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Vagal Innervation and Early Postoperative Ileus in Mice

Mario H. Mueller · Martina Karpitschka · Zhirong Gao · Sarah Mittler · Michael S. Kasparek · Bernhard Renz · Andrej Sibaev · Jörg Glatzle · Yongyu Li · Martin E. Kreis

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Abstract
Introduction Postoperative ileus is characterized by infiltrates of leukocytes in the gut wall 24 h after surgery, which is subject to vagal modulation. We hypothesized that vagal modulation is irrelevant during earlier hours of postoperative ileus and aimed to determine whether afferent neuronal feedback to the central nervous system is altered by vagal innervation during this early period.
Methods C57BL6 mice were laparotomized and received standardized small bowel manipulation to induce postoperative ileus. Subgroups were vagotomized 3–4 days prior to experiments while control animals were sham-operated. Three or 9 h later a 2-cm jejunal segment was harvested for multi-unit mesenteric afferent nerve recordings in vitro. Intestinal motility was monitored continuously and intestinal muscularis was stained for myeloperoxidase to determine infiltration of leukocytes.
Results Peak amplitudes of intestinal motility and afferent nerve discharge at baseline were not different in all subgroups. Afferent discharge to 5-HT (500 μM) was virtually absent following vagotomy at 3 and 9 h of postoperative ileus (POI) compared to controls ($p<0.05$). Maximum afferent nerve discharge to bradykinin and peak firing during maximum distension at 60 mmHg was not different in all subgroups while luminal distension from 10 to 30 mmHg was lower at 3 h of POI following vagotomy compared to controls ($p<0.05$). The number of myeloperoxidase positive cells was similar at 3 h of POI in both subgroups; however, at 9 h of POI, ileus counts were increased to 713±99 cells following vagotomy compared to 47±6 cells per square millimeter in control animals.
Conclusions Vagal afferents mediate sensitivity to low-threshold distension and 5-HT during postoperative ileus but not to high-threshold distension and bradykinin. Vagal inhibition of the intestinal immune response is present at 9 h but not detectable earlier, i.e., at 3 h of postoperative ileus when spinal reflex inhibition may prevail.

Mario H. Mueller and Martina Karpitschka contributed equally to the study.

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M. H. Mueller · M. Karpitschka · Z. Gao · S. Mittler · M. S. Kasparek · A. Sibaev
Walter-Brendel Institute for Surgical Research,
Ludwig-Maximilian's University,
Munich, Germany

M. H. Mueller (✉) · M. S. Kasparek · B. Renz · M. E. Kreis
Department of Surgery, Hospital Grosshadern,
Ludwig-Maximilian's University,
Marchioninistrasse 15,
81377 Munich, Germany
e-mail: mario.mueller@med.uni-muenchen.de

Z. Gao · Y. Li
Department of Pathophysiology, Tongji University,
Shanghai, China

A. Sibaev
Department of Internal Medicine II, Hospital Grosshadern,
Ludwig-Maximilian's University,
Munich, Germany

J. Glatzle
Department of Abdominal and Transplantation Surgery,
Eberhard-Karls University,
Tuebingen, Germany

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Introduction

Postoperative ileus (POI) is characterized by inhibition of intestinal motility after abdominal surgery which potentially entails prolonged parenteral nutrition, abdominal pain, a longer hospital stay and, therefore, increased total treatment cost.[1] Understanding POI is difficult as several factors contribute to this condition.[2,3] It seems, furthermore, that POI is characterized by different phases and that the different factors are of varying relevance in each phase.[4] One reason for limited success to treat postoperative ileus with traditional prokinetics[5] and newer attempts to use anti-inflammatory agents[6,7] may be that these regimen are usually not adjusted to the different phases of postoperative ileus. However, to design differential treatments according to potentially time-dependent prevailing pathophysiological mechanisms during postoperative ileus, understanding its development is of paramount importance.

In addition to autonomic neuronal reflex circuitry, which is known for decades to contribute to postoperative ileus,[8] inflammatory mechanisms were shown to be pivotal for postoperative ileus in the late nineties. Kalff et al. observed that the extent of inhibition of intestinal motility 24 h after surgery depends on leukocyte infiltration into the muscularis layer of the intestinal wall.[9] According to recent research, systemic[10] and local inflammatory responses, e.g., during postoperative ileus[11] are subject to regulation by the vagus nerve. The efferent vagus releases acetylcholine in the periphery during inflammation which has an attenuating effect on cytokine release of intestinal macrophages.[10,12] Evidence also exists that acetylcholine binds to the α-7 subunit of acetylcholine receptors on intestinal macrophages.[12] This potential of the vagus nerve to attenuate peripheral inflammation was named the "cholinergic anti-inflammatory reflex."

Interestingly, triggering POI by manipulating the intestine is followed by immediate inhibition of intestinal motility[2] which is unlikely to be attributed to a local inflammatory response as this requires more time to develop.[13] Indeed, this immediate inhibition of intestinal motility works via spinal afferent and efferent nerve fibers that form the substrate for this autonomic reflex.[8] While these two events, i.e., spinal reflex inhibition and later inflammatory response at the level of the intestinal muscularis certainly represent key mechanisms, the contribution of the vagus nerve in the early hours of postoperative ileus is unknown.

Based on previous evidence that the intestinal inflammatory response and vagal modulation was observed 24 h after surgery,[9,11] we hypothesized that the vagus nerve is not involved in modulation of POI early, i.e., 3 h after surgery via alteration of spinal afferent sensitivity nor via modulation of immune cells. This question is of importance since if—contrary to our hypothesis—the vagus nerve plays a role in the early development of postoperative ileus, treatment strategies to modulate the vagal innervation might be useful within the first 24 h after surgery.

The aim of the present study was, therefore, to identify potential vagal influence on POI and afferent sensitivity within a few hours after surgery.

Methods

Model of Postoperative Ileus

Postoperative ileus was induced in male mice (C57BL6, body weight, 20–30 g). Animals were maintained on a 12-h light/dark cycle and fed a standard laboratory diet. The animal research committee in charge of our institution approved of the research protocol prior to experiments. The procedure of postoperative ileus induction was described previously.[14] In brief, animals were deeply anesthetized with enflurane inhalation and a midline laparotomy was performed. The small bowel was pulled out onto moist gauze, and the small bowel was systematically manipulated from the ligament of Treitz to the terminal ileum for 15 min with two moist cotton applicators. Then, the laparotomy was closed with a running suture. All animals recovered quickly from surgery and generally began to eat and drink. Different subgroups of animals were sacrificed 3 or 9 h following bowel manipulation. The rationale to choose the 3-h time point was that at this stage the postoperative leukocyte infiltration into the intestinal muscularis layer is known to have not yet developed.[15] The second time point at 9 h after small bowel manipulation was chosen because we previously observed that leukocyte infiltration into the muscularis is present at this time point (unpublished observation).

Chronic Subdiaphragmatic Vagotomy

In order to explore the role of the vagal innervation, afferent and efferent vagal denervation was performed by subdiaphragmatic vagotomy 3–4 days before afferent recordings were made. For this aim, animals underwent laparotomy under sterile conditions during general anesthesia. Then, the subdiaphragmatic branches of the vagal nerve were identified at the abdominal esophagus

and cut. In control animals (sham operation), the subdiaphragmatic vagal nerve was dissected free but not cut. The time interval of 3–4 days between vagotomy and afferent nerve recordings was chosen in order to allow degeneration of the efferent and afferent vagal fibers innervating the intestine.[16]

Technique of Afferent Nerve and Motility Recordings

The experimental setup for afferent nerve and motility recordings is shown in Fig. 1. Following laparotomy, a 2-cm segment of proximal jejunum with the mesentery attached was resected, placed into a perfusion chamber and superfused with bicarbonate buffer equilibrated with 95% O_2 and 5% CO_2 (composition (millimolar) Na^+ 143.5, K^+ 5.9, Cl^- 126, Ca^{2+} 2.5, Mg^{2+} 1.2, H_2PO_4 1.2, SO_4 1.2, HCO_3^- 25, glucose 10, and sodium butyrate 1, pH 7, at 7 ml min^{-1}, chamber temperature 32°C). The jejunal segment was continuously perfused with Krebs at a rate of 1.5 ml min^{-1} with the help of a perfusion pump (IVAC 711, IVAC Corp., San Diego, USA). For this purpose the proximal end of the segment was cannulated and the other end left open to the atmosphere. Intestinal motility was continuously monitored by intraluminal pressure recordings which were established by connecting the cannula inserted into the intestinal lumen to a pressure transducer (CED single channel 1902 preamplifier/filter; Cambridge Electronic Design, Cambridge, UK). Changes in intraluminal pressure (millimeter of mercury) are given as peak amplitudes over a period of 200 s minus baseline. For afferent nerve recordings, the mesenteric arcade was introduced into a separate recording chamber through an aperture that was sealed with vaseline before the chamber was filled with colorless heavy liquid paraffin pre-warmed to 32°C to insulate the recording electrodes. A single paravascular nerve bundle was prepared from a mesenteric arcade. It was then attached to one of a pair of platinum recording electrodes, with a strip of connective tissue wrapped around the other to serve as a reference. The electrodes were connected to a CED single channel 1902 preamplifier/filter (CED, as above), and the signal was amplified 10.000 times and filtered with a bandwidth of 100 to 1 kHz. Signals from electrophysiological recordings and signals from the pressure transducer were passed into a power Micro 1401 interface system (CED), saved, and viewed online by Spike 2 software (version 4.01; CED) on a personal computer (Fig. 1).

Following a 15-min period for stabilization of the recordings, afferent sensitivity to serosal serotonin (5-HT, 500 μM) and bradykinin (0.5 μM) was determined. For this purpose, the perfusion was stopped and the agent added directly into the organ bath with a pipette to reach the mentioned final concentration. After a minimum recovery period of 10 min, the jejunal segments were mechanically distended up to a maximum pressure of 60 mmHg by continuous infusion of normal saline with the perfusion pump (IVAC 711, as above, perfusion rate, 1.5 ml min^{-1}). 5-HT, bradykinin and mechanical ramp distension were chosen as test stimuli as they were previously well characterized to stimulate different subpopulations of mesenteric afferent nerve fibers in the small intestine (5-HT predominantly vagal afferents,[17,18] bradykinin predominantly spinal afferents[19,20] and mechanical ramp distension vagal and/or spinal afferents depending on the distension pressure).[19] Furthermore, these stimuli are likely to act on intestinal

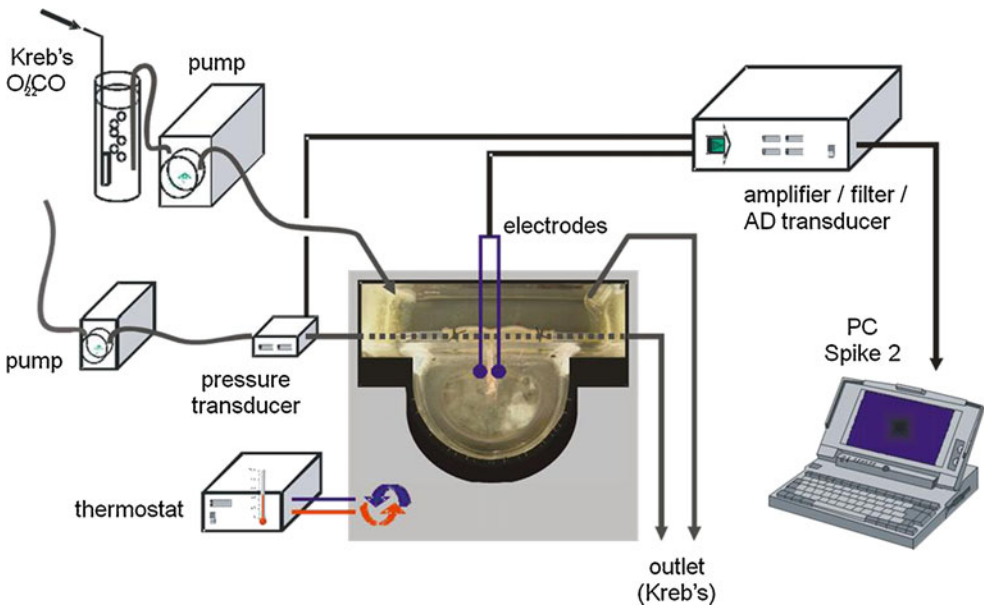

Fig. 1 Experimental setup for extracellular multiunit nerve discharge recording and intraluminal pressure recording. A segment of jejunum was cannulated from both ends to monitor luminal pressure and a single paravascular nerve bundle was dissected out from a mesenteric arcade, then attached to platinum electrodes to record afferent nerve discharge

afferents during postoperative ileus secondary to mast cell activation[21] and spontaneous intestinal distension.[22]

Myeloperoxidase Staining

A separate piece of jejunum was stained for myeloperoxidase (MPO) in order to visualize leukocytes. This histological workup was performed on whole mounts of the intestinal muscularis to determine the extent of postoperative intestinal inflammation. MPO-positive cells were stained by a mixture of 10 mg Hankers-Yates reagent, 10 ml Krebs-Ringer buffer, and 100 μL 3% hydrogen peroxidase for 10 min. Leukocytes were counted in 30 consecutive areas in each specimen at 200 times magnification. Counts are given as positive cells per square millimeter.

Drugs

Enflurane was purchased from Abbott, Wiesbaden, Germany; 5-HT and bradykinin, 5-HT from Sigma Chemicals, St. Louis, MO, USA.

