2004 SSAT/SAGES Joint Symposium—Contributions to the Treatment of GI Disease by Laparoscopy, Endoluminal, and Transluminal Therapies

Summary Statement

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Summary Statement

During the 1990s, we were fortunate to observe the rapidly expanding field of laparoscopic surgery made possible by new imaging and instrument technology. This era had been preceded by a rapid development in endoscopic technology and techniques. Although laparoscopy implied a transabdominal approach, endoscopy implied a transluminal approach to both diagnosis and therapy. Today, transluminal could mean via a laparoscopic approach through the stomach wall to resect a local tumor or it could mean a transluminal endoscopic approach to the transgastric route to remove an appendix.

This symposium was designed to look back at the contributions all of these technologies have made to patient care. Laparoscopy has provided better staging of cancer for gastrointestinal malignancy, such as of the liver, bile ducts, and pancreas, than is provided by just computed tomography (CT) scanning. We no longer rely on CT alone for staging these cancers. In combination, CT and laparoscopy will improve results simply by providing better staging in selected patient groups. Two lectures outlined these contributions.

Laparoscopy has also entered the realm of complex resections, such as with the esophagus, liver, and pancreas. The trocar-delivered devices for stapling and hemostasis have allowed these resections to be developed at advanced laparoscopic centers that already have experience with this complex surgery. The rules primarily involve adhering to the principles of surgery already known for these organs and then making the outcomes better. Two of these lectures review the outcomes that have been possible so far.

Endoscopy-based treatment for gastroesophageal reflex disease (GERD) has been quite controversial. Here we find that the Stretta procedure has led the way to the endoscopic reversal of GERD in selected patients. Finally, the ampulla of Vater has yielded to endoscopy-based resection for selected patients with benign or premalignant disease. These techniques and their follow-up are reviewed and are surprising. There is no role for endoscopic resection of the ampulla for invasive cancer in a patient fit for surgery.

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Laparoscopic Staging for Hepatobiliary Carcinoma

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KEY WORDS: Laparoscopy, staging, liver neoplasms, biliary tract neoplasms

Hepatobiliary malignancies as a group are associated with particularly high rates of unresectability. Preoperative imaging is always improving but is relatively insensitive for small liver lesions, peritoneal disease, and major vascular invasion. The goal of staging laparoscopy is to avoid unnecessary laparotomy by identifying disease that precludes resection.

Staging laparoscopy is generally associated with less morbidity, shorter hospital stays, and decreased recovery time compared with laparotomy. However, complications related to trocar placement and pneumoperitoneum have been described, and the time and equipment costs of laparoscopy are not trivial. Furthermore, port site recurrence is thought to be an uncommon but real risk of laparoscopic procedures for malignant disease. Like other staging modalities, laparoscopic staging is most effectively used in patients whose management will be affected by its outcome. Whether staging laparoscopy should be performed routinely, selectively, or not at all, depends on the disease.

EXTRAHEPATIC BILIARY CARCINOMA

Gallbladder carcinoma and extrahepatic cholangiocarcinoma are aggressive malignancies that usually present with unresectable disease. Despite preoperative imaging, patients are often found to have occult metastatic disease at the time of exploration. Because the median survival of patients with metastatic disease is less than 6 months, adequate palliation can usually be achieved with radiographically or endoscopically placed stents. The nontherapeutic laparotomy and its morbidity might be avoided by laparoscopy.

In one recent series of patients with radiographically resectable extrahepatic biliary malignancies, the yield of laparoscopy for occult unresectable disease in gallbladder carcinoma was approximately 50%.[1] As expected, the yield of laparoscopy was lower (20%) but not zero in patients whose gallbladder carcinoma had been incidentally identified after recent cholecystectomy. Following negative laparoscopy, approximately one third of patients undergoing laparotomy with intent to resect were resected, which is higher than expected on the basis of the literature. Hilar cholangiocarcinomas tend to cause symptoms earlier but have correspondingly lower rates of unresectability at the time of diagnosis. The yield of staging laparoscopy for hilar cholangiocarcinoma was 25% in this study, and over half of patients undergoing laparotomy were resected.

For gallbladder and hilar cholangiocarcinoma, the yield of staging laparoscopy is high and the value of surgical palliation is low. We therefore recommend routine laparoscopy. Possible exceptions include relatively healthy patients with incidentally identified gallbladder carcinoma at recent cholecystectomy, as these patients not only are less likely to harbor occult disease but may also have local inflammation that makes staging laparoscopy more difficult and less accurate.

HEPATOCELLULAR CARCINOMA

Despite the availability of numerous nonresectional therapies, surgical resection is still the mainstay of treatment for hepatocellular carcinoma (HCC). Resectability is dependent on several factors, including the size and location of the tumor, the presence and location of multifocal disease, and the quality of the remnant liver. Even after high-quality imaging, only about two thirds of patients explored with the intention to resect actually have hepatectomy. Laparotomy is unnecessary in the other third of patients, as most patients do not need surgical palliation.

One important difference from most other abdominal malignancies is that peritoneal disease is rare in HCC. The value of surface laparoscopy for HCC is mainly the identification of additional visible liver lesions and the assessment of cirrhosis. Occasionally,

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because patients with HCC often do not have a preoperative tissue diagnosis, laparoscopy may reveal an unsuspected primary malignancy elsewhere in the abdomen. Most studies of laparoscopy for HCC have also selectively used laparoscopic ultrasonography (LUS) for the more sensitive identification of additional liver lesions and assessment of major vascular invasion.

Whether the information provided by laparoscopy renders a patient unresectable is obviously somewhat subjective and surgeon dependent. However, in two of the largest studies focusing on HCC, from Memorial Sloan-Kettering Cancer Center (MSKCC)[2] and from Hong Kong,[3] the use of staging laparoscopy/LUS for identifying unresectable disease avoided laparotomy in approximately 20% of patients, and almost 90% of patients undergoing laparotomy were resected.[2,3] In the MSKCC study, laparoscopy/LUS was significantly more likely to identify unresectability in patients if imaging suggested they had cirrhosis or stage IVa disease, that is, major vascular invasion or bilobar tumors. The initial use of laparoscopy avoided unnecessary laparotomy in almost 29% of patients with these features but in only 5% of patients if neither factor was present preoperatively. In the Hong Kong study, laparoscopy/LUS was less accurate in patients with tumors greater than 10 cm. In both studies, the most commonly missed reasons for unresectability were major vascular and adjacent organ invasion.

Laparoscopy/LUS should therefore be used more selectively in patients with HCC. Noncirrhotic patients with peripheral lesions are much less likely to benefit from laparoscopy/LUS than are patients with cirrhosis and patients with suspected major vascular invasion or bilobar disease. In patients with large tumors and possible adjacent organ invasion, determination of resectability often requires laparotomy for palpation and close dissection.

METASTATIC COLORECTAL CANCER

Complete resection of colorectal metastases to the liver offers the potential for long-term survival. Despite preoperative imaging, up to 40% of patients are found to be unresectable at exploration. Two important differences between HCC and metastatic colorectal cancer are that, for the latter, cirrhosis is uncommon and extrahepatic disease—including local recurrence, nodal metastases, and peritoneal implants—is common.

In one of the largest studies focusing on colorectal metastases, 103 patients at MSKCC [4] were prospectively evaluated with staging laparoscopy/LUS. Only 14% of patients overall had unresectable disease identified by laparoscopy/LUS, and only 10% were spared laparotomy. An additional 8% of patients had unresectable disease missed by laparoscopy. In particular, laparoscopy was not helpful in identifying regional lymph node metastases. Patients were stratified by a previously described clinical risk score that assigned points for node-positive primary disease, disease-free interval less than 1 year, number of hepatic lesions greater than one, largest hepatic tumor greater than 5 cm, and CEA greater than 200 ng/ml. Only 4% of patients with a score of 2 or less were found to have unresectable disease at laparoscopy, whereas 27% of patients with a score of greater than 2 had unresectable disease identified at laparoscopy.

As for HCC, staging laparoscopy/LUS should be used selectively for colorectal metastases. Patients at higher risk for having unresectable disease, based on the clinical risk factors listed here, are more likely to benefit from staging laparoscopy/LUS.

LAPAROSCOPIC ULTRASONOGRAPHY

Open intraoperative ultrasonography is considered the gold standard for the determination of resectability of liver tumors, allowing the visualization of lesions as small as 3−5 mm and the identification of vascular invasion with high sensitivity. The introduction of LUS probes in the early 1980s allowed the addition of this valuable modality to staging laparoscopy. The chief limitations of LUS are that it is very operator dependent and that, even with the most experienced operator, it is difficult to obtain a biopsy sample of small, deep lesions.

Most studies of LUS have included a mix of primary and metastatic tumors. In a Cleveland Clinic study[5] comparing LUS with triphasic spiral CT, LUS identified all tumors seen on preoperative CT plus at least one additional tumor in 20% of patients. Although CT did not miss any lesions larger than 3 cm, 28% of lesions smaller than 1 cm were missed. Most of the missed lesions were in segments III and IV near the falciform ligament.

SUMMARY

For gallbladder carcinoma and extrahepatic cholangiocarcinoma, staging laparoscopy is high yield and should be performed routinely. For HCC and metastatic colon cancer, a more selective approach is warranted, reserving staging laparoscopy for patients in whom unresectable disease is more likely to be identified. The exact role of LUS in these patients is not yet determined but likely extends the advantages of

staging laparoscopy. Staging laparoscopy spares patients with unresectable disease from nontherapeutic laparotomy, decreasing their recovery time and, it is hoped, allowing earlier initiation of nonsurgical therapy.

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Laparoscopic Staging Should Be Used Routinely for Locally Extensive Cancer of the Pancreatic Head

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Since the 1980s, computed tomography (CT) has been the primary staging method for pancreatic cancer. New technology has increased the scan speeds for CT, whereas slice thicknesses have decreased. A 10-second scan of the 1980s is now done in less than half a second. CT findings of a pancreatic cancer have been clinically useful. Patients have been grouped by the CT extent of the disease; then the best therapy is chosen. Three staging groups are defined using CT: *local disease* (resection is the initial treatment), *locally extensive disease* involving adjacent major vessels (radiation-based protocols are to be considered), and *distant disease* (only systemic chemotherapy would be indicated). However, even the best CT scan does not detect peritoneal dissemination very well. Some patients with local or locally extensive tumors will also have occult disease not seen by CT. These occult metastases are small (<2–3 mm) liver or peritoneal implants and are too small to visualize even for high resolution helical CT.

Cuschieri et al.[4] described the use of laparoscopy as an adjunct to the management of pancreatic cancer in 1978. At a time when the usefulness of CT scans was still being explored, laparoscopy provided a means of directly providing accurate diagnostic information about the pancreas and intra-abdominal cavity. Laparoscopy could be used to detect occult deposits. Since then, laparoscopy has been validated as a means of improving the CT assessment of tumor staging.[1–3] Jimenez and colleagues[1] looked at potentially resectable cases of pancreatic cancer with no distant disease by CT and found that laparoscopy observed gross metastases in the peritoneal cavity in 24%. The percentage was higher for distal lesions (36%) than for head lesions (17%). Surprisingly, positive peritoneal lavage cytology (PLC) was the only finding in 9% of the patients. (Note that the sixth edition of the AJCC *Cancer Staging Manual*[5] has deemed positive peritoneal cytology as stage IV, M1 disease [distant metastases].)

Most studies of laparoscopy and pancreatic cancer have focused on the patients deemed "resectable" by CT, but these represent a minority of the cases. To better direct nonoperative therapies, we focused on those patients who were deemed unresectable and had no distant disease by CT. They underwent outpatient diagnostic laparoscopy, peritoneal lavage for cytology, and biopsies as necessary. Our goal was to discover occult disease and to avoid unnecessary radiation-based treatments that focus the treatment on the primary tumor, that is, local-regional chemoradiotherapy protocols. By detecting patients with occult distant disease, we would accomplish two items. First, these patients would have the best chance of a treatment response using newer multidrug chemotherapy regimens. Second, the results of the local-regional treatments would not be falsely lowered. In the past we have not had an effective treatment for distant disease, but recent gemcitabine-based and other combination drug treatments have resulted in increased response rates in the range of greater than 30%, whereas the previous rates were less than 10%.[6]

In our study[7] of 74 patients with "locally unresectable disease by CT," the use of laparoscopy found unsuspected metastases in 34% (28% in the head and 53% in the tail). PLC was the most common unsuspected finding in 27% of all patients and in 80% of the patients with positive findings. Positive PLC was the only finding in 12%. All patients with peritoneal metastasis had positive PLC; that is, no patient had isolated peritoneal metastasis without PLC being positive. Of the 25 patients with positive findings, 20% had isolated liver metastasis without positive PLC. Interestingly, we observed no difference in the prevalence of positive PLC between patients undergoing preoperative percutaneous biopsies and those not undergoing this type of biopsy (31% versus 35%). Comparison with the "resectable by CT" patients of the Jimenez et al.[1] study is illustrated in Table 1. Distal tumors in the pancreatic body or tail were more likely to have unsuspected gross metastasis than were tumors in the head of the pancreas. Positive PLC followed the same trend. Both gross

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Table 1. Comparison of studies of patients with pancreatic cancer and no disease seen on computed tomography (CT) that used laparoscopy and peritoneal lavage cytology (PLC)

Study type	Gross metastases head/distal lesions (%)	Positive PLC head/distal lesions (%)	Postive PLC but no observed metastases (%)
Jiminez et al.,[1] 2000 (N = 125), resectable by CT	17/36	9/36	9
Liu and Traverso, 2004 (N = 74), unresectable by CT	28/53	19/53	12

metastases and PLC appeared to be more common in the "unresectable by CT" cases.

The value of PLC deserves some comment. Even the human eye looking through a laparoscope would have missed about 10% of these metastatic lesions. Twelve percent of our patients had positive PLC but no gross metastasis, which is similar to the 9% observed in the Jimenez et al. study.[1] The latter study, as well as ours (unpublished results), documented that patients with unsuspected metastasis have significantly shorter median survival than patients who did not. Positive PLC is associated with aggressive disease and dismal outcomes. Might more accurate staging to direct newer systemic chemotherapy treatments improve these results? As emphasized earlier, patients with positive PLC would most likely not benefit from local-regional therapy directed at the primary tumor.

The high rate of unsuspected metastasis observed in these laparoscopic studies implies a median survival of 6–9 months. There are significant implications here. Use caution when interpreting results from studies that do not stage their patients with laparoscopy and PLC. Currently, patients with locally advanced disease are treated with radiation-based protocols aimed at local-regional control. The protocols for these studies assume that there are no metastatic deposits in their patients. Our study suggests that up to 53% of patients may be inappropriately considered for these protocols. Diagnostic laparoscopy will also affect patients who are considered for "down-staging" protocols. Many patients are considered unresectable because of portal vein or superior mesenteric vein involvement. Our study indicates that these patients require staging with diagnostic laparoscopy and PLC to rule out metastatic disease before the initiation of neoadjuvant therapy.

Another benefit of diagnostic laparoscopy is that this staging method is uniquely applicable in a community setting. Patients who present with unequivocal locally unresectable disease can undergo diagnostic laparoscopy before referral.

There is an inherent weakness if staging relies entirely on CT. Many distant metastases of pancreatic cancer are small or apparent only on cytology. In patients thought not to have distant disease by CT but with locally extensive "unresectable" tumors, diagnostic laparoscopy with PLC has shown that one quarter of patients with head lesions and half of patients with body/tail lesions have unsuspected metastasis. These patients would have been inappropriately enrolled in therapies directed at the primary tumor. Also, studies that do not use laparoscopy with PLC in staging would be expected to have falsely low survival statistics.

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Laparoscopic Resections of Liver and Pancreas

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Hepatobiliary and pancreatic (HBP) surgery and minimally invasive surgery (MIS) appear, at first glance, to represent disparate fields of surgical science. For 50 years, surgeons have been developing safer methods of performing ever larger and more technically demanding hepatic and pancreatic resections. For 15 years, minimally invasive surgeons have been striving to make surgery safer with increasingly less traumatic interventions. The benefits of each discipline are well documented in the literature. The crossover lies in our ability to achieve the goals of an open HBP procedure using minimally invasive surgery techniques and thereby receive the benefit of both practices.

In 1991, the first peer-reviewed reports of laparoscopic liver resections were published. They described a wedge resection for a focal nodular hyperplasia. Similarly, the first laparoscopic distal pancreatic resection for an insulinoma was described in 1993, and the first pancreaticoduodenectomy was described in 1994. Subsequently, more than 500 journal articles have been published describing a full spectrum of resections of benign and malignant liver and pancreatic disease.

The descriptions of these laparoscopic procedures give great technical detail and outline potential clinical applications. While caution is advised to avoid broadly condoning such procedures until their appropriate role has been better elucidated, such procedures must not be prematurely condemned, as it seems clear that the diminution of maximally invasive procedures is on the horizon.

SURGICAL TEAM AND OPERATING ROOM REQUIREMENTS

The foremost requirement for surgeons who wish to perform laparoscopic HBP procedures is that they be knowledgeable and experienced open HBP surgeons. Second, they must either be, or work closely with, skilled laparoscopic surgeons. Appropriate operating room (OR) staff and hospital resources need to be in place for both open and laparoscopic procedures. The importance of specialized OR teams in improving efficiency of the OR and patient outcomes in these highly technical surgical specialties has been well documented.

Our institution has made an effort to maintain a simple and standardized laparoscopic instrument tray so that our OR staff is expert in maintenance and use. A standard instrument pan includes a strong, smooth-edged retractor; a suction device; atraumatic graspers; needle drivers; a curved dissector and scissors with monopolar electrocautery attachments; and a fascial closure device. Stapling devices, harmonic shears, clip appliers, and hand assist devices are available on request. Laparoscopic ultrasound equipment is always available in the room.

SURGICAL TECHNIQUES

Laparoscopic liver resection techniques have been developed for benign cyst fenestration, wedge resection, and anatomic segmental resection. Laparoscopic pancreatic resection techniques have been described for tumor enucleation, distal pancreatectomy, and pancreaticoduodenectomy.

The basic tenets of such resections mimic those of open resections. Dissection and mobilization are technical exercises similar to the open counterpart. A number of different laparoscopic parenchymal transection technologies have been developed, including stapling devices, harmonic shears, water jets, ultrasonic dissectors, and others. Most surgeons adapt the technique used in their open practice. Each method has pros and cons, and its success is dependent on the familiarity of the surgeon with that method.

LIVER RESECTIONS

Limited laparoscopic liver resections are increasingly being reported at specialty centers throughout the world.[1,2] They are technically feasible and can be performed with a low morbidity profile. If a program has appropriately trained surgeons and the facility requirements in place, it is reasonable to consider such an approach for benign disease. In patients with

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malignant disease, however, a larger barrier remains to be overcome.[3] First, it must be proved that we can reliably comply with the defined tenets of oncologic surgery. Staging must be complete, tumor manipulation minimal, and margins and lymphadenectomies adequate. It must also be determined whether carbon dioxide pneumoperitoneum or other aspects of minimal access techniques have a harmful effect on the progression of cancer. Finally, we will need to demonstrate through controlled trials that a minimally invasive approach achieves similar or improved outcomes with regard to quality of life and survival.

PANCREATIC RESECTIONS

Laparoscopic pancreatic resections are also being reported with an increasing frequency. Laparoscopic pancreaticoduodenectomies do not at this point appear to be a reasonable undertaking.[4] The morbidity of the procedure is less associated with the incision and more dependent on the physiologic impact of the resection, anastomoses, and re-plumbing of the bowel. The added technical difficulty of a laparoscopic approach is not made up for by the reduced invasiveness. Pancreatic enucleations and distal resections, however, are technically easier, and the reduced impact of the surgery may prove beneficial.[4,5] The same concerns are at play regarding benign versus malignant disease.

SUMMARY

It is our belief that there will be a significant role for minimally invasive approaches to HBP surgery in the future. There is great potential for reduction of perioperative morbidity, possibly more so than in other fields of surgery, due to the major impact on the patient after the maximally invasive open procedures. At this early stage in development, however, we must be cautious about too rapid an assimilation of the techniques without proper evaluation of the outcomes. High-quality data, collected prospectively, from randomized trials where possible, remain the key to implementing proper utilization.

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Minimally Invasive Resection and Mechanical Cervical Esophagogastric Anastomotic Techniques in the Management of Esophageal Cancer

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Standard esophagectomy for carcinoma of the esophagus is associated with significant morbidity and mortality rates. Although reports from some individual surgical centers have reported exceptional survival results with standard esophagectomy, a recent report summarizing nationwide statistics identified mortality rates from esophagectomy that ranged from 8% in high-volume centers to as great as 23% in low-volume centers.[1] Morbidity related to the procedure has also been high. The most important morbidity seen is related to the failure of the esophagogastric anastomosis.

Minimally invasive esophagectomy (MIE) has the potential to lower the morbidity of open operation and allow quicker return to normal function compared with the use of open surgical approaches. Now that advanced minimally invasive surgical procedures are more frequently being performed, detailed results and outcomes must be reported to the surgical community, to assess potential advantages and disadvantages. Also, the use of mechanical stapled techniques may assist in reducing the postoperative leak and stricture rate with cervical esophagogastric anastomosis after esophagectomy.

PATIENTS AND METHODS

During a 5-year period from June 1996 through August 2002, we performed MIE in 222 patients. The primary inclusion criterion for esophageal cancer patients fit for operation was the presence of a resectable lesion after evaluation with endoscopic ultrasound and computed tomography staging of the malignancy.

Initially, we used a laparoscopic transhiatal approach for patients with smaller tumors or with high-grade dysplasia as an indication for esophagectomy (n = 8). We have converted to the combined thoracoscopic and laparoscopic approach to esophageal dissection and gastric mobilization as our procedure of choice for MIE in the last 214 patients. We believe that mobilization of the intrathoracic esophagus is safer and that a more complete lymph node dissection can be accomplished with the inclusion of the thoracoscopic approach. Our current technique of MIE is similar to our previously reported description.[2]

The difficulty with anastomotic leak and postoperative stricture after cervical esophagogastric anastomosis has led us to explore the use of a totally mechanical stapled technique. It is appreciated that this anastomotic difficulty is primarily related to ischemia of the fundic tip and to the inherent imprecision of a hand-sewn anastomosis compared with mechanical stapled anastomosis.[3,4] Later in this lecture summary, we report a comparison of our mechanical stapled technique versus the hand-sewn or partially stapled approach to cervical esophagogastric anastomosis.

Our 222 patients included 186 (83.8%) men and 36 (16.2%) women (median age, 66.5 years; age range, 39–89 years). Preoperative indications for operation included carcinoma in 175 (78.8%) and high-grade dysplasia in 46 (21.2%). Neoadjuvant chemotherapy was used in 78 (35.1%) and radiation in 36 (16.2%). Before MIE, expandable esophageal stents had been placed in 13 patients (5.9%) during induction therapy, and 19 patients (8.6%) had undergone unsuccessful photodynamic therapy to treat high-grade mucosal dysplasia. There was a history of previous open abdominal surgery in 55 (24.8%) of patients.

The stomach was used as the esophageal substitute in all patients. The esophageal bed was used for the gastric conduit in 213 cases, and the substernal route was selected in 9 cases to allow postoperative radiation to the esophageal bed without irradiation of the gastric pull-up. Pyloromyotomy was performed in 28 and pyloroplasty in 136 patients (74%). A laparoscopic feeding jejunostomy was placed in 202 patients at the time of MIE (91%).

RESULTS

MIE was successfully completed in 206 (92.8%) patients. Minithoracotomy was required in 12 (5.4%) and laparotomy in 4 (1.8%) patients due to

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Table 1. Major and minor complications following minimally invasive esophagectomy

Minor complications	No. (%)	Major complications	No. (%)
Atrial fibrillation	26 (11.7)	Anastomotic leak—overall	26 (11.7)
		Normal gastric tube	10 (6.1)
		Narrow gastric tube	16 (25.9)
Atelectasis with mucus plug requiring bronchoscopy	10 (4.5)	Myocardial infarction	4 (1.8)
Pleural effusion requiring tube	14 (6.3)	Gastric tip necrosis	7 (3.2)
J-tube infection	1 (0.5)	Delayed gastric emptying	4 (1.8)
Clostridium difficile colitis	2 (0.9)	Pancreatitis	3 (1.4)
Wound infection	2 (0.9)	Chylothorax	7 (3.2)
Intraoperative tracheal perforation (1–2 mm)	2 (0.9)	Tracheal tear	2 (0.9)
Miscellaneous (others)	5 (2.25)	Deep vein thrombosis	3 (1.4)
		Pulmonary embolus	3 (1.4)
		Pneumonia	17 (7.7)
		ARDS	4 (1.8)
		Vocal cord palsy	8 (3.6)
		Renal failure	2 (0.9)
		Miscellaneous (others)	4 (1.8)
Total	55 (24)	Total	71 (32)

ARDS = acute respiratory distress syndrome.

the presence of significant cavitary adhesions impeding the progress of the procedure.

The 30-day operative mortality rate was 1.4% (n = 3). The three deaths resulted from pneumonia and multisystem organ failure in one patient, postoperative myocardial infarction in one patient, and pericardial tamponade occurring 3 days after MIE in another patient.

Major and minor morbidities are outlined in Table 1. The anastomotic leak rate was affected by the size of the gastric tube. In those patients with our standard diameter gastric tube of 6 cm, anastomotic leaks occurred in 10 of 164 (6.1%). In those patients in whom a narrow tube (3–4 cm) was used (n = 58), the leak rate was significantly increased ($P < 0.001$), occurring in 15 (25.9%) of patients.

The median stay in an intensive care unit was 1 day (range, 1–30 days), time to oral intake was 4 days (range, 1–40 days), and length of hospital stay was 7 days (range, 3–75 days). The mean follow-up was 19 months (range, 1–68 months), with cancer-related survival similar to that seen after open esophagectomy on a pathologic stage analysis.

DISCUSSION

We recently reanalyzed our anastomotic results with a hand-sewn or partial mechanical stapled cervical esophagogastric anastomosis (n = 56) versus the use of a totally mechanical stapled anastomotic technique (n = 125).[5] The technique involves the use of the Endo Stapler to create the posterolateral side-to-side union between the cervical esophagus and the mobilized stomach. A standard TA stapler is then applied across the anterior walls of the esophagus and stomach to complete the anastomosis. The results of this anastomotic comparison are depicted in Table 2, where we noted a significant reduction in anastomotic failure and postoperative stricture among our patients

Table 2. Results of total mechanical (TMA) versus hand-sewn/partial mechanical (HSM) anastomotic techniques[5]

	TMA (n = 125)	HSM (n = 56)	*P* value
Median ± SEM operative time (min)	259 ± 89 (range, 84–480)	347 ± 94 (range, 115–555)	<0.0001
Postoperative leak (%)	5.6	23.2	0.001
Median length of hospital stay (days)	11	13	0.002
Anastomotic stricture (%)	17.6	44.6	0.002

undergoing a totally mechanical stapled anastomotic approach.

We have demonstrated that MIE is feasible and can produce therapeutic outcomes comparable to those reported in most open surgical series. The use of a full gastric tube and a totally mechanical stapled cervical esophagogastric anastomotic technique can reduce anastomotic morbidity following esophagectomy. It is important to note that our results with MIE originate from a center with extensive experience in both benign and malignant esophageal surgery and daily exposure to advanced minimally invasive surgical techniques. It will be important to determine whether MIE can be developed in other centers with similar outcomes. A phase II INTERGROUP study (Eastern Cooperative Oncology Group, E2202) is currently being developed with plans to study this issue.

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Gastroesophageal Reflux Disease and the Truth About Endoluminal Therapy

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There are four Food and Drug Administration–approved endoscopic modalities for the treatment of gastroesophageal reflux disease (GERD): the Bard EndoCinch Procedure suturing system (Bard, Billerica, MA), the Full-Thickness Plicator System (NDO Surgical, Inc., Mansfield, MA), Enteryx, an injectable liquid of biocompatible ethylene-vinyl alcohol copolymer (Boston Scientific, Natick, MA), and the Stretta System (Curon Medical, Sunnyvale, CA). All are in varying stages of development and clinical trials in the United States. At the Vanderbilt Surgery Clinic, I have performed the Stretta procedure since August 2000. Because of my familiarity with the radiofrequency energy treatment of the gastroesophageal junction, here I focus exclusively on the usefulness of this specific modality.

SHAM VERSUS STRETTA RANDOMIZED TRIAL

There is only one endoscopic treatment of GERD that has undergone rigorous evaluation with a sham-versus-treatment trial.[1] The investigators randomized 64 patients with gastroesophageal reflux to either Stretta treatment (n = 35) or to a sham procedure (n = 29). The principal outcome tested at 6 months after treatment with Stretta was GERD-specific symptoms and quality of life as assessed by health-related quality-of-life questionnaires. There was a significant improvement in GERD quality-of-life score in active treatment patients and no improvement in sham-treated patients. The improvement in symptoms continued 12 months after treatment in the active group. There was, however, no improvement in daily medication use or in esophageal acid exposure times as measured by 24-hour pH monitoring 6 months after Stretta or sham treatment. Patients who crossed over to active treatment 6 months after the sham treatment had a significant improvement in GERD-related symptoms, acid exposure time, and decrease in medication use. The authors concluded that there was no sham effect with the Stretta procedure, but there was significant improvement in GERD quality of life 6 months after active treatment.

COMPARISON OF LAPAROSCOPIC FUNDOPLICATION TO STRETTA

At the Vanderbilt Surgery Clinic, 140 patients presenting for surgical or endoscopic treatment of their GERD were selected for either Stretta or laparoscopic fundoplication (LF).[2] Patients who fit stringent criteria for application of the Stretta (hiatal hernia $\leqslant$2 cm, no Barrett's esophagus, lower esophageal sphincter pressure $>$8 mm Hg, and manometry acceptable for LF) were offered Stretta or LF. Sixty-five of the 140 patients selected the Stretta procedure for their treatment. The operative time for Stretta was approximately one third that for LF. Stretta was generally done under conscious sedation rather than general anesthesia and was a true outpatient procedure compared with a 1.5-day average hospitalization for LF. Although 97% of the patients undergoing LF were able to completely stop the use of proton pump inhibitors (PPIs), there was a significant number, 58%, in the Stretta group who were able to completely stop the use of PPIs with good symptom control of their GERD. Among the STRETTA group, 11% had no response to treatment and remained on the same twice-daily dose of PPIs. After Stretta, approximately one third of the patients were able to provide a postoperative 24-hour pH study, which revealed that there was a significant decrease in acid exposure time in the distal esophagus after Stretta treatment. Of the patients who were studied, 36% had a normal pH study after the Stretta treatment. Even though far fewer numbers of patients responded completely to the Stretta treatment compared with LF patients, there was an overall high rate of satisfaction in both groups, with 89% of the Stretta patients and 96% of the LF patients expressing satisfaction with their treatment. We concluded that patients undergoing Stretta in this prospective study

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were satisfied with treatment outcomes and a new treatment paradigm was proposed. The new paradigm suggests that patients with medically refractory GERD symptoms, who fit the mentioned selection criteria, can be treated with the Stretta procedure. The 10% of the patients who do not respond to the Stretta treatment at all can be treated with LF, because LF can be performed after Stretta without an increase in the technical difficulty of the procedure.

The Stretta procedure is technically much easier to perform. Our studies have demonstrated that outcomes are no different for a physician who has done 10 or more procedures than it is for physicians who have done fewer than 10 procedures.[3] There also is no increase in technical difficulty in the performance of the Stretta on a patient who weighs more than 500 pounds than there would be in a supermodel with a subnormal body mass index. The same cannot be said for LF, and a number of articles have pointed out the higher rate of complications and the failure rate after LF in morbidly obese individuals. It is virtually impossible to consider LF in patients who have previously undergone gastric bypass, but the Stretta procedure is only marginally more difficult in the patient who has previously undergone gastric surgery. It also is no more difficult to perform a Stretta in someone who has undergone 10 previous laparotomies with extensive adhesions than it is in someone who has had no previous abdominal surgery. There is a significant difference in the technical ease, operative time, learning curve, and usefulness in specific patient cohorts between the Stretta procedure and LF.

MEDICAL TREATMENT VERSUS SURGICAL TREATMENT

The advent of the PPIs for treatment of GERD has changed the practice of surgical treatment for GERD. Many of the patients presenting to the Vanderbilt Surgery Clinic have achieved control over their active erosive esophagitis and many of their heartburn symptoms only to find that they experience progressive symptoms from nonacid regurgitation and that their quality of life is impaired because of their inability to eat foods, perform physical activities such as bending, sleep at night, or go out and socialize with friends. The changing face of people seeking surgical or endoscopic treatment therefore dictates much of their desire is to obtain better symptom relief than current medical therapy can provide. Thus, for most patients seeking surgical treatment of GERD, it is the GERD-specific quality of life, as well as the overall quality of life, that is the most important facet of their satisfaction with surgical or endoscopic treatment. We find that satisfaction after Stretta in a large number of patients collected in a Stretta registry[3] was extremely high at 88%, and in the comparison between LF and Stretta, patient satisfaction with the Stretta procedure at our institution was 89%.[2] Many patients opt out for an incisionless outpatient treatment that does not require a prolonged period of time off work and is accompanied by an extremely low rate of complications compared with LF, which has a higher postoperative complication rate, longer recovery, pain, and discomfort associated with the procedure.

SUMMARY AND FUTURE

The data support a recommendation for the use of Stretta in well-selected patients who fit strict guidelines. Improvements in efficacy have to be made to put aside some of the nagging questions regarding these procedures, specifically the Stretta, because only 36% of our patients have normalized acid exposure times in the distal esophagus on 24-hour pH monitoring after performance of the Stretta.[2] Furthermore, the sham-versus-Stretta trial did not demonstrate a significant improvement in acid exposure time between the active and sham-treated groups at 6 months.[1] The long-term studies are out to only 27 months,[4] which is not long enough to completely satisfy the skeptic regarding the long-term usefulness of these procedures. I have no doubt that there will be improvements in technique and outcomes, which suggests the future of endoscopic treatment of GERD is promising. Further studies are needed to help determine the best treatment for patients, whether that be medical, surgical, or endoscopic.

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Endoscopic Resection of Ampullary Neoplasms

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Historically, ampullary neoplasms were often found late, presented with symptoms of chronic gastrointestinal bleeding or pancreaticobiliary obstruction, and were treated with radical surgery or, less commonly, local resection. Currently, the majority of ampullary adenoma patients seen in my practice are referred from other gastroenterologists and found incidentally during evaluation of gastroesophageal reflex disease (GERD) or dyspepsia. Alternatively, they may be found during screening in Gardner/familial polyposis patients or at time of endoscopic retrograde cholangiopancreatography (ERCP) for mild elevations of liver function levels or amylase and lipase elevations. Patients who present with obstructive jaundice, relapsing pancreatitis, extremely large lesions, or those that encompass more than one third of the luminal circumference have been less amenable to endoscopic cure in my practice, as have those with submucosal lesions. The latter include islet cell tumors, carcinoids, and gut stromal tumors.

ENDOSCOPIC TREATMENT

Endoscopic options for invasive, malignant ampullary lesions are limited to palliation. This includes preoperative decompression of the pancreaticobiliary tree and stent insertion (usually placement of a self-expandable metal stent into the bile duct for surgically unfit patients or those with metastatic disease.

Endoscopic options for patients with potentially curable lesions include thermal ablation (Nd:YAG laser or argon plasma coagulation [APC]) or papillectomy in conjunction with sphincterotomy and/or stent placement into the pancreaticobiliary tree. Diagnostic prerequisites before attempting ampullary resection or ablation include baseline laboratory studies to include tumor markers (CEA/CA19-9), a pancreas protocol computed tomography (CT) scan, multiple biopsies (if there is a question of invasive malignancy), and selective endoscopic ultrasonography or intraductal ultrasound.

ENDOSCOPIC TECHNIQUE

Although there have been multiple techniques for endoscopic removal of the papilla that have been described, I undertake baseline ERCP in all patients followed by piecemeal or single-snare papillectomy and immediate retrieval of the tissue specimen. Smaller fragments can be suctioned through the scope, but larger ones require endoscopic removal.

Dual sphincterotomies and stent placements minimize the acute risk of cholangitis and pancreatitis, respectively, and the subsequent risk of stenosis of the pancreaticobiliary outlets. APC or Nd:YAG laser can be used for lateral extensions of tumor, although saline injection should be considered to preclude transmural burn.

Variations of the above technique include (1) papillectomy first, ERCP, sphincterotomy, and stents next and (2) piecemeal polypectomy after initial sphincterotomy or stent placement through tumor.

Alternatively, extensive thermal ablation of the papilla by means of laser, APC, or bipolar cautery has been undertaken after multiple biopsy samples have been taken. I have limited this therapy in my practice to patients with a limited recurrence postpapillectomy in the setting of widely patent pancreatobiliary sphincterotomies.

RESULTS OF PAPILLECTOMY

The literature is replete with reports that papillectomy is associated with both acute and subacute or chronic complications.[1] The former may include bleeding, perforation (usually limited to excision or treatment of lateral tumor extension), and acute pancreatitis or cholangitis from edema of the pancreatic duct or biliary orifices, respectively. Delayed complications can include bleeding, usually at 7–10 days postprocedure, and duodenal or papillary stenosis. The latter may present as pancreatitis or biliary colic.

Thermal destruction of the papilla appears to be both riskier and less successful for cure compared with papillectomy. Saurin et al.[1] treated 25 patients

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with Nd:YAG laser, of whom two thirds had endoscopic and histologic remission. There was one recurrence at a median follow-up of 66 months. Five patients (21%) had their endoscopic treatment discontinued because of advanced age or severe unrelated disease, and three patients (12.5%) had treatment failure because of either severe pancreatitis or tissue ingrowth into the pancreaticobiliary tree. At a median follow-up of 81 months, two patients had undergone pancreaticoduodenectomy, one third had died of unrelated causes, and 58% were alive, in whom one cancer developed.

Familial polyposis (FAP) patients seem to fare less well. In a retrospective review of 59 FAP patients and 32 with sporadic ampullary adenomas treated at the Mayo Clinic, ablation was successful in 44% of sporadic and 34% of FAP adenomas at a median follow-up of 24 months.[2] Complications occurred in 15 (19%), of which 3 (4%) were severe and included duodenal stenosis, necrotizing pancreatis, and transmural burn (1 each). Thirteen patients (16%) were referred for surgery during follow-up.

There have been a number of series that have looked at a small number of patients treated with papillectomy for ampullary adenoma.[3,4] Combining the results of four series published in 2000 and 2001, 47 of 49 patients had successful papillectomy.[1,3,5,6] The complication rate in these series ranged from 0% to 25%, and there was recurrence in four (8.5%), of whom one was ultimately shown to have cancer. Three patients (6.3%) had subsequent surgery.

In the largest, multicenter retrospective review of patients with endoscopically removed ampullary adenomas, 103 patients who underwent papillectomy at four referral centers were reviewed.[7] Seventy-two of these had sporadic adenoma and the remaining patients had a variant of FAP. Presenting symptoms were jaundice/cholangitis/pain (n = 59), pancreatitis (n = 18), and bleeding (n = 12). Twenty-six patients were asymptomatic. Long-term endoscopic treatment was successful in 83 patients (80%). Older patients, smaller lesions (21 versus 30 mm, $P < 0.0001$), and sporadic lesions (63 of 72 [86%] versus 20 of 31 [67%], $P = 0.02$) were more likely to be treated successfully. There were 10 complications (10%), which included acute pancreatitis (n = 5), bleeding (n = 2), and late papillary stenosis (n = 3). Acute pancreatitis was more common in those who did not have pancreatic duct stents inserted (17% versus 3.3%), whereas papillary stenosis was also more common (15.4% versus 1.1%) in patients who did not have pancreatic stent placement.

Studies have also been published looking at thermal ablation of ampullary adenomas compared with papillectomy. For instance, Vogt et al.[3] retrospectively reviewed 36 patients treated with papillectomy (n = 18) or thermal ablation (n = 18). Median follow-up was 75 and 33 months, respectively. The incidence of subsequent ampullary cancer was calculated to be threefold higher in the ablation group (1:15.5 patient-years compared with 1:52.8 patient-years), and there was a significant decrease in the incidence of cancer-related death in the papillectomy group ($P = 0.0045$). The authors correctly concluded that snare resection of ampullary adenomas was preferable to thermal destruction.

FOLLOW-UP

Despite an increased willingness for endoscopists to treat ampullary tumors, a word of caution is in order. On the one hand, patients with Gardners/FAP have a field defect, and despite papillectomy and treatment with sulindac or a cyclooxygenase 2 inhibitor, recurrence at the papillectomy site is a real concern. Because of this, the malignant potential for other C-loop polyps, and the absence of conclusive data showing that papillectomy decreases the rate of periampullary cancer in this patient population, lifelong follow-up at 6- to 12-month intervals is mandatory. I also follow-up patients with sporadic adenomas who have undergone papillectomy yearly for at least 3–5 years and sporadically thereafter and suggest screening colonoscopy to rule out concomitant colorectal polyps.

Indications for subsequent surgery in my practice include evidence of invasive cancer, significant residual adenomatous tissue which involves the distal bile or proximal (head) pancreatic duct, or development of significant lateral extensions in which risk of perforation or subsequent duodenal stenosis appears prohibitive.

SUMMARY

The endoscopic treatment of benign ampullary neoplasm in expert hands carries at least a 10% procedural or postprocedural risk. As such, consider referral to a center with more experience. Papillectomy (plus sphincterotomy/stent) is both safer and more effective than thermal therapies alone. Stent therapy alone is acceptable (preferably metal stents) in patients of high surgical risk or those with unresectable cancer. Surgery is the treatment of choice for all other good-risk patients with demonstrable invasive cancer. Finally, long-term follow-up is mandatory for patients with benign disease who are treated endoscopically to rule out recurrence or residual intraductal disease.

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Pancreaticoduodenectomy With Vascular Resection: Margin Status and Survival Duration

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Major vascular resection performed at the time of pancreaticoduodenectomy (PD) for adenocarcinoma remains controversial. We analyzed all patients who underwent vascular resection (VR) at the time of PD for any histology at a single institution between 1990 and 2002. Preoperative imaging criteria for PD included the absence of tumor extension to the celiac axis or superior mesenteric artery (SMA). Tangential or segmental resection of the superior mesenteric or portal veins was performed when the tumor could not be separated from the vein. As a separate analysis, all patients who underwent PD with VR for pancreatic adenocarcinoma were compared to all patients who underwent standard PD for pancreatic adenocarcinoma. A total of 141 patients underwent VR with PD. Superior mesenteric-portal vein resections included tangential resection with vein patch (n = 36), segmental resection with primary anastomosis (n = 35), and segmental resection with autologous interposition graft (n = 55). Hepatic arterial resections were performed in 10 patients, and resections of the anterior surface of the inferior vena cava were performed in 5 patients. PD was performed for pancreatic adenocarcinoma in 291 patients; standard PD was performed in 181 and VR in 110. Median survival was 23.4 months in the group that required VR and 26.5 months in the group that underwent standard PD ($P = 0.177$). A Cox proportional hazards model was constructed to analyze the effects of potential prognostic factors (VR, tumor size, T stage, N status, margin status) on survival. The need for VR had no impact on survival duration. In conclusion, properly selected patients with adenocarcinoma of the pancreatic head who require VR have a median survival of approximately 2 years, which does not differ from those who undergo standard PD and is superior to historical patients believed to have locally advanced disease treated nonoperatively. (J GASTROINTEST SURG 2004;8:935–950)

KEY WORDS: Pancreaticoduodenectomy, pancreatic cancer, vascular resection

The first resection and reconstruction of the superior mesenteric vein (SMV) as part of pancreaticoduodenectomy (PD) was reported by Moore et al. from the University of Minnesota in 1951.[1] Subsequent reports described various techniques to reconstruct the SMV and/or portal vein (PV),[2] with Symbas et al.[3] concluding that autologous vein grafts remained patent, while synthetic prostheses had a high rate of occlusion. PV resection at the time of PD was reported by surgeons from Japan in an attempt to improve survival duration by performing an en bloc resection of the pancreas and surrounding structures.[4]

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This concept was also supported in the United States in 1973 when Fortner[5] proposed "regional pancreatectomy," which involved the systematic resection of major peripancreatic vascular structures together with wide soft tissue clearance. Contrary to the beliefs of Fortner and others, radical or extended PD has not been demonstrated to confer a survival benefit.[6]

A more contemporary debate continues regarding the use of vascular resection (VR) for isolated invasion of the SMV, PV, or superior mesenteric–portal vein (SMPV) confluence. It is important to emphasize the distinction between this procedure and regional pancreatectomy. The first operation incorporates venous resection during PD only when, in the opinion of the operating surgeon, the involved segment of SMV or SMPV confluence cannot be separated from the pancreatic tumor. PD with segmental resection of the SMV, PV, or SMPV confluence is not performed in an effort to achieve a greater extent of lymphatic clearance. However, similar to regional pancreatectomy, isolated resection of the SMV or SMPV confluence is technically challenging, and therefore consensus has not been reached on whether the risk of operation may outweigh the potential oncologic benefit of tumor resection. In 1946, Waugh and Clagett[7] modified the Whipple operation to its current form and, most important, outlined the goals of surgical therapy for pancreatic cancer: (1) there should be reasonable opportunity for cure, (2) the risk of death should not outweigh the prospects for cure, and (3) the patient should be left in as normal a condition as possible. The debate over whether major vascular resection should be performed at the time of PD can be framed in the context of these goals. Ongoing questions regarding VR at the time of PD include the following: Is the survival of patients who require VR different than that of patients who undergo standard PD? Can VR and reconstruction be accomplished with acceptable morbidity and mortality?

We address these important issues in this article, which represents the largest reported experience with VR and reconstruction at the time of PD in the Western literature.

METHODS

Patients

Data on all patients who underwent PD at The University of Texas M. D. Anderson Cancer Center between July 1, 1990, and July 31, 2002, were retrieved from a prospective pancreatic tumor database. Patients who underwent pancreatic operations other than PD (e.g., distal pancreatectomy or total pancreatectomy) were excluded. All patients who underwent major VR at the time of PD for any histologic diagnosis were identified, and data on patient demographics, treatment, histopathology, and follow-up were recorded. This retrospective study was approved by the institutional review board. Venous resection involving the SMV, PV, or SMPV confluence included tangential resection with a saphenous vein patch (V1), segmental resection with splenic vein ligation and either primary anastomosis (V2) or interposition grafting (V3), or segmental resection without splenic vein ligation and either primary anastomosis (V4) or interposition grafting (V5) (Fig. 1). For purposes of this analysis, the occasional patient who underwent minor tangential resection of the SMV or PV, which did not require at least a patch, was considered not to have undergone venous resection as there was no way to determine the extent of the tangential resection, which usually was insignificant. Other VRs and reconstructions performed at the time of PD included resection of the hepatic artery (usually at the origin of the gastroduodenal artery or a replaced right hepatic artery) or the anterior surface of the inferior vena cava (IVC). As a separate analysis, all patients who underwent PD for pancreatic adenocarcinoma were identified to allow comparison of those who did and did not undergo VR.

Preoperative Evaluation

Preoperative evaluations included physical examination, routine laboratory testing, chest radiography, and contrast-enhanced computed tomography (CT). To be considered for PD, patients had to fulfill objective radiographic criteria for resectability, which included (1) the absence of extrapancreatic metastatic disease; (2) no evidence of tumor extension to the superior mesenteric artery (SMA) or celiac axis, as defined by the presence of a fat plane between the tumor and these arteries; and (3) a patent SMPV confluence with a suitable segment of SMV and PV to allow venous resection and reconstruction if necessary. The extent of venous involvement by the primary tumor was not a contraindication for operation as long as there was no CT evidence of tumor extension to the celiac axis or SMA and there was a suitable SMV (below) and PV (above) the site of venous involvement.

Pancreaticoduodenectomy

PD was performed in a standard fashion, with six clearly defined steps, as previously described.[8] The most oncologically important and difficult part of the operation was the sixth and final step, which involved division of the pancreas and completion of the

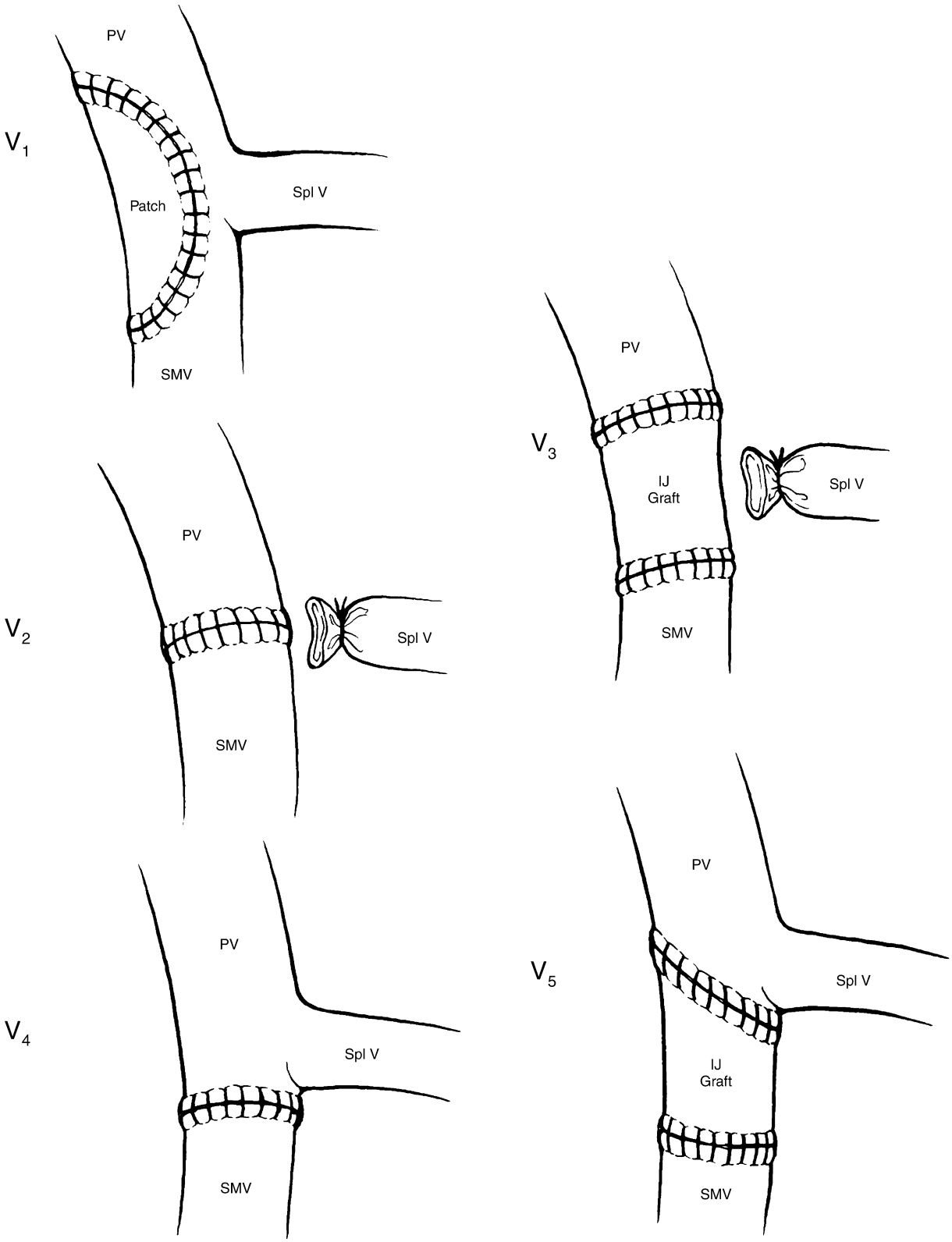

Fig. 1. Illustration of the five forms of venous resection and reconstruction.

retroperitoneal (RP) dissection. In standard PD that did not require venous resection, the surgeon was able to separate the pancreas from the SMPV confluence by reflecting the specimen laterally and dividing the small venous tributaries to the uncinate process and pancreatic head. The uncinate process was completely removed from the SMV and its first jejunal branch to ensure full mobilization of the SMPV confluence and subsequent identification of the SMA. The first jejunal branch of the SMV originates from the posteromedial aspect of the SMV (at the level of the uncinate process), travels posterior to the SMA, and enters the proximal aspect of the jejunal mesentery. This first jejunal branch usually gives off one or two venous tributaries to the uncinate process that require division. If tumor involvement of the SMV (at the level of the first jejunal branch) prevented dissection of the uncinate process from the SMV, the first jejunal branch was divided. Division of the first jejunal branch of the SMV was not considered venous resection for the purposes of this study. After full mobilization of the SMPV confluence and retraction of it medially (to the patient's left), the SMA was then exposed. In more complicated situations, such as those that typically required venous resection, the SMA was identified medial to the SMV. The specimen was then removed from the right lateral border of the SMA in a distal-to-proximal direction up to the celiac ganglion. All soft tissue to the right of the adventitia of the SMA was removed with the PD specimen. The soft tissue adjacent to the right lateral border of the proximal 3–4 cm of the SMA represented the RP margin, also known as the mesenteric or uncinate margin [9] (Fig. 2).

Venous Resection

Tangential or segmental resection of the SMV, PV, or SMPV confluence was performed when, in the opinion of the operating surgeon, the pancreatic head and/or uncinate process could not be dissected free of the SMPV confluence without either leaving gross tumor on the vein or creating a venotomy. Elective venous resections in the absence of tumor adherence were not performed. The standard technique for segmental venous resection has historically involved transection of the splenic vein. Division of the splenic vein allowed complete exposure of the SMA medial to the SMV and provided increased SMV and PV length (as these structures were no longer tethered by the splenic vein) for a primary venous anastomosis following segmental vein resection. With the splenic vein divided, the RP dissection was then completed by sharply dividing the mesentery and RP tissues anterior to the aorta and to the right of the exposed SMA; the specimen was then attached only by the SMPV confluence. Vascular clamps were placed 2–3 cm proximal (on the PV) and distal (on the SMV) to the involved venous segment, and the vein was transected, allowing tumor removal. A generous 2- to 3-cm segment of SMPV confluence could be resected without the need for interposition grafting if the splenic vein was divided. Venous resection was always performed with inflow occlusion of the SMA to prevent small bowel edema, which makes pancreatic and biliary reconstruction more difficult. Systemic heparinization (2500–5000 units) was used before occluding the SMA. The free ends of the vein were reapproximated using interrupted sutures of 6-0 Prolene.

Splenic vein preservation was possible only when tumor invasion of the SMV or PV did not involve the splenic vein confluence. Preservation of the splenic vein–SMV–PV confluence significantly limits mobilization of the PV and prevents primary anastomosis of the SMV following segmental SMV resection unless segmental resection is limited to 2 cm or less. Therefore, in most patients who underwent SMV resection with splenic vein preservation, an interposition graft was required. Our preferred conduit for interposition grafting was the internal jugular vein.[10] Preservation of the splenic vein adds significant complexity to venous resection because it prevents direct access to the most proximal 3–4 cm of the SMA (medial to the SMV). In such cases, therefore, venous resection and reconstruction were performed either before the specimen was separated from the right lateral wall of the SMA or after complete mesenteric dissection by separating the specimen first from the SMA.[8,11]

Five types of venous resection were performed, as illustrated in Fig. 1. A tangential resection of the SMPV confluence (V1) was performed for tumor adherence that was limited to a small aspect of the lateral or posterior wall of the SMPV confluence and repaired using a patch from the greater saphenous vein. When tumor involved the SMV–splenic vein–PV confluence, splenic vein ligation was necessary. If the SMPV confluence could be reapproximated without tension, an end-to-end primary anastomosis was performed (V2); if the SMPV confluence could not be reapproximated without tension, autologous internal jugular vein was used for an interposition graft (V3). When tumor involvement was limited to the SMV or PV such that the splenic vein could be preserved, a primary end-to-end anastomosis of the SMV or PV was occasionally possible (V4). However, with preservation of the splenic vein, an interposition graft (V5) was usually necessary because of the

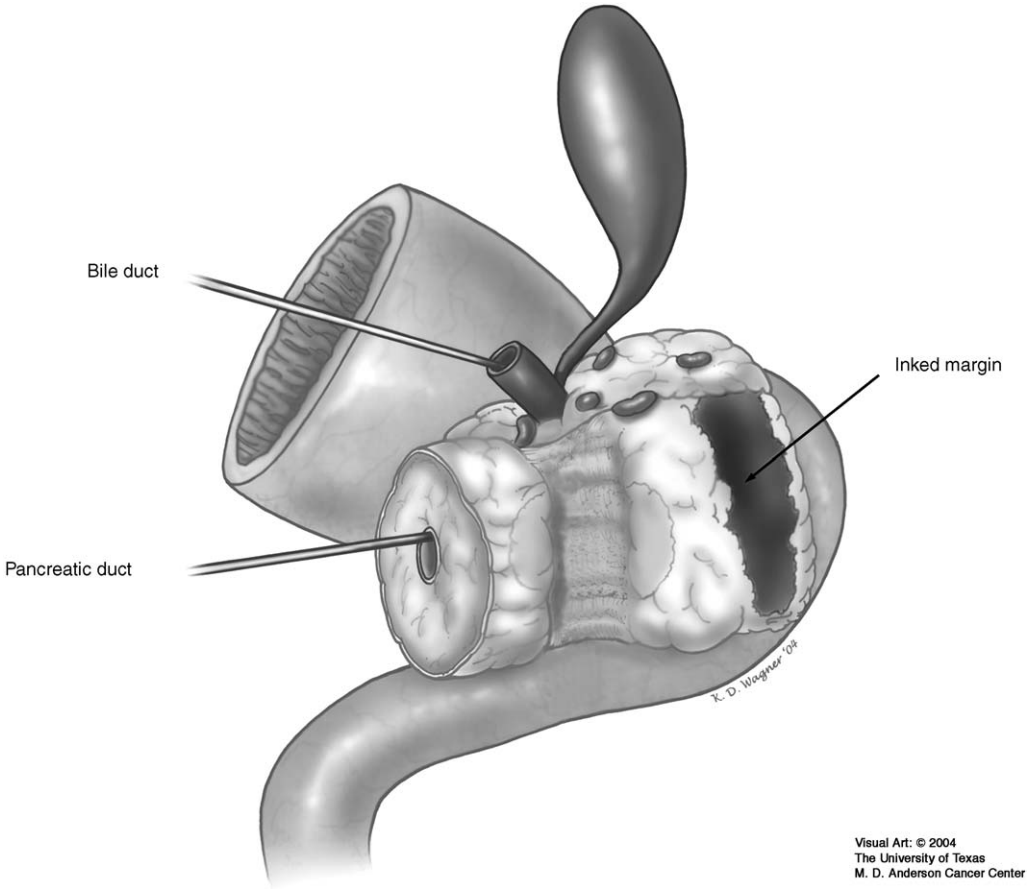

Fig. 2. Illustration of a pancreaticoduodenectomy specimen demonstrating how the retroperitoneal margin (tissue adjacent to the superior mesenteric artery) should be inked at the time of permanent section pathologic examination. This is the only way to determine the status of this important margin of excision; this margin cannot be retrospectively evaluated if the margin was not inked for identification at the time of gross inspection. A small probe is in both the bile duct and the pancreatic duct.

limited mobility of the PV caused by an intact PV–splenic vein confluence.

Other Vascular Resections

Other than resection and reconstruction of the SMPV confluence, VRs were rarely performed. Hepatic artery resection and reconstruction were performed at the time of PD when limited tumor involvement of the origin of the gastroduodenal artery necessitated resection of a short segment of the common and/or proper hepatic arteries. A primary anastomosis or a reversed saphenous vein graft was used to repair the hepatic artery. If a replaced right hepatic artery (arising from the SMA) was inseparable from the primary tumor, it was resected. The need for revascularization was based on the underlying hepatic arterial anatomy and the extent of back-bleeding in the distal artery. Resection of the anterior wall of the IVC was performed when, at the time of Kocher maneuver, the surgeon could not separate the posterior aspect of the tumor from the IVC.

Pathologic Analysis

Since July 1990, a standardized system for the pathologic evaluation of PD specimens has been used at our institution.[12] Tumor size (maximal transverse diameter) was recorded at the time of pathologic evaluation of the PD specimen. This measurement was difficult or impossible to make in some cases due to preoperative treatment if gross tumor could not be distinguished from uninvolved adjacent pancreatic parenchyma. Evaluation of the status of the RP margin of resection was performed prospectively in all surgical specimens. The RP margin was defined as the soft-tissue margin directly adjacent to the proximal 3–4 cm of the SMA. Early in our experience, the

RP margin was evaluated by microscopic examination of an en face section measuring approximately 2 mm in thickness; the margin was recorded as positive (R1, microscopically positive following a gross complete resection) or negative (R0, microscopically negative) for carcinoma. For the past 5 years, the RP margin was evaluated according to the sixth edition of the AJCC *Cancer Staging Manual*[9] and illustrated in Fig. 2. The RP margin was recorded as positive (R1) or negative (R0) for tumor; if negative, the distance in millimeters from the tumor to the inked margin was recorded. Margins interpreted as suspicious for carcinoma (n = 2) were considered positive (R1) for the purposes of this analysis. Histologic evidence of tumor cell invasion of the segment of resected vein was defined as tumor cell infiltration of the tunica adventitia and/or tunica media of the vein wall. Tumor cell abutment without histologic evidence of invasion of at least the tunica adventitia was not considered vein invasion.

Operative Details and Perioperative Complications

Surgical time was recorded from the anesthesia record and defined as the time from incision to application of the final wound dressing. Intraoperative blood loss and intraoperative transfusions of red blood cells were also recorded from the anesthesia record, not the operative report. Major postoperative complications were defined as previously described and included perioperative death (within the first 30 days after surgery or during the original hospital stay if longer than 30 days); need for reoperation; clinically evident pancreaticojejunal anastomotic leak (as defined by drain amylase >2.5 times the upper limit of normal and clinical symptoms including fever, leukocytosis, fistula, or abscess); intra-abdominal hemorrhage; intra-abdominal fluid collection (sterile collection or abscess); myocardial infarction or sudden cardiac death; pulmonary complications including pneumonia; gastrointestinal bleeding; and sepsis syndrome.[13] Regarding the incidence of clinical pancreatic anastomotic leaks, it is important to note that the pancreatic anastomosis was rarely drained in our more contemporary experience. Therefore, a pancreatic anastomotic leak would not be clinically evident in the absence of the need for percutaneous drainage or reoperation. Hospital stay was calculated by considering the day of surgery as day 1, and the day of discharge was not counted as a hospital day.

Adjuvant Therapy

Preoperative and/or postoperative chemoradiation included either protocol-based or off-protocol treatment. Radiation therapy was delivered using either a standard-fractionation (50.4 Gy in 28 fractions) or a rapid-fractionation (30 Gy in 10 fractions) regimen. Concomitant chemotherapy included 5-fluorouracil, paclitaxel, or gemitabine.[14–16]

Statistical Analysis

All data analyses were performed with SPSS version 11.5 software (SPSS, Inc., Chicago, IL). The χ^2 test was used to compare categorical variables. Independent *t* tests and Mann Whitney μ tests were used to evaluate continuous variables. Survival and follow-up were calculated from the time of initial cytologic or histologic diagnosis to date of death or last follow-up. All deaths from any cause, including perioperative deaths, were included in our survival analysis and subsequent multivariate analysis. Overall survival was estimated using the method of Kaplan and Meier.[17] The log-rank test was used to evaluate differences between survival curves. A value of $P < 0.05$ was considered statistically significant.

Univariate and multivariate analyses of the effects of potential prognostic factors on survival were done using a Cox proportional hazards regression. Covariates included age, gender, tumor size, T and N status, need for reoperative PD, presence of a major complication, operative blood loss, RP margin status, use of adjuvant therapy (preoperative and/or postoperative), need for major VR and histologic evidence of venous invasion.

To evaluate the unconfounded effect of VR on RP margin status, a logistic regression was performed to examine the impact of age, gender, performance of VR, tumor size, N status, and use of preoperative therapy on RP margin status.

RESULTS

During the study period, 572 patients underwent PD for all histologic diagnoses, and 141 (25%) required major VR (Table 1). Resection of the SMV, PV, or SMPV confluence was performed in 126 (89%) of the 141 patients. These venous resections included V1 in 36, V2 in 24, V3 in 15, V4 in 11, and V5 in 40. Segmental resection of the hepatic artery, with or without interposition grafting, was performed in 17 (12%) of the 141 patients; 7 of these also underwent concomitant venous resection and reconstruction. Resection of the anterior wall of a portion of the IVC was performed in six patients (4%), of whom one also underwent concomitant venous resection and reconstruction.

Operative characteristics and perioperative complications for the 141 patients who required VR are listed in Table 2. There were three perioperative

Table 1. Demographics, tumor histology, and type of vascular resection in the 141 patients who underwent pancreaticoduodenectomy with vascular resection

Variable	No. of patients (%)
Gender	
Male	89 (63)
Female	52 (37)
Age (yr)	
Median	62.4
Range	23–81
Histology	
Adenocarcinoma	
Pancreas	111 (79)
Bile duct	3 (2)
Duodenum	3 (2)
Neuroendocrine carcinoma	9 (6)
Other carcinoma	5 (3.5)
Sarcoma	4 (3)
Lymphoma	1 (1)
Benign disease	5 (3.5)
Type of vascular resection	
V1	36 (26)
V2	24 (17)
V3	15 (11)
V4	11 (8)
V5	40 (28)
Hepatic artery	17 (12)*
Inferior vena cava	6 (4)*

*Concomitant venous resection and reconstruction was performed in 7 patients who underwent hepatic arterial resection and 1 patient who underwent inferior vena cava resection. See text for explanation of V1–V5.

Table 2. Operative characteristics and perioperative complications in 141 patients who underwent pancreaticoduodenectomy with vascular resection

Variable	
Median estimated blood loss (range)	1675 ml (250–14,200)
Median operative time (range)	603 min (244–1340)
Median hospital stay (range)	13 days (2–108)
	No. of patients (%)
Perioperative death	3 (2)
Major perioperative complication*	29 (21)
Reoperation	4 (3)
Pancreaticojejunal anastomotic leak	2 (1)
Intra-abdominal hemorrhage	3 (2)
Intra-abdominal fluid collection: sterile	3 (2)
Intra-abdominal fluid collection: abscess	5 (2)
Myocardial infarction	3 (2)
Pulmonary complications	14 (10)
Gastrointestinal bleeding	6 (4)
Sepsis syndrome	1 (1)

*Some patients had more than one complication.

deaths, for a mortality rate of 2.1%. Major perioperative complications occurred in 29 patients (21%). Reoperation was necessary in four patients; three of them had intra-abdominal hemorrhage. In the other patient, the biliopancreatic and gastrointestinal reconstruction was delayed for approximately 36 hours (necessitating a second laparotomy) because of bowel edema caused by a prolonged period of venous occlusion at the time of venous resection and reconstruction. At reoperation, there was no evidence of intestinal ischemia, and the patient had an uneventful recovery. A clinically evident pancreatic anastomotic leak occurred in 2 (1.4%) of 141 patients. This low number is clearly influenced by the infrequent placement of an abdominal drain in proximity to the pancreatic anastomosis (and therefore the inability to measure drain fluid amylase) in our more recent experience. The median hospital stay was 13 days.

PD was performed for adenocarcinoma of pancreatic origin in 291 (51%) of the 572 patients: 181 (62%) of the 291 patients underwent standard PD, and 110 (38%) required VR and reconstruction. A comparison of the two groups appears in Table 3. The median age in both groups was 63.9 years (range, 30.0–83 years for the two groups). Among the 110 patients who required VR, venous resection was performed in 100 patients and consisted of V1 in 29, V2 in 21, V3 in 10, V4 in 10, and V5 in 30. Of these 100 patients, 3 required concomitant resection of the hepatic artery, and 1 required concomitant resection of the anterior surface of the IVC. In the remaining 10 patients, 8 required isolated hepatic artery resection and reconstruction, and 2 required isolated resection of the anterior surface of the IVC. The performance of VR and reconstruction was associated with a greater likelihood of the surgery being a reoperative PD after an unsuccessful attempt at PD before referral, increased intraoperative blood loss, larger tumor size, and a greater likelihood of having a microscopically positive RP margin (R1 resection). Not shown in Table 3 is that T3 tumors were more common in the VR group (82% versus 64%, $P = 0.001$), consistent with the larger tumor diameter in the patients who required VR.

A multivariate logistic regression was used to examine the impact of the following variables on the outcome of RP margin status: age, sex, reoperative status, estimated blood loss, performance of VR,

Table 3. Univariate analysis of demographic, operative, pathologic, and treatment characteristics for 291 patients who underwent pancreaticoduodenectomy (PD) for pancreatic adenocarcinoma

	Vascular resection	Standard PD	*P* value
Total No. of patients	110	181	
Gender, n (%)			
Male	69 (63)	106 (59)	0.48
Female	41 (37)	75 (41)	
Median (mean) age (yr)*	63.9 (62.1)	63.9 (63.4)	0.23
Range	41–81	30–83	
Reoperative PD, n (%)	27 (25)	25 (14)	0.02
Operative blood loss (ml) *†			
Median (mean)	1600 (1829)	800 (923)	<0.001
Range	250–6000	100–2900	
Tumor size (cm)*‡			
Median (mean)	3.0 (3.2)	2.8 (2.8)	<0.001
Range	1.0–6.0	0.2–6.0	
Positive retroperitoneal margin (R1), n (%)	24 (22)	21 (12)	0.02
Positive lymph nodes (N1), n (%)	50 (45)	95 (52)	0.25
Major perioperative complication, n (%)	20 (18)	39 (22)	0.43
Perioperative death	1 (1)	2 (1)	0.86
Hospital stay (days)*§			
Median (mean)	13.5 (15.5)	12.0 (13.9)	0.01
Range	7–108	5–70	
Intraoperative radiation therapy, n (%)	31 (28)	73 (40)	0.04
Adjuvant therapy (preoperative or postoperative), n (%)	97 (88)	165 (91)	
Preoperative chemoradiation	82 (75)	127 (70)	0.51
Postoperative chemoradiation	25 (23)	50 (28)	0.34

*Mann-Whitney U test used.
†Estimated blood loss was not recorded in the anesthesia record for 8 patients.
‡Tumor size could not be accurately assessed at the time of pathologic evaluation of the pancreaticoduodenectomy specimen in 27 patients due to the effect of neoadjuvant therapy.
§$P > 0.05$ if the 10% of patients with the lowest and highest lengths of hospital stay are excluded from analysis.

tumor size, N stage, and use of neoadjuvant therapy. After adjusting for these variables, only tumor size and the use of neoadjuvant therapy, not the performance of VR, had significant effects on RP margin status. Tumor size was associated with a significantly increased risk of a microscopically positive RP margin (odds ratio [OR] = 1.5, 95% confidence interval [CI] = 1.1–2.0), and neoadjuvant treatment was associated with a significantly decreased risk of a microscopically positive RP margin (OR = 0.47; 95% CI = 0.23–0.96).

Log-rank tests were used to compare Kaplan-Meier survival curves for each prognostic factor of interest among the 291 patients with pancreatic adenocarcinoma (Table 4). The median overall survival for the 291 patients was 24.9 months. On univariate analysis, the only significant predictor of decreased survival was the presence of lymph node metastases, with a median survival of 21.1 months for patients with N1 disease compared with 31.9 months for patients with N0 disease ($P = 0.005$). The median survival was 23.4 months for the group who required VR and 26.5 months for the group who underwent standard PD ($P = 0.18$). Kaplan-Meier survival curves for patients who underwent VR and who underwent standard PD are displayed in Fig. 3.

Histopathologic evaluation of the resected segment of SMV, PV, or SMPV confluence was performed in 62 of the 100 patients who underwent venous resection. In the remaining 38 patients, the surgeon did not identify the specimen as containing a portion of the SMPV confluence and so an evaluation was not done, or the pathologist did not include information about this evaluation in the final pathology report. Such information cannot be retrieved retrospectively. Among the 62 patients who had histopathologic evaluation of the resected venous segment, tumor cell invasion of the vein wall was present in 38 patients (61%) and absent in 24 (39%). The median survival did not differ between patients who did and did not have histopathologic evidence of vein invasion (Table 4).

Table 4. Univariate analysis of factors influencing median survival in 291 patients who underwent pancreaticoduodenectomy (PD) for pancreatic adenocarcinoma

Prognostic variable	No. of patients	Median survival (mo)	95% Confidence interval	*P* value
Overall	291	24.93	21.40–28.46	—
Gender				
Male	175	23.10	19.05–27.15	0.47
Female	116	26.97	22.43–31.50	
Extent of PD				
Standard PD	181	26.50	21.11–31.89	0.18
PD with vascular resection	110	23.43	19.50–27.37	
Reoperative PD*				
No	238	24.93	20.68–29.18	0.23
Yes	52	25.13	17.88–32.39	
T status				
Tis†	4	—	—	0.22
T1	25	30.77	16.61–44.92	
T2	56	25.87	20.27–31.46	
T3	206	23.70	19.94–27.46	
N status				
N0	146	31.93	24.57–39.30	0.005
N1	145	21.07	17.40–24.73	
Retroperitoneal margin				
R0	246	26.50	22.29–30.71	0.14
R1	45	21.37	17.05–25.68	
Histopathologic evidence of vein invasion‡				
No	24	19.67	10.30–29.03	0.66
Yes	38	19.83	13.34–26.33	
Major complication*				
No	228	26.50	20.18–32.82	0.11
Yes	59	24.40	18.51–30.29	
IORT				
No	187	24.93	20.60–29.27	0.85
Yes	104	24.47	16.78–32.16	
Adjuvant therapy*				
No	29	18.50	9.48–27.52	0.92
Yes (preoperative or postoperative)	261	25.13	21.42–28.85	
Preoperative (neoadjuvant) therapy				
No	80	25.37	19.56–31.18	0.46
Yes	209	24.93	20.44–29.43	
Postoperative therapy				
No	215	24.47	20.39–28.54	0.42
Yes	75	26.50	13.30–39.70	

*Missing data were secondary to transition from paper to electronic medical record when the prospective database was being developed and included reoperative PD (1 patient), major complication (4 patients), preoperative therapy (2 patients), and postoperative therapy (1 patient).
†For Tis, 3 of 4 patients censored (1 patient died at 39 months), so a median survival was not calculated. All 4 patients received preoperative (neoadjuvant) therapy; 2 of the 4 had a complete pathologic response and 2 had only in situ carcinoma at the time of permanent pathologic evaluation of the surgical specimens. All 4 patients had biopsy proved invasive adenocarcinoma before neoadjuvant therapy. If Tis (n = 4) is excluded, $P = 0.52$ for remaining T1–T3 stages.
‡Of 100 patients, 62 had information available.
IORT = intraoperative radiation therapy.

We performed a multivariate analysis of the effect of all potential prognostic factors on survival in patients undergoing PD for pancreatic adenocarcinoma. To assess the impact of prognostic variables such as VR, all variables except histopathologic evidence of vein invasion were included in the Cox proportional hazards model for the first analysis (Table 5). Evidence of vein invasion was excluded from the multivariate analysis because results were available for only 62 patients. Thus, inclusion of vein invasion in the multivariate analysis would have dramatically decreased its power to assess the effects of other

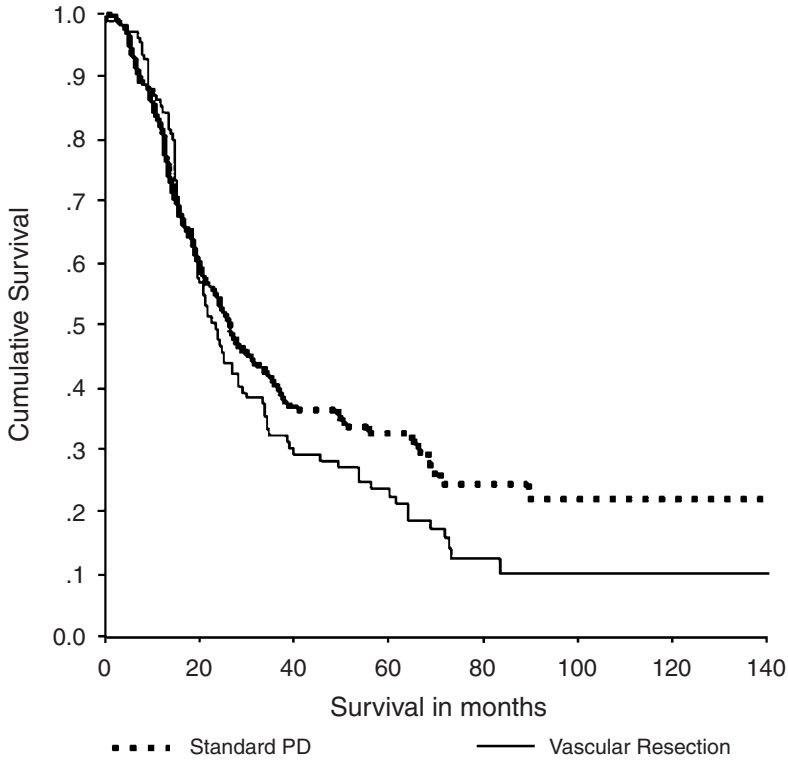

Fig. 3. Kaplan-Meier survival curves in patients with pancreatic adenocarcinoma who underwent standard pancreaticoduodenectomy (PD) or PD with vascular resection and reconstruction. Median survival for standard PD was 26.50 months. Median survival for PD with vascular resection was 23.43 months. Log-rank test: $P = 0.18$.

covariates. In the first multivariate analysis performed (n = 291), the presence of N1 disease was identified as a significant predictor of decreased survival (hazard ratio [HR] = 1.50, $P = 0.01$). The only other prognostic factor of significance was the occurrence of one or more major perioperative complication(s) (including perioperative deaths), which was associated with a significantly decreased survival (HR = 1.52, $P = 0.024$). These results were consistent when the multivariate analysis was repeated for the 229 patients for whom vein invasion data were not available; presence of lymph node metastases (HR = 1.6, $P = 0.012$) and major perioperative complication(s) (HR = 1.8, $P = 0.007$) were the only statistically significant covariates. VR was not associated with decreased survival in either multivariate analysis (for n = 291 analysis, HR = 1.1, $P = 0.499$; for n = 299 analysis, HR = 0.759, $P = 0.276$). Finally, a third multivariate analysis was performed including only the 62 patients who had histologic vein invasion data available. The results were also similar. However, in this small subgroup, any preoperative or postoperative adjuvant treatment proved protective (HR = 0.049, $P = 0.007$), and nodal metastases lost significance (HR = 0.90, $P = 0.78$). Because all 62 patients who had histologic vein invasion data available had VRs, the effect of VR could not be assessed in this smaller group.

Finally, all postoperative CT scan reports within the first year of operation were reviewed to detect the presence of early occlusion of the SMV, PV, or SMPV confluence. Of the 126 patients who underwent SMV-PV reconstruction, at least one postoperative CT scan, transabdominal ultrasound, or vascular radiographic study was available for 116 (92%); 8 patients (6.9%) were noted to have venous occlusion.

DISCUSSION

VR and reconstruction at the time of PD remains controversial because of the complexity and magnitude of the operative procedure combined with the aggressive biologic behavior of pancreatic adenocarcinoma, which results in a short survival duration for most patients, even those who undergo a potentially curative PD. Therefore, physicians are appropriately hesitant to accept an operative approach

Table 5. Multivariate analysis of factors that may influence survival in 291 patients who underwent pancreaticoduodenectomy (PD) for pancreatic adenocarcinoma

Covariate	No. of patients	No. of deaths	Hazards ratio	95% Confidence interval	*P* value
Gender					
Male	175	126	1.00		
Female	116	84	0.93	0.67–1.29	0.64
Age of surgery (yr)	—	—	1.01	0.99–1.03	0.35
Reoperative PD					
No	238	166	1.00		
Yes	52	43	1.09	0.72–1.66	0.67
Vascular resection					
No	181	120	1.00		
Yes	110	90	1.13	0.79–1.63	0.50
Blood loss (ml)	—	—	1.0	1.00–1.00	0.45
Tumor size (cm)	—	—	0.95	0.82–1.11	0.54
T stage (AJCC)*					
Tis	4	1	1.00		
T1	25	15	0.77	0.10–6.12	0.81
T2	56	43	1.12	0.15–8.34	0.92
T3	206	151	1.13	0.15–8.35	0.90
Nodal metastasis					
No	146	97	1.00		
Yes	145	113	1.50	1.10–2.05	0.01
Retroperitoneal margin					
No	246	173	1.00		
Yes	45	37	1.16	0.77–1.76	0.47
Major perioperative complication					
No	228	159	1.00		
Yes	59	47	1.52	1.06–2.19	0.02
Any adjuvant treatment					
No	29	21	1.00		
Yes	261	188	0.96	0.41–2.24	0.93
Preoperative treatment					
No	80	60	1.00		
Yes	209	149	1.18	0.62–2.25	0.62
IORT					
No	187	126	1.00		
Yes	104	84	1.00	0.72–1.38	0.98
Postoperative treatment					
No	215	154	1.00		
Yes	75	55	0.95	0.54–1.66	0.85

*Hazards ratios not significantly changed when Tis category is excluded (n = 4) and T1 category is used as reference.
IORT = intraoperative radiation therapy.

that may be associated with increased patient morbidity and mortality. In addition, confusion remains over the indications for vascular resection of the SMV or SMPV confluence. Venous resection was initially performed as part of a regional pancreatectomy in an effort to maximize lymphatic and soft tissue resection.[5] Subsequent clinical investigation demonstrated that a wider or more extensive lymphadenectomy at the time of PD for pancreatic adenocarcinoma has little impact on survival duration.[6,18] Therefore, many physicians simply classify patients with suspected isolated tumor involvement of the SMV, PV, or SMPV confluence (on CT or at the time of laparotomy) as having locally advanced disease. Such patients have a median survival of 10–12 months,[19] far inferior to the survival duration of almost 2 years reported herein.

Our experience with resection and reconstruction of the SMV, PV, or SMPV confluence has been restricted to those patients in whom tumor adherence to these venous structures prevented the surgeon from mobilizing the SMPV confluence from the pancreatic head and uncinate process, as is necessary for standard PD. The assessment of tumor adherence to the

SMPV confluence is a judgment made at the time of surgery, and final pathologic evaluation of the surgical specimen will not demonstrate tumor infiltration of the vein wall in all cases (61% in this series). If dissection along the periadventitial plane of the SMV or PV is unable to separate the vein from the tumor, venous resection and reconstruction is the only way to accomplish a complete resection. To what degree peritumoral inflammation is enhanced secondary to the neoplastic process or endoscopic intervention is not known. Similarly, little is known about the effect of chemoradiation on the tumor–vessel interface. Importantly, venous resection was not performed in an effort to achieve a greater degree of lymphatic clearance. In addition, the pancreatic tumors in all patients in this series fulfilled a CT-based definition of resectability that includes the absence of tumor extension to the celiac axis and SMA. Our series is unique in incorporating strict preoperative radiographic criteria for local tumor resectability; patients whose primary tumors did not meet this definition did not undergo PD. Such criteria are necessary to avoid the inclusion of patients with grossly incomplete resections (gross residual disease; R2 resection). Survival duration in this group will be affected more by the failure to remove all gross tumor than by other potential prognostic variables.

VR and reconstruction were required in 141 (25%) of 572 patients who underwent PD during a 12-year period. Although the proportion of patients who required VR might seem high, patients were not refused operation based on the presence of tumor-induced narrowing of the SMV, PV, or SMPV confluence as long as there was no evidence of tumor extension to the celiac axis or SMA. Previous studies from our institution have demonstrated that approximately 80% of patients taken to surgery for planned PD are able to undergo PD, even those with pancreatic adenocarcinoma; this finding attests to the high accuracy of radiographic imaging studies and the value of strict objective anatomic criteria in predicting resectability.[20] If objective criteria for resectability, as used in this and other studies from our institution, become more widely adopted, especially in the conduct of future clinical trials, it should not be unexpected that 20–25% of patients with otherwise resectable pancreatic cancer will be found to have isolated venous involvement. As demonstrated in this report, such patients have a survival duration similar to those without venous involvement if treated with PD as part of a multimodality approach to their disease.

