 6–20%, and up to 42%, respectively. In our current study, one (5%) of the 19 patients with pCR did go on to suffer a local recurrence.

High rates of recurrence and resultant poor outcomes have been demonstrated in patients with only a pPR.[48,50] Five (26%) of our observed recurrences (seven total recurrences) occurred in the 19 patients with pPR. Our standard recommendation in the setting of pPR is to proceed with radical resection with the reasoning that a similar incomplete response may exist in the regional lymph nodes. Of the 19 pPR patients, five had immediate radical surgery with two subsequent recurrences. Of the remaining 14 patients electing not to undergo radical resection, only three patients developed recurrent disease. Although the completeness of pathologic response appears to represent the current best surrogate marker for locoregional and systemic response, there appear to be additional, yet understood biologic factors that may contribute to this determination.

Despite relatively long-term follow-up, one of the limitations of our study is the possibility of recurrences occurring beyond 5 years, a scenario previously noted following local excision, particularly in the setting of chemoradiation. In a long-term follow-up of patients with rectal cancer treated with local excision, Paty et al.[56] have reported recurrences occurring close to 10 years and beyond. A follow-up study of CALGB 8984 also has shown a number of recurrences beyond 5 years.[9] Accurate determination of the efficacy of local excision for rectal cancer following NCR will likely require additional long-term surveillance.

The retrospective nature of our study introduces inherent biases in patient selection and follow-up. However, our study, coupled with the other aforementioned studies support prospective strategies such as the current American College of Surgeons Oncology Group (ACOSOG) Z6041 study, which is a multi-institutional cooperative group trial that is assessing the use of local excision for T2N0 rectal cancers treated initially with NCR. We would anticipate that, with results from prospective trials, future recommendations for locally advanced rectal cancer will include local excision as part of the therapeutic algorithm. It is also essential that future studies incorporate quantitative and comprehensive assessments of critical patient measures such as anorectal function and quality of life.

In conclusion, local excision for T2 and T3 rectal cancer following NCR in highly selected patients is associated with minimal morbidity and outcomes comparable to radical resection. Although radical resection remains the standard of care, our data suggest that in patients with a complete or near-complete response to NCR, local excision may be considered. Such an approach may be particularly reasonable for those individuals who are poor surgical candidates due to significant co-morbidities. Patients subsequently noted to have a pCR may be most likely to experience a favorable outcome. The completion of large-scale prospective studies is still required to provide patient selection guidelines and validate the efficacy of this approach.

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Alessandro Fichera, M.D. (Chicago, IL): The group from Moffitt really needs to be congratulated for a very provoking study, a timely study as well. They have reviewed a large series of patients with T2/T3 rectal cancer that underwent transanal excision after combined modality therapy at their institution in the neoadjuvant setting. As I said, this is a very timely study, since this is a topic of prospective, randomized evaluations at this point.

To take a little bit of a step back and give you a little bit of an historical background, the Memorial Sloan-Kettering and the University of Minnesota group have shown that the recurrence rate after local excision alone for T2 rectal cancer could be as high as 47%, obviously not an acceptable rate. In the CALGB 8984 trial, chemoradiation therapy was utilized in the adjuvant setting after local excision for patients found to have a T2/T3 lesion with a significant improvement in local recurrence rate. Now, with the results of the German rectal cancer trial that was presented during the talk, it is clear that neoadjuvant chemoradiation therapy offers significant advantages and should be considered the treatment of choice for locally advanced rectal cancer. The authors have applied this approach to T2 and T3 lesions in their series. The study I thought was very well presented, and their pathologic complete response rate of 25 out of 44 patients is quite impressive.

There are some limitations to the study. Obviously, this is a retrospective evaluation, and there is obviously a selection bias inherent to the group of 29 patients that were opposed to a colostomy. Furthermore, during the 20 years of the study, our ability to stage rectal cancers and to deliver radiation therapy has changed dramatically. Notwithstanding these limitations, their results are very impressive. I would like to ask the authors a few questions.