Data Analysis

Electrophysiological recordings were evaluated by determination of baseline discharge frequency (imp per second) which was calculated by averaging afferent nerve discharge per second for the 60-s period immediately prior to administration of the test stimuli. The response to bradykinin and 5-HT was expressed as the peak increase in the number of afferent impulses above baseline over a period of

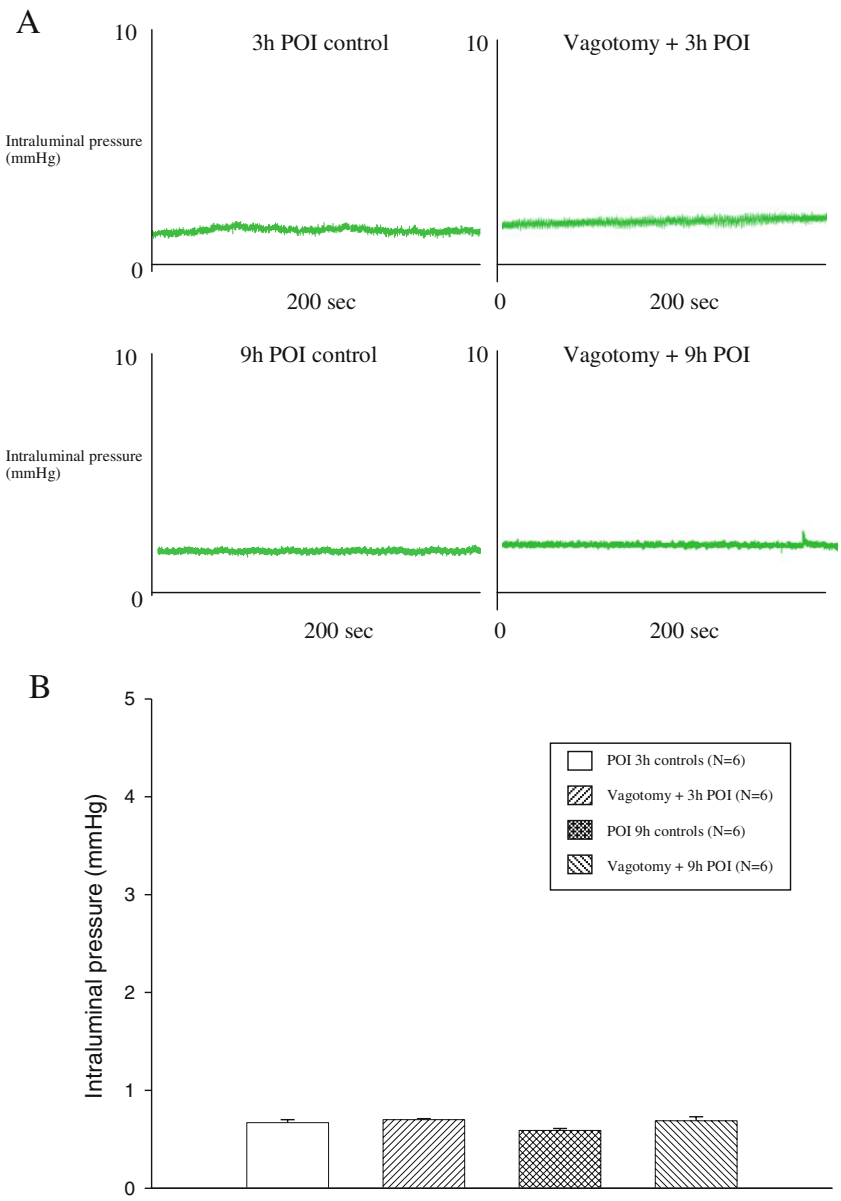

Fig. 2 **a** Shows representative intraluminal pressure recordings from a segment of an animal 3 and 9 h after induction of POI after previous sham operation and from an animal 3 and 9 h after induction of POI following previous vagotomy. **b** The peak pressures above baseline were determined during a period of 200 s and are given as mean± SEM

5 s, while the response to distension was quantified over a 5-s period at pressure increments of 10 mmHg. Intestinal motility was quantified at baseline by determining amplitudes of phasic changes in intraluminal pressure over a period of 200 s. Data are presented as mean±SEM and were compared by one-way ANOVA and post hoc Bonferroni correction. A probability of $P<0.05$ was considered statistically significant. *N* refers to the number of segments except in the MPO experiments where this represents the number of animals. Evaluations of all parameters were performed in a blinded fashion wherever technically possible.

Results

Intestinal Motility

Segments from ileus animals were characterized by complete inhibition of intestinal motor events with peak amplitudes of 0.67±0.03 mmHg in non-vagotomized animals 3 h after induction of POI and 0.59±0.01 mmHg in non-vagotomized animals 9 h after induction of POI. No difference in intestinal motility was observed in chronically vagotomized animals 3 h after induction of POI with 0.70± 0.02 mmHg and at the 9 h time point with 0.69± 0.04 mmHg (each $n=6$, Fig. 2).

Afferent Nerve Sensitivity

Recordings of multi-unit mesenteric afferent nerve discharge demonstrated spontaneous afferent nerve firing with spikes of different waveforms and frequencies. At baseline, peak afferent firing was 12±1 imp sec^{-1} in vagotomized 3-h POI animals, which was not different from 14± 1 imp sec^{-1} in 3 h POI controls. In vagotomized animals at 9 h of POI, baseline discharge was 10±2 imp sec^{-1} which was again not different compared to 11±1 imp sec^{-1} in controls at 9 h of POI (ns, each $n=6$).

Peak afferent discharge to 5-HT was reduced to 0.7± 1 imp sec^{-1} in chronically vagotomized animals 3 h after POI induction compared to 8±1 imp sec^{-1} in 3-h POI controls ($p<0.05$). In chronically vagotomized animals 9 h after POI induction, peak afferent discharge was 3±

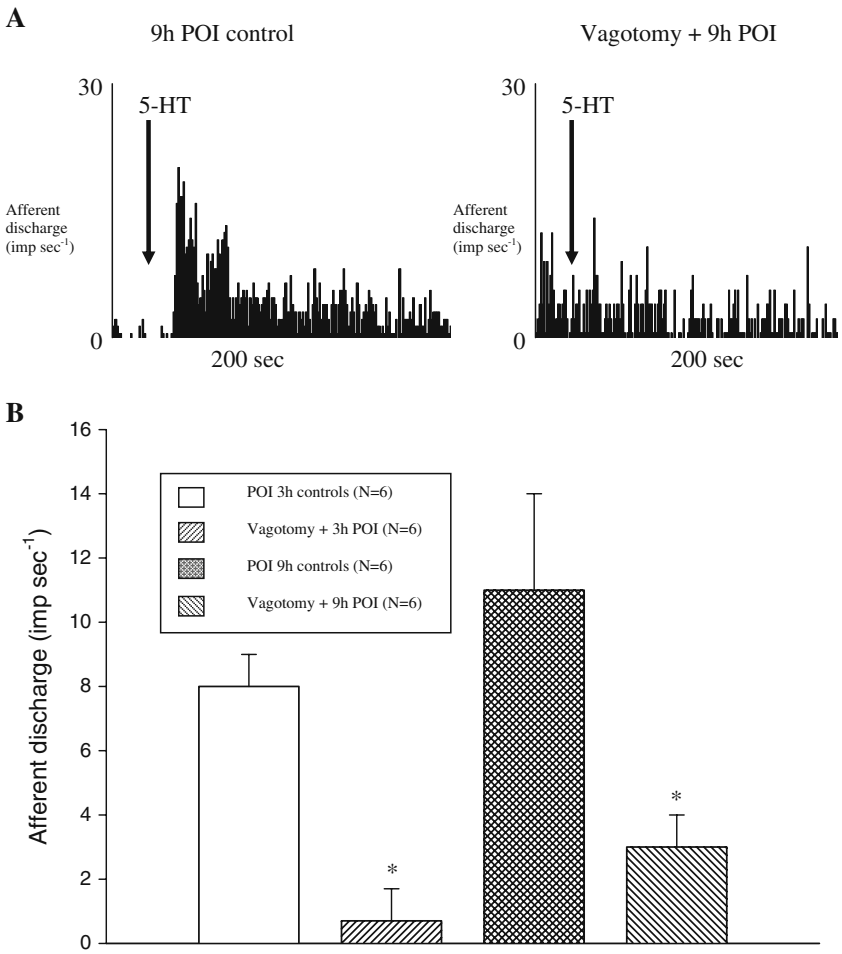

Fig. 3 **a** Representative recordings of the response to serosal 5-HT (500 μM) in small intestinal segments from animals 9 h after induction of POI and at 9 h POI following previous vagotomy. **b** Group data are shown in this *bar diagram*. Peaks in mesenteric afferent nerve discharge following serosal bradykinin are given as mean±SEM, $*P<0.05$ versus POI 3- and 9-h controls. Note the decreased afferent response after vagotomy in 3- and 9-h POI animals

1 imp sec^{-1} compared to 11±3 imp sec^{-1} in 9-h POI controls ($p<0.05$, Fig. 3).

No difference in afferent firing following serosal bradykinin was observed following chronic vagotomy 3 h after POI induction 20±1.6 imp sec^{-1} compared to 22±1.1 imp sec^{-1} in controls at 3 h of POI. Afferent discharge to bradykinin was 47±9 imp sec^{-1} in vagotomized animals at 9 h of POI which was not different compared to 41±5 imp sec^{-1} in controls at 9 h (Fig. 4). However, a difference was present comparing the 3-h groups and the 9-h groups ($p<0.05$).

Mechanical Stimulation

During mechanical ramp distension afferent nerve discharge was characterized by a pressure-dependent rise in afferent firing frequency. At the maximum intraluminal pressure of 60 mmHg in the intestinal segment, no difference in afferent firing was present in the 3-h subgroups during POI which was 83±9 imp sec^{-1} following chronic vagotomy and 80±8 imp sec^{-1} in 3-h controls. In chronically vagotomized animals 9 h after POI induction, peak afferent discharge after ramp distension at 60 mmHg was 107±16 imp sec^{-1} and 115±21 imp sec^{-1} in control animals at 9 h of POI which was again not different. At luminal distension from 10 to 30 mmHg, afferent discharge was lower in vagotomized animals compared to sham controls in the 3-h groups ($p<0.05$); however, in the 9-h animals, no difference occurred at these low-pressure levels (Fig. 5).

Myeloperoxidase Stains

The number of leukocytes infiltrating the intestinal muscularis was 27±7 positive cells per square millimeter in control animals at 3 h of POI and 47±6 positive cells per square millimeter in control animals at 9 h of POI. Following vagotomy, the number of leukocytes in the muscularis of 3-h ileus animals was 29±5 positive cells per square millimeter which was equal to the sham POI animals. However, in vagotomized animals at 9 h of POI, leukocyte count increased to 713±99 positive cells per square millimeter ($N=6$, $P<0.05$; Fig. 6).

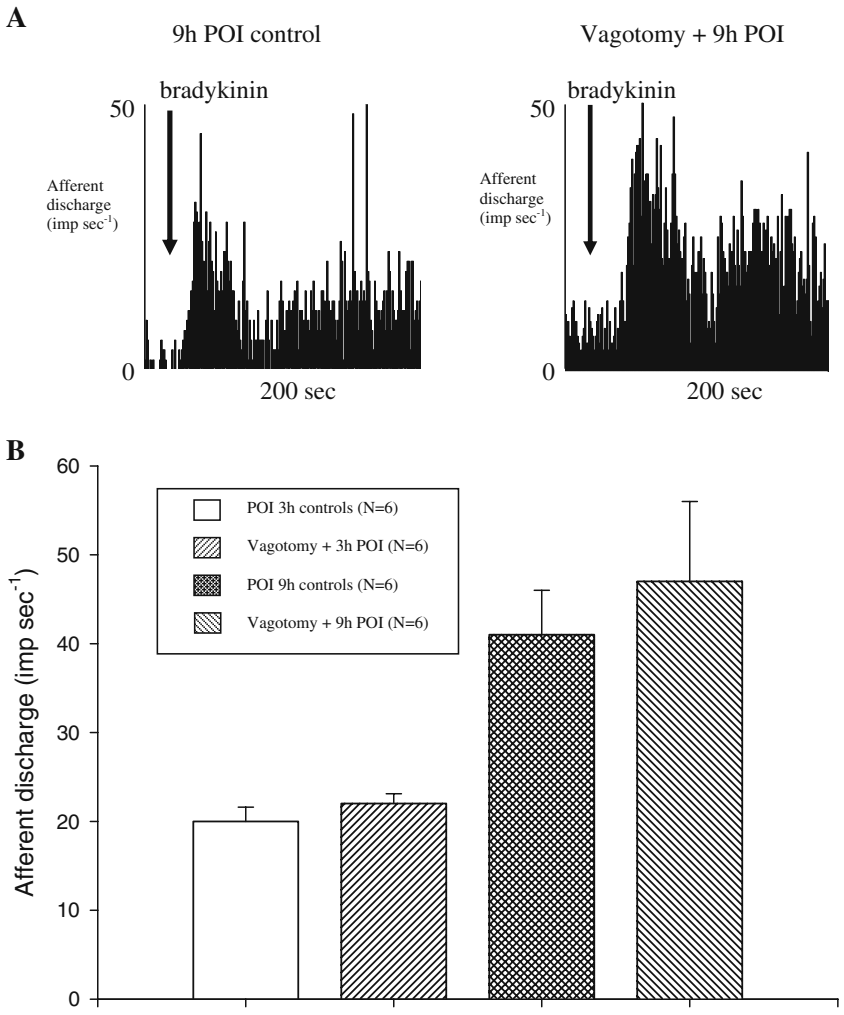

Fig. 4 **a** Representative recordings of the response to serosal bradykinin (0.5 μM) in small intestinal segments from animals 9 h after induction of POI and at 9 h POI following previous vagotomy. **b** Group data are shown in this *bar diagram*. Peaks in mesenteric afferent nerve discharge following serosal bradykinin are given as mean±SEM. Note that responses were not different in vagotomized animals compared to the corresponding control animals at the 3- and 9-h time point