Perioperative death occurred in 3 (2%) of 141 patients who underwent VR and reconstruction. Although 21% of VR patients experienced a major perioperative complication, only 3% required reoperation and only 1.4% experienced a clinically significant pancreaticojejunal anastomotic leak. Importantly, this report represents our entire institutional experience with such complicated pancreatic surgeries. As can be seen in Figs. 4 and 5, perioperative blood loss and operative time have declined over the past decade as we have gained experience and refined various technical aspects of the operative procedure. PD with VR and reconstruction is clearly more complicated than standard PD and should be undertaken only by surgeons and at institutions experienced with this operation.

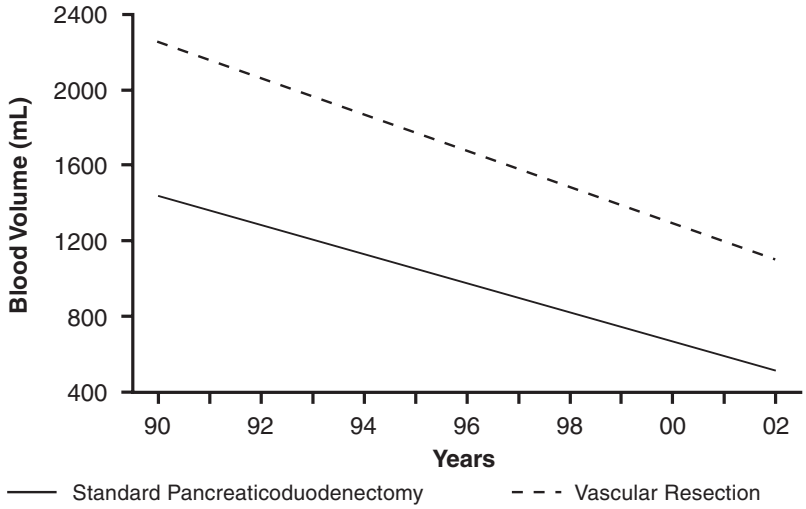

Fig. 4. Median estimated blood loss per year for standard pancreaticoduodenectomy and pancreaticoduodenectomy with vascular resection during the time of this report.

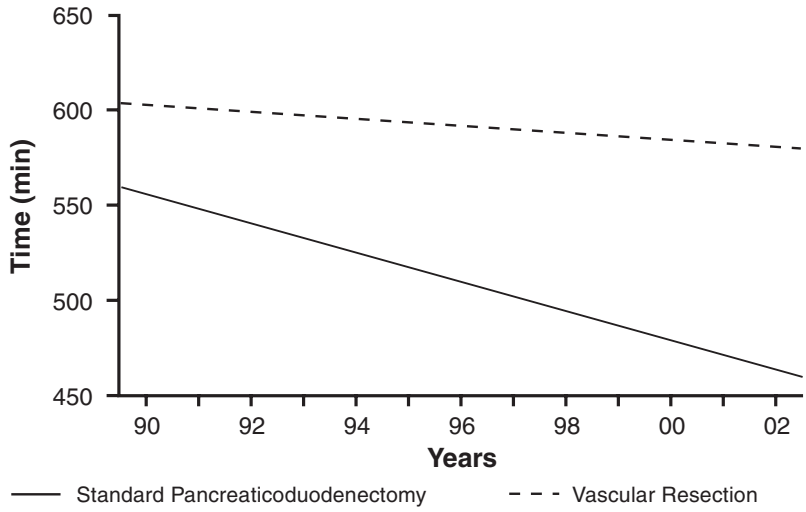

Fig. 5. Median operative time per year for standard pancreaticoduodenectomy and pancreaticoduodenectomy with vascular resection during the time of this report.

In the analysis of 291 patients who underwent PD for adenocarcinoma of pancreatic origin, we found no difference in survival duration between those who did and those who did not require VR and reconstruction (see Fig. 3). This finding is particularly striking given the fact that patients who underwent VR and reconstruction were more likely to have undergone a previous unsuccessful attempt at PD before referral and had larger tumors (see Table 3). In contrast to our previous report, reflective of our early experience, the larger sample size presented in this report revealed that R1 resections were more common in patients who required VR (22%) compared to standard PD (12%).[21] However, after adjustment for tumor size, no significant difference in R1 resections remained, suggesting that the finding of a positive RP margin was a function of tumor size. Importantly, the increased frequency of R1 resections in those who required VR did not translate into a significant survival difference compared to patients who underwent standard PD.

Unique to this series was the absence of grossly incomplete (R2) resections (attributable to accurate preoperative determination of resectability) and the prospective evaluation of the RP margin to allow accurate determination of R status. Such data are not available in many other reports of venous resection at the time of PD[22–31] (Table 6). In the absence of prospective evaluation of the RP margin, reports of venous resection during PD are impossible to interpret. When venous involvement is an unexpected finding at the time of PD, surgeons will often attempt to separate the SMPV confluence from the pancreatic

Table 6. Report of pancreaticoduodenectomy with venous resection in the Western literature

First author (year)	No. of patients	Operative mortality (%)	Median survival (mo)	No. with positive margin (%)
Capussotti[22] (2003)	22	0	NA	5/6 (83)*
Howard[23] (2003)	13	8	13	3 (23)
van Geenen[24] (2001)	34	0	14	20 (59)
Bachellier[25] (2001)	21	3.2	12	8 (38)
Roder[26] (1996)	22	0	8	15 (68)
Harrison[27] (1996)	42	2	13	10 (24)
Yeo [28] (1995)	10	NA	NA	NA
Launois[29] (1993)	9	0	6.1†	NA
Trede[30] (1990)	12	0	NA	NA
Sindelar[31] (1989)	20	20	12	NA

NA = not available/not reported.
*Retroperitoneal margin assessed in 6 of 22 patients.
†Value is given as mean.

head. When this maneuver is unsuccessful, the surgeon is left with either a grossly positive margin or an inadvertent venotomy. Venous injury often results in uncontrolled hemorrhage and the necessity for rapid removal of the tumor without proper attention to the SMA dissection; such cases often also result in an R2 resection. Therefore, studies that retrospectively examine the presence or absence of VR as a prognostic factor for survival should include only those patients who have undergone a complete gross resection (R0 or R1). Patients who have undergone an R2 resection have a predictable course: local and eventually distant recurrence will limit survival duration. It is inappropriate, therefore, to include such patients in an analysis of prognostic factors predictive of survival duration under the assumption that they have undergone complete tumor removal. The current edition of the AJCC *Cancer Staging Manual*[9] emphasizes the importance of the R designation in all pathology reports. The surgeon should document the presence or absence of a complete gross resection at the time of PD, and if the RP margin is microscopically positive, the histopathology report should not be finalized until the pathologist has reviewed the operative note and can accurately apply an R1 or R2 designation.

In interpreting the RP margin results in this series, it must also be noted that multimodality therapy was delivered to almost all patients with adenocarcinoma (88% of the VR group and 91% of the standard PD group). The extent to which chemotherapy and/or chemoradiation may abrogate the negative biologic effect of an R1 resection is unknown at the present.

Multivariate analysis demonstrated that only positive regional lymph nodes and major perioperative complications were associated with a significant decrease in survival duration. All potential prognostic factors were included in this analysis except for the presence or absence of histologic evidence of invasion of the vein wall, which was available for only 62 patients. To confirm that excluding vein invasion did not change the results of the multivariable analysis, two additional analyses were performed: the multivariate analysis was repeated excluding the 62 patients for whom vein invasion data were available and again for these 62 patients. In all applicable multivariate analyses performed, the need for VR and the status of the RP margin did not have a significant effect on survival.

In summary, VR and reconstruction at the time of PD add significant complexity to an already lengthy operation associated with significant morbidity and occasional mortality. However, our data suggest that with proper patient selection and surgeon experience, VR and reconstruction can be performed safely. In patients with adenocarcinoma of pancreatic origin, the need for VR does not significantly impact survival duration. The median survival of patients who underwent VR was 23 months, approximately 1 year longer than the survival of patients believed to have locally advanced, surgically unresectable pancreatic cancer and treated nonsurgically.

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Discussion

Dr. John Hoffman (Philadelphia, PA): Jennifer, you did a marvelous presentation of a very difficult set of data. I thank you for giving me the manuscript early. You have shown these data representing, to my knowledge, the largest series of major vascular resections for pancreatic cancer in the world. We have certainly come a long way since the 1970s, when any kind of tumor encroachment upon the vein indicated unresectability. I have a few questions.

For patient selection, you have not totally defined what would make a tumor unresectable with respect to venous involvement. What about the small segmental occlusion with collaterals? What about severe bilateral vein narrowing? How many cases of advanced venous involvement such as this were included in your series? The outstanding results probably mean that this is a group with less advanced cancer than most other series of vein resection. Second, what was the accuracy of CT prediction of venous involvement and the need for resection? How many with resection had normal CT scans? You had patients with histologic venous invasion and those without. Was the CT able to predict histologic involvement? You have shown in a subset of patients with histologic investigations that there is no survival difference between those with actual microscopic invasion and those without. Are the numbers really large enough to give you enough statistical power to determine that?

Did you look at depth or extent of venous invasion as a risk factor? Were any of the veins involved at the cut margin of the vein? You have shown that those with vein resection have a statistically significant increase in positive retroperitoneal or SMA margins, at least in the univariate analysis. Could you tell us if the correlation was any stronger between those with histologic vein involvement and retroperitoneal (RP) margin involvement positivity? In other words, did those veins that were actually involved have a higher incidence of SMA margin involvement?

Are any of the other RP surgical margins examined at M. D. Anderson such as the portal, SMV, and posterior RP margins, and do they make a difference if you have looked at them? Most series of vein resections show them to comprise maybe 10% or 15% of the total resectional experience. You have major venous resections in 38% of your operations, plus other operations where you are actually resewing the vein where you have resected some of it, and you counted those as nonvenous resections. So it is a

higher likelihood to have a vein resected at M. D. Anderson than I believe at other hospitals.

Last question. This may be unfair to ask of you, but Dr. Evans has preached for years that a positive RP margin means stage IV disease in which the resection shouldn't have been done, yet you have shown no difference in survival between those with positive and negative margins. Has this finding changed his thinking and advice?

Dr. Tseng: Thank you, Dr. Hoffman. Regarding short segment vein occlusion, it is uncommon to see occlusion of the SMV or SMV–portal vein confluence in the absence of SMA involvement due to the close proximity of the SMV to the SMA. In addition, it is very rare to see short segment occlusion of the SMV with an adequate venous segment above (portal vein) and below (SMV) to allow successful venous interposition grafting. Therefore, because patients with isolated short segment SMV occlusion, and no extension to the SMA and suitable anatomy for interposition grafting, are so rare, the majority of patients with an occluded SMV or SMV–portal vein confluence have a locally advanced, surgically unresectable primary tumor.

The second question referred to CT prediction of vein invasion. In our previous publication by Bold et al. that was also presented to this society, CT predicted the need for venous resection in 84% of patients. Pathologic analysis revealed tumor invasion of the vein wall in 71% of resected specimens. In the current report, of the 62 patients who had histopathologic evaluation of the resected venous segment, tumor cell invasion of the vein wall was present in 38 patients (61%) and absent in 24. Median survival did not differ between patients who had histopathologic evidence of vein invasion and those who did not. This is not surprising considering that the tumor has access to the systemic circulation long before the patient is diagnosed. We did not look at the depth of the vein invasion.

Third, regarding the RP margin analysis, after adjusting for age, gender, reoperative status, estimated blood loss, performance of vascular resection, tumor size, N stage, and use of neoadjuvant therapy, only tumor size, with an odds ratio of 1.5, and the use of neoadjuvant therapy, with an odds ratio of 0.47, were associated with a positive RP margin (neoadjuvant therapy protective), but not the performance of vascular resection.

Regarding our pathologic evaluation of pancreaticoduodenectomy specimens, we follow the sixth edition of the AJCC *Cancer Staging Manual*, and prospectively evaluate the RP margin as outlined by the AJCC and the UICC. While I agree that it is reasonable to also focus on other margins such as the soft tissue margin posterior to the pancreatic head and anterior to the IVC, the RP margin (you will agree) is the margin most likely to be positive when an R1 or R2 resection is performed.

With respect to your question that vein resections are more commonly performed at M. D. Anderson, as you know, we use an objective CT-based definition of "resectable." We do not refuse patients who have significant venous involvement if their primary tumor is resectable based on the absence of metastatic disease and the absence of arterial involvement (as discussed in detail in the manuscript). I suspect these patients would not be offered an operation at many other institutions. The substantial number of reoperative pancreaticoduodenectomies performed at our institution supports this hypothesis.

You are correct that we defined venous resection as the need for a patch or a segmental resection. Nonsegmental resections repaired without a patch were not considered venous resections. This thereby removed subjective analysis from the definition of venous resection.

Last, you asked about the significance of a positive RP margin. Our data on R1 resections, as discussed in detail by Dr. Raut yesterday at the meeting of the Pancreas Club, are unique, due to the prospective evaluation of the RP margin. We are not aware of any other large series, which has such rigorous margin analysis. Our data do suggest that multimodality therapy and careful attention to surgical technique may abrogate the negative biologic effect of an R1 resection. However (and this is an emphatic however), this does not mean that R2 resections with gross tumor left at the RP margin would achieve the same result.

Thank you very much.

Dr. Sukamal Saha (Flint, MI): If I am not mistaken 70% of your patients in both groups had neoadjuvant treatment?

Dr. Tseng: Yes.

Dr. Saha: So at this point, what is your criteria for not giving somebody neoadjuvant, and from your historical control, is it because your data are better because neoadjuvant treatment has been given to 70% of the patients or because of your surgical techniques?

Dr. Tseng: Our goal at M. D. Anderson is to treat all patients with pancreatic cancer on a clinical trial. We always have an open neoadjuvant and adjuvant trial; if a patient with resectable pancreatic cancer has tissue confirmation of adenocarcinoma, they are encouraged to enroll in the current neoadjuvant protocol.

Dr. Saha: But 30% of patients you did not.

Dr. Tseng: If we do not have a positive diagnosis of pancreatic adenocarcinoma, then we proceed with surgery first.

Risk Factors and Outcomes in Postpancreaticoduodenectomy Pancreaticocutaneous Fistula

John W. Lin, M.D., M. Eng., John L. Cameron, M.D., Charles J. Yeo, M.D., Taylor S. Riall, M.D., Keith D. Lillemoe, M.D.

A significant fraction of patients undergoing pancreaticoduodenectomy develop a postoperative pancreaticocutaneous fistula. To identify risk factors for this complication and to delineate its impact on patient outcomes, we conducted a retrospective review of 1891 patients undergoing pancreaticoduodenectomy between 1981 and 2002. Overall, 216 patients (11.4%) developed a postoperative pancreaticocutaneous fistula. In univariate analysis, gender, coronary disease, diabetes mellitus, operative times, blood loss, radical lymphadenectomy, gland texture, and specimen pathology correlated with fistula rates. In a multivariate model, however, only gland texture and coronary disease were statistically predictive. A soft gland was associated with a 22.6% fistula rate, a 20.4-fold increase in fistula risk over those patients with a medium or firm gland (95% confidence interval, 4.7–90.9). No patient with a firm gland developed a fistula. Although 30-day postoperative mortality was not different between those patients with and those without fistula (1.4% versus 1.5%), the mean length of stay was longer (26.0 days versus 13.2 days) and the rates of certain complications were increased in those patients with fistula. In this single-institution experience, pancreaticocutaneous fistula was most strongly predicted by pancreatic texture. Choice of anastomotic technique did not correlate with fistula rates. Pancreaticocutaneous fistula increases postoperative length of stay and morbidity but was not directly associated with increased postoperative mortality. (J GASTROINTEST SURG 2004;8:951–959)

KEY WORDS: Pancreaticoduodenectomy, pancreatic fistula, morbidity, mortality

Despite recent advances in operative technique and postoperative patient management, a significant fraction of patients undergoing pancreaticoduodenectomy develop a postoperative pancreaticocutaneous fistula. Recent large series have reported failure of the pancreaticoenteric anastomosis in 9%–18% of patients worldwide,[1–6] a complication rate not far improved from Dr. Whipple's report of a 19.5% fistula rate more than 50 years ago.[7] Although the physiologic and anatomic characteristics of the pancreas confer unique difficulties in anastomotic healing, the fact that leak rates of 10% would be considered intolerable for most other gastrointestinal anastomoses has stimulated continuing investigation into this surgical problem.

A number of methods for reducing the incidence of pancreaticocutaneous fistula have been proposed and tested. Many of these involve technical features of the anastomosis, including site of reconstruction (pancreaticogastrostomy versus pancreaticojejunostomy),[8,9] anastomotic technique (e.g., continuous versus interrupted sutures; duct-to-mucosa anastomosis versus stump invagination),[10,11] use of biologic adhesives,[12,13] and use of intraoperative transanastomotic stents[14] or drains.[15] In addition, given the likely contribution of zymogen-rich pancreatic juice to

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anastomotic failure, several groups have performed clinical trials evaluating the use of the somatostatin analogue octreotide as a prophylactic measure.[16,17] Some investigators have even advocated omitting the anastomosis entirely, with occlusion of the remnant duct[18] or drainage to create a predicted, controlled pancreaticocutaneous fistula.[19]

Most recent reports utilize conservative measures for the treatment of pancreaticocutaneous fistulas,[2,6] reflecting the increasing capabilities of interventional radiologists.[20] Prior retrospective analyses of surgical series have lent considerable insight into the risk factors predisposing a patient to a postpancreaticoduodenectomy pancreaticocutaneous fistula. Accumulated experience with pancreaticoduodenectomy has implicated several important risk factors: general health, pancreatic fibrosis, exocrine reserve, surgical volume, and pancreatic pathology.[8,21] Two factors, however, have complicated these retrospective analyses: (1) most reports describe fewer than 200 patients, thus limiting the statistical power and ability to interpret negative results; and (2) investigators have used a range of definitions for pancreaticocutaneous fistula, varying from very broad definitions (e.g., drainage of $\geqslant$10 ml/day of amylase-rich fluid after postoperative day 3[22]) to narrowly inclusive definitions (e.g., continued drainage of amylase-rich fluid after postoperative day 20[3]).

To further understand the risk factors contributing to this complication and to define its effects on patient outcomes, we conducted a retrospective review of the Johns Hopkins experience with postpancreaticoduodenectomy pancreaticocutaneous fistula.

METHODS

Of the 2149 patients undergoing pancreaticoduodenectomy at the Johns Hopkins Hospital between 1981 and 2002 inclusive, data regarding the presence or absence of postoperative pancreaticocutaneous fistula were available for 1987 patients. The 96 patients who underwent total pancreatectomy were excluded, with the remaining 1891 patients considered in further analyses. *Pancreaticocutaneous fistula* was defined as either a radiologically proved anastomotic leak or the continued drainage of amylase-rich fluid on or after postoperative day 10,[8] a clinically relevant definition similar to that used in other reports.[15,23] Operative technique, as well as standard prepancreaticoduodenectomy and postpancreaticoduodenectomy patient management at the Johns Hopkins Hospital, have been reported previously.[6] It has been our general practice to perform a two-layer pancreatic-enteric anastomosis and to leave operatively placed closed suction drains near, but not in contact with, the anastomosis.

Potential risk factors considered in univariate analysis included demographic factors, comorbidities, technical decisions by the surgeon, operative measures, and gland features. Primary outcome measures considered included 30-day postoperative mortality, postoperative length of stay, and the rate of common complications.

As complete demographic, operative, and outcome data were not available for all 1891 patients, numerator and denominator values are reported for each data point. The χ^2 tests were performed for univariate analyses of categorical values; mean values were compared by Student's t tests and medians by Mann-Whitney rank sum analyses. Logistic regression was performed for multivariate models with P values and 95% confidence intervals estimated by the Wald method. Statistics were computed with the StatView (SAS Institute) software suite. Statistical significance was defined at the $P < 0.05$ level. Data are presented, when appropriate, as mean $\pm$ SEM.

RESULTS

Of the 1891 evaluable patients undergoing pancreaticoduodenectomy between 1981 and 2002, 216 patients (11.4%) developed a postoperative pancreaticocutaneous fistula. Overall operative mortality (30-day mortality) was 1.6%, and overall morbidity (any complication) was 40.0%. Mean postoperative length of hospital stay was 13.5 $\pm$ 0.6 days in this longitudinal cohort of patients. Although surgical volume increased dramatically over the study period, there was no statistically significant change in the pancreaticocutaneous fistula rate over time (Mann-Whitney rank sum test, $P = 0.08$; Fig. 1).

Putative risk factors for the development of a postoperative pancreaticocutaneous fistula were conceptually divided into three broad categories: (1) patient-related factors, such as age, gender, race, and the presence of comorbidities; (2) surgeon-related factors, such as operative time, estimated blood loss, intraoperative blood transfusions, pancreatic anastomotic technique, and preoperative and postoperative biliary stenting; and (3) disease-related factors, including the texture of the pancreatic remnant at the transection site and the pathologic diagnosis.

Among *patient-related factors*, patients with a postoperative fistula (n = 216) were older, with a mean age of 65.4 $\pm$ 0.8 years, compared with 63.3 $\pm$ 0.3 years for patients without fistula ($P = 0.02$). Additional univariate analyses of patient-related factors are summarized in Table 1. Figures in parentheses

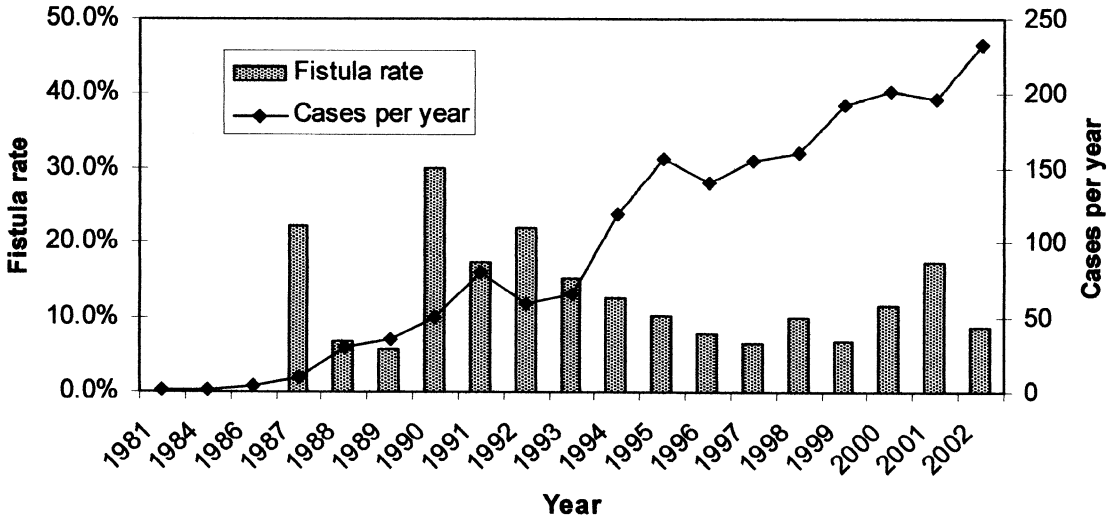

Fig. 1. Longitudinal trends in rates of pancreaticocutaneous fistula and pancreaticoduodenectomy case volume. There is no statistically significant change in fistula rates over time ($P = 0.08$, Mann-Whitney rank sum test). Included in these data are only the patients who underwent pancreaticoduodenectomy and had a pancreas remnant (body and tail) left behind. Thus, patients undergoing total or completion pancreaticoduodenectomy are excluded.

represent the ratio of patients developing fistula to total at-risk patients within that category; due to missing data, the denominators within each group do not always total 1891.

Male gender correlated with an increased fistula rate (13.2% versus 9.4% for female gender, $P = 0.01$). Rates of fistula among African Americans were only 6.0%, compared with 11.8% among whites and 13.6% among those of other races, but this difference was not statistically significant ($P = 0.11$). Among comorbidities, a history of acute or chronic pancreatitis tended to be associated with lower pancreaticocutaneous fistula rates, but again this difference was not statistically significant given the low prevalence of these comorbidities. Neither hypertension nor smoking correlated with fistula rates. Diabetes mellitus appeared to be a protective comorbidity; that is, patients without a history of diabetes were at higher risk for postoperative fistula (12.0% versus 7.7% in patients with diabetes, $P = 0.03$). In addition, a history of coronary artery disease correlated with increased fistula rates (14.9% versus 9.7% in the absence of coronary disease, $P = 0.02$).

Univariate analyses of *surgeon-related factors* are summarized in Table 2. Although it is difficult to quantify operative complexity, gross surrogates of increased complexity such as operative time, estimated blood loss, and units of intraoperative red blood cell transfusions did correlate with increased rates of postoperative pancreaticocutaneous fistula.

In addition to overall operative complexity, we also searched for correlations between specific technical decisions and fistula rates. All patients underwent a pancreatic anastomosis. No difference was found in fistula rates between patients with a pancreaticojejunal or a pancreaticogastric reconstruction (11.1% versus 11.3%, $P = 0.91$). Data regarding incorporation of the pancreatic duct in the inner layer of the anastomosis (i.e., a "mucosa-to-mucosa" or "duct-to-mucosa" anastomosis) were recorded for 280 recent patients. There was no statistically significant difference in fistula rates with or without duct incorporation, although the small number of patients undergoing duct-to-mucosa anastomosis limits the statistical power of this negative result. There also was no correlation between fistula rates and the performance of an end-to-end versus an end-to-side pancreaticojejunal anastomosis. Data on pancreatic duct stenting were not available, as it is not a routine component of pancreaticoduodenectomy at the Johns Hopkins Hospital. Differences in use of preoperative biliary drainage (via endoprostheses or percutaneous transhepatic route), intraoperative biliary drainage (via T-tube), or no biliary drainage did not affect postoperative pancreaticocutaneous fistula rates (Table 2).

In a subset of 293 patients enrolled in a randomized clinical trial that tested standard pancreaticoduodenectomy versus radical resection, patients undergoing radical resection had a significantly higher fistula rate (12.6%) than did patients undergoing standard lymphadenectomy[24] (5.6%, $P = 0.04$) (Table 2). This subgroup of patients all had periampullary cancers, most being pancreatic, with a correspondingly low

Table 1. Patient-related risk factors for pancreaticocutaneous fistula

		P value
Age (yr)*		
Patients developing fistula (n = 216)	65.4 ± 0.8	0.02
Patients with no fistula (n = 1675)	63.3 ± 0.3	
Overall fistula rate†	11.4 (216/1891)	
Gender†		
Male	13.2 (134/1017)	0.01
Female	9.4 (82/873)	
Race†		
White	11.8 (194/1649)	NS (*P* = 0.11)
Black	6.0 (8/134)	
Other	13.6 (11/81)	
Comorbidities†		
Acute pancreatitis		
Yes	9.2 (7/76)	NS (*P* = 0.56)
No	11.4 (201/1770)	
Chronic pancreatitits		
Yes	6.3 (7/111)	NS (*P* = 0.09)
No	11.6 (201/1737)	
Hypertension		
Yes	11.6 (77/661)	NS (*P* = 0.63)
No	10.9 (129/1183)	
Smoking		
Yes	10.8 (55/510)	NS (*P* = 0.70)
No	11.4 (152/1331)	
Diabetes mellitus		
Yes	7.7 (25/326)	0.03
No	12.0 (182/1519)	
Coronary artery disease		
Yes	14.9 (39/262)	0.02
No	9.7 (81/832)	

Advanced age, male gender, lack of diabetes mellitus, and presence of coronary artery disease correlate with increased fistula rates.
*Values given as mean ± SEM.
†Values are percent who develop fistulas (n/total).

fistula rate among the latter subgroup. Conversely, although the fistula rate among radical resection patients compares favorably with the historical fistula rate of 11.4% for all resections, it nevertheless represents a 2.4-fold increase in risk (odds ratio 95% confidence interval, 1.0–5.7) over the corresponding control group (standard resection).

Univariate analyses of *disease-related factors* are summarized in Table 3. The texture of the gland remnant at the site of transection correlates strongly with subsequent postoperative fistula rates. Data on gland texture were recorded for 235 recent patients. Of these 235, 93 were classified by the operating surgeon as a soft (normal, friable) gland, 78 as intermediate in texture, and 64 as firm (fibrotic, sclerotic). Among patients with a soft gland, 22.6% developed a fistula, compared with a fistula rate of only 2.6% among patients with an intermediate gland. No patient with a firm gland developed a pancreaticocutaneous fistula. Compared with patients with a moderate or firm gland, patients with a soft gland were 20.4-fold more likely to develop a fistula (odds ratio 20.4; 95% confidence interval [CI], 4.7–90.9).

Impressive trends in fistula rate were also evident for individual pathologic diagnoses (Table 3). Fistula rates were lowest among patients with pancreatic adenocarcinoma, at only 4.9%. Fistula rates were higher for the other periampullary cancers: distal cholangiocarcinoma, 15.8% (29 of 183); duodenal carcinoma, 15.4% (12 of 78); and ampullary carcinoma, 18.4% (41 of 223). Patients with chronic pancreatitis developed postoperative pancreaticocutaneous fistulas in 10.1% of cases, whereas fistula rates for other benign diseases were significantly higher: intraductal papillary mucinous neoplasia, 18.2% (10 of 55); pancreatic cystadenomas, 22.4% (13 of 58); benign islet cell tumors, 18.2% (4 of 22); and duodenal adenomas, 32.8% (21 of 64).

Given the likely covariance of individual factors, such as pancreatic texture and inflammatory pancreatic pathologies, we next examined a multivariate model (Table 4) incorporating all nine factors with statistically significant correlations in univariate analysis (age, gender, diabetes, coronary disease, operative time, blood loss, transfusions, pancreatic texture, and pathology). The type of retroperitoneal lymphadenectomy was excluded, as that data only pertained to patients with periampullary tumors. Complete data on all 9 variables were available for 218 patients. In this multivariate model, only two factors, a history of coronary artery disease (odds ratio, 3.7; 95% CI, 1.2–12.1) and a soft gland (odds ratio, 10.0; 95% CI, 2.1–47.6) were predictive. Gland texture was the strongest predictor. A soft pancreatic gland implied a 10-fold elevation in risk for postoperative pancreaticocutaneous fistula (versus an intermediate or hard gland).

The development of a postoperative pancreaticocutaneous fistula complicates a patient's postoperative hospital course, although previous reports emphasize its transformation from the dreaded, mortal complication in the early era of pancreatic surgery to one that is currently typically managed conservatively, or with nonoperative techniques.[2,20] To better quantify the effects of pancreaticocutaneous fistula, we examined several measures of patient outcomes (summarized in Table 5).

Overall mortality was not affected by the development of pancreaticocutaneous fistula. Patients with a

Table 2. Surgeon-related risk factors for pancreaticocutaneous fistula

	Mean ± SEM and (median [and 10th–90th percentiles])	*P* value
Operative time (min)*		
Patients developing fistula	412 ± 8.4 [386.5 (300–560)]	0.001
Patients without fistula	386 ± 2.3 [369 (287–503)]	0.006
Estimated blood loss (ml)*		
Patients developing fistula	1150 ± 87 [725 (300–2300)]	0.03
Patients without fistula	914 ± 26 [700 (300–1600)]	0.03
Intraoperative transfusions (units)*		
Patients developing fistula	1.16 ± 0.19 [0 (0–4)]	0.03
Patients without fistula	0.84 ± 0.05 [0 (0–2)]	0.78
Pancreatic-enteric anastomosis†		
Pancreaticojejunostomy (PJ)	11.1 (145/1311)	NS ($P = 0.91$)
Pancreaticogastrostomy (PG)	11.3 (22/194)	
Duct incorporation in PJ (n = 278)†		
Mucosa-mucosa anastomosis	11.6 (29/249)	NS ($P = 0.44$)
Duct not incorporated	6.9 (2/29)	
End-to-end or end-to-side in PJ (n = 598)†		
End-to-end	10.4 (7/67)	NS ($P = 0.71$)
End-to-side	9.0 (48/531)	
Retroperitoneal lymphadenectomy (n = 293)†‡		
Standard	5.6 (8/142)	0.04
Radical	12.6 (19/151)	
Billiary stenting†		
Preoperative stent (either endoscopic or PBD)		
Yes	11.3 (136/1203)	NS ($P = 0.96$)
No	11.4 (61/536)	
Postoperative HJ stent		
None	10.4 (37/357)	NS ($P = 0.66$)
PBD	8.5 (38/448)	
T-tube	9.3 (34/365)	

Increased operating times, blood loss, and intraoperative red blood cell transfusions all correlated with increased fistula rates. The performance of a radical lymphadenectomy correlated with increased fistula rates, but other technical features of the anastomosis or of biliary stenting did not.
*Value given as mean ± SEM [median (10th–90th percentile)].
†Values given as percent developing fistulas (n/total).
‡All 301 patients in this group had periampullary cancers and were randomized in a clinical trial testing benefits of radical lymphadenectomy.[36]
PBD = percutaneous biliary drain.

fistula, however, were more likely to undergo reexploration: 7.9% (17 of 216) versus 2.7% (45 of 1674) in patients without fistula ($P < 0.0001$). Six of the 17 reexplorations in patients with fistula were for management of sepsis from a leaking pancreaticojejunostomy or drainage of an intra-abdominal abscess. In these six patients, four were treated by anastomotic repair and wide drainage and two were treated by completion pancreatectomy. The other reexplorations were for bleeding (6 of 17) or for wound problems (5 of 17). Mean length of stay in patients without pancreaticocutaneous fistula was 13.2 ± 0.4 days compared with 26.0 ± 1.0 days for patients who developed a postoperative fistula ($P < 0.0001$).

Rates of certain postoperative complications were also increased in patients with a pancreaticocutaneous fistula (Table 5). Patients with a fistula were more likely to have postoperative acute pancreatitis, bile leak, intra-abdominal abscess, and wound infection. Rates of other complications, such as delayed gastric emptying, cholangitis, and pneumonia were not statistically different in patients with or without a pancreaticocutaneous fistula.

Despite the increase in early postoperative complications in patients with pancreatic fistula, the overall survival in patients with pancreatic cancer was not affected by the development of a pancreaticocutaneous fistula. Of the 796 patients with pancreatic cancer in this study, long-term survival information was available for 758 (38 of 39 patients with fistula; 720 of 758 patients without fistula). Kaplan-Meier survival curves for these two groups of patients are not statistically different (Fig. 2; Mantel-Cox log-rank $P = 0.79$). The median survival for patients with

Table 3. Specimen-related risk factors for pancreaticocutaneous fistula

	% Developing fistula (n/total)	*P* value
Texture of gland at transection site (n = 235)		
Soft	22.6 (21/93)	<0.0001
Moderate	2.6 (2/78)	
Firm	0.0 (0/64)	
Pathology		
Pancreatic adenocarcinoma	4.9 (39/796)	<0.0001
Distal cholangiocarcinoma	15.8 (29/183)	
Duodenal carcinoma	15.4 (12/78)	
Ampullary carcinoma	18.4 (41/223)	
Pancreatic cystadenocarcinoma	12.5 (1/8)	
Malignant islet cell tumor	15.3 (11/72)	
Cancer metastatic to pancreas	6.7 (1/15)	
Chronic pancreatitis	10.1 (17/168)	
IPMN (without cancer)	18.2 (10/55)	
Ampullary/duodenal adenoma	32.8 (21/64)	
Pancreatic cystadennoma	22.4 (13/58)	
Benign islet cell tumor	18.2 (4/22)	
Gastrointestinal stromal tumor	4.8 (1/21)	
Other benign processes	12.5 (16/128)	

Both pancreatic texture and pathology correlate with fistula rates. IPMN = intraductal papillary mucinous neoplasia.

a postoperative fistula was 19 months, whereas the median survival for patients without a postoperative fistula was 17 months.

DISCUSSION

Pancreaticocutaneous fistulas continue to be a common complication following pancreaticoduodenectomy, occurring in 11.4% of patients operated on at the Johns Hopkins Hospital between 1981 and 2002. Patients who develop a postpancreaticoduodenectomy pancreaticocutaneous fistula do not incur any increase in operative mortality, nor does it negatively affect long-term survival in patients with pancreatic cancer. Postoperative length of stay, however, is significantly increased, associated with an increased reoperation rate and an increase in the rates of postoperative pancreatitis, bile leak, intra-abdominal abscess, and wound infection.

During the past two decades, increasing experience with this procedure at our institution and others has contributed to improvements in important patient outcomes such as postoperative mortality and inpatient length of stay.[2,24] Despite a growing body of evidence that high surgical volumes translate into improved patient outcomes for complex gastrointestinal procedures,[25] we have not observed a significant decline in the rate of pancreaticocutaneous fistula, even while surgical volume exceeds 200 pancreaticoduodenectomies per year. Similar rates of pancreaticocutaneous fistula are observed in other large institutional series,[1,4] although fistula rates of 2–3% have been reported in smaller series.[26,27]

Part of the reported differences in fistula rates clearly arise from heterogeneities in diagnosis, indications for operation, and fistula definition. Univariate analysis of correlations between risk factors and pancreaticocutaneous fistula identified a number of statistically significant correlates. Some risk factors are easily accepted as correlating with increased fistula rates, such as advanced age, absence of diabetes, longer operations, and higher blood loss. The correlation between pancreatic pathology and fistula is also intuitively accepted, because pancreatic adenocarcinoma and chronic pancreatitis are typically associated with firm, fibrotic, easily sutured glands, whereas other benign processes or other periampullary cancers are often associated with a soft, normal, friable gland. Other risk factors, though, are less easily understood, such as male gender (also observed in the 1995 report of Marcus and colleagues[3]) and the presence of coronary artery disease.

Table 4. Multivariate logistic regression model (N = 218)

	P value	Odds ratio	95% Confidence interval
Coronary arterial disease	0.03	3.73	1.15–12.1
Soft pancreatic texture	0.004	10.0	2.10–47.6
Age (per decade)	NS ($P = 0.62$)	1.12	0.72–1.74
Male gender	NS ($P = 0.76$)	0.99	0.33–2.97
Diabetes mellitus	NS ($P = 0.85$)	0.84	0.15–4.75
Operative time (per hour)	NS ($P = 0.78$)	1.08	0.62–1.89
Estimated blood loss (per deciliter)	NS ($P = 0.48$)	0.95	0.83–1.09
Transfusions (per unit)	NS ($P = 0.20$)	1.37	0.85–2.20
Pathology: other than pancreatic cancer	NS ($P = 0.07$)	4.65	0.89–24.5

In this multivariate model, only a history of coronary artery disease or a soft (normal) pancreatic texture was predictive for the development of a pancreaticocutaneous fistula.

Table 5. Pancreaticocutaneous fistula and surgical outcome measures

	Patients without fistula	Patients with fistula	*P* value
Operative mortality*	1.5 (25/1670)	1.4% (3/213)	NS ($P = 0.92$)
Reexploration rate*	2.7 (45/1674)	7.9% (17/216)	<0.0001
Postoperative length of stay (days)†	13.2 ± 0.4 [10 (7–21)]	26.0 ± 1.0 [22 (12–44)]	<0.0001
median (10th–90th percentile)			<0.0001
Complications*			
Pancreatitis (postoperative)	1.3 (21/1674)	4.2 (9/216)	0.001
Bile leak	2.7 (45/1675)	7.0 (15/215)	0.007
Intra-abdominal abscess	4.4 (73/1675)	21.3 (46/216)	<0.0001
Wound infection	6.6 (110/1674)	25.1 (54/215)	<0.0001
Delayed gastric emptying	14.7 (245/1672)	18.1 (39/215)	NS ($P = 0.18$)
Cholangitis	2.7 (46/1674)	3.7 (8/216)	NS ($P = 0.43$)
Pneumonia	1.5 (25/1674)	2.8 (6/216)	NS ($P = 0.16$)
Cardiac complication	3.8 (33/879)	2.8 (3/106)	NS ($P = 0.63$)
Lymphatic or chylous leak	0.7 (9/1294)	0.7 (1/143)	NS ($P = 0.99$)

Although there was no effect on operative mortality, patients with fistula were more likely to undergo reoperation and had higher lengths of stay and rates of certain complications.
*Value given as percent (n/total).
†Value given as mean ± SEM [median (10th–90th percentile)].

In our multivariate analysis, only two risk factors were independent correlates of increase pancreaticocutaneous fistula: history of coronary artery disease and pancreatic texture. An important limitation of this multivariate analysis is the sample size (n = 218), made necessary as complete model data were only available on this subgroup of patients. Nevertheless, the impact of gland texture was dramatic. No patient with a firm gland developed a postoperative fistula, whereas 23% of patients with a normal, soft gland developed a postoperative fistula.

The explanation for increased fistula rates with soft gland texture appears obvious, because a normal, soft pancreatic remnant holds sutures poorly and has normal exocrine function (two easily understood reasons for anastomotic failure). The reasons why previous coronary artery disease would be associated with pancreatic fistula are not as obvious. Perhaps previous coronary artery disease is a surrogate for decreased visceral perfusion leading to anastomotic ischemia, or perhaps some of the various medications typically prescribed to such patients (aspirin, other antiplatelet

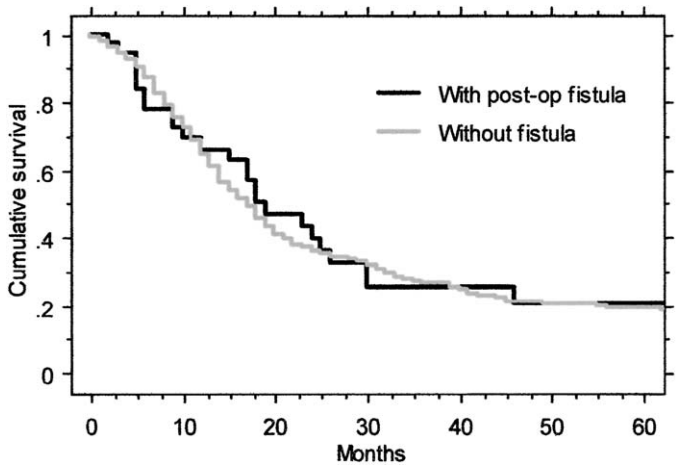

Fig. 2. Long-term survival of patients with pancreatic adenocarcinoma is not influenced by the development of a postpancreaticoduodenectomy pancreaticocutaneous fistula. Kaplan-Meier survival curves for patients with and patients without a postoperative fistula are not statistically different (Mantel-Cox log rank test, $P = 0.79$).

agents, β-blockers, etc.) compromise anastomotic healing.

The results of the current study also indicate that, at least at our institution, many of the factors that are in fact controllable by the surgeon do not have a statistically significant impact on the development of a postoperative fistula. For example, choices regarding anastomotic technique (pancreaticojejunal versus pancreaticogastric; end-to-end versus end-to-side; duct-to-mucosa or not) did not statistically correlate with fistula rates. Similarly, choices regarding preoperative and intraoperative biliary stenting also did not correlate with postoperative fistula rates. One notable exception is the extent of lymphadenectomy in patients with cancer. In a subgroup of patients with periampullary tumors enrolled in a randomized clinical trial testing the benefit of radical resection with retroperitoneal lymphadenectomy, we observed a higher rate of pancreaticocutaneous fistulas in patients undergoing the more extensive procedure.

Reoperation was not performed simply to repair a failed pancreatic-enteric anastomosis, because the majority of leaking anastomoses will heal with appropriate nonoperative measures (drainage, total parenteral nutrition, octreotide, etc.). However, should reoperation be necessary for control of a leaking pancreatic anastomosis, the options include anastomotic repair or revision with wide drainage, conversion to isolated Roux-en-Y pancreaticojejunostomy with wide drainage, conversion to pancreaticogastrostomy with wide drainage, or completion pancreatectomy.

The significant dependence of postoperative pancreaticocutaneous fistula rates on patient comorbidity and gland characteristics has significant implications for investigational trials of methods to decrease fistula rates. In evaluating the suitability of a control group, it will be imperative to assess uniformity in distribution of gland textures and patient health status (e.g., coronary disease, American Society of Anesthesiologists status). Furthermore, comparison of fistula rates between institutions, or even among different subgroups of patients within a single institution, is unlikely to be highly informative unless variations in risk factors are accounted for. Finally, given the dramatic risk conferred by a soft gland (odds ratio, 10.0; 95% CI, 2.1–47.6), future clinical trials may wish to restrict their analyses to this subgroup of patients. Inclusion of patients with moderate or firm glands, in which fistulas are already rare, would only dilute the statistical power of the trial.

An additional source of heterogeneity in pancreatic fistula research is the variation among criteria defining a pancreaticocutaneous fistula. These definitions range from the very liberal, such as the presence of 10 ml/day or more amylase-rich fluid in postoperative drains after postoperative day 3,[22] to the very stringent, such as the continued drainage of amylase-rich fluid after postoperative day 20.[3] Other investigators require evidence of systemic inflammation (e.g., fever, leukocytosis),[4] whereas others do not. The current divergence in fistula definition has significantly weakened otherwise important meta-analyses regarding pancreaticocutaneous fistulas.[11,21] To help remedy the differences in fistula definitions, an International Study Group for pancreatic fistula definition met in Athens, Greece, in March 2004. While a formal report of this group remains pending at this time, it is hoped that a consensus definition can be accepted, allowing more uniform assessment and reporting of patient outcomes after pancreatic resection (Bassi; unpublished data).

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Mechanisms of Resistance to Erbitux (Anti–Epidermal Growth Factor Receptor) Combination Therapy in Pancreatic Adenocarcinoma Cells

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We previously demonstrated that pancreatic adenocarcinoma BxPC-3 xenografts display resistance to treatment with Erbitux, gemcitabine, and radiation, whereas MIA PaCa-2 xenografts are highly sensitive to the same therapy. Here, we elucidate in vitro mechanisms that may explain the observed differential response of epidermal growth factor receptor (EGFR) expressing pancreatic adenocarcinoma xenografts to Erbitux-based combination therapy in vivo. MIA PaCa-2 and BxPC-3 protein lysates were probed with antibodies to EGFR, ErbB2, ErbB3, and ErbB4. Constitutive ErbB3 activity was visualized by immunoblot analysis using anti-phosphotyrosine antibodies and receptor-specific immunoprecipitates. *erbB2* and *erbB3* gene expression in both cell lines was quantified with real-time polymerase chain reaction. Erbitux-induced internalization of EGFR was determined by flow cytometry following Erbitux treatment for different incubation times at 0°C and 37°C. MIA PaCa-2 and BxPC-3 protein extracts were also probed with anti–phospho–mitogen-activated protein kinase antibody after stimulation with EGF and in the presence of Erbitux. Although both cell lines expressed EGFR and ErbB2 protein, ErbB3 protein was selectively expressed by BxPC-3 cells, where it also showed evidence of constitutive phosphorylation. There was a 10-fold increase of *erbB3* transcript levels in BxPC-3 cells compared with MIA PaCa-2. ErbB4 protein was not detectable in either cell line. Erbitux mediated EGFR internalization in MIA PaCa-2 cells after 2 hours of incubation, whereas it did not promote EGFR internalization in BxPC-3 cells. Likewise, EGF-dependent phosphorylation of MAPK p44/42 was blocked by Erbitux treatment in MIA PaCa-2 but not BxPC-3 cells. Erbitux selectively interfered with EGF-induced MAPK activation in MIA PaCa-2 but not BxPC-3 cells. Persistent MAPK activation and impaired in vitro internalization of EGFR by BxPC-3 pancreatic cancer cells may be due to constitutive ErbB3 signaling, facilitated by heterodimerization with EGFR, which may explain resistance to Erbitux-based combination therapy in vivo. (J GASTROINTEST SURG 2004;8:960–970)

KEY WORDS: EGFR, Erbitux, pancreatic cancer

The epidermal growth factor (EGF) family of receptors has been implicated in the pathogenesis and prognosis of solid tumors. The epidermal growth factor receptor (EGFR, also known as ErbB1/HER1) and its downstream signaling mediators, which include Ras, Raf kinase, and the mitogen-activated protein kinases (MAPK), are integral components of the principal signaling cascade involved in regulating carcinoma growth.[1] EGFR is encoded by the c-*erbB-1* proto-oncogene and is a transmembrane growth factor receptor with tyrosine kinase activity. EGFR is overexpressed in up to 60% of human pancreatic adenocarcinomas and its blockade is, therefore, a rational therapeutic approach to treating pancreatic cancer.[2,3] Increased EGFR expression levels have been correlated with poor survival among pancreatic cancer patients.[4,5] Pancreatic cancers also display high protein levels of other members of the ErbB receptor

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family, such as ErbB2 and ErbB3.[3] The ability of ErbB family receptors to undergo heterodimerization is an important basis for the system's diversity and flexibility in signal amplification on ligand binding.[6] ErbB2 is the preferred dimerization partner for ErbB1, and the presence of ErbB2 has been correlated with enhanced tumor cell proliferation and invasion.[7,8] ErbB1 can also form heterodimers with ErbB3, which contains several binding motifs in its COOH terminus for the p85 subunit of phosphatidylinositol-3'-kinase (PI3K).[9–11] In addition, there are important signaling complexes that may not include EGFR, such as the potent ErbB2/ErbB3 heterodimer, which functions as an oncogenic unit to drive tumor cell proliferation.[12,13]

Erbitux (also known as cetuximab or IMC-C225; ImClone Systems Incorporated, New York, NY) is a human-mouse chimerized IgG_1 antibody with high affinity to the EGFR, derived from the murine anti-EGFR monoclonal antibody 225. Erbitux blocks binding of the natural ligands EGF and transforming growth factor-α to EGFR and is able to induce dimerization, internalization, and downregulation of EGFR. This antibody blocks the activation of the tyrosine kinase domain of the EGFR after stimulation with a specific ligand. Erbitux interferes with cell cycle progression and has been shown to be effective at producing in vitro inhibition of tumor cell proliferation and inhibiting tumor growth in vivo. The potential mechanisms involved in Erbitux inhibition of tumor growth include arrest in cell cycle progression, activation of apoptosis, inhibition of angiogenesis, inhibition of invasion/metastasis, and activation of immune responses.[14] Preclinical data suggest that the antitumor effect of targeting EGFR with specific antibodies produces delayed tumor growth without significant tumor regression. Achievement of tumor cytoreduction using agents targeting EGFR in combination with radiation and/or chemotherapy therefore represents a logical developmental strategy.[14,15] Erbitux was shown to enhance the antitumor effects of several chemotherapeutic agents, including gemcitabine.[16,17] A recent clinical trial of Erbitux in combination with gemcitabine showed promising activity against pancreatic cancer.[18] The combination of Erbitux and radiation therapy also resulted in a positive interaction between the two modalities with enhanced antitumor effects.[19]

We previously demonstrated that Erbitux in combination with gemcitabine therapy and radiation therapy has greater efficacy against human pancreatic cancer cells and tumor xenografts than either treatment alone, or any combination of two treatments. Short-term (4 days) Erbitux-based therapy was effective in vitro, inhibiting tumor cell proliferation and promoting apoptosis, in both the BxPC-3 and MIA PaCa-2 pancreatic cancer cell lines, with more pronounced effects in the former cell line.[20] These in vitro results, however, did not completely correlate with in vivo findings, where long-term Erbitux-based multimodality therapy was more efficient against MIA PaCa-2 xenografts (complete regression after 6 weeks of treatment), in contrast to BxPC-3 xenografts, which displayed only partial inhibition of growth and quickly regrew after discontinuation of treatment.[20] We subsequently demonstrated that this apparently paradoxical phenomenon was explained by the duration of in vitro Erbitux treatment. A 6-week in vitro Erbitux treatment, followed by exposure to EGF, resembled in vivo conditions more closely and was shown to enhance the effect of gemcitabine and radiation on MIA PaCa-2 cells but not in BxPC-3 cells, in concordance with our previous xenograft findings.[21]

The mechanisms that determine pancreatic cancer cell response to Erbitux-based therapy remain largely unknown. The objective of the present study was to determine the expression of the ErbB family of receptors in MIA PaCa-2 and BxPC-3 pancreatic cells as well as to analyze differential EGFR internalization and activation of downstream signaling mediators in response to in vitro Erbitux therapy. This in vitro analysis of signaling pathways and potential mediators of response may provide valuable further insight into the mechanisms that determine xenograft resistance to this kind of therapy.

MATERIAL AND METHODS

Cell Lines

Human pancreatic adenocarcinoma cell lines MIA PaCa-2 and BxPC-3 were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 2 mmol/L glutamine in a humidified 37°C incubator supplied by 5% CO_2.

Reagents and Antibodies

Humanized mouse anti-human EGFR antibody (Erbitux) was obtained as a gift from ImClone Systems Incorporated. Anti-EGFR antiserum E7 and anti-ErbB2 antiserum M6 have been previously characterized and were raised in rabbits against synthetic peptides of the respective human coding sequence.[11,12,22] Rabbit anti-ErbB3, rabbit anti-ErbB4, mouse anti-phosphotyrosine, rabbit anti-phospho-MAPK p44/42, and rabbit anti-MAPK p44/42 antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz,

CA). Horseradish peroxidase–conjugated goat anti-rabbit antibody was purchased from Southern Biotechnology (Birmingham, AL). Anti-phosphotyrosine p85 PI3K binding motif antibody was obtained from Cell Signaling (Beverly, MA). TriZOL reagent was obtained from InVitrogen (Carlsbad, CA). Alexa-conjugated goat anti-human IgG was obtained from Molecular Probes (Eugene, OR). Protein A agarose beads were obtained from Upstate Biotechnology (Lake Placid, NY). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Western Blotting of Cell Lysate From MIA PaCa-2 and BxPC-3 Cells to Detect EGFR and Its Isotypes

MIA PaCa-2 and BxPC-3 cells were cultured in DMEM containing 10% FBS and glutamine until confluent. Cells were washed with cold phosphate-buffered saline (PBS) twice and lysed in radioimmunoprecipitation assay lysis buffer (RIPA) (consisting of 1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 1% sodium deoxycholate, 50 mmol/L Tris-HCl, pH 7.5, and 1 mmol/L EDTA) with 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin. Samples of cell lysate were collected after centrifugation at 15,000 rpm for 15 minutes at 4°C. Fifty micrograms of protein from each sample was separated in 7.5% SDS–polyacrylamide gel electrophoresis (PAGE) and electronically transferred to a polyvinylidine difluoride (PVDF) membrane overnight. The membranes were probed with E7 rabbit anti-EGFR antibody (1:2000), M6 rabbit anti-ErbB2 (1:2000), rabbit anti-ErbB3 (1:400), and rabbit anti-ErbB4 (1:400) followed by horseradish peroxidase–conjugated goat anti-mouse or rabbit antibodies. Membranes were developed using an ECL kit after washing with PBS containing 1% bovine serum albumin (PBS/BSA).

Immunoprecipitation of ErbB3 and ErbB3 Phosphorylation

MIA PaCa-2 and BxPC-3 cells were plated onto 100-mm Petri dishes and cultured until confluent. Cells were treated with EGF, 60 ng/ml, at 37°C (10 minutes). Untreated cells on a different dish were used as control. Cells were washed with PBS and lysed in RIPA buffer containing 10 mmol/L sodium orthovanadate and protease inhibitors, with detergent conditions that ensured disruption of heterodimers and receptor-specific immunoprecipitates. Supernatants were harvested after further centrifugation. One milligram of protein from each sample was incubated at 4°C overnight with 10 μg of rabbit anti-ErbB3 antibody. Twenty microliters of 50% Protein A beads were added to each tube and incubated with cell lysate at 4°C (2 hours). One milligram of protein from each sample was mixed with beads only as control. Beads were centrifuged and washed three times with RIPA buffer. Beads were then resuspended in sample buffer and boiled for 5 minutes. Supernatant was loaded onto 4–15% gradient gels for SDS-PAGE and transferred to PVDF membrane. Mouse anti-phosphotyrosine antibody was mixed with membrane at room temperature (2 hours) after blocking, followed by horseradish peroxidase–conjugated goat anti-mouse antibody. Membranes were developed with ECL kit.

The membrane was stripped with 7 mol/L guanidine hydrochloride for 20 minutes and reprobed with rabbit anti-ErbB3 antibody after blocking to confirm the presence of ErbB3. The membrane was then reacted with horseradish peroxidase–conjugated goat anti-rabbit antibody after washing. The membrane was developed with ECL kit.

A similar method was used to detect the ErbB3-phosphorylated YXXM site, which is the binding motif for the p85 subunit of PI3K. Total cell protein lysate was obtained as described earlier. In addition, protein samples were immunoprecipitated with anti-ErbB3 antibody after treatment with and without EGF as described earlier. Samples were transferred to a PVDF membrane after SDS-PAGE and then incubated with rabbit anti-phosphotyrosine p85 PI3K binding motif antibody at room temperature (2 hours). This was followed by incubation with horseradish peroxidase–conjugated goat anti-rabbit antibody and development with ECL kit.

Real-time PCR for *erbB2* and *erbB3* Gene Expression

MIA PaCa-2 and BxPC-3 cells were grown as described above until confluent. Cells were harvested and RNA was extracted using TriZOL according to the manufacturer's instructions. Total RNA was subjected to quantitative real-time polymerase chain reaction (PCR) with *erbB2* and *erbB3* primers and probes obtained from Applied Biosystems (Foster City, CA). Reactions were assembled in duplicate using the TaqMan one-step PCR kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. The β-actin expression was quantified as the housekeeping gene. Nontemplate controls for each primer/probe set included water in place of RNA. The reaction was performed on the ABI 7900 with the following protocol: 30 minutes at 48°C, followed by 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

Measurement of Erbitux Binding Affinity and EGFR Expression Levels in MIA PaCa-2 and BxPC-3 Cells

MIA PaCa-2 and BxPC-3 cells (1×10^6/tube in triplicates) were incubated with serial dilutions (0.65–334 ng) of ^{125}I-Erbitux in 0.1% PBS/BSA for 1 hour at room temperature with agitation. Cells were washed with 0.1% PBS/BSA and centrifuged, and isotope activity in the cell pellets was measured with a gamma counter. Cell-associated EGFR numbers were calculated by Scatchard analysis.

Internalization of EGFR on MIA PaCa-2 and BxPC-3 Cells

MIA PaCa-2 and BxPC-3 cells were plated onto six-well plates at 5×10^5/well and incubated for 18 hours. Plates were kept at 4°C for 15 minutes. Fresh medium containing 60 ng/ml of EGF or 16 μg/ml of Erbitux was added to designated wells. Cells were incubated with Erbitux at 37°C or 0°C for 1, 2, 3, and 4 hours. In the EGF group, cells were incubated with EGF at 37°C or 0°C for 2 hours. Cells were washed with cold PBS (1 ml/well) twice. One milliliter of cold 0.2 mol/L acetic acid and 0.5 mol/L NaCl (pH 3.0) was added to each well and incubated on ice for 6 minutes. Cells were then washed with cold PBS (1 ml/well) once, followed by PBS containing 1% BSA and 0.1% NaN_3. Cells were scraped in 1 ml of PBS with 1% BSA and 0.1% NaN_3 (fluorescent activated cell sorter [FACS] buffer), centrifuged, and resuspended in 100 μl of FACS buffer. Erbitux (0.1 μg) was added to the tubes and incubated with cells for 30 minutes at 0°C. Cells were washed with FACS buffer and incubated with 20 μl/tube of Alexa-conjugated goat anti-human IgG antibody (1:40 dilution). Cells were incubated with the secondary antibody for 30 minutes at 0°C and fixed with 300 μl/tube of 2% paraformaldehyde in PBS. Cells were stored at 4°C until examination using flow cytometry (Becton Dickinson, San Jose, CA).

Probing MAPK on MIA PaCa-2 and BxPC-3 Cells

MIA PaCa-2 and BxPC-3 cells, approximately 95% confluent, were cultured in serum-free DMEM for 36 hours. Cells were incubated with Erbitux (5 μg/ml) for 24 hours. An equal amount of human IgG was used as control. Cells were treated with EGF, 60 ng/ml, for 10 minutes at 37°C. Samples without EGF were used as control. Cells were washed twice with cold PBS containing 2 mmol/L sodium orthovanadate and 10 mmol/L sodium pyrophosphate. Cells were scraped, and cell pellets were resuspended in RIPA buffer containing protease inhibitors, 2 mmol/L sodium orthovanadate and 10 mmol/L sodium pyrophosphate. Samples were centrifuged at 15,000 rpm for 15 minutes at 4°C using an Eppendorf centrifuge. Supernatant of the samples was harvested, and protein concentration was measured.

One hundred micrograms of protein from each sample was loaded onto 10% gels for SDS-PAGE after samples were treated with three times sample buffer and boiled. Samples were transferred electronically to PVDF membrane. Rabbit polyclonal antibodies anti-p44/42 or anti–phospho-p44/42 were added to the membranes respectively and incubated overnight at 4°C. Membranes were reacted with horseradish conjugated goat anti-rabbit antibody after washing.

RESULTS

Both MIA PaCa-2 and BxPC-3 cells expressed EGFR and ErbB2 protein. For EGFR, protein expression was relatively higher in BxPC-3 cells, whereas the two cell lines displayed similar levels of ErbB2 protein. ErbB3 protein, however, was expressed only in BxPC-3 cells. ErbB4 protein was not detectable in either cell line under conditions in which ErbB4 was readily visualized in a positive control (Fig. 1).

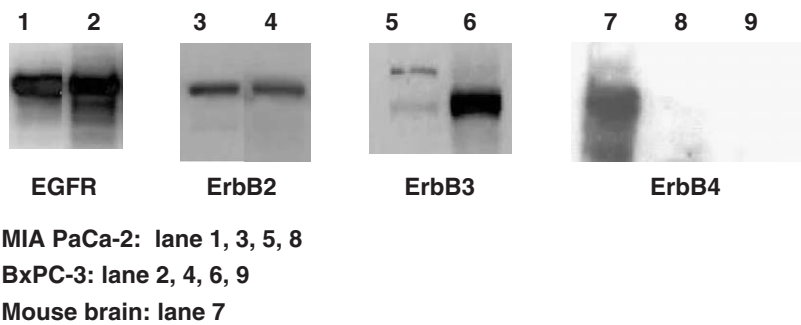

Fig. 1. Expression of ErbB protein family of receptors in MIA PaCa-2 (lanes 1, 3, 5, and 8) and BxPC-3 (lanes 2, 4, 6, and 9) pancreatic cancer cells. Mouse brain was used as positive control for ErbB4 (lane 7).

To analyze the state of functional activation of ErbB3 protein in BxPC-3 cells, ErbB3 immunoprecipitates were probed with anti-phosphotyrosine antibody. The ErbB3 protein showed constitutive phosphorylation in BxPC-3 cells, and this was enhanced by EGF treatment (Fig. 2). The membrane was stripped and reprobed with ErbB3 antibody to confirm comigration of ErbB3 and the phosphotyrosine epitope in question.

BxPC-3 total cell protein lysates expressed phosphotyrosine at the consensus YXXM site, which is the binding motif for the p85 subunit of PI3K and which was absent in MIA PaCa-2 cells (Fig. 3). BxPC-3 ErbB3 immunoprecipitates were probed with the same antibody, confirming that expression of the p85 binding motif was ErbB3 specific (Fig. 3).

Expression levels of *erbB2* and *erbB3* transcripts were compared by dividing the target threshold cycle (C_T) value by the reference (β-actin) C_T value for the RNA from each cell line. This calculated target/reference ratio was used to compare the relative gene expression levels of both *erbB2* and *erbB3* between the two cell lines. RNA from HT-29 colon cancer cells, known to overexpress the *erbB3* gene, was used as positive control in the assay (data not shown).[23] There was a 10-fold higher baseline *erbB3* transcript level in BxPC-3 cells compared with MIA PaCa-2. A smaller difference (threefold) was detected between the *erbB2* transcript levels for those two cell lines (Fig. 4).

We sought to determine the EGFR density and Erbitux binding affinity on MIA PaCa-2 and BxPC-3 cells by using ^{125}I-labeled antibody and Scatchard analysis. Our results indicated that while both cell lines have similar affinity for the antibody, BxPC-3 cells exhibited a relative twofold increase in EGFR density at intermediate levels of receptor overexpression (Fig. 5). At saturation, the estimated absolute EGFR numbers were 120,000 for BxPC-3 cells and 57,000 for MIA PaCa-2 cells. Thus, EGFR was overexpressed at intermediate levels in both cell lines with a twofold difference in relative receptor levels. This observation was consistent with immunoblot analysis shown in Fig. 1.

Erbitux induced internalization of up to 50% of EGFR in MIA PaCa-2 cells after 4 hours of incubation at 37°C compared with cells where the internalization process was prevented by low temperature (Fig. 6, *A*). Erbitux did not promote any EGFR internalization in BxPC-3 cells, even after incubation for 4 hours at 37°C (Fig. 6, *B*).

When downstream signal transduction mediators were analyzed, EGF induced phosphorylation of MAPK p44/42, and this was partially blocked by Erbitux treatment in MIA PaCa-2 cells. EGF also induced phosphorylation of MAPK p44/42 in BxPC-3 cells, but Erbitux did not counteract this effect (Fig. 7).

DISCUSSION

Human carcinomas frequently express one or more members of the EGFR family. These different ErbB receptors may homodimerize or heterodimerize on activation with ligand and trigger cellular proliferation. Some of these receptor heterodimers may not be recognized by EGFR antibodies, and that process could then become a mechanism of resistance. Pancreatic adenocarcinoma cells have been shown to display increased mRNA and protein expression levels of different members of the EGF family of receptors, including ErbB2 and ErbB3.[3] Because multiple ErbB receptor combinations and heterodimers may be active in a tumor, this may influence its response to ErbB-targeted therapy.[21] Heterodimerization leads to signaling diversity as indicated by adaptor recruitment or signaling outcome.[6,24] EGF-driven EGFR homodimers are destined for intracellular degradation, whereas the corresponding heterodimers with

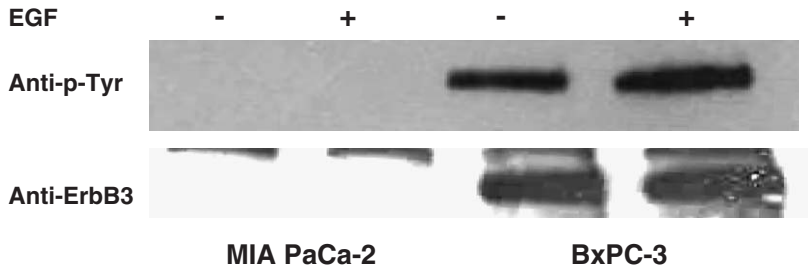

Fig. 2. Tyrosine phosphorylation of ErbB3 immunoprecipitates with and without EGF (60 ng/ml) from MIA PaCa-2 and BxPC-3 pancreatic cancer cells. The membrane was stripped and reprobed with ErbB3 antibody (*bottom*) to confirm specificity.

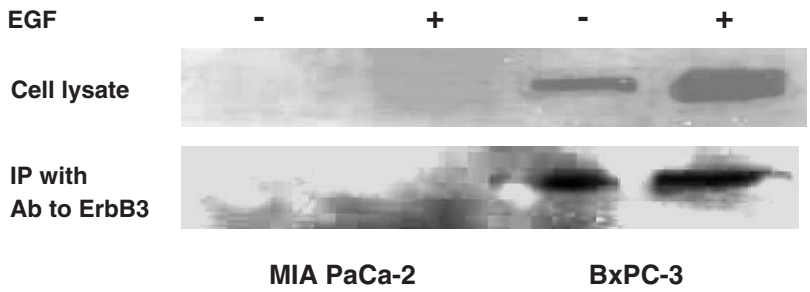

Fig. 3. Tyrosine phosphorylation of the p85 PI3K regulatory subunit binding motif, with and without EGF (60 ng/ml), in total cell lysate (*top row*) and ErbB3 immunoprecipitates (*bottom row*) from MIA PaCa-2 and BxPC-3 pancreatic cancer cells.

ErbB2 or with ErbB3 are recycled to the cell surface and their signaling is enhanced.[25] In those cases, EGF ligand may still activate EGFR/ErbB2 or EGFR/ErbB3 heterodimers, bypassing blockage with Erbitux.

ErbB2 has been recently identified as a critical component of EGF signaling to the Gab1/Gab2-PI3K-Akt pathway in colon cancer cells.[26] The ErbB-2/ErbB3 complex is the most active ErbB dimer and may function as a potent unit that drives tumor cell proliferation. The basis for the potency of its signaling lies in its capacity to stimulate both the Ras/Raf/MAPK pathway for proliferation and the PI3K-Akt pathway for survival.[6]

ErbB3, however, has also been shown to mediate EGF responses in cells expressing both ErbB3 and EGFR.[10] On stimulation with EGF, ErbB3 can be phosphorylated and activate the PI3K pathway via the regulatory p85 subunit.[9,11] More recent reports have provided further evidence for the ErbB3/PI3K interaction, addressing the ability of ErbB3 to act in a nonlinear fashion, facilitating the diversification of downstream signals.[27]

In our study, we characterized the malignant phenotype of the MIA PaCa-2 and BxPC-3 pancreatic cancer cells and their response to Erbitux therapy in vitro. As described in earlier publications, BxPC-3 xenografts showed only partial response to Erbitux-based combination therapy and regrew after discontinuation of treatment. In contest, MIA PaCa-2 xenografts displayed complete and permanent inhibition of growth after treatment with Erbitux, gemcitabine, and radiation. As mentioned, although in vitro responses may not be necessarily predictive of in vivo effects, careful analysis of cell surface receptors and signal transduction mediators may provide valuable insights into the underlying mechanisms of resistance. The possible cellular mechanisms that could

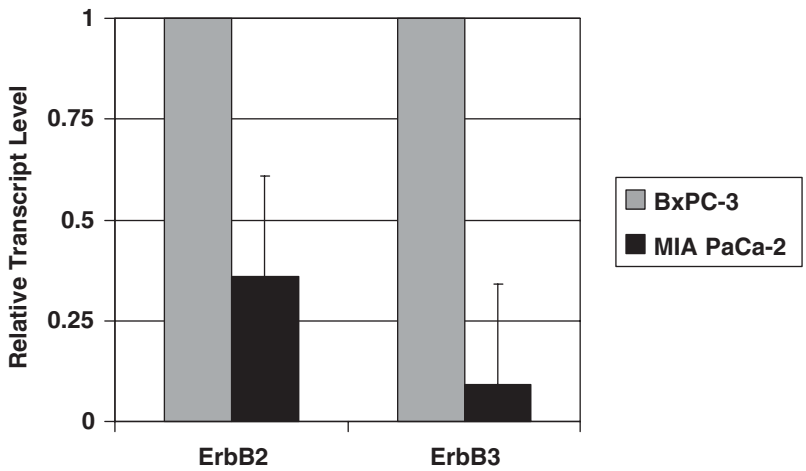

Fig. 4. MIA PaCa-2 and BxPC-3 cell relative gene expression levels of *erbB2* and *erbB3*, measured by real-time polymerase chain reaction.

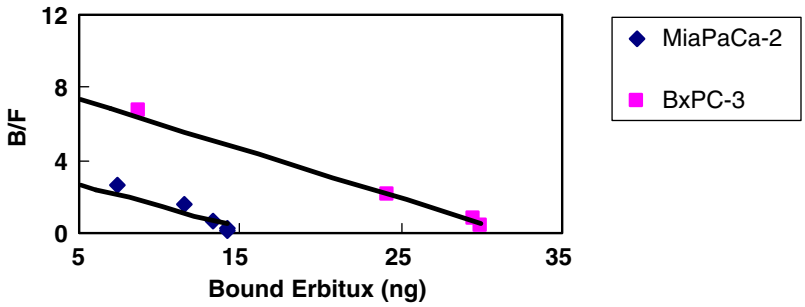

Fig. 5. ^{125}I-Erbitux binding affinity and epidermal growth factor receptor (EGFR) density in MIA PaCa-2 and BxPC-3 pancreatic cancer cells. The bound-to-free Erbitux antibody ratio (measured as ^{125}I isotope activity in cell pellets and supernatants) is presented as a function of increasing ^{125}I-Erbitux protein concentrations. The slope of the curves represents ^{125}I-Erbitux affinity for EGFR, and the curve–x-axis intersection value serves as an estimation of EGFR density in both cell lines. Scatchard analysis showed that the number of EGFRs per cell was 57,000 for MIA PaCa-2 and 120,000 for BxPC-3.

explain this differential response include, but are not limited to, alterations in EGFR internalization, expression of alternative downstream signal transduction mediators, and heterodimerization with other ErbB receptors. Additional mechanisms of resistance to Erbitux-based treatment were not addressed in our experiments and may involve persistence of EGFR stimulation by alternative ligands such as transforming growth factor-α and amphiregulin.[28,29]

In the present analysis, we established the baseline expression of ErbB family members in MIA PaCa-2 and BxPC-3 cells to evaluate possible heterodimer formation. BxPC-3 cells selectively express ErbB3 both at the transcript and protein levels, whereas this receptor is absent from MIA PaCa-2 cells. Both cell lines express ErbB2 protein at similar levels, and neither of them displays the ErbB4 receptor. Furthermore, the ErbB3 protein is constitutively phosphorylated in BxPC-3 cells, and this steady-state activation is enhanced by EGF. We also demonstrated that BxPC-3 ErbB3 contains phosphotyrosine at the binding motif for the PI3K p85 subunit. ErbB1 and ErbB2 only weakly bind the p85 subunit of PI3K [30] A strong association has been established by prior studies, however, between ErbB3 and PI3K activity, and ErbB3 contains several p85-binding motifs in its COOH terminus.[9–11] The Akt proteins are an important downstream target of PI3K activity, but we detected no differences in total Akt or phosphorylated Akt protein levels after stimulation with EGF and treatment with Erbitux (data not shown), suggesting involvement of alternative pathways such as protein kinase C.[31]

Although we provide no direct evidence of EGFR/ErbB3 heterodimerization in BxPC-3 cells or its causal effects on resistance to Erbitux-based therapy in vivo, our results suggest that these cells differentially express activated ErbB3 compared with the MIA PaCa-2 cells. The effects of Erbitux treatment on EGFR/ErbB3 cross-linking, ErbB3 phosphorylation, and the PI3K pathway remain to be determined. Experiments that include silencing ErbB3 expression with small interfering RNA (siRNA) in BxPC-3 cells and transfection of ErbB3 expression vectors in MIA PaCa-2 cells are under way and may provide additional information on the role of EGFR/ErbB3 heterodimers and resistance to Erbitux-based therapy.

To further investigate alternative mechanisms of resistance, we evaluated Erbitux binding affinity and the density of EGFR expression in both cell lines. Based on our prior publications, we hypothesized that observed differences in Erbitux-mediated effects in vitro, including proliferation and apoptosis, could be mediated by differences in affinity and/or EGFR density in these pancreatic cancer cells. Alternatively, we proposed that EGFR internalization and signal transduction mediation were different for the two cell lines and that those differences contributed, at least in part, to response to Erbitux-based treatment in vivo.

Our results confirmed that BxPC-3 cells exhibit greater EGFR density (twofold) with similar Erbitux binding affinity between the two cell lines. A twofold difference in receptor numbers at intermediate levels of EGFR overexpression (57,000–120,000) may have greater functional impact than at high levels of receptor overexpression. This is in concordance with our prior publications, where we have demonstrated that tumor cell augmentation of EGFR expression using adenoviral vectors increases the antiproliferative and radiosensitization effects of Erbitux in vitro.[32,33] This is also in agreement with previous in vitro findings

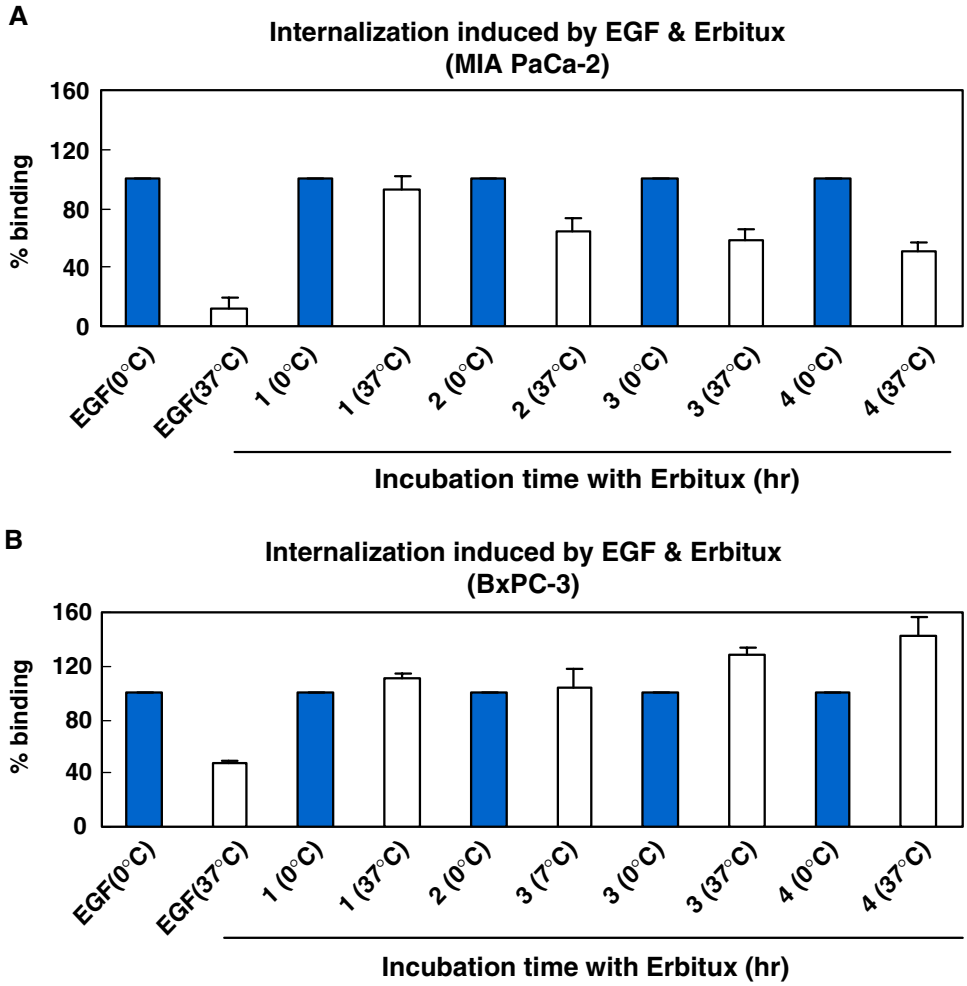

Fig. 6. (*A*) epidermal growth factor (EGF)-induced (2-hour incubation) and Erbitux-induced (1- to 4-hour incubation) internalization of EGF receptor, measured by flow cytometry at 0°C and 37°C in MIA PaCa-2 pancreatic cancer cells. The rate of internalization is in inverse relationship to the EGF and Erbitux binding percentage. Results are relative to the EGF and Erbitux binding values at 0°C, which were considered as 100% (no internalization present) and are shown without error bars. (*B*) EGF-induced (2-hour incubation) and Erbitux-induced (1- to 4-hour incubation) internalization of EGFR, measured by flow cytometry at 0°C and 37°C in BxPC-3 pancreatic cancer cells. The rate of internalization is in inverse relationship to the EGF and Erbitux binding percentage. Results are relative to the EGF and Erbitux binding values at 0°C, which were considered as 100% (no internalization present) and are shown without error bars.

where we had shown that BxPC-3 cells, with greater EGFR density, initially display increased inhibition of proliferation and apoptotic death after short-term Erbitux-based treatment compared with MIA PaCA-2 cells.[20] Greater EGFR density, however, does not explain the observed BxPC-3 cell resistance to in vitro Erbitux-based therapy when in vivo conditions are resembled by adding EGF after longer treatment intervals (6 weeks).[21]

To characterize cellular mechanisms that could explain BxPC-3 cell and BxPC-3 xenograft resistance, we analyzed the kinetics of Erbitux-induced EGFR internalization using flow cytometry after incubating pancreatic cancer cells with Erbitux for several time intervals and at two different temperatures (0°C and 37°C). EGF-induced EGFR internalization was used as reference. In the MIA PaCa-2 cells, Erbitux induced EGFR internalization after 4 hours that was comparable to that induced by EGF. BxPC-3 cells showed no internalization of EGFR after Erbitux treatment. Absence of receptor internalization and subsequent degradation may lead to persistent

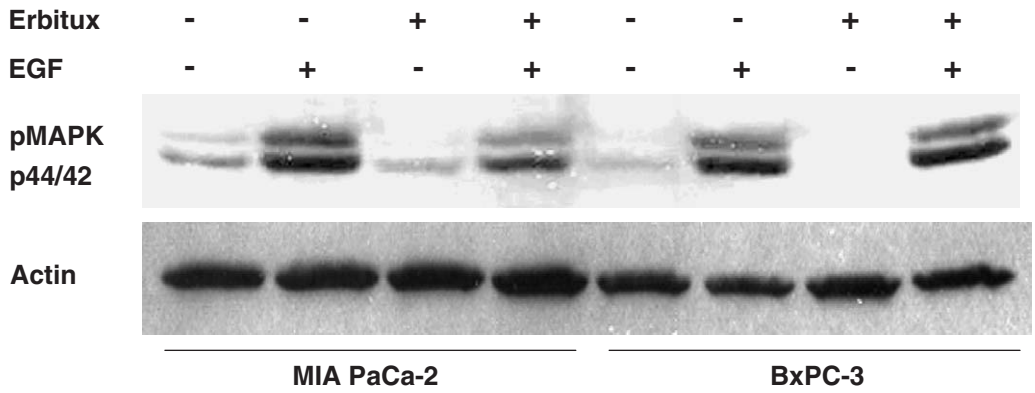

Fig. 7. Immunoblots for p44/42 phosphorylated MAPK in MIA PaCa-2 and BxPC-3 pancreatic cancer cells, incubated with Erbitux (5 μg/ml for 24 hours) and epidermal growth factor (EGF) (60 ng/ml for 10 minutes). Cells incubated with human IgG (5 μg/ml for 24 hours) and without EGF were used as negative controls, respectively.

activation of the EGFR signal transduction pathway and resistance to Erbitux-based therapy in vivo. We provided further evidence of the Erbitux effects on the EGFR pathway state of activation by demonstrating that EGF induces MAPK phosphorylation in MIA PaCa-2 cells and that this effect is partially blocked by Erbitux. On the contrary, strong MAPK phosphorylation in BxPC-3 cells was not inhibited with Erbitux treatment, leading to persistent EGFR pathway activation, which may lead to lack of response to Erbitux treatment. We conclude that impaired internalization of EGFR on BxPC-3 pancreatic cancer cells may be due to ErbB3 protein expression, which results in heterodimerization of EGFR, persistent MAPK activation, and, possibly, resistance to Erbitux-based combination therapy in vivo.

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Discussion

Dr. R. Daniel Beauchamp (Nashville, TN): This is an important area of study, as noted from the recent headline-grabbing articles that came out in *The New England Journal of Medicine* and *Science* related to studies of the EGF tyrosine kinase inhibitor Iressa. Unfortunately, these biologically targeted therapies have been fairly disappointing in their clinical application, with a relatively small number of patients actually benefiting from the effects, and I think these types of studies, to actually uncover the mechanism behind that resistance to these treatments, are very important. A lot of these studies have led to the conclusion that one really has to target multiple components of this pathway to really affect cell proliferation and survival. You have anticipated one of my questions related to knocking down the expression of *erbB3* and seeing if that will actually have the anticipated result.

I would also ask whether you have tried just using an EGF receptor tyrosine kinase inhibitor or a more promiscuous tyrosine kinase inhibitor to see if that alone is enough to abrogate the effect of the *erbB3* in your system?

Dr. Arnoletti: Thank you, Dr. Beauchamp. We have not tried other tyrosine kinase inhibitors. These studies are limited to just two cell lines, but, as you metioned, obviously trying to knock out *erbB3* expression, I think, would be the most useful experiment or mechanism to try to prove that indeed there is a heterodimer being formed and that that plays a role in resistance. We have also seen that, as expected, in vitro results do not always predict an in vivo response, and the results in vitro may not necessarily correlate with what is seen in vivo, but at least we hope to provide some further insight into the mechanism.

Dr. Joerg Haier (Muenster, Germany): You presented very interesting in vitro data; however, we know that several receptors for cytokines or growth factors and so on are differently expressed in the in vivo situation. Do you have any data about regulation of the *erbB* receptors in vivo, and can the antibodies affect it and result in internalization in vivo?