Although the numbers are small, it appears that disease-free survival is somewhat reduced, 79% versus 88% at 5 years and 92% at 3 years, in the groups with positive lymph nodes. Should a local excision be offered to these patients at all, accepting a failure rate of 21%? On the same line, we do know that the risk of local recurrence is delayed by radiation therapy. The Brazilian study has also shown a slight increase in the number of recurrences as the follow-up continues. Even though your disease-free survival changes only slightly between 3 and 5 years, what are you expecting to see 5 years from now, and how are you going to follow these patients up? In view of the results of the study, what options are you offering to a patient that presents to your clinic with a T3 N0 rectal lesion at the dentate line at this point?

I truly enjoyed your presentation that was kindly provided to me ahead of schedule. Thank you also for the opportunity to discuss the paper.

Rajesh Nair, M.D. (Tampa, FL):Dr. Fichera, thank you very much for your review of our study and your critical appraisal. I will try and answer the questions in order.

In terms of patients with node-positive disease, the setup of the current prospective trial with ACOSOG includes patients with early stage, T2 node-negative cancers, and we agree, ideally, that this approach should be limited to node-negative patients. As we and others have demonstrated, the rates of recurrence with node-positive disease are too high. I think attempts at local excision can be made in patients, again, who are completely opposed to radical surgery and/or colostomy or are medically unfit for surgery. However, they need to understand that they will be accepting a higher rate of recurrence utilizing this approach.

In terms of long-term follow-up, the data from the Memorial group has clearly shown that local recurrences can occur more than 5 years beyond the initial time of treatment. Therefore, we will need to change our approach in follow-up, especially if this approach becomes utilized, extending from 5 to 10 years and maybe longer.

And in terms of a patient who presents to us with a T3 N0 cancer, our approach, again, would be to offer them neo-adjuvant chemoradiation and then make a decision based upon the clinical response. In a patient who has a complete clinical response, our recommendation would still be to tell them that the standard of care is to undergo full radical surgical resection. However, if they are, again, opposed to colostomy and/or radical surgery or are medically unfit, we can offer this procedure, again, with the understanding that there may be an increased rate of local recurrence with this technique.

Bruce A. Orkin, M.D. (Washington, DC): I enjoyed your presentation very much. This is actually a very critical area that we are trying to evaluate ourselves. I have probably done over 200 of these cases, about a third of them for malignancies. Our experience has been that those patients who have had preoperative radiation and chemotherapy have a much, much higher complication rate. We primarily use transanal endoscopic microsurgery, and we have seen a lot of failures in terms of the wounds falling apart. I actually had a discussion about that with one of our colleagues yesterday.

Are you seeing such an increase in complications? Are you using TEM for any of these cases now? If so, are you prospectively evaluating it?

Dr. Nair: In terms of TEM, none of the patients in this particular study underwent TEM. However, within the last 12 months, almost all of the patients who have undergone local excision have been treated using TEM. In terms of complications, we have not noticed a significant change in our overall morbidity rate. In this current cohort, there was one patient who had a disruption of their suture line and subsequently required dilatation for an anorectal stenosis.

In a group of patients treated too recently to have been included in this study, we have seen two patients with rectal drainage secondary to minor suture line dehiscence. In both of these cases, symptoms were self-limited and completely resolved.

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Laparoscopy-Assisted Distal Gastrectomy for Gastric Cancer

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Abstract
Objective The purpose of this study was to evaluate the safety and value of laparoscopy-assisted distal gastrectomy (LADG) for early stage gastric cancer (stages IA, IB, and II).
Materials and Methods We retrospectively assessed 101 cases treated by LADG and compared to 49 contemporaneous cases treated by open distal gastrectomy (DG) between 2001 and 2006. Clinical variables, such as tumor diameter, operation time, blood loss, number of lymph nodes dissected, and length of stay were investigated.
Results Tumor size (mm) was significantly smaller in the LADG group ($p < 0.0001$). Although operation time (min) in the two groups was similar (278 ± 57 vs. 268 ± 55), mean blood loss was significantly higher in the DG group (139 ± 181 vs. 460 ± 301, $p < 0.0001$). Fewer lymph nodes were harvested in the LADG group (27 ± 14 vs. 34 ± 19, $p = 0.012$). Hospital stay was longer in the DG group (13.3 ± 8.5 vs. 16.7 ± 10.5, $p = 0.034$). There was no mortality in either group. Postoperative surgical complications occurred in six (6%) of the LADG and four (8%) of the DG.
Conclusions The authors conclude that laparoscopy-assisted distal gastrectomy is a safe and useful operation for early-stage gastric cancers. If patients are selected properly, laparoscopy-assisted distal gastrectomy can be a curative and minimally invasive treatment for gastric cancer.