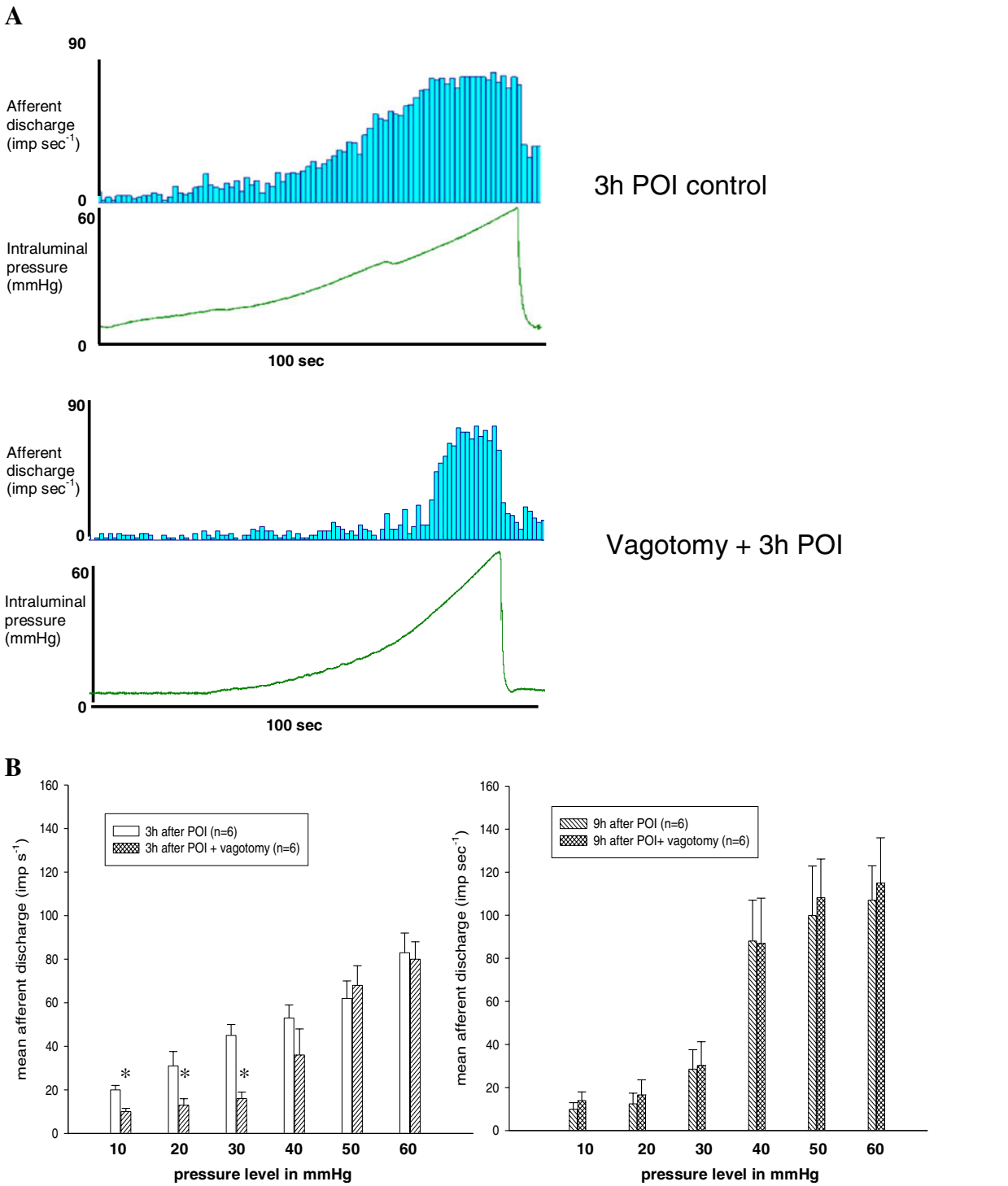

Fig. 5 **a** Representative recording of mesenteric afferent nerve discharge during mechanical intestinal distension in a segment taken from a mouse 3 h after induction of postoperative ileus and 3 h after induction of postoperative ileus following previous vagotomy. The *upper* trace displays sequential rate histograms of mesenteric afferent nerve discharge frequency in imp per second. The *lower* trace gives the recording of the increasing pressure in the intestinal segment during distension. Note the different response profiles of the afferent nerve, i.e., the greater rise in afferent firing at low distension pressures in 3-h postoperative ileus animals compared to vagotomized 3-h postoperative ileus animals. **b** This table shows the increase in afferent nerve discharge above baseline at different distension pressures during ramp distension at 3 and 9 h after induction of postoperative ileus compared to 3 and 9 h after induction of postoperative ileus following previous vagotomy. Note that vagotomy in postoperative ileus animals only altered the afferent nerve response to distension from 10 to 30 mmHg at 3 h. This effect did not occur at 9 h after induction of postoperative ileus ($p<0.05$ vs. 3-h POI controls)

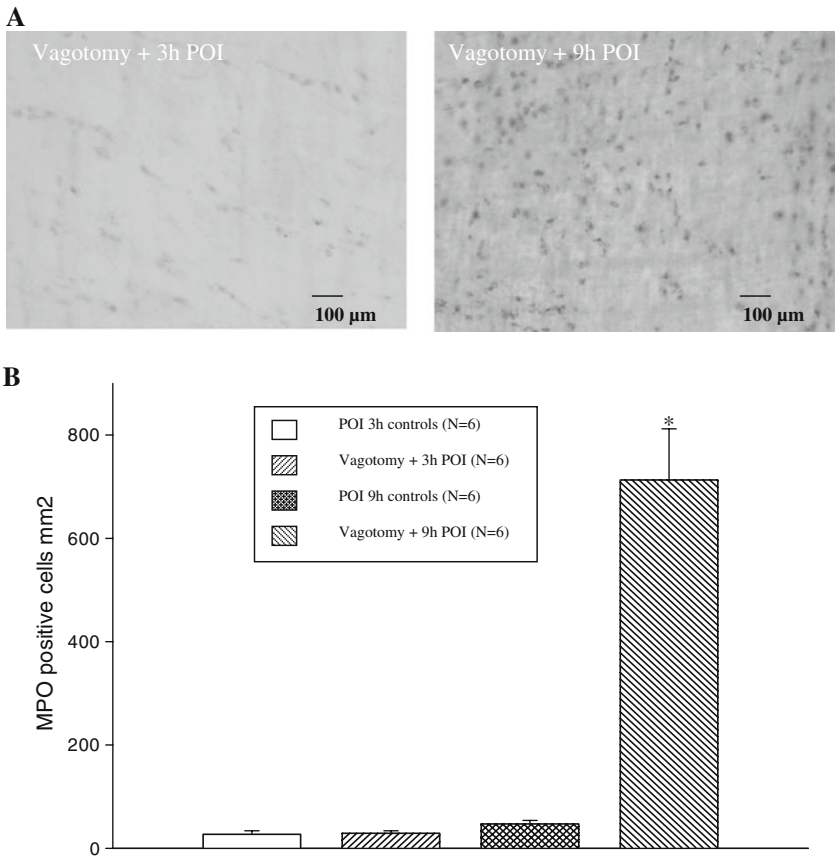

Fig. 6 **a** MPO-stained leukocytes in whole mounts of intestinal muscularis. Surgical manipulation was followed by a leukocyte infiltration of muscularis layer that was increased by vagotomy. **b** Histogram of the number of leukocytes infiltrating intestinal muscularis in whole mounts. Leukocyte counts are given as positive cells per square millimeter (mean±SEM, *$P<0.05$ vs. 9-h POI controls)

Discussion

During the time course of postoperative ileus, an acute phase early after surgery and a prolonged phase later on may be differentiated.[5] It seems that different pathophysiological mechanisms may be at play during different phases of postoperative ileus which is likely to have therapeutic implications.

In the present study, postoperative ileus was investigated 3 and 9 h following surgery which was simulated by intestinal manipulation. Intestinal motility was inhibited to a similar extent in all subgroups, i.e., independent of time point or vagal denervation by chronic subdiaphragmatic vagotomy 3 to 4 days before intestinal manipulation. Visceral sensitivity to 5-HT was absent following vagotomy as was mechanosensitivity to low-threshold distension. Bradykinin sensitivity was increased at the 9-h time point independent of vagotomy. Interestingly, detection of leukocytic infiltrates in the intestinal muscularis layer was similar in the different subgroups except for the chronic vagotomy group at 9 h after surgery when a dramatic increase in MPO staining was seen.

The small intestine is connected to the central nervous system via efferent and afferent spinal nerve fibers projecting to the spinal cord and by efferent and afferent vagal nerve fibers reaching the brain stem (Fig. 7). Recordings of intestinal motility in the different subgroups at 3 and 9 h after surgery demonstrated that a complete inhibition persists during this period which was uninfluenced by vagotomy. Thus, the vagus nerve obviously does not trigger inhibition of intestinal motility at this early time point. This is in keeping with previous evidence that spinal afferents are responsible for early neuronal reflex inhibition of intestinal motor events (e.g., eight).

We aimed to investigate afferent sensitivity via vagal and spinal pathways for two reasons: The absence of vagal afferent sensitivity documents successful vagal denervation by subdiaphragmatic vagotomy and subsequent degeneration of the vagal innervation to the small intestine. The second reason was that we aimed to explore whether the vagus has a modulatory action on spinal afferent pathways which are important for the development of POI immediately after surgery.[8]

Afferent sensitivity to 5-HT was absent following chronic vagotomy. This is in accordance with previous observations that the 5-HT afferent nerve response is mediated by vagal afferents via the 5-HT_3 receptor.[17,18] Another parameter to test vagal afferent sensitivity is low-threshold distension of the intestinal loop.[19] This was also reduced in chronically vagotomized animals at the 3-h time

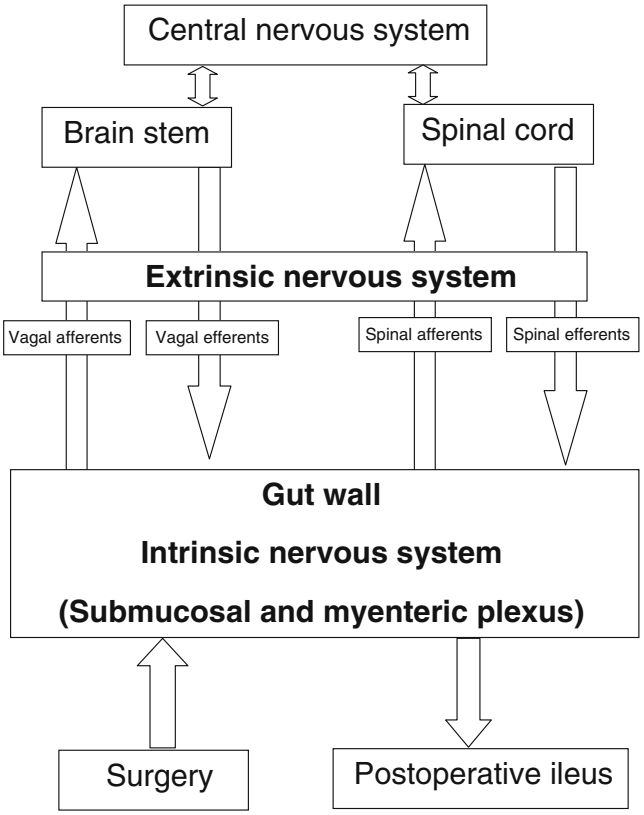

Fig. 7 Summary of pathways involved in postoperative ileus. After surgery extrinsic spinal and vagal nerve fibers are differentially activated which results in inhibition of intestinal motility known as postoperative ileus

point, and, therefore, also documents that vagal innervation was absent. The rationale behind this is that vagal innervation is considered to be responsible for the organization of physiological functions such as normal motility which involves sensing of low-pressure motor events, while high pressure or—in other words—noxious stimulation by high pressure luminal distension sensitizes predominantly serosal afferents which are of spinal origin.[19] The latter kind of afferent sensitivity was uninfluenced by chronic vagotomy which shows that sensitivity to noxious mechanical stimulation is not vagally mediated and, therefore, relayed via the second large population of afferents in the mesenteric nerve bundle which is spinal. Furthermore, this spinal afferent sensitivity was not modulated by the absence of vagal fibers. These observations were confirmed by testing afferent sensitivity to bradykinin which is also predominantly mediated via spinal afferents.[20,23]

Interestingly, reduced afferent discharge to low-pressure distension was only observed at the 3-h and not the 9-h time point. It seems that after 9 h, the inflammatory response is a lot more developed in the vagotomy group when compared to controls as shown in this study by MPO staining of the muscularis layer in the intestine. Increased release of inflammatory mediators in the vagotomy group at 9 h may have sensitized afferents more than in controls which may have blurred the difference in afferent nerve discharge at low distension pressures seen between both groups at 3 h. This possible explanation is supported by the observation that there is a general trend to increased discharge in the vagotomy group at 9 h compared to controls and generally increased afferent firing responses at 9 h compared to the 3-h time point.