Dr. Arnoletti: I don't think studies have been done in vivo as far as the internalization process goes. The reports on the *ErbB* expression pattern in pancreatic cancer are scant. It is also highly variable depending on which antibody they have used and what criteria are defined as to what constitutes EGFR positive or *erbB3* positive or negative. There is one report from Germany published by Friess in the *Annals of Surgery* in 1999, and that is the one I cited, that describes up to 60% of pancreatic cancers as expressing *erbB3* in addition to EGFR. No other studies have addressed expression of *erbB3*, and that is certainly an important point; one of our goals is to further characterize pancreatic cancer tumor specimens to be better able to predict which patients may or may not respond to therapy.

Dr. Syed Ahmad (Cincinnati, OH): The flip side of your experiment is most phase III clinical trials nowadays don't require EGFR positivity for targeted therapy, so could you comment on why you think patients without EGFR positivity respond to therapy?

Dr. Arnoletti: In the data from the colon cancer studies, even when one of the criteria for entry in the published study was EGFR expression, the amount of expression did not seem to correlate with the degree of response. Both cell lines that we tested expressed EGFR. We have not tested cell lines that do not express EGFR with antibody-based therapy. They may respond. EGFR receptor expression may not correlate necessarily with response, but I do not know in this particular model what would happen, and I think one interesting experiment would be to test it in cell lines that are EGFR negative.

Gene Variants and Binge Eating as Predictors of Comorbidity and Outcome of Treatment in Severe Obesity

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Melanocortin-4 receptor gene (*MC4R*) variants are associated with obesity and binge eating disorder (BED), whereas the more prevalent proopiomelanocortin (*POMC*) and leptin receptor gene (*LEPR*) mutations are rarely associated with obesity or BED. The complete coding regions of *MC4R*, *POMC*, and leptin-binding domain of *LEPR* were comparatively sequenced in 300 patients (233 women and 67 men; mean ± SEM age, 42 ± 1 years; mean ± SEM body mass index, 43.5 ± 0.3 kg/m^2) undergoing laparoscopic gastric banding. Eating behavior, esophagogastric pathology, metabolic syndrome prevalence, and postoperative weight loss and complications were retrospectively compared between carriers and noncarriers of gene variants with and without BED during 36 ± 3-month follow-up. Nineteen patients (6.3%) carried 8 *MC4R* variants, 144 (48.0%) carried 13 *POMC* variants, and 247 (82.3%) carried 11 *LEPR* variants. All *MC4R* variant carriers had BED, compared with 18.1% of noncarriers ($P < 0.001$). BED rates were similar among *POMC* and *LEPR* variant carriers and noncarriers. Gastroscopy revealed more erosive esophagitis in bingers than in nonbingers before and after banding ($P < 0.04$), regardless of genotype. *MC4R* variant carriers lost less weight ($P = 0.003$), showed less improvement in metabolic syndrome ($P < 0.001$), had dilated esophagi ($P < 0.001$) and more vomiting ($P < 0.05$), and had fivefold more gastric complications ($P < 0.001$) than noncarriers. Overall outcome was poorest in *MC4R* variant carriers, better in noncarriers with BED ($P < 0.05$), and best in noncarriers without BED ($P < 0.001$). *MC4R* variants influence comorbidities and treatment outcomes in severe obesity. (J GASTROINTEST SURG 2004;8:971–982)

KEY WORDS: Melanocortin-4 receptor gene, binge eating, severe obesity, metabolic syndrome, treatment outcome

Obesity affects the health of populations worldwide[1] and is rapidly reaching epidemic proportions. As a multifactorial disease, caused by the interaction of genetic factors and the environment,[2] sedentary lifestyles, high-fat energy-dense diets, and a genetic predisposition to obesity all play a part in the epidemic.[3]

The brain pathways involving leptin, proopiomelanocortin, α-melanocyte stimulating hormone (α-MSH), and their respective receptors regulate energy homeostasis and appetite control.[4–8] Approximately 30 melanocortin-4 receptor gene (*MC4R*) variants have been associated with the obese phenotype[9,10] and account for about 5% of severe human obesity,[9,11,12] making *MC4R* mutations the most common known cause of monogenic obesity. In children, mutations resulting in complete loss of function of the melanocortin-4 receptor in vitro were associated with the

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most severe phenotype (a more severely obese phenotype).[11] Moreover, correlations between the impaired signaling properties of these mutant receptors with hyperphagia, as well as their association with binge eating disorder (BED), point to a significant role for *MC4R* in the control of human[9,11] and porcine[13] eating behaviors, although this has been disputed recently.[14] The proopiomelanocortin gene (*POMC*) and the leptin receptor gene (*LEPR*) have also been implicated in the development of monogenic obesity in humans.[15–18] Bulimia, anorexia, and binge eating may cause gastrointestinal symptoms,[19,20] but there is no information on the effects of BED on esophagogastric pathology.

The growing epidemic of obesity mandates effective prevention and treatment to achieve weight loss and reduce comorbidities, such as the very costly components of the metabolic syndrome.[21] The most common primary method of treatment is dietary, requiring life-long volitional control of the quantity and/or composition of ingested nutrients. Results are disappointingly ineffective.[1,22] Operations restricting the capacity and outflow from the stomach, thus imposing a low-calorie diet with less need for volitional control,[23] are relatively effective for treating severe obesity.[24] However, just as for conventional dietary treatment, reliable predictors of poor weight loss and complications are lacking, although BED has been shown to predict poor weight loss after dietary[25] and surgical[26] treatment.

Gene abnormalities are known in several metabolic diseases[27,28] and have been demonstrated to have predictive value in the treatment of myocardial infarction[29] and colorectal cancer.[30] Here we demonstrate the influence of gene variants in three obesity-associated candidate genes (leptin-binding domain of *LEPR*, *POMC*, and *MC4R*) on comorbidities and severity of the obese phenotype, and treatment outcomes after laparoscopic gastric restrictive surgery, in an attempt to further investigate the role of abnormal molecular physiology in linking genes and behavior.[31]

PATIENTS AND METHODS

Patients

The first 300 patients undergoing laparoscopic adjustable banding over a 5-year period, among 469 consecutive severely obese patients described earlier,[9] were entered into this study. Patient characteristics were mean ± SEM age of 42 ± 1 years, 77% female, mean ± SEM height of 167 ± 1 cm, mean ± SEM body mass index (BMI) of 43.5 ± 0.3 kg/m^2, and 33% smokers. Exclusion criteria were BMI less than 35 kg/m^2, age younger than 18 or older than 70 years, and alcoholism or drug abuse determined at interview or through information from family or referring physician.

A multidisciplinary team consisting of a physician specializing in obesity, a bariatric surgeon, a dietician, and a psychologist assessed each patient before laparoscopic gastric banding. Data were entered prospectively into our computerized database (Obesity Base 2000; Zürich, Switzerland). Patients were fully informed about all procedures and gave written consent. The study was approved by the local ethics committee and complied with the Declaration of Helsinki.

Blood Chemistry

After a 12-hour overnight fast, patients had blood drawn for measurement of serum concentrations of total cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, albumin, alkaline phosphatase, γ-glutamyl transpeptidase, and blood glycosylated hemoglobin (Hb_{A1c}) using standard hospital laboratory techniques. Leptin was determined by radioimmunoassay (Linco Research, Prof. Krech und Partner AG, Kreuzlinger, Switzerland). Blood testing was performed preoperatively and annually postoperatively.

Gene Analysis

At least 18 months postsurgery, 20 ml of EDTA venous blood was collected for comparative sequencing of the complete coding and flanking 5′ and 3′ untranslated regions of *MC4R*, *POMC*, and the leptin binding domain of *LEPR*. Both DNA strands were routinely amplified and sequenced to guarantee maximum accuracy in variation analysis. The *MC4R* reference sequence (GenBank accession No. S77415), the two coding exons defining the *POMC* structural sequence (GenBank accession No. V01510), and the respective sequences of *LEPR* (assembled in-house with the use of GenBank accession Nos. AC097063.2 and U59257.1) were dissected into suitable polymerase chain reaction fragments.[9] The cycling parameters for *MC4R*, *POMC*, and *LEPR* fragments have been detailed previously.[9] Investigators were blinded to the genotype of the patients throughout this study.

Body Composition

Body fat and lean body mass were determined by dual-energy x-ray absorptiometry (DEXA) (QDR 4500A; Hologic, Bedford, MA) preoperatively and after about 3 years.[9] Central obesity was defined using fat distribution data obtained by DEXA, as previously described,[32] to derive waist circumference. Percentage of central fat mass (trunk fat divided by the sum

of total lean and body fat mass) and peripheral fat mass (fat of arms and legs divided by total lean and body fat mass) were calculated, and fat distribution was expressed as the ratio of central fat mass to peripheral fat mass. Waist circumference in our operated patients was derived from determinations of body fat distribution by DEXA and waist circumference in 402 normal weight and obese subjects (men: n = 79, BMI 38.5 ± 1.2 kg/m^2 [range, 21.1–71.1 kg/m^2], age 41 ± 1 years; women: n = 323, BMI 38.1 ± 0.6 kg/m^2 [range, 18–67 kg/m^2], age 41 ± 1 years).

Metabolic Syndrome

Metabolic syndrome components (type 2 diabetes mellitus, dyslipidemia, hypertension, and central obesity) were defined according to guidelines of the National Cholesterol Education Program (NCEP) Expert Panel (ATP III),[21] with minor modifications. Thus, type 2 diabetes mellitus was diagnosed in patients taking hypoglycemic medication or exhibiting elevated serum Hb_{A1c} (≥7%). Dyslipidemia was defined as elevated serum triglycerides (≥1.69 mmol/L [150 mg/dl]) and/or reduced HDL cholesterol (≤1.04 mmol/L [40 mg/dl] in men and ≤1.29 mmol/L [50 mg/dl] in women), or use of lipid-lowering drugs. Hypertension was diagnosed in patients taking antihypertensive drugs or having elevated systolic and/or diastolic blood pressure (≥130 or ≥85 mm Hg, respectively). Central obesity followed the ATP criteria[21] of waist circumference greater than 102 cm for men and greater than 88 cm for women, corresponding to the measured DEXA body fat distribution of 0.84 and 0.65, respectively (see Body Composition). The metabolic syndrome was considered present in patients having three or more of the components listed.[21]

Eating Behavior

Patients were classified as binge eaters or non–binge eaters, as described previously.[9] Diagnostic criteria for BED included at least twice-weekly bingeing over a minimum of 6 months. A *binge* was defined as rapid consumption of an unusually large amount of food in the absence of hunger, causing the subject to feel embarrassed, depressed or guilty, and out of control. There was no purging behavior. Only subjects who fulfilled all criteria for BED, determined independently by three members of the multidisciplinary team, were described as "bingers." Subjects not fitting these criteria were termed "nonbingers."

Gastric Banding

Laparoscopic adjustable gastric banding was performed as described earlier.[33,34] Briefly, the inflatable band encircled the cardia of the stomach and was sutured in place to form a small pouch (volume <20 ml; Fig. 1). The injectable subcutaneous port was sutured external to the lower third of the sternum and attached via tubing to the inflatable gastric band. Four to 6 weeks after surgery, the band was inflated for the first time using contrast (Iopamiro 200, iopamidol; Bracco, Milan, Italy). Indications for inflation were less than 1-kg weight loss per month or absence of fullness reported during a semistructured interview after a standard meal (half the size of a typical preoperative meal). Deflation was indicated for obstruction, nightly aspiration, or vomiting more than twice per week.

Complications, reoperations, blood pressure, and weight were recorded at each office visit. Weight loss is expressed as percentage of preoperative BMI. Adjustments of filling volume of the band were made as necessary every 2 months, based on vomiting, eating behavior, and weight development, recording the number of adjustments as a measure of adaptation of eating behavior to gastric restriction.[23] Follow-up was 36 ± 3 months.

If insufficient weight loss occurred, medical treatment was added using sibutramine (Reductil; Abbott, Baar, Switzerland) or orlistat (Xenical; Roche, Basel, Switzerland). *Insufficient* is defined as

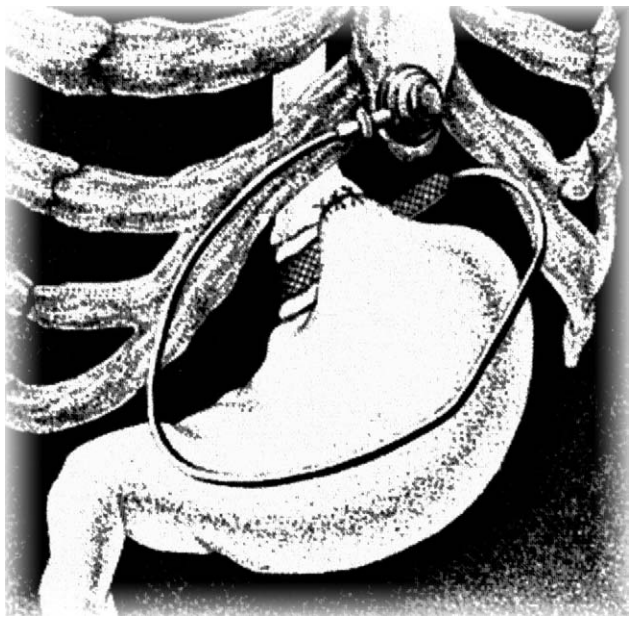

Fig. 1. Gastric band with port and tube system. The adjustable gastric band encircles the cardia of the stomach, forming a small pouch. The tube leads from the gastric band to the port, which is sutured subcutaneously external to the lower third of the sternum, allowing transcutaneous adjustment of the band.

(1) less than 50% excess weight loss after primary operation, or (2) plateau weight for 3 consecutive months, or (3) initial excess weight loss of more than 50% but greater than 10% of nadir weight regained, reducing excess weight loss to less than 50%.

Gastroesophageal Studies

Before and 2 years after gastric banding, gastroscopy was performed for evaluation of mucosal appearance, and barium swallow radiographs were obtained using a standard volume of barium sulfate suspension (Micropaque; Delpharm, Bretigny-sur-Orge, France). Maximum esophageal diameter was measured using the radiopaque gastric band as a reference on anterior and lateral images, choosing the greater of the two. Similarly, the area of the gastric pouch was expressed in terms of the area of the rectangular image of the band.

Complications and Reoperations

One patient died of myocardial infarction during her third postoperative year, and data up to her death were included in the analysis. Complications were categorized as either gastric or port/tube related, expressed per patient per treatment year. Port/tube complications consisted of infection, port site discomfort, and tube or port leakage. Gastric complications were band leakage or slippage, band intolerance, or band erosion confirmed radiologically or by endoscopy. *Band intolerance* was defined as nocturnal aspiration or coughing necessitating deflation of the band followed by weight regain of 5% or more, or a loss of less than 2 BMI units per year (≈5–6 kg). At each office visit, vomiting history was evaluated and scored according to frequency and/or severity (0 = absence of vomiting; 1 = vomiting associated with rapid eating or stressful circumstances; 2 = vomiting once or less weekly, and 3 = more than once weekly, both unrelated to eating rate or stress).

Band replacement was performed for leakage or slippage; conversion to gastric bypass was elected for band erosion, intolerance, or recurrent slippage.

Outcome Score

To facilitate intergroup comparisons, an aggregate "outcome score" was created, combining postoperative persistence of obesity and metabolic syndrome components with side effects and complications. Each patient was given 1 point for the presence of each of 14 characteristics (gastric complications, reoperations, conversion to gastric bypass, band-filling adjustments, esophageal diameter, erosive esophagitis, central/peripheral fat mass ratio, type 2 diabetes mellitus, hypertension, low HDL cholesterol, elevated triglycerides [see Patients and Methods], belonging to the top tertile of vomiting score and to the bottom tertile of weight loss).

Statistical Analysis

Statistically significant differences were analyzed between patient groups matched for age, gender, and BMI with and without gene variants and with and without BED. Analysis of variance for repeated measures or Mann-Whitney *U* test, where appropriate, were corrected for multiple comparisons (SSPS version 10.0 for Windows). All results are presented as mean ± SEM in text, tables, and figures. Reported *P* values were two-sided, accepting levels of $P \leqslant 0.05$ as significant, $P \leqslant 0.10$ as a strong trend, and $P < 0.20$ as a weak trend.

RESULTS

Genotype

Nineteen patients carried eight different *MC4R* variants (6.3%; 13 women and 6 men; age, 47 ± 3 years [mean ± SEM]; BMI, 45.0 ± 1.0 kg/m^2 [Table 1]), whereas 281 patients were noncarriers (220 women and 61 men; age, 41 ± 1 years; BMI, 43.4 ± 0.3 kg/m^2). All genetic variants were heterozygous and have been described previously.[9]

Thirteen different *POMC* variants were carried by 144 patients (48.0%; 111 women and 33 men; age, 41 ± 1 years; BMI, 43.6 ± 0.4 kg/m^2), but none were located in the α-MSH region of the gene (see Table 1). Four variants were localized in the noncoding region, four were synonymous, and five variants affected the amino acid sequence (see Table 1). One hundred fifty-six patients were noncarriers of *POMC* variants (122 women and 34 men; age, 42 ± 1 years; BMI, 43.5 ± 0.4 kg/m^2).

Eleven different variants were found in the leptin-binding domain of *LEPR* (see Table 1) carried by 247 patients (82.3%; 194 women and 53 men; age, 42 ± 1 years; BMI, 43.6 ± 0.3 kg/m^2), whereas 53 patients were noncarriers of *LEPR* variants (38 women and 15 men; age, 41 ± 1 years; BMI, 43.2 ± 0.6 kg/m^2).

Phenotype

MC4R variant carriers (n = 19) were significantly older than noncarriers ($P = 0.02$) and more often men. Therefore, *MC4R* variant carriers (n = 19) were retrospectively matched individually for age (±3 years), presurgery BMI (±1 BMI unit), and gender with *MC4R* variant noncarriers who had completed about 3 years of follow-up (n = 155; Table 2). This matched subgroup of 174 patients consisted of 85 *POMC* variant carriers (58 women and 27 men; age, 44 ± 1 years; BMI,

Table 1. Variants in the melanocortin-4 receptor (*MC4R*), proopiomelanocortin (*POMC*), and leptin-binding domain of the leptin receptor (*LEPR*) genes in the study population (N = 300)

	Base change*	Effect on amino acid sequence*	Allele frequency
MC4R variants	C408T	Thr5Thr	0.002
	C728T	Thr112Met	0.003
	C886T	Arg165Trp	0.002
	A700G	Val103lle	0.015
	A1144C	lle251Leu	0.005
	T544C	Phe51Leu	0.002
	A991G	Met200Val	0.002
	A1419G	3′-UTR	0.002
POMC variants	C4512T	Cys6Cys†	0.007
	C7662T	Ser94Ser§	0.003
	Ins between codon 99 and 100 (AGCAGCGGC) 1–2×	SerSerGly§	0.060
	C7965T	Ala195Ala‡	0.003
	C4335G	5′-UTR††	0.003
	A7429G	Intron 2††	0.022
	C7726T	Leu116Leu§	0.002
	C7774G	Pro132Ala§††	0.002
	A8021G	Glu214Gly‖	0.015
	A8042G	Tyr221Cys¶††	0.002
	C8086G	Arg236Gly**	0.002
	del T 8211	3′-UTR††	0.002
	C8246T	3′-UTR	0.212
LEPR variants	T88641C	Ser343Ser	0.225
	G88642A	Val344lle	0.002
	G88917A	Intron 9	0.013
	T88928C	Intron 9††	0.057
	C95778T	Intron 11††	0.003
	T95869C	Intron 11††	0.002
	T96008C	Thr548Thr	0.003
	T96135C	Intron 12††	0.002
	A96215G	Intron 12††	0.002
	A97118G	Intron 12††	0.073
	G97244A	Arg612His	0.002

*Position numbers are given relative to GenBank reference sequences: S77415 for *MC4R*; V01510 for *POMC*; AC097063.2 for *LEPR*. UTR denotes untranslated region. Region in *POMC* protein:
†Signal peptide.
‡After β-endorphin.
§Between γ-MSH and α-MSH.
‖Between ACTH and β-MSH.
¶β-MSH.
**Between β-endorphin and β-MSH.
††Novel variants.

44.3 ± 0.5 kg/m^2) and 89 noncarriers (62 women and 27 men; age, 45 ± 1 years; BMI, 44.2 ± 0.6 kg/m^2), and 138 carriers (98 women and 40 men; age, 45 ± 1 years; BMI, 44.2 ± 0.4 kg/m^2) and 36 noncarriers (22 women and 14 men; age, 42 ± 2 years; BMI, 44.6 ± 0.7 kg/m^2) of variants in the leptin-binding domain of *LEPR*.

Binge eating disorder. All *MC4R* variant carriers (n = 19) had BED (100%) compared with 18.1% of matched noncarriers (n = 28; $P < 0.001$; see Table 2). These 28 bingeing noncarriers were matched for age, BMI, and gender with 85 noncarriers without bingeing (see Table 2). The frequency of binge eating was similar between carriers and noncarriers of *POMC* variants (27% for both) and tended to be higher among *LEPR* variant carriers than noncarriers (29.7% versus 16.7%; $P = 0.11$).

Esophagogastric pathology. Overall, bingers had a higher frequency of erosive esophagitis than non-bingers ($P = 0.04$) but a similar prevalence of gastritis. However, the rate of esophagitis between carriers and noncarriers of *MC4R* variants was not statistically different (see Table 2), whereas noncarriers without bingeing demonstrated a lower rate of esophagitis than noncarriers with bingeing ($P = 0.02$; see Table 2).

Metabolic syndrome. The prevalence of metabolic syndrome was 68.4% among *MC4R* variant carriers, compared with 49.0% in matched noncarriers ($P = 0.08$; see Table 2): central to peripheral fat mass ratio tended to be higher ($P = 0.06$), type 2 diabetes mellitus more frequent ($P = 0.03$), and dyslipidemia (triglycerides, $P = 0.01$; HDL cholesterol, $P = 0.07$) and hypertension showed trends toward higher levels in *MC4R* variant carriers ($P < 0.15$; see Table 2). Preoperatively, significantly more carriers of *MC4R* variants took antihypertensive and hypoglycemic drugs than did noncarriers (63.2% versus 29.7%, $P = 0.003$; 15.7% versus 4.5%, $P = 0.05$; respectively), with no difference in lipid-lowering medication. All baseline metabolic phenotypic data were similar between *MC4R* variant noncarriers with and without bingeing (see Table 2) and between carriers and noncarriers of *POMC* and *LEPR* variants.

Outcome

Weight loss. *MC4R* variant carriers lost about 18% (7 kg, or 5% of preoperative BMI) less weight than did noncarriers during 3-year follow-up (analysis of variance for repeated measures, $P = 0.003$; Fig. 2, *A*). However, after conversion to gastric bypass for complications (n = 20), weight loss during the year after reoperation was similar between *MC4R* variant carriers and noncarriers (18.4 ± 2.4 kg versus 16.9 ± 2.9 kg, respectively; $P = 0.74$). In the 81.0% of patients without reoperations (42.1% of *MC4R* variant carriers versus 85.8% of noncarriers; $P < 0.001$),

Table 2. Baseline characteristics for groups matched according to *MC4R* variant status (n = 174) and binge eating disorder (BED) status among noncarriers of *MC4R* variants (n = 113)

	MC4R variant carriers (n = 19)	*MC4R* variant noncarriers (n = 155)	Noncarriers with BED (n = 28)	Noncarriers without BED (n = 85)
Age (yr)	47 ± 3	44 ± 1	43 ± 1	44 ± 1
Gender (% female)	68.4	69.0	82.1	82.4
Body mass index (kg/m^2)	45.0 ± 1.0	44.2 ± 0.4	46.4 ± 1.1	45.2 ± 0.4
Binge eating disorder (%)	100.0*	18.1	100.0*	0.0
Erosive esophagitis (%)	53.3	39.4	53.8‡	28.2
Gastritis (%)	20.0	21.9	26.9	20.2
Metabolic syndrome (%)	68.4§	49.0	53.6	48.2
Body fat (kg)	51.7 ± 1.9	54.8 ± 0.8	58.1 ± 2.6	57.1 ± 0.9
Central/peripheral fat mass ratio (%)	1.22 ± 0.07§	1.10 ± 0.02	1.05 ± 0.04	1.02 ± 0.02
Type 2 diabetes mellitus (%)	36.8‡	15.5	14.2	12.9
Dyslipidemia (%)	89.5‖	74.2	78.6	70.6
Hypertension (%)	73.7‖	56.7	64.3	57.6
Triglycerides (mmol/L)	3.0 ± 0.4†	2.2 ± 0.1	2.2 ± 0.2	2.0 ± 0.1
HDL cholesterol (mmol/L)	1.07 ± 0.07§	1.20 ± 0.03	1.20 ± 0.05	1.22 ± 0.03
Glycosylated hemoglobin (%)	6.1 ± 0.3	5.8 ± 0.1	5.7 ± 0.1	5.7 ± 0.1
Total cholesterol (mmol/L)	5.9 ± 0.2	5.8 ± 0.1	5.6 ± 0.2	5.7 ± 0.1
Albumin (g/L)	37.5 ± 0.8	37.9 ± 0.3	38.0 ± 0.6	37.6 ± 0.4
Alkaline phosphatase (U/L)	83.7 ± 4.9	82.3 ± 1.6	85.3 ± 4.0	79.8 ± 2.2
GGT (U/L)	42.2 ± 6.7	38.2 ± 2.1	38.5 ± 4.2	32.1 ± 2.0
Leptin (ng/ml)	41.5 ± 4.6	38.1 ± 1.3	40.8 ± 3.4	41.8 ± 1.7
Systolic blood pressure (mm Hg)	133 ± 3	135 ± 1	138 ± 2	134 ± 2
Diastolic blood pressure (mm Hg)	83 ± 2	87 ± 1	89 ± 2	87 ± 1
Smokers (%)	26.3	34.2	25.9	35.3

*$P < 0.001$.
†$P < 0.01$.
‡$P < 0.05$.
§$P < 0.10$.
‖$P < 0.15$.

carriers lost less weight during 3-year follow-up (percentage of preoperative BMI lost, 18.9% ± 3.8% versus 27.5% ± 0.7% in noncarriers; $P = 0.01$; Fig. 2, *B*). *MC4R* variant noncarriers with bingeing tended to lose less weight compared with nonbingers ($P < 0.10$; Table 3). Weight loss was similar between carriers and noncarriers of *POMC* and *LEPR* variants ($P > 0.32$).

There was no difference in frequency of patients taking sibutramine or orlistat between *MC4R* variant carriers (n = 3) and noncarriers (n = 13; $P = 0.58$) or between noncarriers with (n = 3) and without bingeing (n = 9; $P = 0.47$). Furthermore, weight loss per month of rescue medical treatment was similar between these groups ($P = 0.2$ and 0.76, respectively).

Complications. *MC4R* variant carriers experienced five times more gastric complications per patient and treatment year than did *MC4R* variant noncarriers ($P < 0.001$), whereas port- and tube-related rates were similar. *MC4R* variant noncarriers with and without bingeing had similar rates of gastric and port/tube-related complications per patient and treatment year (see Table 3), as did carriers and noncarriers of *POMC* and *LEPR* variants.

More than fourfold more *MC4R* variant carriers were among the 19% of patients (n = 33) who underwent reoperation for gastric complications. Conversion to gastric bypass was also four times more frequent in *MC4R* variant carriers than noncarriers ($P < 0.001$; see Table 3). Numerically increased rates of reoperation and conversion to gastric bypass in *MC4R* variant noncarriers with and without bingeing did not reach statistical significance (see Table 3). In contrast, *POMC* and *LEPR* variant carriers and noncarriers demonstrated similar reoperation and conversion rates (not shown).

Gastric band adjustments, esophageal diameter, and vomiting scores were all significantly higher in *MC4R* variant carriers compared with noncarriers

Table 3. Three-year outcome of laparoscopic gastric banding for groups matched according to *MC4R* variant status (n = 174) and binge eating disorder (BED) status among noncarriers of *MC4R* variants (n = 113)

	MC4R variant carriers (n = 19)	*MC4R* variant noncarriers (n = 155)	Noncarriers with BED (n = 28)	Noncarriers without BED (n = 85)
Duration (mo)	38.7 ± 1.4	40.2 ± 0.3	40.2 ± 0.5	40.6 ± 0.4
Preoperative body mass index lost (%)	22.0 ± 1.9‡	27.1 ± 0.7	25.0 ± 1.9§	28.2 ± 0.9
Gastric complications††§§	0.303 ± 0.060*	0.057 ± 0.010	0.069 ± 0.026	0.068 ± 0.015
Port/tube-related complications††	0.060 ± 0.047	0.031 ± 0.008	0.024 ± 0.017	0.025 ± 0.010
Reoperation (%)§§	57.9*	14.2	25.0	15.3
Conversion to gastric bypass (%)§§	36.8*	8.4	17.9	8.2
Band-filling adjustments (n/yr)§§	5.3 ± 1.5*	2.7 ± 0.2	3.5 ± 0.7	2.8 ± 0.2
Area of gastric pouch (cm^2)‖‖	13.9 ± 3.0	15.2 ± 1.7	14.1 ± 1.4	16.2 ± 2.5
Esophageal diameter (cm)§§‖‖	2.5 ± 0.4*	1.3 ± 0.1	1.5 ± 0.2	1.3 ± 0.1
Vomiting score‡‡	1.47 ± 0.21‡	1.12 ± 0.06	1.29 ± 0.15‖	1.06 ± 0.08
Erosive esophagitis (%)§§‖‖	46.7‖	29.9	46.1‡	26.3
Gastritis (%)§§‖‖	13.3	7.3	15.4‡	3.9
Body fat lost (%)	31.3 ± 2.8†	40.3 ± 1.1	34.5 ± 2.8	39.9 ± 1.3
Central/peripheral fat mass ratio (%)§§	1.14 ± 0.08‡	0.99 ± 0.02	0.99 ± 0.04	0.93 ± 0.02
Metabolic syndrome (%)	42.1¶	9.0	3.6	9.4
Type 2 diabetes mellitus (%)§§	15.8**	1.9	0	2.4
Dyslipidemia (%)	78.9¶	29.7	28.6	31.8
Hypertension (%)§§	42.1**	12.9	17.9	10.6
Albumin (g/L)	43.4 ± 0.9	41.9 ± 0.3	42.0 ± 0.5	41.9 ± 0.5
Alkaline phosphatase (U/L)	71.9 ± 5.8	65.9 ± 1.4	62.8 ± 2.3	66.9 ± 2.1
GGT (U/L)	31.9 ± 5.1	22.3 ± 1.2	20.1 ± 1.9	20.0 ± 1.4

MC4R = melanocortin-4 receptor gene. Values depict mean ± SEM.
Between *MC4R* variant carriers and noncarriers, or between non-carriers with and without bingeing: *$P \leq 0.001$, †$P \leq 0.01$, ‡$P \leq 0.05$, §$P < 0.10$, ‖$P < 0.20$.
Change in frequency of metabolic syndrome and its components from baseline to 3 years between *MC4R* variant carriers and noncarriers: ¶$P < 0.001$, **$P < 0.01$.
††Complications per patient per treatment year.
‡‡Vomiting score (see Methods).
§§Characteristics used in the Outcome Score (see Methods).
‖‖For patients who underwent conversion to gastric bypass, investigations were before reoperation.

($P < 0.05$; see Table 3). Adjustments and esophageal diameter were similar between *MC4R* variant noncarriers with and without bingeing, but vomiting scores tended to be higher in noncarriers with bingeing ($P = 0.15$; see Table 3). These parameters were similar between carriers and noncarriers of *POMC* and *LEPR* variants (all $P > 0.60$).

Esophagogastric pathology. The postoperative prevalence of erosive esophagitis determined by endoscopy tended to be higher in *MC4R* variant carriers compared with noncarriers ($P = 0.18$; see Table 3), whereas the prevalence of gastritis was similar in carriers preoperatively and postoperatively ($P = 0.72$) but decreased significantly in noncarriers ($P < 0.001$; see Tables 2 and 3). Moreover, erosive esophagitis and gastritis were more prevalent after surgery in *MC4R* noncarriers with bingeing than in those without bingeing (both $P < 0.05$).

Metabolic syndrome and its components. Metabolic syndrome was reduced 80% in *MC4R* variant noncarriers over 3 years ($P < 0.001$) and was more persistent in *MC4R* variant carriers compared with noncarriers ($P = 0.001$; see Table 3) throughout the study period, including the individual components of the metabolic syndrome: central body fat; type 2 diabetes mellitus; dyslipidemia; and hypertension (all $P < 0.02$). The frequencies of type 2 diabetes mellitus, dyslipidemia, and hypertension in *MC4R* variant carriers after 3 years of sustained weight loss were similar to those of noncarriers at baseline (see Tables 2 and 3). Despite a substantial decrease in body fat over the study period, the central-to-peripheral fat ratio remained significantly higher in *MC4R* variant carriers than in noncarriers ($P = 0.02$; see Table 3). After 3 years of weight loss, the frequency of metabolic syndrome and its parameters was similar between *MC4R* noncarriers with and without bingeing (see Table 3) and carriers and noncarriers of *POMC* and *LEPR* variants. Similar relationships were obtained when data were analyzed by gender (data not shown).

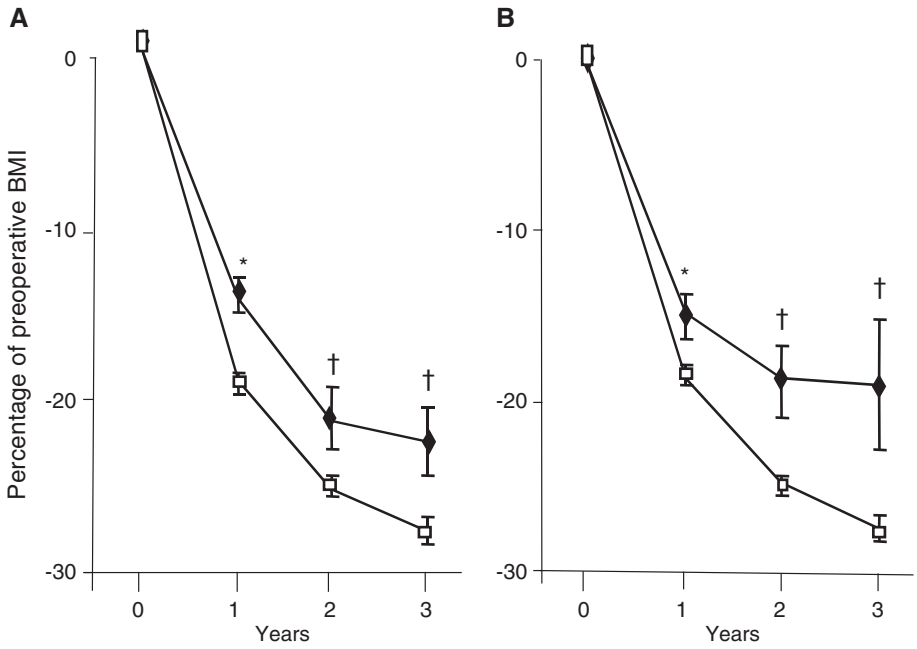

Fig. 2. Percentage of preoperative body mass index lost during 3-year follow-up in melanocortin-4 receptor gene (*MC4R)* variant carriers (♦) and matched *MC4R* variant noncarriers (□) for whole group (**A**) and for patients not undergoing reoperation (**B**). (A, Analysis of variance for repeated measures, $P = 0.003$. *$P < 0.001$, †$P = 0.03$, ♦n = 19, □n = 155. B, Analysis of variance for repeated measures, $P = 0.01$. *$P = 0.08$, †$P = 0.02$, ♦n = 8, □n = 133. Values given as mean ± SEM.)

Outcome score. Overall outcome was best for *MC4R* variant noncarriers without bingeing compared with noncarriers with bingeing ($P < 0.05$). Outcome was worst in *MC4R* variant carriers ($P < 0.001$ versus both other groups; Fig. 3).

DISCUSSION

We demonstrate for the first time that gene variants may determine the outcome of treatment of severe obesity, with respect to both complications

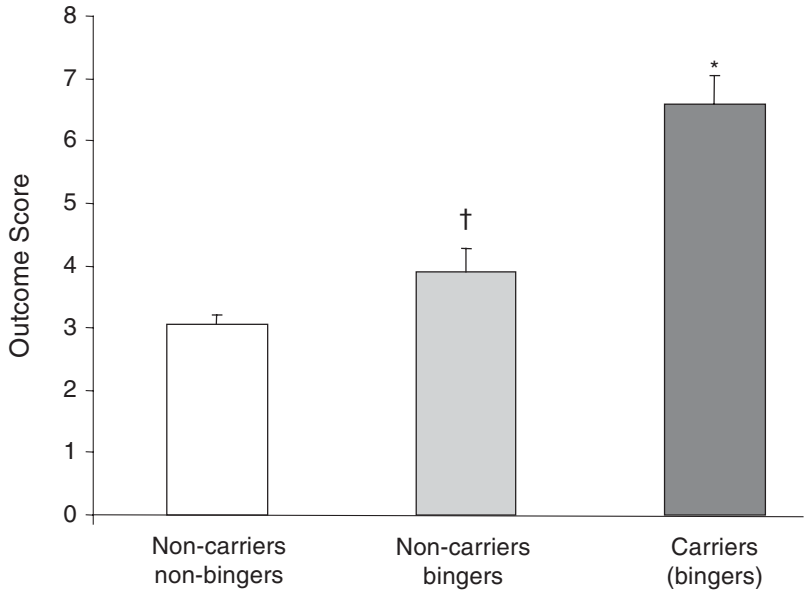

Fig. 3. Mean ± SEM outcome scores for noncarriers of melanocortin-4 receptor gene variants (*MC4R*) without binge eating disorder (n = 127), noncarriers with bingeing (n = 28), and carriers of *MC4R* variants (n = 19). *$P < 0.001$: *MC4R* variant carriers versus noncarrier bingers and noncarrier nonbingers. †$P < 0.05$: *MC4R* variant noncarrier bingers versus noncarrier nonbingers.

of treatment and the response of comorbidities in the presence of a dramatically sustained reduction of the metabolic syndrome and its components. Furthermore, we show the importance of melanocortin-4 receptor gene variants for the severity of BED and describe for the first time esophagogastric pathology diagnosed by endoscopy in such patients. In contrast, we have already shown that variants in the G-protein β3 subunit gene do not predict outcome after gastric banding,[35] in conflict with another study showing that such variants predict benefit of treatment with sibutramine or nonpharmacologic weight loss programs.[36]

Nineteen of the population of 300 severely obese patients (6.3%) exhibited variants in *MC4R*, consistent with previous studies.[9,11,12] *POMC* variants were present in 48% of patients, and variants in the leptin-binding domain of *LEPR* in 82.3%, but the rare *POMC*[15,16] and *LEPR*[17,18] variants associated with early-onset obesity were not found.

With the exception of a trend toward more BED among *LEPR* variant carriers than noncarriers, none of the phenotypic differences distinguishing *MC4R* variant carriers from noncarriers were found among patients with *POMC* or *LEPR* variants. Thus, we hypothesize that variants of the melanocortin-4 receptor gene, whether demonstrated to be functional in vitro or not,[37,38] may disrupt the modulating effect of this gene on eating behavior.[7] However, BED is polygenic and multifactorial. We found a higher baseline prevalence of erosive esophagitis and metabolic syndrome components in bingers overall compared with nonbingers. *MC4R* variant carriers demonstrated an attenuated response to treatment with persistent erosive esophagitis and increased postoperative complication rates compared with noncarriers. Collectively, these findings imply a more severe form of eating disorder among patients with *MC4R* variants compared with bingeing noncarriers, as has been suggested in children.[11] Moreover, in accord with Farooqi et al.,[11] the prevalence of metabolic syndrome and its components was already increased at baseline in carriers compared with noncarriers of *MC4R* variants in the present study.

Most of our findings, including the comorbidities and complications in carriers of *MC4R* variants, are attributable to their more aggressive, uncontrolled overeating, compared with noncarriers with bingeing. *MC4R* variant carriers did not have a preoperative history of vomiting, as evidenced by their diagnosis of bingeing and exclusion of bulimia, yet the prevalence of erosive esophagitis was 50% greater in all bingers than in nonbingers. This prevalence did not improve postoperatively in *MC4R* variant carriers. Furthermore, this conclusion is strengthened by the increased frequency of postoperative vomiting, greater need for band adjustments, dilated esophagi on radiography, and frank gastric band–related complications requiring twice as many reoperations in *MC4R* variant carriers compared with noncarriers with bingeing. Gastrointestinal symptoms of bulimia and anorexia nervosa have usually been attributed to the typical purging behavior associated with those conditions.[19] The gastroesophageal pathology often described in obesity, on the other hand, has been linked to increased intra-abdominal pressure with delayed esophageal clearance and reflux.[39] Our findings of varying frequencies of erosive esophagitis in obese subjects of similar BMI, without vomiting, and with gastritis in the distal stomach (below the constricting band), imply different pathogenetic mechanisms specific to the rapid rate of eating in BED.

Because purely gastric restrictive operations such as banding and gastroplasty, much like conventional dieting, rely on impulse control, it is reasonable to conclude that long-term maintenance of weight loss after such operations will fail in patients lacking such control. Indeed, gastrointestinal bypass, or diversionary operations that incorporate maldigestive mechanisms, associated with rapid transit of nutrients from the stomach into the small intestine, have universally demonstrated superior long-term results.[22] Although it is too early for conclusions, our preliminary finding of similar weight loss in carriers and noncarriers of *MC4R* variants during the first year after conversion to gastric bypass bears this out. One report found similar weight loss in bingers and nonbingers after diversionary operations,[40] whereas one did not.[26] Nevertheless, laparoscopic gastric banding in noncarriers of *MC4R* variants led to a near eradication of type 2 diabetes (15.5% to 1.9%), dramatically better than results of lifestyle changes in a previous study,[41] and a substantial improvement of the metabolic syndrome in general (49.0% to 9.0%; see Tables 2 and 3).

The higher prevalence of metabolic syndrome and its poorer response to significant weight loss in *MC4R* variant carriers merit special attention in the context of elucidating putative mechanisms for insulin resistance. It is true that all antiobesity operations achieve early (even before substantial weight loss) and sustained remission of type 2 diabetes mellitus,[23] with better maintenance after diversionary procedures,[22] but the mechanisms remain controversial. Based on earlier work,[42] we hypothesize that the intrinsically aggressive and rapid eating of the carriers of *MC4R* gene variants overrides the gastric restriction of the adjustable band, initiating the cascade of brisk insulin release, increased free fatty acids, and triglyceridemia, associated with fatty liver, impaired degradation of insulin, and hyperinsulinemia.[43,44]

CONCLUSION

In aggregate, our findings suggest that carriers of *MC4R* gene variants, in contrast to matched severely obese patients without gene abnormalities and binge eating, exhibit an aggressive form of BED associated with the metabolic syndrome and gastrointestinal pathology, with poorer response to massive sustained weight loss from food restriction after gastric banding. Although numerous "behavioral genes" have been described,[45–48] this may be the first demonstration of somatic manifestations of behavioral genes, in fact introducing a genomic background to psychosomatic medicine, in this era of "genomic medicine."[49]

We are indebted to our hard-working data entry team, particularly Beatrice Arn, Diana Strassmann, and Andreas Kinzel; to Monica Scheumann for organizing data collection; to Markus Sprenger, Jürg Mühlemann, and the IT Team for managing ObesityBase; to Praxis Dr. Horber for the ongoing care of our patients; to Professor Münch and Professor Altdorfer for performing gastroscopies; and to the team of GenProfile AG for performing comparative sequencing, genotyping, and data processing.

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Discussion

Dr. Michael Sarr (Rochester, MN): For a long time, bariatric surgeons have been looking for the Holy Grail of bariatric surgical success, i.e., something predictive of outcome. What have we really learned today, and why is this the first paper in the session? I would like you to think of the concept of preemptive surgery. In the past, endocrine surgeons used this concept by looking at phenotypes, increased calcitonin, imaging, etc., to try and take out tumors at an early stage of phenotypic (often presymptomatic) expression. Similarly, the oncologic surgeons have taken molecular biology into practice and screened for genotypes, MEN syndrome, FAP, etc., and operated "preemptively." The group by Kral et al. appears to have merged a phenotypic expression of a disease, that is, binge eating disorder, with a genotypic set of polymorphisms to predict outcome. Yes, this is important in bariatric surgery because although it is just a start, it has ramifications to everybody else in the audience who doesn't do bariatric surgery. So many disciplines are potentially involved. Now, how about the study itself?

The strengths of this study are that the diagnosis of a binge eating disorder was arrived at independently by three members of the group blinded to the genotypic status; second, patients with these polymorphisms were matched for age, sex, and BMI; third, two other putative genes with 24 other polymorphisms were also included to make it a realistic study; fourth, it is a large patient group; and fifth, probably most importantly, this is a well-respected, multi-disciplinary, credible group with unbelievably complete follow-up.

But are there any weaknesses? Yes. There is one potential weakness. We have heard that previously this group reported this phenotypic expression of a binge eating disorder to be associated with the MC4R polymorphism in the *New England Journal of Medicine*. This group took a lot of flack from many groups that said that up to 80% of these polymorphisms when tested in vitro lacked a functional defect in cell signaling. Thus, the polymorphism was present, the phenotype was present, but a functional defect biochemically was absent. So I have three questions for you.

First, if these polymorphisms lack a functional defect in cell signaling, how can they be related to a phenotypic expression? Second, your results are robust for gastric banding, and your title should specify post gastric banding, but how about for Roux-en-Y gastric bypass, duodenal switch, etc.? And third,

I'll give you a chance to speculate. What is the next step for your group in terms of epigenetic phenomenon, polygenomic interactions, and proteomics?

Dr. Bruce Wolfe (Carmichael, CA): Could you comment on the severity of the binge eating disorder between the MC4 variants and the patients who did not have such a variation, and from that can you conclude whether you are predicting poor outcome on the basis of the presence of a binge eating disorder as opposed to an abnormal phenotype? Secondly, is there any impact of psychotherapy pre- or postoperatively in the binge eating disorder patients that may improve their outcome?

Dr. Kral: I thank Dr. Sarr and Dr. Wolfe for the questions and for the nice discussion.

Regarding the validity of in vitro findings: of whether these polymorphisms indeed are active in the test tube versus in the actual phenotype? More information is becoming available. There are new candidate ligands that might explain the absence of in vitro findings. The answer to the question, apart from the obvious, "this is what we found during rigorous testing without knowledge of the genotype"? A paper in 2003 demonstrated that a beta subtype of MSH might be a more significant ligand in this context. I expect that we will learn that the debate circulated around the alpha MSH, but ligands might be more important that explain this discrepancy.

Gastric bypass was used as a rescue operation in a small subset of patients who had explant of their bands. The impression so far, the clinical impression, and I am going to insist, as always, that data not be presented before one has enough duration of observation, is that we don't have enough patients, or long enough observation, but the impression is that patients with rescue operations who have polymorphisms do poorer, even with a diversionary reoperation.

The next step? You will be hearing a paper in this Society by Dr. Potoczna on Wednesday about other genes that are being studied. The group in Zurich is accruing more patients to this study to verify and expand our results together with other groups. As a matter of fact, the database now exceeds 500 patients with genotyping, and there are more than 1,000 patients entered into the total database. This field is a moving target similar to the field of GI peptides, in the past. We will learn new mechanisms and new gene polymorphisms and will have different techniques soon. I doubt whether there is going to be a "magic bullet" for this very complex disease of obesity. But please remember, I am talking about a subgroup that is rather small (<7% of severely obese patients).

Dr. Wolfe, an analysis was not done of the severity of the binge eating disorder because with these very tough criteria, there is very little variance in severity. We did separate analyses for patients with and without binge eating disorder and found independent effects of BED controlling for all relevant factors.

Psychotherapy has not been evaluated because, as with so many obesity-associated conditions, surgery itself provides effective treatment. Furthermore, after gastric restriction, no patient meets the criteria for binge eating—they simply can't binge "enough"!

Laparoscopic Gastric Bypass Results in Decreased Prescription Medication Costs Within 6 Months

Jon Charles Gould, M.D., Michael Joseph Garren, M.D., James Ralph Starling, M.D.

The prevalence of obesity has reached epidemic proportions. The treatment of obesity-related health conditions is costly. Although laparoscopic gastric bypass is expensive, health care costs in obese patients should decrease with subsequent weight loss and overall improved health. Specifically, monthly prescription medication costs should decrease quickly after surgery. Fifty consecutive laparoscopic gastric bypass patients at a university-based bariatric surgery program were enrolled in the study. Medication consumption was prospectively recorded in a database. Patients' monthly prescription (not over-the-counter) medication costs before surgery and 6 months postoperatively were calculated. Retail costs were determined by a query to drugstore.com, an online pharmacy. Generic drugs were selected when appropriate. Costs for diabetic supplies and monitoring were not included in this analysis. Patients were mostly female (86%). Mean body mass index preoperatively was 51 kg/m^2. Mean excess weight loss at 6 months was 52%. Patients took an average of 3.7 prescription medications before surgery compared with 1.7 after surgery ($P < 0.05$). All patients took nonprescription nutritional supplements, including multivitamins, oral vitamin B_{12}, and calcium postoperatively. Laparoscopic gastric bypass resulted in a significant improvement in comorbid health conditions as early as 6 months after surgery. In an unselected group of patients, this led to a substantial overall mean monthly prescription medication cost savings, especially in those with gastroesophageal reflux disease, hypertension, diabetes, and hypercholesterolemia.
(J Gastrointest Surg 2004;8:983–987)

Key words: Morbid obesity, gastric bypass, pharmaceutical savings, bariatric surgery

The prevalence of overweight and obesity is increasing rapidly in the United States. The National Health and Nutrition Examination Survey (NHANES 1999–2000) demonstrates that among U.S. adults surveyed, 64% are overweight and 4.7% are morbidly obese (body mass index [BMI], >40 kg/m^2).[1] Obesity is a significant risk factor for the development of multiple disease states such as diabetes, hypertension, hypercholesterolemia, gastroesophageal reflux disease (GERD), and many other chronic medical conditions.[2] Furthermore, surgically induced weight loss has been demonstrated to lead to significant improvement or even resolution of many of these health problems.[3,4]

The treatment of obesity-related health conditions is costly. A recent study estimates that 9.1% of U.S. health care dollars are spent annually treating the comorbid medical conditions of obesity.[5] In 2002 dollars, this represents a sum of approximately $92.6 billion. Medicare and Medicaid finance approximately half of these costs. The use and cost of medications are markedly increased in the obese compared with nonobese patients.[6] Surgically induced weight loss should lead to decreased monthly medication costs, particularly in obese patients with medical conditions that can be expensive to treat such as diabetes and GERD.

METHODS

This study was performed with the approval of the University of Wisconsin Institutional Review Board and meets all HIPAA requirements. The study group consisted of the first 50 consecutive patients who had undergone laparoscopic Roux-en-Y gastric bypass surgery at our institution between July 2002 and April 2003. Our surgical technique involves the use of a 21-mm circular stapler to create the gastrojejunostomy

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and has been previously described in detail.[7] All patients had at least 6 months of follow-up. The prescription medication consumption of each patient was prospectively recorded in our bariatric surgery database. Preoperative prescription medication consumption was compared with prescription medication consumption at the time of the routine 6-month postsurgery clinic visit.

We developed a protocol for post–gastric bypass medication adjustments. In conjunction with each patient's individual primary care physician, we tapered or stopped medications while monitoring the relevant clinical variable. For example, cholesterol-lowering agents were typically stopped at the time of surgery unless total cholesterol was above 350 or triglycerides were above 750 before these medications were started. Serum cholesterol was checked 3 and 6 months after surgery to confirm the resolution of this condition. These medications were restarted if necessary based on laboratory results.

Patients taking prescription medications for GERD continued on these products for 6 weeks after surgery. These medications were stopped at that time, unless the patient developed a marginal ulcer. If symptoms consistent with GERD recurred after these medications were stopped, they were restarted.

Diuretics and combination antihypertensive medications containing a diuretic were avoided in the postoperative period. Patients were often discharged from the hospital on a decreased dose of high blood pressure medication. Most antihypertensive medications were tapered gradually on an outpatient basis while the patient's blood pressure was monitored closely.

The condition of type 2 diabetes mellitus was found to respond to gastric bypass surgery rapidly or in a more gradual manner. We believe that tight control of blood sugar during the immediate postoperative period can be difficult, and may in fact be detrimental. Significant hypoglycemia and neuroglycopenia are possible, especially with certain medications. Diabetic patients monitored their blood sugars closely while at home. Insulin doses were decreased and tapered depending on the patient's blood glucose levels after surgery. Sulfonylureas and their combinations were avoided due to the risk of hypoglycemia. Metformin is less likely to induce hypoglycemia and was continued in most cases, at least initially.

Depression is a condition with a high prevalence in our patient population. Patients were encouraged to continue pharmacotherapy for depression during the initial 6-month postoperative period. Patients were reevaluated for clinical depression before and after stopping antidepressants. Most medications that do not fall into one of the previous categories were managed by the patient's primary care physician with input from our bariatric surgical team when appropriate.

All patients were placed on ursodiol 300 mg twice a day for 6 months after surgery, unless they had a previous cholecystectomy. Because patients had stopped taking ursodiol by 6 months, this medication was not included in our analysis. The costs of over-the-counter, nonprescription nutritional supplements such as multivitamins, calcium, and vitamin B_{12} were excluded. Costs for diabetic supplies and blood glucose monitoring, as well as costs associated with the treatment of sleep apnea (equipment rental, oxygen tanks), were also not included in this analysis.

Costs of medications were determined by a query to drugstore.com, a popular online pharmacy. All medication costs used in this study were attained online on the same day (October 6, 2003). When appropriate, generic drugs were selected. Statistical analysis on medication cost data was performed using the Wilcoxon rank sum test. Comorbidity prevalence before and after surgery were compared by using the Fisher exact probability test.

RESULTS

Our study group consisted of 7 men and 43 women. The mean BMI preoperatively was 51 ± 7 kg/m^2 (range, 39–67 kg/m^2). The mean patient age was 44.0 ± 9.4 years (range, 23–63 years). Mean percent excess weight loss after 6 months was $52\% \pm 11\%$ (range, 35%–82%). A total of 81 unique medications were used to treat 21 different medical conditions. Among all medical conditions, hypertension was treated with the widest variety of medications, with 25 unique drugs used. The average number of medications per patient preoperatively was 3.7 ± 2.5. After surgery, patients took an average of 1.7 ± 1.6 prescription medications ($P < 0.05$). Four patients were not taking any prescription medications at the time of their surgery.

The prevalence of selected comorbid medical conditions preoperatively and 6 months after surgery are listed in Table 1. Comorbidity-related medication

Table 1. Prevalence of selected comorbid medical conditions before and after surgery

Condition	Before surgery (%)	After surgery (%)	*P* value
Diabetes	26	4	<0.01
Hypertension	46	10	<0.01
Hypercholesterolemia	26	0	<0.01
Gastroesophageal reflex disease	32	4	<0.01
Depression	54	54	NS

expenses and monthly prescription medication cost savings are described in Table 2 and Fig. 1. The greatest cost savings were realized in obese patients with GERD who were treated with a proton pump inhibitor (PPI) medication preoperatively. After 6 months, two patients in this series were taking PPI medications due to the development of a marginal ulcer postoperatively. In one of these patients, this was a new medication. Each of these patients with a marginal ulcer had a normal upper endoscopy after their 6-month clinic visit. Both of these patients subsequently have discontinued this medication. Several patients have gone on to successfully stop their antidepressant medication after the 6-month postoperative time period. None of the patients in this study group have had to restart a cholesterol-lowering agent after it was stopped.

At the time of their gastric bypass, 12 of the 50 patients (24%) had undergone a previous cholecystectomy. The remaining patients were placed on ursodiol at a dose of 300 mg twice a day for 6 months. The cost of generic ursodiol is $71.99 per month. This represents a mean cost per patient of $328 not captured in this analysis. Only 1 of the 38 patients with a gallbladder remaining after surgery has gone on to require a cholecystectomy (2.6%).

DISCUSSION

The prevalence of the examined comorbid medical conditions in our patient group and the response of each condition to surgically induced weight loss are similar to those of other large series of laparoscopic gastric bypass.[8] The overall improvement in health and quality of life after laparoscopic gastric bypass has been well documented.[9,10] Dymek and colleagues[11] demonstrated that in addition to improved health-related quality of life after gastric bypass surgery, patients consistently demonstrate improved self-esteem (Rosenberg Self-Esteem Scale) and depression (Beck Depression Inventory). Some of our patients who continue on antidepressants should be able to eventually discontinue these medications, according to this data.

It is likely that the cost savings we demonstrated in this group of patients would have been more significant had we included the expense associated with treating sleep apnea, as was done in one recent study.[12] The prevalence of sleep apnea in our bariatric surgery practice is 33%. For this study we selected the least expensive generic medication (if available) in all cases; this is not always the case in actual clinical practice. This fact is also likely to have minimized the magnitude of the final cost savings.

Nonprescription vitamin and supplement costs were not included in this analysis. We require that our patients take a chewable multivitamin with iron ($4.00 per month), calcium supplement ($7.00 per month), and oral vitamin B_{12} supplement ($1.00 per month). Many of our patients are encouraged to take protein supplements as well. We often recommend a protein powder containing 21 g of protein per serving at a cost $14 per month. It is possible to take all of the required and recommended supplements for less than $30 per month, although many patients opt for more expensive sources of vitamins and protein.

Studies estimate that of the health care expenses attributable to obesity in affluent countries, approximately 30% are pharmaceutical costs for treating obesity-related disorders.[13–15] Crudely extrapolated to our results, this represents an overall health care cost savings in excess of $400 per month per patient. The presumption that ultimately gastric bypass surgery is a cost-effective health care intervention has proved to be difficult to demonstrate. Intuitively, it would seem that there is a breakeven point in time where the cost of bariatric surgery equals the health care cost savings realized after weight loss and improved overall health. The cost savings are obviously dependent on the nature and extent of each patient's comorbid medical conditions, the surgical complication rate, and the durability of the surgical results. Cost-effective analyses have been performed using deterministic decision models and parameter estimates from multiple

Table 2. Comorbidity-related medication expenses and monthly cost savings

Condition treated with medication	Before surgery ($)	6 mo after surgery ($)	Monthly savings ($)
Diabetes	64.9 ± 88	2.0 ± 7.1*	62.9
Hypertension	45.0 ± 26.7	8.4 ± 19.8*	36.6
Hypercholesterolemia	85.8 ± 34.5	0*	85.8
Gastroesophageal reflex disease	110.6 ± 86.0	19.2 ± 41.7*	91.4
Depression	107.1 ± 85.2	107.1 ± 85.2	0
All prescription medications	217.6 ± 189	97.3 ± 107*	120.3

*$P < 0.05$.

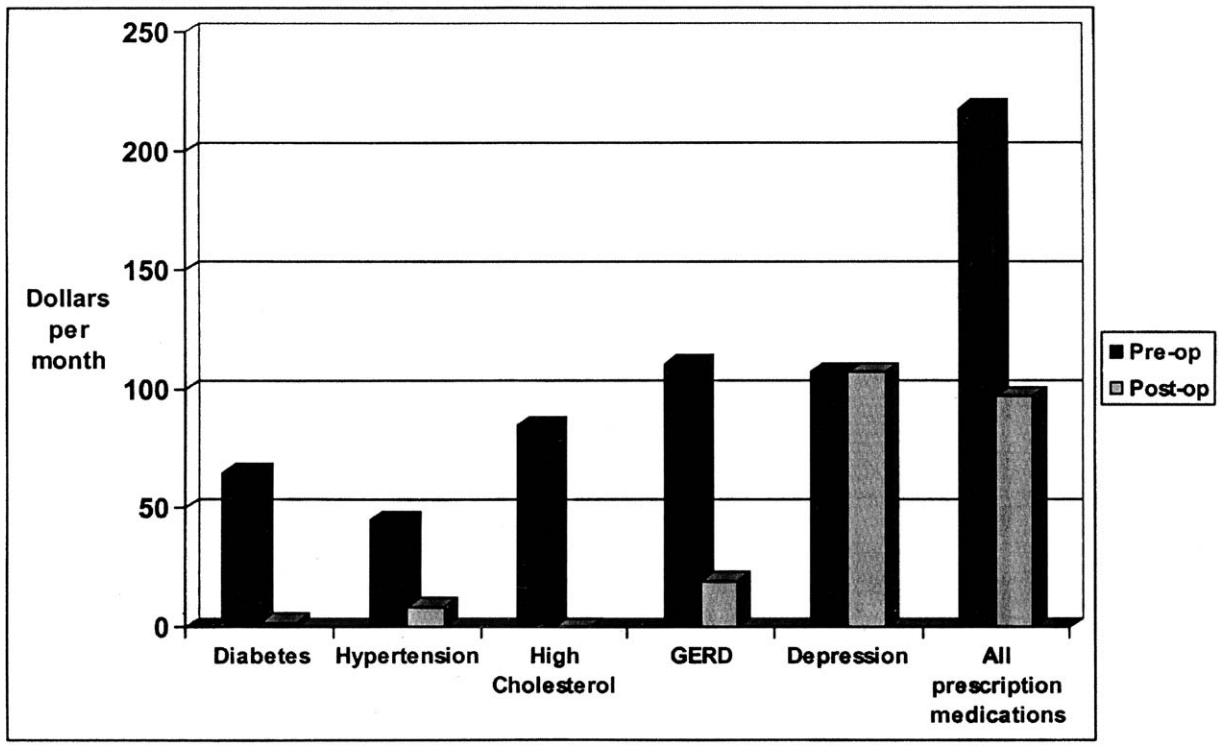

Fig. 1. Comorbidity-related prescription medication expenses before and 6 months after laparoscopic gastric bypass. GERD = gastroesophageal reflex disease.

sources (expert opinion, medical literature).[16,17] Each referenced study reached a similar conclusion—that gastric bypass surgery is a cost-effective alternative to no treatment.

Despite much data documenting the benefits of surgically induced weight loss, many payers continue to exclude bariatric surgery as a benefit. This is a purely financial decision. Bariatric surgery is expensive. Laparoscopic gastric bypass in our program costs in excess of $30,000 for an uncomplicated procedure and hospital stay. Increases in health care costs have led the payers to develop a number of strategies to contain these costs. Denying patients access to bariatric surgery based on policy exclusions or arbitrary definitions of what constitutes a "medical necessity" is a common tactic. As the prevalence of morbid obesity increases, the outcomes improve, and the public awareness of surgery as a viable treatment option increases, the demand for these expensive surgeries is at an all-time high. The breakeven point for the payers on bariatric surgery, if such a point exists, is likely more than 10 years after surgery. Considering the high turnover rate of participants in many health plans, it hardly makes sense from a business perspective to finance this intervention. In fact, the response of many morbidly obese patients denied coverage for bariatric surgery is to switch to a different provider that will pay. Unfortunately, the number of payers willing to cover bariatric surgery is declining rapidly.

Long-term follow-up of these patients will be necessary to determine the durability of their improved health and lower pharmaceutical expenses. It seems likely that laparoscopic gastric bypass represents a cost-effective alternative to conventional obesity treatment, providing substantial lifetime health benefits as well. More extended and comprehensive prospective comparative studies are needed to help prove that the expenses associated with bariatric surgery are justified in this age of limited health care funding.

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Adenocarcinoma of the Esophagus and the Esophagogastric Junction: Positron Emission Tomography Improves Staging and Prediction of Survival in Distant but Not in Locoregional Disease

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In adenocarcinoma of the esophagus and esophagogastric junction for prognostication and treatment allocation, one prerequisite is accurate pretreatment staging. This staging, we hypothesized, would be improved by the use of positron emission tomography (PET). After 55 patients suitable for radical esophageal resection were staged with PET, spiral computed tomography (CT), and endoscopic ultrasonography (EUS), results were compared with histopathology and with survival. Accuracy in detecting locoregional lymph node metastasis did not differ significantly between EUS (72%), PET (60%), and CT (58%). Adding PET to standard staging failed to improve the accuracy of N staging ($P = 0.250$). In M staging, accuracy between CT (75%) and PET (76%) did not differ. The accuracy of combined studies of CT and PET and of EUS, CT, and PET were 87% ($P = 0.016$ versus CT) and 91% ($P = 0.031$ versus EUS and CT), respectively. Of the 55 patients, 19 (35%) had metastatic lesions. By combined use of CT and EUS and by combined use of CT, EUS, and PET, 8 and 14 ($P = 0.031$), respectively, could be detected. In nodal disease without distant metastases, PET did not improve the prediction of survival. However, positive PET for distant metastasis by either positive EUS or CT predicts well the poor survival of these patients. The staging value of PET by itself in adenocarcinoma of the esophagus is limited because of low accuracy for nodal and the lack of specificity for distant disease prognosis. Adding PET to standard staging does, however, improve detection of stage IV disease and its associated poor survival. (J GASTROINTEST SURG 2004;8:988–996)

KEY WORDS: Adenocarcinoma, esophagus, esophagogastric junction, positron emission tomography, staging

The incidence of adenocarcinoma near the esophagogastric (EG) junction in Western countries has increased markedly.[1,2] Accurate staging is a prerequisite for the optimal choice of treatment. Limited resection has been suggested as the optimal treatment for early adenocarcinoma,[3] but in more advanced disease, radical surgery with lymphadenectomy seems to end in better long-term survival and to increase the likelihood of achieving complete resection.[4,5] Multimodal therapy may improve outcome even in more advanced cases.[6]

Staging is usually conducted in accordance with International Union Against Cancer (UICC) TNM staging.[7] Although in experienced hands EUS is considered accurate in predicting local infiltration and lymphatic metastases, T stage in a recent study of adenocarcinoma of the esophagus and EG junction was correct in only 66% and N stage in 72% of patients.[8] N staging, in particular, is currently considered of little importance, because it cannot be assessed with sufficient accuracy.[9] EUS with fine-needle

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aspiration is, however, promising, although it is still undergoing investigation.[10] To search for distant metastases, the problem even with modern CT technology is its inability to detect small (<1-cm-diameter) metastases. The role of laparoscopic and thoracoscopic staging of lymphatic and distant metastases remains controversial.[11,12] It is evident that lack of accuracy in these pretherapeutic staging methods makes it difficult to precisely define completely resectable disease.

Because positron emission tomography (PET) is based on accumulation of fluorinated glucose analog (18-*F*-fluoro-deoxy-D-glucose [FDG]) in malignant cells,[13] it provides the opportunity to detect altered tissue metabolism in malignant tumors.[14] In esophageal cancer, according to recent studies, PET is superior to CT or to combined use of CT and EUS in assessing distant metastases.[15–21] These studies have, however, either been small or retrospective or have included patients with different histologic types of tumors or patients with incomplete lymph node dissection. Despite the suggested superiority of PET over other staging modalities, correlation between PET findings and survival of patients with adenocarcinoma near the EG junction has not been well assessed. In our prospective study, the purposes were to stage adenocarcinoma of the esophagus and the EG junction by PET, EUS, and CT and to compare results with histopathology of the specimens obtained with radical two-field lymphadenectomy. Another aim was to determine the ability of PET to predict survival of these patients.

PATIENTS AND METHODS

In this prospective analysis, patients with histologically proved adenocarcinoma of the esophagus or the EG junction who were treated at the Helsinki University Central Hospital were eligible for inclusion. Exclusion criteria were inoperability for medical reasons or for unresectable tumor by conventional staging. In addition to routine by staging with endoscopy, CT, and EUS, all patients included underwent PET. All patients gave their informed consent, and the study protocol was approved by the Ethics Committee of Helsinki University Central Hospital.

Positron Emission Tomography Imaging

All PET studies were performed after a minimum fast of 6 hours. Radiochemical synthesis of 18-FDG was a modification of the method of Hamacher et al.[22] Imaging was performed with a GE Advance scanner (GE Medical Systems, Milwaukee, WI) with an axial field of view of 15 cm and a spatial resolution of 6 mm. A median dose of 370 MBq of FDG was injected into the vein of the forearm and, after a 50-minute uptake period, PET imaging was started. The emission scan was obtained in four or five bed positions (5 minutes per position), starting from the level of the maxilla down to the mid-abdomen. The first 19 patients were imaged without transmission correction for photon attenuation. After November 2000, all images were corrected for decay, dead time, and photon attenuation and reconstructed in a 128×128 matrix, with an ordered subsets expected maximum likelihood reconstruction algorithm and four iterations. For patients without transmission-corrected scans, standard Hanning-filtered backprojection with a 0.3 cutoff level was applied for image reconstruction.

Transaxial, coronal, and sagittal views were visually evaluated on a high-resolution display monitor (SUN workstation, Sun Microsystems, Inc., Mountain View, CA). Corresponding diagnostic CT scans of the chest and abdomen as well as radiology reports were always available, but no direct co-registration was done of PET and CT images. All focally increased FDG uptake not associated with a known physiologic accumulation of tracer was scored on a 3-grade scale as definitively positive, potentially positive, and unlikely to be positive for malignancy. After co-reading of CT and/or transmission scans, the anatomic localization of the focus was included in the evaluation.

Treatment

In patients with adenocarcinoma of the distal esophagus or EG junction, a radical esophagectomy and two-field lymphadenectomy were planned. Our conventional two-field lymphadenectomy consisted of en bloc esophagectomy with removal of adjacent lymphatic and areolar tissue between the tracheal bifurcation and the superior border of the pancreas. The block of tissue removed included, along with the bronchial, subcarinal, paraesophageal, parahiatal, celiac, left gastric, and splenic artery lymph nodes, the rim of the diaphragmatic muscle around the hiatus, the thoracic duct, both right and left mediastinal pleura, and the lesser curvature of the stomach with a 10-cm distal resection margin. The dissection was bounded anteriorly by the main bronchi and pericardium and posteriorly by the vertebral column and aorta. In addition, in many cases the upper mediastinal nodes were sampled. The lymph node sites were separately sent to the pathologist for site-specific analysis of nodal metastases.

Frozen sections taken during surgery showed 12 patients to have unexpected metastatic disease (stage M1b).[7] After diagnostic sampling, these patients

received palliative treatment. If the preoperative imaging had shown cervical metastases, a biopsy of cervical lymph nodes was done. In most palliative cases, careful sampling was carried out both intra-abdominally and intrathoracically. Every distant metastasis was identified histologically either on biopsy specimens taken during explorative operations (of 12 lesions: organ metastases in 11 and widespread paraaortic lymphatic growth and metastatic left supraclavicular lymph nodes in 1) or from resected specimens obtained in radical surgery (7 lesions). In these seven patients, the location of pM1 disease was celiac axis in six (three suspected on EUS) and the surface of left adrenal gland in one (suspected on PET). False-positive findings were verified either histologically during explorative surgery (cervicotomy in two patients and laparotomy in one) or by 4-year follow-up (one patient).

Data Analysis

The results of imaging modalities were compared with the histopathologic results. The TNM classification was according to the latest edition of the UICC.[7] Sensitivity, specificity, and accuracy were also calculated by standard definitions and compared by use of the McNemar test.[23] Accuracy of diagnosis of distant metastases was studied for the whole group, but diagnostic correctness of staging of regional lymph nodes was determined only after the radical operation. Pathologic and clinical T staging were compared only in cases where the primary tumor was removed.

Survival was measured from date of treatment to death. Follow-up data of all patients were collected until October 21, 2003. This is the date when the last computer-based search to show all deaths in the study group was done at Statistics Finland. Median survival was calculated according to the Kaplan-Meier method, and comparisons of survival times between groups were made by the log-rank test. All statistical calculations were carried out with SPSS software. Significance set at $P < 0.05$.

RESULTS

Patient Characteristics

Between December 1998 and October 2003, 55 patients were included. Of these patients' 55 adenocarcinomas, 20 were located in the distal esophagus (Siewert type 1) and 35 were located at the EG junction (Siewert type 2). An overview of patient characteristics and tumor localization in relation to treatment is provided in Table 1. The Kaplan-Meier estimate of survival at 4 years was 36% (median survival, 19 months) in the entire study group and 47% (median survival, 30 months) after resection with two-field lymphadenectomy (n = 43). For those with radical operations, the total number of analyzed lymph nodes was 1478, with an average number of analyzed lymph nodes per patient of 34 (range, 12−79 nodes).

Primary Tumor

The primary tumor was detected in 45 of 55 patients by PET (sensitivity, 82%). A false-negative finding, however, occurred in 10 patients, of whom 7 had pT1 lesions, with positive lymph nodes in 1, and 3 had pT2 lesions without positive lymph nodes. A significant difference ($P < 0.0001$) existed between the size of tumor in false-negative (14 mm; range, 2−30 mm) and true-positive (54 mm; range, 10−150 mm) studies. In the latter group, all lesions were pT2 or pT3. The patients with undetectable tumors in PET had a 3-year survival rate of 86% compared with 23% for those with detectable tumors ($P = 0.0033$).

CT identified the primary tumor in 38 patients (sensitivity, 69%). The size of undetectable tumors by CT was 27 mm (range, 2−100 mm) compared with 54 mm (range, 5−150 mm) for detectable ones ($P = 0.009$). By adding PET to CT and without EUS, in practice, more primary tumors can be detected (47 versus 38 of 55, $P = 0.004$). EUS could detect all primary tumors except two (5 mm and 20 mm in diameter). Of 43 radically operated patients, passage of the endoscope was impossible because of malignant obstruction in 7 (16%). Nevertheless, the endoscopist could estimate the T stage of the tumor in each

Table 1. Patient characteristics and tumor localization in relation to therapeutic strategy

Patient subset	Age (yr) (mean ± SD)	M/F (n)	Localization
Primary surgery with two-field lymphadenectomy (n = 43)	61.4 ± 11.5	33:10	Siewert type 1 (n = 17) Siewert type 2 (n = 26)
Explorative surgery with palliative treatment (n = 12)	57.4 ± 11.6	9:3	Siewert type 1 (n = 3) Siewert type 2 (n = 9)

case. All tumors where the passage was obstructed were pT3, except for one, which was pT2. The accuracy of EUS for assessing the depth of tumor infiltration in the 43 patients undergoing esophageal resection was 27 (63%). EUS overstaged the T stage in 12 (28%) and understaged it in 4 (9%) of 43 patients.

Locoregional Lymph Node Metastases

At surgery, 26 patients (60%) had metastatic locoregional nodes. Diagnostic sensitivity, specificity, and accuracy of PET, EUS, and CT for detecting locoregional nodal metastases are presented in Table 2.

Accuracy did not differ significantly between EUS, CT, and PET. Although the sensitivity of EUS was significantly higher than that of either CT ($P = 0.001$) or PET ($P = 0.001$), its specificity was poorer (0.008 versus PET and 0.063 versus CT). The high specificity of CT and PET was at least in part due to their low sensitivity. Among the six patients who had obstructing tumors, EUS correctly predicted five with N1 disease and also recognized one without positive locoregional nodal disease.

In adding PET to standard staging (EUS and CT), the accuracy of N staging did not result in improvement (36 versus 39 of 43, $P = 0.250$). N staging did, however, improve upon addition of PET to CT alone (25 versus 30 of 43, $P = 0.063$). Combining these staging techniques did not improve prediction of survival. In pN- or clinically N-negative disease, median survival level was not reached (Fig. 1, *A*). Median survival in pN-positive disease and in clinically N-positive disease is shown in Fig. 1, *B*.

Metastatic Disease

Of the 55 patients, 19 (35%) had metastatic lesions (9 distant lymph node metastases and 12 organ metastases). Of these patients, PET could identify 10, and CT, 6. By combined use of CT and EUS 8, and by combined use of CT, EUS, and PET, 14 of the 19 ($P = 0.008$ versus CT and 0.031 versus EUS and CT) could be detected. Although widespread peritoneal carcinomatosis could be detected by none of the imaging modalities for three patients, PET showed one to have metastasis in a rib and CT showed the other to have metastasis in celiac lymph nodes, suggesting stage IV disease. The location of undetectable M1 disease by EUS, PET, or CT in five patients was carcinomasis in one, celiac lymph nodes in three, and celiac lymph nodes and pancreas surface in one. In two of these patients, passage of EUS was impossible. In three patients, PET showed false supraclavicular metastasis on the left side, and liver metastasis in one; CT showed a false positive finding in the spleen in one patient. The sensitivity, specificity, and accuracy of different staging methods for distant metastases are listed in Table 3. Adding PET to CT ($P = 0.004$) or to combined use of EUS and CT ($P = 0.031$) improved the accuracy of M staging.

Median survival was not reached in pM-negative disease (Fig. 2, *A*). In clinical M-negative disease, median survival was 24 months when disease was detected by any of the imaging techniques alone or in combination with each other: CT (95% confidence interval, 13−36 months), PET (14−35 months), EUS or CT (13−36 months), EUS, CT, or PET (14−35 months), and EUS or CT and PET (14−35 months). Median survival in clinically or histopathologically stage IV disease is shown in Fig. 2, *B*. Either positive EUS or CT with positive PET for distant metastasis predicted well the poor survival of these patients.

DISCUSSION

In this prospective study, by combined use of CT and EUS, 42%, and by combined use of CT, EUS,

Table 2. Results of positron emission tomography (PET), computed tomography (CT), endoscopic ultrasonograpy (EUS), and a combination of these in identifying locoregional nodal metastases among patients undergoing two-field lymphadenectomy

	Sensitivity		Specificity		Accuracy	
	n/total	%	n/total	%	n/total	%
PET	9/26	35	17/17	100[†]	26/43	60
CT	11/26	42	14/17	82[‡]	25/43	58
CT and PET	13/26	50	17/17	100[†]	30/43	70[§]
EUS	22/26	85*	9/17	53	31/43	72
EUS and CT	22/26	85*	14/17	82[‡]	36/43	84[‖¶]
EUS, CT, and PET	22/26	85*	17/17	100[†]	39/43	91[‖**]

For sensitivity: *$P = 0.001$ versus PET or CT and $P = 0.004$ versus CT and PET.
For specificity: [†]$P = 0.008$, [‡]$P = 0.063$ versus EUS.
For accuracy: [§]$P = 0.063$, [‖]$P = < 0.0001$ versus CT; [¶]$P = 0.063$ and **$P = 0.008$ versus EUS.

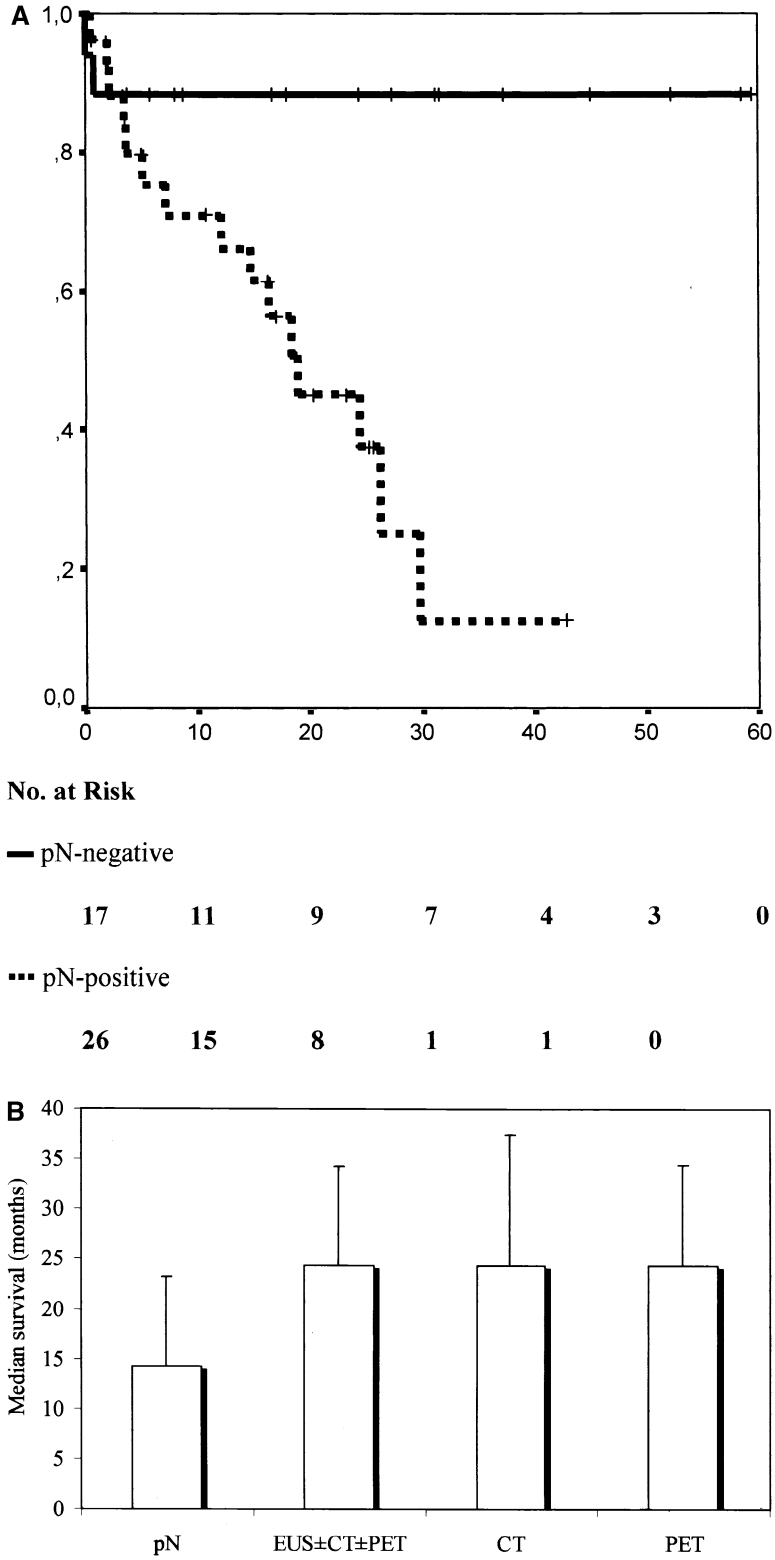

Fig. 1. (**A**) Kaplan-Meier estimate of survival by pathologic N stage. (**B**) Median survival in pN-positive or in clinically positive disease detected by different staging methods. Survival is shown as months with 95% confidence interval.

Table 3. Detection of distant metastases by positron emission tomography (PET), computed tomography (CT), endoscopic ultrasonography (EUS), and a combination of these in 55 patients with adenocarcinoma of the esophagus or the esophagogastric junction

	Sensitivity		Specificity		Accuracy	
	n/total	%	n/total	%	n/total	%
PET	10/19	53	32/36	89	42/55	76
CT	6/19	32	35/36	97	41/55	75
CT and PET	12/19	64†	36/36	100	48/55	87§
EUS and CT	8/19	42	36/36	100	44/55	80
EUS, CT and PET	14/19	74*	36/36	100	50/55	91‡

For sensitivity *$P = 0.008$ versus CT and $P = 0.031$ versus combination of EUS and CT and †$P = 0.031$ versus CT.
For accuracy: ‡$P = 0.004$ versus CT and $P = 0.031$ versus combination of EUS and CT and §$P = 0.016$ versus CT.

and PET, 74% ($P = 0.031$) of patients with adenocarcinoma of the esophagus and EG junction stage IV disease could be detected. Accuracy of nodal staging by addition of PET to standard staging (EUS and CT) did not improve. N staging was improved, however, by addition of PET to CT alone. For patients with adenocarcinoma near the EG junction, only positive PET with either positive EUS or CT for stage IV disease identified, comparable to pathologic M1 disease, their poor survival (median, 6 months; 95% confidence interval, 4−7 months). In staging, this is a decisive factor to exclude patients from unnecessary surgical treatment. Our results are, therefore, valuable in estimating the usefulness of PET as an additional investigation.

Although a change was made in the image reconstruction method in PET midway through the trial, a similar change has previously not affected rate of tumor detection.[24] We therefore analyzed all PET findings together. PET could neither identify very small primary tumors nor detect small metastatic lesions such as intra-abdominal carcinomatosis; the primary indication for PET is not to diagnose tumors. Nevertheless, the overall ability of PET to detect malignant tissue of esophageal origin is relevant. If PET cannot detect very small primary tumors, it is unlikely to identify small-volume metastatic lesions. Other authors have confirmed the low sensitivity of PET in detecting esophageal tumors of small volume (Tis or T1).[15,25] In our study, with PET, even three pT2 tumors were not visible. PET spatial resolution is 6 mm, but some tumors of up to 30 mm (mean diameter, 14 mm) went undetected. Obviously, spatial resolution is not the only limitation in PET accuracy. Therefore, PET in its current form has limitations in detecting small volumes of adenocarcinoma tissue of esophageal origin. This limitation can, however, offer benefits in clinical practice. According to our findings, patients with PET-detectable primary tumor were unsuitable for the new less radical approaches such as endoscopic mucosa resection[26] or limited resection,[3] because all of them had pT2 or pT3 lesions, with a 4-year survival rate of 23%.