Keywords Gastric cancer · Laparoscopic surgery · Gastrectomy · Lymphadenectomy

Although the incidence of gastric cancer is decreasing worldwide, it remains one of the most common causes of cancer deaths in Japan.[1] The rate of detection of early gastric cancer in Japan has increased more than 50% as a result of periodic gastric endoscopy and that has resulted in an improvement in the survival rate of gastric cancer patients.[2] Since early gastric cancer is associated with a low recurrence rate and a long survival time after surgical treatment, attention should be directed to patients' quality of life after surgery. Minimally invasive therapy has been implemented since 1990s as a means of the management of patients with early gastric cancer.[3] After the first successful case of laparoscopy-assisted distal gastrectomy (LADG) was reported in 1994,[4] LADG became widely adopted as a means of treating early gastric cancer. Since the field of view of the surgical site is limited and it was still difficult to systematically dissect regional lymph nodes, no standardized procedure has been established.[5] Flexible laparoscopes were developed for gastrointestinal endoscopy to expand the field of vision by allowing overhead or side-arm laparoscopy procedures and may facilitate standardization of the LADG procedures.[6] The purpose of this study was to clarify the safety and value of laparoscopic surgery for gastric cancer.

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Materials and Methods

We retrospectively reviewed 101 cases of LADG and compared them to 49 contemporaneous cases treated by open distal gastrectomy (DG), all treated at the Nippon Medical School Hospital between February 2001 and October 2006. Laparoscopic surgery was selected based on the results of a preoperative assessment of depth of wall invasion by endoscopy, barium radiology, and endoscopic ultrasonography. In accordance with the Japanese guideline for gastric cancer therapy, laparoscopic surgery for gastric cancer was indicated when the clinical diagnosis was tumor-limited to the mucosa or submucosa layer.[7]

Every LADG procedure was performed by the method as outlined below.[8] The patient was positioned on the operating table in the reverse Trendelenburg position. After creating a CO_2 pneumoperitoneum of 8 mmHg by the open technique, a flexible laparoscope (Olympus, Tokyo, Japan) was introduced through the infraumbilical port, and four other ports were placed in the upper abdomen. The gastrocolic ligament was dissected with laparoscopic coagulating shears (Sonosurg, Olympus, Tokyo). The left gastroepiploic vessels were exposed below the spleen and cut with a bipolar vessel sealing system (Ligasure, Tyco, Norwalk, CT, USA) or surgical clips to dissect the lymph nodes station (number 4sb). The right gastroepiploic vessels were divided in order to dissect the subpyloric lymph nodes station (number 6). The duodenum was transected 0.5 cm distal to the pylorus with an endoscopic stapling device. The suprapyloric lymph nodes station (number 5) was dissected after dividing the right gastric artery. The common hepatic artery was exposed toward the trunk of the splenic artery. The left gastric vein was identified and cut near the left gastric artery to dissect additional lymph nodes station (number 8a). The left gastric artery was divided, and the lymph nodes stations around the celiac artery (number 7 and 9) were dissected. The left cardiac and superior gastric lymph nodes stations (number 1 and 3) were dissected. The stomach was transected intracorporeally with a linear stapler, and the en-bloc resection of the stomach and lymph nodes (D1 + β) was completed. The stomach and perigastric lymph nodes were removed through a mini-laparotomy (4–7 cm) and placed on the upper abdomen. Reconstruction was performed either by the Billroth I method with a double-stapling device (29 mm in diameter), Roux-en Y anastomosis with a linear stapler, or Billroth II and Braun anastomosis by the hand-sewn method.