While we found that the vagus nerve does not influence postoperative ileus in its early phase 3 and 9 h hours after surgery, a completely different situation exists during the prolonged phase at 24 h when The et al.[11] described a modulatory action of the vagal innervation. Apparently, this modulatory action of the vagus nerve is not relevant for the early phase; since then, the inflammatory response just starts to develop as was shown by our myeloperoxidase stains that were essentially negative in non-vagotomized animals 3 and 9 h after surgery, while it is fully established at 24 h in the study by The et al.[11] In other words, at 3 h, the inflammatory response as a substrate for vagal modulation would not have been established, so that it could not be altered by vagal innervation and postoperative ileus subsequently. Interestingly, the count of MPO-positive cells went up almost tenfold at the 9-h time point in vagotomized animals. The most likely explanation here is that the attenuating effect of the vagus nerve[10] was missing, so that a more robust inflammatory response developed, albeit this did not relate in altered motility or afferent sensitivity at this early time point after surgery yet, although it was observed by others later on.[11]

In the present study, there was no effect of complete vagotomy on early postoperative motility. Thus, a contribution of the efferent and the afferent vagus to early postoperative ileus was ruled out. Furthermore, selective ablation of afferents by capsaicin was tested in a previous study at the 24-h time point and did not show any effect on motility compared to controls.[24] We, therefore, believe that it is very unlikely that vagal efferent or afferent fibers contribute selectively to the early inhibition of intestinal motility brought on by spinal reflexes.[8]

This study supports current understanding of postoperative ileus as a condition consisting of two phases.[11] While the first phase starting immediately after surgery and lasting for a few hours is characterized by neuronal reflex inhibition via spinal innervation, the subsequent second phase is dominated by leukocyte infiltration in the muscularis layer of the gut wall which results in a prolonged period of inhibited intestinal motility. Thus, stimulation of intestinal motility via the vagal axis, e.g., by gum chewing,[25] agonists at the α-7 subunit of acetylcholine receptors on intestinal macrophages[5] or acetylcholine esterase inhibitors [21] is unlikely to improve

this condition early after surgery but potentially in the prolonged phase of postoperative ileus. Furthermore, the prolonged phase of postoperative ileus may be influenced by anti-inflammatory agents, while the acute phase may be improved by reducing inhibitory spinal reflexes. Thus, a phase adapted differential therapeutic concept seems necessary for optimal treatment of postoperative ileus.

We conclude from our study that sensitivity to 5-HT and low-threshold distension is mediated via vagal afferents, while sensitivity to high-threshold distension and bradykinin is independent of intact vagal innervation—observations that confirm previous evidence. Inhibition of intestinal motility at 3 and 9 h after surgery does not depend on the vagus nerve. The dramatically increased number of leukocytes infiltrating the intestinal muscularis at 9 h in vagotomized animals suggests that vagal modulation may start around this time point which may affect motility later on subsequent to an increased local inflammatory response.

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Discussion

DR. BRIAN J. DUNKIN (Houston, TX): I want to congratulate you on really an elegant model and an elegant piece of work. When I'm reviewing this, it seems to me that the one of the essentials of the presentation is that you confirm that this model works by vagotomizing the animals and then, three days later, by your measurements with 5HT and a low-pressure luminal distension, you are not seeing the expected results for the vagus nerve. So that's a nice confirmation of the model.

And then it seems you also define the early point for leukocyte infiltration, in that it happens at nine hours, but not at three hours, and the vagus nerve seems to modulate that.

I have a few questions.

One starts with the leukocyte modulation. You're not seeing really any difference in leukocyte infiltration at the three-hour mark. And at the nine-hour mark, when your vagus nerve is intact, that's modulated. Are we not seeing that three hours because of a role of the vagus nerve, or is it really that your immune response can't react that fast by the three-hour time point and it kind of catches up with you at nine?

A second is that, to me, this would suggest that anti-inflammatories could help reduce postoperative ileus, and I would love to hear your comments on that, either with a Cox-2 inhibitor, which seems to have an effect on the spinal, or with just steroids.

The last is, has this model been tested in other animal models to confirm that it works as well?

Closing Discussant

DR. MARIO HELMUT MUELLER:

1: Leukocyte infiltration takes some time to develop.

First, macrophages release cytokines like IL-6 and only thereafter leukocytes are entering the gut wall. Control experiments proved that there is a gap between three and nine hours experiments (this is not shown on the data slide). In fact, there is a seven fold increase.

2: Anti inflammatory drugs, Cox 2 inhibitors could be effective, but you will probably have to use the drug in advance.

If postoperative ileus has been fully developed, it might be difficult to modulate it. Besides the inflammatory reaction there is a neuronal component which might be COX-2 dependent. No clinical data is available on this case, but it might be worth using a Cox 2 in advance.

3: The model was first established in rats.

The reason for choosing the mouse model is that mice can easily be genetically modified and therefore are more 'divers'(knockout mouse).

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2010 SSAT POSTER PRESENTATION

Tissue Compression Analysis for Magnetically Anchored Cautery Dissector During Single-Site Laparoscopic Cholecystectomy

Lauren B. Mashaud · Wareef Kabbani · Angel Caban · Sarah Best · Deborah C. Hogg · Jeffrey A. Cadeddu · Richard Bergs · Heather Beardsley · Raul Fernandez · Daniel J. Scott

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Abstract
Introduction The purpose of this study was to evaluate the histological effects of dynamic abdominal wall compression using the magnetic anchoring and guidance system (MAGS) platform.
Methods Cholecystectomy was performed in two nonsurvival and two survival pigs using a single-site laparoscopic (SSL) approach. A deployable MAGS cautery dissector was used to perform the entire dissection in conjunction with a laparoscope and other instruments. The abdominal wall areas corresponding to the region occupied by the MAGS platform were examined grossly and microscopically for signs of tissue damage. Gallbladder dissection time was 36 min with no complications. Compressed abdominal wall thickness was 1.4 cm.
Results In all four animals, a very mild skin erythema was noted immediately postprocedure but was nonvisible within 20 min. Mild peritoneal blanching was noted in two animals, and one animal exhibited a 5-mm area of petechiae. Necropsy demonstrated no adhesions. Light microscopy documented no evidence of tissue injury for all specimens.
Discussion This study demonstrated that the use of the MAGS cautery dissector for a SSL cholecystectomy was advantageous in providing triangulation and did not result in any significant gross or microscopic tissue damage despite the thin abdominal wall of the porcine model.

This study was presented as a poster presentation at the Society for Surgery of the Alimentary Tract, May 1–5, 2010, New Orleans, LA.

L. B. Mashaud · A. Caban · D. C. Hogg · D. J. Scott (✉)
Southwestern Center for Minimally Invasive Surgery,
University of Texas Southwestern Medical Center,
5323 Harry Hines Blvd,
Dallas, TX 75390-9156, USA
e-mail: Daniel.Scott@utsouthwestern.edu

W. Kabbani
Department of Pathology, University of Texas Southwestern Medical Center,
Dallas, TX, USA

S. Best · J. A. Cadeddu
Department of Urology, University of Texas Southwestern Medical Center,
Dallas, TX, USA

R. Bergs · H. Beardsley · R. Fernandez
Texas Manufacturing Assistance Center,
Automation and Robotics Research Institute, University of Texas,
Arlington, TX, USA

Keywords Magnetic anchoring and guidance systems (MAGS) · Magnets · Laparoscopy · Natural orifice translumenal endoscopic surgery (NOTES) · Laparoendoscopic single-site surgery (LESS) · Histology

Introduction

Over the last two decades, laparoscopic surgery has surpassed conventional open surgery for many procedures in terms of patient benefit.[1–3] The current shift towards minimal invasive surgery has not abated, and new ground is constantly being paved towards techniques that reduce the number of laparoscopic port sites. Two approaches have emerged, including natural orifice translumenal endoscopic surgery (NOTES) and single-site laparoscopic surgery (SSL). These techniques propose different advantages as the number of port sites are reduced, thereby reducing risk of port site complication rates.[4–6] However, these techni-

ques present a new set of technical challenges that have yet to be overcome by the limitations of current technology. The central theme plaguing these techniques revolves around multiple instruments being passed through a single access port, which creates poor working angles, unavoidable instrument collisions, and inadequate reach, or the inability to vigorously manipulate tissue and retract organs.[7]

Realizing these limitations, our group developed the magnetic anchoring and guidance system (MAGS), which consists of an external handheld magnetic instrument that is coupled across the abdominal wall to a magnetic device that is deployed intraabdominally through an access port incision.[8–10] In this manner, instruments required to perform a variety of surgical procedures can be deployed as needed (e.g., camera, cautery, retractors, etc.) in either a NOTES or SSL environment.[11,12] By coupling the instruments across the abdominal wall, an otherwise occupied access port is freed for use by other instruments. Angles needed to perform dissection (sharp or blunt) can be attained even with associated space constraints of current platforms. Instrument collisions are eliminated due to increased degrees of freedom and the loss of "parallel" instrument working environment.

Our group has previously published data regarding the performance of the MAGS devices in porcine models[9,10] and to a limited extent in actual human cases.[13] In our cumulative experience of 59 live porcine, and two human cases, we have not detected gross evidence of any significant adverse tissue effects due to abdominal wall compression between the internal and external magnets. However, anticipating potential regulation concerns regarding this novel instrument platform, we have embarked on studying the tissue effects related to MAGS. Our prior study documented no gross or histological adverse effects after 4 h of stationary compression in a survival porcine model.[14] The aim of this study was to examine the gross and histological effects of abdominal wall compression during an operative procedure using the MAGS platform.

Methods

Cholecystectomy was performed in four porcine models (41–53 kg, two nonsurvival and two survival (72 h and 14 days). All animals underwent general anesthesia and were placed in a supine position. For all procedures, a SSL approach with percutaneous gallbladder retraction sutures was used (Figs. 1 and 2). The models were prepped and draped using sterile technique. Initial entry was established by making a 2.5-cm transumbilical incision. The MAGS cautery dissector was inserted through the fascial defect with the electrical cable exiting the abdomen through this

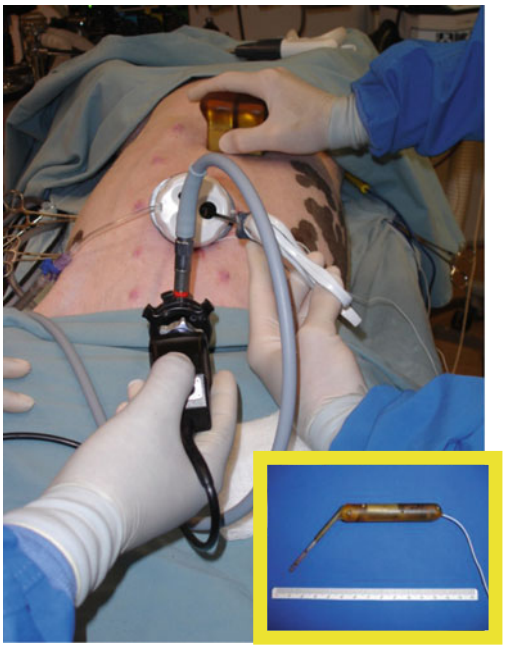

Fig. 1 External view of the operative setup, including the multiport access device and the external handheld magnet for the MAGS cautery dissector and MAGS cautery instrument (*inset*)

defect. In expectation of future device repositioning, additional tether length (approximately 15 cm) for the device was inserted to ensure adequate length for intra-abdominal maneuvering. The wound retractor portion of the multiport access device (Uno Port, Ethicon Endosurgery, Cincinnati, OH) was fixed into place, and the cap was sealed. Pneumoperitoneum of 15 mmHg was established, and a 5-mm 30° standard length rigid laparoscope was inserted for visualization. The MAGS device was visualized, and the external handheld magnet was used to couple the instrument across the abdominal wall by slightly

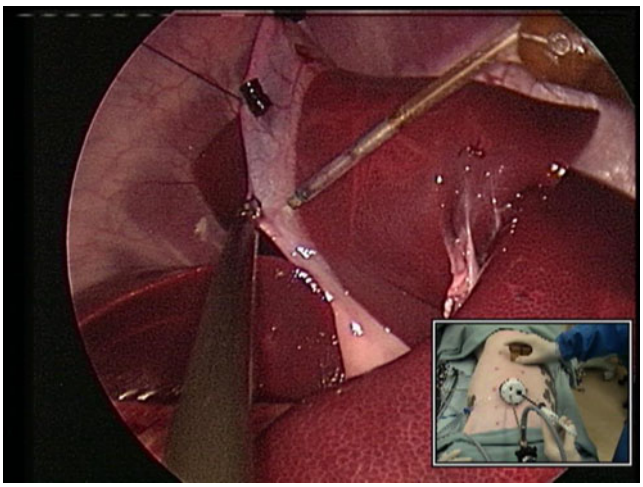

Fig. 2 Internal (laparoscope) view of the operative field, including the MAGS cautery dissector and suture retraction of the gallbladder; *inset* shows external view

deforming the abdomen. To retract the gallbladder and expose the area of dissection, a 2–0 prolene on a Keith needle with a rubber syringe stopper tied to the end of the suture was employed (Fig. 2). The needle was inserted into the abdominal cavity using a laparoscopic needle driver. Similar to our clinical experience, the dome of the gallbladder was then transfixed, and a suitable location was chosen high in the right subcostal area in the midclavicular line; the suture was percutaneously retrieved and fixed externally with a hemostat.[6] In a similar fashion, a second Keith needle was placed at the mid-body of the gallbladder to provide further exposure; this suture was retrieved near the anterior axillary line. A standard rigid laparoscopic grasper was used through the access port to provide additional gallbladder retraction. The MAGS cautery device was used to perform the dissection and to skeletonize the cystic duct artery. Once a critical view of safety was achieved, a clip applier was used to clip these structures, and they were transected using laparoscopic scissors. The MAGS cautery instrument was used to remove the gallbladder from the bed of the liver. This was accomplished by using blunt dissection and monopolar electrocautery energy. Once all attachments of the gallbladder were removed, the retraction sutures were released, and the organ was removed from the abdomen through the SSL port and inspected for perforations. Areas on the skins where magnetic coupling occurred were delineated with a permanent marker and also marked with staples to ensure accurate future tissue harvesting. These areas were immediately examined internally using the laparoscope and externally by direct visualization for any signs of tissue damage. Nonsurvival animals (n=2) were immediately sacrificed. For survival animals (n=2), the fascia was closed, and the skin was approximated; these animals were then recovered and observed prior to sacrifice at 72 h and 14 days.