Many reports[27–29] have advocated the use of EUS for the prediction of T and N stages in esophageal carcinoma. Although EUS is the only effective method for detecting tumor invasion depth,[8] it has limitations even for this purpose. Our results with 63% accuracy for T stage and 72% accuracy for N stage support the recent findings of Salminen et al.,[8] who included only patients with adenocarcinoma of the esophagus and EG junction. Our results certainly differed from the 80% accuracy in T stage achieved by Richards et al.[27] Perhaps the low accuracy can be explained by selection bias, because we included as patients only those who were candidates for radical surgery, and only resected tumors were included in the final analysis. Although PET achieved a high specificity (100%) in detecting locoregional nodal metastases, its accuracy differed nonsignificantly from that of EUS or CT (60%, 72%, and 58%, respectively). This was mostly because in its sensitivity for detecting lymph node metastases, PET was inferior to EUS (85% versus 35%, $P = 0.001$). The ability of PET to detect metastatic locoregional lymph nodes (N1 disease) was in our study less than that in reports of Flanagan et al.[15] or Kole et al.[16] Our results were, however, very similar to those of Flamen et al.[25] The explanation for this is that the extensive two-field lymphadenectomy performed in the present study and that of Flamen et al.[25] produced more lymphatic tissue for histopathologic analysis and therefore provided a more reliable reference for imaging studies. These kinds of comparisons between different imaging modalities are, however, of limited benefit for clinical practice, because PET, like the other techniques, is rarely the sole staging modality. Adding

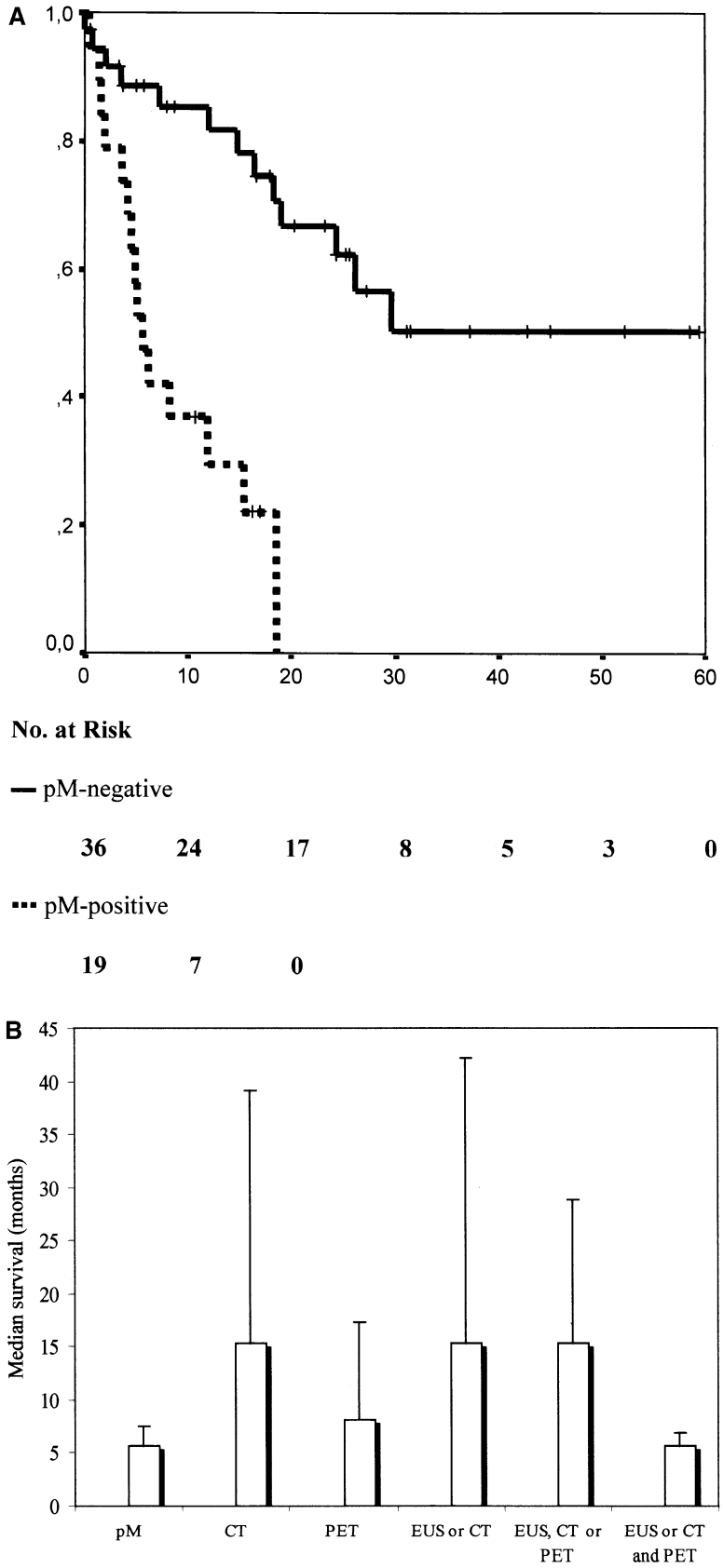

Fig. 2. (**A**) Kaplan-Meier estimate of survival by pathologic M stage. (**B**) Median survival in stage IV adenocarcinoma: pM-positive or in clinically detected disease (computed tomography [CT], positron emission tomography [PET], endoscopic ultrasonography [EUS], or a combination of these). Survival is shown as months with 95% confidence interval.

PET to standard staging (EUS and CT) failed to improve the accuracy of N staging (36 versus 39 of 43, $P = 0.250$). N staging was improved, however, after addition of PET to CT alone (25 versus 30 of 43, $P = 0.063$). The value of PET in the nodal staging of adenocarcinoma near the EG junction is thus limited to cases when or to units where EUS is unavailable.

Promising reports have appeared concerning the ability of PET to detect stage IV disease in esophageal cancer.[15,16,19,25] Kole et al.[16] stated that the accuracy of PET and CT to predict resectability was 88% and 65%, respectively. These findings are in line with reports by Flamen et al.[25] Luketich et al.,[30] in their series of 91 patients, found PET to have an accuracy of 84% in identifying distant metastases. They observed that all metastatic sites missed by PET were less than 1 cm in diameter, in accord with our results of undetected peritoneal carcinomatosis in three patients with small lesions. In adenocarcinoma of the esophagus and EG junction, according to our series, the overall accuracy of PET in detecting stage IV disease was no better than the ability of CT. This was mostly because of the lack of sensitivity in the finding of distant lymph node metastases and false-positive cervical lymph nodes in three and of the liver in one. Adding PET to CT or to combined use of CT and EUS improved the accuracy of detecting stage IV disease. Of 19 patients with pathologic stage IV disease, combining EUS, CT, and PET, 14 patients (compared with 8 with EUS and CT) could have their disease detected. In locally advanced tumors, PET is therefore recommended to exclude patients from unnecessary surgery.

None of the staging modalities used in this study by itself or in any combinations were as efficient estimators of outcome as was pathologic N staging in positive nodal disease. Detection of N1 disease by any of these modalities was, however, associated with such poor survival that patients like these should be considered for neoadjuvant therapy. It can be argued that even though the survival differences for patients with clinically positive or negative M1 disease by these imaging techniques was in most cases insignificant, the difference is of clinical relevance. Only those patients with either positive CT or EUS with positive PET for metastases have as poor an outcome as do patients with pathologic M1 disease, and the former should be treated accordingly. Even if CT or EUS stage IV disease cannot be confirmed by less invasive techniques, positive PET can exclude patients from surgery. Findings of distant disease even with PET alone must in most cases be verified by histology or cytology because of the high rate of false-positive findings.

The rate of stage IV disease among potentially resectable patients seems quite high (35%) in this study. In our recently published study, this rate at the population-level in Finland was 21.5%.[5] In the same time period, the rate was 18.6% in our unit. In this study, there are several reasons for the increase. First, PET results suggested stage IV disease in locations (e.g., adrenal glands) that would not be explored during normal surgery. Second, the number of exploratory operations was quite high because our patients did not consider the new method, PET, to be trustworthy. Third, there were some young patients whom we, together with the patients, wanted to explore surgically although even the standard staging with CT and EUS suspected stage IV disease. Last, our en bloc technique with two-field lymphadenectomy includes celiac nodes together with the retroperitoneal tissue covering the left adrenal gland. Of seven patients with stage IV disease who underwent resection, six had celiac node metastases. With less systematic techniques, these nodal metastases might be unforeseen. Regardless of the high rate of patients with stage IV disease, the long-term results of resected patients in this study and in our recently published series show a 5-year survival rate close to 50%.[5]

In conclusion, all of the staging methods we used were far from perfect. EUS has problems with accuracy in detecting both T and N stages. The weakness of PET is its inability to identify locoregional metastases and its poorer specificity in M1 disease. Combining PET with standard staging (EUS and CT) improves, however, the accuracy of staging; it provides valuable information on prognosis and long-term outcomes in patients with adenocarcinoma near the EG junction. In this disease, PET seems to be an important additional modality for staging.

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The Role of Botulinum Toxin Injection and Upper Esophageal Sphincter Myotomy in Treating Oropharyngeal Dysphagia

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The aims of this study were to assess the efficacy and safety of botulinum toxin (BoTox) injection in the cricopharyngeus muscle (CP) and CP myotomy in patients with oropharyngeal dysphagia (OPD) and to identify factors predicting the outcome of these treatments. The study involved patients with persistent OPD despite 2–6 months of rehabilitation, who all underwent clinical evaluation, esophageal manometry, upper gastrointestinal endoscopy, and videofluoroscopy (VFS). Patients received 5–10 BoTox units injections in the CP, identified by electromyography. Surgical myotomy of the upper esophageal sphincter was performed when dysphagia persisted after two BoTox injections. After treatment, patients were reevaluated with clinical interviews and VFS. The study population included 21 patients (15 mean and 6 women; median age, 68 years), classified into three groups, based on the etiology of their OPD: eight (38%) had central nervous system abnormalities, five (24%) had peripheral nerve disease, and eight (38%) were classified as idiopathic. The median time since the onset of dysphagia was 18 months. Thirteen of 21 patients (62%) needed supplemental/total gastrostomy feeding, and 5 of 21 (24%) had tracheostomy. One patient died, on posttreatment day 7, due to massive aspiration. No other BoTox-related complications were observed. After BoTox injection, dysphagia improved in 9 of 21 (43%) patients. Severely altered VFS findings and CP incoordination or low activity predicted BoTox failure at multivariate analysis. Dysphagia improved in 8 of 11 (72.7%) patients who failed to respond to BoTox and underwent myotomy. A mild impairment of VFS findings and a higher pressure of pharyngeal contractions best predicted response to BoTox with or without myotomy. BoTox injection can be used as the first therapeutic option in patients with OPD: it is safe and simple and relieves dysphagia in 43% of cases. If BoTox fails, CP myotomy can be offered to patients with preserved oral and tongue activity at VFS and an intact bolus propulsion ability on manometry. (J GASTROINTEST SURG 2004;8:997–1006)

KEY WORDS: Oropharyngeal dysphagia, BoTox, myotomy

Swallowing is a highly complex sensory motor process: when it is disrupted, oropharyngeal dysphagia (OPD) is experienced. The consequences of OPD can be devastating: regurgitation of material from the nostrils and mouth, pulmonary aspiration, malnutrition, and even death.

In some patients affected by OPD, the main abnormality lies in an impaired upper esophageal sphincter

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(UES) relaxation with a consequent outflow obstruction. The UES is mainly (but not exclusively) composed of the cricopharyngeus muscle (CP), which keeps a constant basal tone and luminal occlusion at rest, enabling rapid relaxations during swallowing. In patients with OPD, surgical CP myotomy or, more recently, its chemical paralysis using BoTox has been advocated. Most reports on the treatment of OPD with surgical myotomy[1] or BoTox paralysis of the CP[2–5] are single case reports or small retrospective series, and the evidence to support these treatments remains limited, especially in patients with neurogenic OPD.[6] Hence, this study was conducted to evaluate the efficacy of BoTox and/or surgical CP myotomy in patients with OPD of varying severity and etiology and to assess the potential role of BoTox as a means for identifying patients who would benefit from UES myotomy.

This study was approved by the Ethical Committee of Padova University Hospital, and informed consent was obtained from all patients.

MATERIAL AND METHODS

Patient Population

Patients with OPD unresponsive to 2–6 months of swallowing therapy were selected from a larger group of patients (N = 91) observed from 1999 to 2003 at the Swallowing Unit of Padova University Hospital. Patients with Parkinson's disease, amyotrophic lateral sclerosis, or terminal disease or who were unfit for surgery were ruled out.

Study Design

All patients underwent clinical evaluation, esophageal manometry, upper gastrointestinal endoscopy, videofluoroscopic study of swallowing, electromyography (EMG), and BoTox injection. In cases of persistent OPD after 2 BoTox injections, CP myotomy was performed and a specimen of the muscle was retrieved for histopathological analysis. A new rehabilitation cycle was started within 2 weeks of the operation. Patients unable to cope with oral feeding had a percutaneous gastrostomy and a protective tracheostomy was performed in the event of aspiration.

Clinical Evaluation. Patients rated their swallowing problem using the same 8-point scale considering severity (0–5 points) and frequency (0–3 points) of dysphagia, before and after any treatment, and as part of their follow-up.

Esophageal Manometry. This was performed as described in detail elsewhere.[7] Briefly, an 8-lumen low-compliance infused system with computerized data acquisition and analysis was used. A high-frequency data acquisition mode (50 Hz) was adopted in view of the rapidity of events occurring during swallowing. UES pressure was measured as the catheter was withdrawn at a constant rate of 5 mm/sec. The maximum amplitude recorded by each probe during its passage through the UES was averaged and considered as the UES pressure. To evaluate the pharyngoesophageal segment, the manometric probe with four radially oriented side holes was positioned at the upper edge of the UES, with two other side holes situated 5 and 10 cm above (in the distal and proximal pharynx, respectively) and one situated 5 cm below (in the cervical esophagus). Five swallows of 5 ml water were evaluated or, if patients were unable to swallow properly, they were asked to perform five "dry swallows." The following pharyngeal contraction parameters were considered: amplitude, duration, and intrabolus pressure (i.e., the pressure generated by the passage of the bolus in the distal pharynx and seen at manometry as a slow pressure increase, or shoulder, before the major upstroke generated by pharyngeal wall contraction, as described by Cook et al.[8]) (Fig. 1). UES relaxation (expressed as the residual pressure at swallowing) and the coordination of UES opening with pharyngeal contractions were calculated as described by Knuff et al.[9]

Videofluoroscopy. The swallowing study was performed before and after treatment according to the protocol adopted at the Radiology Unit of Padova University Hospital, using a Prestige VH (General Electric Company, North St. Paul, MN) while the patient was sitting on a chair or a wheelchair. The examination was performed in both a lateral and a frontal position while the patient was asked to swallow a standardized amount of liquids with different viscosities. In general, the first swallow was obtained with a water-soluble medium; then, if no aspiration was observed, barium sulfate was given as a contrast agent. The motility of tongue, soft palate, and pharynx; any aspiration; and the passage of the fluid through the UES into the esophagus were separately assessed and scored (each of these five factors contributed to the score with a score of 1 for abnormality present or 0 point for no abnormality found).

Electromyography and BoTox Injection. EMG activity was recorded on a portable two-channel EMG/EP EBNeuro Myto (Florence, Italy) connected to a Windows notebook PC. EMG of the inferior constrictor (IC) muscles of the pharynx and CP was evaluated simultaneously, whereas the patient was lying supine, with the trunk slightly raised and the head extended. A 35-mm-long (26-gauge) concentric needle electrode was inserted along the superolateral

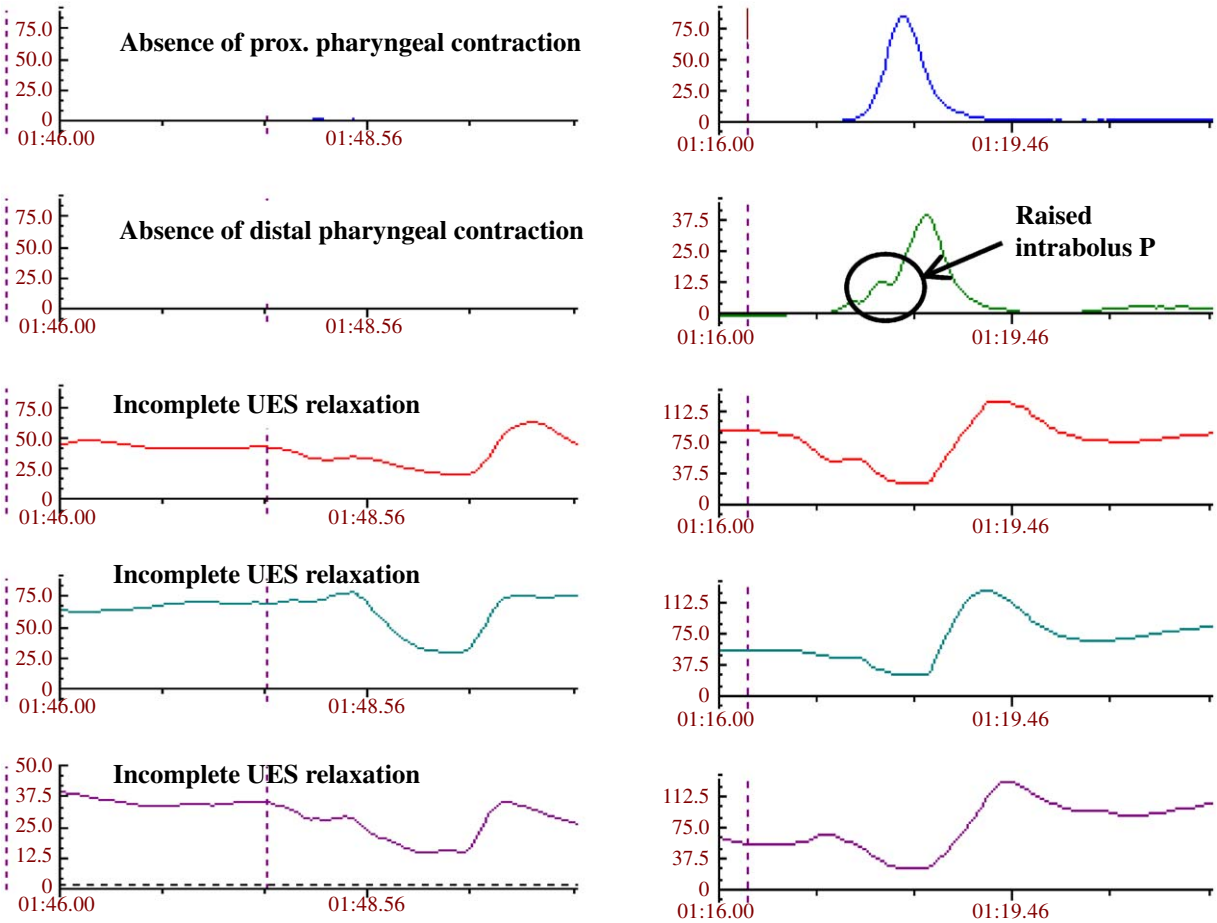

Fig. 1. Motility tracing in a patient with absence of pharyngeal contraction and virtually no upper esophageal sphincter (UES) relaxation and in a patient with raised intrabolus pressure and incomplete UES relaxation.

border of the thyroid cartilage, to record the IC muscles electric activity. A 50-mm-long (26-gauge) concentric needle electrode (Mendelec Myoject, Witney, UK) was inserted at the inferolateral aspect of the cricoid cartilage and rotated medially after insertion, to record the CP electric activity (Fig. 2). Then the EMG trace of both muscles was shown on the monitor and a sound like crumpling crêpe paper was heard during swallowing. With the needle in place, patients were invited to swallow, say a vowel, breathe vigorously through their nose, and lift their head to rule out any insertion in nearby muscles. Then, the activity of the IC and CP muscles was recorded during dry swallowing. Pharyngoesophageal incoordination at swallowing (i.e., simultaneous electric activity in the two muscles), low pharyngeal electric activity, permanent CP activity unrelated to swallowing (spasm), and low CP electric activity were considered abnormal findings. After confirming pharyngoesophageal dysfunction by EMG, the 50-mm-long Teflon-coated EMG needle inserted in the CP muscle was connected to a tuberculin syringe and, after a further check on the proper insertion of the needle in an active area of the CP muscle, 4–10 units of BoTox (BoNT/A, Botox; Allergan Inc., Irvine, CA) was injected.[10] If there was any doubt as to the effectiveness of the injection, due to a poor EMG activity of the CP, the injection was repeated within 48–96 hours. A second injection was also administered if there was no improvement in dysphagia after the first injection. A new cycle of rehabilitation was started within 48 hours of the last BoTox injection. BoTox treatment was considered effective if the patient's dysphagia improved (score $\leqslant 3$) and the improvement persisted for at least 6 months. BoTox treatment was considered ineffective when dysphagia persisted (score >3) after the injection and at least 2 weeks of swallowing therapy.

CP Myotomy. The operation was performed under general anesthesia with a left laterocervical incision

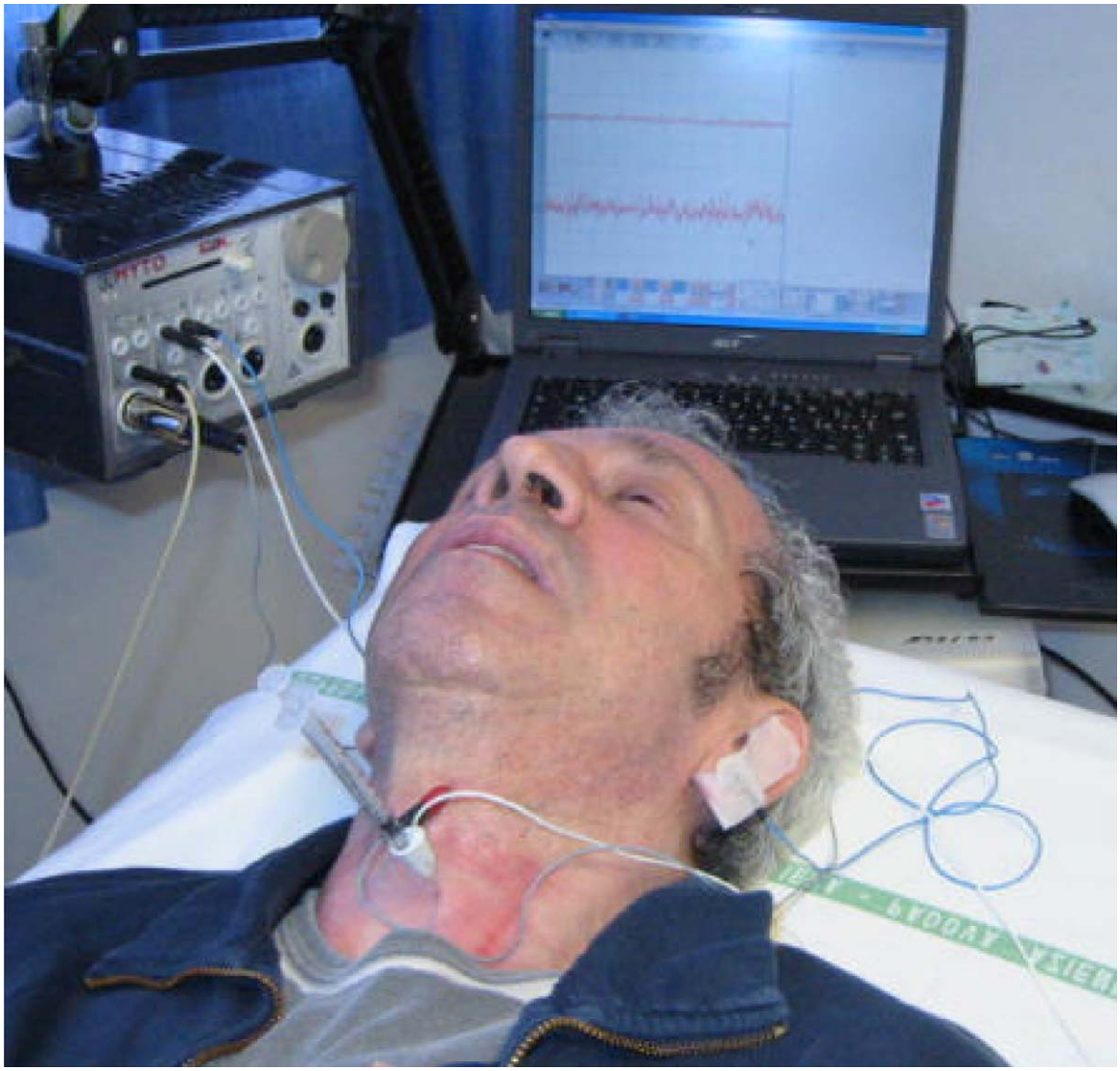

Fig. 2. Portable two-channel electromyogram, connected to a notebook PC. A 50-mm-long needle electrode is inserted along the inferolateral aspect of the cricoid cartilage (see text for details).

along the anteromedial border of the sternocleidomastoid muscle. The omohyoid muscle was divided, and the carotid sheath was retracted laterally. The inferior thyroid artery was divided, and the thyrolaryngeal block was retracted medially and rotated to expose the posterior part of the esophagus. The CP was identified visually, and its fibers were divided medially until the submucosal plane was reached. A plane between the submucosa and the muscle layer was easily identified, and the myotomy was prolonged across the CP 5 cm down the cervical esophagus. A 25 Foley catheter was inserted in the esophagus, and its balloon was gently inflated with 2–3 ml of water and carefully withdrawn. The remaining circular fibers, adjacent to the submucosal layer, were exposed and stretched to facilitate the completion of the myotomy, which was extended 1–1.5 cm in the IC muscle, or until the Foley catheter with the inflated balloon could pass easily through the esophagus into the pharynx.[11]

Histologic and Morphometric Studies. Biopsy samples were fixed in 10% buffered formalin and embedded in paraffin. Sections of 7 μm in thickness were selected for morphometric analysis and processed according to Masson's trichrome staining method. Three sections were randomly selected from each specimen for morphometric analysis. In each section, the muscle–to–connective tissue ratio was evaluated using a computer-assisted image analysis system (QWin; Leica Imaging Systems, Cambridge,

UK). Five specimens of CP collected from gender- and age-matched cadavers with no history of dysphagia served as a control group.

Statistical Analysis

All data are expressed as median and interquartile range. Nonparametric tests were used to compare data (Mann-Whitney *U* test and paired *t* test, as appropriate). Categorical data were analyzed using the Fisher exact test. A logistic regression was performed to correlate predictors of BoTox and BoTox with or without myotomy outcome. The regression procedure was conducted on the variables that were statistically significant at univariate analysis or those statistically different at the comparison between the two groups. Covariates in the model were required to reach the significant 0.05 level.

RESULTS

Patient Population and Clinical Features of Oropharyngeal Dysphagia

Twenty-one patients (15 men and 6 women; median age, 68 years; interquartile range [IQR], 60–71) were entered into the study. Based on OPD etiology, they were divided into three groups: 8 (38%) (group 1) had central nervous system abnormalities (cerebrovascular accident in five patients, trauma in one, surgery for brain tumor in one, and Dandy-Walker in one); 5 (24%) (group 2) had peripheral nerve disease (postradiotherapy neuropathy in two patients, polyneuropathy in three), and 8 (38%) (group 3) in whom no neurologic abnormalities were found and were classified as idiopathic. The median time since the onset of dysphagia was 18 months (7–36 months). The median pretreatment dysphagia score was 8 (7–8). Thirteen of 21 (61.9%) patients required supplemental or total gastrostomy feeding; 5 of 21 (23.8%) required a tracheostomy.

Manometry and Videofluoroscopy

The manometric and videofluoroscopic characteristics of the pharynx and UES in the three groups of patients are summarized in Table 1.

Electromyographic Findings and Outcome of BoTox Injection

Eight patients had CP spasm at EMG: five had CP spasm alone, and three had CP spasm and incoordination. Nine patients had CP incoordination alone, and two had low CP activity. CP activity was normal in two patients.

All patients received EMG-guided BoTox injection as first treatment: a median of 2 injections was performed.[1–4] One patient died, on posttreatment day 7, during rehabilitation, due to massive aspiration. No other BoTox-related complications were observed. Dysphagia improved in 9 of 21 patients (42.8%). The gastrostomy was removed from four of five patients whose dysphagia improved. The tracheostomy was likewise removed from two of three patients. Of the nine patients who improved after BoTox injection, one was in group 1, four were in group 2, and four were in group 3 (Table 1). The data on dysphagia and VFS scores before and after BoTox injection are shown in Table 2.

In two of the nine (22.2%) patients who responded to BoTox injection, OPD recurred within 1 year of the last BoTox injection (one patient had a third BoTox injection and one refused further treatment); one patient died 15 months later of unrelated causes, one had a progression of her polyneuropathy, and

Table 1. Manometric and videofluoroscopic characteristics of pharynx and upper esophageal sphincter in the three groups

	Group 1 (n = 8)	Group 2 (n = 5)	Group 3 (n = 8)	*P* value
Manometry				
Resting pressure (mm Hg)	53.5 (80–104.5)	81.5 (47–115)	67 (15–193)	0.97
Length (mm)	36* (30–44)	31 (23–39.5)	39 (20–52)	0.58
Relaxation (%)	80† (57–84)	77.5 (55.5–99)	68 (42–99)	0.93
Pharyngoesophageal coordination (%)	100 (48–100)	100 (50–100)	100† (0–100)	0.91
Pharyngeal contraction amplitude (mm Hg)	8† (0–45)	71.5 (40–114)	20 (0–161)	0.18
Videofluoroscopy				
Impaired oral phase	5	3	2	NS
Impaired tongue motility	3	2	0	NS
Score	4 (3–4)	3 (3–4)	3 (2–3)	0.08

Data are expressed as median (interquartile range). Data are unavailable for *one patient and †four patients.
Group 1 includes central nervous system abnormalities, group 2 includes peripheral nerve disease, group 3 includes idiopathic.

Table 2. Clinical and videofluoroscopic characteristics before and after botulinum injection

	Before botulinum (n = 21)	After botulinum (n = 21)	*P* value
Dysphagia score*	8 (8–8)	0 (0–6)	<0.0001
Mild (1–3)†	1 (4.7)	12 (57.1)	
Moderate (4–6)†	1 (4.7)	4 (19)	
Severe (7–8)†	19 (90.6)	5 (23.9)	
Videofluoroscopy score*	4 (3–4)	1 (0–2)	<0.0001

Data are expressed as *median (interquartile range) and †n (%).

five are still asymptomatic at 18 months or longer (Table 3).

Factors Predicting Outcome of BoTox Injection

The clinical, videofluoroscopic, and manometric features of patients who did or did not respond to BoTox injection are summarized in Table 4.

Peripheral nerve OPD etiology ($P < 0.01$), CP spasm—alone or in combination with CP incoordination ($P < 0.0001$), and a high VFS score ($P = 0.03$) were predictive of a good outcome after BoTox treatment (Table 5).

No relationship was found between response to BoTox injection and the duration of dysphagia, any presence of gastrostomy or tracheostomy, or dysphagia score. Response to BoTox injection was also unrelated to any of the UES parameters.

Multivariate analysis was performed with the following variables: EMG remarks and VFS score ($R^2 = 1$). Both variables significantly predicted a good outcome after BoTox injection ($P < 0.01$).

Outcome After Upper Esophageal Sphincter Myotomy

Eleven of the 12 patients who failed to respond to Botox injection underwent surgical myotomy. The median time between the last BoTox injection and surgery was 58 days (range, 15–90 days). Operative mortality and morbidity were nil. After a median follow-up of 17 months (range, 6–31 months), swallowing improved in 8 of 11 patients (72.7%). After myotomy, gastrostomies were removed from three additional patients and a tracheostomy from one; three additional patients resumed near-total oral feeding, but their gastrostomy was retained to facilitate liquid intake.

Patients who responded to neither BoTox nor myotomy were in group 1 (three patients, two with acute cerebrovascular disease, and one with severe trauma) or group 2 (one patient with postradiotherapy neuropathy).

On the other hand, all patients with idiopathic OPD (group 3) improved after either BoTox injection (three patients) or subsequent myotomy (four patients).

Factors Predicting Outcome After Myotomy or Chemical Paralysis of the Upper Esophageal Sphincter

Patients who responded to BoTox or myotomy had a lower VFS score ($P = 0.027$) and higher of pharyngeal contraction pressure ($P < 0.05$) than those who did not (Table 6).

Table 6 shows the parameters that predicted a good outcome after BoTox/myotomy treatment on univariate analysis. Multivariate analysis was performed with the following variables: pharyngeal contraction pressure, VFS score. and OPD etiology ($R^2 = 0.48$). The VFS score was significant in predicting a good outcome after BoTox–myotomy ($P < 0.05$) (Table 7).

Table 3. Clinicopathologic characteristics and follow-up details for the nine patients with successful botulinum treatment

Patient	Etiology	Electromyography remarks	Videofluoroscopy score	Follow-up (mo), outcome
1	CNS	CPS	4	24, Good
2	ID	PEI	1	15, Died of unrelated causes
3	ID	CPS	2	<12, Recurrence
4	ID	CPI/CPS	1	<12, Recurrence
5	ID	CPS	3	18, Good
6	PND	CPI/CPS	3	38, Good
7	PND	CPS	2	31, Good
8	PND	CPS	3	31, Good
9	PND	CPI/CPS	4	17, Progression of polyneurophaty

CNS = central nervous system abnormalities; PND = peripheral nerve disease; ID = idiopathic; CPS = cricopharyngeal spasm; CPI = cricopharyngeal incoordination.

Table 4. Comparison of patients with good versus poor outcome after botulinum

Characteristic	Outcome		
	Good (n = 9)	Poor (n = 12)	*P* value
Age	69 (59–71)	67 (59–70)	0.80
Gender (M/F)	6/3	9/3	
Duration of dysphagia (mo)	13 (6–24)	21 (8–39)	0.48
Dysphagia score	8 (7–8)	8 (8–8)	0.44
Upper esophageal sphincter			
Resting pressure (mm Hg)	68 (26–125)	81 (66–96)	0.75
Length (mm)	24 (21–39)	40 (33–43)	0.14
Relaxation (%)	68 (50–89)	80 (57–98)	0.47
Pharyngoesophageal coordination (%)	100 (0–100)	100 (0–100)	0.21
Pharyngeal contraction amplitude (mm Hg)	74 (17–123)	30 (0–69)	0.13
Videofluoroscopy score	3 (2–3)	4 (3–4)	0.03

Data are expressed as median (interquartile range). *Data unavailable in one patient.

Histologic and Morphometric Studies

Histology and morphometric studies on seven tissue specimens (after myotomy) showed a marked decrease in the amount of muscle fibers in the CP. The muscle–connective tissue ratio was significantly lower in patients than in controls (Fig. 3).

DISCUSSION

OPD is a protean condition varying considerably in severity from a lumpy sensation in the throat to complete inability to swallow and aspiration. It can be a manifestation of specific oropharyngeal diseases or of systemic or neurologic disorders of various etiologies. The treatment of OPD, especially of neurologic etiology, is often difficult. Given the wide and heterogeneous spectrum of situations that can lead to OPD, a single management strategy is impracticable. Most patients benefit from controlled diet, nonoral feeding, and rehabilitation therapies, however.[12]

A subset of OPD patients could benefit from enlargement of the UES opening and a lower resistance to trans-sphincter flow,[13,14] achievable by sectioning the CP fibers (myotomy) or by BoTox injection. Kaplan[15] first reported on the beneficial effect of CP myotomy for the treatment of severe OPD, and several years later, Dunne et al.[16] reported using BoTox to treat dysphagia caused by CP achalasia. The potential role of BoTox as an alternative to surgery is appealing: it is used in a variety of hypertonic muscular disorders with minimal or no side effects[17]; it is a virtually noninvasive procedure, relatively simple to implement, and certainly less costly than surgery. The main drawback of the therapeutic use of BoTox is that its effect is relatively short-lived, but it could be useful in identifying patients who would benefit from definitive surgical treatment: it has been suggested that, if the toxin fails to improve CP disorders, then myotomy is also unlikely to be helpful.[6]

The results of the present study show that BoTox is effective in nearly 40% of OPD patients: a less severely damaged swallowing function, as evaluated by VFS and the presence of CP spasm at EMG, are the best predictors of a successful outcome.

In our experience, BoTox injection was a safe procedure, although we recorded one complication: just 1 week after BoTox treatment, one patient died of aspiration pneumonia. Abolishing the CP's protective mechanism may theoretically have caused the patient's aspiration, but we believe that this death was

Table 5. Univariate analysis: Predictive factors of good outcome after botulinum treatment

Characteristic	Outcome		
	Good (n = 9)	Poor (n = 12)	*P* value
Upper esophageal sphincter			
Resting pressure (>70 mm Hg)	4 (44.4)	9 (75)	0.20
Relaxation (>70%)	3 (66.7)	7 (58.3)*	0.37
Pharyngoesophageal coordination (>90%)	8 (88.9)	7 (58.3)*	0.32
Pharyngeal contraction amplitude (>40 mm Hg)	5 (55.6)	5 (45.4)*	1.00
Videofluoroscopy score (>3)	2 (22.2)	8 (75)	0.08
Electromyography:			
Cricopharyngeus muscle spasm or spasm and incoordination	8 (88.9)	0 (0)†	<0.0001
Cricopharyngeus muscle incoordination or low activity	1 (11.1)	10 (100)†	
Gastrostomy	5 (55.5)	8 (75)	0.67
Tracheostomy	3 (33.3)	2 (16.7)	0.61

Data are expressed as n (%). Data are unavailable for *one patient and †two patients.

Table 6. Differences between patients with good versus poor outcome after botulinum with or without myotomy

Characteristic	Outcome		
	Good (n = 17)	Poor (n = 4)	*P* value
Age	68 (60–71)	64 (51–70)	0.62
Gender (M/F)	11/6	4/0	0.28
Duration of dysphagia (mo)	18 (7–38)	19 (11–26)	0.72
Dysphagia score	8 (7–8)	8 (8–8)	0.72
Upper esophageal sphincter			
Resting pressure (mm Hg)	82 (41–130)	74 (62–80)	0.45
Length (mm)	34 (23–41)	28 (15–40)	0.94
Relaxation (%)	68.5 (55–89)	90.5 (51–99)	0.37
Pharyngoesophageal coordination (%)	100 (0–100)	100 (96–100)*	0.91
Pressure of pharyngeal contraction (mm Hg)	64 (19–89)	4 (0–17)	0.05
Videofluoroscopy score	3 (2–4)	4 (4–4)	0.03

Data are expressed as median (interquartile range). *Data are unavailable for one patient.

more likely related to his previous condition than to the BoTox treatment. Other authors have already suggested that destroying the protective effect of the CP by means of BoTox injection or surgical myotomy usually carries self-limiting risks, and serious complications are usually related to the patient's underlying disease.

The second finding of this study is that most patients in whom BoTox injection failed nonetheless responded to UES myotomy, indicating that BoTox injection cannot be used to discriminate between patients who may or may not benefit from surgery.

There are several hypotheses as to why myotomy can still be useful even where BoTox fails. First, there are numerous methods for delivering BoTox to the CP: rigid and flexible endoscopes with CT, fluoroscopy, and EMG guidance have been used,[2–6] as well as direct injections in the muscle during surgery. The quantity of BoTox injected also varies considerably, from 5 to 100 units in single or multiple shots. Such a variability in both the delivery system and the dosage means that no standardized method has emerged so far, meaning that the BoTox may not reach the CP consistently. Unfortunately, no methods are available for detecting the presence of BoTox in human muscle. Although the injection method and the amount of toxin were decided on the basis of previous experience,[10] it is impossible to say for sure that the BoTox reached the CP muscle and that the amount of BoTox was adequate. A second possibility concerns the fact that some subjects have a resistance to BoTox A. There are seven distinct immunologic botulinum toxins, and it has occasionally been reported that some patients who were unresponsive to botulinum toxin type A responded well to other botulinum toxins (B, C, or F). When the present

Table 7. Univariate analysis: predictive factors of good outcome after botulinum with or without myotomy

Characteristic	Outcome		
	Good (n = 17)	Poor (n = 4)	*P* value
Upper esophageal sphincter			
Resting pressure (>80 mm Hg)	10 (62.5)	0 (0)	0.09
Relaxation (>70%)	11 (68.7)*	3 (75)	1.00
Pharyngoesophageal coordination (>80%)	12 (70.6)	3 (100)*	0.54
Pressure of pharyngeal contraction (>20 mm Hg)	12 (75)*	1 (25)	0.10
Videofluoroscopy score (>3)	6 (35.3)	4 (100)	0.03
Electromyography			
Cricopharyngeus muscle spasm with or without incoordination	8 (47)	0 (0)†	0.49
Cricopharyngeus muscle incoordination or low activity	11 (53)	2 (100)†	

Data are expressed as n (%). Data are unavailable for *one patient and †two patients.

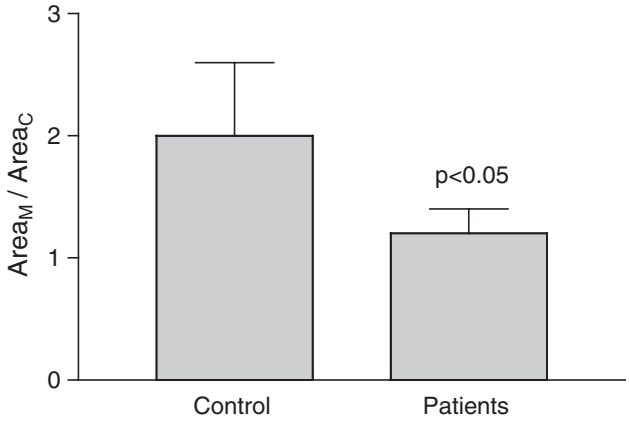

Fig. 3. Muscle/connective tissue ratio in specimen of cricopharyngeus muscle in oropharyngeal dysphagia patients and controls. A statistical difference was found between patients and controls ($P < 0.05$).

study was designed, however, only BoTox A was commercially available and our Ethical Committee rejected the use of different botulinum toxins. In any case, given the relative rarity of immunologic botulinum toxin A resistance, it is unlikely that this mechanism could explain all of the cases in which BoTox A proves ineffectual. The third and most likely explanation for BoTox failing to affect the CP of patients with OPD lies in structural changes in the muscle itself, with a reduction in the muscle fiber content. BoTox is probably ineffective when injected in a rigid, inelastic muscle where connective tissue is prevalent—a hypothesis supported by histologic findings of a higher muscle–connective tissue ratio in patients whose BoTox treatment failed and by EMG findings.

The data from the present study again confirm that the best candidates for BoTox injection or myotomy for OPD are patients still capable of propelling the bolus through the mouth and pharynx, as indicated by a lower VFS score and high pharyngeal contraction pressure: in these cases, increasing transsphincter outflow and facilitating the passage of the bolus in the gullet probably suffice to solve their swallowing difficulties. On the other hand, if the bolus does not get beyond the mouth or its propulsion through the pharynx fails, increasing the UES opening is unlikely to have any effect.

Other pharyngoesophageal manometry parameters have recently been suggested for selecting OPD patients for surgery, such as the lack of a subatmospheric pressure drop on swallowing and a high intrabolus pressure.[18] These parameters require the patient's cooperation to be monitored, however: OPD patients—and particularly those with neurologic disorders—are often unable to perform the 5–10 "wet swallows" required to obtain this information. In this setting, pharyngeal pressure is much easier to record and, combined with clinical data (OPD etiology) and VFS score, it is easy to use to select candidates for BoTox or surgery.

The first step in treating OPD patients could be an EMG-guided BoTox injection in the CP: it is safe and relatively straightforward, and it offers a relatively lasting improvement in dysphagia symptoms. Patients with CP spasm at EMG have a better chance of responding to this treatment. If BoTox fails, CP myotomy should be offered to patients who still have the ability to propel the bolus from the mouth to the esophagus.[19–21]

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Quantitative, Tissue-Specific Analysis of Cyclooxygenase Gene Expression in the Pathogenesis of Barrett's Adenocarcinoma

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Cyclooxygenase (Cox-2) is implicated in the pathogenesis of many cancers including esophageal adenocarcinoma (EAC), whereas the role of the isoform *Cox-1* in carcinogenesis is not well understood. To further elucidate the role of these factors in the development of EAC, we measured the gene expressions (mRNA levels) of *Cox-2* and *Cox-1* by real-time quantitative polymerase chain reaction (QRT-PCR) in tissues from normal esophagus with and without erosive gastroesophageal reflux disease (GERD), Barrett's esophagus (BE), dysplasia, adenocarcinoma, and in healthy gastric antrum. All tissues were purified by laser capture microdissection from endoscopic or surgical resection specimens. Median *Cox-2* gene expression did not differ significantly among the esophageal control groups but was elevated 5-fold in BE, 8-fold in dysplasia and 16-fold in EAC compared to normal esophageal controls with no erosive GERD. Erosive GERD tissue had slightly higher median *Cox-2* expression but *Cox-2* expression in normal antrum was much higher than that in a normal esophagus, close to that of dysplasia. In contrast to that of *Cox-2*, *Cox-1* expression was significantly decreased in all neoplastic tissues compared to normal controls. *Cox-1* and *Cox-2* expression varied over a wide range in the neoplastic tissues but over a relatively narrow range in the esophageal normal tissues. The occurrence of substantial alterations in *Cox-1* and *Cox-2* expression at the BE stage indicates that these are early events in the development of EAC. These results confirm the important role of Cox-2 amplification in the pathogenesis of esophageal adenocarcinoma, but the unexpected down-regulation of Cox-1 raises questions about its role in carcinogenesis. (J GASTROINTEST SURG 2004;8:1007–1017)

KEY WORDS: *Cox-1*, *Cox-2*, gene expression, Barrett's esophagus, esophageal adenocarcinoma

Barrett's esophagus (BE), a condition in which the normal squamous epithelium of the distal esophagus is replaced with metaplastic specialized intestinal-type epithelium as a sequela of chronic gastroesophageal reflux disease,[1,2] is known to be the first stage of a multistep progression from metaplasia to dysplasia to adenocarcinoma.[1,3] The ability to isolate tissue representing each of these stages by endoscopy or after surgery has made it possible to study and identify many molecular events associated with the pathogenesis of esophageal adenocarcinoma. Changes at the genomic level, such as p53 and p16 mutation, gene methylation and aneuploidy, as well as abnormal expression of growth factors, cell adhesion molecules, and cell signaling factors have been reported.[4,5] These changes result in de-regulation of key cellular processes, including proliferation, apoptosis, and cellular differentiation.[6,7]

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One of the most studied molecular events linked to gastrointestinal carcinogenesis is the up regulation of cyclooxygenase-2 (Cox-2). Cox-2 and the isoform *Cox-1* are rate-limiting enzymes in the conversion of arachidonic acid to prostaglandins. Epidemiologic studies have shown that the long-term use of non-steroidal anti-inflammatory drugs, which inhibit cyclooxygenases, are associated with a reduced risk of developing cancer, especially digestive cancers.[8–10] These findings have focused much interest on the role of cyclooxygenases in the pathophysiology of cancer and in the use of cox inhibitors as chemopreventive and chemotherapeutic agents.[10] Data from most studies suggest that *Cox-1* is constitutively expressed, whereas *Cox-2* is inducible by various factors and is itself a transcriptional regulator of a number of genes involved in carcinogenesis.[11]

A number of previous studies have addressed the role of Cox-2 in the pathogenesis of esophageal adenocarcinoma.[12–20] These studies have all reported increased Cox-2 levels at various stage of progression to EAC, but are quantitatively inconsistent as to the frequencies and extents of *Cox-2* overexpression in the various tissue types. For example, reported detection frequencies of Cox-2 immunoreactivity in BE have ranged from 0% [19] to 81%.[16] Such discrepancies may have arisen in part from the use of semi-quantitative immunohistochemistry (IHC) methodology, which has standardization issues related to using different antibodies, staining and scoring protocols,[12] and from isolating RNA without separation of tumor tissue from surrounding normal tissue.[15,16] None of the previous studies concurrently analyzed *Cox-1* expression.

The aim of the present study was to establish accurate ranges of *Cox-1* and *Cox-2* gene expressions in clinical tissue specimens representing metaplasia-dysplasia-EAC sequence in order to better characterize the role of these genes in each of the stages of progression to EAC. We utilized two recent technological advances—laser-capture microdissection (LCM)[21] and quantitative real-time polymerase chain reaction (QRT-PCR)[22] to overcome some of the previous methodologic limitations and to improve the accuracy of gene expression measurements in specific tissue types.

MATERIAL AND METHODS

Definition of Erosive Esophagitis Using Endoscopy

We used the Skinner-Belsey classification to grade the different steps of mucosal injury when performing endoscopy. The term ersosive esophagitis was used when the patients had grade II (linear ulcerations) or grade III (cobblestone esophagitis) mucosal changes.

Definition of Histologic Injury/Reflux Esophagitis of the Squamous Epithelium

We defined reflux esophagitis in the squamous epithelium-lined mucosa by the presence of intraepithelial eosinophils and maturation abnormalities including basal cell hyperplasia and papillary elongation.

Defintion of a Positive 24h pH-monitoring

Twenty-four-hour pH monitoring was performed by positioning a glass pH electrode (Mui Scientific, Toronto, Ontario, Canada) 5 cm above the manometrically measured upper border of the lower esophageal sphincter. The electrode was connected to a digital recording device (Microdigitrapper, Synectics Medical, Irving, TX), and pH was continually monitored for 24 hours. The patients' diets were limited to foods having a pH in the range of 5 to 7. The stored data were transferred to a computer and analyzed with the use of a standard software package (Multigram, Gastrosoft, Irving, TX) according to our standard protocol. The following parameters were measured: total percent time in which the pH was less than 4, percent time the pH was less than 4 when the subject was upright, percent time the pH was less than 4 when the subject was supine, total number of reflux episodes longer than 5 minutes, time of the longest reflux episode, and a composite score based on these parameters (DeMeester score). The study was defined positive when the DeMeester score was greater than 14.72.

Tissue Samples for Real-Time PCR

Esophageal tissue samples (n = 91) were obtained from endoscopy or surgical specimens from 47 patients with Barrett's metaplasia, dysplasia (high- or low-grade) or cancer and were immediately snap-frozen in liquid nitrogen. Through histologic evaluation, the samples were separated into 3 different groups:

1) 37 samples containing specialized intestinal metaplasia on biopsy from 22 patients (BE group)
2) 17 samples containing specialized intestinal metaplasia and either low- or high-grade dysplasia from 11 patients (dysplasia group)
3) 37 samples with confirmed adenocarcinoma of the esophagus from 28 patients (carcinoma group). Because we planned to use laser-capture microdissection (LCM) to separate tissue types, 12 patients were classified into separate

groups depending on whether their tissue samples showed metaplasia next to dysplasia or adenocarcinoma. A single specimen was analyzed from 31 patients and multiple specimens were analyzed from 16 patients. In those cases when multiple samples from one patient were analyzed, the average value was taken for the statistical analysis.

For normal tissue controls, 48 specimens of the squamous epithelium of the esophagus were taken 3 cm above the gastroesophageal junction. These tissues were taken from a total of 48 different patients and the following criteria (see definitions above) were used to classify them:

1) samples of patients with upper gastrointestinal symptoms but normal endoscopy, no histologic injury, and normal 24 hour esophageal pH monitoring (pH− group)
2) samples of patients with upper gastrointestinal symptoms and abnormal 24 hour esophageal pH monitoring but normal endoscopy and no histologic injury (pH+, non esophagitis group)
3) samples of patients with upper gastrointestinal symptoms, endoscopic evidence of erosive esophagitis, evidence of histological injury and abnormal 24 hour esophageal ph monitoring (pH+, erosive esophagitis group).

In these three control groups, one sample was analyzed for each patient and patients were classified in just one group. None of the patients of the control groups had evidence for BE, dysplasia, or carcinoma. Additionally, from 11 patients of the pH- group, a biopsy from the antrum was taken as another normal tissue control. All patients included in this study had undergone no prior foregut-operation and no prior neoadjuvant therapy, if they were cancer patients.

Tissue samples were obtained from a total of 95 patients. There were 34 women and 61 men in this group, with a median age of 56 years (21–86).

Approval for this study was obtained from the Institutional Review Board of the University of Southern California Keck School of Medicine and written informed consent was obtained from participating patients.

Microdissection

For microdissection, frozen samples were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek U.S.A., Inc., Torrance, CA) and cut into serial sections with a thickness of 20 µm. Sections were mounted on uncoated glass slides and stored at −80°C. For histologic diagnosis, three representative sections, consisting of the beginning, middle, and end of sectioning, were stained with hemotoxylin and eosin (H&E) by the standard method.

Before microdissection, sections were air-dried, fixed in 70% ethanol for 3 minutes and washed in H_2O for 2 min. Afterwards, they were stained with nuclear fast red (NFR, American MasterTech Scientific, Inc., Lodi, CA) for 10 seconds and again washed in H_2O for 30 seconds. Samples were then dehydrated in stepwise manner with 70% ethanol, 95% ethanol and 100% ethanol for 30 seconds each, followed by incubation in xylene for 5 minutes and complete air-drying. All H&E stained sections were evaluated by a pathologist. Normal esophageal samples or normal gastric samples were dissected from the slides using a scalpel, if the histology of the samples was homogeneous and contained more than 90% tissue of interest. All other sections were selectively isolated by LCM (P.A.L.M. Microsystem, Leica, Wetzlar, Germany) according to the standard procedure.[21] The dissected flakes of tissue were transferred to a reaction tube containing 400 µl of RNA lysis buffer.

RNA Isolation and cDNA Synthesis

RNA isolation from OCT-embedded samples was done according to a proprietary procedure of Response Genetics, Inc. (Los Angeles, CA; United States patent number 6,248,535). Afterwards, cDNA was prepared as previously described.[23]

Real-Time PCR Quantification of mRNA Expression

Quantification of *Cox-1* and *Cox-2* and an internal reference gene (ß-actin) was done using a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence detection System (TaqMan) Perkin-Elmer (PE) Applied Biosystems, Foster City, CA, USA), as described.[26] The PCR reaction mixture consisted of 1200 nmol/of each primer, 200 nmol/probe, 0.4 U of AmpliTaq Gold Polymerase, 200 nmol/each dATP, dCTP, dGTP, dTTP, 3.5 mM $MgCl_2$ and 1x Taqman Buffer A containing a reference dye, to a final volume of 20 µl (all reagents from PE Applied Biosystems, Foster City, CA, USA). Cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 46 cycles at 95°C for 15s and 60°C for 1 min. The primers and probes used are listed in Table 1.

TaqMan measurements yield Ct values that are inversely proportional to the amount of cDNA in the tube; i.e., a higher Ct value means that more PCR cycles are required to reach a certain level of detection. Gene expression values (relative mRNA levels) are expressed as ratios (differences between the Ct values) between the genes of interest (*Cox-1*

Table 1. Primers and probes

GenBank accession: NM_000962
Forward primer *cox*-1
Sequences: 5′-CGCTGGTTCTGGGAGTTTGTC-3′
Reverse primer: *cox*-1
Sequence: 5′-GGGACTGGGGATAAGGTTGGA-3′
TaqMan probe: *cox*-1
Sequence: 6FAM 5′-CGAGAGATGCTCATGCGCC TGG-3′ TAMRA
GenBank accession: NM_000963
Forward primer *cox*-2
Sequence: 5′-GCTCAAACATGATGTTTGCATTC-3′
Reverse primer: *cox*-2
Sequence: 5′-GCTGGCCCTCGCTTATGA-3′
TaqMan probe: *cox*-2
Sequence: 6FAM 5′-TGCCCAGCACTTCACGCAT CAGTT-3′ TAMRA
Gen BAM 5′-ACCACCACGGCCGAGCGG-3′ TAMRA

and *Cox-2* in this case) and an internal reference gene (β-actin) that provides a normalization factor for the amount of RNA isolated from a specimen.

Statistical Analysis

Cox-1 and *Cox-2* mRNA expression levels in samples with Barrett's metaplasia, dysplasia, and carcinoma were compared to the pH−, pH+, non-esophagitis and pH+, erosive esophagitis groups and the antrum of the pH− group by using the Mann-Whitney *U* test to identify significant differences in the expressions of those groups. Also, gene expression levels among the control groups were compared to each other. Because of the larger number of tests undertaken, the Benjamini and Hochberg multiple comparison correction was performed afterward. Statistical significance was set at the 0.05 level for the corrected *P*-value.

RESULTS

The median values and ranges of the gene expression of *Cox-1* and *Cox-2* determined by QRT-PCR (Taqman) analysis of all of the different tissue types comprising 139 tissue samples from 95 patients are listed in Table 2 and shown in Fig. 1. In Fig. 2, the results using a "patient-group-classification" instead of a "tissue-group-classification" for the Barrett's, dypslasia and EAC group are shown. Thereby, a patient was classified in only one of those three groups, and Barrett's and dysplasia samples of cancer patients for the analysis were excluded.

Cox-2 mRNA expression was lowest in the pH− group (median 0.11). Median *Cox-2* gene expression levels were 0.12 in pH+, non esophagitis, 0.21 in pH+, erosive esophagitis, 1.01 in BE, 1.11 in dysplasia, and highest in the EAC group with 1.77. The increased *Cox-2* mRNA gene expression in the BE, dysplasia, and carcinoma groups was significant compared to the pH− and pH+, non esophagitis group. The pH+, erosive esophagitis group, had a non-significantly decreased median *Cox-2* expression compared to the BE and dysplasia group ($p = 0.09$), whereas compared to the carcinoma group a significant difference was detected. Within the control groups, there was no significant difference detectable, but a trend toward higher *Cox-2* gene expression was present in the pH+, erosive esophagitis group. This group contained a number of high *Cox-2* expression values not seen in either the pH− or pH+, non esophagitis group.

Cox-1 mRNA expression was highest in the pH+, non esophagitis group (median 4.39). Median gene expression levels of *Cox-1* were 3.38 in the pH− group, 2.66 in the pH+ group, erosive esophagitis, 1.05 in the carcinoma, 0.82 in the BE group, and lowest in the dysplasia group with a value of 0.81. The apparent down-regulation of *Cox-1* expression in the BE, dysplasia and EAC groups of the *Cox-1* gene expression levels was significant compared to the three control groups, as listed in Table 3. There was no significant difference in the *Cox-1* expression within the control groups.

Median *Cox-2* gene expression in antrum tissues (median = 1.27) was significantly higher than in the squamous epithelium, similar to those of the BE, dysplasia and EAC groups. However, median *Cox-1* expression in the antrum tissues (median = 3.58) was more similar to that of the control tissue and was significantly higher than in BE, dysplasia, and EAC tissues.

There was no statistically significant difference in median *Cox-2* or *Cox-1* gene expressions between BE samples of patients with only intestinal metaplasia compared to BE tissues and tissues from dysplasia or cancer patients, and also dysplasia samples of patients with just dysplasia compared to dysplastic tissue of cancer patients.

Discussion

The study reports quantitative analysis of *Cox-1* and *Cox-2* gene expressions in tissues of the metaplasia-dysplasia-carcinoma sequence in the esophagus. We made use of two recent technological developments, laser capture microdissection (LCM)[21] and real-time quantitative PCR,[22] in order to maximize the tissue-specific accuracy and reliability of the gene expression data. LCM makes it possible to effectively separate different types of tissues found in heterogeneous clinical specimens, thus providing greater

Table 2. *cox-1* and *cox-2* mRNA expression levels in the different tissue groups

Tissue group	No. of samples	mRNA expression (median) (range)	
		Cox-1 × 100/β-actin	*Cox-2* × 100/β-actin
Squamous, pH−	17	3.38 (0.6–7.1)	0.11 (0.01–2.94)
Squamous, pH+, NERD	17	4.39 (0.01–8.71)	0.12 (0.01–3.33)
Squamous, pH+, esophagitis	14	2.66 (0.01–13.48)	0.21 (0.04–6.08)
Barrett's	22	0.82 (0.19–2.22)	0.61 (0.01–13.90)
Dysplasia	11	0.81 (0.35–2.09)	1.11 (0.28–3.03)
Carcinoma	28	1.05 (0.07–4.15)	1.77 (0.1–14.20)
Gastric antrum (squamous, pH−)	11	3.58 (1.35–16.92)	1.27 (0.1–15.59)

NERD = nonerosive reflux disease.

assurance that any data generated are authentically representative of particular tissue types. Real-time QRT-PCR permits a more accurate and reproducible quantification of gene expression (relative mRNA level) and generates a range of numerical values, thus allowing more precise delineation of the difference in gene expressions between tissues.

Our data show progressively increased *Cox-2* expression at each successive stage of esophageal carcinogenesis. Consistent with most previous studies,[13–18] we found that *Cox-2* is significantly elevated in BE compared to normal tissue, demonstrating up-regulation of *Cox-2* to be an early event in carcinogenesis. The study of Zimmerman et al.,[19] who failed to detect any *Cox-2* immunoreactivity in BE mucosa, is a lone exception. However, although qualitatively similar, our results and those reported in previous studies differ in some quantitative aspects. We found a 16-fold difference in median *Cox-2* mRNA expression between normal esophagus and EAC, but the greatest increase occurred in the transformation of normal tissue to BE (5-fold) with only a further 3-fold greater increase thereafter. Morris et al.,[13] using IHC to measure *Cox-2* protein, found the majority of the increase in *Cox-2* (5-fold) to occur in the progression of BE to high grade dysplasia. The IHC data of Cheong et al [20] showed no difference between *Cox-2* expressions in BE and in EAC, but *Cox-2* in high grade dysplasia was higher than that of EAC. It is not yet clear whether the source of the quantitative

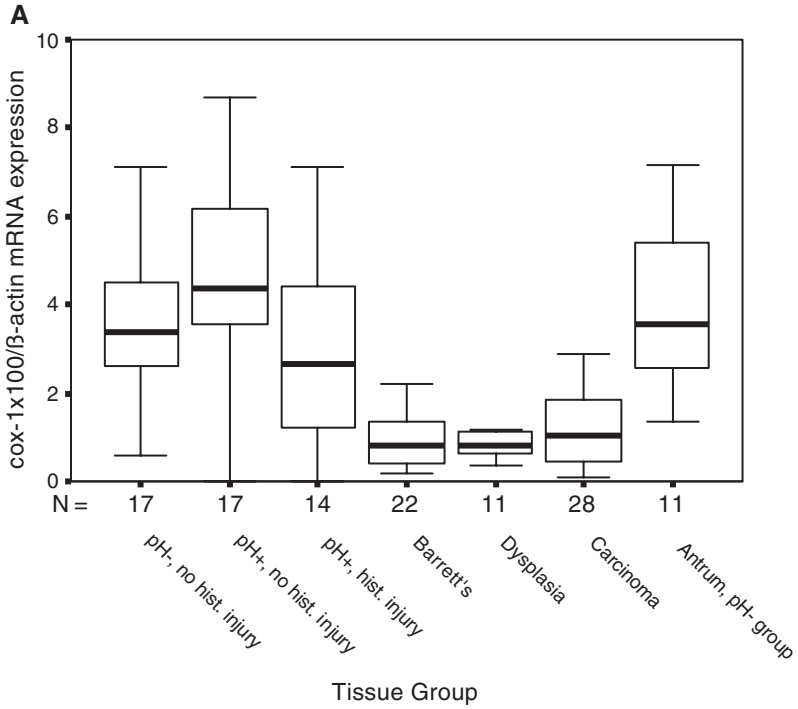

Fig. 1 (A). Relative cox1 mRNA in the different tissue groups. The boxes show the 25th and 75th percentile (interquartile) ranges. Median values are shown as a horizontal black bar in each box. The whiskers show levels outside the 25th and 75th percentile.

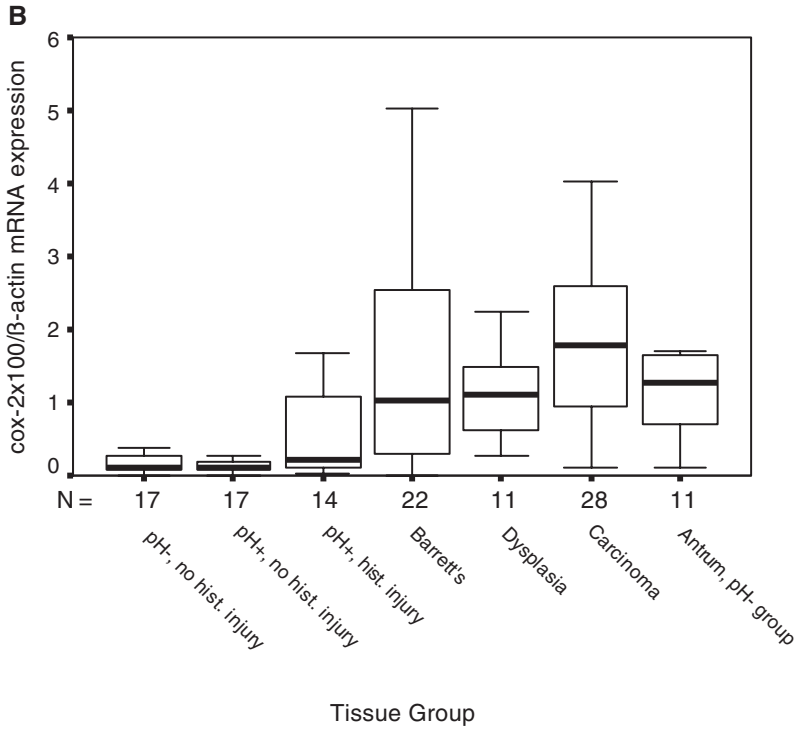

Fig. 1 (B). Relative *Cox-2* mRNA expression in the different tissue groups.

discrepancies among these studies is methodological or whether, in fact, gene expression levels do not always correspond to protein levels because of post-translational processing. However, the fact that the various studies using IHC reported widely different values for over-expression frequencies of *Cox-2* in BE suggests considerable inherent variability in the IHC methodology.

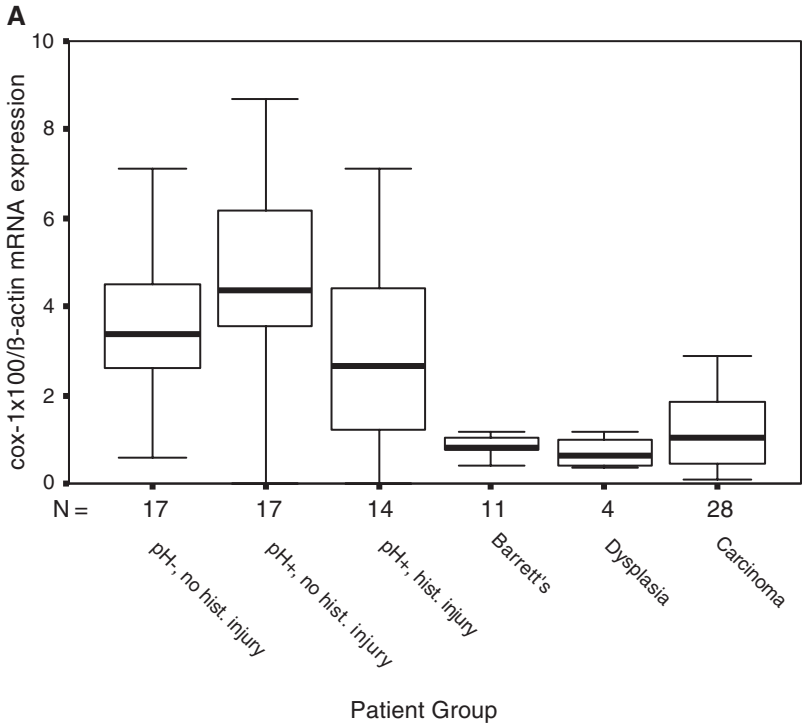

Fig. 2 (A). Relative *Cox-1* mRNA in the different patient groups.

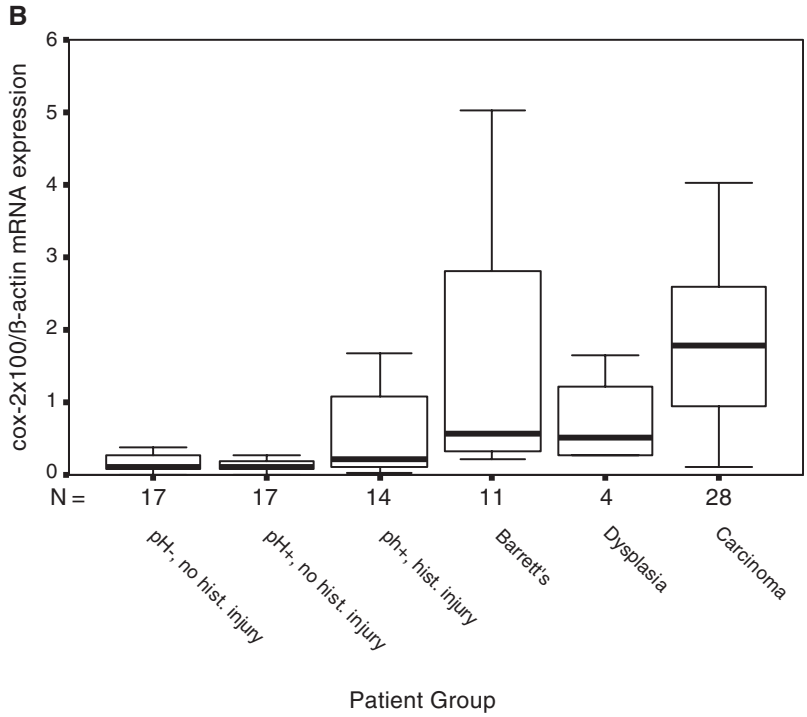

Fig. 2 (B). Relative Cox-2 mRNA in the different patient groups.

Only one[20] of the previous studies that used IHC methodology reported detectable Cox-2 protein in normal esophagus. That we were able to detect and quantitatively measure *Cox-1* and *Cox-2* gene expression in all specimens illustrates the greater sensitivity of RT-PCR methodology. Consistent with the low Cox-2 protein levels suggested by the negative IHC results, we found uniformly low *Cox-2* gene expressions in normal tissues with a relatively narrow range of expression values.

In addition to normal esophagus as a baseline gene expression control, we also included tissues from patients with GERD as controls for possible gene expression changes caused by acid reflux. Erosive

Table 3. Comparing the gene expression levels of different tissue groups with use of the Mann-Whitney *U* test and the corrected *P* value using the Benjamini and Hochberg correction

Tissue group		*P* value	
		For *Cox-1*	For *Cox-2*
Squamous, pH−	Squamous, pH+, NERD	0.21 (0.22)*	0.78 (0.78)
Squamous, pH−	Squamous, pH+, esophagitis	0.46 (0.46)	0.12 (0.16)
Squamous, pH−	Barrett's	<0.0001 (<0.002)	<0.0001 (<0.002)
Squamous, pH−	Dysplasia	<0.0001 (<0.002)	<0.0001 (<0.002)
Squamous, pH−	Carcinoma	<0.0001 (<0.002)	<0.0001 (<0.002)
Squamous, pH+, NERD	Squamous, pH+, esophagitis	0.13 (0.14)	0.06 (0.09)
Squamous, pH+, NERD	Barrett's	<0.0001 (<0.002)	<0.0001 (<0.002)
Squamous, pH+, NERD	Dysplasia	<0.0001 (<0.002)	<0.0001 (<0.002)
Squamous, pH+, NERD	Carcinoma	<0.0001 (<0.002)	<0.0001 (<0.002)
Squamous, pH+, esophagitis	Barrett's	0.003 (0.004)	0.06 (0.09)
Squamous, pH+, esophagitis	Dysplasia	0.009 (0.01)	0.06 (0.09)
Squamous, pH+, esophagitis	Carcinoma	0.01 (0.01)	0.002 (0.004)
Gastric antrum (squamous, pH−)	Barrett's	<0.0001 (<0.002)	0.74 (0.79)
Gastric antrum (squamous, pH−)	Dysplasia	<0.0001 (<0.002)	0.6 (0.69)
Gastric antrum (squamous, pH−)	Carcinoma	<0.0001 (<0.002)	0.31 (0.38)

NERD = nonerosive reflux disease.
**P* value in parentheses indicates a collective value after using the Benjamini and Hochberg collection for multiple comparisons.

GERD, likely the immediate precursor condition to the development of Barrett's esophagus, is characterized by endoscopically visible injury to the distal esophagus.[24] All patients included in the pH+, erosive esophagitis group had an abnormal 24 hour esophageal pH monitoring and both endoscopic evidence of erosive esophagitis and histologic evidence of tissue injury. It is interesting to note that, although pH+, non esophagitis samples had the same median *Cox-2* as normal epithelium, the pH+, esophagitis group had greater median *Cox-2* expression and also contained a number of high *Cox-2* expression values not seen in either of the two other control groups, but close to some values in the BE group. Because *Cox-2* is known to be a mediator of inflammation[25,26] the elevation of *Cox-2* seen in erosive esophagitis specimens may be up-regulation linked to inflammation associated with gastroesophageal reflux. In fact we have recently reported a relationship between *Cox-2* expression and 24-hour pH parameters.[27] Whether this represents a transitory up-regulation secondary to inflammation or a permanent up-regulation that sets the stage for the subsequent transformation of normal tissue to intestinal metaplasia is unknown. Investigations of *Cox-2* expressions following treatment by either drugs or antireflux surgery may provide insight into the mechanisms at play.

We also isolated tissue from the antrum with the idea of possibly using it as a normal control tissue for columnar epithelium below the gastroesophageal junction. However, although *Cox-1* expression in the antrum was similar to that of normal esophagus (Fig. 2), *Cox-2* expression was very high, about 10-fold greater than normal esophagus and exceeding that of BE and dysplasia (Fig. 1). The antrum may represent an example of normal tissue with permanently up-regulated *Cox-2*, perhaps as a consequence of the extreme pH conditions in the stomach.

Although the up-regulation of *Cox-2* expression at each step of the BE-dysplasia-EAC sequence is generally consistent with previously reported results, the approximately 4-fold down-regulation of *Cox-1* expression that we observed in all neoplastic tissues was unexpected and, to our knowledge, has only one precedent in the literature: a study by Wiese et al.[28] in which it was reported that COX-1 protein levels were significantly reduced in colorectal tumors compared to matched normal tissues. Most previous studies have reported that *Cox-1* expression does not change during carcinogenesis, thus giving rise to the often-expressed belief that *Cox-1* expression is constitutive and, in contrast to that of *Cox-2*, non-inducible. Although down-regulation of a gene during tumorigenesis is a *prima facie* indication of tumor suppressor activity, studies showing that both *Cox-1* as well as *Cox-2*–deficient mice have reduced intestinal and skin papilllomas suggest that Cox-1 also plays a positive role in tumorigenesis (at least in mice), albeit possibly by a somewhat different mechanism than *Cox-2*.[29] If indeed *Cox-1* does promote tumorigenesis, it is difficult to see how tumorigenic progression would thereby benefit from decreased *Cox-1* expression. Thus, at this point, we can only speculate as to the significance of *Cox-1* down-regulation in esophageal carcinogenesis. Possibly, because the decline of *Cox-1* expression occurs almost entirely at the transition from normal tissue to BE, reduced *Cox-1* expression specifically facilitates only the differentiation of esophageal epthelium to intestinal metaplasia. Among other possibilities are a) that the down-regulation of *Cox-1* is not functional for tumorigenesis but *Cox-1* is being co-regulated with some other gene that is the real tumor suppressor; b) the effect of *Cox-1* in tumorigenesis is different in humans than in mice and it really does act as a tumor suppressor. In erosive GERD (pH+, erosive esophagitis group) tissues, the expression of *Cox-1* tended in the same direction (downward) as in the BE tissues, supporting the idea that this tissue represents in some respects an intermediate stage of conversion to metaplasia.

One of our longer-term goals is to determine if it is possible to obtain unique gene expression profiles characteristic of each different stage of EAC carcinogenesis in order to test the feasibility of the concept of "molecular pathology," i.e., histological characterization based on quantitative measurement of gene expression values, because current tests, including endoscopy and histopathologic examination, do not allow accurate diagnosis of each stage of the development of Barrett's–associated adenocarcinoma. Because of the considerable overlap among the gene expression ranges for each tissue type, none of the intermediate stages of progression can be unequivocally identified based on just *Cox-1* or *Cox-2* expressions. However, as shown in Fig. 1, *B* the range of *Cox-2* expression in normal esophagus is relatively narrow with a definite upper limit. Thus, finding high *Cox-2* expression in the esophagus is likely to indicate the presence of some abnormal tissue that would call for further examination. Although the *Cox-1* expression ranges in normal tissue do not appear to have a definite lower limit, most of the values in normal tissue are relatively high and thus a low *Cox-1* expression might also indicate an abnormal condition, especially in conjunction with a high *Cox-2* value. Additionally the ratio of *Cox-2* and *Cox-1* could therefore be used in the future as a more effective method to highlight/distinguish the different stages of disease, as shown in Fig. 3.

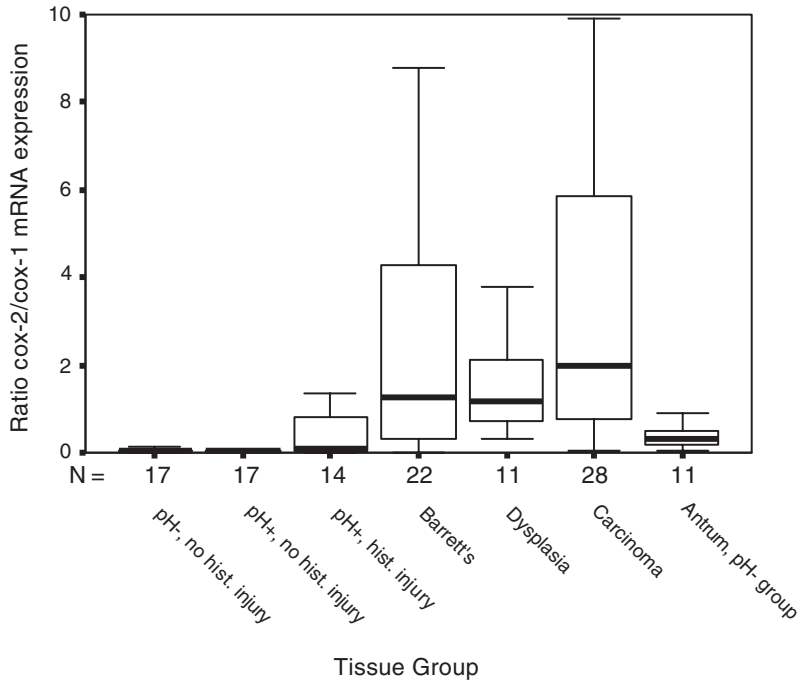

Fig. 3. Ratio of cox-2/cox-1 mRNA expression in the different tissue groups.

The determination of accurate ranges of *Cox-1* and *Cox-2* gene expressions in EAC and the other tissues will allow a more precise estimate of these gene expressions as risk factors for progression, recurrence, and survival. High *Cox-2* expression is known to be an unfavorable prognostic factor in a number of cancers, including esophageal cancer.[14,30–32] We have previously shown in lung cancer patients that if a data base of accurate quantitative values for gene expressions is generated and matched to clinical outcome data, it is possible to determine specific cut-off points of gene expression values that separate low- and high-risk groups.[33] (In this group of lung cancer patients, those with *Cox-2* gene expression > 0.6 had a 31% 5-year survival compared to 62% for those with lower *Cox-2* gene expression).[34] When clinical data for the set of patients in the present study mature and become available, it should be possible eventually to answer a number of questions, such as: 1) Are the patients with erosive GERD who have high *Cox-2* more likely to progress to BE? 2) Are the patients with BE who have high *Cox-2* values more likely to progress to dysplasia and cancer? 3) Can we quantitatively delineate a range of *Cox-2* expression values that signify worse survival of EAC patients?

Drs. Kuramochi, Vallböhmer, and Uchida contributed equally to this work.

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Discussion

Dr. G. Sarosi (Dallas, TX): That was a very interesting paper, and you are to be applauded for making a strong effort to get just the tissue you want by laser microdissection, because that has always been a problem with these tissue-based studies—you get a mix of tissue. I wanted to ask you a couple of questions about your methodology, and then I am going to push you to speculate a little bit about what the inverse relationship of Cox-1 and Cox-2 means.

How thick were your sections? And the reason that I ask is if you use very thin sections, you tend to just get the cells that you see; if you use very thick sections, you might conceivably get things that are underneath the sort of cells that you see.

The second question I wanted to ask is, in my experience with patient specimens, one of the great challenges is that individual variations between patients are sometimes as large as the individual variations between groups, and do you have any patients where you actually have the progression of Barrett's, about whom you can actually make comments about the progression of *Cox-1* and *Cox-2* in an individual patient?

And finally, could you speculate about what this means for chemo prevention? We sort of constantly think of *Cox-1* as a steady state and *Cox-2* as sort of the important gene, and do you think that this may matter for chemo prevention? People advocate aspirin alone or specific *Cox-2* inhibitors, and do you think that that is going to matter?

Dr. Vallböhmer: To the first question, the thickness of our sections was 20 micrometers. We made

some experiments and tested lower and higher thicknesses, and that was the best thickness we could take and get really enough tissue. As you know, when you are performing laser capture microdissection, it takes sometimes about one hour to just work on one sample of one patient, so you have to play with that to better it a little bit.

To the second question, the study is now in progress. We included so far over 200 patients in the study at different stages of disease, and we have patients right now in follow-up whose stage of disease might change.

And about chemo prevention, it is very suggestive. I think *Cox-2* inhibitors are the right way for chemo prevention, but why is *Cox-1* downregulated? There is only one paper in the literature that describes the same effect, looking at colorectal cancer, that also describes a *Cox-1* downregulation, and they didn't find the right answer. We have three suggestions why it could be.

Cox-1 could be downregulated to facilitate the difference or the development from squamous to columnar epithelium, but it could also be a tumor-suppressor gene itself or related to other tumor-suppressor genes. We are not sure. Even the *Cox-2* upregulation with inflammation; is that just transient because of the reflux and it goes back after, let's say, a Nissen? Or is it already the first step of the beginning of the development of Barrett's metaplasia? We will look at that.

Dr. J. Svanvik (Linkoping, Sweden): Thank you for a nice presentation. Is there a risk that you could capture some inflammatory cells despite this microdissection, and that could influence the results, too?

Dr. Vallböhmer: When you laser capture, as you saw on the video clip, you can really be very sure that you just get the tissue you are looking for. You sit with a pathologist and look at an H&E slide, then mark the area that you would like to get, go to the machine, and ensure that you just get the area that you would like to analyze.

Dr. J. Fischer (Cincinnati, OH): Let me ask you a question. You are doing a preventative study with the Cox-2, and you have the Cox-1/Cox-2 system, but there have been a series of other abnormalities observed in Barrett's esophagus at the molecular level. Where do you think that the Cox-1/Cox-2 system fits in? For example, with p53 or p27 abnormalities that have been explored in the area of Barrett's, if you are right and this is "the big deal," then you will see an effect, and if this is just one of a number of other molecular changes that you see in a disordered epithelium, manipulating the Cox-1/Cox-2 system won't have any effect. So, do you have any thoughts about that?

Dr. Vallböhmer: First of all, I think the cyclooxygenase system has a very important role in the development of Barrett's and finally of cancer. So I think Cox inhibitors will have an important role in that field. But you are right, there are other pathways that are involved in that development, like, for example, the apoptotic pathways. There are members of the apoptotic pathway that are important and perhaps there is another key or another way to block that system too. So it could be that not just Cox-1 or Cox-2 will be involved through chemotherapy—perhaps other pathways or parameters too.