Data were collected from medical charts, operation records, and pathology reports. The following variables were evaluated: age, sex, co-morbidity, tumor size, location, gross type, histological type, depth of wall invasion, and presence or absence of lymph node metastasis. The clinical variables evaluated were operation time, blood loss, postoperative complications, and length of postoperative stay. Complications were classified as intraoperative or postoperative. All data are expressed as means ± standard deviation. Categorical variables were analyzed by the χ^2 test, and differences in continuous variables were analyzed by Student's t test. A P value of less than 0.05 was considered indicative of statistical significance. All statistical analyses were performed with the StatView software package (version 5.0, SAS Institute, Cary, NC, USA).

Results

The clinical characteristics of the patients are shown in Table 1. Half of the patients had concurrent disease, including cardiovascular disease (n = 23) and diabetes mellitus (n = 19). The mean body mass index (BMI) values of both groups were similar. The tumors were located in the middle third and/or lower third of the stomach. Macroscopic examination revealed that all of the tumors in the LADG group were superficial, flat tumors with or without minimal elevation or depression (type 0) and 75 tumors of them were superficial depressed type (type 0–IIc; Table 2). Tumor size was significantly smaller in the LADG group than in the DG group.

The surgical data of the patients are shown in Table 3. Although operation times were similar, mean blood loss was significantly less in the LADG group than in the DG group. The extent of lymph node dissection in the LADG was the perigastric lymph nodes (D1) in three cases, systemic perigastric lymph nodes (D1 + α) in 28 cases, additional lymph node dissection along the common hepatic artery (D1 + β) in 67 cases, and extended lymph node dissection (D2) in three cases. The extent of lymph node dissection in the DG group was mainly D2 in 34 cases

Table 1 Clinical Data of the Patients who Treated by Gastrectomy

Factor	LADG (n=101)	DG (n=49)	p value
Age (years)	63.1±11.5	65.2±10.8	n.s.
Male/female	63/28	34/15	n.s.
Height (cm)	159±8	160±9	n.s.
Weight (kg)	56.8±9.6	57.1±10.8	n.s.
BMI (kg/m^2)	22.3±3.1	22.2±3.6	n.s.
Co-morbidity	57 (56%)	27 (55%)	n.s.
Cardiovascular disease	23	11	
Respiratory insufficiency	1	2	
Liver cirrhosis	4	3	
Renal failure	4	2	
Cerebral infarction	8	3	
Diabetes mellitus	19	9	
Rheumatic arthritis	3	1	

Table 2 Macroscopic Findings in the Cases Treated by Gastrectomy

Factor	LADG (n=101)	DG (n=49)	p value
Tumor size (mm)	22.0±13.9	33.1±18.6	<0.001
Macroscopic findings			<0.001
Type 0			
0-I (Protruded type)	9	1	
0-IIa (Superficial elevated type)	7	7	
0-IIb (Flat type)	7	0	
0-IIc (Superficial depressed type)	75	13	
0-III (Ecavated type)	3	0	
Type 2	0	10	
Type 3	0	16	
Type 5	0	2	
Histologic type			n.s.
Well differentiated	73	37	
Poorly differentiated	28	12	

(69%). The number of lymph nodes dissected in the LADG group was significantly smaller than in the DG group.

The most common reconstruction method was the Billroth I method, which was performed in half of the cases in the both groups. The next most common method of reconstruction was the Roux-en Y method in the LADG group (37%) and the Billroth II method in the DG group (38%). There were 12 simultaneous operations in the LADG group, consisting of nine cholecystectomies, two inguinal hernias, and one colectomy, and there were eight simultenous operations in the DG group, consisting of six cholecystectomies and two colectomies. Intraoperative complications occurred in three cases: bleeding from the mid-colic vein (940 ml) in one case, bleeding from the left gastric artery (1040 ml) in one case, and a duodenal stump injury in one case. In the duodenal stump injury case, the procedure was converted to laparotomy in order to repair the duodenal stump.