At necropsy, the abdominal wall areas corresponding to the region occupied by the MAGS platform (previously marked locations) were grossly examined internally and externally for signs of tissue damage. Adhesions were graded according to a previously established adhesion grading system described by Diamond and colleagues.[15] Compressed and uncompressed abdominal wall thicknesses were measured. A total of four abdominal wall segments were harvested within the confines of the previously marked borders. Representative samples were sent for histological analysis. Additionally, multiple random tissue sections of skin, subcutaneous tissue, and muscle were submitted for as controls. Histological evaluation was performed by a board-certified pathologist who was blinded to the specimen source (control or treatment group). A thorough examination of areas for necrosis or discoloration was performed. Paraffin-embedded, H&E sections were examined to evaluate necrosis, acute inflammation, ulceration, and vascular congestion. In addition, nicotinamide adenine dinucleotide dehydrogenase (NADH) staining was performed to evaluate cellular viability (nonsurvival animals only). The following histological metrics were used to assess muscle necrosis: sarcolemmal nuclear displacement or loss, mononuclear infiltration, muscle fiber splitting, variation in muscle size (ischemic atrophy), hyaline degeneration, and cytoplasmic vacuolization. All findings were scored using standardized grading scales. Each parameter was graded as absent, focally present (one focus), multifocal, and diffuse (>20% of muscle fibers involved). In addition, the presence of acute inflammation, fat necrosis, skin ulceration, and vascular congestion was recorded and graded as: absent, mild (<5% of the section area), moderate (5–20%), and marked (>20%). NADH staining was graded as: absent, focal (one focus), multifocal, and diffuse (<20% of fibers with loss of NADH staining).

Results

All cholecystectomies were completed using a SSL approach. A small gallbladder perforation occurred in pigs 1, 2, and 4, but bile spillage was minimal, and there were no complications. The total operative time was 73 min (66–82). The average gallbladder dissection time (start of dissection until completion of gallbladder removal from the liver bed) was 36 min (15–49). The remaining operative time accounted for access, MAGS device insertion and arm deployment, gallbladder suture retraction, specimen and device retrieval, peritoneal surface inspection, photodocumentation, data collection, and closure. No difficulties were encountered with introduction, deployment, or retrieval of the MAGS device, which took 1–2 min at the beginning and end of the procedure.

The MAGS cautery instrument facilitated excellent triangulation with minimal instrument conflicts when used in conjunction with only one laparoscopic instrument, a laparoscope and suture retraction; a clear critical view was achieved in all cases. In all cases, MAGS coupling strength was excellent and suitable instrument control was afforded. Uncompressed and compressed abdominal wall thicknesses were 2.1 cm (1.4–2.5), and 1.4 cm (1.1–1.8), respectively. The average percent compression was 29.7±6.0%.

On postoperative visual inspection of the skin, mild erythema was noted in all four animals immediately postprocedure, the areas corresponding to the outer edges of the rectangular external magnet; there was no bruising seen immediately and during follow-up. This erythema dissipated and became nonvisible within 20 min. In two animals (3 and 4), mild blanching was noted on the

peritoneal surface. This blanching very rapidly became nonvisible within 30 s. One animal (pig 3) exhibited a 5-mm area of petechiae on the peritoneal surface which was no longer apparent upon sacrifice at 72 h.

At necropsy, no signs of infection, no adhesions to the peritoneal surface areas, and no gross signs of tissue damage where the magnet had been coupled were observed. All tissue sections demonstrated no light microscopic evidence of skin, subcutaneous and muscle necrosis or atrophy, and no loss of the normal NADH staining (Figs. 3 and 4). No ulceration, acute or inflammation or granulation tissue formation, fat necrosis or vascular congestion was noted.

Discussion

With the advent of NOTES and SSL, surgeons, investigators, and device manufacturers have realized that traditional endoscopic and laparoscopic platforms are not well suited for some aspects of these novel procedures. Deployable instruments may be one solution. Only one mechanically activated deployable device has been introduced clinically; this device uses a grasper to retract tissue by attaching a second grasper to the peritoneal surface.[16] Alternatively, several groups, including ours, have explored the use of magnets for organ retraction. We reported a MAGS gallbladder retractor which facilitated transgastric, transcolonic, and transvaginal cholecystectomy in porcine models.[17] Others have reported similar magnetic retraction strategies for NOTES and SSL cholecystectomy in animals and live humans.[7,18–22] Our group has also introduced the concept of deployable instruments for other tasks, such as dissection and visualization. Both our MAGS cautery dissector and camera instruments have proven valuable in facilitating NOTES and SSL procedures in animal models.[9,11–14,17] More recently, we successfully used the MAGS camera for visualization during two SSL human cases (nephrectomy and appendectomy).[13] These reports document the utility of magnetically anchored instruments in overcoming numerous hurdles associated with limited access approaches.

Much of our work to date has focused on ensuring that the magnets generate sufficient coupling strength to overcome the distances associated with the abdominal wall. A large amount of engineering has been required to optimize current MAGS configurations, such that these instruments may be suitable for clinically relevant abdom-

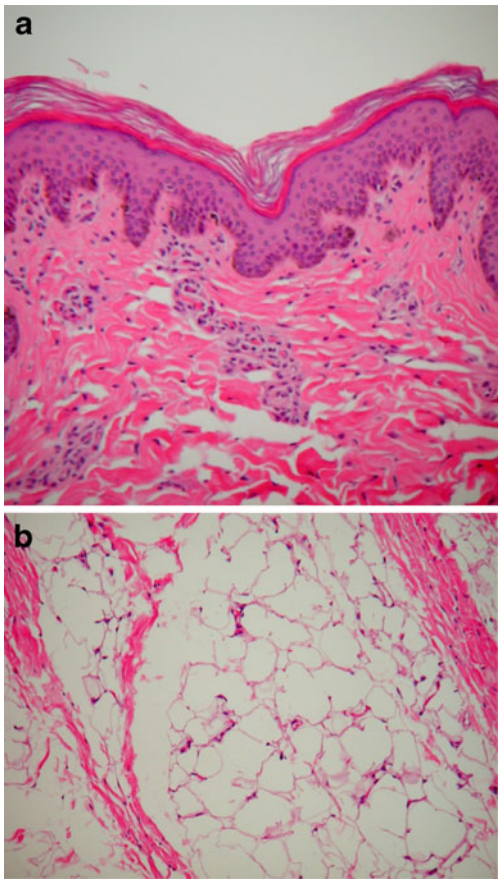

Fig. 3 Histopathology (H&E ×200) of the skin (**a**) and subcutaneous tissue (**b**) showing no ulceration, fat necrosis, or inflammation

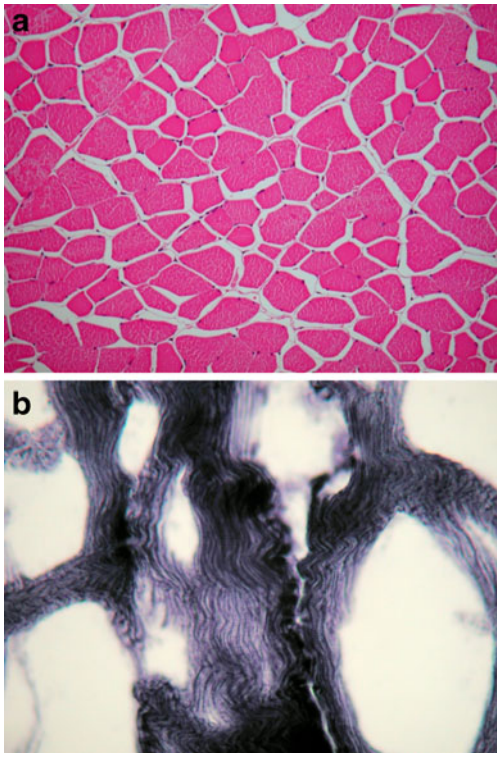

Fig. 4 Histopathology (×200) of skeletal muscle. **a** H&E stain showing no necrosis, inflammation, or atrophy, and **b** NADH staining (for tissue viability, nonsurvival subjects only), demonstrating normal staining pattern (completely viable tissue)

inal wall thicknesses. Milad et al. documented that only 1.5% of patients with a BMI ≤40 kg/m^2 had abdominal thicknesses >4.0 cm.[23] In a prior study by our group, we documented that the optimal MAGS configuration was capable of suspending our heaviest internal instrument (remote-controlled cautery dissector, 39g) at a distance of 4.78 cm.[24] Our recent human experience suggests that the MAGS platform generates sufficient coupling forces to facilitate accurate camera navigation in nonobese adult and adolescent patients.[13]

On the other hand, one concern that has been repetitively raised by reviewers of our work has been the potential for tissue injury due to magnetic compression forces. Numerous reports of animal and human work using magnetically anchored instruments have not documented any adverse tissue effects during the use of available instruments.[8–12,17–22,24] However, there has been very little work done to document the safety of these instruments from a histological perspective. It is this paucity of literature that has driven our group to explore these areas and publish our findings.

Our prior study documented no significant gross or microscopic tissue damage after static MAGS compression.[14] In that study, external and internal MAGS devices were applied across the abdominal wall for up to 4 h in pigs that were survived up to 14 days. In the 12 specimens collected from three animals, only mild, transient peritoneal blanching was observed, and there was not any skin bruising; histologic evaluation showed no necrosis, atrophy, or ulceration. These data were reassuring in supporting MAGS safety since relatively thin abdominal walls (1.5 cm compressed) exhibited no significant adverse effects over a relatively long period of time. However, the question still remained whether repetitive manipulations of a MAGS instrument during the performance of an actual procedure would result in different tissue effects. Thus, the goal of our study was to replicate the conditions relevant to an actual operation and to examine these same tissue interactions. The SSL cholecystectomy model provided a suitable procedure whereby the MAGS cautery dissector could be extensively used during the whole dissection. The procedures were accomplished by gently rocking the external component to accomplish blunt and sharp dissection with accuracy. The external component was also translated over short distances to keep tissues within reach. Although operative times were relatively short, given the extensive manipulations of the instrument in conjunction with a thin abdominal wall (1.4 cm compressed), this model seemed appropriate. Importantly, the only adverse effects that we detected were similar to the prior static study. Namely, mild, transient peritoneal blanching and skin erythema were detected; however, no bruising occurred, and no adverse histological effects were evident. Additionally, no complications were noted at necropsy, and there were no adhesions in the areas corresponding to MAGS instrument manipulation, which corroborates our prior findings in other survival animal studies.

This study also provided us with additional information regarding the use of the MAGS cautery dissector to facilitate a SSL cholecystectomy. By using the MAGS cautery dissector as the primary operative device, excellent triangulation was afforded; the cautery dissector was freely mobile and allowed a 90° angle of approach from the umbilical port instruments. Coupling strength in all of our procedures was excellent, and precise movements were afforded, such that a critical view was achieved. Moreover, using the MAGS cautery instrument in conjunction with suture gallbladder retraction strategy freed up an otherwise occupied port; thus, only two laparoscopic instruments (a grasper and the laparoscope) were used. This technique nearly eliminated the cumbersome internal and external instrument conflicts that normally are associated with a SSL approach. Additionally, the MAGS cautery dissector with deployed electrically tethered across abdominal between the multiport access device and the abdominal wall did not result in problems maintaining the pneumoperitoneum. While perforations during the dissection occurred in pigs 1, 2, and 4, bile spillage was minimal. We attribute the perforations to the difficult nature of the SSL approach, even with the improved triangulation afforded by the MAGS cautery dissector, and to the very thin-walled nature of the porcine gallbladder with nearly nonexistent fat planes. Undoubtedly, there will be a learning curve associated with the MAGS device; our operative times were somewhat long (73 min). However, we attribute this primarily to the investigational nature of our experiments, as much time was spent for photodocumentation and data collection. MAGS instrument deployment and retrieval took very little time (1–2 min), and the dissection phase was reasonably short (36 min).

In conclusion, this paper adds to our understanding of tissue interactions related to the MAGS platform. Similar to our prior static study, this study which examined dynamic interactions detected no significant gross or microscopic tissue damage after a realistic porcine SSL procedure. Additionally, the MAGS platform proved advantageous in performing these operations. These findings support important safety and efficacy aspects of the MAGS platform for planned use in humans.

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ORIGINAL ARTICLE

Does Impaired Gallbladder Function Contribute to the Development of Barrett's Esophagus and Esophageal Adenocarcinoma?