Dr. R. Wong (Washington, D.C.): I have two questions. The first question is, when you did your microdissection, I think it is becoming more recognized that the lamina propria versus the epithelium may have a major role in terms of the Cox-2 stimulation, and the question is, did you actually try to microdissect the epithelium and compare that to the lamina propria, because there are many stromal cells in the lamina propria that may also produce Cox-2?

Dr. Vallböhmer: We didn't do that comparison directly.

Dr. Wong: So you had a combination of both epithelial and lamina propria cells?

Dr. Vallböhmer: We did.

Dr. Wong: The other question was, if you take sections from a similar biopsy and compare the difference between one section versus another microdissection, how much variation do you have between these sections?

Dr. Vallböhmer: Sometimes there were variations. We took multiple biopsies from one section of a patient and analyzed if the gene expression is different. They were sometimes mainly in Barrett's tissue variations, and we are looking now if that perhaps has something to do with the height; you know, when you take it a higher or a lower level, the gene expression is sometimes different. We have to collect more of these samples or take multiple biopsies to answer this question.

Dr. C. Pellegrini (Seattle, WA): Do you have any data on patients from whom you obtained the tissue as to whether the patients were treated with proton pump inhibitors, and if so, for how long? There is some data that has suggested in the past that pulses of acid have a different chance of stimulating Cox-2 expression, at least in isolated cells, as opposed to constant acid perfusion.

Dr. Vallböhmer: When patients come to us we perform a pH study and endoscopy. They have to stop taking proton inhibitors two weeks before. Now you can ask if there will still be an influence of the PPIs. So we have data for each patient if they took PPIs before our studies, and we are right now analyzing if this has an influence at the gene expression level. We don't know right now, but we hopefully will have an answer.

Proteomic Analysis of SEG-1 Human Barrett's-Associated Esophageal Adenocarcinoma Cells Treated With Keyhole Limpet Hemocyanin

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Keyhole limpet hemocyanin (KLH) is an immune stimulant derived from a circulating glycoprotein of the marine mollusk *Megathura crenulata*. We previously reported that KLH inhibited the growth of human Barrett's-associated esophageal adenocarcinoma in vitro via apoptotic and nonapoptotic mechanisms. We hypothesize that KLH reduces the growth of Barrett's cancer cells by altering protein expression profiles. A cell line (SEG-1) derived from Barrett's-associated adenocarcinomas of the distal esophagus was selected. Cells were administered KLH (500 μg/ml) or vehicle. After 24 hours, cytosolic fractions were separated through two-dimensional gel electrophoresis. Statistical analysis was performed with Evolution Pro software to identify spots that were differentially expressed between the KLH and control groups. Proteins displaying a twofold or greater change in expression levels were selected for identification. In a total of 420 spots, 31 were differentially expressed between the KLH and control groups. In all, 12 were upregulated and 19 were downregulated. Of the 31, 17 were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Proteomic evaluation shows downregulation of proteins associated with metabolic processes (glycolysis, protein synthesis). KLH also induced proteins indicative of oxidative stress (heat shock 70 family and UDP-glucose 6-dydrogenase). Our results indicate that growth arrest by KLH is accompanied by a cellular stress response and attenuation of metabolic processes. The use of KLH as adjuvant or topical therapy for Barrett's adenocarcinoma provides a promising development in the treatment of this disease. (J GASTROINTEST SURG 2004;8:1018–1023)

KEY WORDS: Esophagus, adenocarcinoma, proteomics, keyhole limpet, hemocyanin

Esophageal cancer is one of the most rapidly increasing cancers in incidence in North America. An estimated 14,250 people in the United States developed esophageal cancer in 2003, with 13,300 reported deaths, and most of these cases started with Barrett's esophagus. Barrett's esophagus occurs when the distal esophagus becomes partially lined with columnar epithelium of the intestinal metaplasia subtype. The use of proteomics as a tool in screening and prognosis for gastrointestinal malignancies is a rapidly developing area. Single-protein biomarkers have been identified in colorectal cancer; however, their sensitivity and specificity to date have made them unreliable and thus limited their clinical use. On a wider scale, biomarkers and molecular targets are currently being identified by analysis of the proteome "fingerprint" characteristic of gastrointestinal cancers.[1] To date, however, there have been no comprehensive studies of esophageal cancer protein profiling or protein expression patterns associated with anticancer therapy. Barrett's esophagus is an excellent model in which to study the early events of neoplastic progression.[2] Specifically, our interest is in the response of Barrett's cancer cells to treatment with the novel therapeutic agent keyhole limpet hemocyanin (KLH) and the resulting alterations in protein expression.

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KLH is a high-molecular-weight, copper-containing protein found in the hemolymph of the sea mollusk *Megathura crenulata*.[3] This extracellular respiratory protein has many immunostimulatory properties, including the ability to enhance the host's immune response by interacting with T cells, monocytes, macrophages, and polymorphonuclear lymphocytes.[4] KLH has been used primarily as a carrier for vaccines and antigens and as adjuvant treatment in regimens such as antimicrobial therapy. KLH is regarded as a safe and highly effective immunotherapy for superficial bladder cancer.[5] It is also effective when applied topically. We previously reported that KLH has significant antiproliferative effects in vitro against breast, pancreas, and prostate cancers.[6] In breast cancer cells, KLH reduced cell proliferation without significant changes in apoptosis; in fact, apoptosis was decreased by 20–30% compared with controls.[7] However, in pancreatic cancer cells, apoptosis was increased almost sixfold. Significant decreases were seen in a number of proinflammatory cytokines.[7] In breast cancer cell lines, there was a 40% inhibition of growth induced by KLH and more cytokines were inhibited, including interleukin (IL)-8, -6, and -2, and this occurred in the absence of significant changes in apoptosis. The direct growth inhibition of multiple tumor cell lines along with induction of apoptosis and alterations in cytokine production provided sufficient preliminary data to test KLH as a growth inhibitor for esophageal cancers. Its efficacy in the treatment of epithelial derived adenocarcinomas remains unknown.

In preliminary in vitro experiments, we found that KLH had beneficial effects in Barrett's-associated esophageal adenocarcinoma by inhibiting cell proliferation.[8] Using stable tissue cultures of Barrett's cancer cells, we tested the effects of KLH on cell growth and apoptosis. The results showed that KLH inhibits the proliferation of two esophageal cancer cell lines in vitro by an average of more than $29 \pm 18\%$ after 72 hours of exposure. Our studies also revealed that treatment with KLH enhanced apoptosis in Barrett's-associated adenocarcinoma cell lines.[8] The molecular mechanisms that underlie these changes in response to KLH have not been identified. Based on these preliminary in vitro data, we hypothesized that KLH reduced the growth of Barrett's esophageal adenocarcinoma by altering protein expression profiles.

MATERIAL AND METHODS

Cell Culture and Reagents

A human esophageal adenocarcinoma cell line, SEG-1, derived from Barrett's-associated adenocarcinomas of the distal esophagus, was cultured as previously described.[8] SEG-1 tumor cells were selected as a model to study the in vitro effects of KLH because they possess a lower baseline rate of apoptosis and a higher expression of cyclooxygenase (COX)-2 compared with other adenocarcinoma cell lines as reported by Souza et al.[9] Cells were cultured in Dulbecco's modified Eagle's medium with L-glutamine (Invitrogen, Inc., Carlsbad, CA) supplemented with 10% fetal bovine serum (American Type Culture Collection, Manassas, VA), penicillin G (100 units/ml), and streptomycin (100 μg/ml; BioWhittaker, Inc., Walkersville, MD) and maintained in monolayer culture at 37°C in humidified air with 5% CO_2. The esophageal cell line was grown to confluency in $3 \times$ T75 flasks to obtain approximately 5×10^7 cells. KLH was supplied as a lyophilized powder (Calbiochem, La Jolla, CA). Cells were incubated for 24 hours with KLH at 500 μg/ml. This concentration was selected based on our previously published results showing reduced Barrett's cancer cell proliferation and increased apoptosis after treatment with KLH for 16 hours at concentrations ranging from 2.0 to 500 μg/ml.[8] As a negative control, an equal volume of vehicle (100% tissue culture medium) was added.

Two-Dimensional Electrophoresis

Cytosolic cell extracts were prepared by scraping cells on ice in 2 ml of two-dimensional electrophoresis digitonin solution (0.01% digitonin, 0.83% carrier ampholytes [CA], pH 3–10, 1:1000 protease inhibitors [Sigma, St. Louis, MO]). After 5 minutes on ice, extracts were centrifuged at 16,000*g* for 1 minute at 4°C. Protein concentrations were determined using the BCA protein assay kit (Pierce Chemical, Indianapolis, IN). The supernatant was adjusted to 8 mol/L urea, 1% Triton X-100, 0.5% dithiothreitol, 0.5% CA (pH 3–10), and 0.0025% bromophenol blue. For two-dimensional electrophoresis, 160 μg of protein was separated by pI on immobilized pH gradient gel strips (pH 3–10) and by size on 12% polyacrylamide gels and then stained with silver. Two-dimensional gels corresponding to three replicates each of untreated and KLH-treated SEG-1 cytosolic extracts were digitally imaged using Quantity One software (BioRad, Hercules, CA) and a GS-800 Calibrated Densitometer (BioRad) and analyzed using Evolution Pro (Nonlinear Dynamics Ltd. [Nonlinear USA, Durham, NC]) two-dimensional analysis software. Proteins displaying a twofold or greater change in expression levels were selected for identification.

Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry

Protein identities were established using matrix-assisted laser desorption ionization time of flight

mass spectrometry (MALDI-TOF-MS). For this, protein spots were excised and destained in 350 µl of 1:1 (v/v) 100 mmol/L ammonium bicarbonate/methanol for 30 minutes with rotation followed by 350 µl of 100 mmol/L ammonium bicarbonate for 30 minutes and 350 µl of acetonitrile for 15 minutes. The acetonitrile was removed and the gels were dried in a SpeedVac (Savant Instruments, Farmingdale, NY) for 5 minutes. Proteins were digested by the addition of 50 µl of 2 µg/µl trypsin (Promega, Madison, WI) in 25 mmol/L ammonium bicarbonate, and reactions were allowed to proceed at 37°C for 18 hours. Peptides were extracted with 100 µl of 25 mmol/L ammonium bicarbonate and 8 µl of acetonitrile with sonication for 15 minutes. Peptides were purified using a C18 ZipTip (Millipore Corp., Bedford, MA) according to the manufacturer's recommendation. The peptides were eluted in 3 µl of 50% acetonitrile, 2% acetic acid. For MALDI-TOF-MS analysis, 0.5 µl of extracted peptides was mixed with 0.5 µl of 10 mg/ml α-cyano-4-hydroxycinnamic acid and spotted onto a MALDI plate. MALDI mass spectra were acquired with a MALDI-R instrument (Waters Micromass, Milford, MA). Proteins were identified from their peptide mass fingerprint using ProteinLynx database searching software (Waters Micromass).

RESULTS

A proteomic approach was used to determine the differential expression of proteins 24 hours after treatment of a Barrett's cancer cell line with the immunostimulant KLH. The cytosolic proteins from cells were separated by two-dimensional electrophoresis in three replicate gels per treatment. Figure 1 shows the gel images of the proteomic profile pretreatment and post-treatment with KLH. A total of 420 proteins and polypeptides were detected on each gel. All the identified spots were localized in the pI 3–10 range with a molecular mass range of 5–200 kDa. After analysis for spot detection, background subtraction, and volume normalization, Evolution Pro software selected 31 spots as being regulated (Table 1). In all, 12 were upregulated and 19 downregulated. Of the 31, 17 were identified by MALDI-TOF-MS. The rest are still unknown.

Proteomic evaluation shows downregulation of proteins associated with metabolic processes such as glycolysis and protein synthesis. Eleven spots were identified by MALD-TOF-MS analysis to be differentially expressed in Barrett's cancer cells treated with KLH; pyruvate kinase M1 (corresponding to three different protein spots) and α-enolase (two different protein spots) after 24 hours of treatment with KLH. Downregulation of three spots were identified as proteins involved in protein synthesis: 40S ribosomal proteins S21, S12, and 60S ribosomal protein P2. Nucleoside diphosphate kinase B, guanine nucleotide-binding protein, and tubulin-β2 were also downregulated with KLH treatment.

Six proteins were upregulated in response to KLH treatment. KLH induced proteins indicative of cellular oxidative stress. Two protein spots belong to the heat shock 70 family (Hsp70 and Hsp cognate 71 protein) and two to the upregulation of UDP-glucose 6-dehydrogenase (UDP-G6DH). Profilin and prefoldin were also upregulated in Barrett's cancer cells after treatment with KLH.

DISCUSSION

In proteomics, expression profiling is the identification of cellular proteins as a function of a particular state (e.g., differentiation, developmental state, or disease state) or as a function of exposure to a drug or physical stimulus.[10] Diseased cells and drug-treated cells can be compared to determine which proteins are differentially expressed. This approach collectively offers a means of detecting potential molecular targets for therapeutics in disease. In this study, the response of Barrett's cancer cells to treatment with the novel therapeutic agent, KLH was tested for alterations in protein expression. We have identified for the first time a host of protein alterations in Barrett's cancer cells, both upregulated and downregulated, in response to KLH via a proteomic approach.

Approximately 420 protein spots were detected. Proteins displaying a twofold or greater change in expression levels were selected for further identification. A total of 31 were differentially expressed between the KLH and control groups. In all, 12 were upregulated and 19 were downregulated. This observation agrees with the expectation that over- and under-expression of certain proteins result in Barrett's cancer cells exposed to an anticancer agent. The numbers of proteins detected and differentially expressed between KLH-treated and control Barrett's cancer cells are comparable to other studies. Using similar technology, Zhang et al.[11] found 600 differentially secreted proteins or polypeptides in presurgery and postsurgery sera collected from patients with esophageal squamous cell carcinoma. More recently, Bernstein et al.[12] detected 454 proteins, of which 241 were different in colon adenocarcinoma cell lines resistant to deoxycholate-induced apoptosis with 82 proteins either overexpressed or underexpressed. We found that proteins with altered expression in response to KLH fall into metabolic and signal

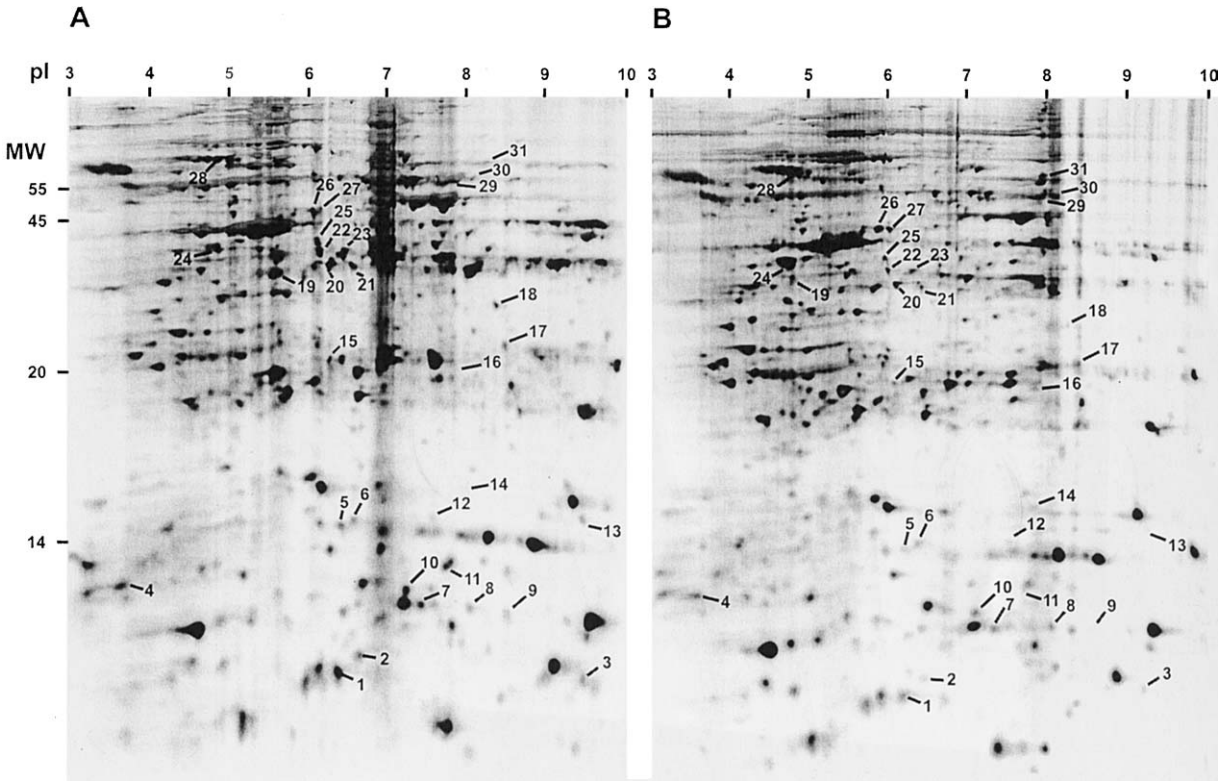

Fig. 1. Two-dimensional electrophoresis profiles of the control and keyhole limpet hemocyanin (KLH)-treated Barrett's adenocarcinoma cells (SEG-1) after 24 hours. One hundred sixty micrograms of protein was separated by two-dimensional electrophoresis (Isoelectric focusing at pH 3−10, 12% SDS-PAGE) and stained by silver staining. (**A**) Control. (**B**) KLH treated.

transduction pathways used by other anticancer agents and therapeutics.

More proteins were downregulated with KLH than were upregulated, as revealed by proteomic analysis of Barrett's cancer cells. Eleven spots were identified by MALD-TOF-MS analysis to be differentially expressed pyruvate kinase M1 (corresponding to three different protein spots) and α-enolase (two different protein spots) after 24 hours of treatment with KLH. Both of these proteins play an important role in glycolysis. Glycolysis is known to be the primary energy source in most cancer cells, and therapies that inhibit both cell proliferation and glycolytic supply are common. For example, the antifungal azole derivative clotrimazole decreases glycolysis and the viability of colon adenocarcinoma cells.[13] Similarly, metabolic changes are associated with chemotherapy-induced apoptosis in colorectal cells treated with doxorubicin.[14] Our previous studies show that treatment with KLH enhances apoptosis in Barrett's-associated adenocarcinoma cell lines.[8] In this study, the downregulation of glycolytic proteins with KLH suggests that esophageal adenocarcinoma cells respond to this chemotherapy by the diminution of oxidative phosphorylation and/or glycolytic enzymes. This would explain the reductions in cell growth and the induction of apoptosis we have observed in our previous studies with KLH. Downregulation of three spots were identified as proteins involved in protein synthesis: 40S ribosomal proteins S21, S12, and 60S ribosomal protein P2. The ribosomal proteins play an important role in the elongation step of protein synthesis, transcription, DNA repair, and the antigenic response. This function makes ribosomal proteins potential molecular targets for therapies of human malignancies. For example, the anticancer drug rapamycin works by regulating protein degradation and ribosome biogenesis, thus reducing cancer growth.[15] We found that KLH has beneficial effects in Barrett's-associated esophageal adenocarcinoma by inhibiting cell proliferation and enhancing apoptosis.[8] Downregulation of these ribosomal proteins indicates that KLH may be slowing the growth of Barrett's cancer cells by affecting protein translation machinery. Nucleoside diphosphate kinase B, guanine nucleotide-binding protein, and tubulin-β2 were also downregulated with KLH treatment. Nucleoside diphosphate kinase is a transcriptional activator

Table 1. Table of differentially expressed proteins in Barrett's esophageal cancer cells treated with keyhole limpet hemocyanin

Spot No.	Swiss protein accession No.	Protein name	Biological function
Upregulated			
8	P07737	Profilin I	Cytoskeletal regulator
9	Q9UHV9	Prefoldin subunit 2	Protein folding/chaparone
17	P11142-2	Heat shock cognate 71 kDa protein	Protein folding/chaparone
24	P08107	Heat shock 70-kDa protein 1	Protein folding/chaparone
29	O60701	UDP-glucose 6-dehydrogenase	UDP-glucuronate biosynthesis
30	O60701	UDP-glucose 6-dehydrogenase	UDP-glucuronate biosynthesis
Downregulated			
3	P35265	40S ribosomal protein S21	Protein biosynthesis
4	P05387	60S ribosomal protein P2	Protein biosynthesis
7	P22392	Nucleoside diphosphate kinase B	Nucleoside biosynthesis, transcription regulation
10	P25398	40S ribosomal protein S12	Protein biosynthesis
15	P14618	Pyruvate kinase M1	Glycolysis
18	P25388	Guanine nucleotide–binding protein β subunit-like protein 12.3	Signal transduction
19	P05217	Tubulin β-2 chain	Microtubule-based process
20	P14618	Pyruvate kinase M1	Glycolysis
21	P14618	Pyruvate kinase M1	Glycolysis
22	P06733	α-Enolase	Glycolysis
23	P06733	α-Enolase	Glycolysis

of c-Myc, whereas guanine nucleotide protein binds protein kinase C. In cancer therapy, disruption of guanine-binding proteins (i.e., Ras proteins) and tubulin formation are two rational therapeutic strategies that target cancer growth.[16,17] Reduced proliferation and survival of Barrett's esophageal cancer cells with KLH treatment may be mediated through multiple proteomic changes at these levels.

Six proteins were upregulated in response to KLH treatment. Two protein spots belong to the heat shock 70 family (Hsp70 and Hsp cognate 71 protein) and two belong to the upregulation of UDP-glucose 6-dydrogenase (UDP-G6DH). Profilin and prefoldin were also upregulated. In response to stress, heat shock proteins cooperate with chaperones to stabilize preexistent proteins and to protect against nonnative conformations by other proteins. Heat shock proteins are also involved in cell proliferation, apoptosis, and the immune system.[18,19] In studies where Hsp70 was stably overexpressed in cells and subsequently injected into mice, tumor growth was reduced.[20] Our observation that KLH upregulates heat shock proteins in Barrett's cancer substantiates this idea. In preliminary in vitro experiments, we found that KLH has beneficial effects in Barrett's-associated esophageal adenocarcinoma by inhibiting cell proliferation.[8] Our studies also revealed that treatment with KLH enhanced apoptosis in Barrett's-associated adenocarcinoma cell lines.[8] The involvement of heat shock proteins in response to KLH suggests that the novel anticancer therapeutic is targeting heat shock protein function. The enzyme UDP-G6DH was also upregulated in response to KLH. Its biological function is to convert UDP-glucose to UDP-glucuronate, a critical component of glycosaminoglycans, hyaluronan, chondroitin sulfate, and heparan sulfate. UDP-G6DH is also involved in phase II detoxification (glucuronidation of xenobiotics). Previous studies show that proinflammatory cytokines, such as IL1-β, increase the expression of UDP-G6DH in human fibroblasts.[21] We have observed the inhibition of cancer cell growth induced by KLH to be accompanied by cytokine inhibition, including IL-8, -6, and -2, without substantial changes in IL-1β.[7] Other proteins, profilin and prefoldin, were also upregulated by KLH. Profilin helps to mediate cellular response to oxidative stress and proliferation as a cytoskeletal regulator, whereas prefoldin acts as a cytosolic chaperone to deliver nonnative proteins to target sites for folding. These ubiquitous proteins are highly conserved and little is known about their function in cancer cells. Taken together, KLH may be inducing the oversecretion and release of heat shock 70 proteins and UDP-G6DH, along with cytosolic protein

regulators, to protect against tumor growth in Barrett's adenocarcinoma.

In summary, Barrett's esophageal cancer cells expressed proteins that were both upregulated and downregulated in response to treatment with the experimental therapeutic agent KLH. Proteomic evaluation shows downregulation of proteins associated with metabolic processes (glycolysis, protein synthesis). Treatment with KLH also induced proteins indicative of cellular stress (heat shock 70 family and UDP-G6DH). Identification of candidate proteins altered with KLH-treated cells via proteomic analysis provides clues to the molecular mechanisms behind the anticancer effects of KLH. Based on these results, the use of KLH as adjuvant or topical therapy for Barrett's adenocarcinoma offers a promising development in the treatment of this disease.

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Molecular Biology of Squamous Cell Carcinoma of the Anus: A Comparison of HIV-Positive and HIV-Negative Patients

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The molecular mechanisms involved in progression of squamous cell carcinoma of the anus (SCCA) are poorly elucidated, as well as the potential role of HIV infection. Loss of heterozygosity (LOH) is one of the mechanisms responsible for inactivation of tumor suppressor genes. We hypothesized that HIV-induced immunosuppression may contribute to an alternate molecular pathway in SCCA progression, through persistence of human papillomavirus infection within the anal canal. This study was undertaken to compare the molecular biology of SCCA in HIV-positive (HIV+) and HIV-negative (HIV−) patients. We retrieved tumor specimens from 18 HIV− and 10 HIV+ patients diagnosed with SCCA in two institutions. DNA from tumor and normal tissues was extracted and then amplified by polymerase chain reaction. LOH was investigated at 14 loci: three at 18q (*DCC*), two at 13q (*Rb*), three at 17p (*p53*), three at 11q, one at 2p, and two at 5q (*APC*). LOH was defined by a tumor DNA-to-normal tissue DNA ratio of >2. HIV+ patients were younger (36 ± 7 years versus 53 ± 13 years, $P = 0.001$) and showed a trend toward tumors of larger size (3.7 ± 1.6 cm versus 2.6 ± 1.5 cm, $P = 0.09$). The median $CD4^+$ count in HIV+ patients at the time of diagnosis was 74×10^6/L (range, 5−900). The overall frequency of LOH was 17.3% (41 LOH of 236 informative loci). Tumors in HIV− patients were more likely to present LOH than were tumors in HIV+ patients (24.1% versus 6.6%, $P = 0.0004$). Differences between the two groups with regard to allelic losses were also observed at specific loci, such as 18q (41% [HIV−] versus 0% [HIV+], $P = 0.05$), 17p (43% versus 10%, $P = 0.09$), and 5q (33% versus 0%, $P = 0.12$). Consistent LOH on chromosomes 17p, 18q, 5q, and 11q were observed in HIV− patients with SCCA. By contrast, allelic losses at 17p, 5q, and 18q seem to be rare in tumors of HIV+ individuals. These data suggest that immunosuppression may promote SCCA progression through an alternate pathway and that persistence of HPV infection within the anal canal may play a central role in this process. (J GASTROINTEST SURG 2004;8:1024–1031)

KEY WORDS: Anal cancer, biology, HIV, genes, HPV, loss of heterozygosity

Squamous cell carcinoma of the anus (SCCA) includes 1.5% of all digestive system cancers in the United States, with 4,000 new cases and 500 deaths in 2003.[1] SCCA is considered a sexually transmitted disease that is clinically related to infection with high-risk human papillomaviruses (HPVs).[2,3] The best-characterized factor in the molecular biology of SCCA is the integration of HPV types 16/18 DNA into anal canal cell chromosomes.[4,5] However, in an experimental model of neoplasm, the presence of high-risk HPV was not sufficient to induce transformation and tumor progression.[6] Studies on squamous cell carcinomas of the cervix have demonstrated that in addition to HPV integration, the neoplastic process requires the loss of tumor suppressor gene (TSG) function.[7] Thus, HPV infection is thought to be a necessary factor but insufficient on its own to promote malignant transformation in SCCA.

Since 1990, numerous epidemiologic studies from the United States have demonstrated a high incidence

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of anal cancer among AIDS patients.[8] Although most immunocompetent individuals experience spontaneous regression of anogenital HPV infection, HIV-positive (HIV+) patients are more likely to have persistent HPV infection than are HIV-negative (HIV−) patients.[9] In the case of male homosexuals, it has been possible to investigate the effects of HIV-mediated immune suppression on HPV infection, as dual infection with both viruses is relatively common in this population.[10] Although the mechanisms of the HPV–HIV interaction are poorly understood, it has been suggested that HIV-related immune suppression is responsible for an enhanced expression of HPV infection in the anal canal, which may lead to HPV-induced epithelial abnormality.[11]

Persistence of high-risk HPV within the anal canal of immunosuppressed individuals has important biological implications, because high-risk HPV (mostly types 16 and 18) encode for three oncoproteins with growth-stimulating and transforming properties (E5, E6, and E7).[12] The E6 protein contributes to cell transformation and the development of malignancy through the binding and inactivation of p53 protein, an important negative regulator of cell growth.[13] It has also been demonstrated that the binding of E7 to retinoblastoma (Rb) protein results in degradation of this protein via ubiquination.[14] Unlike many cancers in which LOH at 17p is demonstrated in 40–60% of cases, the *p53* gene is frequently wild-type in HPV-related cervical cancers.[15] Thus, the notion has arisen that E6-mediated abrogation of p53 protein–dependent apoptosis is equivalent to inactivating mutation of the *p53* gene.[16]

We hypothesized that, first, HIV-induced immune suppression may promote SCCA progression through an alternate molecular pathway and, second, that LOH at various loci harboring known TSGs may not be a necessary step in the progression of SCCA in HIV+ patients due to the persistence of HPV infection in this population. Thus, the aim of this study was two-fold: to identify specific patterns of LOH involved in SCCA progression and to correlate these molecular alterations with HIV status.

METHODS

Patients

Through a preliminary database search, a total of 124 patients who received treatment for SCCA at Mayo Clinic Rochester between 1980 and 2000 were identified. The following parameters were assessed for entry in a computerized database: patient demographics, tumor stage, tumor location (anal canal or anal margin), HIV status, history of anal warts, modalities of treatment including toxicity and side effects, response to chemoradiation therapy, and clinical outcome. A pathologist reviewed all specimens for confirmation of the initial diagnosis and to delineate the area of normal and tumor tissues on specimen. The tumor percentage had to be 70% or greater for our analysis parameters. All patients had given prior approval to have their specimen used for research. The Institutional Review Board approved the study protocol.

Tumor Microdissection and DNA Extraction

Genomic analyses were performed and interpreted by individuals who were blinded to patients' HIV status. A total of 18 specimen from HIV− patients (all from the Mayo Clinic) were deemed appropriate for DNA analysis. Tumor specimens originating from 10 HIV+ patients were retrieved from the Department of Pathology at the University of South California in Los Angeles. The tissues were routinely collected from the endoscopy suite or from the operating theatre, fixed in buffered formalin, embedded in paraffin, and stored for a variable number of months before selection for analysis. Paraffin-embedded blocks were cut with a microtome into 5-μm-thick sections and affixed to glass microscope slides. Using a sterile scalpel blade, areas of normal (nontumor) and cancer tissue were microdissected under a dissecting microscope using a hematoxylin and eosin–stained section as a guide. Care was taken to prevent admixture of microdissected domains and to ensure that identified tissue were removed in a precise manner. Genomic DNA was then extracted from the microdissected tissue using the QIAamp DNA Mini Kit No. 51306 (Qiagen, Venlo, The Netherlands). The specimens were deparaffinized in xylene, purified with absolute alcohol, and centrifuged at 14,000 rpm. The pellets were dried under reduced pressure in a DNA SpeedVac (Savant, Inc., Farmingdale, NY), resuspended in 15 ml of Genereleaser (BioVentures Inc., Murfreesboro, TN), used according to the manufacturer's protocol, and incubated at 55°C overnight in 200 mg/ml proteinase K (Sigma, St. Louis, MO). The specimens were used directly for polymerase chain reaction (PCR) analysis.

Tumor Loss of Heterozygosity Assays

The following microsatellite primers tightly linked to TSGs were used for LOH analyses: D18S35 (*dcc*) and D18S46 (*DCC/Smad4*); D13S270 and D13S319 (*Rb*); tp53, D17S786, and D17S513 (*p53*); D11S29, D11S4127, and D11S925 (*11q*); D5S346 and D5S421 (*APC*); and D2S123 (*2p*). Each pair was optimized

for efficient amplification. One primer from each pair was labeled with a phosphoramidite dye. Each 15-μL reaction contained 2 μL of genomic DNA, 200 μmol/L dNTPs, 1.33 μmol/L each primer, 0.5 U Ampl*Taq* Gold (PE Biosystems), and 1.5−2.5 mmol/L $MgCl_2$. Reactions were cycled in either a Perkin Elmer (Wellesley, MA) 9600 GeneAmp PCR System or an MJR (Waltham, MA) Tetrad Cycler as follows: 10 minutes at 95°C; then 35 cycles of 30 seconds at 95°C, 30 seconds at 58°C or 55°C, and 30 seconds at 72°C; and then a final 10-minute extension at 72°C. Reactions were held at 5° C until analysis. PCR products were resolved on an ABI (Applied Biosystems, Foster City, CA) 3100 DNA sequencer. Genotypes were analyzed using ABI Genotyper 2.5 software.

Definition of Loss of Heterozygosity

Loss of heterozygosity was assessed by comparing the ratio of the peak heights of each allele between normal and tumor DNA samples (N1/N2*T2/T1). For each marker, ratios of 2 or greater were defined as clear evidence for LOH; ratios of 1.5–2 were interpreted as suggestive evidence for LOH; and ratios of 1.5 or lower were considered as absence of LOH.

The results for each marker were then collected to determine LOH at individual loci according to the following: A normal DNA–to–tumor DNA ratio of 2 or greater at one marker at least was considered a strong indication of LOH for the specific locus. A ratio greater than 1.5 but less than 2 at one marker at least was considered indicative for "indeterminate" LOH at the specific locus. When ratios were less than 1.5 for all informative markers, this was considered a clear indication for no LOH at the specific locus.

Statistical Analysis

Statistical analyses were undertaken by means of the software package STATGRAPH 3.0 software for Windows (Statgraph Software Inc., San Diego, CA). Quantitative data were expressed as mean ± SD or median (range). Group comparisons were made using χ^2 or Fisher's exact test for categorical variables, and Student's *t* test for continuous variables. *P* values less than or equal to a two-sided α level of .05 were considered statistically significant.

RESULTS

A total of 18 tumors from HIV− and 10 tumors from HIV+ patients were available for DNA analysis. The distribution of tumors according to location within the anal margin or the anal canal was similar between groups. HIV+ patients with SCCA were exclusively males and were younger than HIV− patients (36 ± 7 years versus 53 ± 13 years, $P = 0.001$). Eight of 10 of the HIV+ patients had $CD4^+$ counts that were below 200×10^6/L at the time of diagnosis. Seventy-two percent of HIV− and 77% of HIV+ patients were alive without evidence of recurrence at the time of last follow-up. Overall, two thirds of patients were treated initially with radiation therapy or a combination of chemotherapy and radiotherapy. However, DNA analysis was performed on pretreatment biopsies to avoid any potential bias due to the effect of adjuvant treatment. The clinical characteristics of patients included in the study are summarized in Table 1.

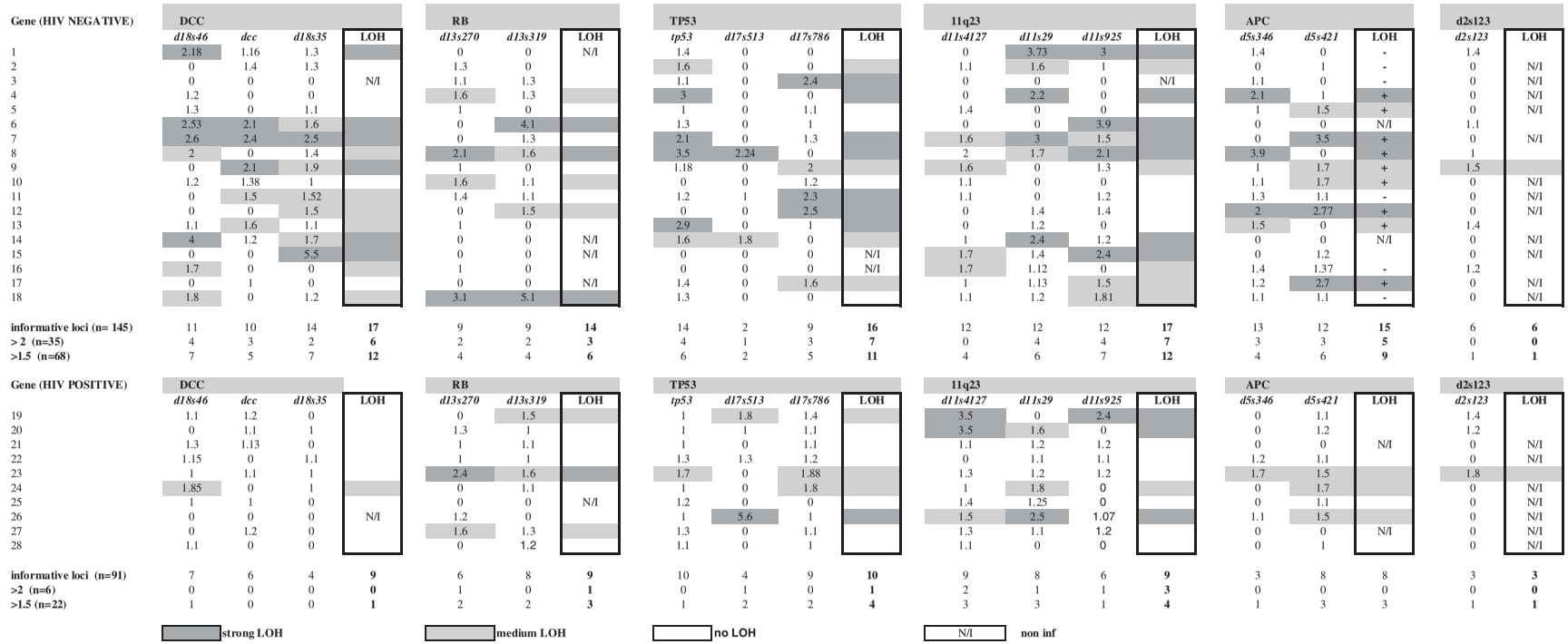
Loss of Heterozygosity at Individual Loci

Of 14 loci tested, 57.5% (145 of 252) were considered informative for LOH in HIV− patients, and 65% (91 of 140) were informative in HIV+ patients. A total of 41 instances of LOH (ratio ⩾2) were observed in the entire group: 35 in HIV− and 6 in HIV+ patients. Thus, when considering all informative loci, SCCAs in HIV− patients were more likely to present with LOH than were tumors in HIV+ patients (35 LOH/145 loci [24.1%] versus 6 LOH/91 loci [6.6%], $P = 0.0004$). When considering a ratio greater than 1.5 for the definition of LOH, a total number of

Table 1. Clinical characteristics of patients with SCCA

Parameter	HIV negative	HIV positive	*P**
Gender (M/F)	5/13	10/0	<0.001
Age (mean ± SD yr)	53 ± 13	36 ± 17	<0.001
Tumor location			0.67
Anal canal	14	7	
Anal margin	4	3	
Tumor size (mean ± SD cm)	2.6 ± 1.5	3.7 ± 1.6	0.09
$CD4^+$ count (median [range])	—	74 (5–900)	
Follow-up (median mo [range])	47 (1–440)	29 (3–67)	0.26
Radiation therapy			1.00
Yes	13	6†	
No	5	3	
Status at last follow-up			1.00
Alive without recurrence	13	7†	
Alive with recurrence	0	1	
Dead	5	1	

*Fisher's exact test or Student's *t*-test, when indicated.
†One patient lost to follow-up.



Gene (HIV NEGATIVE)	DCC				RB			TP53				11q23				APC			d2s123	
	d18s46	*dcc*	*d18s35*	LOH	*d13s270*	*d13s319*	LOH	*tp53*	*d17s513*	*d17s786*	LOH	*d11s4127*	*d11s29*	*d11s925*	LOH	*d5s346*	*d5s421*	LOH	*d2s123*	LOH
1	2.18	1.16	1.3		0	0	N/I	1.4	0	0		0	3.73	3		1.4	0	-	1.4	
2	0	1.4	1.3		1.3	0		1.6	0	0		1.1	1.6	1		0	1	-	0	N/I
3	0	0	0	N/I	1.1	1.3		1.1	0	2.4		0	0	0	N/I	1.1	0	-	0	N/I
4	1.2	0	0		1.6	1.3		3	0	0		0	2.2	0		2.1	1	+	0	N/I
5	1.3	0	1.1		1	0		1	0	1.1		1.4	0	0		1	1.5	+	0	N/I
6	2.53	2.1	1.6		0	4.1		1.3	0	1		0	0	3.9		0	0	N/I	1.1	
7	2.6	2.4	2.5		0	1.3		2.1	0	1.3		1.6	3	1.5		0	3.5	+	0	N/I
8	2	0	1.4		2.1	1.6		3.5	2.24	0		2	1.7	2.1		3.9	0	+	1	
9	0	2.1	1.9		1	0		1.18	0	2		1.6	0	1.3		1	1.7	+	1.5	
10	1.2	1.38	1		1.6	1.1		0	0	1.2		1.1	0	0		1.1	1.7	+	0	N/I
11	0	1.5	1.52		1.4	1.1		1.2	1	2.3		1.1	0	1.2		1.3	1.1	-	0	N/I
12	0	0	1.5		0	1.5		0	0	2.5		0	1.4	1.4		2	2.77	+	0	N/I
13	1.1	1.6	1.1		1	0		2.9	0	1		0	1.2	0		1.5	0	+	1.4	
14	4	1.2	1.7		0	0	N/I	1.6	1.8	0		1	2.4	1.2		0	0	N/I	0	N/I
15	0	0	5.5		0	0	N/I	0	0	0	N/I	1.7	1.4	2.4		0	1.2		0	N/I
16	1.7	0	0		1	0		0	0	0	N/I	1.7	1.12	0		1.4	1.37	-	1.2	
17	0	1	0		0	0	N/I	1.4	0	1.6		1	1.13	1.5		1.2	2.7	+	0	N/I
18	1.8	0	1.2		3.1	5.1		1.3	0	0		1.1	1.2	1.81		1.1	1.1	-	0	N/I
informative loci (n= 145)	11	10	14	**17**	9	9	**14**	14	2	9	**16**	12	12	12	**17**	13	12	**15**	6	**6**
> 2 (n=35)	4	3	2	**6**	2	2	**3**	4	1	3	**7**	0	4	4	**7**	3	3	**5**	0	**0**
>1.5 (n=68)	7	5	7	**12**	4	4	**6**	6	2	5	**11**	4	6	7	**12**	4	6	**9**	1	**1**

Gene (HIV POSITIVE)	DCC				RB			TP53				11q23				APC			d2s123	
	d18s46	*dcc*	*d18s35*	LOH	*d13s270*	*d13s319*	LOH	*tp53*	*d17s513*	*d17s786*	LOH	*d11s4127*	*d11s29*	*d11s925*	LOH	*d5s346*	*d5s421*	LOH	*d2s123*	LOH
19	1.1	1.2	0		0	1.5		1	1.8	1.4		3.5	0	2.4		0	1.1		1.4	
20	0	1.1	1		1.3	1		1	1	1.1		3.5	1.6	0		0	1.2		1.2	
21	1.3	1.13	0		1	1.1		1	0	1.1		1.1	1.2	1.2		0	0	N/I	0	N/I
22	1.15	0	1.1		1	1		1.3	1.3	1.2		0	1.1	1.1		1.2	1.1		0	N/I
23	1	1.1	1		2.4	1.6		1.7	0	1.88		1.3	1.2	1.2		1.7	1.5		1.8	
24	1.85	0	1		0	1.1		1	0	1.8		1	1.8	0		0	1.7		0	N/I
25	1	1	0		0	0	N/I	1.2	0	0		1.4	1.25	0		0	1.1		0	N/I
26	0	0	0	N/I	1.2	0		1	5.6	1		1.5	2.5	1.07		1.1	1.5		0	N/I
27	0	1.2	0		1.6	1.3		1.3	0	1.1		1.3	1.1	1.2		0	0	N/I	0	N/I
28	1.1	0	0		0	1.2		1.1	0	1		1.1	0	0		0	1		0	N/I
informative loci (n=91)	7	6	4	**9**	6	8	**9**	10	4	9	**10**	9	8	6	**9**	3	8	8	3	**3**
>2 (n=6)	0	0	0	**0**	1	0	**1**	0	1	0	**1**	2	1	1	**3**	0	0	0	0	**0**
>1.5 (n=22)	1	0	0	**1**	2	2	**3**	1	2	2	**4**	3	3	1	**4**	1	3	3	1	**1**

Fig. 1. DNA ratios and loss of heterozygosity.

90 instances of allelic imbalance were observed. The differences between groups, however, remained statistically significant for all loci (HIV−; 68 LOH/145 loci [46.9%] versus HIV+; 22 LOH/91 loci [24.1%], $P = 0.0005$). The detailed data of DNA analysis for each marker are summarized in Figures 1 and 2.

When looking at specific TSGs, there were again significant differences between groups for *DCC* (9 instances of LOH of 35 informative loci [25.7%] in the HIV− group versus no instances of LOH of 17 informative loci in the HIV+ group, $P = 0.02$) and for *p53* (8 instances of LOH of 25 informative loci [32%] in the HIV− group versus 1 instance of LOH of 23 informative loci in the HIV+ group, $P = 0.02$). By contrast, detailed DNA analysis for each individual marker failed to reveal any difference at 11q23 (8 instances of LOH of 36 informative loci [22.2%] in the HIV− group versus 4 instances of LOH of 23 informative loci [17.3%] in the HIV+ group, $P = 0.74$).

Loss of Heterozygosity at Specific Loci

Again, LOH was the most consistent between the two groups at 11q23. A total of 10 instances (38.5%) of LOH at that site were identified: 7 (41%) in the HIV− group versus 3 (33%) in the HIV+ group. The differences in allelic imbalance between groups were the most obvious on chromosomes 18q (41% [HIV−] versus 0% [HIV+], $P = 0.05$), 17p (43% versus 10%, $P = 0.09$), and 5p (33% versus 0%, $P = 0.12$). The differential distribution of chromosomal aberrations between groups is detailed in Figure 3.

Using a cutoff value of 1.5, differences between groups for LOH at specific loci remained significant for *DCC* (68.7% [HIV−] versus 30% [HIV+], $P = 0.01$). However, differences between groups were no longer significant at 17p (61% [HIV−] versus 25% [HIV+], $P = 0.10$) and 5q (60% [HIV−] versus 25% [HIV+], $P = 0.19$).

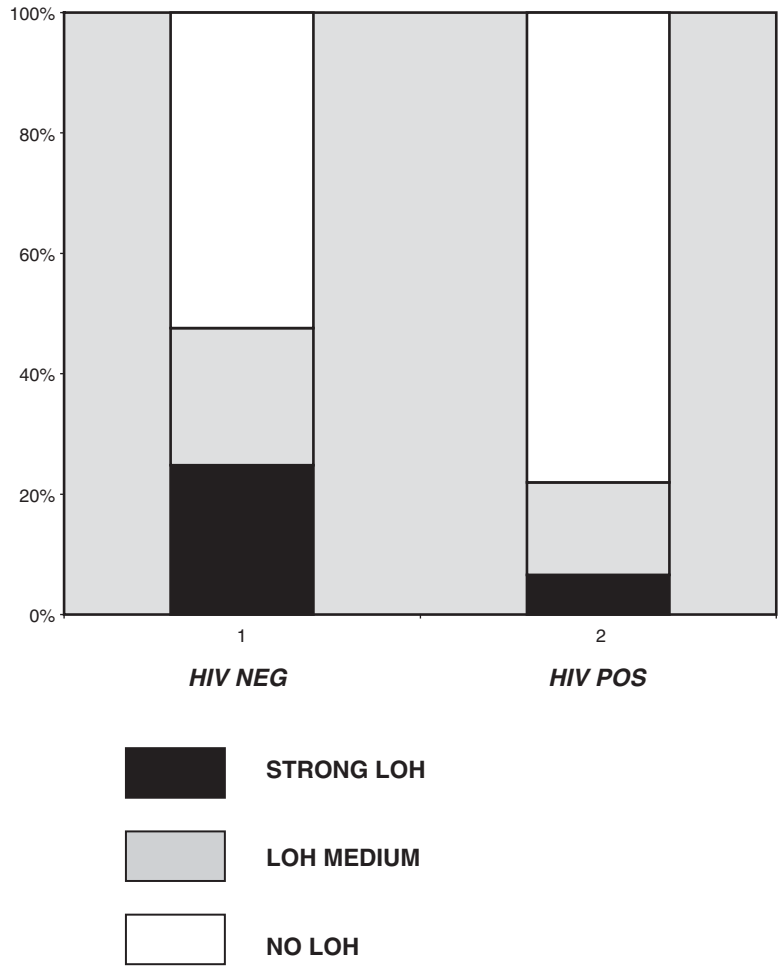


Fig. 2. Percentage of loss of heterozygosity (LOH) for all loci in HIV-negative and HIV-positive patients. *P* value = 0.0004 (strong LOH) and 0.0005 (strong plus medium LOH).

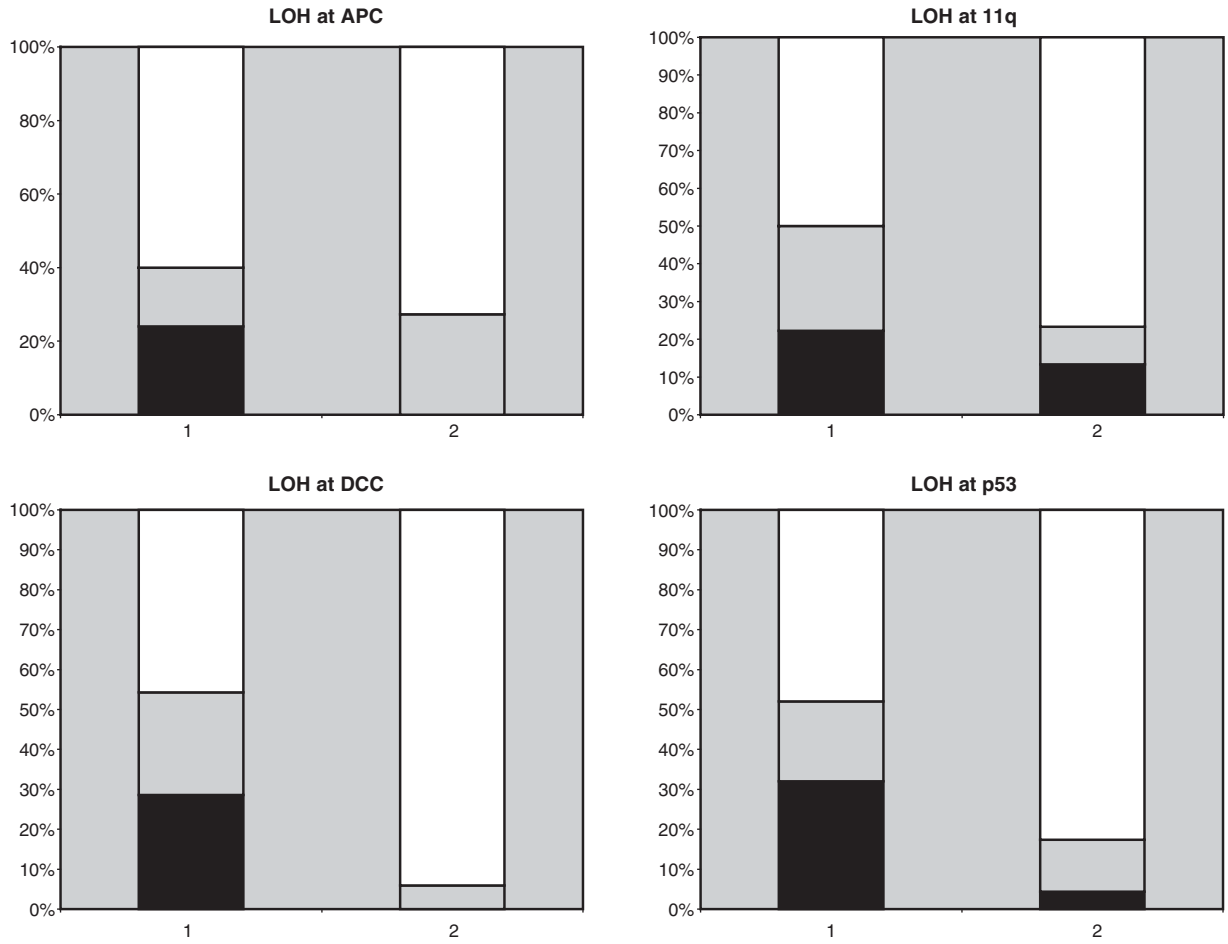


Fig. 3. Comparison of loss of heterozygosity at specific loci between HIV-negative (1) and HIV-positive (2) patients.

DISCUSSION

It is accepted that genetic events, whether induced by or independent of HPV infection, are required for progression of anal cancer. The data presented here indicate that the molecular mechanisms involved in SCCA progression markedly differ between HIV+ and HIV− individuals. Consistent LOH on chromosomes 17p, 18q, 5q, and 11q were observed in HIV− patients with SCCA. By contrast, allelic imbalance in general and, more specifically, LOH at 17p, 5q, and 18q was less likely to occur in tumors of HIV+ individuals. A similar distribution of LOH at 11p was documented in SCCA of HIV+ and HIV− patients.

Although the association between HIV infection and HPV-related anogenital neoplasia has been repeatedly demonstrated through epidemiologic studies, these findings so far have not been substantiated by genetic analysis. In addition, the molecular mechanisms involved in progression of SCCA are poorly elucidated, with relatively few data available.[17,18] In this study, consistent LOH at 11q was documented in 38.5% of SCCA, which is in accordance with a previous series, in which Heselmeyer et al.[19] identified allelic imbalance at 11q in 9 of 23 cases (40%). In fact, the 11q23 region has received much scrutiny, since Hampton et al.[20] demonstrated that 62% of cervical carcinomas had allelic deletions on chromosome 11q. Furthermore, in vitro studies have demonstrated that HPV-mediated immortalization of human keratinocytes requires LOH at 11q and/or 18q.[21] In summary, a TSG on chromosome 11q23 seems to be implicated in carcinogenesis SCCA, and this chromosomal aberration appears to be relatively independent from HIV status.

Our results also indicate that allelic imbalance on chromosomes 17p, 18q, and 5q differ markedly between HIV+ and HIV− patients. In the latter group, LOH (defined by ratio >2) at these loci were documented in 43%, 41%, and 33% of cases, respectively. It is interesting to note that the same TSGs (*p53*, *DCC*, and *APC*, respectively) have been previously implicated in the progression of colorectal cancer, a

neoplasia that is independent of HPV infection.[3,22] Thus, in HIV− patients, the sequence of genetic events seems similar to what is observed in non–HPV-related digestive cancers. An immediate implication is that the necessary accumulation of multiple allelic losses requires a long latency period between the time of HPV infection (peak incidence in the 20s) and the appearance of SCCA in this population (mid-50s).

Our data also indicate that although LOH at 17p and 18q is quite common in immunocompetent individuals, mutations at these loci are rarely observed in HIV-infected patients. First, it is important to note that our patient population was severely immunosuppressed at the time of diagnosis, with $CD4^+$ counts of less than 200×10^6/L in 8 of 10 patients. Previous studies indicate that there is an inverse relationship between $CD4^+$ level and HPV DNA level in the anal canal.[23] It is likely that HIV+ patients in this study had persistent infection with high-risk HPV. HPV-infected cells in the epithelium of the anal canal have the functional equivalent of mutations in *p53* and *Rb*, due to the interaction of their respective proteins with viral oncoproteins E6 and E7.[24] On the basis of our data and the existing literature, we can distinguish three key events in the pathogenesis of SCCA in HIV+ patients: 1) the integration of HPV DNA in the cellular genome; 2) E6-mediated functional inactivation of p53 protein; and 3) LOH on chromosome 11q.

In conclusion, the data presented here demonstrate that *DCC* and *p53* mutations are not required for SCCA progression in HIV+ patients; persistence of HPV infection within the anal canal may play a central role in this process and explain the differences observed with the molecular patterns of these tumors in immunocompetent individuals. In the future, administration of an HPV-16/18 vaccine might help in reducing the incidence of SCCA in HIV-infected patients.[25]

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Discussion

Dr. Robert Beart (Los Angeles, CA): Is it a different mechanism or do you think it is a subset of the other mechanism? Is that mechanism in play in non–HIV-positive patients?

Dr. Gervaz: I think it is the same mechanism, and probably the aim of the mechanism is to inhibit the function of the p53 protein. But this may be achieved either through a mutation of the gene or through an inhibition of the protein, and it is obviously much easier to have an inhibition of the protein than to have a mutation, because it takes much longer.

Dr. Walter Koltun (Hershey, PA): I may have missed it, but did you subtype your HPV infections in each of your patients?

Dr. Gervaz: No, we did no HPV testing in this population. So we have no data regarding which subtypes of HPV were implicated in these tumors.

Lymph Node Metastasis in T1 Adenocarcinoma of the Colon and Rectum

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The biology of colorectal cancer differs according to location within the large intestine. To evaluate the clinical significance of tumor location as a risk factor for lymph node metastasis (LNM), we performed a detailed pathological review of T1 adenocarcinomas of the colon and rectum. T1 adenocarcinomas of the colon and rectum treated by radical resection (n = 428) were identified from prospective clinical databases at two institutions. Tumor location was assigned as right colon (cecum to transverse), left colon (splenic flexure to sigmoid), or rectum (0–18 cm from AV). Pathology slides were reviewed, extent of submucosal invasion (sm width, sm depth) was quantified using an optical micrometer, and morphologic features of the cancer and its infiltrating margin were recorded. The overall rate of LNM was 10%. On univariate analysis, LNM was significantly more common in the rectum (27/176, 15%) compared to the left colon (13/160, 8%, p = .04) or right colon (3/92, 3%, p = .003). However, on multivariate analysis, deep submucosal invasion and lymphovascular invasion were independent and significant risk factors, whereas tumor location was not. T1 colorectal cancers have a progressively higher risk of LNM as their location becomes more distal. However, the increasing rate of LNM observed in cancers of the left colon and rectum is explained by a higher prevalence of high-risk pathologic features. In early colorectal cancers, tumor morphology is the strongest clinical predictor of metastatic behavior. (J GASTROINTEST SURG 2004;8:1032–1040)

KEY WORDS: Colorectal cancer, lymph node metastasis, local therapy, pathology

The term "early colorectal cancer" is used to describe primary adenocarcinomas of the colon and rectum in which the invasive component is limited to the submucosa. These superficial cancers are defined by the TNM system as stage T1 NX M0 and include cancers that are both lymph node negative and lymph node positive. T1 cancers are heterogeneous in their clinical presentation and can appear as malignant polyps, villous tumors, polypoid cancers, and sessile cancers. Similar in appearance but excluded from the definition of early colorectal cancer are the precancerous lesions that do not truly invade the submucosa, the "intramucosal carcinomas" and "in-situ carcinomas." The prevalence of T1 cancers can be estimated from published series in which they represent up to 12% of colorectal tumors removed via endoscopic polypectomy and up to 10% of cancers removed with surgery.[1–6]

Local therapy for early colorectal cancer has gained popularity over the past two decades. Through the avoidance of bowel resection, local therapy substantially lowers the cost and morbidity of cancer treatment. However, successful use of local therapy relies on the ability to select cases with a very low risk of lymph node metastasis (LNM). For pedunculated malignant polyps removed via endoscopic polypectomy, the risk of LNM is low and the cure rate is high, provided no high-risk pathologic features are identified.[5–7] However, the use of local excision for T1 rectal cancers is more controversial. Early published series reported excellent results, but recently several large retrospective series have reported high local failure rates (11%–18%) even for T1 rectal cancers with no adverse pathology.[8–10] Of particular concern is the report by Nascimbeni and colleagues[11]

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identifying a sixfold increase in risk of LNM for T1 colorectal cancers located in the lower rectum. These data imply that local excision of early cancers located in the rectum may be unsafe even in the absence of other high-risk pathologic features.

To evaluate the importance of tumor location as an independent risk factor for LNM in early colorectal cancer, we retrospectively reviewed a large series of T1 adenocarcinomas treated by bowel resection. Pathology slides were reviewed to identify pathologic features that may be predictive of LNM. Cases from two institutions were combined to provide adequate statistical power for multivariate analysis.

METHODS

Case Selection

T1 adenocarcinomas of the colon and rectum treated by bowel resection were identified at two institutions from prospective clinical databases. At Memorial Sloan-Kettering Cancer Center, a database maintained by the colorectal surgery service identified 313 cases of primary T1 adenocarcinoma treated by surgical resection as initial therapy between 1987 and 2001. Original pathology reports and, when required, discharge summaries and operative reports were reviewed to confirm patient age, gender, and diagnosis; surgical resection; tumor size and location within the large intestine; TNM stage; and absence of preoperative chemotherapy or radiotherapy. For 78 cases, pathology slides were not available, leaving a study group of 235 cases from New York. At Tokyo Medical and Dental University, a database of 193 T1 adenocarcinomas of the colon and rectum treated by surgical resection between 1990 and 2001 was maintained by the authors from that institution (S.O., K.S.). Data including patient age and gender, diagnosis, tumor size and location, type of resection, and TNM stage were available. Location of carcinoma within the colon was defined as right colon (proximal to the splenic flexure), left colon (descending colon and sigmoid colon), and rectum (0–18 cm above the anal verge).

The cases from the two institutions were stripped of all patient identifiers and combined to make an anonymous study group of 428 cases. The study was approved by the institutional review board of Memorial Sloan-Kettering Cancer Center to ensure the safety and privacy of all patients.

Pathology Slide Review

All pathology slides (hematoxylin and eosin stained) were reviewed and scored by one surgical pathologist (S.O.). All 428 cases were sessile carcinomas. The gross configuration of the tumor was protruded in 291 cases, depressed in 41 cases, and indeterminate in 96 cases. For all 428 carcinomas, slides were assessable for the following morphologic features: grade (well, moderate, or poor differentiation), lymphovascular invasion (LVI) (present or absent), and single cell infiltration (present or absent). Other microscopic features assessed included growth pattern (expansive versus invasive edge), high-grade component (high-grade cancer cells present or absent from the invasive edge), mucinous component (mucinous cancer cells present or absent from the invasive edge), desmoplastic change (slight versus extensive desmoplasia in the submucosa adjacent to the invasive edge), cellular stromal reaction (presence versus absence of mononuclear cell infiltration at the submucosal edge), and abscess formation (presence versus absence of neutrophil infiltration at the submucosal edge).

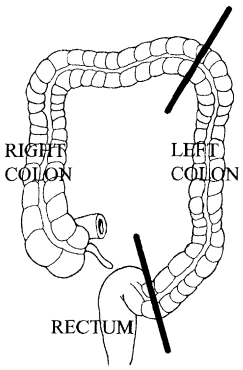
Tumor size was the maximal tumor diameter reported on gross assessment of the tumor on the original pathology report. The extent of submucosal invasion was measured directly from the pathology slides using an optical micrometer. The depth of submucosal invasion (SM depth) was measured in 400 carcinomas as a vertical distance in millimeters from the muscularis mucosa to the deepest point of tumor invasion into the submucosa. The width of submucosal invasion (SM width) was measured as the greatest transverse diameter of the submucosal component of the cancer. Because adequate transverse sections were not available for all cases, SM width could be assessed for only 304 of the 428 carcinomas.

Statistical Analysis

All data were evaluated as categorical variables. The effect of each clinical and pathologic variable on the rate of LNM was evaluated univariately using the χ^2 test. For each variable, only evaluable cases were considered in the χ^2 test. Variables found to be significant ($P < 0.05$) in the univariate analysis were evaluated in a multivariate analysis using the Cox proportional hazards method. The statistical analyses were performed using SPSS Version 10 software.

RESULTS

Among the 428 evaluable patients with T1 colorectal cancer, there were 170 women and 258 men. The median age at the time of bowel resection was 64 years (range, 26–89 years). The median number of lymph nodes removed in each surgical specimen was 10 (range, 1–41 lymph nodes). In 385 cases, all lymph nodes were benign. A total of 43 patients (10%)



Location	TOKYO	NEW YORK	All Cases
Right Colon	1 /35 2.9%	2 /57 3.5%	3/92 3.0%*
Left Colon	3 /85 3.8%	10 /75 13%	13/160 8.0%**
Rectum	13/73 19%	14/103 14%	27/176 15%
Total	17/193 8.8%	26/235 11%	43/428 10%

* P = .003 right colon versus rectum
** P = .04 left colon versus rectum

Fig. 1. Distribution of T1 adenocarcinomas of the colon and rectum evaluated at Tokyo Dental and Medical University in Tokyo and at Memorial Sloan-Kettering Cancer Center in New York. The proportion of carcinomas with lymph node metastasis is indicated for each region of the colon.

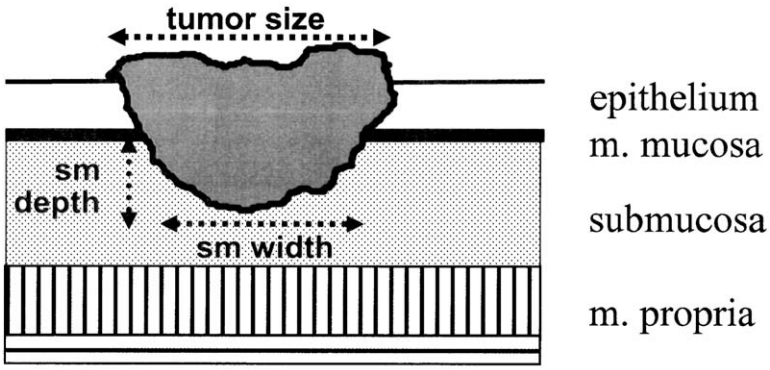
had at least one LNM, and in these cases the number of malignant lymph nodes found ranged from one to five. The number of LNMs per specimen varied as follows: one LNM for 26 patients, two LNMs for 10 patients, three LNMs for 3 patients, four LNMs for 3 patients, and five LNMs for 1 patient.

Location of Carcinoma

The distribution of colorectal carcinomas within the large intestine and the proportion of lymph node–positive carcinomas within each region of the colon are shown in Fig. 1. LNMs were most common in the rectum (15%) at a rate that was statistically higher than that for the left colon (8.0%, $P = 0.04$) and the right colon (3.0%, $P = 0.003$). The cases from Tokyo and from New York showed a similar trend of increasing LNM rate from right colon to left colon and to rectum.

Tumor Size and Submucosal Invasion

The median values and range for tumor size, SM depth, and SM width for T1 colorectal cancers are shown in Fig. 2. The median tumor size was 2.0 cm.



	median	range
tumor size (cm)	2.0	0.3 - 15
sm width (mm)	7.3	0.14 – 23.5
sm depth (mm)	3.0	.02 – 13.3

Fig. 2. Measurement of tumor size, width of submucosal invasion (sm width), and depth of submucosal invasion (sm depth).

For 90% of the carcinomas, the largest gross diameter was 4 cm or less. However, some carcinomas had a large adenomatous component and measured as large as 15 cm. The SM width covered a wide range, from 0.02 to 23.5 mm, and was statistically linked to tumor size by linear regression ($P = 0.02$). The SM depth had a smaller range, from 0.2 to 13.3 mm, and showed a less strong but statistically significant correlation to tumor size ($P = 0.05$). For tumors greater than 4 cm, the average SM width was 9.5 mm and the average SM depth was 4.1 mm.

On univariate analysis, tumor size was not a significant predictor of LNM. Smaller cancers (≤2.0 cm, n = 236) and larger cancers (>2.0 cm, n = 212) had a similar rate of LNM (9% versus 11%, respectively; $P = 0.3$). In addition, among the cancers where the gross configuration could be determined, the protruded carcinomas (n = 291) and the flat or depressed carcinomas (n = 41) had similar rates of LNM (13% versus 9%, respectively; $P = 0.6$).

For the 304 carcinomas for which SM width could be measured, the relationship of this statistic to LNM is shown in Table 1. Of the nine breakpoints used to stratify the tumors, all showed a slightly higher rate of LNM for tumors with greater SM width. However, none of these differences were statistically significant. Because it showed a trend toward significance, the 5-mm breakpoint was selected for inclusion in the multivariate model.

In contrast to SM width, SM depth was found to be a strong predictor of LNM. Of the seven breakpoints examined for SM depth, four values (1.0, 2.0, 2.5, and 3.0 mm) each defined two groups of cancers with superficial versus deep submucosal invasion that had significantly different rates of LNM (Table 2). The 3.0 mm breakpoint showed the strongest significance ($P = 0.018$) and was selected for multivariate analysis.

Table 1. Analysis of submucosal width as a predictor of lymph node metastasis in 304 T1 adenocarcinomas

Submucosal width	No. of cases	Cases with lymph node metastasis (%)	*P* value
2 mm			
<	24	0	
>	280	37 (13)	0.055
3 mm			
<	44	2 (5)	
>	260	35 (13)	0.13
4 mm			
<	67	6 (9)	
>	237	31 (13)	0.36
5 mm			
<	85	6 (7)	
>	219	31 (14)	0.09
6 mm			
<	118	12 (10)	
>	186	25 (13)	0.4
7 mm			
<	143	15 (10)	
>	161	22 (14)	0.4
8 mm			
<	170	16 (19)	
>	134	21 (16)	0.10
9 mm			
<	197	20 (10)	
>	107	17 (16)	0.14
10 mm			
<	216	23 (11)	
>	88	14 (16)	0.2

Histopathologic Features

Three morphologic features were scored in all 428 cases. Grade was assessed as well differentiated, moderately differentiated, or poorly differentiated based on the proportion of the cancer exhibiting diffuse, nonglandular growth versus gland-forming cancer.[12] LVI was scored as present whenever cancer cells were identified within an epithelium-lined channel thought to represent either a lymphatic vessel or a blood vessel. In this study, the generic term "lymphovascular invasion" was used because it was often not possible to distinguish reliably between lymphatic and

Table 2. Analysis of submucosal depth as a predictor of lymph node metastasis in 304 T1 adenocarcinomas

Submucosal depth	No. of cases	Cases with lymph node metastasis (%)	*P* value
1.0 mm			
<	28	0	
>	372	41 (11)	0.044
1.5 mm			
<	59	2 (3.4)	
>	341	39 (11)	0.060
2.0 mm			
<	96	4 (4)	
>	304	37 (12)	0.024
2.5 mm			
<	143	9 (6.3)	
>	257	32 (12)	0.044
3.0 mm			
<	196	13 (6.6)	
>	204	28 (14)	0.018
3.5 mm			
<	241	22 (9.1)	
>	159	19 (12)	0.36
4.0 mm			
<	272	27 (10)	
>	128	14 (11)	0.76

vascular invasion. Single cell infiltration was scored as present when individual cancer cells free of gland formation were identified anywhere along the invasive edge of the carcinoma.

Six morphologic descriptors of the deep, invasive edge of the carcinoma were scored in only a portion of cases based on the adequacy of the available slides: growth pattern (expansive versus invasive edge, n = 230), high-grade component (high-grade cancer cells present or absent from the invasive edge, n = 235), mucinous component (mucinous cancer cells present or absent from the invasive edge, n = 285), desmoplastic change (slight versus extensive desmoplasia in the submucosa adjacent to the invasive edge, n = 233), cellular stromal reaction (presence versus absence of mononuclear cell infiltration at the submucosal edge, n = 252), and abscess formation (presence versus absence of neutrophil infiltration at the submucosal edge, n = 233). Photomicrographs illustrating selected pathologic features are show in Fig. 3.

The relationship of histologic features to LNM in univariate analysis is shown in Table 3. Tumor grade showed a strong correlation. Well-differentiated carcinomas had a low rate of LNM (2%) compared with all other tumors, and poorly differentiated carcinomas had a high rate (42%) compared with all other tumors. LVI was identified in 135 (32%) of the T1 adenocarcinomas and was a strong predictor of LNM. Single cell infiltration at the invasive edge (38% of evaluable cases) and high-grade component at the invasive edge (61% of evaluable cases) were common histologic features that were positively correlated with LNM. Among the remaining histologic features, two were negative predictors of LNM on univariate analysis: expansive growth pattern (0% LNM rate in 21 cases) and abscess formation (2.6% LNM rate in 38 cases). Each of these features were included in the multivariate analysis. Mucinous component, desmoplastic change, and cellular stromal reaction had no correlation with LNM and were not analyzed further.

Multivariate Model of Lymph Node Metastasis

Tumor location, SM depth, and four histologic features (grade, LVI, high-grade component, and single cell infiltration) were assessed as predictors of LNM in a multivariate analysis using the Cox proportional hazards model (Table 4). The only statistics that remained significant predictors on multivariate analysis were SM depth (odds ratio 2.7, $P < 0.05$) and presence of LVI (odds ratio 4.4, $P < 0.003$). Tumor location in the rectum was not significantly correlated with LNM on multivariate analysis and was therefore found not to have independent predictive value. The addition of SM width as an additional variable in the multivariate model did not alter the findings (data not shown).

DISCUSSION

Early colorectal cancers are of considerable interest to scientists, pathologists, and surgeons.[13] Because of their relatively small size compared with more advanced cancers, it is easier to assess their morphologic features and to quantify their extent of invasion. As documented in this study, these early cancers are heterogeneous in their size, degree of submucosal invasion, and histologic appearance. Because they frequently contain preinvasive as well as invasive components and because they may also contain one or more LNMs in the resected specimen, they can provide a biological record of the transition from polyp to primary cancer to metastasis. From a clinical perspective, understanding the risk of LNM is of critical importance as this knowledge is required for successful use of local therapies as definitive treatment.

There have been multiple published reports addressing the question of predictors of LNM in colorectal cancer. Cancer arising in pedunculated polyps has been well studied.[1–3,7] The risk of LNM is low (<1%) provided (1) the cancer is confined to the head, neck, or stalk of the polyp and (2) no high-grade cancer or LVI is identified. However, the rate of LNM is less predictable in sessile lesions in which the cancer invades the submucosal layer of the large intestine. The overall risk of LNM for sessile T1 cancers is approximately 8%–15%.[11,14–18] However, defining the independent predictors of LNM in this setting is difficult because of the small sample size and absence of multivariate analysis in most studies. Of particular concern are recent reports of high local recurrence rates (11%–18%) following transanal excision of T1 adenocarcinomas of the rectum with no adverse histologic features.[8–10]

Fig. 3. Pathologic findings in selected T1 adenocarcinomas of the colon and rectum. **(A)** Poorly differentiated adenocarcinoma with loss of glandular architecture. *Arrowhead*, signet ring cell. **(B)** Lymphovascular invasion. *Arrowhead*, cluster of tumor cells within an endothelium lined vascular channel. **(C)** Single cell infiltration at the invasive edge. *Arrowhead*, individual tumor cells invading submucosa in linear pattern. **(D)** Expansive growth pattern at tumor submucosal edge. **(E)** Mucinous component at tumor submucosal edge. *Arrowhead*, extracellular mucin accumulation. **(F)** Desmoplastic change at tumor submucosal edge. *Arrowhead*, eosinophilic collagen fibers at tumor invasive edge.

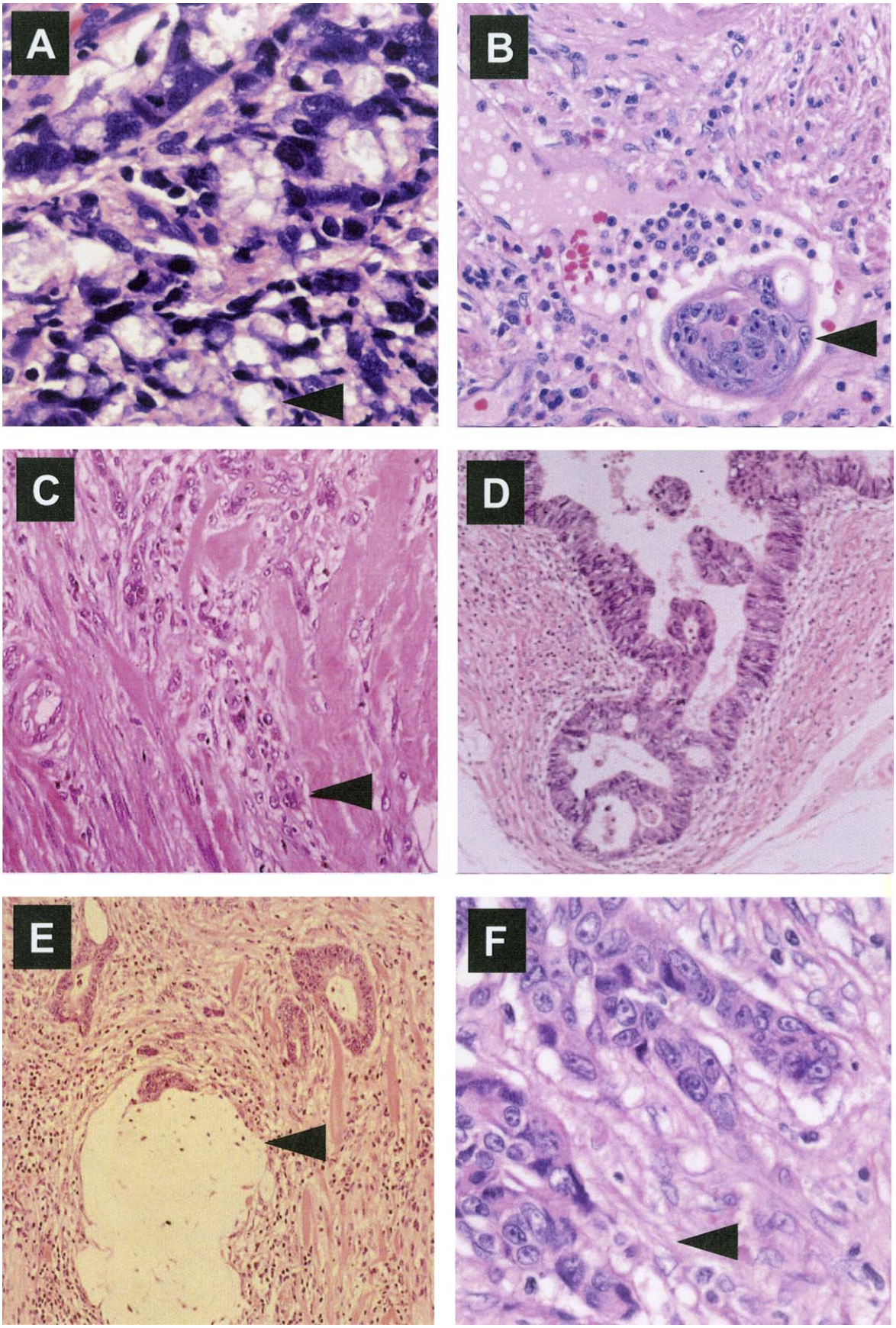

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Table 3. Evaluation of histopathologic features as predictors of lymph node metastasis in 428 T1 adenocarcinomas

Histopathologic feature	No. of cases	Cases with lymph node metastasis (%)	*P* value
Grade			
Well differentiated	86	2 (2)	<0.001
Moderately differentiated	330	36 (11)	
Poorly differentiated	12	5 (42)	<0.007
Lymphovascular invasion			
Present	135	28 (21)	
Absent	293	15 (5.1)	<0.0001
Single cell infiltration			
Present	233	38 (16)	
Absent	195	5 (2.6)	<0.0001
Growth pattern			
Expansive	21	0 (0)	
Infiltrative	209	26 (12)	<0.0001
High-grade component			
Present	143	21 (15)	
Absent	92	5 (5.4)	0.027
Mucinous component			
Present	224	29 (13)	
Absent	61	8 (13)	0.97
Desmoplastic change			
Slight	36	4 (11)	
Extensive	197	22 (11)	0.99
Cellular stromal reaction			
Present	57	4 (7)	
Absent	195	25 (13)	0.25
Abscess formation			
Present	38	1 (2.6)	
Absent	195	25 (13)	0.068

With the addition of our study, there are now six published studies that evaluate tumor location in the colon and rectum as a predictor of LNM in T1 adenocarcinomas (Table 5). All six studies report a higher rate of LNM in the rectum, and in five of six studies the LNM rate in the rectum is 14% or higher. Therefore, it is well established that the rate of LNM of T1 rectal cancers is high. In addition, LVI and SM depth are well-established risk factors.[11,16,17] What is not clear is how strong rectal location is as a risk factor when it is assessed in a multivariate model that includes other pathologic features.[11,16]

Our data reveal that T1 colorectal cancers have a progressively higher risk of LNMs as their location in the large intestine becomes more distal. However, when histopathologic features are assessed in combination with tumor location, the data show that the LVI (relative risk, 4.4×) and depth of submucosal invasion (relative risk, 2.7×) are the two most important predictors of LNM in T1 cancers. In contrast, rectal location is not an independent predictor. These findings are supported by other correlative studies in the literature. The data also fit with genetic studies that document the biology of colorectal cancer varies by location, with a high rate of microsatellite stability, aneuploidy, chromosomal deletions, and *p53* mutations in rectal and sigmoid cancers but not in more proximal cancers.[19,20] Our data strongly suggest that the increased risk of LNM seen in T1 rectal cancers is due to their intrinsic biology rather than to their location. This leaves hope that careful morphologic analysis can identify high-risk and low-risk cancers and can significantly improve patient selection for transanal excision of T1 rectal cancers.

As mass screening for colorectal cancer becomes more widely used, the prevalence of early colorectal cancer will likely increase. The biology and metastatic potential of these cancers vary. At present, careful histopathologic assessment of the entire excised lesion is the best guide to risk of regional LNM. This knowledge remains essential for the successful use of

Table 4. Multivariate analysis of risk of lymph node metastasis in T1 adenocarcinomas of the colon and rectum

		Univariate			Multivariate		
	No. of cases	Odds ratio	95% CI	*P* value	Odds ratio	95% CI	*P* value
Location (rectum versus colon)	176 versus 252	2.7	1.4–5.1	0.003	0.96	0.39–2.4	0.93
Submucosal depth (>3 mm versus <3 mm)	204 versus 196	2.2	1.1–4.5	0.022	2.7	1–7.5	<0.05
Grade (not well versus well)	342 versus 86	5.7	1.4–24	0.018	5.4	0.54–54	0.15
High-grade component (present versus absent)	143 versus 92	3	1.1–8.2	0.036	1	0.27–4	0.95
Lymphovascular invasion (present versus absent)	154 versus 274	4.3	2.2–8.4	<0.0001	4.4	1.7–11	<0.003
Single cell infiltration (present versus absent)	233 versus 194	7.3	2.8–19	<0.0001	1.2	0.33–4.5	0.77

CI = confidence interval.

Table 5. Recent publications evaluating the percentage of T1 adenocarcinomas that have lymph node metastasis according to location in the colon or rectum

Study	N	Colon (%)	Rectum (%)	*P* value
Kikuchi et al., 1995[14]	182	3.8	14	0.04
Oh-e et al., 2001[15]	254	12	17	NS
Okuyama et al., 2002[16]	101	7	22	0.02
Nascimbeni et al., 2002[11]	353	13	16	NS
Sakurgai et al., 2003[17]	278	7.3	8.3	NS
Current study	428	6.3	15	0.01

endoscopic ablation, transanal excision, and other local therapies for T1 adenocarcinomas of the colon and rectum.

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Discussion

Dr. Sukamal Saha (Flint, MI): Very elegant study. I have two questions for you. Can you tell us the average number of lymph nodes you found in the rectum, especially the low rectum, compared to the right-sided colon? And did you do APR in those patients? How were lower-third rectal lesions treated? What operation did you do for the lower-third rectal T1 lesions?

Dr. Paty: Regarding the average number of lymph nodes resected, I don't have the actual number for patients in this series of T1 cancers. I have previously reviewed this at our institution, and our average lymph node yield for colon cancer is about 15 lymph nodes. I've never reviewed the data for rectal cancer.

Low rectal cancer in healthy patients is managed at our institution primarily by radical surgery. We

do preoperative ultrasound staging. If the tumor is T1, low and accessible, we will offer local excision. But only if the final pathology shows no adverse features would we not recommend a subsequent resection.

Dr. James Becker (Boston, MA): I would like to follow up on the previous question. I wanted to know how many of your patients were treated with local excision, because that could skew the analysis of the rest of the T1 patients? Also, perhaps you could comment on how these findings correlated with either endoscopic or transrectal ultrasound.

Dr. Paty: I did not collect ultrasound data for these cases. I agree with you that there is a huge selection bias, and in fact not only in the rectal cases but also in the colon cases. How aggressive the endoscopists are in removing small T1 lesions is going to affect the denominator, i.e., the number of very early T1 cases that ultimately get resected. So I think all these studies are very much denominator studies. In other words, patient selection will affect the results. That is not such a problem with the overall data, but can be a big problem with subset analyses, such as evaluating the distal third rectal lesions.