Table 3 Surgical Data of Patients Treated by Gastrectomy

Factor	LADG (n=101)	DG (n=49)	p value
Operation time (min)	278±57	268±55	n.s.
Mean blood loss (ml)	139±181	460±301	<0.001
Lymph nodes dissected (n)	27±14	34±19	0.012
Lymph node dissection			<0.001
D1	3	1	
D1+α	28	6	
D1+β	67	8	
D2	3	34	
Reconstruction			0.001
Billroth I	44	25	
Billroth II	20	19	
Rou-en Y	37	5	
Simultaneous surgical procedure	12 (12%)	8 (16%)	n.s.
Cholecystectomy	9	6	
Colectomy	1	2	
Inguinal hernia	2	0	

Table 4 Pathological Findings in the Cases Treated by Gastrectomy

Factor	LADG (n=101)	DG (n=49)	p value
Histologic type			n.s.
Well differentiated	73	37	
Poorly differentiated	28	12	
Depth of wall invasion			<0.001
T1 (mucosa)	48	5	
T1 (submucosa)	44	18	
T2 (proper muscle)	7	15	
T2 (subserosa)	1	10	
T3 (serosal exposure)	1	1	
Lymph node metastasis			0.003
Absent	90	34	
Present	11	15	
Final stage			<0.001
IA	85	19	
IB	10	13	
II	6	17	

Pathological examination of the surgical specimens from the LADG group revealed tumor invasion of the mucosa and submucosa layers (T1) in 92 cases, of the proper muscle and subserosa (T2) in eight cases, and serosal exposure (T3) in one case (Table 4). There were no lymph node metastases (N0) in 89 cases, but perigastric lymph node metastasis (N1) was present in 11 cases and regional gastric lymph node metastasis (N2) in one case. The final stage in the LADG group was IA in 85 cases, IB in ten cases, and II in six cases. The rate of lymph node metastasis and the mean number of positive lymph nodes were significantly lower in the LADG group. Pathologically, there were more tumors in stage IA in the LADG group, whereas tumors in stage IB and stage II were more frequent in the DG group.

Postoperative hospital stay was significantly shorter in the LADG group than the DG group (Table 5). There was

Table 5 Postoperative Length of Hospital Stay and Complications of Patients Treated by Gastrectomy

	LADG (n=101)	DG (n=49)	p value
Length of hospital stay (day)	13.3±8.5	16.7±10.5	0.034
Morbidity	6 (6%)	4 (8%)	n.s.
Anastomotic bleeding	3	0	
Leakage	1	1	
Anastomotic stenosis	0	1	
Roux-Y stasis	1	0	
Wound infection	1	1	
Cholangitis	0	1	

no hospital mortality. Postoperative surgical complications occurred in six cases (6%) in the LADG group and consisted of anastomotic bleeding in three patients, wound infection in one patient, Roux-en Y stasis in one patient, and anastomotic leakage in one patient. Postoperative complications occurred in four cases in the DG group and consisted of anastomotic stenosis, wound infection, cholangitis, and anastomotic leakage in one patient each.

Discussion

Laparoscopic surgery for early gastric cancer is gaining acceptance among surgeons in Japan.[9] According to the Japanese guidelines for gastric cancer treatment, it can be used to treat cases of early staged gastric cancer, in which tumor depth and lymph node metastasis are limited to T1 and N0/N1 or T2 and N0.[10] However, it remains an investigational procedure because of its technical difficulty and the lack of level I evidence based on randomized data.[9] Analysis of the learning curve for laparoscopic colectomy has suggested that performance of over 30–50 procedures is required to achieve technical proficiency.[11] In this study, we compared the outcome of patients who underwent LADG with that of patients treated by DG.

Mean blood loss during LADG was 139 ml and less than in the DG group. However, intraoperative vascular injury during LADG caused massive bleeding in two cases, and injury of the mid-colic vein and left gastric artery resulted loss of over 900 ml of blood. The vessels were tied with sutures through the mini-laparotomy. There were intraoperative complications of LADG in three cases (3%), consisting of bleeding in two cases and a slight injury of the duodenum while suturing the stump through a small abdominal incision that required conversion to laparotomy and repair of the duodenal stump. There was no surgical mortality in either group, and the postoperative morbidity rate was 6% (6/101) in the LADG group. Postoperative surgical complications after LADG consisted of anastomotic bleeding after double stapling of the gastroduodenal anastomosis in three cases, and all three occurred among the first 27 cases and thus may have been related to the learning-curve period. Complications in the LADG group consisted of anastomotic leakage, Roux-en Y stasis, and wound infection in one case each. The incidence of complications in the LADG group was comparable to the incidence associated with open surgery and other studies of LADG in Japan (14.8%)[12] and Korea (9.8%).[13]