Ayman O. Nassr · Syeda Nadia Shah Gilani · Mohammed Atie · Tariq Abdelhafiz · Val Connolly · Neil Hickey · Thomas Noel Walsh

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Abstract
Introduction Esophageal adenocarcinoma is aetiologically associated with gastro-esophageal reflux, but the mechanisms responsible for the metaplasia–dysplasia sequence are unknown. Bile components are implicated. Impaired gallbladder function may contribute to duodenogastric reflux (DGR) and harmful GERD.
Aims This study aims to compare gallbladder function in patients with Barrett's esophagus, adenocarcinoma, and controls.
Methods Three groups of patients, all free of gallstone disease, were studied. Group 1: (n=15) were normal controls. Group 2: (n=15) were patients with >3-cm-long segment of Barrett's esophagus. Group 3: (n=15) were patients with esophageal adenocarcinoma. Using real-time ultrasonography unit, gallbladder volume was measured in subjects following a 10-h fast. Ejection fraction was calculated before and after standard liquid meal and compared between the groups.
Results The mean percentage reduction in gallbladder volume was 50% at 40 min in the adenocarcinoma group compared with 72.4% in the control group (p<0.001). At 60 min, gallbladder filling had recommenced in the control group to 64.1% of fasting volume while continuing to empty with further reduction to 63% in the Barrett's group and to 50.6% (p=0.008) in the adenocarcinoma group. The mean gallbladder ejection fraction decreased progressively from controls to Barrett's to adenocarcinoma and was significantly lower in Barrett's group (60.9%; p=0.019) and adenocarcinoma group (47.9%; p< 0.001) compared with normal controls (70.9%).
Conclusion Gallbladder function is progressively impaired in Barrett's esophagus and adenocarcinoma. Gallbladder malfunction increases duodenogastric reflux, exposing the lower esophagus to an altered chemical milieu which, in turn, may have a role in promoting metaplasia–dysplasia–neoplasia sequence in the lower esophageal mucosa.

Keywords Gallbladder function · Barrett's esophagus · Adenocarcinoma esophagus

A. O. Nassr · S. N. S. Gilani · M. Atie · T. Abdelhafiz · V. Connolly · T. N. Walsh (✉)
Department of Surgery, Academic Centre, Connolly Hospital, Blanchardstown,
Dublin 15, Ireland
e-mail: twalsh@rcsi.ie

N. Hickey
Department of Radiology, Academic Centre, Connolly Hospital, Blanchardstown,
Dublin 15, Ireland

Introduction

The chief risk factor for esophageal adenocarcinoma is Barrett's esophagus, which in turn results from mucosal injury secondary to gastro-esophageal reflux disease (GERD). Impaired lower esophageal sphincter function and the composition of the refluxate are key factors in any resultant mucosal injury. Disturbance of lower esophageal sphincter function allows reflux of gastric contents into the esophagus.[1–4] In addition to gastric acid,[5–7] other components of the refluxate such as pancreatic enzymes,[8–12] intestinal enzymes,[8,13–15] and bile, in particular,[8,16] are all implicated in esophageal mucosal injury.

Bile is stored in the gallbladder between meals and is expelled into the duodenum by contraction of the gallbladder in response to meal-stimulated CCK secretion from the duodenum (Fig. 1). In addition to inducing gallbladder contraction, CCK causes relaxation of the sphincter of Oddi and relaxation of the lower esophageal sphincter (LES). The resultant bile bolus in the duodenum switches off CCK release by a negative feedback mechanism. (Fig. 2). Thus, when the gallbladder is functioning normally, bile is mixed with food in the duodenum and little is free to enter the stomach or come into contact with the esophageal mucosa.[18,19]

This orderly sequence of bile processing can be disturbed in a number of circumstances,[20–23] such as a non-functioning gallbladder or following cholecystectomy. When the gallbladder is removed or full of gallstones, the storage facility is destroyed. Instead of storage followed by meal-stimulated release, bile trickles constantly from the liver into the duodenum permitting retrograde reflux into the stomach.[24–26] Furthermore, as the bile is delivered constantly into the duodenum the mechanism for switching off CCK secretion is impaired. Plasma CCK levels remain elevated after meals[27] which in turn may contribute to altered cardia function[28–32] and increased gastro-esophageal reflux. When the gallbladder is non-functioning or poorly functioning bile storage and release by the gallbladder is compromised. The absence of a bolus fails to provide a negative switch-off signal. Gallbladder function may be altered by increasing volumes of gallstones[33,34] chronic cholecystitis, chronic pancreatitis,[35] and by diabetes mellitus[36] amongst other diseases.[37–39]

There is evidence that implicates duodenogastric-esophageal reflux in the pathogenesis of Barrett's esophagus[40–42] and adenocarcinoma. We have previously shown that cholecystectomy is associated with an increased incidence of GERD.[22,27,70] Others have shown a link between

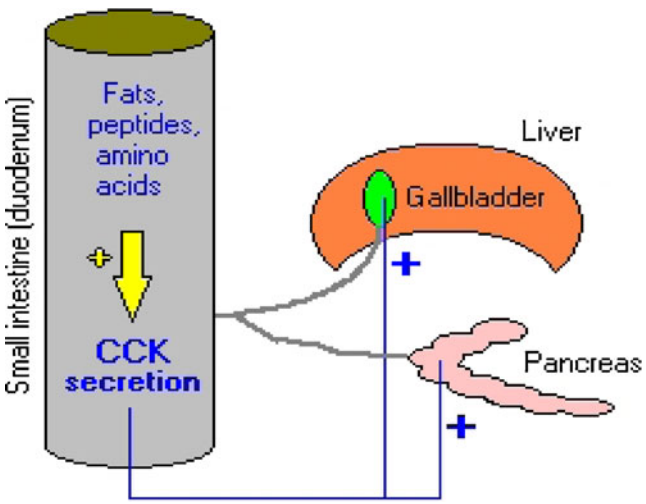

Fig. 1 Normal mechanism of CCK release. Meal-stimulated CCK release from the duodenum results in gallbladder emptying. Impaired CCK release is a potential cause of impaired gallbladder emptying

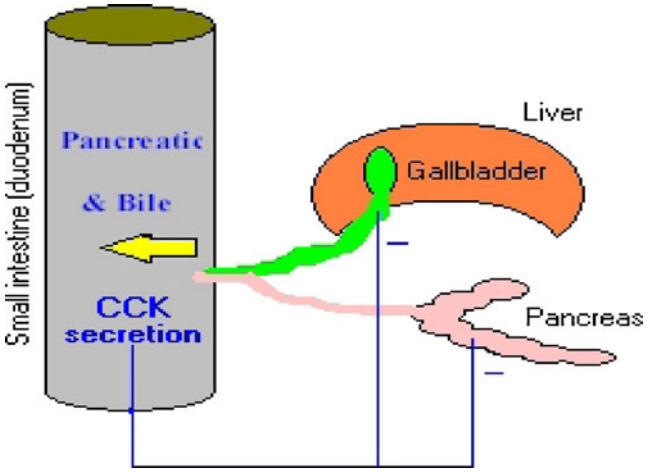

Fig. 2 Normal mechanism of CCK inhibition. CCK release from the duodenum is inhibited by the negative feedback of a bile bolus in the duodenum. Modulation of CCK release is the chief mechanism of control of gallbladder emptying

cholecystectomy and adenocarcinoma.[20,22,24] Studies have also demonstrated that foregut and gallbladder function are impaired in Barrett's esophagus.[33,34] There are no studies on gallbladder function in esophageal adenocarcinoma.

As a unifying concept to weave together these strands of evidence, we hypothesized that gallbladder function may be impaired in patients with Barrett's esophagus and adenocarcinoma of the esophagus, which may in turn contribute to duodenogastric reflux and to the metaplasia–dysplasia–neoplasia sequence. The aim of this study, therefore, was to compare gallbladder function between patients with Barrett's esophagus, adenocarcinoma, and controls.

Patients and Methods

Study Groups

Three study groups were enrolled. Since the prevalence of gallstone disease in patients with Barrett's is higher than patients without Barrett's[43], we screened all patients prior to study to exclude gallstone disease.

Group 1 (n=15) were healthy volunteers attending the radiology department for non-GI radiological investigations. None had symptoms of GERD.

Group 2 (n=15) were patients with histologically confirmed long segment Barrett's esophagus (>3 cm from the OGJ) who were off all medication that might impact on the gastrointestinal tract for at least 14 days.

Group 3 (n=15) were patients with newly diagnosed adenocarcinoma of the esophagus without evidence of metastatic disease, all of whom were able to swallow fluids without a difficulty.

Ethics Approval

Approval for the study was obtained from the ethics committee in the hospital before enrolment. All participants were interviewed and a study information proforma was completed. Informed consent was signed by each participant prior to recruitment to the study.

Exclusion Criteria

Participants with conditions known to affect gallbladder motility were excluded from the study. These included patients with diabetes, chronic liver disease or cholelithiasis. Also excluded were patients being prescribed pharmacological agents known to affect acid secretion or GI or gallbladder motility. A previous history of esophageal, gastric, duodenal, hepatic, pancreatic, or biliary surgery was also an exclusion factor. Esophageal adenocarcinoma patients with advanced cancer, an esophageal stent in situ, severe dysphagia that might compromise the ingestion of the test meal, or patients receiving chemoradiotherapy or post-chemoradiotherapy were also excluded. Apart from the adenocarcinoma group none had a history of cancer.

Sample Size

Sample size was calculated using "PS: Power and Sample Size Calculation© software (version 2.1.31, 2003, Department of Biostatistics, Vanderbilt University School of Medicine, Nashville, TN).

The Standard Test Meal

All patients were given a standard test liquid meal to stimulate gallbladder contraction. The meal used as the standard test meal was the commercially available Fortisip® Strawberry (Nutricia Clinical NZ, Auckland, New Zealand). Fortisip® Strawberry is a commercially available nutritionally complete food commonly used for patients with increased energy or protein requirements or for those who have little appetite for food. A liquid preparation was chosen as standard to facilitate rapid ingestion into the stomach for patients with some degree of stenosis. The meal provided 300 kcal in each 200 ml fluid preparation (1.5 kcal/ml) in the form of 10.2% saturated fatty acids, 9.4% monounsaturated fatty acids, and 30.4% polyunsaturated fatty acids. It was felt that this would facilitate gallbladder emptying in a more physiological manner than using a purely fatty meal which may have resulted in exaggerated emptying. The standard test meal (Fortsip) was ingested at a rate of 50–100 ml/min via a straw.[44,45]

Measurement of Gallbladder Function

A real-time ultrasonography unit (Model: GE Logiq 9, GE Healthcare Technologies, Clinical Systems Information Technologies, Hatfield, UK) was used for measuring gallbladder volume.[46] The transducer used was a real-time multi-frequency (2–4 MHz) sector transducer.

Ultrasonographic scanning was performed after a 10-h overnight fast to ensure adequate gallbladder distension. The fasting gallbladder volume (FGV) was calculated and the common bile duct caliber was measured before meal ingestion. The gallbladder volume (GBV) was also calculated at 20, 40, and 60 min following the standard test meal.

Study participants remained in the sitting position after meal and between scans to facilitate passage of the meal through the stomach to the duodenum. Any participant who was unable to tolerate sitting comfortably adopted the right lateral position. Images and measurements were obtained in suspended deep inspiration.

Gallbladder volume was calculated using software capable of automatically capturing the gallbladder length, width and depth (Model: GE Logiq 9, GE Healthcare Technologies, Clinical Systems Information Technologies, Hatfield, UK). To verify the software produced readings, duplicate readings were also obtained to calculate the gallbladder volume manually by using the ellipsoid formula:[39,46,47]

$$\frac{\text{Volume} = \pi \times \text{Length} \times \text{Width} \times \text{Depth}}{6}$$

The FGV was taken as the base line volume.

Calculation of Gallbladder Emptying

Gallbladder emptying (GBE) was taken as the difference between the FGV and the GBV at a specific time, expressed as a percentage of the basal gall bladder volume:

$$\frac{\text{GBE}(X_{\min}) = (\text{FGV} - \text{GBV}(X_{\min})) \times 100}{(\text{FGV})}$$

Data Collection and Statistical Analysis

Data were collected using a computer-generated database (Microsoft Office Access 2003, Microsoft Windows XP Professional™, Microsoft Corporation, Redmond, WA). Statistical analysis was performed using SPSS 10.0 software for Windows™ (SPSS Inc., Chicago, IL) to calculate the mean, the standard deviation (SD) and the any significant difference between the different groups. The t test for independent samples was used to compare gallbladder emptying and ejection fraction between the study groups and the control group using a p value of <0.05 and a power of 0.8.

Results

Demographic Data

A total of forty-five participants had gallbladder emptying assessed with a male/female ratio of 2.4:1. There was no significant difference between the ages of the different groups. The mean (SD) age of the control group was 69.7 (8.7) years which compared with 66.0 (9.9) years for the Barrett's group (p=0.542) and 64.5 (14.9) years for the adenocarcinoma group (p=0.622).

Gallbladder Volume

The mean resting gallbladder volume was 26(1.1) mls in controls, compared with 38.1 (26.8) ml in the Barrett's group (p=0.005) and 27.6(16.1) ml in the adenocarcinoma group (p=0.054) (Table 1).

Gallbladder volume decreased gradually after the standard meal in all groups. This decrease was more significant at 20-min posttest meal in the control group, where gallbladder volume fell to 10.5 (5.1) ml than in the Barrett's group 22.7 (21.2) ml compared with the control group (p<0.001), or the adenocarcinoma group 15.5 (15.7) ml compared with the control group (p=0.003).

Gallbladder volume reached its lowest level in the control group at 40 min to 7.5[4] ml and had started to fill again by 60 min to 9.4 (4.3) ml. Both the Barrett's and the adenocarcinoma groups continued to show significant decrease in volume at 60 min confirming continuation of emptying with volumes of 14.9 (14.5) ml (p=0.007) and 14.4[13] ml (p=0.01), respectively. The gallbladder volume decrease was slower in the Barrett's group and the cancer group than in normal controls and continued up to 60 min with no recovery.