Preoperative Serum Albumin Level Is a Prognostic Indicator for Adenocarcinoma of the Gastric Cardia

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Among patients with adenocarcinoma of the gastric cardia, we noted that patients with higher preoperative serum albumin levels appeared to survive longer than patients with lower levels. Thus, we evaluated serum albumin as a prognostic factor for patient survival. From 1987 to 1997, 314 patients with adenocarcinoma of the gastric cardia underwent curative resection. Patient serum albumin levels were evaluated on the second day after admission, before any nutritional support. Patients were divided into two groups: those with normal serum albumin levels (>3.5 g/dl) and those with abnormal serum albumin levels. The perioperative mortality and morbidity were 5.7% (18 of 314) and 22.3% (70 of 314), respectively. The surgical resectability rate was significantly better among patients with normal serum albumin levels ($P < 0.001$). The 5-year overall survival rate of patients with normal serum albumin levels was also better than those with abnormally low serum albumin levels (38.4% versus 19.1%, $P = 0.0003$). In each cancer stage, the 5-year survival rate of patients with normal serum albumin levels was better than that among those with hypoalbuminemia. By multivariate analysis, serum albumin level and the pathologic T, N statuses were independent factors correlated with prognosis. Preoperative serum albumin level correlated highly with resectability and survival. Patients with abnormal serum albumin levels had worse survival than did those with normal serum albumin levels. We recommend that postoperative adjuvant therapy be given to all patients with hypoalbuminemia preoperatively. (J GASTROINTEST SURG 2004;8:1041–1048)

KEY WORDS: Serum albumin, adenocarcinoma, gastric cardia

Recent epidemiologic studies clearly indicate that the prevalence of adenocarcinoma of the gastric cardia is rising in Western countries.[1–3] The long-term survival rate remains poor, probably due to late stage at presentation, high incidence of lymph node involvement, and esophageal invasion.[4,5] Beside the pathologic classification (pTNM) system, there are no other better factors to predict the prognosis, based on a recent literature review.[4,6,7] Because the location of the tumor is just between the esophagus and the stomach, the management of this type of cancer is a controversial subject.

Serum albumin is the most abundant of the liver secretory proteins and has a pool size of 5000 mg/kg of body weight.[8] The serum albumin level is still the traditional, standard factor by which to assess a patient's nutritional status. Decreasing serum albumin levels may be due to malnourishment (poor intake or malabsorption), overconsumption, or bleeding. Cancer of the gastric cardia often causes lumen obstruction, dysphagia, and bleeding, which can lead to loss of serum albumin. On the other hand, low serum albumin levels are a good predictive candidate of increased risk of mortality and morbidity among hospitalized patients.[9–12] Because there was insufficient data concerning the relationship of serum albumin level and outcome of adenocarcinoma of the gastric cardia in the current English literature, the aim of this research was to study whether serum albumin

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Table 1. Characteristics in different serum albumin (SA) patients

	Normal SA patients (n = 328)	Abnormal SA patients (n = 239)	*P* value
Age (yr)	67.1	70.4	<0.001
Gender (M/F)	311:17	229:10	0.691
Diet at presentation (n)			<0.001
None	3 (1.0%)	18 (7.9%)	
Liquid	40 (13.2%)	62 (27.1%)	
Soft	157 (51.8%)	105 (43.9%)	
Normal	103 (34.0%)	44 (19.1%)	
Resectability	231 (70.4%)	83 (34.7%)	<0.001

levels could be used to predict the outcome of adenocarcinoma of the gastric cardia.

MATERIAL AND METHODS

From 1987 to 1997, a total of 567 patients with adenocarcinoma of the gastric cardia were treated in the Department of Surgery at Taipei-Veterans General Hospital. All patients had pathologically confirmed as adenocarcinoma of the gastric cardia. Until 2003, all living patients were monitored for at least 5 years.

The preoperative work-up included routine blood tests, biochemistry, chest radiography, computed tomography, upper gastrointestinal barium studies, upper gastrointestinal panendoscopy with biopsy, and radioisotopic scanning of whole body bone. The preoperative serum albumin level was measured in blood sampled on the morning of the second day after admission, with at least 8 previous hours of gastric emptying. No patient received intravenous nutrition, including total parental nutrition, partial parental nutrition, or exogenous albumin before blood sampling. Patients were divided into two groups based on preoperative serum albumin levels: normal (>3.5 g/dl) and abnormal (≤3.5 g/dl).

The degree of dysphagia was recorded as the diet tolerance of patients when they were admitted to the ward. Dysphagia was classified into four dietetic categories: none (patient cannot tolerate any food), liquid (patients tolerated a liquid diet), soft (patient tolerated a soft diet), and normal diet. The degree of dysphagia was evaluated relative to serum albumin level.

The surgical procedures were recorded as total gastrectomy or proximal subtotal gastrectomy with radical lymph node dissection. During surgery, frozen

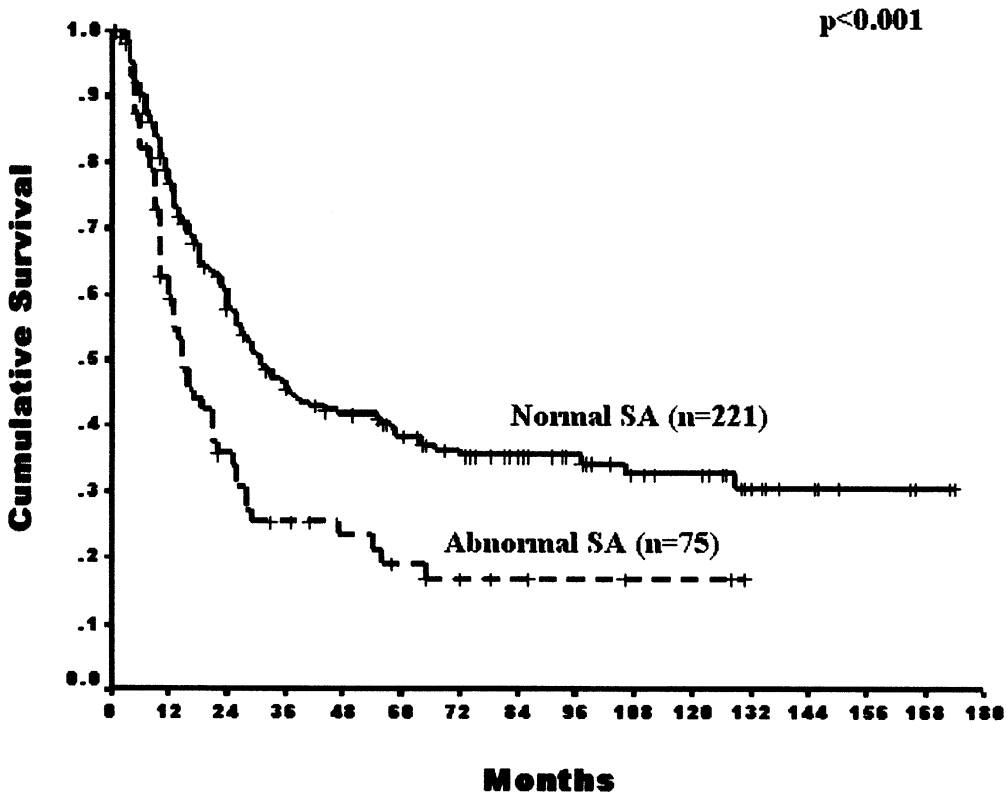

Fig. 1. Overall survival curves in different serum albumin (SA) groups.

sections of the proximal and distal margins of the resected tumor were examined by a pathologist to ensure there was no residual tumor on the margins. *Curative resection* was defined as removal of all gross disease and negative pathologic findings on the tumor margins of standard tissue sections. The surgical specimens were also examined and staged according to the pathologic classification (pTNM) of the International Union Against Cancer and the American Committee on Cancer for gastric cancer.[13,14]

All patients were routinely monitored every 3–6 months in the outpatient department. Tumor recurrence and metastasis were identified when chest radiography, abdominal sonography, whole-body bone scan, upper gastrointestinal panendoscopy, and computed tomography of the abdomen showed any evidence of the disease.

Statistical Analysis

Relationships between the level of preoperative serum albumin and clinicopathologic parameters were analyzed using the χ^2 test for qualitative parameters or Student's *t* test for quantitative parameters, respectively. Survival curves were plotted with using the Kaplan-Meier method and compared using the log-rank test. Cox regression analysis was used to test the significant parameters for their value as prognostic indicators. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) for Windows. Statistical significance was set at $P < 0.05$.

RESULTS

Of the 567 patients with adenocarcinoma of the gastric cardia, the mean age was 68.5 years (age range, 31–93 years). The ratio of males to females was 20:1 (540/27). According to the level of serum albumin, there were 328 (57.8%) patients with normal albumin level (serum albumin >3.5), and 239 (42.2%) patients showed hypoalbuminemia (serum albumin ⩽3.5). The clinical characteristics of the two groups are listed in Table 1. There were significant differences in age, dysphagia at presentation, and resectability. The patients with hypoalbuminemia had a poor tumor resectability compared with the patients with normal serum albumin levels ($P < 0.001$).

Of the 567 patients admitted for adenocarcinoma of the gastric cardia, 314 (55.4%) patients underwent curative surgery, including 268 total gastrectomies and 46 proximal subtotal gastrectomies. The perioperative mortality and morbidity rates were 5.7% (18 of 314) and 22.3% (70 of 314), respectively. Both of these rates were relatively lower among patients with normal serum albumin levels than among those with low serum albumin levels (4.3% versus 9.6% for mortality, $P = 0.075$; 20.3% versus 27.7% for morbidity, $P = 0.168$). The other 253 (44.6%) patients did not undergo curative surgery and had only a 5.6-month mean survival. This was significantly worse than that for patients undergoing curative surgery (34.7 months versus 5.6 months, $P < 0.001$).

Excluding perioperative mortalities (18 of 314), the 5-year survival rate for the 296 patients undergoing curative resection was 33.7%. When stratified by preoperative serum albumin level (normal or decreased) (Fig. 1), the 5-year survival of patients with normal serum albumin levels was significantly better than that of patients with lower serum albumin levels (38.4% versus 19.1%, $P < 0.001$).

Using univariate analysis, we examined gender, age, dysphagia, extent of resection, pathologic T and N status, and serum albumin levels (Table 2). In the survival analysis, gender and age showed no significant difference. Dysphagia at presentation and preoperative albumin level significantly influenced survival.

Table 2. Survival in patients with adenocarcinoma of the gastric cardia after curative resection

Prognostic factor	n	5-Year survival rate (%)	*P* value
Age (yr)			0.137
⩾ 65	209	28.8	
< 65	87	44.4	
Sex			0.877
Male	285	33.5	
Female	11	36.3	
Extent of resection (n)			0.564
Total gastrectomy	251	34.3	
Proximal subtotal gastrectomy	45	30.3	
Diet at presentation (n)			<0.001
None	5	0	
Liquid diet	24	4.9	
Soft diet	142	33.3	
Normal diet	104	45.0	
Serum albumin (n)			<0.001
Normal	221	38.4	
Abnormal	75	19.1	
Depth of penetration (T) (n)			<0.001
pT1	22	89.7	
pT2	31	78.3	
pT3	196	23.6	
pT4	47	14.2	
Nodal involvement (N) (n)			<0.001
pNØ	96	63.4	
pN+	200	19.0	

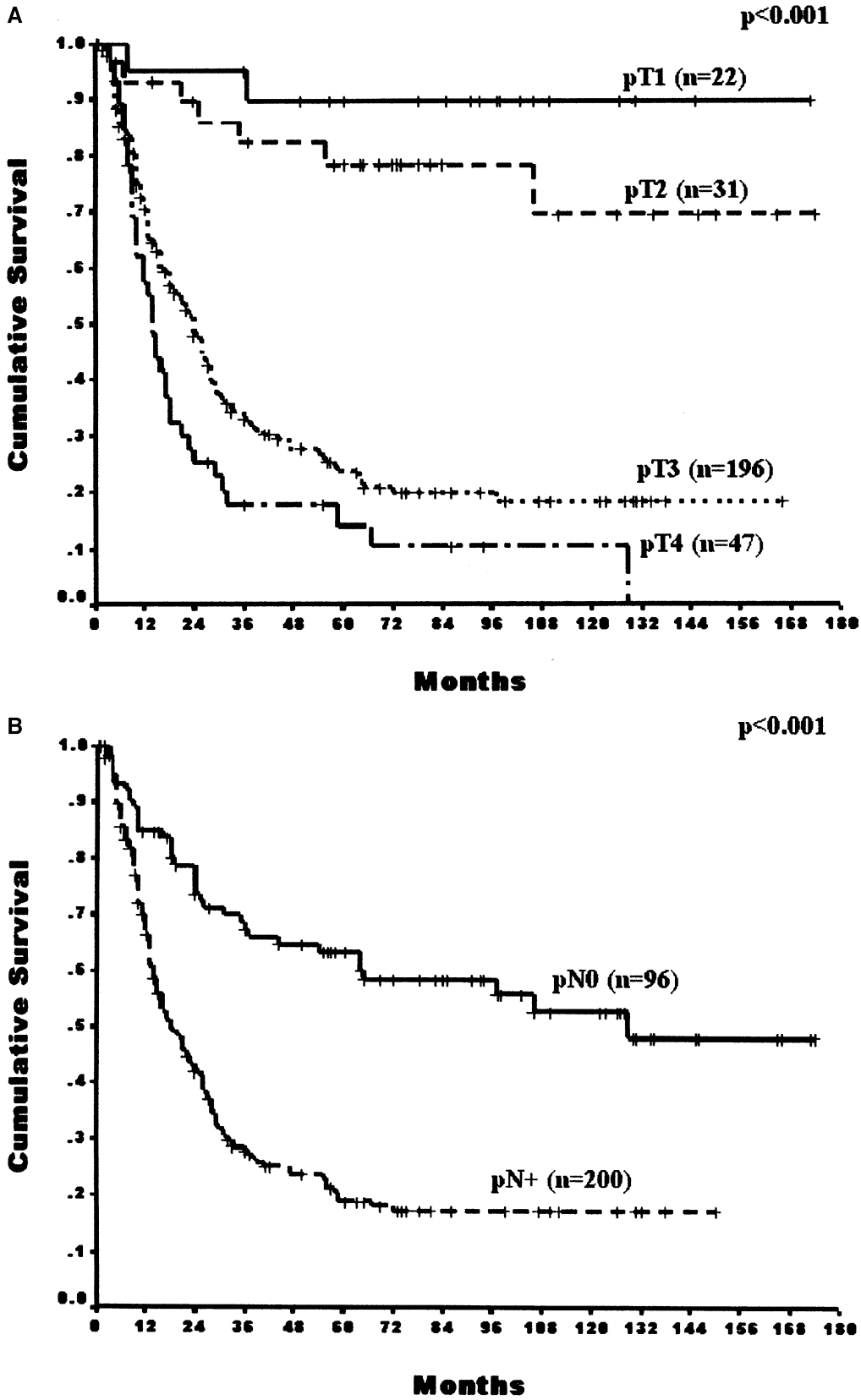

Fig. 2. (**A**) Survival curves for postoperative patients with different T stages. (**B**) Survival curves for postoperative patients with different N stages. pNØ = patients without lymph node involvement; pN+ = patients with lymph node involvement.

For extent of resection, different operative methods did not influence the survival ($P = 0.564$). Pathologically, both depth of penetration (T) and nodal involvement (N) influenced survival (Fig. 2, *A* and *B*). In the multivariate analysis, diet at presentation, preoperative serum albumin level, and the pathologic T and N status were independent factors predicting prognosis.

In our study, the distribution of patients in different pT and pN stages was uneven. Only 22 (7.4%) patients were in the early (pT1) stage, whereas most (82.1%) patients were in the local advanced stage (pT3 or pT4) of disease. The percentages of pNØ and pN+ patients were not the same; 67.6% patients had lymph node involvement (pN+) when they underwent surgery. Within the same pT and pN stages, we compared the 5-year survival based on different serum albumin levels (Table 3). In the pT1/pT2 stage, most of the patients (44 of 53, 83.0%) had a normal serum albumin levels. Among patients with pT3 disease (66.2%), patients with normal serum albumin levels had significantly better 5-year survival than those with decreased serum albumin levels (Fig. 3). Patients with stage pT4 disease (15.9%) had similar results to those with pT3 disease (Fig. 3). According to lymph nodes status and serum albumin level, patients were divided into four groups; survival is shown in Fig. 4. The 5-year survival rate for patients with pNØ and normal serum albumin was 65%, the rate for pNØ and decreased serum albumin was 44%, the rate for pN+ and normal serum albumin was 23%, and the rate for pN+ and decreased serum albumin was 5%. Among patients with stage pNØ disease, 5-year survival of those with normal serum albumin levels was better than that of hypoalbuminemic patients ($P = 0.035$). The result was similar for patients with stage pN+ ($P = 0.002$).

Table 3. Five-year survival rate in different T and N stage with different serum albumin (SA) levels

	Normal SA patients		Abnormal SA patients		
	n	5-Year survival (%)	n	5-year survival (%)	*P* value
T category					
pT1	20	89	2	100	0.627
pT2	24	78	7	80	0.919
pT3	144	27	52	13	0.015
pT4	33	20	14	0	0.003
N category					
pNØ	74	65	22	44	0.035
pN+	147	23	53	5	0.002

DISCUSSION

The incidence of adenocarcinoma of the proximal stomach including the gastroesophageal junction has shown a large increase in numerous studies of Western countries.[1–3] This trend is in contrast to a decrease in the incidence of the distal gastric adenocarcinomas. According to the classification of Siewert et al.[7] and De Manzoni et al.,[15] *adenocarcinoma of the gastric cardia* is defined as a tumor center within 5 cm proximal and distal of the gastroesophageal junction. Undoubtedly, the prognosis of patients with adenocarcinoma of the gastric cardia is worse than that among those with distal gastric cancer.[4] The possible explanations included patients with late-stage disease at presentation, a high incidence of lymph node involvement, and distal esophageal invasion.[16] In our study, most of the patients had a locally advanced tumor (82.1%) and lymph node involvement (67.6%). In the literature review, 5-year survival rate among patients undergoing curative surgery ranged from 15% to 33%.[6,7,15–21] Accordingly, our 5-year survival rate was 33.7%, with acceptable mortality and morbidity.

With the exception of that reported by Kodera et al.,[18] the percentage of early-stage gastric cardiac adenocarcinoma (pT1) is reported in the range of 6–15%.[6,7,15,17] In our study, 7.4% of patients were diagnosed in the early stage (pT1) and, of these, 89.7% enjoyed a 5-year survival. Data regarding survival of patients with stage pT1 disease are scarce and inconsistent.[6,22,23] The patients in stage pT2 had similarly high 5-year survival, in contrast to the poorer outcomes of patients in stages pT3 and pT4. Two hundred (67.6%) patients had nodal involvement in our study, which is comparable with previous reports of 62–81%.[6,7,15,17,18] Overall, the subgroup with positive lymph nodes had a 5-year survival rate of only 19.0% compared with 63.4% of the pNØ subset. On univariate analysis, nodal involvement was a significant prognostic factor for adenocarcinoma of the gastric cardia ($P < 0.001$).

Dysphagia was a major complaint of gastric cardiac cancer and led to malnutrition. Dysphagia is also a common complaint among patients with lumen obstruction due to distal esophageal tumors. In fact, it is difficult to differentiate among patients with distal esophageal tumor and those with adenocarcinoma of the gastric cardia with distal esophageal invasion. We examined dietary tolerance at presentation to evaluate the relationship of dysphagia in correlation to serum albumin level. Most patients with severe dysphagia had lower albumin levels, especially patients who could not tolerate any food or tolerated only a liquid diet. However, serum albumin level is more reliable, objective, and easily measurable than the evaluation of dysphagia.

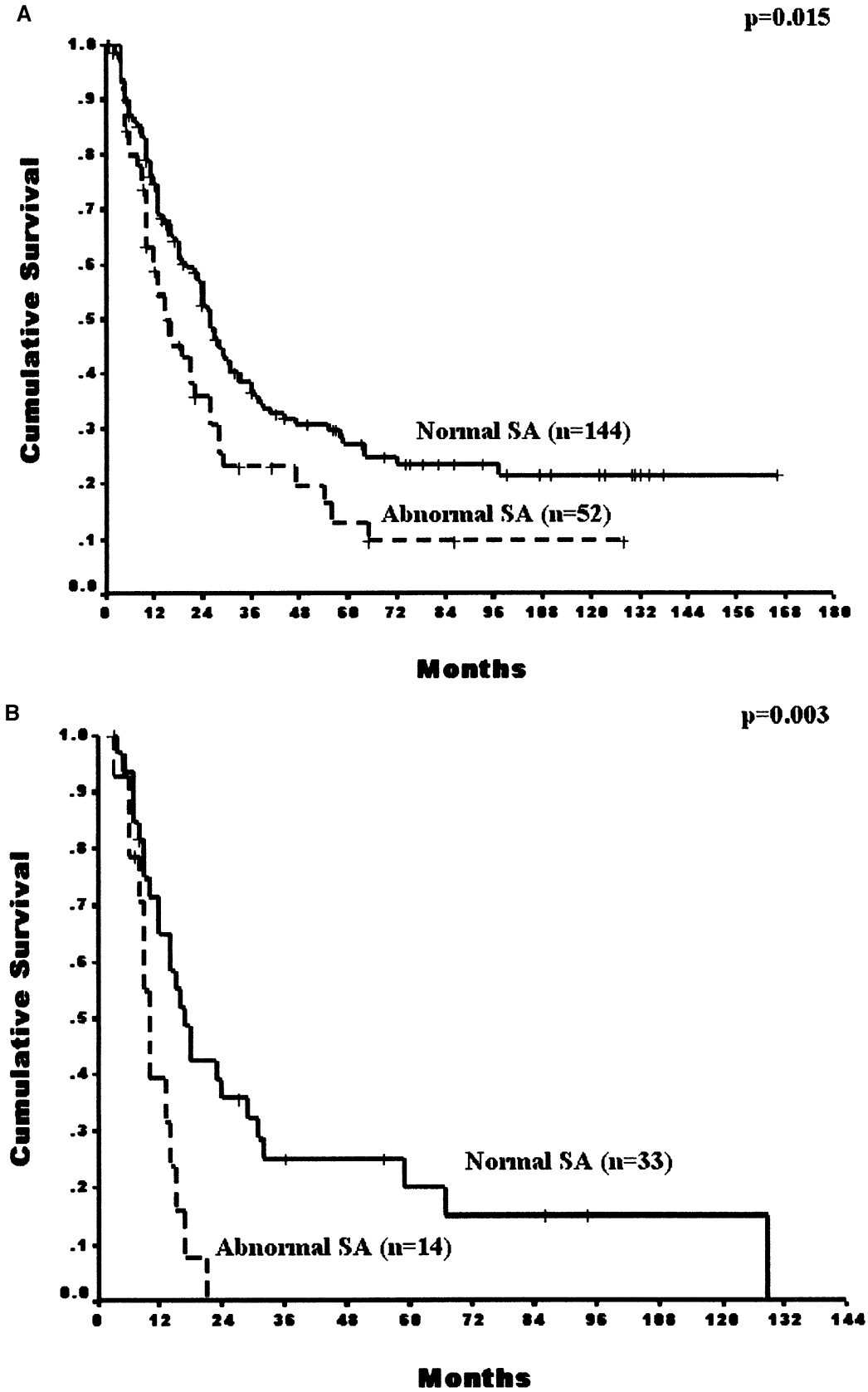

Fig. 3. (**A**) Survival curves for patients with stage pT3 disease with different serum albumin (SA) levels. (**B**) Survival curves for patients with stage pT4 disease with different SA levels.

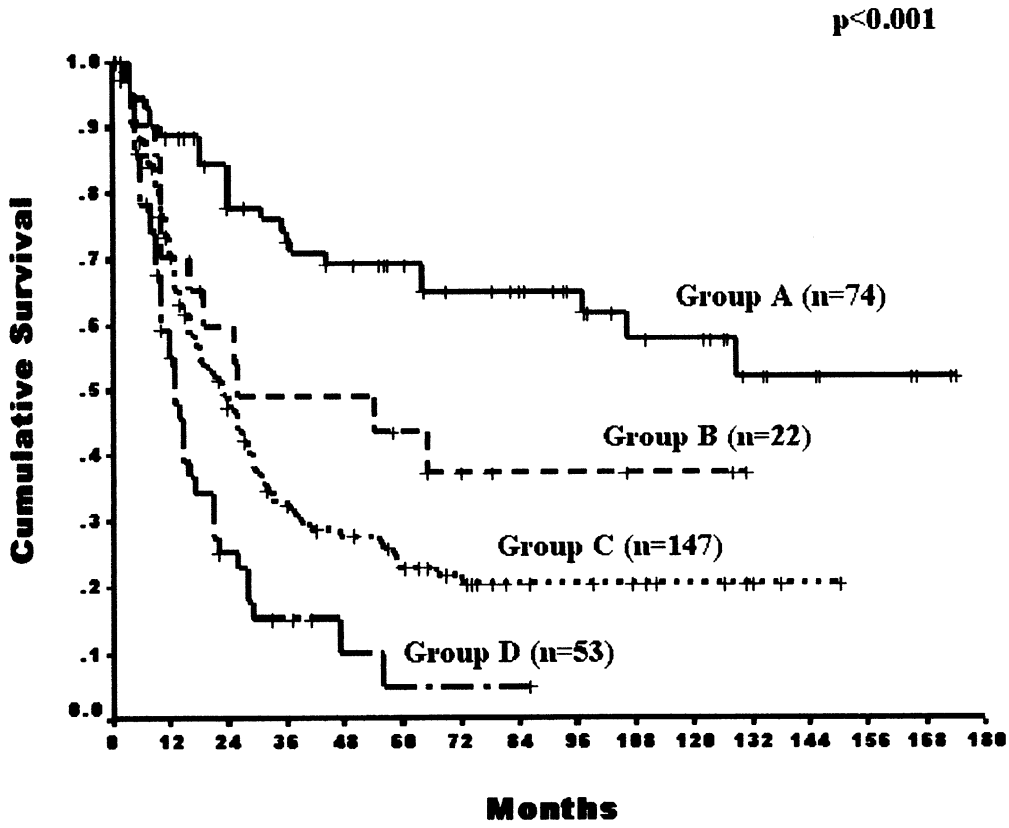

Fig. 4. Survival curves for each pN group with different serum albumin levels. Group A = pNØ and normal serum albumin; group B = pNØ and abnormal serum albumin; group C = pN+ and normal serum albumin; group D = pN+ and abnormal serum albumin. (A versus B, $P = 0.035$; A versus C, $P < 0.001$; A versus D, $P < 0.001$; B versus C, $P = 0.192$; B vs. D, $P = 0.005$; C vs. D, $P = 0.002$.)

Hypoalbuminemic patients had higher perioperative mortality and morbidity in many pervious studies,[7–10] whereas other studies reported no higher incidence of mortality and morbidity.[24] In our study, the perioperative mortality and morbidity in hypoalbuminemic patients tended to be higher than in those with normal serum albumin level, but the difference was not significant. The differences in results may be due to the differences in the cutoff value of serum albumin within these studies.

In our study, we used serum albumin levels to divide patients into two groups and compared the survival differences between both groups under the same pathologic parameters. In patients with stage pT1/pT2 disease, survival did not differ. This may be due to inadequate patient numbers. However, among patients with pT3/pT4 stage, the survival difference was significant (Fig. 3, *A* and *B*). Consistent with this, patients with a normal level of serum albumin also had better outcomes than did those with hypoalbuminemia and the same pN status. According to a study by Lewis et al.,[8] hypoalbuminemia could lead to cellular immunity impairment, which could result in the poor prognosis. Thus, serum albumin level is not only a window into the patient's nutritional status but also a useful factor for predicting patient prognosis after resection of the adenocarcinoma.

Due to poor long-term prognoses, patients with advanced carcinoma were traditionally suggested to receive adjuvant chemotherapy to improve their survival.[25] According to our results, the 5-year survival rate among patients with hypoalbuminemia was poorer than that among those with normal serum albumin levels under the same pathologic T and N statuses. That is, multimodal therapy should also be offered to patients with preoperative hypoalbuminemia, even if they have earlier-stage disease.

The level of preoperative serum albumin not only reflected the nutritional condition of the patient but also was a predictor of the prognosis of adenocarcinoma of the gastric cardia. Our study demonstrated that the preoperative level of serum albumin, in conjunction with the pathologic TNM stage, provided

information to predict the long-term outcome and the need for further adjuvant treatment postoperatively.

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Integrins Can Directly Mediate Metastatic Tumor Cell Adhesion Within the Liver Sinusoids

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Tumor cells can show different malignant properties regarding their ability for organ-specific metastasis formation. Their adhesive and invasive characteristics mediated by various cell adhesion molecules appear to be crucial for this process. Using intravital fluorescence microscopy, we analyzed the adhesive and invasive interactions of circulating human colon carcinoma cells within the microvasculature of the liver in rats. The involvement of different cell adhesion molecules in specific tumor cell–host organ interactions was investigated. Single-cell suspensions of human colon carcinoma with low (HT-29P) and high (HT-29LMM) metastatic potential were fluorescence labeled with calcein-AM and intra-arterially injected into Sprague-Dawley rats. Initial interactions between different cell lines and the microvasculature of the liver were observed over 30 minutes and semiquantitatively analyzed. Different integrin subunits, carbohydrate ligands, and vascular cell adhesion molecule-1 were inhibited using function-blocking antibodies or by enzymatic removal. Inhibition of sialyl-Lewis$_a$ (sLe$_a$) or enzymatic removal of selectin carbohydrate ligands significantly reduced metastatic cell adhesion. In addition, α6-, β1-, and β4-integrins can directly mediate cell adhesion within the hepatic microcirculation. Furthermore, α2-, α6-, β1-, and β4-integrins are involved in early tumor cell extravasation into the liver parenchyma. Organ-specific formation of colorectal metastases appears to be mainly mediated by specific interactions between circulating carcinoma cells and the vessel wall of target organs but not mechanical entrapment. Selectin–sLe$_a$ interactions with sinusoidal endothelial cells can play a key role in organ-specific targeting, but direct integrin-mediated cell adhesion to extracellular matrix components in the space of Disse appears to be required for the successful formation of liver metastases. (J GASTROINTEST SURG 2004;8:1049–1060)

KEY WORDS: Metastasis, liver, lung, intravital microscopy, colon carcinoma, cell adhesion, invasion, integrins, selectins

The formation of distant metastasis is an extraordinarily complex process. To successfully colonize secondary organs, a tumor cell must complete a sequential series of steps before it becomes a clinically detectable metastatic lesion. These steps include separation from the primary tumor; invasion through surrounding tissues and basement membranes; entry and survival in the circulation, lymphatics, or peritoneal space; arresting in a distant target organ, usually, but not always,[1,2] followed by extravasation into the surrounding tissue; survival in the foreign microenvironment; proliferation; and induction of angiogenesis, while evading apoptotic death or immunologic response (see reviews[3–5]).

Tumor cells isolated from metastases are highly migratory and invasive. Despite the prevalence of secondary tumors in cancer patients, however, the metastatic process appears to be very inefficient. To successfully colonize a distant organ, a tumor cell must complete all steps of the cascade. Failure to complete any step results in the failure to colonize metastatic target organs. Organ specificity and success rates of metastasis formation, however, are not

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solely determined by tumor characteristics but also are influenced by various factors of the host organs.[6]

Tumors can release very high numbers of cells into the blood circulation,[7] but only very few clinically detectable metastases are usually formed.[8] Although many steps in the metastatic process are thought to contribute to metastatic inefficiency, our incomplete understanding of this process suggests that we are aware of some, but not all, of these key regulatory points. For instance, killing of intravasated cells by hemodynamic forces and sheering has been thought to be a major source of metastatic inefficiency.[9] However, recent evidence suggests that the destruction of tumor cells by hemodynamic forces within the vascular tree may not always be a major source of metastatic inefficiency.

An important and early step during formation of distant metastasis is the arrest of circulating tumor cells within the host organ.[10] Various types of cell adhesion molecules appear to be involved in the complex processes of metastatic tumor cell adhesion to the microvasculature. They can mediate successful cell arrest that appears to be dependent on the balance between adhesive and antiadhesive forces, and the rate at which adhesive interactions are broken.[11] Recent reports suggested that stabilization of tumor cell adhesion to the microvessels of host organs is very important for further steps of secondary tumor formation (see review[12]).

Colorectal carcinomas with increased metastatic potential and with a poor prognosis are characterized by specific expression patterns of cell adhesion molecules, such as a high content of certain carbohydrate antigens or different members of the integrin family. For example, levels of the carbohydrate selectin ligands apparently increase during colorectal carcinoma progression from nonmetastatic to metastatic tumors.[13,14] Increased sialylation of mucin-associated carbohydrates, such as sLe_x, seems to be generally characteristic for colon cancer cells with a high potential to metastasize.[15] Metastases have also been found to express decreases in mucin core structures, reciprocal increases in sialylated mucins, and increases in peripheral sLe_x compared with the primary tumors from which they arose.[16]

Several integrins are important mediators promoting different steps of the metastatic cascade, such as cell adhesion, migration, proliferation, and survival at secondary sites.[17] Antagonists of these integrins can suppress cell migration and invasion of primary and transformed cells into the blood or lymphatic circulation and thus enable tumor cells to reach distant organ sites. These antagonists can also block tumor angiogenesis and tumor metastasis. Although integrin ligation by the extracellular matrix (ECM) stimulates cell migration, integrin antagonists (antibodies, peptides, or small molecules) can inhibit this cell property. Blocking integrin ligation can prevent cell attachment to the ECM, and recent studies have shown that antagonized integrins can actively inhibit signal transduction that is a prerequisite for cell migration.[18] For example, the inhibition of $\alpha5\beta1$-integrins negatively regulates fibroblast, endothelial cell and tumor cell migration even when other integrin receptors for ECM components are ligated.[18]

A broad spectrum of integrin expression in certain patterns was found in normal colorectal mucosa, primary tumors, and metastases.[19,20] For example, colon carcinomas tend to have weaker integrin staining than do adenomas or normal cells; however, they also show considerable heterogeneity of $\alpha2\beta1$-integrin expression.[21] In addition, $\alpha5$ subunit was frequently found to be expressed in invasive colon carcinomas, whereas the expression of this integrin subunit is usually poor or absent in normal epithelium.[22] Various combinations of α and β subunits are found only in transformed cells. For example, the $\alpha6$-integrin subunit is normally paired with $\beta1$ subunits, but in colon carcinoma cells, coexpression of $\alpha6$ and $\beta4$ subunits was frequently found.[23]

The liver is the most important host organ for metastasis of colorectal carcinomas. This organ is unique, because it lacks a basement membrane underneath the sinusoidal endothelium.[24] However, ECM components, such as fibronectin, laminin, and collagen types I and IV are present in the space of Disse between endothelial cells (ECs) and hepatocytes. Due to an incomplete layer of hepatic ECs, these ECM components are directly accessible for circulating cells.[25,26] Various studies have shown that interactions between these ECM components within the liver and tumor cells are crucial for the formation of hepatic metastasis.[27–29]

However, most experimental data were obtained using in vitro systems that likely simplify biological responses and reduce their complexity within the host organs or using in vivo end point assays with macroscopic tumors as target structures. These end point assays are unable to differentiate between different steps of the metastatic cascade, such as cell adhesion, survival, and proliferation. Intravital microscopy technologies have recently been used to investigate metastatic tumor cell adhesion within host organ microcirculation, such as liver and lung.[30–32] In these studies, contradictory results were reported regarding the type of entrapment (mechanical entrapment versus active cell adhesion) and the requirement of invasion into host organ parenchyma (invasion versus intravascular proliferation). Using colon carcinoma cells, we observed a lack of mechanical entrapment

in rat liver sinusoids and a rapid extravasation into the liver parenchyma.[33]

Using a direct observation of metastatic tumor cell adhesion and invasion of colon carcinoma cells into the host liver parenchyma by intravital microscopy, we investigated the role of different cell adhesion molecules that are overexpressed in colorectal carcinomas and their secondary tumors for organ-specific formation of liver metastasis. In our study, enzymatic removal of selectin ligands almost completely inhibited metastatic tumor cell adhesion. Additionally, inhibition of α6, β1, and β4 subunits resulted in a significant reduction of tumor cell adhesion within the sinusoids, suggesting direct binding of tumor cells to ECM components in the space of Disse. Furthermore, these integrin subunits and the α2-integrins appear to mediate their extravasation into the liver parenchyma. In contrast, blockade of other integrins, such as α1, α3, and α5 subunits, or vascular cell adhesion molecule (VCAM-1) did not interfere with early steps of organ colonization. These results support our previous observations that specific tumor cell–host organ interactions are required for successful formation of distant metastases, whereas mechanical entrapment is less important in this process.

METHODS

Material and Cells

Media (RPMI1640) and fetal bovine serum (FBS) were purchased from GIBCO-BRL (Karlsruhe, Germany). All other chemicals were purchased from Sigma (Deisenhofen, Germany).

Colon carcinoma cells with low (HT-29P) or high (HT-29LMM) metastatic potential were cultured in RPMI1640 medium containing 10% FBS without antibiotics in humidified 5% CO_2/95% air at 37°C. Confluent cell monolayers were used during the log-phase of growth. For experiments, cells were rinsed with calcium/magnesium-free phosphate-buffered solution (CMF-PBS), trypsinized, and kept in serum-free adhesion medium (RPMI1640, bovine serum albumin [BSA] 1%) for 60 minutes. Trypsinized cells were resuspended as single-cell solutions in CMF-PBS at a final concentration of 1×10^6 cells/ml. This preparation allowed reconstitution of cell surface molecules and did not interfere with adhesive and migrative properties in vitro.[34] The reconstitution and pretreatment of cells did not interfere with their viability as determined by dye exclusion. In addition, because calcein-AM requires intracellular activation, it labels only viable cells.

Intravital Fluorescence Video Microscopy

Male Sprague-Dawley rats (200–250 g; Charles River) were cared for in accordance with standards of the German Council on Animal Care, under an approved protocol of the local Animal Welfare Committee. Rats were anesthetized using inhalation of isofluorane (Curamed, Karlsruhe, Germany) and N_2O. Permanent catheters were introduced into the left heart via the carotid artery and into the right heart via the jugular vein. After a wide median laparotomy was performed, the left liver lobe was carefully mobilized without disturbing hepatic microcirculation. Using a heated operating table, animals were fixed under an upright fluorescence microscope and positioned on their left side. This positioning allowed a partial luxation of the mobilized left liver lobe that was placed on a specific holder to investigate its lower surface. During the experiments, the liver was continuously irrigated with isotonic saline solution.[33]

An upright epifluorescence microscope (Zeiss, Oberkochen, Germany) was used containing a 20-fold objective that was located over a glass slip covering the organ surfaces. The microscope was connected with a video enhancer–zoom lens system and a low-light CCD-video camera (Peiper, Düsseldorf, Germany), allowing real-time imaging via a separate monitor. Fluorescence images were recorded using a timer-containing S-VHS video system for further analysis.[35]

In Vivo Observation of Metastatic Tumor Cell Adhesion

For intravital observation of adhesive interactions between circulating tumor cells and the host organ microcirculation, single-cell suspensions (1×10^6 cells) were injected intracardially over 60 seconds. Previously, we have shown that the route of cell application (left heart, right heart, portal vein) did not influence the adhesive or migratory behavior within the liver sinusoids.[33] This technique did not interfere with cardiocirculatory or pulmonary functions of the animals, which were continuously monitored during the experiments.

Various parameters were used for further investigation and semiquantitative analysis of these interactions. The localization of stable tumor cell adhesions within the vascular tree and in relation to the diameter of the involved vessels was evaluated. If tumor cells were able to arrest within the microvessels, the diameter of the involved vessel was determined compared with the diameter of the adherent tumor cell. Furthermore, remaining blood flow within this vessel

or its occlusion was investigated using fluorescence-labeled dextran. A semiquantitative analysis of tumor cell adhesions and extravasation was performed over a 30-minute observation period, and the numbers of adherent cells were counted for each of the 5-minute intervals. Using a standardized procedure, all microscopic fields were analyzed in each observation period, and average numbers of adherent cells, migrated cells, and total cells observed were counted. In addition, the latency of tumor cell invasion into host organ parenchyma was determined. The relative migration rates were calculated as percentage of cells within the host organ parenchyma in relation to the total numbers of observed cells. Some experiments were independently evaluated by two or three observers in a blinded matter. A strong correlation between the observers ($r > 0.9$) was achieved.

Involvement of Cell Adhesion Molecules

To investigate different cell adhesion molecules regarding their involvement in early steps of the metastatic cascade, their function was specifically inhibited using blocking antibodies or enzymatic approaches.

Integrins from the β1 and β4 family can directly interact with ECM components within the liver but not with sinusoidal ECs. Therefore, the following integrins were inhibited using subunit-specific monoclonal antibodies (mAbs) that are function blocking in vitro: α1 (clone P4C10; Chemicon, Hofheim, Germany), β4 (clone ASC-8; Chemicon), α1 (clone 05-246; Upstate Biotechnology, Hamburg, Germany), α3 (clone ASC-1; Chemicon), α5 (clone JBS5; Serotec, Eching, Germany), and α6 (clone GoH3; gift from J. Eble, Münster, Germany). Furthermore, an inhibiting mAb against the α2β1-heterodimer (clone BHA2.1; Chemicon) was used. These integrins have been previously shown to mediate cell adhesion of HT-29 cells to ECM components in vitro that can be inhibited using the mAb accordingly.[28] Control cells were pretreated with isotype-specific IgG (R&D Systems, Wiesbaden; Germany).

Because tumor cells can adhere to microvascular ECs mainly mediated by members of the selectin family or VCAM-1,[36–38] we investigated if these cell adhesion molecules are also involved in the metastatic cell adhesion of circulating tumor cells. For VCAM-1, cells were pretreated with a function-blocking mAb (clone 1G11; Santa Cruz, Heidelberg, Germany). Selectins can mediate the recognition of sialylated carbohydrates, such as sLe_a, sLe_x, and the MECA-70 antigen. These ligands were enzymatically removed using neuraminidase/sialidase digestion.[39,40] After trypsination and reconstitution of cell surface molecules, cells were incubated with 0.01 U/ml type V-neuraminidase (from *Clostridium perfringens*, EC 3.2.1.18; Sigma, Deisenhofen, Germany) for 60 minutes. This neuraminidase hydrolyzes terminal sialic residues with α2β3, α2β6, and α2β8 glycosidic linkages. Alternatively, control cells were enzymatically pretreated with hyaluronidase (from *Clostridium histolyticum*, Sigma, Deisenhofen, Germany), which does not interfere with cell surface structures on tumor cells. Cells were subsequently washed and immediately used for the in vivo experiments. In further experiments, the most important selectin ligands on tumor cells, sLe_a (clone KM93; Chemicon) and sLe_x (clone KM231; Chemicon), were inhibited using function-blocking mAbs.

Detection of 5-Bromo-2-Deoxyuridine–Labeled Cells Within the Liver

In some experiments, cells were preincubated with 5-bromo-2-deoxyuridine (BrdU) 24 hours before injection into the rats. Intravital microscopy of the hepatic microcirculation was performed as described above. After the 30-minute observation period, the portal vein was cannulated and the liver was perfused with formalin solution using physiologic hydrostatic pressure (8–10 cm H_2O). Finally, prefixed organs with maintained microvascular structures were removed, completely fixed, and paraffin embedded. Serial sections of the liver were used for anti–BrdU fluorescence staining.

RESULTS

Metastatic Tumor Cell Adhesion to Liver Sinusoids

Circulating HT-29P and HT-29LMM, and Caco-2 colon carcinoma cells were usually able to easily pass the microvascular vessels of potential target organs without mechanical entrapment. Even at the end of the observation period, circulating cells were observed passing the capillary system of the liver. Within 2 minutes after injection of the cell suspensions, the first cells were observed that arrested within the hepatic microvessels. Small numbers of adherent cells were observed that rapidly started to migrate from the intravascular compartment into the hepatic parenchyma within the 30-minute observation period. First migrated cells were observed within 5–10 minutes after injection of the cell suspension in all cell lines, but HT-29LMM cells showed the highest rates of cell migration.

Selectins Mediate Adhesive Interactions

Highly liver metastatic HT-29LMM cells were pretreated with neuraminidase for enzymatic removal of potential selectin carbohydrate ligands. This treatment resulted in a dramatic loss of adhesive interactions between circulating tumor cells and the sinusoidal vessel wall ($P < 0.05$–0.005). The numbers of adherent cells significantly decreased, but 10–20% of the cells were still able to establish stable cell adhesions within the hepatic microcirculation. Due to the small numbers of adherent cells, the absolute numbers of migrated cells were very small after neuraminidase pretreatment. Therefore, the migration rates were difficult to compare between neuraminidase-treated and control cells. Considering this limitation, these rates were similarly in both groups (maximum rates, 14% versus 18%). In contrast, pretreatment with hyaluronidase showed a slight nonspecific effect on the numbers of adherent and totally observed cells but to a highly significant lesser extent than neuraminidase-treated cells. In addition, cell migration rates were not affected by hyaluronidase (Fig. 1).

Because ligand removal for selectin binding of tumor cells resulted in inhibited cell adhesion within the hepatic microcirculation, further experiments were performed to identify the sialylated carbohydrate Lewis antigens involved in metastatic cell adhesion within the liver. If sLe_a-ligands were inhibited using a function-blocking mAb, adhesion of circulating tumor cells within the sinusoids was significantly reduced to less than 40%. Although only a small numbers of cells were able to successfully establish these adhesions, the relative numbers of migrated cells remained comparable between untreated and sLe_a-inhibited cells. In contrast, cells that were pretreated with anti-sLe_x mAb did not show significant alterations in metastatic tumor cell adhesion or migration into the liver parenchyma (Fig. 2).

VCAM-1 and its ligands—the α4-integrin—are not expressed on colon carcinoma cells. Consequently, pretreatment of HT-29LMM cells with a function-blocking VCAM-1 mAb did not modify early interactions between circulating tumor cells and vessel walls of the hepatic microcirculation (see Fig. 2).

Integrins Can Directly Mediate Metastatic Cell Adhesion Within the Liver

Low metastatic HT-29P and highly metastatic HT-29LMM cells were pretreated with function-blocking mAb against the β1-integrin subunit. In both cell lines, this treatment resulted in a substantial reduction of the numbers of adherent cells ($P < 0.05$–0.005) to about one third compared with IgG control cells (Table 1). In addition, the numbers of migrated and totally observed cells were significantly decreased after inhibition of the β1-integrin ($P < 0.05$–0.005). Furthermore, the relative migration rates of HT-29LMM cells showed a tendency of β1-integrin–mediated inhibition, but significant differences were not found in both cell lines (Table 1). Therefore, using the highly liver metastatic HT-29LMM cell line, we investigated various α subunits that form heterodimers with the β1-integrin and that show altered expression in colorectal carcinomas or their distant metastases. Inhibition of α6-integrins resulted in a significant reduction of the numbers of adherent and

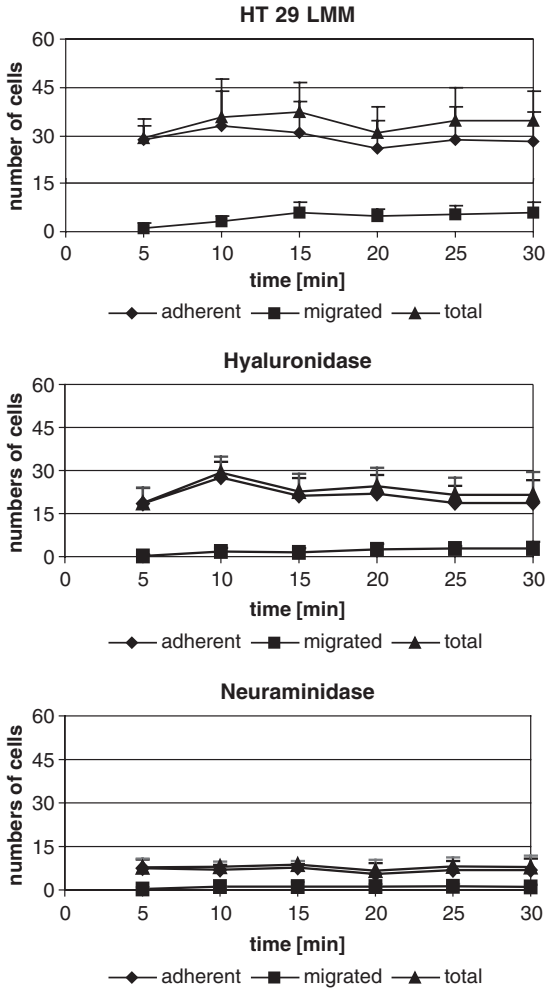

Fig. 1. Neuraminidase can inhibit metastatic tumor cell adhesion. Fluorescence-labeled HT-29LMM cells were either untreated or pretreated with neuraminidase or hyaluronidase for 1 hour before intravital microscopy. Enzymatic digestion of selectin carbohydrate ligands significantly reduced cell adhesion within the liver sinusoids ($P < 0.05$–0.005).

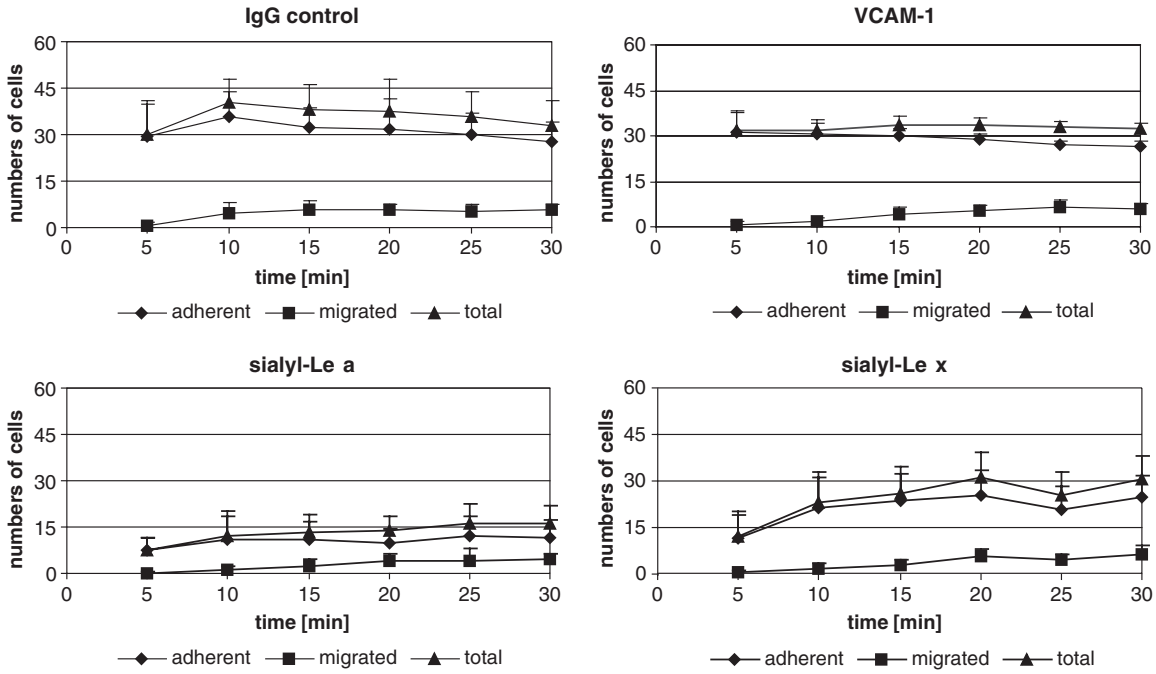

Fig. 2. sLe_a can mediate tumor cell adhesion within the hepatic microcirculation. Selectin-ligands sLe_a and sLe_x were inhibited in HT-29LMM cells using function-blocking mAb against these carbohydrate molecules. Blockade of sLe_a resulted in a significant reduction of adhesive binding of the colon carcinoma cells within the hepatic sinusoids ($P < 0.001$), whereas inhibition of sLe_x did not affect these cellular properties. The numbers of migrated cells and cell migration rates were also not influenced using both mAbs. In contrast, function-blocking mAbs against the vascular cell adhesion molecule VCAM-1 did not interfere with early steps of metastasis formation in this model.

totally observed cells to about 50% of the untreated or control cells ($P < 0.05$–0.001). In contrast, function-blocking mAbs against α1-, α2-, α3-, or α5-integrins did not influence the numbers of adherent or totally observed cells. Furthermore, blockade of the α2- or α6-integrins significantly reduced cell migration rates ($P < 0.05$). In addition to heterodimers with β1-integrins, the α6 subunit can form heterodimers with β4-integrins. This subunit has therefore also been blocked in HT-29LMM cells, resulting in an almost identical reduction in cell adhesion and migration rates within the liver sinusoids as found after α6-integrin inhibition ($P < 0.05$–0.001) (Fig. 3; Table 2).

Localization of Cells Within Liver Parenchyma

During the intravital observation, we were able to differentiate between adherent cells that were arrested within the microvessels and migrated cells that had left the blood circulation. To validate the location of arrested tumor cells, they were perfusion-fixed within the target organ, maintaining its microvascular structure. Using a BrdU-staining technique, adherent tumor cells were visualized within the liver sinusoids, and remaining vessel lumens were confirmed using serial sections. In addition, some cells were found that had left the microcirculation within 30 minutes after cell injection and were clearly located between the hepatocytes (Fig. 4).

DISCUSSION

Metastasizing tumor cells have to successfully complete a number of steps to form secondary tumors at distant organs.[41] Once these cells enter the blood circulation, they have to survive the conditions of fluid flow and arrest in secondary host organs. Mechanical entrapment[30,31] and receptor-specific "seed-and-soil" adhesions[1,33] are still controversially discussed as determining factors for this cell arrest. It has long been accepted that most malignant tumors show an organ-specific pattern of metastasis. For example, colon carcinomas usually metastasize to liver and lung but rarely to bone and brain and almost never to other organs, such as kidneys. In contrast, other tumor entities, such as malignant melanomas or prostate cancer, usually prefer other organs, such as bones and central nervous system. However, experimental data using different types of assays revealed

Table 1. β1-integrins mediate metastatic cell adhesion of colon carcinoma cells within the liver

	Interval (min)					
	5	**10**	**15**	**20**	**25**	**30**
HT-29P						
Adherent	23.7 ± 5.7	24.4 ± 6.7	20.3 ± 7.1	22.6 ± 5.7	21.9 ± 4.4	20.5 ± 5.0
IgG	29.0 ± 9.8	32.0 ± 6.5	32.0 ± 5.7	28.6 ± 6.4	31.9 ± 7.2	27.1 ± 11.6
Anti-β1	12.3 ± 5.7‡	12.9 ± 4.6‡	11.6 ± 5.8‡	12.6 ± 5.2‡	14.0 ± 5.4‡	12.4 ± 6.5‡
Migrated	1.0 ± 1.4	2.2 ± 1.6	3.1 ± 1.8	3.1 ± 1.2	3.2 ± 1.4	3.21 ± 2.7
IgG	2.5 ± 2.0	4.7 ± 2.9	4.9 ± 3.1	4.4 ± 2.4	4.4 ± 1.9	3.1 ± 1.6
Anti-β1	1.5 ± 1.5	2.5 ± 1.6	1.9 ± 1.7*	1.9 ± 1.6*	2.5 ± 2.0	1.7 ± 1.4
Totally arrested	23.8 ± 6.4	25.0 ± 8.4	25.8 ± 6.9	24.2 ± 7.9	23.6 ± 5.7	22.8 ± 6.7
IgG	31.5 ± 10.2	37.8 ± 7.7	36.9 ± 7.6	33.0 ± 8.5	36.3 ± 7.4	30.2 ± 11.6
Anti-β1	13.7 ± 6.8‡	15.4 ± 5.3‡	13.5 ± 7.2‡	14.4 ± 5.5‡	16.5 ± 6.1‡	14.1 ± 7.5‡
Relative migration rate (%)	4 ± 6	9 ± 5	14 ± 10	13 ± 4	14 ± 7	14 ± 9
IgG	8 ± 7	13 ± 8	13 ± 6	13 ± 5	12 ± 6	11 ± 6
Anti-β1	9 ± 8	16 ± 10	12 ± 8	13 ± 9	16 ± 9	11 ± 7
HT-29LMM						
Adherent	28.4 ± 4.7	32.8 ± 10.8	31.0 ± 9.5	26.1 ± 8.5	28.7 ± 10.1	28.4 ± 9.1
IgG	28.4 ± 9.9	34.0 ± 9.0	30.6 ± 7.3	29.2 ± 11.5	27.4 ± 10.1	25.2 ± 8.6
Anti-β1	16.5 ± 7.2†	15.5 ± 6.0‡	15.5 ± 9.2‡	17.5 ± 13.2*	16.4 ± 10.8†	16.3 ± 11.7*
Migrated	0.9 ± 1.6	3.1 ± 1.8	6.0 ± 4.8	4.8 ± 1.9	5.7 ± 2.6	6.0 ± 3.1
IgG	0.5 ± 0.9	4.5 ± 3.0	5.5 ± 2.8	5.3 ± 2.1	4.8 ± 2.5	5.0 ± 2.5
Anti-β1	1.4 ± 1.8	1.4 ± 1.2†	1.7 ± 2.0‡	2.1 ± 1.1‡	1.2 ± 1.5‡	1.6 ± 1.3‡
Totally arrested	29.3 ± 6.0	35.9 ± 11.4	37.0 ± 9.7	30.9 ± 9.7	34.4 ± 10.7	34.4 ± 9.2
IgG	28.9 ± 10.4	38.4 ± 9.3	36.1 ± 9.4	34.5 ± 13.1	32.3 ± 12.1	30.2 ± 10.7
Anti-β1	17.9 ± 6.7	16.9 ± 6.0‡	17.2 ± 9.3‡	19.6 ± 13.3‡	17.6 ± 10.4‡	17.9 ± 11.8†
Relative migration rate (%)	0 ± 0	12 ± 9	21 ± 13	23 ± 10	22 ± 6	23 ± 10
IgG	2 ± 2	17 ± 9	17 ± 6	18 ± 5	17 ± 5	18 ± 3
Anti-β1	9 ± 11	8 ± 6	10 ± 12	14 ± 11	9 ± 13	10 ± 7

Numbers of adherent cells, migrated cells, and totally arrested cells and relative migration rates were pairwise compared after pretreatment with anti-β1 monoclonal antibody or control IgG in HT-29P and HT-29LMM cells using Student's *t* test (*$P < 0.05$, †$P < 0.01$, ‡$P < 0.005$).

contradictory results regarding the specificity of initial interactions between host organs and circulating tumor cells.

Our results provide evidence that the successful colonization of distant organs by circulating colon carcinoma cells is mainly mediated by specific adhesive interactions between these cells and the microvasculature of the host organs. Inhibition of selectin-mediated cell binding as well as blockade of integrin-mediated cell adhesion specifically reduced adhesive properties of colon carcinoma cells within the hepatic microcirculation. Although cell rolling as known from activated leukocytes has never been observed in our experiments, the data suggest that selectin–carbohydrate interactions between circulating cells and sinusoidal ECs can support the formation of stable tumor cell adhesions. We hypothesize that the larger diameter of circulating tumor cells

Table 2. Cell migration rates after pretreatment with different anti–α-integrin monoclonal antibody

	Interval (min)					
	5	**10**	**15**	**20**	**25**	**30**
IgG	1 ± 2	12 ± 9	16 ± 5	16 ± 4	15 ± 4	16 ± 4
anti-α1	3 ± 3	9 ± 2	11 ± 2	11 ± 13*	12 ± 4	15 ± 3
anti-α2β1	6 ± 5	8 ± 5	7 ± 7*	8 ± 5†	11 ± 6	9 ± 7*
anti-α3	3 ± 4	8 ± 4	9 ± 4*	14 ± 2	11 ± 4	15 ± 5
anti-α5	4 ± 3	9 ± 5	11 ± 4	14 ± 4	13 ± 2	10 ± 3†
anti-α6	0 ± 0	2 ± 4*	9 ± 6	5 ± 5†	6 ± 4†	13 ± 4

HT-29LMM cells were pretreated with a different function-blocking monoclonal antibody for 1 hour before intravital microscopy. Relative cell migration rates (%) cells were determined using standardized procedures in 5-minute intervals for 30 minutes (*$P < 0.05$, †$P < 0.01$).

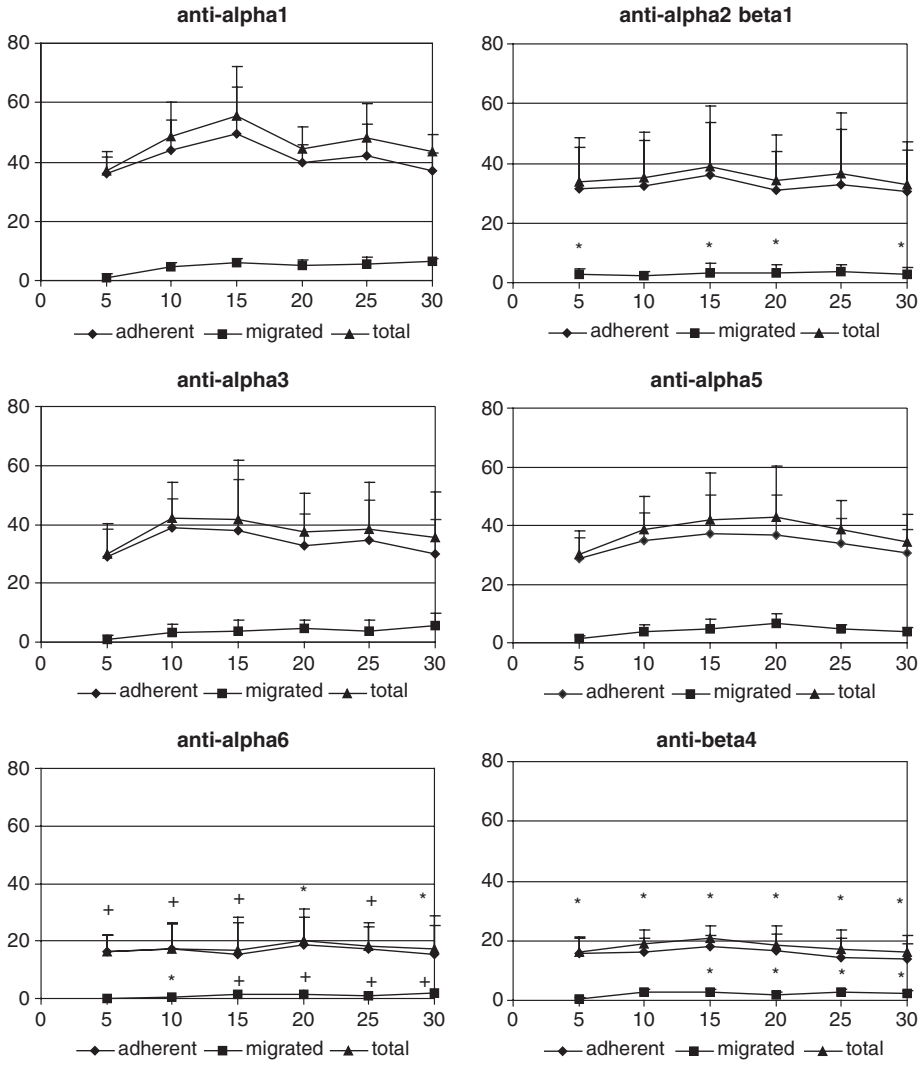

Fig. 3. Effects of α-integrin inhibition on early steps of metastasis formation. Highly metastatic HT-29LMM cells were pretreated with different function-blocking mAbs. Inhibition of α6-integrins resulted in reduced cell adhesion and migration within the hepatic sinusoids, whereas α2-blockade significantly affected migration into the liver parenchyma. In addition, mAbs against β4-integrins caused a significant reduction of cell adhesion and migration similar to the inhibition of α6-integrins ($^{*}P < 0.05$; $^{\dagger}P < 0.01$).

with resulting higher shear forces caused by the fluid flow may be responsible for the lack of rolling. In addition, the sinusoidal EC lining shows the unique feature of incomplete layers with direct accessible ECM components. Therefore, direct binding of integrins on circulating tumor cell surfaces to ECM within the space of Disse can mediate stable cell adhesions within this target organ. The ability of direct binding of circulating colon carcinoma cells to structures within the space of Disse has been demonstrated using electron microscopy showing cell adhesion within gaps of the sinusoidal EC lining.[42] Supporting this hypothesis, we found that inhibition of α2-, α6-, β1-, and β4-integrins can interfere with adhesive interactions in our model. Binding to laminin by α6β1- and/or α6β4-integrins appears to be crucial for cell adhesions and invasion into the hepatic parenchyma, whereas α2β1-integrins, the main ligands for collagens on tumor cells, seem to be required only for early invasion into the liver parenchyma, but not cell adhesion. Recently, we showed that αvβ1-, αvβ5-, and αvβ6-integrins, but not αvβ3-integrins, can also mediate cell adhesion and early migration of HT-29 colon carcinoma cells within the liver sinusoids (Enns et al., unpublished data, 2004). Because integrin blockade only results in an incomplete reduction of cell adhesions, we assume that inhibition of a single integrin subunit can probably be replaced,

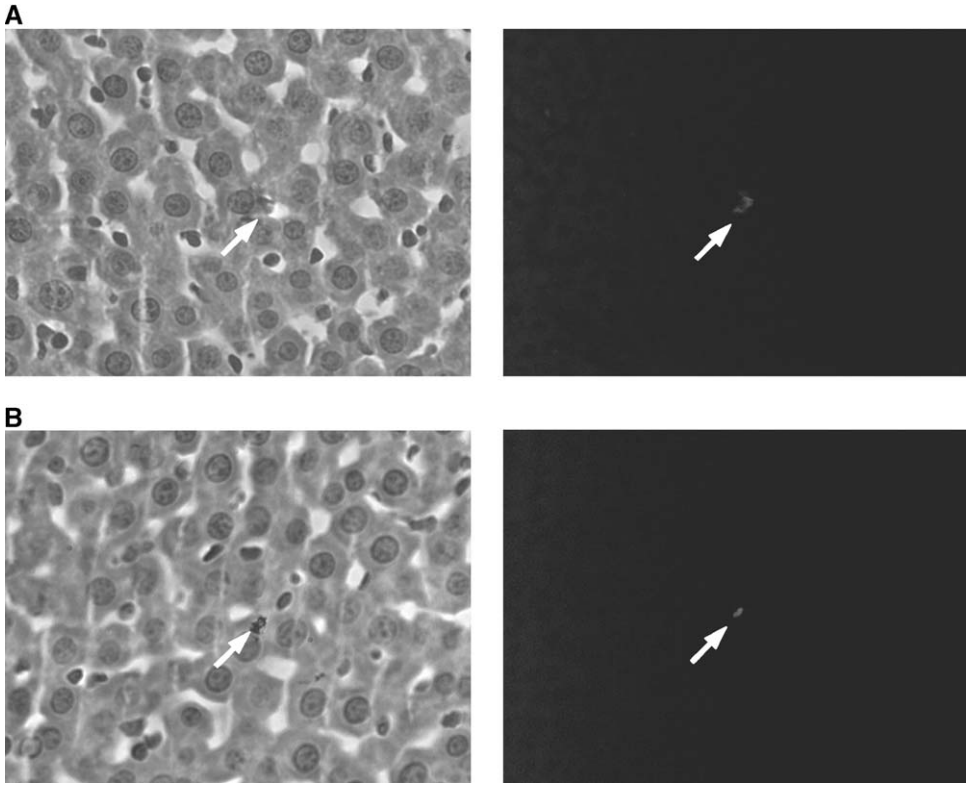

Fig. 4. Validation of tumor cell location within the liver. HT-29LMM cells were labeled using BrdU for 24 hours and injected intracardially as described. After 30 minutes, the liver was fixed using formalin infusion via the portal vein. BrdU was visualized within the nuclei of an invaded cell (*left*); conventional hematoxylin and eosin counterstaining was performed (*right*). *Arrows* are located at identical positions on the right and left sides. (**A**) Tumor cell adherent within a liver sinusoid leaving remaining vessel lumen. (**B**) Migrated cell within the liver parenchyma.

at least in part, by other integrin subunits and is not sufficient to completely block adhesive properties. This suggests that interactions of different cell adhesion molecules may be required for these early steps of metastasis formation.

The identification of cell surface molecules using function-blocking mAbs can be limited by their interference with cellular signaling events. For example, simple monomeric ligation of integrins has minimal effects on focal adhesion organization and the phosphorylation status within these complexes. In contrast, ligand occupancy and integrin-clustering have been shown to result in certain signal transduction, such as acquisition of vinculin, talin, or α-actinin.[43,44] Therefore, although function-blocking mAbs usually act as antagonists of cell adhesion molecules, they can have intrinsic agonistic capacity with partial stimulatory effects on cell adhesion–mediated signal transduction and subsequent cellular reactions. All antibodies used in this study are function blocking in vitro for cell adhesion under static conditions. However, their exact activity under in vivo conditions is not known. Thus, some effects of the inhibition of various cell adhesion molecules might by underestimated in our experimental setup.

Indeed, similar to our results, Kikkawa et al.[45] described an increased arrest of CHO cells within sinusoids if they injected cells de novo transfected with αvβ3-integrins. In addition, the expression of sLe ligands, α2- and α6-integrins in colon carcinomas correlated with their metastatic potential and patient's prognosis.[20] Moreover, using our animal model we found that the migration of adherent tumor cells into the hepatic parenchyma was related to their metastatic potential in different colon carcinoma cell lines (Schlüter et al., unpublished data, 2004).

Previously, we observed cell arrest in microvessels with diameters larger than the diameters of the tumor cells and the remaining perfused lumen suggesting that mechanical size restriction is not important for cell arrest in host organs.[33] Additionally, cell arrest occurred only in organs that are usually targets for colorectal metastases. For example, in renal capillaries, colon carcinoma cell arrest was not found,

whereas circulating tumor cells were able to successfully adhere to the vessel wall of pulmonary capillaries and liver sinusoids (Schlüter et al., unpublished data, 2004). Similar results of specific cell adhesions within the microvasculature of target organs were reported by Glinsky et al.[46] and Al-Mehdi et al.[1] Comparable to others,[45,47] we found an early extravasation of these cells, but intravascular proliferation has also been reported.[1,49] The velocity of this process might be different depending on the cell lines and/or tumor entities.

In our experimental system, some modifications were used compared with what was used by other investigators. Steinbauer et al.[48] reported size restriction in a mouse system, but used fluorescent beads (>10 μm) for comparison that appear to be more rigid than circulating tumor cells. In contrast to Chambers' group,[30,31] who has also found mechanical cell arrest of circulating tumor cells as the main mechanism, we used an upright microscope, which enabled us to investigate the upper surface of the liver. This approach can avoid additional tissue pressure on the lower side of the liver lobe caused by the weight of the tissues above. This additional tissue pressure might be responsible for increased intravascular pressure and/or reduced size of the microvessels subsequently interfering with tumor cell adhesion. Mook et al.[49] injected very high numbers of cells (5×10^6 in 0.5 ml) directly into the portal vein. In our previous study[33] using a similar technique, we found that this procedure can result in severe disturbances of the hepatic microcirculation with subsequent occlusion of sinusoids with cell clusters. In our opinion, these findings do not reflect the physiologic conditions during metastasis formation. Therefore, we used the injection of smaller numbers of tumor cells into the left heart. We had previously compared this approach with other routes of cell application and found that cellular adhesive interactions were independent of whether the cells were injected intracardially (left or right heart) or into the portal vein.[33] Due to the better reproducibility and lesser complications, the intra-arterial application was preferred in our model. Furthermore, although immunocompetent rats were used, human and rat carcinoma cells with comparable malignant properties showed similar patterns of adhesive interactions within the liver sinusoids.[33] In addition, visualization of metastatic tumor cell arrest and extravasation was similar in immunocompetent and immunodeficient mice, whereas further progression was influenced by immunologic phenomena.[48]

In summary, our study suggests that organ-specific formation of colorectal metastases is mainly mediated by specific interactions between circulating carcinoma cells and the vessel wall of potential target organs but not mechanical entrapment. Selectin–sLe interactions with sinusoidal ECs can play a key role in this organ-specific targeting, but direct integrin-mediated cell adhesion to ECM components in the space of Disse also appears to be required for initial steps of hepatic metastasis formation. We hypothesize that this fact can explain, at least in part, the high frequency of liver metastases in colorectal carcinomas.

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Discussion

Dr. J. Howard (Toledo, OH): I think this may prove to be a tremendous contribution. Have you studied the passage of cancer cells through the lymph nodes?

Dr. Gassmann: No, using this model we were not able to study passage of tumor cells through the lymph node.

Dr. F. Moody (Houston, TX): In an attempt to relate this to the human situation, does it relate to the number of tumor cells you introduce? Have you looked at that in terms of possibly overwhelming whatever is trying to prevent that from happening because the body doesn't want to see those cells?

Dr. Gassmann: Actually, you are addressing the problem of immunologic interactions between tumor cells and the human body. In fact, here we used human cells injected into the rat. In previous studies we injected rat colon carcinoma cells originating from the same species, and during this first period of 30 minutes we were observing here, we found basically similar behavior of rat colon carcinoma cells and human colon carcinoma cells. So we think for the initial adhesion and extravasation during the first 30 minutes, this may not be relevant at that point. Later on, it is possible.

Dr. G. Telford (Milwaukee, WI): I am curious, is this the only mechanism that determines whether there are metastases? Every once in a while you see large tumors that don't seem to be metastasizing anywhere. Do some tumors not shed tumor cells or is the most important factor adhesive interactions?

Dr. Gassmann: Unfortunately, metastases formation is a multistep process, and we all know the situation of big tumors not metastasizing to distant organs. This may have two reasons. First of all, the tumor cell infiltration occurs at a primary tumor site. So tumor cells must get access to the vascular lumen. The second point, here we only showed adhesion and extravasation. So these are two basic features that tumor cells show, and we know from clinical observation that these processes have some kind of relation to the clinical course. Also, later on there are other features occurring like dormancy, proliferation, angiogenesis that contribute to the formation of a clinically evident metastases. So here we are only looking at a very narrow time range.

Dr. James Becker (Boston, MA): Perhaps you can clarify your model. As I understand it, the study utilized intra-arterial infusions of cells. At least in terms of colon cancer, perhaps more relevant would be intravenous or intra-portal infusion, especially as it relates to liver metastases. The route of infusion might impact the whole adhesion process. Can you comment on that?

Dr. Gassmann: Thank you for this question. Of course when we started using this model, we investigated all these routes of application: we used intraportal injection, we used intravenous injection, and then used intra-arterial injection. And we found that the basic behavior of the cells within the liver does not differ between intra-arterial, intravenous, and intraportal injection, but what we actually found were more complications while using the intraportal injection and more pulmonary complications using the intravenous injection. We did not find differences in the basic behavior of the cells but better reproducibility and less hemodynamic changes in the animals; we decided to go on with the arterial route.

Evaluation of Charlson-Age Comorbidity Index as Predictor of Morbidity and Mortality in Patients With Colorectal Carcinoma

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The Charlson-Age Comorbidity Index (CACI) is a validated tool used to predict patient outcome based on comorbid medical conditions. We wanted to determine if the CACI would predict morbidity and mortality outcomes in patients undergoing surgery for colorectal carcinoma. Records of 279 consecutive colorectal cancer patients who underwent laparotomy by a single surgical group between 1997 and 2001 were reviewed in a retrospective fashion for patient demographics, stage at diagnosis, operation, surgeon, perioperative complications, tumor characteristics, comorbid diseases, performance status, length of stay (LOS), disposition, and mortality. Using the preoperative history and physical, all patients were assigned a score for the CACI. Perioperative morbidity and mortality were recorded and graded to account for severity. The University Statistical Consulting Center and SPSS software were used to analyze the results. The patients were primarily white (97.1%) with a male-to-female ratio of 1:1.2 and a median age of 72 years. AJCC stage at presentation was stage 0 (3.2%), stage I (28.3%), stage II (24.4%), stage III (24.4%), or stage IV (19.7%). Median LOS was 7.0 days. Perioperative mortality was 17 of 279 (6.1%), and overall mortality was 32.6% at a median follow-up of 18.5 months. Higher CACI scores and AJCC stage at presentation correlated with longer LOS and overall mortality. Only the CACI correlated with perioperative mortality and disposition. No correlation was observed with location of tumor, type of surgery, or surgeon. Patients with higher cumulative number of weighted comorbid conditions as indicated by the CACI are at higher risk for perioperative and overall mortality. This simple scoring system is also a significant predictor of disposition (home versus extended care facility) and LOS. The CACI can be a useful preoperative tool to assess and counsel patients undergoing surgery for colorectal carcinoma. (J GASTROINTEST SURG 2004;8:1061–1067)

KEY WORDS: Colon cancer, comorbidity, mortality

Identification of patients at high risk for postoperative morbidity and mortality is difficult. There has been new interest in improving patient selection because outcome measures are now being used by consumers to determine hospital and surgeon suitability for the management of various diseases (e.g., Leapfrog initiative[1]). On a practical level, better preoperative counseling for patients and their families is needed to accurately guide expectations. Several authors have created grading systems to predict outcomes of both medical and surgical patients.[2–10] To be useful in clinical practice, a system must be simple to use and be easily comparable between patients. Most important to surgeons, it should be useful to predict outcomes based on the preoperative score.

One such system, called the Charlson Age-Comorbidity Index (CACI), was developed by Mary Charlson, M.D., at New York Hospital in the late 1980s. This index was later validated in a cohort of patients with breast cancer and since then has been used in many different settings, including with other types of cancer patients.[2–6] This number scoring system

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is based on weighted comorbid medical conditions and includes a factor for age by decade. An absolute number can be obtained and used for comparison. The CACI is probably the most widely used comorbidity index to date.[2] Data were gathered from the inpatient medical service at New York Hospital. Mortality at 1 year was analyzed as a function of various comorbidities. This resulted in a list of 19 conditions to be used for the scale. Any disease generating a relative risk of death greater than 1.2 was retained and weighted (Table 1). An additional factor adjusting for age was also created.

In the current era of multidisciplinary treatment of cancer, many options for the treatment and palliation of oncology patients exist. The risk-to-benefit ratio of the various treatment modalities should be evaluated separately as part of the complete determination of care. If patients and physicians have a better understanding of risk, or the likelihood of complications, then patient selection will favor those most likely to have a benefit from our interventions.

Surgical management of colon cancer disproportionately affects the older patient, who typically has a number of comorbid diseases. Thus, we used the CACI to determine if this system was useful to predict perioperative risk of morbidity and mortality and compared it with another commonly used and simple to administer scale, the Karnofsky Performance Status Scale (KPS).[11]

Table 1. Charlson Index

Weight*	Conditon
1	Myocardial infarction (MI)
	Congestive heart failure (CHF)
	Peripheral vascular disease (PVD)
	Cerebrovascular disease (CVD)
	Dementia
	Chronic obstructive pulmonary disease (COPD)
	Connective tissue disease
	Ulcer disease (PUD)
	Mild liver disease
	Diabetes (DM)
2	Hemiplegia
	Moderate/severe rental disease
	Diabetes with end-organ damage
	Any tumor
	Leukemia
	Lymphoma
3	Moderate/severe liver disease
6	Metastatic solid tumor
	AIDS

*Age index adds 1 point for each decade over 40 years.

METHODS

Records of 279 consecutive colorectal cancer (CRC) patients who underwent laparotomy by a single surgical group between 1997 and 2001 were reviewed. Patients were identified using the Oncology Outcomes Management Database maintained for hospital cancer program accreditation by the American College of Surgeons. The institutional review board approved the study. Patients were treated at a single institution and followed to death or time of analysis in August 2002. Complete medical records were available during this time frame from both office and hospital charts. Initial history and physical examination, laboratory values, radiographic image reports, and operative and pathology reports were reviewed for clinical and pathologic staging based on the AJCC *Cancer Staging Manual*, Fifth Edition, and comorbid diseases. Information regarding perioperative morbidity, local tumor recurrence, development of metastatic disease, and mortality (perioperative and disease specific) was obtained.

CACI scores (Table 1) were calculated by weighting each comorbid disease separately and adding to the age factor as described in the original article by Charlson. Specifically, determination of a CACI score involves an additive method in which each comorbid disease corresponds to a weighted number. By adding all numbers, including 1 point for each decade over the age of 40, a final score can be determined. Type and severity of perioperative complications were recorded based on the standard morbidity and mortality grading system as developed by Clavien et al.[12] Additional points of evaluation such as length of stay (LOS) and disposition from hospital (home, home with visiting nurse, nursing home [NH], and death) were performed as part of an overall outcome measurement. Follow-up information was obtained for each patient, including current state of health, time to death, and death related to disease or from other factors. Only three patients were lost to follow-up.

Data were analyzed using SPSS software with the assistance of the Wright State University Statistical Consulting Center. Statistical analysis using the λ coefficient was chosen for nominal and ordinal data. We used χ^2 analysis for nominal data alone. A Spearman correlation was chosen when continuous variables were used. The Cox proportional hazards model was used to determine the effect of each Charlson age group (≤ 7 or >7) and (≤ 10 or >10) on the hazard rate of measurements for perioperative and overall mortality from colon cancer. In addition, the Kaplan-Meier method was applied to generate survival analysis for patient groups divided at a CACI score of 7

and a CACI score of 10. These two cutoff points were chosen because 7 is the median CACI for the cohort and 10 divides the cohort into similar numbered groups for analysis of perioperative mortality.

RESULTS

The median age at the time of treatment of 72 years (range, 22–95 years) with a median follow-up of 18.5 months (range, 0–65 months). Other demographics are given in Table 2. The cohort was mostly white (97%), with a male-to-female ratio of 1:1.2. Initial AJCC stage[13] was based on laparotomy, clinical, and radiologic findings and were evenly distributed for invasive cancers (Table 2). Choice of surgical procedure was performed at the discretion of the attending surgeon based on preoperative and intraoperative findings. The vast majority of patients (73.1%) underwent segmental resection of the colon with primary anastomosis regardless of stage. The median number of lymph nodes recovered was 7 (range, 0–64), implying limited mesenteric resections in some cases.

Tumor distribution was predominantly right-sided colon (42.9%), followed by left/sigmoid colon (36.6%) and rectal (10.4%). Synchronous primary malignant lesions were found in 3.9% of patients. Metachronous lesions were noted in two patients, identified at 20 and 30 months after the initial primary tumor was resected. Distant metastases were present at diagnosis in 19.7% of patients. Recurrent disease (local or distant) developed in 12.2% of patients.

Table 2. Patient* and tumor characteristics

Characteristic	n	%
Gender		
Male	127	45.5
Female	152	54.5
Race		
White	271	97.1
Black	5	1.8
Other	3	1.2
Stage (AJCC)		
0	9	3.2
1	79	28.3
2	68	24.4
3	68	24.4
4	55	19.7
Pathology		
Adenocarcinoma	227	81.4
Mucinous	44	15.8
Signet ring	3	1.1
Carcinoma in situ	5	1.8

AJCC = American Joint Commission on Cancer.
*Medium age, 72 years (range, 22–95 years).

Adjuvant therapy was administered to 44.4% of patients. Chemotherapy alone was administered to 35.4%, whereas both chemotherapy and radiation therapy were administered to 8.6%. Only one patient received radiation therapy alone, and he died at home 2 years after initial resection from an unknown cause. Type of chemotherapy was not standardized and therefore not reported separately. Only a small proportion of patient refused adjuvant therapy when indicated (6.8%). External beam radiotherapy was administered to 9% of patients and no patient refused indicated therapy.

The median CACI score was 7.0 (range, 2–17). The most common comorbid conditions encountered were diabetes (15.4%), cerebrovascular disease (15.1%), and chronic pulmonary disease (12.5%). No single comorbid condition was a significant predictor of morbidity or mortality.