LADG plus systemic lymph node dissection (D1+α and D1+β) was performed in 98 cases in the LADG group. The number of lymph nodes retrieved according to the extent of lymph node dissection was 13 in the D1cases, 16 in the D1+α cases, 31 in the D1+β cases, and 33 in the D2 cases. The mean number of lymph nodes retrieved in the LADG group was 27, and less than in the DG group, because the extent of lymph node dissection in the LADG group was mainly D1+β, as opposed to D2 in the DG group. We previously reported a retrospective analysis of 483 patients with early gastric cancer treated by gastrectomy plus D1 or D2 lymph node dissection;[14] however, the 5-year survival rate of the patients with n1-positive submucosal cancer who underwent D2 dissection was 91%, as opposed to a rate of 80% among those who underwent D1 dissection. The criteria for lymph node dissecting numbering have been changed in 1999, and the D2 dissection in our previous study is comparable to D1+β dissection in this series.[15] Based on the above results, the proportion of patients treated by LADG plus D1+β dissection increased in the latter half of this series and in Japan.[12]

The patients were followed up by physical examination, blood tests, upper gastrointestinal tract endoscopy, abdominal ultrasonography, and abdominal computed tomography scanning 3 months, 1 year, and 2 years after surgery. Heterotopic submucosal cancer of the remnant stomach without lymph node metastasis was diagnosed in two cases, 1 and 2 years, respectively, after laparoscopic surgery, and they were treated by total remnant gastrectomy. After a mean follow-up period of 29 months, there was only one tumor death. It occurred 18 months after surgery and was attributable to liver metastasis by a neuroendocrine carcinoma [T1(sm), N1(1/20), P0, H0, f-stage IB]. The long-term oncologic outcome after LADG was reported as a good 5-year disease-free survival rate as conventional open surgery from a multicenter study in Japan.[12]

The minimal invasiveness of laparoscopic surgery has been reported to be associated with less pain, fewer pulmonary complications, and better quality of life after surgery.[5] In the porcine model, manipulation of the small intestine as a cause of increased inflammation might minimize during laparoscopic surgery compared to open surgery.[16] In this study, the mean postoperative hospital stay of the LADG group was shorter than in the group that underwent open surgery. Since the same clinical pathway was implemented in both groups and allowed discharge 8 days after surgery and, thereafter, patients decided the discharge from the hospital, the shorter hospital stay after LADG may be related to early recovery from surgery. However, mean body weight loss in both groups was 2.4 kg at 2 weeks, 3.9 kg at 6 months, and 4.5 kg at 1 year after surgery, and there were no differences between the two groups (data not shown), reflecting the same volume of the remnant stomach and degree of nutritional disturbance after gastrectomy.[17]

Conclusion

The authors conclude that laparoscopy-assisted gastrectomy is a safe and useful operation for most early gastric cancers. If patients are selected properly, laparoscopy-assisted gastrectomy can be a curative and minimally invasive treatment for gastric cancer.

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Re: An Antecolic Roux-En-Y Type Reconstruction Decreased Delayed Gastric Emptying After Pylorus-Preserving Pancreatoduodenectomy by Murakami et al.

Moritz N. Wente · Markus W. Büchler

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Dear Editor:

We read with great interest the article by Murakami et al.[1] from the Department of Surgery, Hiroshima University, Japan in the June issue of the *Journal of Gastrointestinal Surgery*. The authors presented a retrospective series of 132 consecutive pylorus-preserving pancreatoduodenectomies performed at their institution between 1994 and 2006. All patients received a pancreatogastrostomy, but two different reconstruction methods to obtain the digestive continuity: either a retrocolic Billroth I type reconstruction (1994–2000) or an antecolic Roux-en-Y reconstruction (2001–2006). In a multivariate analysis, the reconstruction method was the only factor influencing the occurrence of delayed gastric emptying with a significant benefit for the antecolic reconstruction (81% versus 10%; $P<0.03$). To our knowledge, this is the first comparative study to clarify the beneficiary effect of an antecolic reconstruction method in patients with pancreatogastrostomy. A recent meta-analysis of three randomized controlled trials comparing pancreatojejunostomy with pancreatogastrostomy showed an overall comparable delayed gastric emptying (DGE) rate for both reconstruction techniques [15.8% versus 13.9%; OR 0.85 (0.50; 1.44), $P=0.54$].[2] However, the authors should clearly state why they have used an end-to-end reconstruction compared to the most commonly used method of an end-to-side gastrojejunostomy as described by Delcore et al.[3] and why they have changed their operative strategy in 2001. The given rate of 81% delayed gastric emptying in the retrocolic group with the used definition of the need of a nasogastric tube ≥10 days or an inability to tolerate ≥14 days seems to be extremely high. Even in the randomized controlled trial by Tani et al.,[4] which has been terminated due to the fact that an interim analysis revealed a clear benefit for the antecolic reconstruction method, the retrocolic reconstruction showed a DGE rate of *only* 50% using a bit stronger definition for DGE. This resembles again the necessity of clear definitions and grading of DGE, as it has recently been proposed by the International Study Group of Pancreatic Surgery (ISGPS).[5]

Furthermore, the authors seem to have overlooked the previously published article by Hartel et al.[6] which has already clearly outlined the superiority of an antecolic compared to a retrocolic reconstruction following pancreatoduodenectomy.

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Reply to Comments on "An Antecolic Roux-en-Y Reconstruction Decreased Delayed Gastric Emptying After Pylorus-Preserving Pancreatoduodenectomy"

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To the Editors:

We are grateful to Dr. Wente and Dr. Buchler for their interest in our article, "An antecolic Roux-en Y reconstruction decreased delayed gastric emptying after pylorus-preserving pancreatoduodenectomy". Our responses to the Dr. Wente's comments are as follows.

In our institution, a Billroth I type reconstruction with pancreaticogastrostomy had been routinely used after pylorus-preserving pancreatoduodenectomy (PPPD) up to 2001. The reason is that a Billroth I type reconstruction after PPPD has been reported to have an advantage over a Roux-en-Y reconstruction because it simulates the normal anatomic arrangement and provides a physiologic mixture of food, pancreatic juice, and bile in the portion of the jejunum.[1,2] However, delayed gastric empting (DGE) frequently occurred in patients undergoing a Billroth I type reconstruction after PPPD, as described in this paper (44/54, 81%). According to the previous reports, the incidence of DGE in a Billroth I type reconstruction has been reported to be high.[3,4] Goei et al.[3] reported that the rate of DGE in a Billroth I type reconstruction with pancreaticojejunostomy was 76% (39/51) using the same definition of DGE as our study, which was almost similar to our results. Based on these results, we have changed our reconstruction procedure into a Roux-en-Y reconstruction in 2001. As a result, the incidence of DGE decreased to 10% (8/78). We believe that the occurrence of DGE is strongly affected by a Billroth I reconstruction after PPPD.

An antecolic reconstruction has been reported to reduce the rate of DGE by several investigators.[5,6] In this series, we have also used an antecolic reconstruction since 2001. However, we believe that the most important practice for minimizing the incidence of DGE is to put the stomach at as vertical a position as possible.[7] In order to put the stomach at a vertical position, we routinely divide the right gastric artery and suture the remnant pancreas to the body of the stomach, not to the antrum. With these procedures, the stomach is set at a vertical position after PPPD. Further studies concerning relationship between the incidence of DGE and a vertical stomach reconstruction after PPPD are needed.

Various definitions of DGE have been used to investigate clinical and surgical factors influencing DGE after PPPD.[8] In this series, we chose the DGE definition of either gastric suction for more than 10 days or the inability to tolerate a solid diet on or before the 14th postoperative day. However, because of a lack of a uniform definition of DGE, a reliable comparison of different study reports is not possible. Recently, an internationally accepted consensus definition of DGE has been proposed by the International Study Group of Pancreatic Surgery (ISGPS).[9] Future studies on the occurrence of DGE after PPPD should be analyzed based on this definition.

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