Percentage of Gallbladder Emptying

Because the mean starting fasting gallbladder volume showed slight variability among the three groups, the percentage gallbladder emptying was used to provide better comparison between the three study groups. The fasting gallbladder volume was taken as the base line volume from which subsequent emptied volumes were calculated as the percentage of gallbladder emptying at a specific point of time.

The mean percentage gallbladder volume emptied at 20 min following a standard meal in the control group was 60.5% (12.5%) which was not significantly different from the 48.5% (18%) in the Barrett's group. The percentage emptying in the adenocarcinoma group was 41% (26%) which was significantly reduced compared with the control group (p=0.021).

At 40 min, the mean percentage gallbladder volume reduction in the control group was 72.4% (6.9%), which was similar to the Barrett's group 58.4% (11.6%; p=0.213) but was significantly different from the adenocarcinoma group 44.9% (20.7; p<0.001).

At 60 min, gallbladder filling had recommenced in the control group to 64.1% (10.2). Both the Barrett's and the adenocarcinoma groups continued to show progression of emptying with further reduction to 63%[13] (p=0.427) in the Barrett's group and 50.6% (26.5; p=0.008) in the adenocarcinoma group.

The gallbladder emptying was faster and more significant in the control group compared with the cancer group but not the Barrett's group, mirroring the volume changes, and reaching maximal emptying at 40 min after which recovery was seen. Gallbladder emptying was delayed in the Barrett's and the cancer group particularly as seen after 40 min and continued up to 60 min posttest meal.

Gallbladder Ejection Fraction

The gallbladder ejection fraction was taken as gallbladder emptying at 40 min. The ejection fraction was taken as the study's end-point at which the statistical test for the study power was taken. The ejection fraction was calculated as 72.4% (6.9) in the control group, 58.4% (11.6) in the Barrett's group (p=0.213), and 44.9% (20.7) in the adenocarcinoma group (p<0.00 1) using the Student's t test for independent samples.

Table 1 Gallbladder volumes

	Fasting GBV (ml)	20 min (ml)	40 min (ml)	60 min (ml)
Controls	26	10.5	8	9.4
Barrett's group	38.1	22.7	15	15
Adenocarcinoma	27.6	15.5	18	14.4

Gallbladder volume decreased gradually after the standard meal in all groups. The gallbladder volume decrease was slower in the Barrett's group and the cancer group and continued up to 60 min with no recovery

Discussion

The increase in the incidence esophageal adenocarcinoma is multifactorial, but the contribution of Barrett's esophagus is beyond question.[48] The aetiology of Barrett's mucosa, which represents the severest end of the reflux spectrum, is in turn a consequence of altered gastrointestinal anatomy and physiological mechanisms[49] the most significant of which are the alteration in intestinal motility[50] and the

toxicity of the resultant refluxate.[16,51,52] The motility disturbances identified in Barrett's include esophageal body dysmotility leading to impaired clearance[53,54] lower mean basal LES pressure and increased transient lower esophageal sphincter relaxation episodes, which allow increased gastro-esophageal reflux, gastric dysmotility leading to delayed gastric emptying which could promote reflux and prolong contact with toxic refluxate[55–60] and possible antro-duodenal motility disorders.[61,62]

The toxicity of the refluxate is clearly a co-contributing factor to the resultant injury. While we are unclear about the exact chemical components which inflict most injury, it is clear that injury may result from gastric acid alone[52] or from acid combined with duodenal refluxate.[63,64] The role of acid and bile in the genesis of esophageal mucosal damage and reflux symptoms is complex. Acid combined with pepsin and unconjugated bile acids are critical to the development of esophagitis and Barrett's esophagus.[40,42] Bile alone or duodenal refluxate alone may be the principal factor in determining the severity of esophagitis as Barrett's has been described following total gastrectomy.[65,66] Patients with reflux disease have an increased concentration of bile acids in their esophageal aspirates.[12] Nearly half of the patients with reflux symptoms have combined pathological acid and bile reflux.[67]

Duodenogastric reflux (DGR) of duodenal content into the stomach occurs physiologically but anything that alters the structure or function of the duodeno-pancreatobiliary system will promote DGR. Thus, surgical destruction of the pylorus after distal gastrectomy,[68] pyloroplasty or Whipples procedure increase DGR.[17] Similarly, anything that disturbs the orderly sequence of bile secretion, storage, and release may contribute to DGR.[69] The most dramatic alteration in this environment occurs after cholecystectomy when bile is no longer stored between meals but streams continuously into the duodenum and the stomach.[22,23] We, and others, have previously shown that cholecystectomy results in increased gastro-esophageal reflux[70] and elevated levels of CCK.[27] Amongst the effects of CCK is the reduction in LOS pressure.[27] These combined disturbances may contribute to an abnormal concentration of bile refluxate for a longer duration in the lower esophagus. These findings support a possible contribution of gallbladder malfunction in the development of Barrett's esophagus and adenocarcinoma.[21]

In this study we have shown that gallbladder emptying is progressively impaired in the Barrett's esophagus and adenocarcinoma groups. In normal subjects gallbladder emptying in response to a meal was complete by 40 min after which it began to fill again. Approximately two thirds of resting volume had emptied within the first 20 min and three quarters by 40 min and the filling process had commenced within 60 min of a meal, which is consistent with the literature.[71] The pattern of emptying was altered in the Barrett's group and even more so in the adenocarcinoma group where both groups continued to show significant decrease in volume to 60 min confirming continuation of emptying suggesting abnormal gallbladder motility. Thus, patients with Barrett's and adenocarcinoma had an impaired response to meals suggesting that their gallbladder function was impaired and incapable of storing and releasing bile normally, a condition approaching non-functioning gallbladder. Patient following cholecystectomy[21–23] or patients with poorly functioning gallbladders have increased bile reflux into the stomach and increased potential toxicity.

The cause of the abnormal gallbladder function in Barrett's and cancer is unclear. It may be that there is a common motility disorder that predisposes to both Barrett's esophagus and gallbladder malfunction. This may have a neural basis or a hormonal basis, most likely through CCK secretion. Whatever the cause, it is likely that a lifetime of altered gallbladder function may predispose to chronic toxic bile exposure in the stomach and lower esophagus. Barrett's esophagus is associated with motility disorders of the esophagus and of the stomach and a recent report has observed an association between Barrett's and gallbladder function.[43] Our study is the first report to describe an association between gallbladder malfunction and adenocarcinoma. It is possible that there is a primary motility disorder in Barrett's esophagus affecting the entire upper gastrointestinal tract affecting the esophagus, stomach and the biliary system.

In conclusion, patients with Barrett's esophagus and esophageal adenocarcinoma have abnormal gallbladder function. Whether this is cause or consequence is unclear. Further studies are needed to determine whether impaired gallbladder function contributes significantly to the reflux milieu and in particular to the mucosal change of Barrett's esophagus or adenocarcinoma.

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ORIGINAL ARTICLE

Linear Stapled Esophagogastrostomy is more Effective than Hand-Sewn or Circular Stapler in Prevention of Anastomotic Stricture: a Comparative Clinical Study

Qi-Rong Xu · Kang-Ning Wang · Wen-Ping Wang · Kun Zhang · Long-Qi Chen

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Abstract
Objective The aim of this study was to retrospectively compare the operative effects of linear stapled intrathoracic esophagogastrostomy with hand-sewn or circular stapled anastomosis in prevention of anastomotic stricture.
Method Between October 2007 and October 2009, 293 patients with esophageal or gastric cardia cancer underwent a curative intent resection. Patients received either a linear stapled (LS group, n=166), conventional hand-sewn (HS group, n=59), or circular stapled intrathoracic esophagogastric anastomosis (CS group, n=68). The patients were followed-up and compared at 3 months after the operation.
Result Three groups of patients were comparable on clinical baseline characteristics. There was one operative death in the HS group. The operative complications were documented in 15 patients (5.1%), with no difference among three groups (χ^2=2.215, P=0.330). The follow-up rate was 96.9%. The anastomotic diameter was 1.6±0.4 cm in the LS group, 1.2± 0.3 cm in the HS group, and 1.0±0.4 cm in the CS group, respectively (F=58.110, P<0.001). The anastomotic stricture rates were 1.9% (3/162) in the LS group, 9.3% (5/54) in the HS group, and 20.9% (14/67) in the CS group, respectively (χ^2=24.095, P<0.001). The reflux score in LS group was lower than other two groups (H=6.995, P=0.030).
Conclusion The linear stapled esophagogastrostomy could decrease anastomotic stricture without increasing gastroesophageal reflux.

Keywords Esophagogastrostomy · Linear stapled anastomosis · Anastomotic stricture · Comparative study

Context

The organ mostly used for reconstruction after esophagectomy is the stomach.[1,2] There are some advantages such as ease of construction and the tension-free substitute with sufficient length. However, anastomotic leakage, stricture formation, and gastroesophageal reflux following esophagectomy continue to be major challenges after resection of esophageal carcinoma. They are the main causes of postoperative morbidity and poor quality of life. So, the technique of gastroesophageal anastomosis remains crucial. In 2000, Orringer et al.[3] introduced a novel anastomotic technique and the use of Endo-GIA stapler devices. That procedure was called side-to-side stapled anastomosis and was associated with a lower rate of anastomotic complications compared to the conventional hand-sewn anastomosis. Recently, others also reported that the side-to-side esophagogastric anastomosis could decrease the rate of anastomotic leakage and stricture following esophagectomy.[4–7] However, most reports about this anastomotic technique were applied in the neck. We modified this method as linear stapled anastomosis and used it in the intrathoracic esophagogastric anastomosis. This study was designed to compare the results of different anastomotic techniques, including linear stapled anastomosis (LS group), conventional hand-sewn anastomosis (HS group), and circular

Q.-R. Xu · K.-N. Wang · W.-P. Wang · K. Zhang · L.-Q. Chen (✉)
Department of Thoracic Surgery,
West China Hospital of Sichuan University,
No. 37, Guoxue Alley,
Chengdu, Sichuan 610041, China
e-mail: drchenlq@gmail.com

stapler anastomosis (CS group). The institutional review board of the hospital approved this retrospective study.

Materials and Methods

Clinical Data

Between October 2007 and October 2009, 293 patients with esophageal or cardia carcinoma underwent curative intent resection by one surgical team in our department. There were 166 patients enrolled in LS group, 59 patients in HS group, and 68 patients in CS group. There were 253 men and 40 women, ranging in age from 40 to 80 years (with an average of 60.0 years). The patients' demographic data for the three groups were summarized in Table 1. The tumor was staged and recorded according to AJCC Cancer Staging System (2009, 7th edition).[8]

The diagnosis was established by barium esophagogram and endoscopic biopsies with documented carcinoma as histological evidence. Other preoperative workup included physical examination, laboratory tests, chest and upper abdomen computed tomography, and pulmonary function test. Since the neoadjuvant therapy might markedly increase the postoperative complications, all recruited patients did not receive any preoperative chemotherapy and/or radiotherapy. Anastomotic leakage was defined as the extravasation of water-soluble contrast medium and/or the appearance of oral ingested methylene blue in the thoracic drainage.

Based on the result of our previous randomized clinical trial comparing those three anastomotic methods,[9] the linear stapled anastomosis demonstrated significant reduction of stricture formation while not increasing anastomotic leak and reflux. Therefore, in later on practice, we firstly chose linear stapled method when possible. We chose hand-sewn anastomosis if the esophageal lumen was obviously dilated at operation, and we chose a circular stapled anastomosis when the site of anastomosis was at the apex of thorax because of poor exposure.

Patients were followed-up at the third postoperative month. The assessments included routine barium swallow, evaluating the grade of dysphagia, and gastroesophageal reflux. Anastomotic stricture was defined as the diameter of the anastomotic orifice on barium swallow ≤0.8 cm. The anastomotic diameter was measured at the third postoperative month by averaging the two measurements of anastomotic orifice on crossed directions on still images with scale aside. This work was done by radiologists without knowing the study design and anastomotic approach used. We used validated questionnaires to assess dysphagia and reflux symptoms at the third postoperative month. The dysphagia was evaluated using the Stoller's dysphagia scoring system[10]: 0=able to eat normal diet/no dysphagia; 1=able to swallow some solid foods; 2=able to swallow only semisolid foods; 3=able to swallow liquids

Table 1 Demographic data of the patients

	LS group (N=166)	HS group (N=59)	CS group (N=68)	Statistics	P value
Age (years), mean±SD	60.2±8.4	59.0±7.9	61.3±7.6	F=1.221	0.296
Sex (M:F)	143:23	49:10	61:7	X^2=1.200	0.549
Site of tumor				X^2=6.153	0.406
Upper thoracic	1 (0.6%)	2 (3.4%)	2 (2.9%)		
Middle thoracic	112 (67.5%)	42 (71.2%)	44 (64.7%)		
Lower thoracic	42 (25.3%)	10 (16.9%)	19 (27.9%)		
Gastric cardia	11 (6.6%)	5 (8.5%)	3 (4.5%)		
Staging of tumor				X^2=17.382	0.361
0	0 (0.0%)	1 (1.7%)	0 (0.0%)		
IA	2 (1.2%)	0 (0.0%)	0 (0.0%)		
IB	15 (9.9%)	2 (3.4%)	8 (11.8%)		
IIA	21 (12.7%)	2 (3.4%)	9 (13.2%)		
IIB	35 (21.1%)	23 (39.0%)	24 (35.3%)		
IIIA	53 (31.9%)	13 (22.0%)	13 (19.1%)		
IIIB	17 (10.2%)	10 (17.0%)	8 (11.8%)		
IIIC	23 (13.9%)	8 (13.5)%	6 (8.8%)		
Position of anastomosis				X^2=4.706	0.319
Above the aortic arch	131 (78.9%)	48 (81.4%)	50 (73.5%)		
Under the aortic arch	35 (21.1%)	11 (18.6%)	18 (26.5%)		
Positive margin	5 (3.0%)	4 (6.8%)	2 (7.4%)	X^2=1.589	0.484

M male, *F* female, *LS* linear stapled, *HS* hand sewn, *CS* circular stapled, *SD* standard deviation

only; 4=unable to swallow anything/total dysphagia. The definition of reflux symptoms was that patients had the feeling of heartburn (burning behind the breastbone) and regurgitation (acid taste in the mouth and movement of materials upwards from the stomach). Currently, Carlsson–Dent self-administered questionnaire [11] and reflux symptom questionnaire (RSQ) [12] are usually used in China, with the latter more applicable to Chinese population.[13] We further modified the RSQ: (1) only the most common symptoms, heartburn, and regurgitation were evaluated. Other GERD-related symptoms like epigastric pain or retrosternal pain were excluded in this survey since they could not be easily differentiated from postoperative incisional pain; (2) in accordance with the dysphagia score, we did not use the frequency of reflux but only five-point severity scale was used. The grade of reflux was defined as: 0=no reflux on semi-supine position; 1=postprandial reflux on semi-supine position; 2=reflux on semi-supine position on an empty stomach; 3=postprandial reflux on upright position; and 4=reflux on upright position on an empty stomach. For patients with regurgitation of ingested food, the anastomotic stricture and impaired gastric emptying must firstly be excluded by barium swallow examination.