To evaluate the CACI as an indicator of morbidity and mortality, statistical analysis was carried out with patients divided into two groups with CACI score either 7 or less or greater than 7 using the Cox regression model. This level is chosen as a cutoff based on the median CACI found during analysis. To put this into perspective, a patient with chronic obstructive pulmonary disease, diabetes mellitus, mild/moderate renal disease, and colon cancer (all in this study) would have a CACI score of 6. CACI scores greater than 7 showed a statistically significant difference ($P < 0.011$) and a hazard ratio of 5.1 for perioperative mortality. Similar findings were calculated using cancer-related mortality in the analysis ($P < 0.0001$) with a hazard ratio of 5.3, implying a five times greater risk of colon cancer–related death for patients with CACI scores greater than 7 (Table 3). Because of a low number of deaths (n = 3) in the CACI score of 7 or greater group, a second cutoff point was used, CACI of 10 or less. CACI scores greater than 10 showed a statistically significant difference ($P = 0.0004$) and a hazard ratio of 5.64 for perioperative mortality. Again, using cancer-related mortality in the analysis ($P < 0.0001$) a hazard ratio of 6.04 was found, implying a six-time greater risk of colon cancer–related death for patients with CACI scores greater than 10 (Table 4). Specifically, according to the Cox proportional hazards regression model, for each 1 point increase on the CACI, patients are at a 36% increased risk of perioperative mortality and 32% increased risk of overall cancer-related mortality.

The KPS was evaluated in each patient as a way of comparing the utility of the CACI to a standard oncologic measure of suitability for treatment. Using the admission history and physical 33% of patients

had a KPS of 100. Only 9.4% were below a KPS of 70. Similar to the CACI score, KPS score was also predictive of an increased perioperative and overall mortality. Our analysis suggests that for each unit decrease in KPS score, perioperative mortality increases by 3.5% ($P = 0.0149$, hazard ratio 0.965) and overall mortality increases by 2% ($P = 0.0469$, hazard ratio 0.983). Because the KPS is applied at intervals of 10, 1 unit increases are not particularly useful. Although this well-known measure used to assess oncology patients may be helpful to determine suitability for nonsurgical therapies, it does not seem to be as relevant a predictor of outcome as the CACI in the operative patient.

The common complications were divided into medical and surgical with the most common being pulmonary (6.4%), ileus (4.7%), and *Clostridium difficile* infection (3.6%). Sixty-nine complications were found in 56 patients. A graded list of complications can be seen in Table 4. These were evenly distributed throughout grades 1–3. Patients with high and low CACI scores were also evenly distributed throughout the grades. Therefore, CACI is not a predictor of which patients would have minor versus serious complications.

Median hospital stay for patients in this group was 7.0 days (range, 1–56). One patient died from a massive myocardial infarction on the first postoperative day. However, when considering the entire cohort in the context of increasing CACI scores, there is a significant difference in LOS ($P < 0.001$) based on the Spearman correlation with patients having higher scores requiring longer stay. This is also true based on analysis by stage ($P < 0.005$), which also predicts longer LOS. As patients require longer stays in acute hospitals, focus then turns to disposition. Most patients were able to return home (74%); however, some required additional assistance in the form of a visiting nurse or interval transfer to an NH. We found a correlation between rising CACI scores and disposition to either home, NH, or home with RN ($P = 0.0001$), confirming that patients with more comorbid diseases will need greater assistance at time of discharge from the acute care setting (Table 5). Patients with CACI scores of 7 or less required a visiting nurse or NH in only 1.8 % (n = 5) and 1.4% (n = 4), respectively, whereas those with CACI scores of greater than 7 required visiting nurse (3.6%) or NH (12.5%) much more often. Compared with disease stage alone, the CACI was a better predictor of disposition ($P < 0.031$), where stage was not significant. Thus, the CACI may help to identify those who would benefit from formal preoperative discharge planning.

Concern for practitioner variability in surgical treatment has arisen during recent years. This is mainly a result of outcome studies attempting to determine the varied effects of institution and surgeon experience as well as patient factors. Our analysis also included observations of surgeon effect. A total of six surgeons participated in the care of patients in this group. Using the λ coefficient, we were able to show no significant predictors of morbidity, mortality, LOS, or disposition based on the surgeon alone. Therefore, we are focused on patient characteristics as one of the most important factors.

Survival curves were created using the Kaplan-Meier method for both high- (CACI > 10) and low- (CACI < 10) risk patient groups (Fig. 1). Patients were also divided into groups with CACI scores of 7 or less and greater than 7 (Fig. 2), which also produced significant results. Overall survival for the entire

Table 3. Charlson cutoff points

CACI cutoff point (< or >)	*P* value	Low group*	High group*	Hazard ratio
Mortality				
5	0.0041	8.2 (5/61)	24.3 (53/218)	3.8
6	<0.0001	7.4 (8/108)	29.2 (50/171)	5.4
7	<0.0001	9.7 (14/144)	32.5 (44/135)	5.3
8	<0.0001	11.5 (21/183)	38.5 (37/96)	5.0
9	<0.0001	13.9 (29/208)	40.8 (29/71)	4.8
10	0.0004	15.4 (37/240)	54.9 (21/39)	5.6
Perioperative mortality				
5	0.145	1.6 (1/61)	7.7 (16/218)	4.5
6	0.037	1.8 (2/108)	8.8 (15/171)	4.8
7	0.011	2.0 (3/144)	10.4 (14/135)	5.1
8	0.004	2.7 (5/183)	12.5 (12/96)	4.6
9	0.0034	3.4 (7/208)	14.1 (10/71)	4.2
10	0.0004	3.8 (9/240)	20.5 (8/39)	5.6

*Values given at % (n ≠ total).

Table 4. Complications and mortality

	n	%
Total complications	69	
Medical complications	13	4.6
Surgical complications	56	20.1
Grade of complications		
1	15	5.4
2	17	6.1
3a	14	5.0
3b	1	0.4
4	9	3.2
30-Day mortality	17	6.1
Ovarall mortality	91	32.6

Table 5. Disposition by Charlson-Age Comorbidity Index (CACI) score

	CACI <5/>5	CACI <6/>6	CACI <7/>7	CACI <8/>8	CACI <9/>9	CACI <10/>10
Home	58/158	98/118	132/82	159/57	178/38	199/17
Visiting nurse	1/14	5/10	5/10	6/9	7/8	9/6
Nursing home	1/38	3/36	4/35	13/26	17/22	26/13
Death in hospital	1/8	2/7	3/6	5/4	6/3	6/3

group was 67.4% with a median follow-up of 18.5 months. Disease-free survival was 55.9%, whereas 11.5% are currently alive with evidence of disease. Nonsurvivors were divided into two groups for the purposes of our analysis: death secondary to colon cancer (63.7%) and death from other factors including comorbid conditions (36.2%).

DISCUSSION

CRC is the third leading malignancy diagnosed in the United States and third most common cause of cancer death in both men and women in 2002 as estimated with the American Cancer Society statistics.[13] With the advent of improved screening programs and widespread use of colonoscopy, CRC has shown improved rates of early diagnosis, but mainly in younger patients (<65 years).[14] Late presentations remain a significant problem, and these are more common in elderly patients and result in higher rates of morbidity and mortality.[15,16] However, age alone cannot predict these issues. Multiple factors must be evaluated, including the patient's functional status before surgery and the presence of comorbid conditions. In addition, surgeon- and institution-related factors may play a role.[1]

In our study we chose the CACI as our measure of comorbidity to standardize the evaluation of each patient and place a numbered value on each surgical patient. This scale, as developed by Mary Charlson and colleagues, has been used in prior studies to evaluate patients undergoing surgery for malignancies of the head and neck and breast, as well as in general medical populations. The CACI has been found to be useful in these populations for mortality predictions. Although the initial construct of the index was to predict mortality at 1 year, we believe that the index might be useful in evaluating surgical patients undergoing colon resection for cancer and hoped to generate predictability of end points such as morbidity, mortality, LOS, and disposition. In contrast to previously studied surgical populations, those undergoing abdominal surgery could be considered higher risk as evidenced by longer LOS and higher complication rate compared with neck or breast surgery. We consider this important because colon cancer is a common disease that requires treatment in healthy and chronically ill patients.

Limitations of this study include its retrospective nature and short follow-up with a median of 18.5 months (range, 0–65 months). However, this does represent a homogeneous cohort of consecutive patients who presented for care of CRC in a community

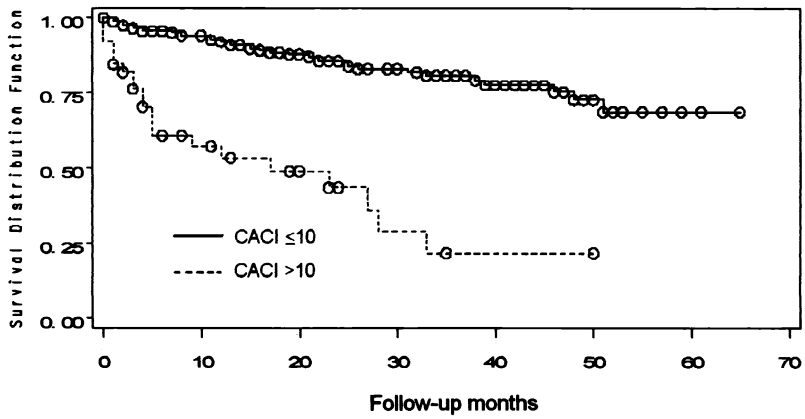

Fig. 1. Depicts survival advantage for patients with Charlson-Age Comorbidity Index score of 10 or less.

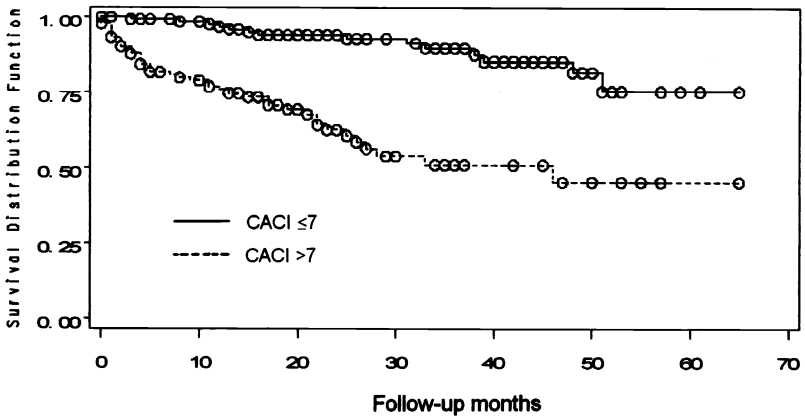

Fig. 2. Cancer-related survival for patients with a Charlson-Age Comorbidity Index (CACI) score of 7 or less and greater than 7, showing significant survival advantage for CACI scores of 7 or less.

hospital. Although the dominant race was white (97%), age, gender, and final pathology were well distributed and comparable to other studies.[15–18]

The goal of a grading system in this group of patients would be to better predict complications and mortality. To date there is no definitive reliable index to help assess patients preoperatively for colon cancer or other abdominal operations as an outcome predictor. In our study, we found the CACI to be a significant predictor of several outcomes. Overall mortality during the study period was 32.6% for all-stage cancer and presumably would be higher if evaluated after longer follow-up. Along with increasing stage of cancer ($P = 0.010$), the CACI was significant ($P = 0.0001$) for cancer-related mortality. Chronologic age itself cannot predict who will die from their disease, but the combination of comorbidity data and age (CACI) may help to separate physiologic from chronologic age effect. For each 1-point increase in the CACI score, patients are at a 32% increased risk of disease-related mortality. This clearly suggests that patients with more and worse comorbid conditions present with a definable increased risk. By using the CACI score, patients can be compared between surgeon and institution to apply an appropriate risk adjustment to patients undergoing surgery for colon cancer.

In addition, perioperative (30-day) mortality was evaluated. In-hospital mortality was only 3.2%. We found our 30-day mortality to be 6.1%, which is similar to previous studies, which noted a 6–10% mortality.[15,17–19] According to our study, perioperative mortality is increased by 36% for each 1-point increase in the CACI scale. Knowledge of this risk may have future implications in defining risk to patients and insurers resulting in increased diagnosis-related groups for these patients.

Based on our findings, higher stage and CACI scores also predicted an increased LOS for patients ($P < 0.0001$). Mean LOS increased incrementally in days for each increase in CACI score. For example, in patients with a CACI score of 6 or less mean, LOS is 6.97 days, whereas in patients with CACI score of greater than 12, the LOS is 9.36 days. This suggests that it may be possible to predict those patients who will require additional hospitalization based on comorbid disease processes and to facilitate discharge planning.

CRC patients are often elderly and, as we have shown, may have multiple medical problems leading to overall disability that could require NH stay or additional resources in the form of visiting nurse assistance. We found that higher CACI scores also correlated with patient disposition. Disposition was scaled from 1 to 4 to identify patient discharge disposition (1 = home, 2 = home with visiting nurse, 3 = NH, 4 = death). As expected, most patients were discharged home (77.4%). The remaining patients were discharged home with visiting nurse (5.4%) or NH (14%). This could not be predicted using only cancer stage. Again the CACI may be helpful with discharge planning earlier in the hospital course or even arranged preoperatively for appropriate patients.

Recent concerns regarding the use of specialized centers for treatment of specific diseases or surgical procedures has lead to a growing concern for identification of those factors related to outcome. Some studies have pointed to center variability, whereas others focus on surgeon or patient characteristics. Although not specifically addressed in our study, it should be noted that no surgeon variability was identified relative to patient outcomes of morbidity, mortality, LOS, or disposition. We therefore consider patient characteristics a more important indicator from our single-institution data.

Previous studies have noted increased complications with increasing age.[15–18,20] Although comorbid conditions were considered, there was no numbered scale that could be used for comparison with other patients or other studies. We identified 69 complications occurring in 56 patients for a complication rate of 20.1%. The grading of complications is important and relates to severity (Table 3). This grading allows us to differentiate minor complications, such as wound infection, from severe complications, such as pulmonary embolus, myocardial infarction, and anastomotic leak. The CACI could not identify patients likely to have complications overall. In fact, for the 56 patients with complications (including perioperative mortality), CACI scores were approximately the same. This was a limitation of our study. Other methods will need to be developed to assist in this regard.

In conclusion, proper patient selection for surgery and prevention of morbidity and mortality are paramount to surgeons. The CACI can be a useful adjunct to clinical evaluation and can be a measurable entity useful for outcome measures. Higher CACI scores for patients undergoing abdominal surgery for CRC can predict a higher perioperative mortality, overall mortality, and longer LOS in the hospital. Furthermore, patients with high CACI scores are more likely to require additional assistance upon discharge in the form of NH or home visiting nurse. In the future, this may facilitate better preoperative patient selection, risk adjustment measurement, and discharge planning.

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Is There a Role for Staging Laparoscopy in Patients With Locally Advanced, Unresectable Pancreatic Adenocarcinoma?

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The study objective was to determine the incidence of laparoscopically detected metastasis in patients with radiographically staged locally advanced adenocarcinoma of the pancreas. Patients with locally advanced pancreatic cancer are considered candidates for novel treatment protocols. Stratification of patients into locally advanced disease versus metastatic disease is imperative to accurately evaluate treatment outcome. Between 1994 and 2000, 100 consecutive patients undergoing staging laparoscopy with radiologic evidence of unresectable locally advanced pancreatic cancer were identified from a prospective database. All patients had preoperative contrast-enhanced, thin-cut computed tomography scanning or magnetic resonance imaging and had no evidence of detectable metastatic disease. There were 53 men and 47 women, with a median age of 64 years. The disease site was the pancreatic head in 69 cases and the body or tail in 31. Radiographic assessment of nonresectability was due to encasement of the celiac or hepatic artery in 37 patients, of the portal vein and superior mesenteric vessels in 56, and extrapancreatic extension in 7. Laparoscopy identified metastatic disease in 37% of patients, not seen on preoperative imaging. Peritoneal disease was noted in 12 cases and liver metastasis in 18 cases, and 7 patients had both. Neither the primary tumor size nor location influenced the incidence of metastatic disease. Standard imaging modalities failed to detect metastatic disease in 37% of patients who were considered to have locally advanced pancreatic cancer. Patients considered for treatment protocols for locally unresectable pancreatic cancer should be staged laparoscopically before initiation of therapy.
(J GASTROINTEST SURG 2004;8:1068–1071)

KEY WORDS: Laparoscopy, pancreatic cancer, staging

Laparoscopic staging for pancreatic cancer is increasingly used as a technique to decrease the number of unnecessary open explorations. Patients with potentially resectable tumors have been found to have unsuspected metastasis 20%–35% of the time despite high-quality preoperative imaging.[1–4] The avoidance of a formal laparotomy allows this group of patients to receive palliative therapy in a more expedient fashion. At many centers, laparoscopic staging has been integrated into the treatment algorithm for radiographically resectable pancreatic cancer. Currently, the role for staging laparoscopy in patients with locally advanced, unresectable pancreatic cancer is less well-defined.

Advanced dual-phase computed tomography (CT) scanning with both arterial and venous timed injection has markedly improved our ability to assess resectability of pancreatic cancer with regard to vascular invasion.[5–7] Patients with portal, celiac, or superior mesenteric vessel encasement are usually considered unresectable. In the absence of distant disease, these patients have been candidates for protocols involving

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combined modality chemotherapy and radiation. This is given in either a palliative[8–10] or potentially neoadjuvant[11–14] setting, depending on the tumor response and subsequent potential for resection. On the other hand, patients with hepatic or peritoneal metastasis have systemic disease and are not routinely considered candidates for local therapies, such as radiation, or for potential resection, but rather may be candidates for other palliative or investigational treatments.

Recently, gene therapy,[15–17] molecular markers,[18] and growth factors[19,20] have been targeted as potential treatment options for patients with unresectable disease. Proper selection of patients for such protocols is imperative to not only define therapy but also accurately assess outcome based on pretreatment stage of disease. Staging laparoscopy, although thoroughly critiqued for patients with potentially resectable pancreatic cancer, may prove to have a role in unresectable patients to accurately stratify before the initiation of treatment. The objective of this study was to determine the incidence of laparoscopically detected distant metastatic disease in patients with locally advanced, unresectable pancreatic cancer who were otherwise considered candidates for chemoradiation based on preoperative imaging.

MATERIAL AND METHODS

From a prospective database maintained by the Department of Surgery, patients with locally advanced pancreatic cancer undergoing laparoscopic staging from 1994 through 2000 were identified. All patients were considered candidates for palliative chemoradiation. Laparoscopic staging was performed to rule out metastatic disease and therefore define the treatment options. Locally advanced, unresectable disease was defined as obvious encasement of the celiac, hepatic, or superior mesenteric artery, or the portal or superior mesenteric vein, or extrapancreatic extension. This study does not include patients with involved portal vein or superior mesenteric vein potentially amenable to resection.

All patients had high-quality thin-cut CT scans (N = 98) or magnetic resonance imaging (N = 2). Patients with inadequate imaging performed elsewhere had the study repeated at our institution. CT scans were considered adequate with slices of 5 mm or smaller through the liver and pancreas. Because many outside films were included in the study, the make and model of the scanners varied. In 1999, Memorial Sloan-Kettering Cancer Center obtained a helical scanner that allowed multiple passes for the liver and higher resolution. Regardless, all radiologic studies were reviewed at Memorial Sloan-Kettering Cancer Center to further confirm the absence of obvious metastatic disease. Those that were noted to have lesions "suspicious for metastatic disease" or "too small to characterize" were recorded. The size of the tumor was noted based on the preoperative imaging. The site of the tumor was considered to be the pancreatic head if it was to the right of the portal vein and the pancreatic body/tail if it was to the left of the portal vein.

The choice of laparoscopic procedure technique was at the discretion of the individual surgeon. In general, a 10-mm laparoscope was placed through a supraumbilical or infraumbilical site or in the right upper quadrant. If metastatic disease was suspected, it was confirmed by laparoscopic biopsy through a separately placed 5-mm trocar. Peritoneal washings and cytology were not routinely performed. Laparoscopic ultrasonography was performed selectively. All pathology and operative reports were reviewed to confirm the presence of metastatic disease. The Kaplan-Meier method was used to estimate and compare survival rates between groups.

RESULTS

Between 1994 and 2000, 3113 patients underwent diagnostic laparoscopy at our institution. Of these, 754 procedures were for pancreatic adenocarcinoma; 100 were locally unresectable without known metastatic disease and were included in the study. Of the 100 patients included in the study, there were 47 women and 53 men (median age, 64 years; age range, 38–84 years). Table 1 outlines patient demographics and reason for unresectability.

A laparoscopic biopsy was performed in 71 cases for diagnostic purposes, including all patients with

Table 1. Patient demographics and tumor characteristics

Characteristic	No. of patients (N = 100)
Median age (yr) (range)	64 (38–84)
Gender (M/F)	47/53
Tumor site	
Head	69
Body/tail	31
Reason for unresectable disease	
Celiac/hepatic artery invasion	37
Portal vein/SMV, SMA invasion	56
Extrapancreatic extension	7

SMA = superior mesenteric artery; SMV = superior mesenteric vein.

Table 2. Laparoscopically detected metastatic disease in patients with pancreatic cancer

	Total	No. with liver metastasis	No. with peritoneal metastasis	No. with liver and peritoneal metastases	Patients with metastatic disease
All patients	100	18 (18%)	12 (12%)	7 (7%)	37 (37%)
Patients with pancreatic head cancers	69	15 (22%)	7 (10%)	4 (6%)	26 (38%)
Patients with pancreatic body/tail cancers	31	3 (10%)	5 (16%)	3 (10%)	11 (35%)

suspected metastatic disease. The remaining 29 patients had a preoperative percutaneous biopsy before referral to our institution and did not have suspected metastatic disease at the time of the laparoscopic staging. Median length of operation was 62 minutes, although in the last 2 years of the study, the median length decreased to 49 minutes (n = 60) and included obtaining frozen section results. Open conversion was required in seven cases, all due to extensive adhesions. There were no major complications in either the open or laparoscopic group. The overall median length of postoperative stay was 1 day. Thirty-nine procedures were performed in the outpatient setting, without an overnight stay.

At the time of laparoscopy, metastatic disease was detected in 37 patients (37%). The site of the metastatic disease is listed in Table 2. The incidence of metastatic disease was similar for pancreatic head cancers and those of the body or tail, as shown in Table 3. Of the 18 patients with hepatic disease, 3 had preoperative CT scans that were read as "suspicious for metastatic disease," but percutaneous biopsies were unable to confirm hepatic disease. Among the entire group, liver lesions were noted as "too small to characterize" in eight cases. However, at laparoscopy, three of the eight cases were without evidence of metastatic disease. As shown in Table 3, the median size of the tumor was 4.0 cm for both the locally advanced and the metastatic group. In addition, the median tumor size was 4.0 cm regardless of the site of the primary. In the majority of the cases the disease was described as 2–5 mm in size.

Survival analysis was performed and compared the patients with and without metastatic disease. The median survival for the 37 patients found to have metastatic disease was 7 months, compared to 9 months for those without metastatic disease ($P = 0.09$).

Table 3. Tumor size and location

	No.	Median tumor size (cm) (range)
Pancreatic head	69	4.0 (2.0–7.5)
Pancreatic body/tail	31	4.0 (2.0–10.0)
Metastatic disease present	37	4.0 (2.0–9.0)
No metastatic disease present	63	4.0 (2.0–10.0)

DISCUSSION

Staging laparoscopy for pancreatic cancer has been shown to increase the resectability rate to as high as 92% following open exploration.[4] For this reason, several centers have incorporated laparoscopy into the treatment and staging algorithm for pancreatic tumors.[1–4] Over the past several years, high-quality CT scans have improved the accuracy of radiographic prediction of resectability.[5–7] This is largely because of improved imaging for the local extension of the tumor into and around the peripancreatic vasculature. These two staging modalities have emerged simultaneously, and therefore the data and the role for laparoscopy in the current literature are conflicting. In the current study, we selected to examine the incidence of metastatic disease exclusively in patients who had high-quality preoperative imaging with radiographic evidence of locally advanced, unresectable pancreatic cancer but without radiographic detection of metastatic disease. Identifying metastatic disease in this group of patients is important as such findings may either alter the treatment options or more accurately assess patient response to various therapies.

Patients with locally advanced pancreatic carcinoma are frequently considered candidates for combined chemoradiation, whereas those with metastatic disease are not usually candidates for the radiation.[8–14] In the current study, we found metastatic disease in 37% of patients who were otherwise considered candidates for combined modality treatment. As a result, this group of patients was spared the use of local radiation in our center.

In the current study, the incidence of metastatic disease was similar regardless of site or size. Other investigators have found that tumors located in the body or tail of the pancreas are more likely to be associated with metastatic disease than are those located in the head.[1] This difference can be explained by the fact that in the current study, all tumors were clearly

unresectable based on preoperative imaging, whereas previous reports found that in patients with potentially resectable disease, the tail tumors are twice as likely as the head tumors to have metastasis.[1–4] This suggests that the biology of locally aggressive pancreatic cancers renders them more prone to metastasis, regardless of the site.

Based on these data, laparoscopic staging should be considered in patients with locally advanced pancreatic cancer that are considered candidates for local radiation treatment or experimental protocols. Those patients not requiring open exploration for palliative bypass are ideal candidates for laparoscopic staging, as are those deemed candidates for laparoscopic bypass. In addition, in novel treatment protocols involving inhibitors of angiogenesis, growth factors, and other molecular markers, laparoscopy should be considered as part of the staging.[15–20] This will more precisely stratify the population as having disease that is either locally advanced only vs. metastatic, and thus more accurately assess tumor response and patient outcome with these novel therapies.

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Caspase-3 Drives Apoptosis in Pancreatic Cancer Cells After Treatment With Gemcitabine

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Pancreatic cancer remains a highly chemoresistant malignancy. Gemcitabine, the most effective first-line agent available, acts by disrupting cellular replication. Caspases belong to a family of proteases that function as key components of the apoptotic death machinery. We investigated the mechanisms by which gemcitabine blocks proliferation and whether it can induce apoptosis in pancreatic cancer cells. Quiescent pancreatic cancer cells (BxPC-3) were stimulated to proliferate (10% fetal calf serum) with or without gemcitabine, PS-341 (26S proteasome inhibitor), or both. Proliferation was measured by MTT assay and apoptosis by propidium iodine staining. To determine activation of the apoptotic regulatory cell proteins, caspase-3 and cleavage of poly(ADP-ribose)polymerase (PARP) into its 85-kDa fragment were assessed by Western blotting. Gemcitabine at even low doses (10 μmol/L) significantly inhibited cellular proliferation, whereas PS-341 (10 nmol/L) had no effect. With combined treatment, PS-341 potentiated the antiproliferative effects of gemcitabine ($P = 0.001$). At 48 hours, the apoptotic fraction was greatly enhanced by the presence of PS-341 compared with gemcitabine alone. Caspase-3 accumulated as early as 30 minutes and was associated with cleavage of PARP to its apoptotic fragment. Gemcitabine, a nucleoside analogue, may in part exert its antiproliferative effects by directing pancreatic cancer cells to a default pathway of apoptosis. 26S proteasome inhibition potentiates this effect, suggesting its potential clinical value against chemoresistance in pancreatic cancer. (J GASTROINTEST SURG 2004;8:1072–1078)

KEY WORDS: Caspase, pancreatic cancer, apoptosis, gemcitabine

Pancreatic cancer remains a highly chemoresistant malignancy. In the United States, 30,700 new cases of pancreatic cancer were diagnosed in 2003.[1] The number of deaths from this disease is almost equal at 30,000, resulting in a depressing survival rate of 4% at 5 years. Surgical resection is the only current modality for cure; however, with most patients diagnosed at advanced stages, the need for adjuvant therapies is paramount.

Gemcitabine (2′,2′-difluorodeoxycytidine) is a pyrimidine analog of cytidine. It achieves its antineoplastic effect by incorporation into both DNA and RNA, resulting in masked DNA termination and inhibiting DNA synthase activity.[2] Gemcitabine is the first chemotherapy agent to show a comparable, if not slightly improved, median survival compared with 5-fluorouracil (5.65 months versus 4.41 months, $P = 0.0025$).[3] For this reason, gemcitabine is generally indicated as a first-line agent for locally advanced and metastatic pancreatic cancer and is prescribed to a majority of patients with this disease.

Our group and others[4–8] investigated the possible mechanisms for chemoresistance in pancreatic cancer. Shah et al.[6] showed that 26S proteasome

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inhibition resulted in block of the activation of the NF-κB pathway, significantly inhibited tumor cell proliferation, and produced highly apoptotic tumors in nude mice. In the current study, we examined whether gemcitabine would inhibit cell growth and induce apoptosis and through which cellular mechanism this would occur. We also studied the effects, if any, proteasome inhibition would have on these cellular activities.

MATERIAL AND METHODS

Cell Culture and Treatments

The BxPC3 human pancreatic adenocarcinoma cell line was obtained from the American Type Culture Collection (Manassas, VA). Cells were grown and propagated in RPMI supplemented with 10% fetal calf serum (FCS), 1% penicillin, and streptomycin and maintained at 37°C and 5% CO_2 atmosphere. PS-341 (Millennium Pharmaceuticals Inc, Cambridge, MA), a dipeptide boronic acid inhibitor, was prepared in dimethylsulfoxide (DMSO) and stored at 4°C. Gemcitabine (Gemzar; Eli Lilly Co., Indianapolis, IN) was reconstituted in sterile water and stored at −20°C. All assays were carried out in triplicate. After serum starvation for 60 hours to induce quiescence, cells were stimulated to proliferate by re-supplementation with media containing mitogen (10% FCS) with or without PS-341 (10 nmol/L) or gemcitabine (10 μmol/L or 100 μmol/L) or in combination.

Cellular Extracts

Cytoplasmic and nuclear extracts were obtained using a Nuclear Extract Kit (Active Motif, Carlsbad, CA). Briefly, the cells were collected in ice-cold phosphate-buffered saline (PBS) in the presence of phosphatase inhibitors to limit further protein modifications. The cellular solution was centrifuged to collect the cell pellet. Next, the cells were resuspended in hypotonic buffer on ice with the addition of detergent. After centrifugation and collection of the cytoplasmic fraction, the nuclei were lysed and nuclear proteins were solubilized in lysis buffer (10% dithiothreitol [DTT], 1% protease inhibitor cocktail, lysis buffer). Both cytoplasmic and nuclear fractions were stored at −80°C until use.

Proliferation Assay

BxPC3 cell proliferation was determined by MTT assay. Cells (2×10^4) were plated onto 96-well plates and allowed to adhere for 6 hours. Cells were then placed in media containing specified treatment regimens. At the end of the specified treatment period, the cells were placed in phenol-free Dulbecco's modified Eagle's medium (DMEM) with 0.1 mg/ml of MTT [3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (Sigma Chemical, St. Louis, MO)] and incubated at 37°C, 5% CO_2 for 3 hours. The medium was aspirated, and the formazan crystals were dissolved in 100 μl of DMSO. An μQuant Microplate Reader (Bio-Tek Instruments Inc., Winooski, VT) measured absorbance at 570 nm, with a reference of 750 nm. Proliferation was defined in relation to that of the untreated cells by the following equation: [Proliferation (% control) = 100 × (absorbance treated sample)/(absorbance of cells before treatment)].

Cell Cycle Progression and Apoptosis

Cells (1×10^6) were plated in parallel in 100-mm^2 culture flasks. After serum starvation for 60 hours, quiescent cells were repleted with 10% FCS with or without specified treatments. Cells were harvested daily by trypsinization, with care to include any floating cells, and then suspended in PBS. They were then fixed with 70% ethanol and stored at −20°C. After 72 hours, the ethanol was aspirated and cells were treated with propidium iodide at room temperature in darkness for 30 minutes. DNA histograms were obtained by fluorescence-activated cell sorting analysis (FACS) using FACScan with CellQuest software for acquisition (Becton Dickinson, San Jose, CA). Cell cycle analysis was performed using the Modfit Program (Verity Software, Topsham, ME). Apoptosis was measured by quantifying the sub-G_0 peak, whereas cell cycle progression was measured with corresponding absorbances for G_0-G_1, S, and G_2-M phases.

Fluorescence-Microscopy

Cells (1×10^6) were plated in parallel in 100-mm^2 culture flasks. After serum starvation for 60 hours, quiescent cells were repleted with 10% FCS with or without specified treatments. After 72 hours of treatment, the medium was removed and 100 μl of the Annexin-V-FLUOS labeling solution (Roche, Germany) was applied. Cells were further incubated for 15 minutes at 25°C. Analysis was performed by fluorescence microscopy with an excitation wavelength of 450 nm and detection wavelength of 565 nm.

Immunoblotting

Equal protein loading was verified by Bradford protein quantification assay (Bio-Rad, Hercules, CA). Samples were solubilized in lithium dodecyl sulfate (LDS) sample buffer containing 0.5 mol/L DTT for 10 minutes at 70°C and loaded onto a NuPage Novex Bis-Tris [bis(2-hydroxyethyl)imino-Tris(hydroxymethyl)methane-HCl] gel (InVitrogen, Carlsbad, CA). After electrophoresis, proteins were

transferred to an Imobilon-P membrane (Millipore, Bedford, MA) at 30 V for 60 minutes. After incubation overnight at 4°C with 1:1000 primary antibody dilution buffer (5% milk with 0.05% [TBS (Tris-buffered saline) and 0.05% Tween]), membranes were developed using a secondary antibody dilution buffer by enhanced chemiluminescence (Cell Signaling Technology, Beverly, MA). Immunoblots were analyzed using primary antibodies for caspase-3 and poly(ADP-ribose)polymerase (PARP) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Statistical Analysis

For all data, effects of treatments and time were evaluated using analysis of variance (ANOVA) for repeated measures. When these effects were significant, pairwise differences were evaluated using Tukey's HSD (Honestly Significant Difference) to compensate for the additive Type I error due to multiple comparisons. Analyses were performed using the XLSTAT-Pro statistical software package.

RESULTS

Gemcitabine Significantly Inhibits Cell Growth and These Effects Are Potentiated by Proteasome Inhibition

BxPC-3 cells were treated with either 10 μmol/L or 100 μmol/L gemcitabine alone, 10 nmol/L PS-341 alone, or in combination for 0, 24, 48, 72, and 96 hours. Cells treated with mitogen (10% FCS) are used for comparison. Cells treated with 10 μmol/L gemcitabine (Fig. 1, *A*) significantly inhibited growth at 72 hours ($P = 0.0001$) and cells treated in combination reach statistically significant decrease in proliferation as early as 48 hours and this effect persists over time ($P = 0.0017$). Cells treated with mitogen proliferate, so that at 72 hours there are approximately 3 times as many cells than were initially plated.

Treatment with 100 μmol/L (Fig. 1, *B*) gemcitabine resulted in inhibition of cell growth at 48 hours ($P = 0.0001$). When cells are treated in combination with 10 nmol/L PS-341, the decline in cell viability is more pronounced and reaches statistical significance at 48 hours ($P = 0.017$). This decline in cell proliferation continues over time so that at 96 hours there is a sixfold decrease in the number of cells compared with mitogen-stimulated cells. This decline in growth is statistically significant at 96 hours compared with the cells treated with 100 μmol/L gemcitabine as well ($P = 0.0001$). As with low-dose gemcitabine treatment, cells treated with mitogen proliferate so that at 96 hours there is fourfold more cells than were initially plated. Treatment with 10 nmol/L PS-341 alone shows little perturbance in the proliferation curves. These results suggest that treatment with gemcitabine and, to a greater degree, cells treated in combination with proteasome inhibition do, in fact, inhibit BxPC-3 cells from proliferating.

Gemcitabine Induces Apoptosis and Cell Cycle Arrest

BxPC-3 cells were treated with 10 μmol/L gemcitabine alone or in combination with 10 nmol/L PS-341 for 48, 72, and 96 hours, and cell cycle analysis was performed as described. As shown in Figure 2,

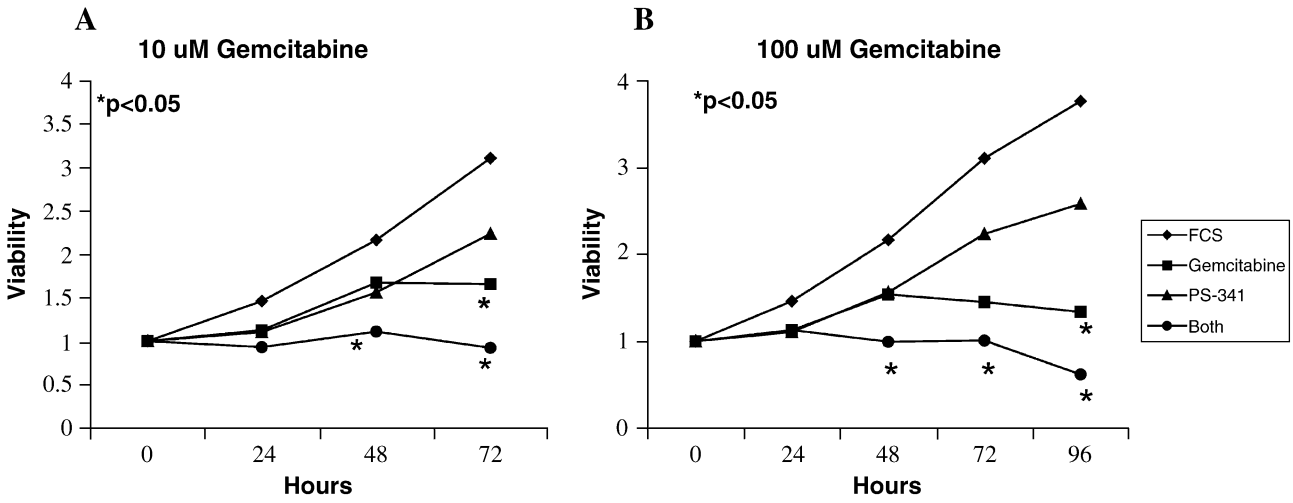

Fig. 1. Proliferation following treatment with 10 μmol/L gemcitabine (**A**) or 100 μmol/L gemcitabine (**B**) over time. Mitogen-stimulated cells (*diamonds*) and PS-341–treated cells (*triangles*) proliferate over time. Gemcitabine-treated cells (*squares*) show significant decrease in proliferation ($P < 0.05$), and combination with PS-341 (*circles*) further abrogates proliferation ($P < 0.05$). (Representative graph from three separate experiments.)

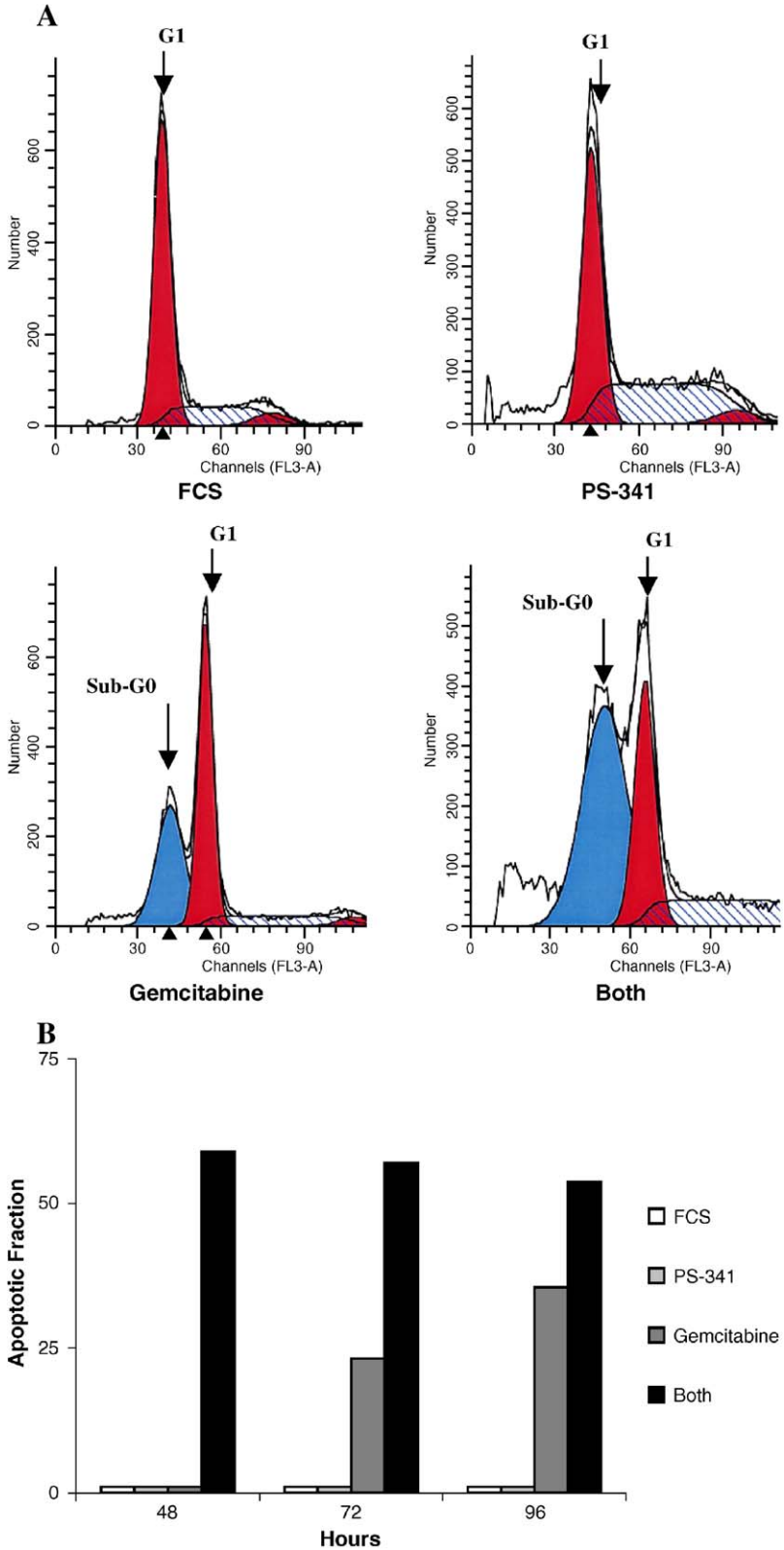

Fig. 2. Sub-G_0 peak at 96 hours. (**A**) Cells treated with either gemcitabine alone or in combination with PS-341 show large numbers of cells in sub-G_0 phase, indicating apoptosis. (**B**) Apoptotic fraction at time points 48, 72, and 96 hours. Cells treated in combination with gemcitabine and PS-341 show increased apoptotic fractions over all time points. (Representative figures from three separate experiments.)

at 96 hours PS-341 did not result in a significant portion of cells in the sub-G_0 population. Gemcitabine treatment alone resulted in 35.52% of cells undergoing apoptosis. The combination of gemcitabine and PS-341 resulted in a significant increase in the number of cells in the sub-G_0 phase (58.85%), suggesting PS-341 is able to enhance the mechanism of gemcitabine-induced apoptosis.

To confirm the effects seen by cell cycle analysis were due to treatment-induced apoptosis, Annexin-V staining was performed. This modality allowed specific differentiation between cells undergoing apoptosis and necrotic cell debris. Cells were treated with 10 μmol/L gemcitabine alone or in combination with 10 nmol/L PS-341 for 72 hours and were processed for Annexin-V labeling. As seen in Figure 3, cells treated with both gemcitabine and PS-341 at 72 hours induced a greater proportion of apoptotic cells than did either modality alone. This finding supports synergistic activity between PS-341 and gemcitabine.

Gemcitabine Induces Caspase-3 Activation

PARP is a 112,000-Da protein that is a substrate for caspase-3. When activated, caspase-3 cleaves PARP to its 85,000-Da form as detected by immunoblotting. Immunoblots of cellular extracts of BxPC-3 cells treated with gemcitabine or PS-341 or in combination for 0, 0.5, 1, and 4 hours were obtained (Fig. 4). Cells treated with gemcitabine alone or in combination with PS-341 show PARP cleavage as early as 0.5 hour of incubation and increases at 1 hour. This was accompanied by a decrease in the noncleaved form of PARP. Cells treated with FCS and PS-341 show some baseline activation of PARP, but the presence of cleaved PARP does not persist over time.

PARP cleavage implies activation of caspase-3. Immunoblots to detect the procaspase-3 form at 32,000 Da and its active form, a 17,000-Da protein, were performed on cells treated under the same conditions as for PARP cleavage (Fig. 5). Cells treated with gemcitabine alone or in combination with PS-341 show evidence of activated caspase-3 at 0.5 hour, and this detection continues through to 4 hours of incubation. PS-341–treated cells also indicate the presence of activated caspase-3 but to a lesser degree than the other treatment groups. These results taken together indicate that gemcitabine is able to induce BxPC-3 cells to undergo apoptosis as evidenced by activation of caspase-3, the cleavage of PARP, and an increase in the population of cells in the sub-G_0 phase.

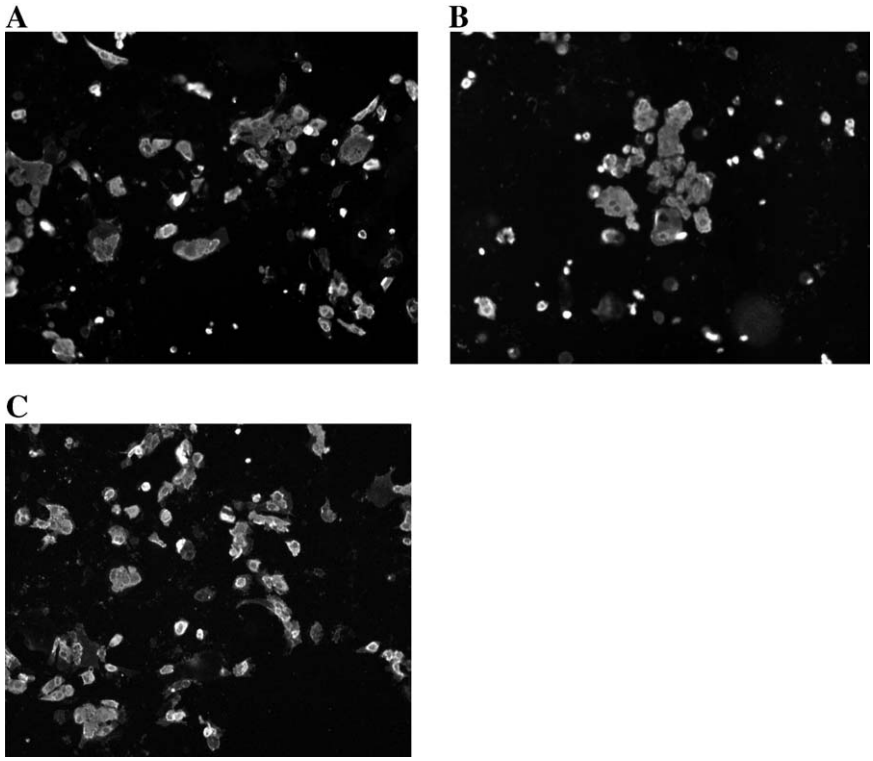

Fig. 3. Annexin-V labeling. (**A**) Gemcitabine alone at 72 hours. (**B**) PS-341 alone at 72 hours. (**C**) Gemcitabine and PS-341 at 72 hours.

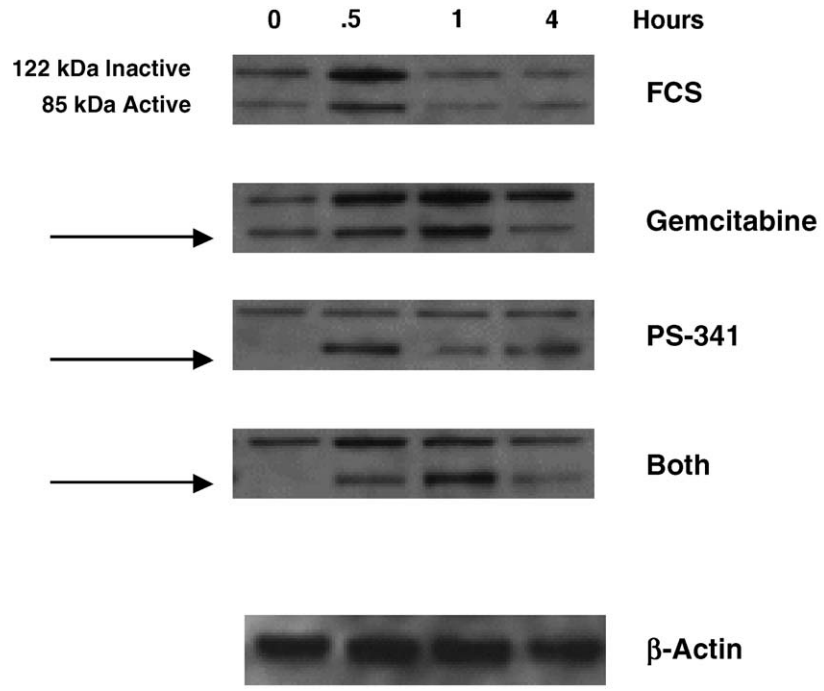

Fig. 4. Poly(ADP-ribose)polymerase (PARP) cleavage. Cells treated with gemcitabine or in combination with PS-341 show PARP cleavage at 0.5 hour seen at 85 kDa. PS-341 alone shows some expression of PARP cleavage. β-Actin serves as internal control.

DISCUSSION

In the present study, we investigated the pathway by which gemcitabine causes apoptosis in a single human pancreatic adenocarcinoma cell line, BxPC-3, showing that caspase activation is required for this activity. Combining treatment with a 26S proteasome appears to intensify these results. Our studies indicate that gemcitabine decreases proliferation through the process of cell death and that, when combined with 26S proteasome inhibition, pancreatic cancer cells are more efficiently directed to this default pathway of apoptosis. Currently, these results are shown only in single pancreatic cell line, and further investigations with multiple pancreatic cell lines are needed to further validate these findings.

Caspases belong to a family of cysteine proteases that have been recognized to play a critical role in programmed cell death. In response to proapoptotic signals, such as cytotoxic stimulation or particular ligand binding to cellular receptors, a set of initiator caspases are activated (caspase-2, -8, -9, and -10). These initiator caspases in turn cleave effector caspases (caspase-3, -6, and -7) from an inactive or procaspase structure to an active form. This is achieved

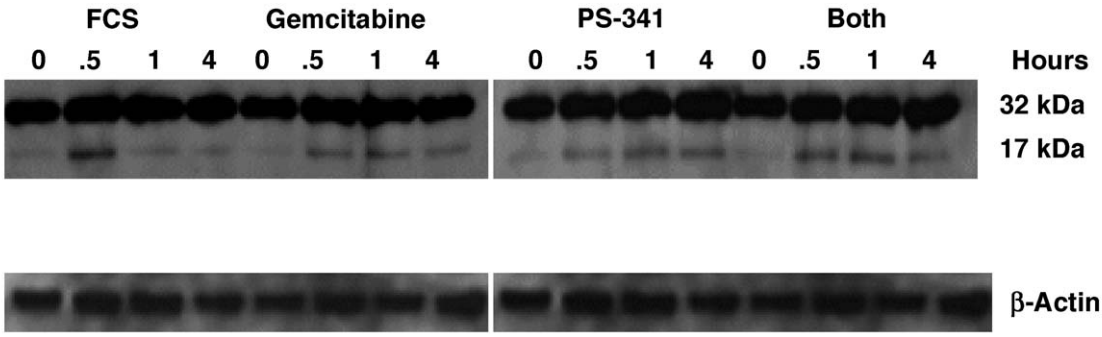

Fig. 5. Caspase-3 activation. Gemcitabine and combination groups show activated caspase-3 expression at 0.5 hour and remain elevated over time. PS-341 treatment results in mild increased expression in activated caspase-3 in a time-dependent manner. β-Actin serves as internal control.

through cleavage at a specific ASP residue.[9] The activated caspase is able to act back on the initiator caspases, in an autoamplification step or lead directly to cell death.

Several groups have investigated the role that caspases play in the apoptotic pathway in pancreatic cancer.[10–12] Satoh et al.[10] examined whether caspase-3 expression reflects the biological behavior in pancreatic ductal carcinoma (PDC) and intraductal papillary mucinous tumor of the pancreas (IPMT). Caspase-3 was overexpressed in PDC and invasive forms of IPMT compared with normal pancreas tissue and benign forms of IPMT. More specifically, caspase-3 expression localized to the cytoplasm of these cells strongly correlated with more invasive tumors. In ASPC-1 pancreatic cancer cells, gemcitabine increased activation of caspase-3 up to ninefold compared with controls.[11] In a third report, Meggiato et al.[12] investigated the correlation of caspase-3 and c-Jun expression in pancreatic duct cancers. Caspase-3 was expressed in 83% of primary tumor samples, and a significant correlation was found between caspase-3 and the proto-oncogene c-Jun ($P < 0.01$).

In a recently published study, caspase activation was shown to be a required step for gemcitabine-induced apoptosis in multiple myeloma (MM) cell lines.[13] After treatment with gemcitabine, MM cells caused apoptosis, as evidenced by an increase in DNA cleavage, activation of caspase-3 activity, and cleavage of PARP. Taken together, these findings confirm that gemcitabine induces apoptosis in a caspase-dependent manner. Several current clinical studies using gemcitabine in patients with relapsed or refractory MM have shown significant efficacy, and phase II studies are currently ongoing.[14,15]

Another highly chemoresistant malignancy is non−small cell lung cancer (NSCLC). A recent study sought to determine the specific apoptotic signaling pathways activated in NSCLC cells after treatment with gemcitabine after inhibition of the NF-κB pathway.[16] Similar to our use of 26S proteasome inhibition to block NF-κB, these authors generated NSCLC cells lacking functional NF-κB activity. It was determined that these cells underwent greater apoptosis and resulted in increased activity of caspase-9 and -3. Inhibition of caspase activity served to "rescue" these cells from cell death, strengthening the argument that gemcitabine-induced apoptosis is caspase dependent.

These studies present strong evidence that caspase-3 is a key mediator in the process of chemotherapy-induced apoptosis in pancreatic and other highly chemoresistant cancers.

In conclusion, our study has shown that gemcitabine-mediated apoptosis is caspase dependent and that additionally blocking the NF-κB pathway with proteasome inhibition results in a greater apoptotic effect. Combination treatment modalities including proteasome inhibition may have therapeutic applications in the enhancement of chemotherapy in human pancreatic cancer.

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Half of the Current Practice of Gastrointestinal Surgery Is Against the Evidence: A Survey of the French Society of Digestive Surgery

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The French Society of Digestive Surgery conducted a survey among its members to assess whether or not the routine practice of gastrointestinal surgery is evidence based. The questionnaire included 13 questions focusing on several aspects of gastrointestinal surgery and for which strong evidence exists. The participants (n = 379) were asked to respond according to their usual practices. The response rate was 75%. Only 57% ± 15% of the responses were in accordance with the evidence. That rate of evidence-based responses did not differ according to the age of participants but was higher at university hospitals ($P = 0.05$). (J GASTROINTEST SURG 2004;8:1079–1082)

KEY WORDS: Surgical practice, evidence-based medicine, survey

In the field of gastrointestinal surgery, an increasing number of randomized trials, meta-analyses, and guidelines are being published.[1] Thus many questions concerning routine surgical practices can be now answered by evidence-based medicine. However, until now very few studies have focused on the impact of such data or guidelines on surgical practices. Thus we tried to determine whether gastrointestinal surgeons in France incorporate the findings from these meta-analyses and randomized trials into their practices.

METHODS

A survey was conducted among members of the French Society of Digestive Surgery (SFCD). The questionnaire included 13 questions for which there are correct answers with a good amount of evidence based on meta-analyses, randomized trials, or well-conducted prospective trials. Four questions were related to specific clinical guidelines published by the SFCD.[2,3] The questions pertained to the following aspects of gastrointestinal surgery (additional references providing evidence for determining the correct answers to these 13 questions are listed in Table 1): preoperative chest radiography, preoperative bowel preparations, intestinal anastomoses (small bowel and colorectal anasotomoses), inguinal hernia repair, laparotomy closure (fascial and skin), intraoperative cholangiography, laparoscopic fundoplication, drainage after colectomy, postoperative use of nasogastric tubes, and postoperative feeding. Participants were asked to choose one of four responses (never, rarely, often, or always); they were also asked to give responses that closely reflected their daily practices. The answers were analyzed using a binary system—that is, the responses "never" and "rarely" were considered together indicating a negative response and the answers "often" and "always" indicated a positive response; the one exception was question 13 where

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Table 1. Thirteen questions presented to French digestive surgeons, with the correct answers and confirming evidence

Question	Evidence-based answer	References
Do you obtain a chest radiograph before operating on a 25-year-old adult for appendicitis?	Never or rarely	7
Do you use postoperative nasogastric aspiration for left colectomy?	Never or rarely	8–10
Do you leave an abdominal drain during right colectomy?	Never or rarely	11
Do you perform a mechanical anastomosis for intraperitoneal colorectal anastomosis?	Never or rarely	12
Do you close the midline laparotomy using an interrupted suture of braided thread?	Never or rarely	13, 14
Do you use staples for cutaneous suturing?	Often or always	15–17
Do you permit enteral feeding on the first post-laparotomy day?	Often or always	18
Do you repair an inguinal hernia in a 45-year-old man using the Shouldice technique?	Never or rarely	19, 20
Do you prepare the colon before elective surgery by means of oral polyethylene glycol?	Never or rarely	21, 22
Do you leave the skin open after an appendectomy for gangrenous appendicitis?	Never or rarely	23
Do you perform a mechanical anastomosis for small intestine resection?	Never or rarely	24–26
Do you perform intraoperative cholangiography in a patient with biliary lithiasis, normal serum liver enzymes, and a small common bile duct?	Never or rarely	27–29
Do you ligate short gastric vessels during laparoscopic total fundoplication?	Rarely or often*	30–33

*Answers indicate that vessel ligation should be performed on demand.

the answers that were grouped together were "never" and "always" in some cases, and "rarely" and "always" in others. The format of the questions varied so that the evidence-based responses could all be either "yes" or "no." Other variables included age (younger or older than 55 years) and activity sectors (university hospital vs. district or private hospital). Questionnaires were distributed by postal delivery or via e-mail. Data were analyzed as means (± standard deviation).

RESULTS

Of the 379 active members of the SFCD, 283 (75%) sent back their completed questionnaires (for a total of 3465 responses); only 57 ± 15% of the answers were found to be in accordance with the evidence. There were four questions for which there was good evidence-based agreement among a majority of the participants: (1) no chest radiograph before an appendectomy in a young male patient (91%); (2) fascial midline closure using a running suture (87%); (3) manual suturing of the small bowel (83%); and skin closure for gangrenous appendicitis (77%). There were another four questions that were answered correctly by slightly more than half of the participants: (1) mesh herniorrhaphy in a 45-year-old man (63%); (2) no drainage after a right colectomy (60%) and (3) no gastric tube after left colectomy (54%); and (4) short gastric vessel ligation "on demand" during fundoplication (51%). However, there were five questions that less than half of the participants answered correctly: (1) skin closure using staples (49%); (2) manual intraperitoneal colorectal anastomoses (42%); (3) no routine cholangiography during cholecystectomy for the case reported in the questionnaire (35%); (4) oral feeding permitted on the first postoperative day after an elective colectomy (30%); and (5) no polyethylene glycol preparation before elective colectomy (25%).

When we analyzed the four questions covering the guidelines published by the SFCD (small bowel and colonic anastomoses, drainage after colectomy, and skin closure), only the guidelines on small bowel manual anastomoses were followed by a majority of participants. The guidelines on drainage, colorectal anasotomosis, and skin closure were followed by only half of the participants.

There was no statistically significant difference according to the age of the participants: 57.3% (±13.8%) of good responses for participants less than 55 years of age vs. 55.4% (±14.6%) for participants older than 55 ($P = 0.34$). However the difference was significant when results were analyzed according to the activity setting; there were 59.2% (±17.6%) good responses for participants working in university hospitals vs. 55.2% (±13.5%) for participants working in other settings ($P = 0.05$).

DISCUSSION

This study was limited to the types of procedures for which strong evidence is available. It became clear, according to the responses in this survey, that approximately half of French gastrointestinal surgeons are

practicing surgery in a way that goes against sound evidence. We are not discussing the numerous surgical questions that have not been answered by meta-analyses or randomized trials; we are specifically referring to evidence-based answers, and this rate of 57% correct responses is disappointing. This low rate of evidence-based responses covering several aspects of the routine practice of gastrointestinal surgery confirms the gap between the "bench" (i.e., evidence-based medicine) and the "bedside" (i.e., daily practice). This has already been reported among general practitioners[4] and colorectal surgeons,[5] and raises the question of whether evidence is incorporated into actual practice. The results of this study raise more questions than they answer. In our opinion, this study demonstrates a general attitude among most surgeons that they are not willing to change their habits following publication of a given meta-analysis or randomized trial. Even published guidelines with specific recommendations failed to change the practices of members of the SFCD. A recent analysis from the United Kingdom[6] showed that the impact of National Institute for Clinical Excellence (NICE) guidance was poor in the field of laparoscopic herniorrhaphy. Greater efforts should be made to bridge the gap between the results of scientific research and routine practices, to promote the culture of evidence-based surgery and to make our guidelines more effective.

CONCLUSION

The present survey demonstrated that at least in France almost half of the routine practice of gastrointestinal surgery goes against evidence reported in the literature. Greater effort should be made to implement the results of randomized trials into the practice of surgery.

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Effect of Ileo-Jejunal Transposition on Ileal Longitudinal Smooth Muscle Contractility In Vitro in Rats

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The aim of the present paper was to study the effects of ileo-jejunal transposition (IJT) on ileal contractile activity in vitro in rats. Male Sprague-Dawley rats were divided into three groups: control, IJT, and sham. In rats with IJT, the distal ileum was interposed isoperistaltically into the proximal jejunum. The jejunoileum was transected and anastomosed at three sites in the sham group. Rats were sacrificed 17–20 weeks postoperatively and the ileal segment was removed. Isometric contractile activity of the isolated ileal longitudinal muscle was measured in tissue chambers. Spontaneous contractile activity was decreased in the IJT group (0.16 ± 0.03 g/min per mg tissue) as compared with the control group (0.25 ± 0.02 g/min per mg tissue, $p < 0.05$). The motor response to cholinergic agonist bethanechol in the IJT group was greater than in the control group above 10^{-6} M dosage. The dose-response curves to adrenergic agonist norepinephrine did not differ between groups. A nitric oxide synthase inhibitor reversed electrical field stimulation-induced inhibition of spontaneous activity in all groups. These results indicate that the response to bethanechol in the IJT group was enhanced in rat ileal longitudinal smooth muscle and this may be an adaptive response to compensate for decreased spontaneous contractile activity.
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Key words: Bethanechol, electrical field stimulation, ileo-jejunal transposition, norepinephrine

Ileo-jejunal transposition (IJT) involves interposing the distal ileum isoperistaltically into the proximal jejunum. We have conducted a series of studies on the effects of IJT[1–4] because this operative procedure seems to be able to improve the severe diarrhea that typically occurs after total proctocolectomy for ulcerative colitis or familial adenomatous polyposis. In dogs with intact colons, IJT leads to marked mucosal hypertrophy along the entire small intestine.[1,2] We also determined that in dogs with intact colons, although IJT delayed gastric emptying with inhibition of post-prandial gastric contractions, small intestinal motility and transit was unchanged.[3] In dogs with IJT and total colectomy, delayed gastric emptying was observed, but we were not able to measure small intestinal contractile activity in this model.[4] These changes in IJT seem to be mediated, at least in part, by increases in plasma concentrations of enteroglucagon and/or peptide YY (PYY), presumably in response to contact of endocrine cells in the ileal mucosa with undigested chyme.[1–4]

We were interested in small intestinal smooth muscle contractility in vitro after IJT, because it might be altered even though IJT in dogs did not affect the global motor patterns. Studying small intestinal contractility in vitro might be of help with regard to understanding small intestinal motility and transit in vivo after IJT. The aim of the present study was to investigate the effect of IJT on ileal longitudinal contractile activity in vitro in rats. We studied spontaneous activity, dose-response to cholinergic agonist bethanechol and adrenergic agonist norepinephrine, and frequency-response to electrical field stimulation (EFS). Our hypothesis was that spontaneous activity in IJT is decreased when compared with a control group.

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MATERIALS AND METHODS

Preparation of Animals

Procedures and animal care were performed according to the guidelines of the Animal Care and Use Committee of the Tohoku University. Sprague-Dawley rats (SLC Co., Tokyo, Japan) weighing 250–300 g were used in all experiments. Rats were anesthetized by inhalation of halothane (Fluothane; Takeda Chemicals Co., Osaka, Japan) and intraperitoneal sodium pentobarbital (Nembutal; Abbott, North Chicago, IL) at the dose of 40 mg/kg. IJT in rats was performed similar to the procedures used in dogs.[3] Briefly, a 15-cm long ileal segment (5 and 20 cm proximal to the ileocecal sphincter) was transected and interposed isoperistaltically into the proximal jejunum 5 cm distal to the ligament of Treitz by an end-to-end jejuno-ileostomy and an end-to-end ileo-jejunostomy. The length of the interposed segment (15 cm) corresponds to 1/4 length of the jejunoileum as in our previous dog study.[3] Continuity of the remaining ileum was restored by an end-to-end ileo-ileostomy. As a sham operated group, the jejunoileum was transected and reanastomosed at three sites: 5 cm distal to the ligament of Treitz and 5 and 20 cm proximal to the ileocecal sphincter, respectively. All rats were allowed free access to water and rat chow from the day of operation onward. Rats were studied 17–20 weeks after IJT (IJT; N =6) and sham operation (sham; N =5). Rats without any surgical procedure that survived for 17–20 weeks after delivery to our institution served as controls (control; N =8).

Recording Contractile Activity

A segment of the distal ileum (10–15 cm proximal to the ileocecal sphincter in control and sham and 15–20 cm distal to the ligament of Treitz in IJT) was removed and cut along the mesenteric border in modified Krebs-Ringer bicarbonate solution (concentrations in mM: NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, calcium disodium edetate 0.26, and glucose 11.1). Eight full-thickness muscle strips per rat were cut in the direction of the longitudinal muscle. Silk loops were tied at both ends of the muscle strips and the muscle strips were suspended vertically in 20 ml tissue chambers filled with modified Krebs-Ringer bicarbonate solution maintained at 37.5°C and bubbled with 95% O_2 and 5% CO_2. The lower end of the muscle strip was connected to a fixed plastic hook in the chamber, while the upper end was attached to a noncompliant force transducer thereby allowing measurement of isometric force.

Experimental Design

After a 90- to 120-minute equilibration period with replacement of the buffer solution in the chamber every 30–40 minutes, each strip was stretched incrementally at 15- to 20-minute intervals to its optimal length (Lo), defined as the length beyond which further stretching did not increase the amplitude of spontaneous activity.[5] All subsequent experiments were performed at this Lo and strips that did not indicate spontaneous activity were not used (less than 5% of strips evaluated).

Two chambers (two muscle strips per rat) were used for the dose-response study to the cholinergic agonist bethanechol and two other chambers were used for the adrenergic agonist norepinephrine. For the experiments with bethanechol, the lowest dose (10^{-7} M) was administered after taking 5 minutes of spontaneous activity as basal activity. The buffer was washed 6 minutes later and the next higher dose was given after a 5- to 6-minute equilibration time. This procedure was repeated until the greatest dose of bethanechol (10^{-4} M) had been given. After recording basal spontaneous activity in two separate chambers, norepinephrine (10^{-8}–10^{-4} M) was administered into the chamber in a cumulative manner every 6 minutes. Four other chambers were used to assess the effect of EFS under nonadrenergic, noncholinergic (NANC) conditions produced by phentolamine (5×10^{-6} M), propranolol (10^{-5} M), and atropine (10^{-6} M). Square wave currents (amplitude =30 V; pulse width =0.5 ms; stimulus duration =10 seconds) were delivered every 4–5 minutes and the responses to varying frequencies (1, 3, 5, 7, 10, 15, 20, and 30 Hz) were studied. The chamber solution was washed after studies of EFS and N^{ω} L-arginine monomethyl ester (L-NAME: 5×10^{-3} M) as well as adrenergic/cholinergic antagonists to produce NANC conditions were administered into the four chambers. Similar experiments with EFS were repeated 30 minutes later to observe the effects of the nitric oxide (NO) synthase inhibitor L-NAME on EFS-induced inhibition of ileal contractile activity. At the conclusion of the experiments, each tissue was blotted and weighed.

Data Analysis

The integral of force generated (area under contractile curve) was measured for 5 minutes in eight chambers at Lo as basal spontaneous activity. Mean amplitude and frequency during this 5-minute duration was analyzed by visual inspection. Responses to bethanechol and norepinephrine were quantitated by measuring the integral of force for 5 minutes immediately after drug administration. The integral of force

was measured by a computer program (MacLab; AD Instruments, Victoria, Australia) and standardized per minute per wet tissue weight of each muscle strip (g/min per mg tissue). The dose-response curves for bethanechol and norepinephrine were obtained by expressing basal spontaneous activity as 100%. To study sensitivity of muscle strips to bethanechol and norepinephrine, the negative log of the equieffective concentration that caused a 50% maximal response was calculated as ED_{half}. Spontaneous contractile activities before and 30 minutes after administration of phentolamine, propranolol, and atropine were compared with the effect of NANC conditions on spontaneous activity. Contractile activity during EFS was quantitated by calculating the force generated during the 10-second stimulus and comparing this value with the force of baseline activity per 10 seconds determined for each strip for a 5-minute period immediately before beginning EFS. The integral of force in response to each frequency was expressed as the percent of basal spontaneous activity. If the percent response was less than 100, EFS was considered to have induced inhibition of spontaneous activity. The frequency-response curve was generated by plotting each percent value (vertical axis) in response to each frequency (horizontal axis). Areas below the horizontal line of 100% and above the frequency-response curve were calculated as "areas of inhibition" to quantitate the inhibitory effect of EFS.

Statistical Comparisons

Student's *t* test was used for comparison of the two groups and analysis of variance. Scheffe's *F* test was used for the analysis of dose-response to bethanechol and norepinephrine. All data were expressed as mean ± SEM.

Drugs

Bethanechol chloride and norepinephrine bitartrate were purchased from the Sigma Chemical Company (Sigma Chemical Co., St. Louis, MO). Atropine sulfate was kindly donated by the Tanabe Seiyaku Company (Tanabe Seiyaku Co., Osaka, Japan). Phentolamine and propranolol chloride were purchased from the Novartis Pharma Company (Novartis Pharma Co., Tokyo, Japan) and the Zeneca Yakuhin Company (Zeneca Yakuhin Co., Tokyo, Japan), respectively.

RESULTS

Spontaneous Contractile Activity

Tracings of representative spontaneous activity in the basal condition in each group are illustrated in Fig. 1. The integral of force was decreased in the IJT group (0.16 ± 0.03 g/min per mg tissue) as compared with the control group (0.25 ± 0.02 g/min per mg tissue, $p < 0.05$), but no statistical difference was noted between the control group and the sham group (0.21 ± 0.04 g/min per mg tissue) or between the sham group and the IJT group. Although mean amplitude was not different between the control group and the sham group, it was reduced in the IJT group as compared with the control group (Table 1). Frequency did not differ between groups (Table 1).

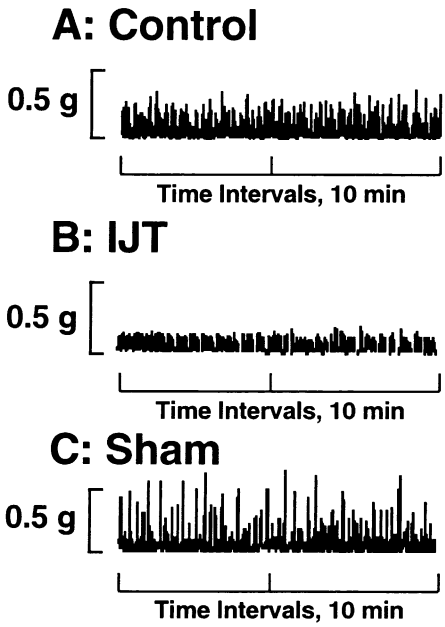

Fig. 1. Representative tracing of basal activity in the control group (**A**), ileo-jejunal transposition (IJT) (**B**), and sham group (**C**). Spontaneous contractile activity was decreased in rats with IJT compared with the other two groups.

Response to Bethanechol and Norepinephrine

Although bethanechol induced a dose-dependent increase in contractile activity in all three groups, a tonic increase of the baseline was observed at 3×10^{-5} and 10^{-4} M in 5 out of 6 rats in the IJT group, but only 1 out of 8 rats in the control group and 1 out of

Table 1. Mean amplitude and frequency in each group

	Control	IJT	Sham
Mean amplitude (mg/mg-tissue)	14.3 ± 3.1	6.7 ± 0.9*	13.2 ± 3.7
Frequency (/min)	8.5 ± 1.0	8.8 ± 1.0	8.3 ± 1.2

*$P < 0.05$, compared to control.

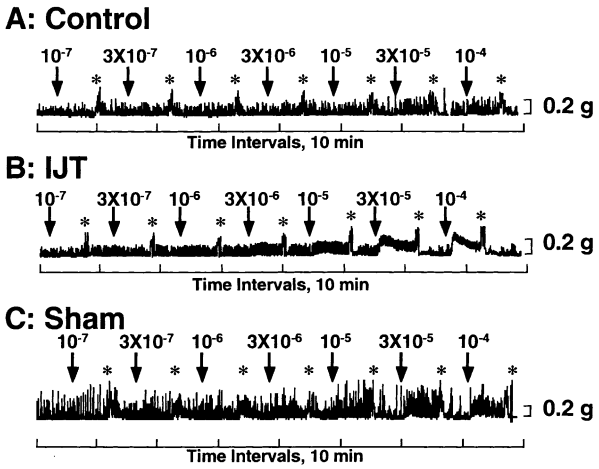

Fig. 2. Representative tracing indicating the effect of bethanechol in the control group (**A**), ileo-jejunal transposition (IJT) (**B**), and sham group (**C**). Bethanechol induced increase in contractility in a dose-dependent fashion in all three groups. Response to bethanechol in the IJT group was greater than the other two groups at the doses of 3×10^{-5} and 10^{-4} M because of tonic increase of baseline. * = washing of the chamber.

5 rats in the sham group (Fig. 2). The motor response to bethanechol in IJT was greater than the control group above 10^{-6} M (Fig. 3, *A*). However, ED_{half} (the negative log of the equieffective concentration that caused a 50% maximal response) in the IJT group did not differ from the control group and the sham group (Table 2), suggesting that the sensitivity to bethanechol is not different between groups. Norepinephrine dose-dependently inhibited spontaneous contractile activity in a similar manner in three groups and the dose-response curve in the IJT group was identical to that in the control group and the sham group (Fig. 3, *B*). ED_{half} was not different between groups (Table 2).

Response to EFS

Administration of phentolamine, propranolol, and atropine to produce NANC conditions did not alter spontaneous activity in all three groups (data not indicated). EFS induced inhibition of spontaneous activity (values less than 100% of baseline) at low frequencies (3–10 Hz) and caused contractions at high frequencies (15–30 Hz) in control rats under basal conditions (Figs. 4, *A* and 5, *A*). This inhibition observed at low frequencies was reversed in the presence of L-NAME (Figs. 4, *B* and 5, *A*) and areas of inhibition were decreased in the presence of L-NAME compared with basal conditions as depicted in Fig. 5, *A* ($p < 0.05$). Inhibition of spontaneous activity at low frequencies and contractions at high frequencies were observed in both the IJT group and the sham group (Figs. 5, *B* and *C*). The effect of L-NAME in reversing inhibition at low frequencies and reducing areas of inhibition was also noted in these groups (Figs. 5, *B* and *C*). These results suggested that EFS-induced inhibitions are induced by NO similarly in all three groups.

DISCUSSION

We determined that after IJT, basal spontaneous activity of rat ileal longitudinal smooth muscle was

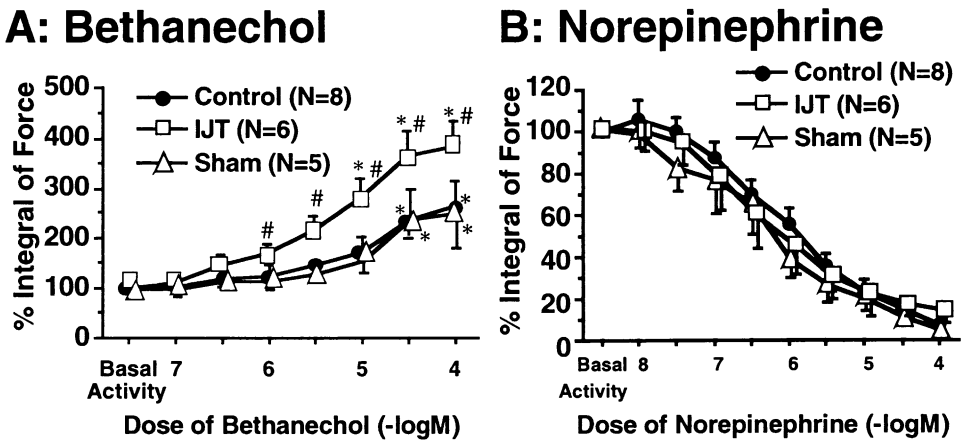

Fig. 3. Dose-response curves to bethanechol (**A**) and norepinephrine (**B**). (**A**) Response to bethanechol in ileo-jejunal transposition (IJT) (N =6) was enhanced compared with the control group (N =8) and the sham group (N =5) above 10^{-6} M dosage. *$p < 0.05$ compared with basal spontaneous activity in the same group. #$p < 0.05$ compared with control of the same dose. (**B**) Dose-response curves to norepinephrine in the three groups were not different from each other (control group: N =8, IJT group: N =6, sham group: N =5). Values are mean ± SEM.

Table 2. ED_{half} for bethanechol and norepinephrine in each group

	Control	IJT	Sham
Bethanechol	6.0 ± 0.2	5.6 ± 0.1	5.8 ± 0.3
Norepinephrine	5.9 ± 0.2	6.3 ± 0.4	6.5 ± 0.4

decreased and the motor response to cholinergic agonist bethanechol was increased without changes in sensitivity. Decreased basal spontaneous contractile activity suggests the possibility that small intestinal transit might be delayed because mechanical activity often correlates with intestinal transit.[5] In dogs with IJT, postprandial gastric motility was inhibited and this inhibition was associated with an increased plasma level of enteroglucagon and PYY.[3] In rats, IJT increased mucosal weight and DNA content in the transposed ileum and increased plasma concentrations of neurotensin.[6] The plasma concentration of glucagon-like peptide-2 was also increased in rats with IJT (unpublished personal data). These results suggest that IJT increases plasma concentrations of peptides mainly released from the ileal mucosa both in dogs and rats. Because those peptides inhibit gastrointestinal contractility and delay transit,[7–9] the decreases in spontaneous contractile activity in the present study might be related to the increase in plasma concentrations of those peptides.

Although the motor response to bethanechol was enhanced in the IJT group as compared with the control group, changes in sensitivity to bethanechol were not obvious. Hypersensitivity or supersensitivity is defined as the phenomenon in which the dose of a substance required to produce a given biological response is less than normal and thus the dose-response curve is shifted to the left.[10] Although the dose-response curve to bethanechol shifted to the left in the IJT group when basal activity was taken as 100%, the negative log of the equieffective concentration that caused a 50% maximal response did not differ between groups. Previous studies reported extrinsic denervation-associated adrenergic hypersensitivity after small bowel transplantation in rat ileum with increased sensitivity to norepinephrine 12 times higher than the control group.[11,12] This hypersensitivity was considered to be caused by an increase of adrenergic receptors on the smooth muscle and not in the enteric nervous system.[11,12] There are five subtypes (M1–M5) for the muscarinic receptors.[13] M2 and M3 receptors are distributed in the rat ileum and the most abundant receptor is the M2 whereas M3 receptors are more minor.[14] Bethanechol seems to stimulate both M2 and M3 receptors. Osinski and Bass[15] reported that active stress generation was increased in response to bethanechol after extrinsic and myenteric denervation. Although we cannot determine the mechanism of enhanced motor response to cholinergic agonist in IJT (increase in cholinergic receptors density, increase in the affinity of cholinergic receptors to bethanechol, or a modification of the receptor signaling), it is possible that the increased response was induced as an adaptive response to compensate for decreased basal spontaneous contractility.

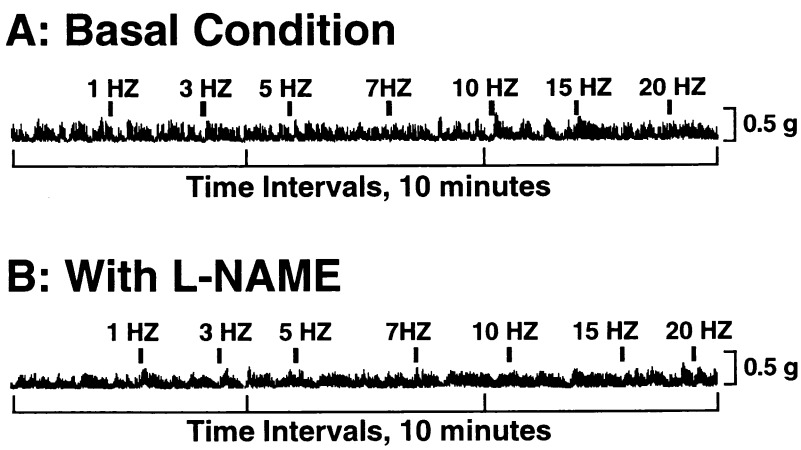

Fig. 4. Representative tracing indicating frequency response to electrical field stimulation (EFS) in a control rat in basal condition (**A**) and with N^{ω} L-arginine monomethyl ester (L-NAME) (**B**). EFS induced inhibition of spontaneous activity at low frequencies (3–10 Hz) and caused contractions at high frequencies (15–30 Hz) in control rats under basal conditions. This inhibition observed at low frequencies was reversed in the presence of L-NAME.

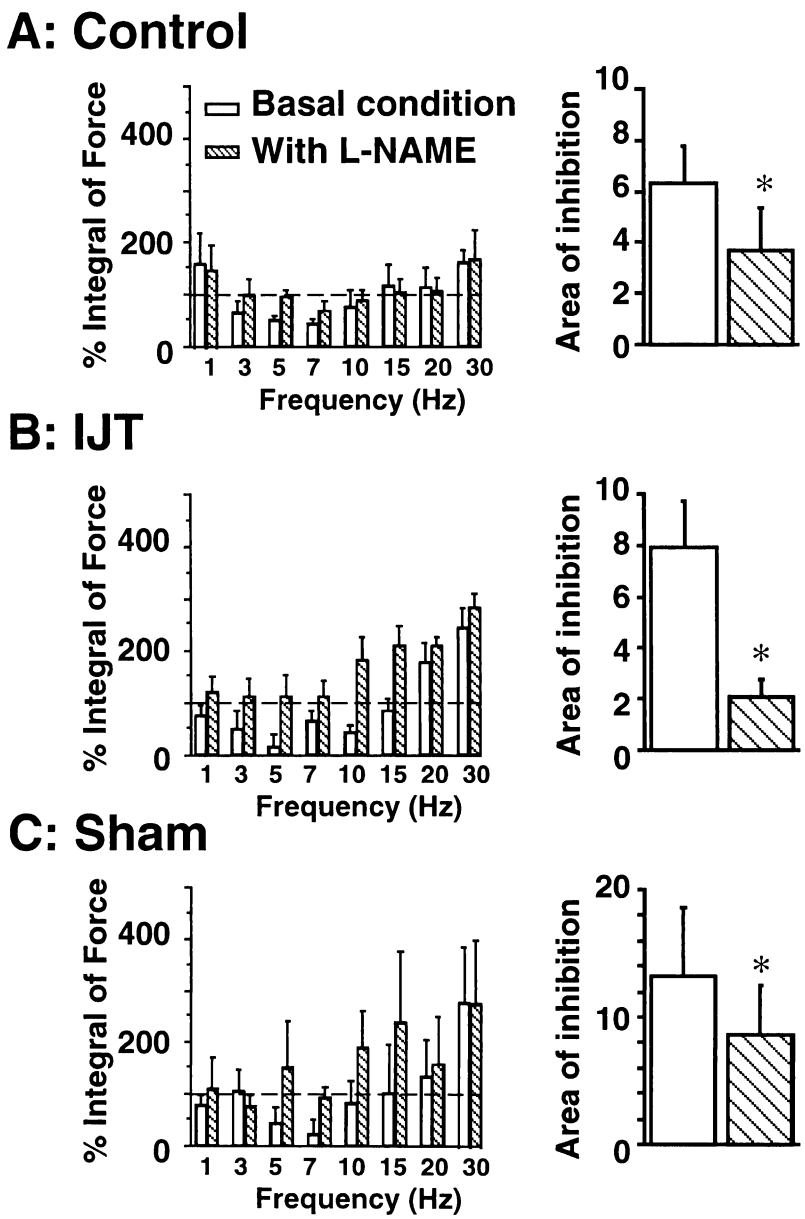

Fig. 5. Frequency-response curves and areas of inhibition in the control group (**A**) N =8, ileo-jejunal transposition (IJT) group (**B**) N =6), and sham group (**C**) N =5 in basal condition and in the presence of N^{ω} L-arginine monomethyl ester (L-NAME). Inhibition of contractility was observed at low frequencies in basal conditions in all groups. This inhibition was reversed by the pretreatment with L-NAME in all groups. Areas of inhibition in the presence of L-NAME were decreased compared with basal conditions in all groups. $^{*}p < 0.05$ compared with basal condition.

EFS induced inhibition of contractile activity at low frequencies, whereas it enhanced contractions at high frequencies. Inhibition at low frequencies was reversed by the NO synthase inhibitor L-NAME. This observation was similar in all three groups suggesting that EFS-induced inhibition at low frequencies is mediated via NO in normal rats and that this inhibitory nitrergic response was not modulated after IJT and sham operation. These results in control rats are consistent with previous studies regarding rat ileal longitudinal muscle.[12,16] Although spontaneous activity was inhibited by NO in jejunal longitudinal muscle, a NO synthase inhibitor did not affect the EFS-induced inhibition.[17] EFS-induced inhibition was not reversed by a NO synthase inhibitor; similarly direct administration of NO into the tissue chamber

did not inhibit basal spontaneous activity in jejunal and ileal circular smooth muscle in rats.[18,19] Thus, the effect of NO or NO synthase inhibitors differs in each site even in the same species. Results in the present study suggest that the decrease in spontaneous contractile activity after IJT does not seem to be caused by changes of nitrergic neurons in the enteric nervous system.

Although we have suggested the possibility that the enhanced motor response to cholinergic agonist in IJT may be an adaptive response to compensate for decreased spontaneous contractile activity in rat ileum, we cannot further clarify the mechanism of decreased spontaneous activity. Most studies regarding postsurgical adaptation focus on mucosal function and changes in serum concentrations of peptides after small intestinal resection.[20] Only a few studies address intestinal adaptation in smooth muscle function postoperatively. We believe our findings contribute to a better understanding of smooth muscle physiology after intestinal surgery.

CONCLUSION

Spontaneous contractile activity was decreased in the IJT group as compared with the control group and the motor response to cholinergic agonist bethanechol in the IJT group was greater than in the control group above 10^{-6} M dosage. These results indicate that the response to bethanechol in the IJT group was enhanced in rat ileal longitudinal smooth muscle and this may be an adaptive response to compensate for decreased spontaneous contractile activity.

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The Beger Procedure—Duodenum-Preserving Pancreatic Head Resection

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Duodenum-preserving pancreatic head resection (DPPHR) was introduced into clinical practice in 1972 after experiments in dogs were completed to elucidate the technique of a segmental resection of pancreatic tissue in regard to early and late histology of the pancreaticojejunostomy, maintenance of adequate vascularization of the pancreatic head rest along the duodenum, and preservation of the exocrine and endocrine function of the pancreas.

THE HEAD OF THE PANCREAS—PACEMAKER OF CHRONIC PANCREATITIS?

The development of an inflammatory mass in the head of the pancreas and the prevalence of pancreatic head enlargement in patients with chronic pancreatitis regarding the epidemiology of the disease are not known exactly. In terms of radiologic diagnosis, using contrast-enhanced CT scanning of the pancreas, approximately 30% to 50% of all patients referred for surgical treatment demonstrate pancreatic head enlargement (>3 to 4 cm). The double-duct system in the pancreatic head—duct of Santorini and duct of Wirsung—is of clinical relevance for the pathomorphogenesis of the inflammation in the pancreatic head, as it is clinically relevant in patients suffering from complete pancreas divisum.[1] Pathomorphologically, patients with an inflammatory mass in the head of the pancreas frequently show focal necrotic lesions, small but rarely large pseudocystic cavities, calcifications of the pancreatic parenchyma, and duct stones in the main duct.[2] The loss of exocrine tissue, mainly the acinar cell component, and the generation of extracellular matrix proteins, including laminin, fibronectin, and collagen, are the main pathomorphologic consequences of the chronic inflammation.[3] The molecular mechanisms that contribute to these changes have been investigated. Overexpression of epidermal growth factor receptor and the c-erb-B2 proto-oncogene, as well as overexpression of transforming growth factor-α are identified as factors influencing the inflammatory process in the pancreatic head.[1,3–7]

In regard to epidemiologic studies, chronic pancreatitis bears the risk of development of ductal pancreatic cancer. In the subset of patients suffering from chronic pancreatitis, after 3 to 20 years of the disease, ductal pancreatic cancer can be found in 2% to 5%.[8,9]

Indications for DPPHR include the following:

- Chronic pancreatitis with an inflammatory mass in the pancreatic head
- Chronic pancreatitis with an intrapancreatic common bile duct stenosis causing cholestasis or jaundice
- Pancreas divisum causing chronic pancreatitis
- Cystic neoplasia of the pancreatic head causing occlusion or compression of the pancreatic main duct and/or the common bile duct
- Adenomatous endocrine neoplasia of the pancreatic head complicating the duct systems
- Chronic pancreatitis causing portal/mesenteric vein compression
- Chronic pancreatitis causing stenosis of the duodenum

Among patients with chronic pancreatitis with an inflammatory mass in the head of the pancreas, 70% to 90% are men, mostly under 40 years of age at the time of diagnosis. The most notable clinical feature is severe medically intractable pain; approximately 75% of the patients suffer from daily severe pain.

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Forty percent to 55% of the patients have a stenosis of the common bile duct, and a severe duodenal stenosis is observed in 5% to 10%.[10]

The presence of a single stenosis of the pancreatic main duct is demonstrated by means of endoscopic retrograde cholangiopancreatography (ERCP) or magnetic resonance imaging (MRI) in approximately 40% of patients; the portal vein system is involved in approximately 12% to 18%.

Diagnostic Workup

The rationale for DPPHR in chronic pancreatitis is the removal of the main inflammatory process, considered to be the pacemaker of the disease, while preserving the upper gastrointestinal tract. The surgical procedure preserves the stomach, duodenum, and biliary tree.

The clinical evaluation includes nutritional status, degree of cholestasis, signs of portal hypertension, and presence of ascites. In each patient, knowledge of endocrine function (i.e., fasting glucose level, oral glucose tolerance test) is mandatory. Regarding exocrine function, most appropriate is the measurement of the fecal elastase, but the pancreolauryl serum test is also sufficient. Laboratory testing includes the tumor markers carcinoembryonic antigen and CA 19-9 in addition to pancreatic and liver function. Regarding invasive investigations, upper gastrointestinal endoscopy and ERCP are indicated. MRI with reconstruction of the intrapancreatic ducts should be done. Pathomorphologic changes of the pancreas are delineated by contrast-enhanced computed tomographic (CT) scanning.

The surgical technique includes the following two major steps:

1. Surgical exposure of the pancreatic head and subtotal resection of the head between the portal vein and the intrapancreatic segment of the common bile duct.[11,12]
2. Reconstruction with Roux-en-Y jejunal loop. Two pancreatic anastomoses must be done.[11,12]

The head of the pancreas is exposed by dividing the gastrocolic ligament, preserving the gastroepiploic vessels. After transection of the duodenocolic ligament, a Kocher maneuver is performed. Exposure of the surgically relevant structures starts with identification of the superior mesenteric vein. Identification of the portal vein in the hepatoduodenal ligament follows. The common hepatic artery and the common bile duct are identified and banded. Starting at the superior mesenteric vein, the groove of the portal vein on the dorsal surface of the pancreatic head is dissected. In most patients it is easy to open this space between the anterior surface of the portal vein and the posterior capsule of the pancreatic head by blunt dissection. In case of an inflammatory process with adhesions to the portal vein, a careful mobilization of the vein from the pancreatic capsule is performed, avoiding injury to the portal or splenic vein. The superior mesenteric vein below and the portal vein above the pancreatic head are looped to control for possible bleeding. After the dissection of the portal vein from the dorsal pancreatic capsule is completed, a silk ribbon is positioned and temporarily tied around the neck of the pancreas resulting in compression of the vessels and enabling lifting of the neck of the pancreas. Before the pancreatic neck is transected, the anterior gastroduodenal artery is identified and ligated near the common hepatic artery. Subtotal resection of the pancreatic head takes place by transecting the neck of the gland, starting at the uncinate edge of the superior mesenteric vein/portal vein. Bleeding vessels are immediately sutured. After the pancreatic main duct is identified, the transection is completed (Fig. 1).

Subtotal resection of the pancreatic head starts after rotation of the head by 90 degrees into an anterior-posterior position (Fig. 2). Blunt dissection of the tissue posterior to the portal vein toward the dorsal pancreatic head capsule eases the exposure of

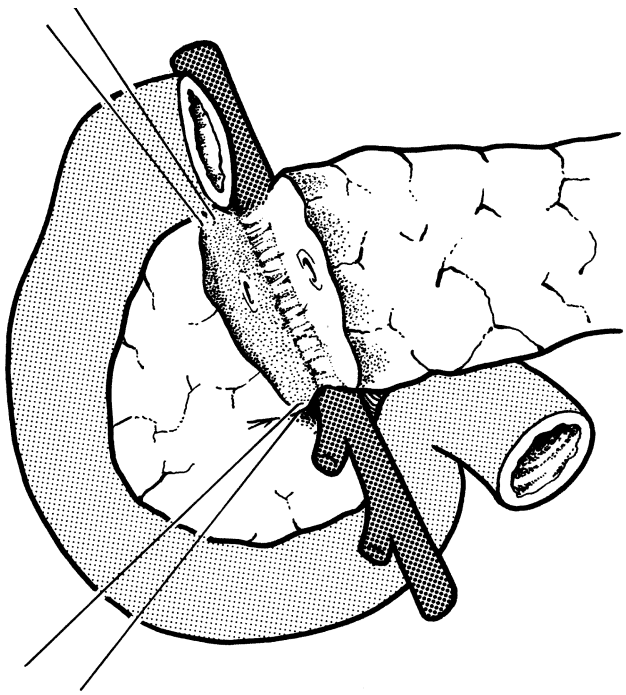

Fig. 1. Duodenum-preserving pancreatic head resection after tunneling of the portal vein behind the pancreas. Transection of the pancreatic neck along the duodenal border of the portal vein.

the pancreatic head. Small branches entering the portal vein directly from the head must be ligated and divided.

The subtotal resection of the pancreatic head starts on the posterior surface of the head after placement of single sutures (3 × 0, resorbable) along the resection line (Fig. 1). The resection along the duodenal wall takes place at a distance of 5 mm from the upper pancreatic head. The transection is completed by dividing the head tissue along the wall of the intrapancreatic portion of the common bile duct toward the papilla (Fig. 3). In some cases, identification of the intrapancreatic common bile duct is difficult. In these cases a Kehr sound is placed toward the papilla in the common bile duct via a small incision in the duct in the hepatoduodenal ligament. It is not necessary to preserve the anterior gastroduodenal artery for adequate blood supply to the duodenum. The supraduodenal vessels as well as the vessels arising from the superior mesenteric artery posterior to the pancreatic head maintain sufficient perfusion of the duodenal wall. Removal of the fibrotic tissue along the common bile duct frequently results in decompression of the duct. If there is inflammation in the wall of the common bile duct, the duct is opened by an incision in the prepapillary duct segment.[11] An internal biliary bypass is performed by including this bile duct incision into the anastomosis between the shell similar

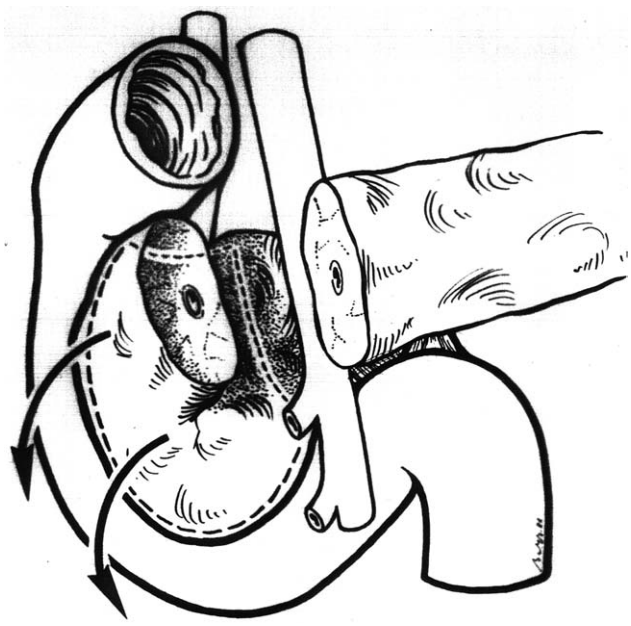

Fig. 2. Subtotal resection of the pancreatic head between the portal vein and the intrapancreatic common bile duct after rotation of the pancreatic head by 90 degrees to the anterior-posterior position.

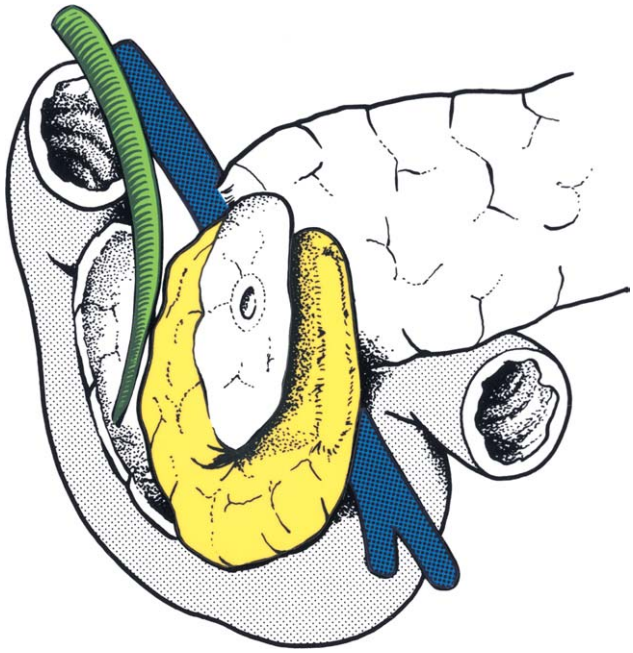

Fig. 3. Subtotal resection results in decompression of the intrapancreatic common bile duct.

to the remainder of the pancreatic head and the jejunum (see below). After subtotal resection of the pancreatic head is completed, meticulous hemostasis, with the use of single stitches (5 × 0, nonresorbable, monofilament sutures), is necessary. Finally, a shell-like remnant of the pancreatic head between the common bile duct and the duodenal wall remains, the distance of the excision line of the pancreatic head to the duodenal wall is 5 mm anteriorly and 2 to 3 cm posteriorly (Figs. 4 and 5).

For reconstruction of the jejunal loop, the jejunum is transacted approximately 20 cm distal to the ligament of Treitz. The jejunal loop is passed through the retrocolic mesenteric cleft to the right of the middle colic artery into the level of the pancreatic head. Reconstruction starts with a side-to-end anastomosis between the jejunal loop and the left pancreas. I prefer to do a mucosa-to-mucosa duct anastomosis (Warren-Cattell anastomosis). In this instance, a duct-to-mucosa anastomosis using six to eight single sutures (6 × 0, resorbable) is carried out (Fig. 6); the anastomosis is completed with sutures (4 × 0, resorbable) between the seromuscularis of the jejunum and the capsule plus tissue of the pancreas.

A side-to-end anastomosis is performed between the jejunal loop approximately 8 cm distal to the left pancreatic anastomosis, with the shell-like remainder of the head (Fig. 7). The jejunal incision is 4 to 5 cm in length. The inner layer of the anastomosis between the jejunum and the pancreas is continuous. The

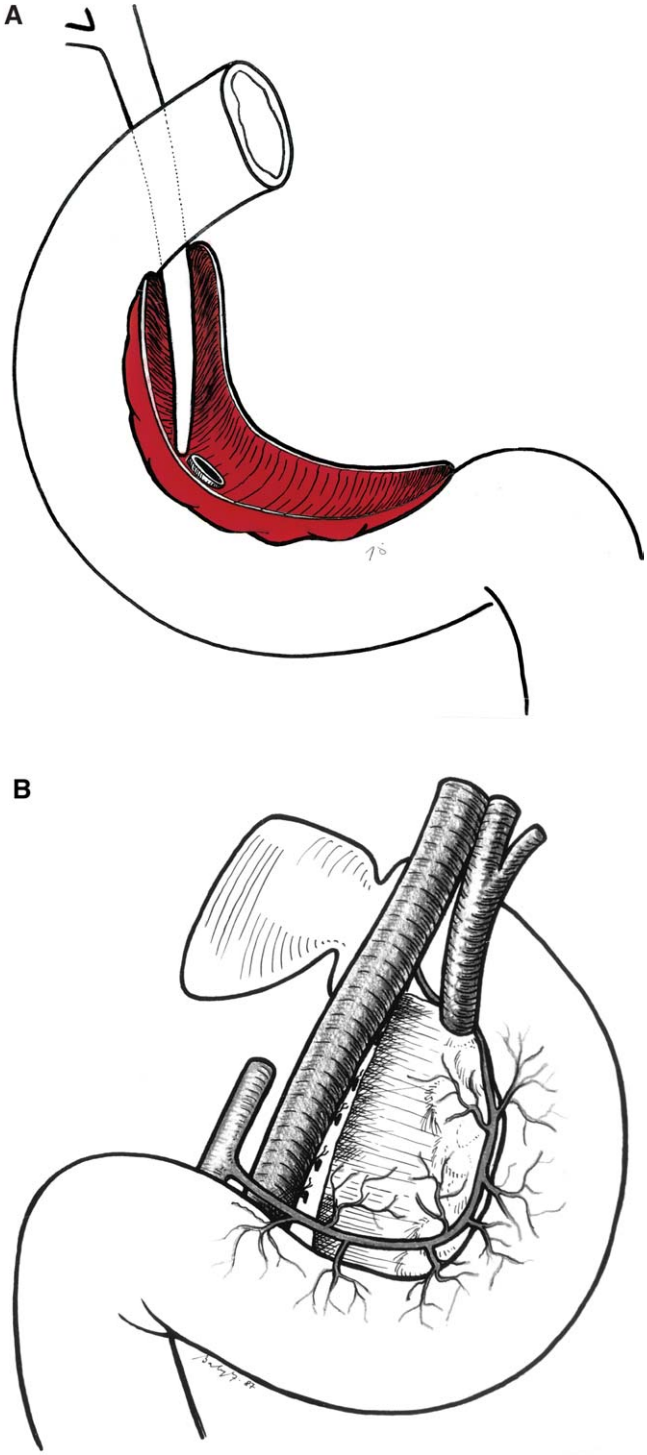

Fig. 4. A, After duodenum-preserving pancreatic head resection, a shell-like structure is created in the remainder of the pancreatic head along the duodenum. The length of the preserved pancreatic head on the posterior side is approximately 2 to 3 cm; on the anterior side it is approximately 0.5 cm. **B**, Posterior view after duodenum-preserving pancreatic head resection. Preservation of the pancreaticoduodenal arteries and the capsule of the pancreas.

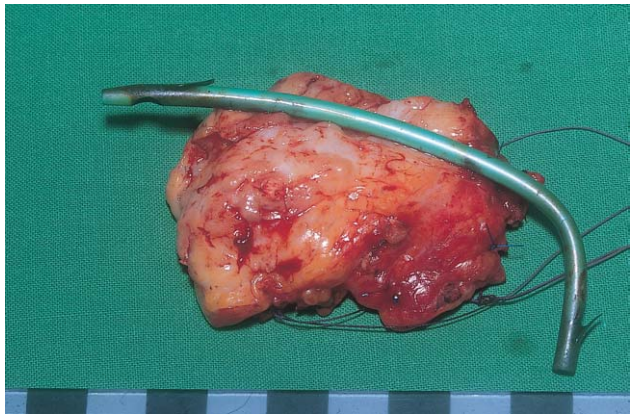

Fig. 5. Duodenum-preserving pancreatic head resection: Operative specimen in a case after failure of stenting of a pancreatic main duct stenosis. Wet weight of the operative specimen was 38 g.

second layer is performed between the pancreatic capsule and the seromuscularis of the jejunum with interruptured sutures. For restoration of intestinal tract continuity, an enteroenteral anastomosis (Roux-en-Y) is carried out 20 cm distal to the pancreatic head anastomosis.

In patients with a prepapillary common bile duct stenosis that persists after subtotal resection of the pancreatic head, an additional anastomosis is created between the suprastenotic portion of the common bile duct and the jejunal loop (Fig. 7). It is not necessary to perform additional side-to-side suturing between the duct and the jejunal wall; the prepapillary incision of the intrapancreatic common bile duct is included in the anastomosis.[12] On the roof of the biliary duct incision, four to six single sutures are placed between the duct and the jejunal wall segment. In

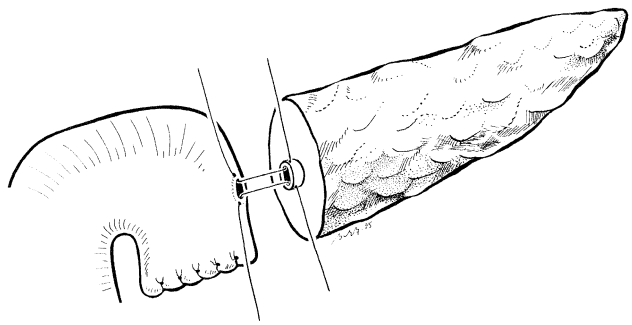

Fig. 6. Reconstruction after duodenum-preserving head resection takes place with a Roux-en-Y jejunal loop; mucosa-to-mucosa anastomosis with the left pancreas is performed (6 × 0, resorbable sutures). For the outer layer, 4 × 0 sutures are used between the pancreatic tissue and the seromuscularis of the jejunal loop (not shown).

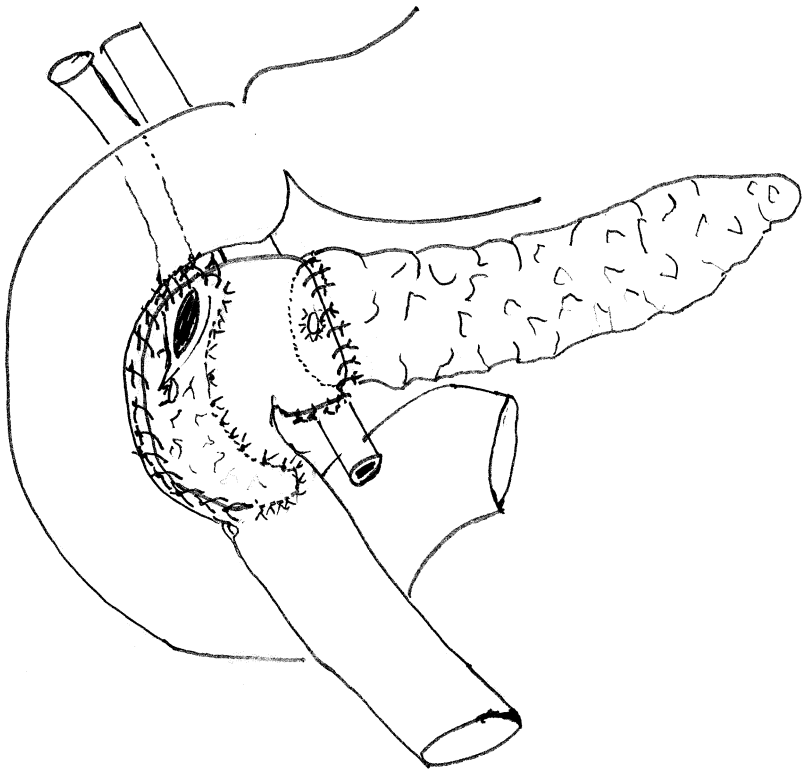

Fig. 7. In cases of severe common bile duct stenosis, an internal biliary anatomosis is performed between the jejunal loop and the prepapillary common bile duct.

case of a biliary bypass, cholecystectomy is recommended to avoid dysfunctioning of the gallbladder.

In patients with a dilated pancreatic duct with multiple stenoses of the pancreatic main duct and absence of side branch duct stenoses, the main pancreatic duct is longitudinally opened on its anterior surface extending toward the tail of the pancreas. A side-to-side anastomosis using the Partington-Rochelle technique for duct-to-jejunal anastomosis is created (Fig. 8).

Postoperative Testing

Postoperatively, after resumption of normal food intake, the patient should be checked for glucose metabolism using an oral glucose tolerance test, and the level of exocrine function should also be determined by means of fecal elastase estimation.

Early Postoperative Course

Many patients surprisingly are free of pain even in the first postoperative days. In the early postoperative period, complications are infrequent; local bleeding, which appears as intestinal blood loss, anastomotic leakage, which is identified by the appearance of intestinal contents in the drainage fluid (3%), and the development of a pancreatic fistula (evacuation of amylase-rich fluid after postoperative day 5 through the drain near the pancreatic head) may be observed. Mild laboratory signs of pancreatitis are frequent but last only 1 to 3 days. Pulmonary complications occur in approximately 10%. Patients are usually on a regular diet by the third to the fifth postoperative day (Table 1).

Regarding long-term outcome and quality of life, the success of the surgical treatment is determined by the pain-free status of the patient and the level of endocrine insufficiency.

Long-Term Outcome After Duodenum-Preserving Pancreatic Head Resection

Preservation of the duodenum using DPPHR is superior to the classical duodenopancreatectomy (Kausch-Whipple resection) in chronic pancreatitis. The most important benefit is the preservation of the duodenum because of the endocrinologic role of the duodenum in the regulation of glucose metabolism. The duodenum, furthermore, regulates gastric emptying of solid and liquid foods. As a result of the preservation of the duodenum, the endocrine function of the pancreas is maintained after DPPHR. The hormones with anti-insulin effects, for example, glucagon and somatostatin, are reduced in the circulation after removal of the pancreatic head.[13] The observed improvement in endocrine function during the

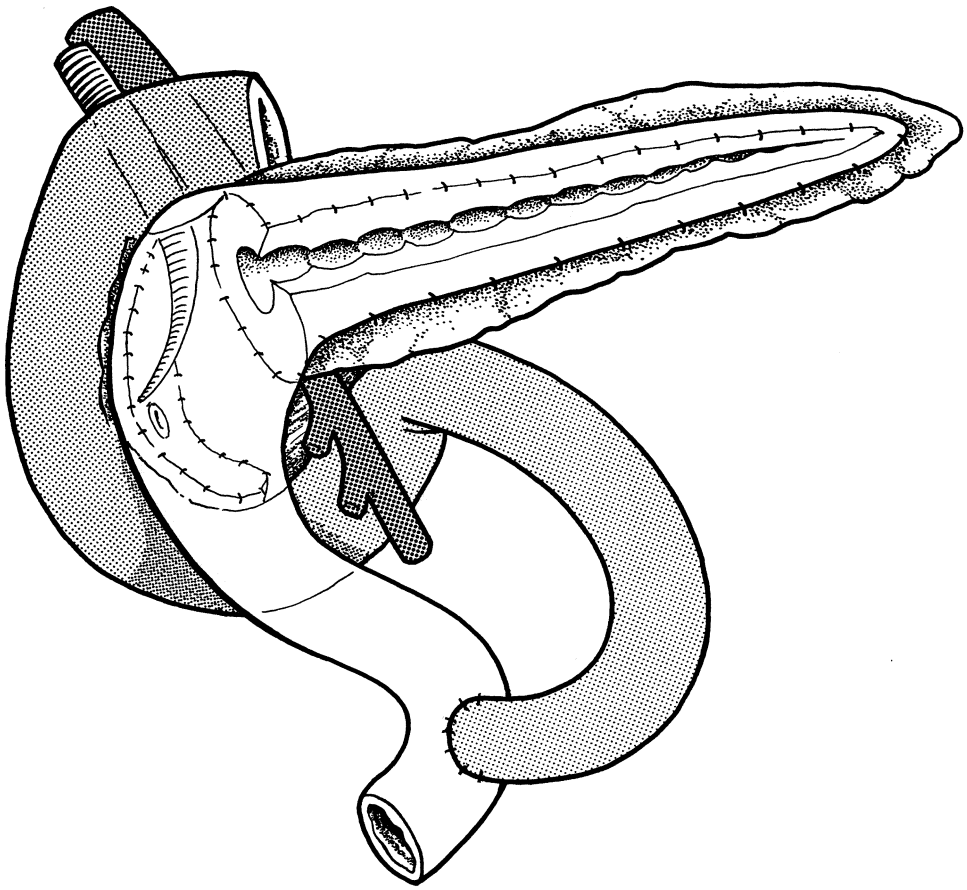

Fig. 8. In cases of multiple stenosis of the pancreatic main duct and absence of side branch duct stenoses, a side-to-side pancreaticojejunal anastomosis is performed (modified from Beger et al.[14]).

postoperative period after DPPHR in some patients is the result of a diminishing glucagon and somatostatin cell compartment.

Table 1. Duodenum-preserving pancreatic head resection: Early postoperative results in 603 patients*

Pancreatic fistula	3.3%	(n = 20)
Breakdown of pancreaticojejunostomy	1.5%	(n = 9)
Intra-abdominal abscess	2.8%	(n = 17)
Delayed gastric emptying	1.5%	(n = 9)
Hospitalization (postoperative)	14.5 days (range 7–87 days) 11.6 days† (range 6–33 days)	
Relaparotomy	4.6%	(n = 28)
Hospital mortality	0.7%	(n = 4)

*December 1972–October 2001: Department of Surgery, Free University Berlin (before May 1982); Department of General Surgery, University of Ulm (after May 1982).
†168 patients: August 1998–September 2001.

After a median observation period of 5 to 7 years and a follow-up of 94%, the control of pancreatic pain is complete and long lasting in approximately 90% of patients.[14] The combination of DPPHR and pancreatic main duct drainage was used only in patients with multiple pancreatic main duct stenoses but absence of side duct obstructions. The use of the side-to-side anastomosis between the pancreatic main

Table 2. Late outcome after duodenum-preserving pancreatic head resection in 388 patients*

Pain free	91.3%
Continuing abdominal pain	8.7%
Complaints, lower abdominal	12.0%
Hospitalization due to pancreatitis	12.5%
Return to work	69%
Glucose metabolism normal	39%
Insulin-dependent diabetes mellitus	44%
Quality of life (Karnofsky score) >80	72%

*Postoperative follow-up 94% after median 5.7 years (range 0.3–14 years).
Modified from Beger et al.[14]

Table 3. Pancreatic head resection in chronic pancreatitis: Duodenum-preserving vs Whipple resection–results of randomized trials

Procedure	Significant or equal differences regarding	Reference
DPPHR >>>> PP Whipple	>Postoperative morbidity >Glucose metabolism >Gastric emptying >Frequency of rehospitalization	Büchler et al.[16]
DPPHR >>> Whipple	>Postoperative morbidity >Glucose metabolism >Frequency of rehospitalization	Klempa et al.[17]
DPPHR = Frey*	= Pain control = Glucose metabolism = Postoperative morbidity < Quality of life	Itzbicki et al.[19]
Frey* >>> PP Whipple	>Postoperative morbidity >Gastric emptying >Quality of life	Itzbicki et al.[20]
DPPHR >>>> Whipple	>Postoperative morbidity >Maintenance of endocrine function >Frequency of rehospitalization >Quality of life	Witzigman et al.[21]

DPPHR = duodenum-preserving pancreatic head resection (Beger); PP Whipple = pylorus-preserving duodenopancreatectomy.
*Frey, modified by Itzbicki ("coring-out" technique of Frey results in a tissue loss of 4 g (wet weight).[20] An operative specimen of 25–40 g tissue results in a subtotal pancreatic head resection as it is the consequence of DPPHR.

duct and the jejunal loop (Puestow procedure) has been shown to fail to benefit almost 40% to 50% of patients over the long term, because of side duct obstructions and persistence of the inflammatory mass in the head of the pancreas. Subtotal resection of the pancreatic head results in removal of 20 to 40 g of pancreatic tissue (see Fig. 5). Approximately 70% of the patients return to their former work[15–18] (Table 2).

Five randomized prospective controlled clinical trials have been published (Table 3)[16–21]. In comparison to the Whipple type of pancreatic head resection, the duodenum-preserving resection is superior in terms of postoperative morbidity, maintenance of glucose metabolism, frequency of delayed gastric emptying, and frequency of rehospitalization.[21] Regarding the Frey procedure,[18] as performed by Itzbicki,[19,20] the level of pain control, maintenance of glucose metabolism, frequency of postoperative morbidity, and quality of life were almost equal to that achieved with DPPHR.

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Review Article

Current Management of Biliary Strictures

Jennifer G. Hall, M.D., Theodore N. Pappas, M.D.

The management of biliary strictures presents a significant challenge to surgeons. If not recognized promptly or if managed improperly, severe complications may result, including cholangitis, portal hypertension, and biliary cirrhosis.[1] With the introduction and widespread use of laparoscopic cholecystectomy in the 1990s, the incidence of biliary injuries and associated bile duct strictures has increased. This increase has led to substantial patient morbidity and impressive financial implications to our health care system,[2] necessitating close scrutiny and review of current management principles of biliary strictures. The estimated incidence of major bile duct injuries, which was fairly constant at 0.1%–0.3% in the open cholecystectomy era, has risen to a rate of 0.2%–0.7% with laparoscopic cholecystectomy.[3–11] In this article, the etiologies of malignant and benign bile duct strictures will be reviewed and the repertoire of approaches for the management of such strictures will be discussed with a focus on those of a benign origin.

ETIOLOGY OF BILIARY STRICTURES

Malignant Strictures

Malignant bile duct strictures are most commonly caused by cholangiocarcinoma, pancreatic adenocarcinoma, ampullary cancer, or tumors metastatic to the porta hepatis[12] (Table 1). Differentiation of malignant bile duct strictures from those of benign origin can be difficult despite the broad repertoire of imaging and diagnostic tools available to the surgeon and radiologist today.[13–16] With the lack of demonstration of a distinct mass lesion, imaging alone using ultrasound, computed tomography (CT), or magnetic resonance imaging (MRI) may not adequately determine malignancy. The diagnosis of malignant biliary strictures depends upon the identification of tumor cells obtained by ultrasound or CT-guided percutaneous fine needle aspiration, bile sampling, endobiliary brushings, or bile duct biopsies.[17]

Management of malignant biliary strictures depends on the resectability of the tumor and often requires a multidisciplinary approach.[18] Radical resection with biliary enteric bypass regarding the anticipation of a long-term cure is the optimal scheme, but resectability rates of such tumors unfortunately lie in the range of 15%–20%.[12,18] The goal for these patients therefore becomes palliation through the use of endoscopic stenting, percutaneous transhepatic stenting, or surgical bypass.[12] Palliative procedures improve the quality of life for these patients by providing relief from the symptoms associated with cholestasis—most notably, jaundice, pruritis, and nausea.[18]

Palliative resection and surgical bypass as a palliative procedure for malignant biliary strictures are generally reserved for the fittest patients, as rates of operative mortality may reach as high as 33%.[19] Placement of transhepatic or endoscopic stents are therefore often preferred and more favorably tolerated in elderly patients, malnourished patients, or in those patients exhibiting advanced disease.

Endoscopic Vs. Percutaneous Placement of Biliary Stents. Although most would agree that internal drainage is preferred over external drainage, the method of stent placement is determined on an individual patient basis to optimize palliation of symptoms and minimize morbidity.[18] Endoscopic drainage offers the advantage of favorable fluid and electrolyte homeostasis, more satisfactory cosmetic results, and greater psychological acceptance, but also carries a higher risk of sepsis and increased morbidity related to stent clogging and subsequent exchange.[18] Nevertheless, Speer and associates demonstrated that endoscopically placed stents were significantly more successful for relieving jaundice compared with percutaneously placed stents (81% vs. 61%, $p = 0.017$)

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Table 1. Causes of malignant biliary strictures

Primary tumors	Metastatic tumors
Cholangiocarcinoma	Pancreatic adenocarcinoma
Pancreatic adenocarcinoma	Colon adenocarcinoma
Gallbladder cancer	Breast adenocarcinoma
Ampullary carcinoma	Lung carcinoma
Hepatoma	Melanoma
Gastric carcinoma	Ovarian adenocarcinoma

and resulted in a lower 30-day mortality rate (15% vs. 33%, $p = 0.016$).[20] With the introduction and widespread use of smaller expandable metal stents, percutaneous placement is now associated with fewer risks and complications including hemorrhage, cholangitis, and pneumothorax.[18] It should also be emphasized, however, that one technique for stent placement does not necessarily exclude the other and improved success rates may be achieved using a combined approach.[18]

Plastic Vs. Metal Stents. The use of both Teflon and self-expandable metallic endoprotheses for the management of malignant biliary strictures has been well reported, with successful relief of obstructive jaundice approaching 90% in most series.[21–31] However, rates as low as 53% have also been reported and are usually associated with Bismuth grade II and III lesions.[23] Stent clogging caused by bacterial contamination with subsequent encrustation and stent migration continue to be the major problems associated with conventional Teflon devices.[21] Rates of occlusion along with the necessity for stent exchange in these types of stents are reported to be as high as 30%–40% in some series.[32–34] A meta-analysis of trials employing the use of antibiotics and/or ursodeoxycholic acid to prevent stent clogging in polyethylene stents failed to show an improvement in stent patency with regard to the use of these agents.[35]

Self-expanding metallic devices can be inserted with relative ease and have lower rates of occlusion compared with plastic devices.[18,27,36] The major cause of metallic stent occlusion is tumor ingrowth into the stent holes. The placement of self-expanding metallic stents may be a particularly attractive option when the percutaneous method is employed, as these stents require a smaller transhepatic tract with less risk of hepatic trauma.[18] In a prospective randomized trial of 20 patients, Wagner and associates compared the use of metallic and plastic stents for the palliative treatment of malignant hilar strictures.[37] This study compared the percutaneous placement of 14 F plastic stents and 24 F self-expanding metallic stents. Successful stent placement was achieved in 100% of the patients who received metallic stents and in 88.9% of those who received plastic stents. Long-term failure (greater than 30 days) was observed in 50% of the plastic stent group and in 18.2% of the metallic stent group. Although these results did not reach statistical significance, the length of hospitalization for the treatment of stent-related complications and reinterventions was determined to be considerably higher in the group receiving plastic stents, suggesting that, despite the initial expense, metallic stents do offer a cost-effective treatment option.[37]

A disadvantage of self-expanding metallic stents for the treatment of malignant biliary stricture arises when the initial stent does not successfully relieve symptoms. Once a stent has expanded across a stricture, it becomes difficult to deploy a second stent.[18] Techniques to overcome such a problem are being developed and include intraductal irradiation to reopen the stricture[38] and prepositioned guidewires for the placement of subsequent stents.[18]

The primary aims of stent placement for malignant biliary strictures is the palliation of symptoms and an improvement with regard to the overall quality of life after stent placement. Questionnaires have confirmed these aims, including benefits in both functional and emotional states.[39,40]

Benign Strictures

Benign biliary strictures pose significant challenges to surgeons in terms of timely and accurate diagnosis, careful evaluation and delineation of the extent of the disease, and appropriate decision-making with regard to the formulation of a treatment plan based upon individual risk assessment. Benign strictures most commonly occur after surgical procedures related to the gall bladder or biliary tree are performed. Noniatrogenic causes of benign strictures include inflammatory conditions and subsequent fibrosis related to chronic pancreatitis, cholelithiasis, choledocholithiasis, sclerosing cholangitis, stenosis of the sphincter of Oddi, or infections of the biliary tract. Additional etiologies of benign biliary strictures are listed in Table 2. The classification of bile duct injuries and strictures according to Strasberg is indicated in Table 3 and Figure 1.

Pathogenesis. With the introduction of laparoscopic cholecystectomy, bile duct injuries have occurred with increased frequency and currently account for over 80% of postoperative biliary strictures.[41] Since the first laparoscopic cholecystectomy was performed in 1985 in Germany, this technique has rapidly replaced the traditional open technique as the method of choice for the treatment of benign gall

Table 2. Benign causes of biliary stricture

Postoperative causes	Inflammatory conditions	Other
Primary biliary surgery	Chronic pancreatitis	Trauma, blunt or penetrating
Laparoscopic cholecystectomy	Cholelithiasis	Ischemia
Open cholecystectomy	Choledocholithiasis	Stenosis of the sphincter of Oddi
Common bile duct exploration	Ascending cholangitis	
Biliary-enteric anastomosis	Primary sclerosing cholangitis	
Cyst excision	Crohn's disease	
Liver transplantation	Duodenal ulcer disease	
	Viral infections	
Other operative procedures	Pancreatic pseudocyst	
Hepatic resection	Hydatid cyst disease	
Portacaval shunt	Liver abscess	
Gastric resection	Postradiation therapy	

bladder disease.[42] The first several years after its introduction to the surgical field, rates of biliary injuries of up to 2% were reported as compared with a fairly constant incidence of 0.1%–0.2% that had been maintained throughout the era of the open technique.[42–44] This was not surprising, given the drastic change from an open easy-access surgical field with a three-dimensional view to a technique with a two-dimensional remote video presentation of the operative field.[42] However, even overcoming the learning curve of the laparoscopic technique, the incidence of bile duct injury has leveled at 0.2%–0.7%, which is still higher than rates seen with the open cholecystectomy operation.[3–7]

A number of factors are associated with bile duct injury during either open or laparoscopic cholecystectomy, including inflammation related to acute or chronic cholecystitis, inadequate exposure, and failure to correctly and completely identify the anatomy before clipping, ligating, and dividing structures.

Table 3. Strasberg classification of biliary injury and stricture*

Class A	Injury to small ducts in continuity with biliary system, with cystic duct leak
Class B	Injury to sectoral duct with consequent obstruction
Class C	Injury to sectoral duct with consequent bile leak from a duct not in continuity with biliary system
Class D	Lateral injury to extrahepatic ducts
Class E_1	Stricture more than 2 cm from right and left bile duct bifurcation
Class E_2	Stricture less than 2 cm from right and left bile duct bifurcation
Class E_3	Stricture at bifurcation of left and right bile ducts
Class E_4	Stricture involving right and left bile ducts; ducts are not in continuity
Class E_5	Complete occlusion of all bile ducts

*Adapted from Strasberg et al.[9]

Hemorrhage into the surgical field from either the cystic artery or a hepatic artery can complicate visualization and constitute accurate identification of structures difficult. The classical error made with regard to laparoscopic cholecystectomy is a misidentification of the common bile duct for the cystic duct, often caused by excessive cephalad retraction of the fundus of the gall bladder in which the cystic and common ducts become closely aligned.[45,46] The common duct is then clipped and divided (Fig. 2). The surgeon, erroneously thinking the cystic duct has been successfully divided, continues to dissect the common duct proximally and eventually transects the proximal biliary system.[45] The right hepatic artery is also typically injured or ligated because of its proximity.[45] Other mechanisms of injury include the generous application of clips to hilar areas that may not be well visualized, thus resulting in the placement of clips across a bile duct with resultant damage.[41] Additionally, failure to recognize congenital anatomic anomalies of the bile duct system, such as direct insertion of the right hepatic duct into the cystic duct or a long common wall between the cystic duct and common hepatic duct, can create an injury.[41]

Preservation of the blood supply to the common bile duct is important for the prevention of subsequent stricture formation. The surgeon must be familiar with the 3-o'clock and 9-o'clock position of the major arteries to the bile duct (Fig. 2) and must limit unnecessary dissection that could potentially compromise the blood supply. Likewise, it is imperative that biliary-enteric anastomoses be performed with careful dissection to preserve vital blood supply to the tissue.

In addition to iatrogenic bile duct injury occurring during cholecystectomy or common bile duct exploration, other operations contributing to benign bile duct stricture formation include gastrectomy for ulcer disease in which excessive inflammation and scarring

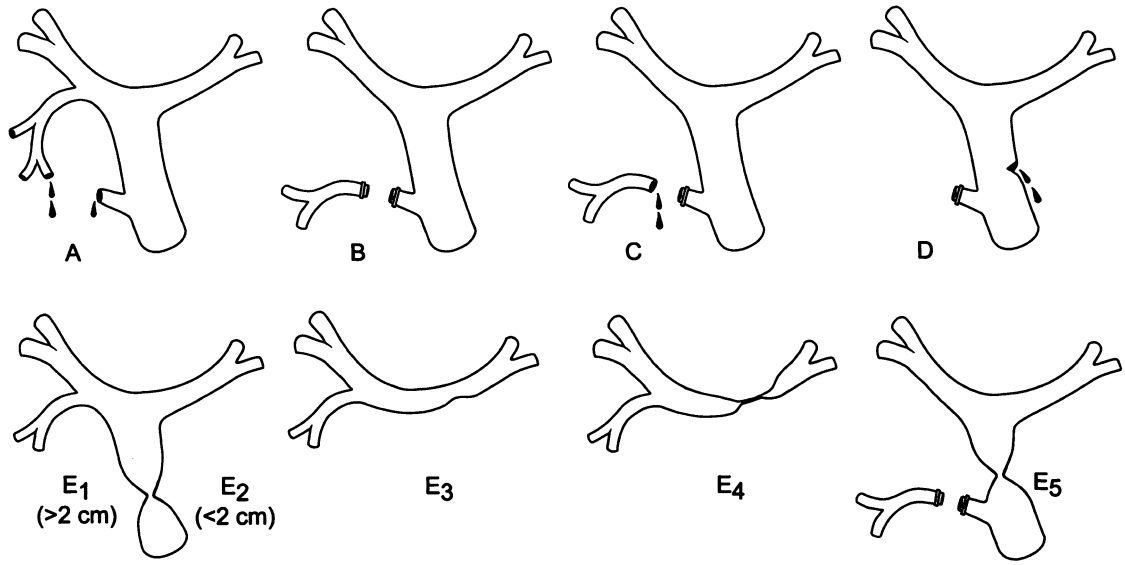

Fig. 1. Classification of bile duct injuries and strictures, adapted from Strasberg et al.[9]

can obscure normal anatomic relationships and hepatic resection in which dissection and mobilization of the hepatic hilum may result in bile duct injury. Postoperative bile duct strictures may also develop at the site of a biliary-enteric anastomosis and, in these cases, is usually the result of ischemia at the site of the anastomosis. Finally, recurrence of bile duct strictures after an attempt at initial repair of these injuries accounts for a fair number of cases.[41,47]

Presentation. The majority of patients with postoperative bile duct strictures will present early after their initial operation with 70% of strictures diagnosed within the first 6 months and 80% diagnosed within the first year[48] (Fig. 3). In cases of laparoscopic injury to the bile duct, recognition may occur during the procedure, but is more commonly discovered in the early postoperative period.[41]

Clinical findings of postoperative bile duct stricture range from subclinical derangement of liver

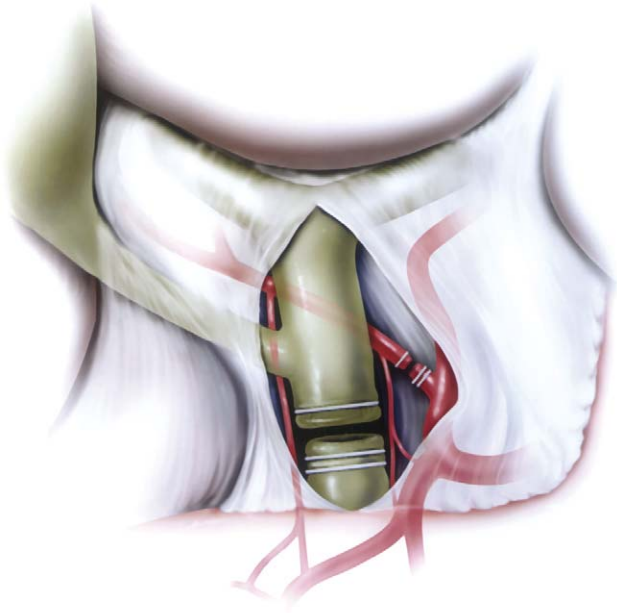

Fig. 2. Classic laparoscopic bile duct injury. The common bile duct is mistaken for the cystic duct and is divided. The right hepatic artery is also subject to injury during this dissection. In addition, the blood supply of the common bile duct is depicted here in the 3 o'clock and 9 o'clock positions.

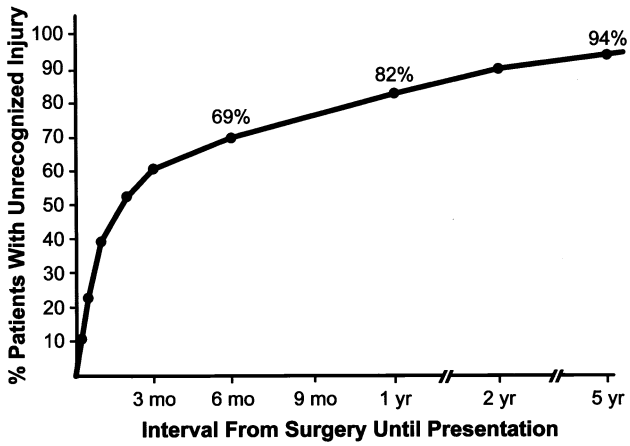

Fig. 3. Graph depicting the cumulative percentage of patients who develop symptoms of bile duct injury from the time of the initial procedure when the injury occurred. More than 50% of patients with bile duct injury will present within the first 3 months and 80% will present within the first year after injury. (Reprinted from Pitt HA, Miyamoto T, Parapatis SK, et al. Factors influencing outcome in patients with postoperative biliary symptoms. Am J Surg 1982;144:14–21, by permission of the publisher.)

function tests to complete hepatic obstruction with jaundice, biliuria, and pruritis.[42] Those who indicate this soon after the initial operation typically manifest symptoms in one of two ways. First, elevated liver tests, specifically total bilirubin and alkaline phosphatase levels, occur as early as 2–3 days postoperatively. Second, and more commonly, bile leakage from the injured duct can manifest in the form of a free leak or a loculated fluid collection. Bile leaking freely into the abdominal cavity may present as biliary drainage from an existing peritoneal drain, as biliary ascites, or as bile peritonitis. Likewise, a loculated fluid collection may result in a sterile biloma or an infected abscess.

Late presentations of postoperative strictures occurring months to years after the initial operation are typified by recurrent bouts of cholangitis that are often treated with repeated courses of antibiotics until the definitive diagnosis is finally established. Advanced biliary cirrhosis and its complications may result in a markedly delayed diagnosis.

Initial Diagnostic Evaluation. Hepatocellular function should initially be assessed in cases of suspected or diagnosed biliary stricture. Readily available and sensitive parameters of hepatic synthetic function include prothrombin time, which monitors the coagulation factors of the extrinsic pathway (factors 2, 7, 9, 10, protein C and S), albumin level, and gamma-glutamyl transferase (GGT), which is the most sensitive marker of hepatocellular damage.[42] The presence of cholestasis can be confirmed and the degree of obstruction quantified if an elevated direct bilirubin and alkaline phosphatase level exists.[42] Complete blood counts and serum electrolytes are usually within the normal range unless associated sepsis is present.

Initial imaging techniques employed regarding the evaluation of patients with postoperative biliary stricture include abdominal ultrasound and CT scan. Abdominal ultrasound readily confirms the presence and level of biliary obstruction. Additionally, the detection of other pathology such as gallstones, ascites, cirrhosis, or portal hypertension can be determined by ultrasonography. CT scan, like ultrasonography, plays an important initial role with regard to the evaluation of postoperative biliary injury and stricture by identifying fluid collections for possible drainage and by identifying the degree of hepatic duct dilatation.

Hepatic imino-diacetic acid (HIDA) scintigraphy demonstrates a high sensitivity for biliary obstruction and leakage for patients in whom injury is suspected, but adds little information concerning biochemical and other more spatially resolute radiographic modalities.[49] HIDA may be used to differentiate between complete and partial biliary obstruction or for confirmation of improved biliary drainage after catheter placement.[50–52] At our institution, however, HIDA is not part of the algorithm for the management of patients with benign strictures.

The gold standard for the evaluation of patients with benign bile duct strictures is cholangiography. Percutaneous transhepatic cholangiography (PTC) and endoscopic retrograde cholangiography (ERC) are two approaches to cholangiography that define the anatomy of the proximal biliary tree and allow for the simultaneous placement of drainage catheters for decompression of the biliary system. These catheters can be used for nonoperative dilation of bile duct strictures and can be of assistance with regard to surgical reconstruction.[53] PTC may be more valuable than ERC in the setting of postoperative bile duct injury, as discontinuity of the injured extrahepatic bile duct prevents adequate filling of the proximal tree limiting complete visualization. However, most patients undergo ERC as the initial procedure. If the stricture can be managed with endoscopic stent and dilation, PTC is not necessary. Recently, magnetic resonance cholangiography (MRC) has increasingly become the method of choice for the diagnosis and delineation of bile duct injuries because of its high sensitivity and low complication rates.[54–57]

Management of Benign Biliary Strictures. Three options for the management of benign biliary strictures are currently available: percutaneous dilation and stenting, endoscopic dilation and stenting, and surgical biliary drainage, most commonly by a Roux-en-Y hepaticojejunostomy. Radiographic vs. surgical therapeutic modalities provide comparable results, with stricture relapse rates for both methods of treatment reported between 15%–45% and mean follow-up times of 4–9 years.[3,58–62] Determining the appropriate management technique and timing for each patient must be planned carefully, taking into account the location and severity of the stricture, the presence of biliary-enteric continuity, the degree of infection and overall health of the individual patient, the length of time anticipated for stenting, and the need for repeated dilation and stent exchange. These decisions are based upon the clinical assessment of both the treating surgeon and the interventional radiologist.

The ultimate goal regarding the management of patients with benign biliary strictures is to correct the increased resistance to biliary flow caused by a reduction in lumen diameter.[42] Obstruction of the biliary tree has been determined to result in rates of bacterobilia in up to 25% of patients, with subsequent higher rates of infectious complications after surgery than in nonobstructed patients.[63,64] Controlling sepsis with organism-specific antibiotic coverage is therefore vital to the initial management of these

patients. In addition, prophylactic antibiotics, typically of the second-generation β-lactam group such as piperacillin, cefuroxamine, or, in the case of penicillin allergy, ciprofloxacin, are given before any manipulation of the biliary tree. Concurrently, an equally important factor with regard to managing these patients is the prevention of cholangitis associated with biliary obstruction through the establishment of appropriate drainage either percutaneously or endoscopically.[42] These techniques often provide not only the diagnosis, delineation of anatomy, and drainage, but are also options for nonoperative management of patients with benign bile duct strictures. In some series, 20%–30% of patients with benign bile duct strictures are managed exclusively by such radiographic techniques.[1,65]

Management of benign biliary strictures via a percutaneous approach has been widely used, such that the largest nonoperative experience in the management of such strictures is with this approach.[41] After intravenous sedation and administration of a local anesthetic, access to the appropriate hepatic duct of the biliary tree, most commonly the right posterior duct,[65] is obtained percutaneously and the stricture is traversed using fluoroscopic guidance (Fig. 4, *A*). If the stricture lies at or near the hepatic bifurcation, both the right and left ductal systems are accessed with transhepatic catheters (Fig. 4, *B*). Stretching of a focal stricture can be achieved by a number of methods, the most common of which is the use of a balloon angioplasty catheter. Balloon catheters based upon the size and location of the stricture and the diameter of the normal duct are used to open the stricture (Fig. 4, *D*). Balloon dilation is quite painful for patients and up to as many as 10%–15% of patients require general anesthesia for this procedure.[66] After dilation, a transhepatic stent is left in place to reduce the rate of restricturing, allow for continued drainage, and provide access for follow-up cholangiography studies and repeat dilation if needed[42] (Fig. 4, *C*).

Transhepatic dilation of benign bile duct strictures has reported initial success rates of 67%–90%, which is higher than that achieved with endoscopic techniques.[66–69] Long-term patency rates using this method have been reported to be between 62% and 78% with follow-up ranging from 3–8 years[66–70] (Table 4). Several series have documented higher patency rates for primary ductal strictures compared with biliary-enteric strictures ranging from 76%–87.5% and 67%–72.5%, respectively.[66,67] Sclerosing cholangitis indicates the worst reported results with a long-term success rate of only 40%, presumably caused by the ongoing inflammatory process and multifocality of the disease.[42,66]

Complications of PTC are not uncommon, occurring in up to 25% of patients, and include cholangitis, hemobilia, bleeding from hepatic parenchyma or adjacent vessels, pleural violation with pneumothorax, bilio-pleural fistula, and inadvertent injury to adjacent structures including the gall bladder and bowel.[66,68,71] Despite these complication risks, however, PTC with dilation is attempted whenever possible in patients presenting with bile duct stricture. At our institution, two to three attempts at stricture dilation may be performed before proceeding to surgical correction.

ERC is often the initial procedure performed for the diagnosis and management of benign biliary strictures. This option requires intact biliary-enteric continuity, usually via the ampulla of the duodenum, and often is not technically feasible after surgical Roux-en-Y reconstruction.[65] Like PTC, the procedure can be performed with intravenous sedation after insertion of an endoscope, sphincterotomy, and cholangiography. The stricture is traversed with a guidewire in retrograde fashion under fluoroscopic guidance (Fig. 5). Once access is obtained, balloon dilatation and stent insertion may follow. If stents have been placed, reevaluation using cholangiography is performed every 3–6 months, with repeat dilatation and stent exchange as needed. There are no convincing data currently available regarding the length of time that stents should remain in place. However, for most studies exemplary results are obtained with larger bore stents (10 F or greater) left in place for at least 6 months and up to 12 months if possible.[3,68,72,73] This allows for adequate biliary drainage while permitting time to plan definitive elective therapy. Most surgeons reevaluate the stricture at the time of routine stent exchange on a regular basis, but there is no consensus regarding the interval at which stents should be replaced to avoid occlusion.[42] Bergmann and associates reported a 70% obstruction rate with resultant jaundice or cholangitis if stents were not changed regularly at 3-month intervals.[74] De Masi and associates, on the other hand, described leaving the stents in place until the patient became symptomatic.[75] With current conflicting data, the risks of stent occlusion with subsequent complications and replacement must be weighed against the risks and expense of repeat procedures.[42]

The success of endoscopic stricture dilatation is reported to be between 27% and 89% with complication rates between 3% and 47%.[3,58,59,61,68,72,74–77] Complications of ERC regarding stent placement include cholangitis, pancreatitis, stent occlusion, migration, dislodgement, and ductal perforation. Stent reocclusion for benign strictures varies depending upon the type of stent used. With the advent

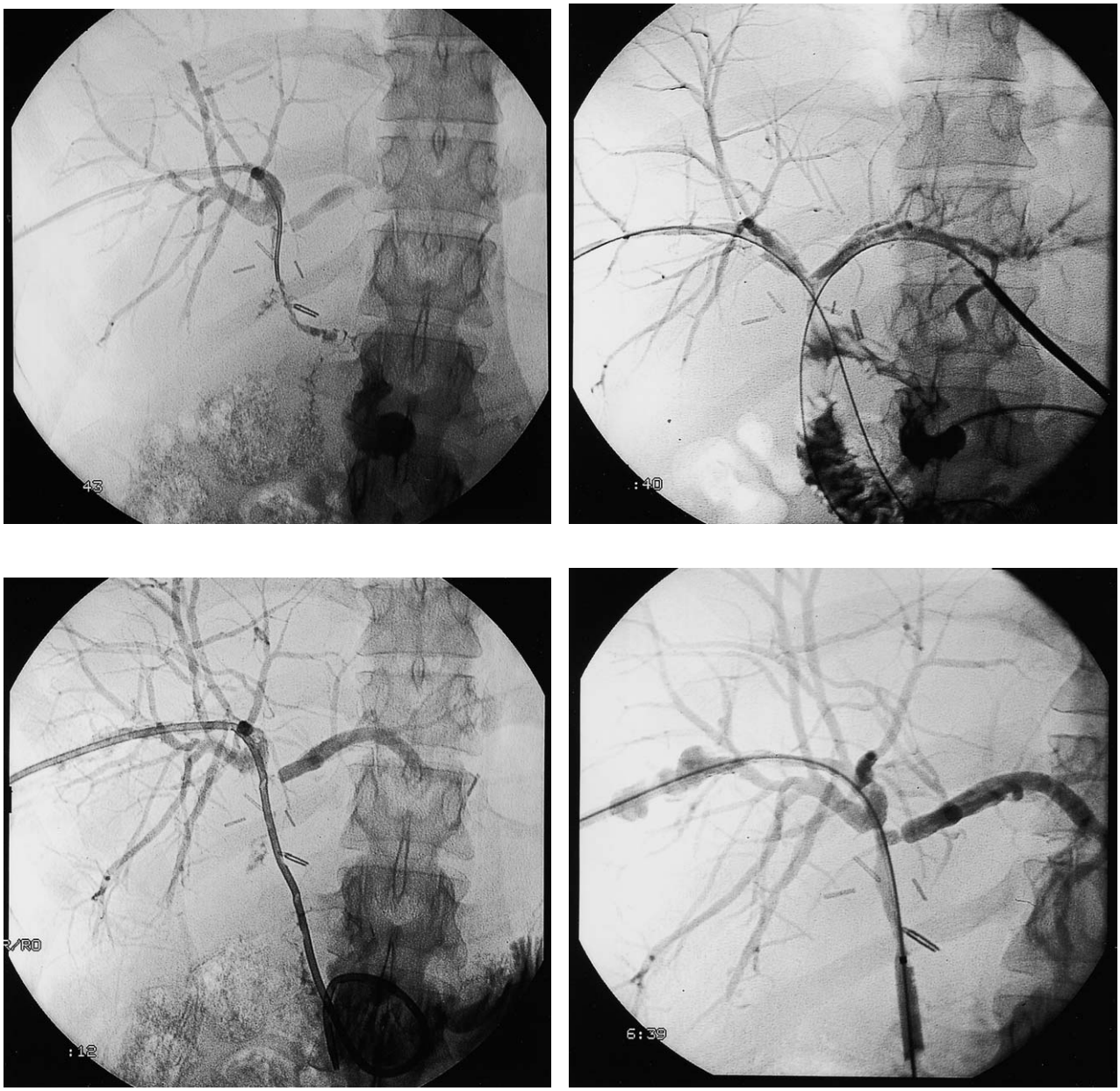

Fig. 4. Percutaneous transhepatic cholangiogram demonstrating (**Top left**) bile duct stricture with proximal duct dilatation. Note the excessive placement of clips in the area of stricture. For strictures at or near the bifurcation of the hepatic ducts, both the right and left ductal systems are accessed with transhepatic catheters (**Top right**). Transhepatic catheters and/or stents may be placed (**Bottom left**) to reduce the rate of restricturing, allowing for continued drainage, and providing access for follow-up cholangiography studies and repeat dilation if necessary. Balloon dilatation (**Bottom right**) is performed allowing for adequate drainage.

of self-expandable metallic stents and with improvements in delivery systems for such stents, early enthusiasm for their use with regard to the management of benign strictures abounded. However, whereas metallic biliary stents have been demonstrated to provide longer periods of patency than plastic stents in patients with malignant strictures, data supporting their use in benign strictures is not as convincing.[75,78–81] One study evaluating the use of plastic and metallic stents for benign biliary strictures reported restenosis in 6 out of 6 (100%) metallic stents and 7 out of 36 (19%) plastic stents, with mean follow-up periods of 50 and 44 months, respectively.[58] This series led to the recommendation against the use of metallic stents for benign stricture disease. Other series have reported similar disappointing long-term results regarding metal stents used for benign strictures with high reocclusion rates caused by

Table 4. Outcomes of percutaneous transhepatic stenting and balloon dilatation of benign bile duct strictures

Author	No. of patients	Success rate (%)	Complication rate (%)	Follow-up (months)
Mueller et al.[66]	61	70	7	36
Williams et al.[67]	74	78	54	28
Born et al.[68]	31	90	26	44
Pitt et al.[3]	20	55	35	59
Lillemoe et al.[70]	25	64	36	28
Suman et al.[69]	56	72	0	36

epithelial hyperplasia and growth into the stent.[78,79,81] Hauseggar and associates reported the results of 20 patients treated with metallic stents for benign stricture in which only a 19% bile duct patency rate remained at 57 months.[79] Finally, metal stents cannot be routinely exchanged or removed and are therefore currently indicated only for the treatment of malignant strictures or, in rare instances, for complicated biliary strictures for which other modalities have failed.[42]

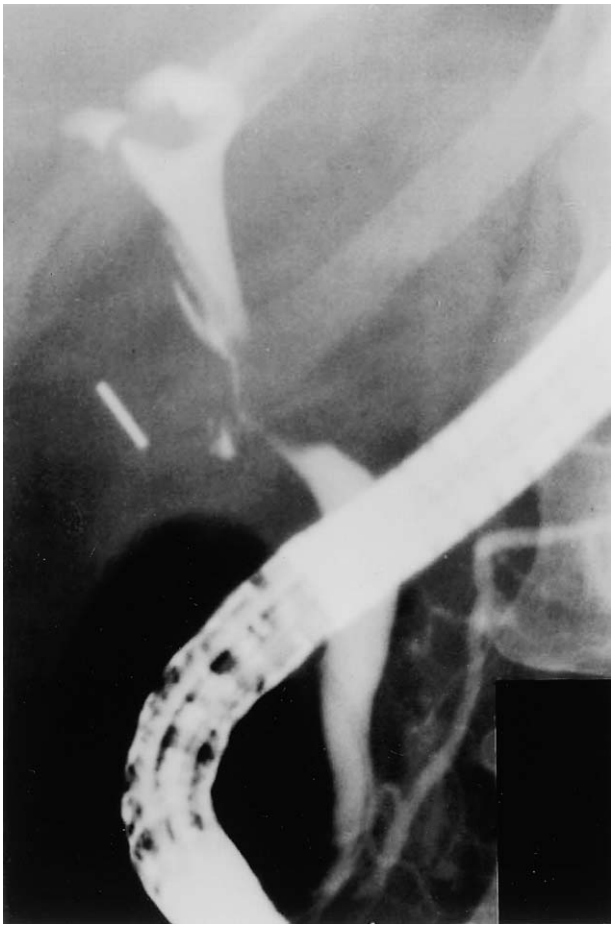

Fig. 5. Endoscopic retrograde cholangiopancreatography (ERCP) demonstrating mid-duct stricture adjacent to the cholecystectomy clip. (Reprinted from Laasch HU, Martin DF. Management of benign biliary structures. Cardiovasc Intervent Radiol 2002;25:457–466, by permission of the publisher.)

The goal of operative management for patients with benign biliary strictures is to reestablish bile flow within the biliary tree and into the proximal gastrointestinal tract in a manner that prevents cholestasis, cholangitis, sludge and stone formation, restricture, or biliary cirrhosis.[82] This goal is successfully accomplished using healthy tissues in a tension-free anastomosis with preservation of blood supply.[82,83] A number of surgical options exist for the repair of benign biliary strictures, including primary end-to-end repair, cholecystojejunostomy, choledochoduodenostomy, Roux-en-Y hepaticojejunostomy or choledochojejunostomy, and mucosal grafting.[3,82,84,85] The choice of repair depends upon the location of the stricture, the degree of obstruction, the timing of the repair, and the experience of the surgeon.

Although the majority of bile duct injuries that occur during laparoscopic cholecystectomy are not realized at the time of initial operation, those that are recognized and properly managed at the time of injury may prevent the development of subsequent bile duct stricture. Acknowledgment of injury at the time of the initial operation requires conversion to an open technique and immediate cholangiography to accurately define the extent of the injury.

Elective repair of biliary strictures indicates exemplary results when compared with immediate reconstruction, especially because such definitive procedures are usually performed at major hepatobiliary centers with surgeons experienced in their management.[72] Excision of the stricture with end-to-end primary repair is rarely successful with regard to establishing continuity of the biliary tract, because of the resultant fibrosis associated with the injury and the invariable loss of ductal length from resection.[82] Likewise, mucosal ileal grafting has not been demonstrated to have wide application or acceptable results and is rarely used as a treatment option for benign strictures.[86]

Cholecystojejunostomy is an option for the bypass of strictures, although it is currently not used frequently compared with the other options available to the surgeon. Laparoscopic cholecystojejunostomy with bypass of the biliary stricture may be an alternative approach with less morbidity than choledochoduodenostomy or hepaticojejunostomy. One series evaluating the potential role of laparoscopic cholecystojejunostomy for the palliation of malignant obstructive jaundice demonstrated that although the

majority of patients are ineligible for this procedure because of prior cholecystectomy, hilar obstruction, or cystic duct obstruction, this method may be used occasionally as an alternative to more complex procedures.[87] This method of biliary bypass should be considered only after cholangiographic documentation of a patent hepatocystic junction that is well separated from the stricture.[87]

Choledochoduodenostomy is commonly used for the repair of benign strictures in the retropancreatic portion, but according to some may not be appropriate for use in other locations because adequate length cannot always be obtained to create a tension-free anastomosis to the duodenum.[82] Concerns also exists with this method because of the risk of bile reflux, ascending cholangitis, and sump syndrome.[88] However, these concerns have not been substantiated by well-designed comparative studies and large-scale cohort studies.[88–91] Recurrent cholangitis after choledochoduodenostomy occurs in only approximately 0%–4% of patients and is more often related to stenosis of the anastomosis than to an ascending cause.[88,92] Similarly, the incidence of sump syndrome varies between 0.14% and 3.3% and can typically be avoided by constructing the anastomosis to the most distal part of the common bile duct to minimize the length of the blind segment.[88,92] This type of repair is most successful in the presence of a dilated common bile duct of at least 15 mm allowing for a widely patent anastomosis.[88,90,93]

Choledochoduodenostomy becomes an ideal choice in cases of a hostile lower abdomen in which the mobilization of a roux jejunal limb in the setting of dense adhesions presents quite a challenge. Choledochoduodenostomy is also technically easier to perform than other more complicated reconstruction methods.[88] In addition, choledochoduodenostomy maintains endoscopic access to the biliary tree.[88] To obtain adequate length for this type of anastomosis, however, generous kocherization is mandatory to prevent excessive tension of the anastomosis. Rates of morbidity and mortality using this approach have been reported to be between 6.6%–13% and 1.3%–6%, respectively,[93–95] establishing this as a safe and effective option for the surgical management of biliary strictures.

The vast majority of surgically repaired benign biliary strictures involve the creation of a Roux-en-Y hepaticojejunostomy.[42,45,70,82] This method of surgical repair has achieved exceptional results in the published series and is the preferable procedure for the surgical management of benign biliary strictures.[25,42,70,82] Principles of repair ensuring success of this technique include the exposure of healthy bile ducts that provide drainage of the entire liver, the preparation of a suitable segment of intestine (usually a roux limb of jejunum that can be anastomosed to the area of stricture in a tension-free fashion), and the creation of a direct mucosal-to-mucosal anastomosis.[41,42] In most series, preoperative transhepatic stents are situated.[1,45,70] Outstanding short-term[45,46,70,72] and long-term[1] results have been reported, including overall success rates of between 72% and 95% with follow-up ranging from 50–133 months (Table 4). Satisfactory results in most series can be defined by the lack of symptoms, jaundice, and cholangitis. In addition, the length of follow-up after operative repair is important to the interpretation of outcome, because although approximately 65% of strictures will recur within 2 years and 90% within 7 years, strictures have been reported to recur up to as many as 20 years after the initial procedure.[47,48]

Despite such positive success rates, these operations are still associated with morbidity and mortality rates higher than those seen with nonoperative techniques. However, as the majority of patients are now referred to large centers for definitive repair of these injuries, morbidity and mortality rates are declining. In 1982, a review of 38 series that had been published since 1900 including 5586 patients and 7643 procedures reported an operative mortality rate of 8.3%.[96] Series published in the last decade reported mortality rates of less than 5%.[1,70,83,97,98] Postoperative morbidity rates still approach 20%–30% with associated complications of hemorrhage, anastomotic leak, cholangitis, and hepatic insufficiency from preexisting biliary cirrhosis, comprising the majority of procedure-related events.[1,41,70,83,97,98] Most anastomotic leaks, as evidenced by bilious drainage from intraoperatively placed drains or by postoperative cholangiography, can be successfully managed by the placement of transhepatic stents to divert biliary secretion, thus avoiding an additional operation.[41] Comparison of mortality and complication rates between the two most commonly performed procedures, choledochoduodenostomy and hepaticojejunostomy, demonstrate similar results in most series, with mortality rates between 0%–2% and complication rates of 20%–30%.[70,82,97,99–102] Restricture rates for the two procedures have also been similarly reported to be 0%–18%, with the majority of restrictures amenable to dilation.[61,70,82,99–102]

Factors that contribute to the successful management of benign biliary strictures have been reviewed and reported, especially because the era of laparoscopic cholecystectomy has demonstrated an increase in biliary injuries. Questions have arisen as to whether the exemplary rates of bile duct stricture repair achieved during the period of open cholecystectomy could be translated to the laparoscopic period given

the complex nature of many of these injuries and the association of considerable inflammation and fibrosis secondary to unrecognized bile leakage. In addition, many of these patients had initially undergone unsuccessful attempts at repair by the primary laparoscopic surgeon, resulting in further fibrosis and scarring and making additional operations even more challenging. In a series of 85 patients with bile duct injury after laparoscopic cholecystectomy, Stewart identified four factors that determined a successful outcome. In this article, the performance of preoperative cholangiography, the method of surgical repair, the details of the operative repair, and the experience of the operating surgeon performing the repair were determined to be notable factors with regard to determining outcome.[103] For this study, when cholangiographic data delineating anatomy was not available preoperatively, 96% of the operations were unsuccessful and when cholangiographic data was incomplete, 69% of the operations were unsuccessful and in some cases led to an inappropriate and harmful operation. In contrast, when complete cholangiographic data were available, successful initial repair was achieved in 84% of patients. With regard to the surgical method of repair, when a primary end-to-end anastomosis was performed over a T-tube in cases of a completely transected duct, no successful repairs were achieved in this series, whereas 63% of Roux-en-Y hepaticojejunostomies were successful. Factors responsible for unsuccessful outcomes in this study included the incomplete excision of the scarred duct, the use of nonabsorbable suture material, the use of a two-layer anastomosis, and the failure to eradicate infection before attempted repair. Initial repair attempted by the primary surgeon was successful in only 17% of cases and no attempts at secondary repair were successful. In contrast, patients whose first attempt at repair was performed by an experienced hepatobiliary surgeon at a tertiary center achieved a 94% success rate.

A comparison of the results of nonoperative procedures with surgery is difficult because of a bias in selecting patients and because of the short follow-up.[82] As interventional procedures gain popularity, more centers are becoming more suitably equipped to manage patients nonoperatively and, as a result, experience with both methods will be more comparable. Currently, however, prospective data are lacking and management decisions must therefore rely on retrospective analyses. One such retrospective comparative study demonstrated a successful repair in 88% of surgically treated patients and in only 55% of balloon dilated patients.[3] David and associates reported an 83% rate of successful outcome after surgical management (n = 35) and a 72% rate of successful outcome with an endoscopic technique (n = 66) over a 4-year follow-up period.[61] Restricture rates were similar between groups in this study (18%), leading the investigators to conclude that stenting should be considered for the initial attempt at definitive management in appropriate patients with the goal of avoiding repeat operation.[61] However, these data should be interpreted carefully and decisions must be based on individual cases until long-term outcomes mature. With sophisticated preoperative diagnosis and with careful operative technique, biliary enteric reconstruction remains the definitive treatment for benign biliary strictures.[42,83]

CONCLUSION

The definitive management of benign biliary strictures depends upon numerous factors, including the complexity and location of the stricture, the degree of inflammation and fibrosis, the presence of ongoing infection or sepsis, and the capability and experience of the surgeon and interventional radiologist at the institution. For some, such as high-risk or compromised patients or those with anastomotic strictures, percutaneous or endoscopic techniques may be most appropriate not only for diagnosis and delineation of the anatomy, but also as initial forms of management. However, successful outcomes regarding the management of most benign biliary strictures requires careful planning by a multidisciplinary team with regard to preoperative preparation, thorough diagnostic studies, and appropriate operative approach. Whatever most appropriate treatment option is selected, the need for careful long-term follow-up must be strongly emphasized, as no therapy is totally free of stricture recurrence.

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Incarcerated Epigastric Hernia, a Rare Cause of Gastric Outlet Obstruction

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KEY WORDS: Hernia, epigastric hernia, gastric outlet obstruction

Epigastric hernia occurs between the xiphoid process and umbilicus in the midline of the abdomen. Herein, we described an unusual case of an incarcerated epigastric hernia causing gastric outlet obstruction.

CASE REPORT

An 80-year-old woman with chronic obstructive airway disease was admitted with a 1-day history of epigastric pain and repeated vomiting. Abdominal examination revealed an upper midline 10-cm subcutaneous epigastric mass. The mass was irreducible and soft in consistency. Bowel sounds were heard on auscultation (Fig. 1).

Laboratory analyses noted marked hypernatremia, hypokalemia, and an elevated urea level (8.5 mmol/L). Upper endoscopy revealed grade C (LA classification) distal esophagitis and a large amount of residual gastric fluid. The antral-pyloric area appeared displaced, and the endoscope could not be passed into the duodenum. Gastric outlet obstruction was diagnosed, and a nasogastric tube was inserted for drainage.

Contrast medium imaging showed complete gastric outlet obstruction without passage of contrast medium into the duodenum. Computed tomography of the abdomen demonstrated incarceration of the distal half of the stomach through a defect in the linea alba, findings compatible with an epigastric hernia complicated by gastric outlet obstruction (Fig. 2).

After optimization of fluid and electrolyte balance, an emergency operation was performed under general anesthesia. An upper midline abdominal incision was made to access the epigastric hernia sac, which contained part of the stomach and attached omentum. The hernia contents were reduced, and the 5-cm defect in the linea alba was closed primarily using interrupted no. 1 nylon stitches. The postoperative course was uneventful. An oral diet was resumed on postoperative day 2, and the patient was discharged from the hospital on day 4.

DISCUSSION

Epigastric hernia is the protrusion of extraperitoneal fat and, occasionally, a peritoneal sac that may contain abdominal viscera through a defect in the linea alba, anywhere between the xiphoid process and the umbilicus. It is rare and comprises about 0.35% to 1.5% of abdominal hernias.[1] The condition usually

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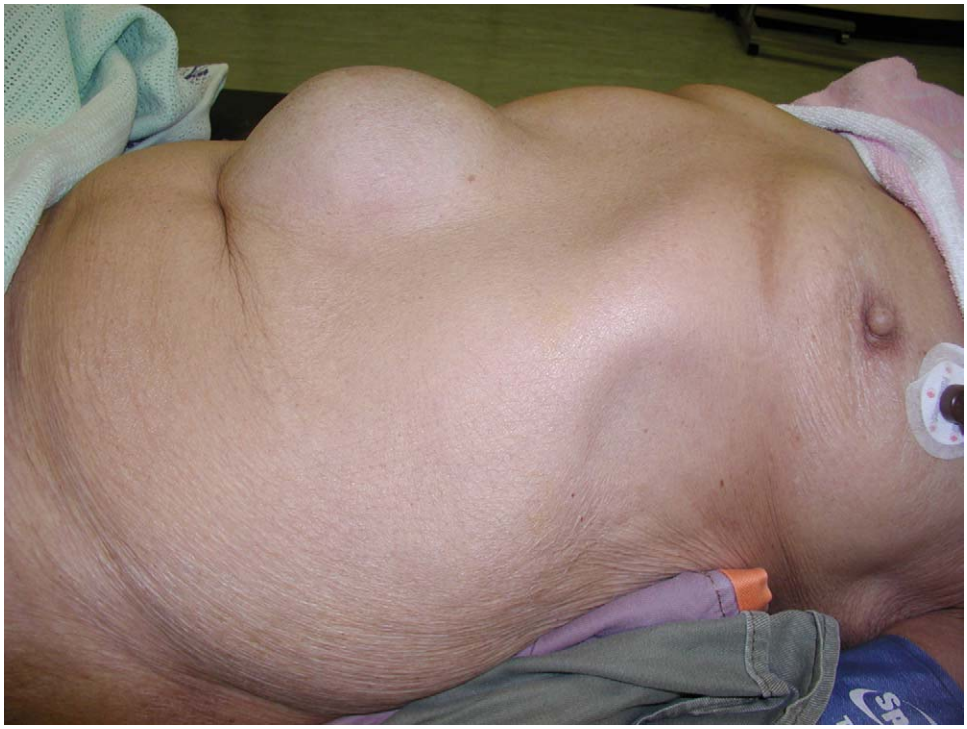

Fig. 1. Clinical photograph of patient with large epigastric mass.

occurs in young, muscular males. Most patients are asymptomatic; however, pain, dyspepsia, and symptoms mimicking peptic ulceration or gallbladder disease can occur.

The most common contents found in the epigastric hernia are preperitoneal fat, falciform ligament, and omentum. Computed tomography facilitates accurate delineation of the linea alba defect and its contents.

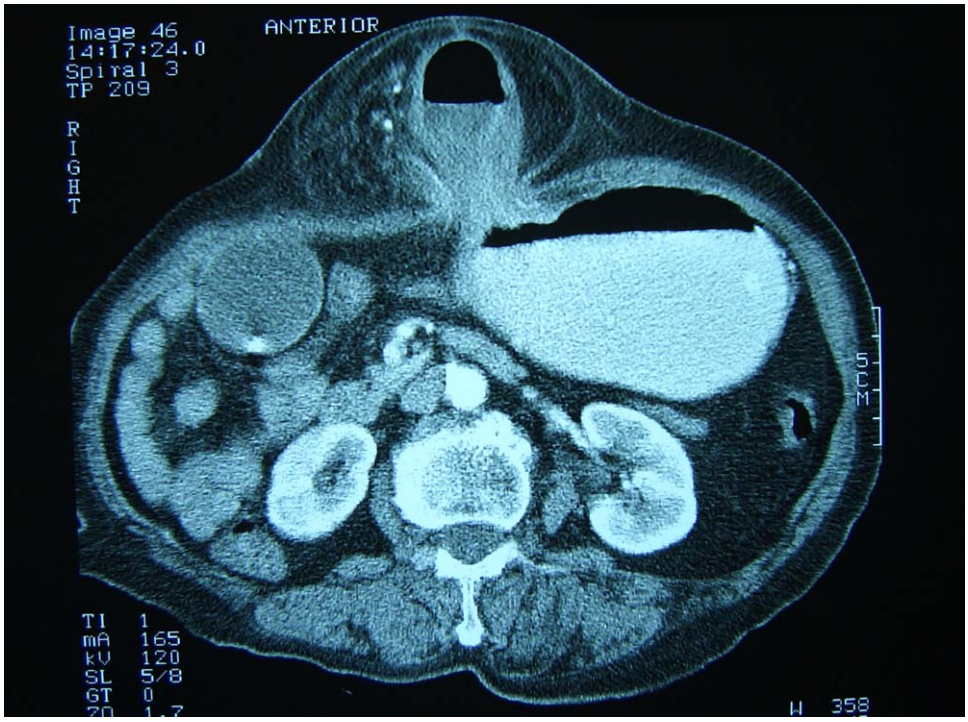

Fig. 2. Computed tomography scan of abdomen documents protrusion of part of the stomach through a defect in the linea alba.

Cases of abdominal wall hernias involving the stomach have been reported in past decades.[2] These reports are exceedingly rare. They describe the stomach herniating through the umbilicus,[3] defects in the linea alba, and even an inguinal opening.[4]

There is no consensus on the best method of surgical repair of epigastric hernia, but the basic principles of a tension-free repair should be observed. Occasionally, there are multiple defects in the linea alba, and cautious intraoperative exploration is advisable to search for occult hernias. The traditional Mayo technique seems to be out of favor because of its high recurrence rate. Tension-free prosthetic repair has been suggested, and studies do show that this approach is safe and effective, with a low complication and recurrence rate.[5]

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