Operative Technique

All patients with thoracic esophageal carcinoma and gastric cardia cancer underwent esophagectomy and lymphadenectomy via a left posterolateral thoracotomy through the fifth intercostal space. The mediastinal pleura were incised. The lesion in the esophagus was explored and then mobilized. Then, the diaphragm was incised. The abdomen was explored and the stomach was mobilized. The gastroepiploic vessels, the short gastric vessels, left gastric vessels were ligated, and the right gastroepiploic vessels were preserved. A gastric tube of 5~6 cm in diameter was created by means of a 75-mm linear cutter stapler [Proximate Linear Cutter-TLC75, Johnson & Johnson Medical (China) Co. Ltd., Shanghai]. Then, the esophagus was continuously mobilized at least 8 cm proximal to the upper edge of the tumor. Lymph nodes were dissected and recorded in groups as AJCC criteria.[8]

For linear stapled anastomosis, the main surgical procedures included: (1) placing two stitches to anchor the esophagus overlaying on the anterior wall of the stomach, followed by making a 2 cm transverse gastrotomy 3 cm below the tip of gastric fundus (Fig. 1a); (2) a linear cutter stapler is used to extend the anastomosis along the posterior wall of the esophagus (Fig. 1b); (3) the newly extended half of the anastomotic orifice was about 2–2.5 cm in length, like an inverted "V" (Fig. 1c); and (4) the gastrotomy and esophageal opening were then closed transversely with 4/0 absorbable Vicryl antibacterial suture (polyglyconate) in an everted interrupted whole-layer anastomotic manner. After finishing the anastomosis, reinforcement with 3–0 absorbable stitches between the muscular layer of the esophagus and the seromuscular layer of the stomach were added, the horizontal part of the anastomosis forms a "C" shape (Fig. 1d). By this technique the diameter of the anastomotic orifice were almost doubled. For stapler anastomosis, the circular stapler of EES 25 (Johnson & Johnson Medical (China) Co. Ltd., Shanghai) was used. For hand-sewn anastomosis, the conventional whole-layer hand-sewn embedded esophagogastrostomy with intermittent 4/0 Vicryl antibacterial suture was performed.

After finishing the anastomosis, a nasogastric tube was placed in the stomach and a feeding tube was placed in the duodenum for early postoperative enteral nutrition support. The feeding usually began on the second postoperative day (POD). Patients were able to take a liquid diet on POD 6, a semi-liquid diet on POD 14, and solid diet on POD 21.

Statistical Analysis

Continues data such as age, the diameter of anastomosis were expressed in mean±standard deviation and analyzed by one-way ANOVA. Categorical data such as the rate of anastomotic stricture were presented by frequency (%) and analyzed by chi-squared test. The ordinal data such as the dysphagia score and reflux score were also presented by frequency (%) and analyzed by Kruskal–Wallis test. Unconditional logistic regression model was used to estimate the factors influencing the anastomotic stricture. The associations of dysphagia score with anastomoic diameter and history of leakage were assessed by Spearman's correlation test. The difference was considered statistically significant if $P<0.05$, and then post hoc test was done for the pairwise comparison accordingly. All statistical analyses were carried out with SPSS 16.0 for windows (SPSS Inc., Chicago, IL, USA).

Results

One hundred and sixty-six (56.7%) patients had linear stapled anastomosis, 59 (20.1%) patients underwent a conventional hand-sewn anastomosis, and 68 (23.2%) patients received circular stapled anastomosis. There was no significant difference among these three groups in terms of age and gender distribution. There was one operative death in the HS group due to myocardial infarction. The anastomotic leakage was observed in two patients in LS group, two in HS group, and one in CS group, respectively ($\chi^2=1.264$, $P=0.721$). Other complications were rare and not different among three groups (Table 2).

Totally, nine patients (2.7%) were lost to follow-up at 3 months, including four from LS group, four from HS

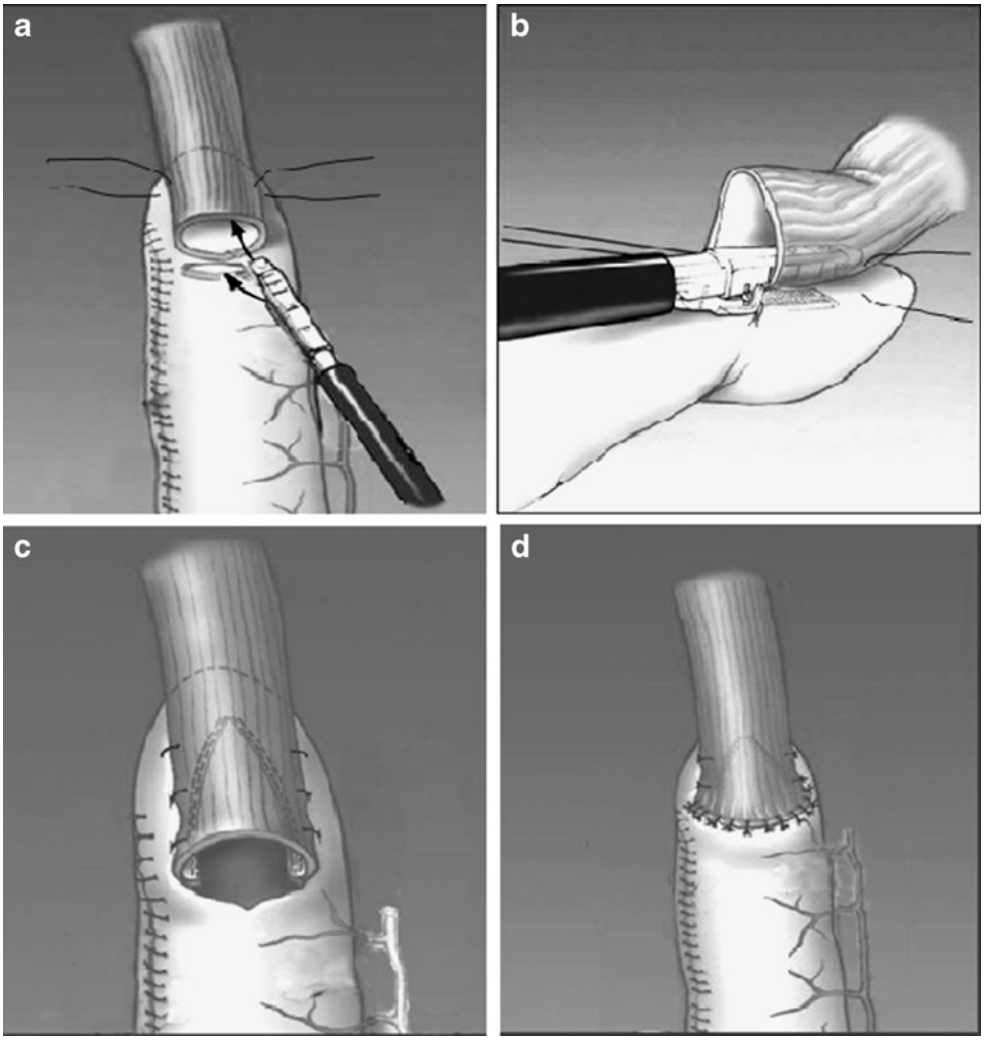

Fig. 1 Linear stapled intrathoracic esophagogastric anastomosis. **a** Place two stitches to anchor the distal esophagus overlaying on the anterior wall of the stomach and make a gastrotomy 3 cm below the tip of gastric fundus. **b** Use a linear cutter stapler to extend the anastomosis along the posterior wall of the esophagus and the anterior wall of the gastric fundus. **c** The shape of the extended anastomosis like an inverted "V". **d** Close the gastrotomy and esophageal opening with 4/0 absorbable Vicryl antibacterial suture (polyglyconate) in an eversion interrupted whole-layer anastomotic manner and add 3–0 absorbable stitches between the muscular layer of the esophagus and then wrap the two corners of the anastomosis back (modified from Behzardi, et al.)[28]

group, and one from CS group (χ^2=3.542, P=0.170). The follow-up rate was 96.9%. The diameter of anastomotic orifice was 1.6±0.4 cm in LS group, 1.2±0.3 cm in HS group, and 1.0±0.4 cm in CS group (F=58.110, P=0.000).

The result of postoperative assessments including dysphagia score, reflux score, and anastomotic stricture were summarized and compared in Table 3. The anastomotic stricture rate in LS group was significantly lower than that in HS group and CS group (χ^2=24.181, P=0.000). Similar result was found when comparing the rate of dysphagia, the LS group had lower rate of dysphagia than HS group (χ^2=15.926, P=0.000), while no difference was found between HS group and CS group (χ^2=0.226, P=0.634). Multivariate analysis revealed that the anastomotic technique and age were the independent factors influencing anastomotic stricture formation (P=0.000, Table 4).

There were five anastomotic leakages in the series, two patients in LS group, two in HS group, and one in CS

Table 2 Complications following esophagogastrostomy in 293 patients

	LS group (N=166)	HS group (N=59)	CS group (N=68)	Statistics	P
Anastomotic leakage	2 (1.2%)	2 (3.4%)	1 (1.5%)	χ^2=1.264	0.721
Chylothorax	0 (0.0%)	1 (1.7%)	0 (0.0%)	χ^2=3.966	0.201
Diaphragmatic hernia	1 (0.6%)	0 (0.0%)	0 (0.0%)	χ^2=0.765	0.682
Recurrent laryngeal nerve paralysis	0 (0.0%)	0 (0.0%)	1 (1.5%)	χ^2=3.309	0.433
Wound infection	2 (1.2%)	0 (0.0%)	0 (0.0%)	χ^2=2.012	0.428
Pulmonary infection	1 (0.6%)	2 (3.4%)	2 (3.0%)	χ^2=4.190	0.148

LS linear stapled, *HS* hand sewn, *CS* circular stapled

Table 3 Outcome of three anastomotic techniques

	LS group (N=162)	HS group (N=54)	CS group (N=67)	Statistics	P
Diameter of anastomosis	1.6±0.4	1.2±0.3	1.0±0.4	F=58.110	0.000[a]
Grade of dysphagia				H=25.621	0.000[b]
0	152	40	47		
1	8	8	7		
2	2	4	8		
3	0	2	5		
Grade of reflux				H=6.995	0.030[c]
0	148	45	53		
1	14	7	12		
2	0	1	1		
3	0	1	1		
Anastomotic stricture	3 (1.9%)	5 (9.3%)	14 (20.9%)	χ^2=24.181	0.000[d]

LS linear stapled, *HS* hand sewn, *CS* circular stapled

[a] Post hoc comparisons for anastomotic diameter: P<0.05 for LS vs HS, HS vs CS, and LS vs CS

[b] Post hoc comparisons for dysphagia score: P<0.05 for LS vs HS

[c] Post hoc comparisons for reflux score: P<0.05 for LS vs CS

[d] Post hoc comparisons for stricture rate: P<0.05 for LS vs HS and LS vs CS

group, respectively. There was one patient with stricture (in LS group) among those four leak patients (one patient with leak in HS group was lost to follow-up). Because of small numbers of leaks (five) and stricture (one) in leak patients, the incidence of stricture between patients with or without leakages were not statistically different (1/5 vs 21/288, Fisher's exact P=0.325).

The dysphagia scores were correlated with anastomotic diameter (rs=−0.587, P<0.001) but not the history of leakage (rs=−0.043, P=0.458). No patients went on dilation based on dysphagia alone without an anastomotic diameter ≤0.8 cm. The dilation was performed only when patients had dysphagia score ≥2 and the anastomotic diameter ≤0.8 cm. Totally, two dilations were performed in LS group, nine in HS group, and 32 in CS group for the relief of dysphagia symptom.

Discussion

The initial gastroesophageal anastomosis was all hand-sewn, eventually giving way to the stapled anastomosis. The disadvantage of the hand-sewn anastomosis was that it requires a long operating time and a higher level of expertise.[14] After the popularization of stapled anastomosis, the hand-sewn approach was only used in incidences of a misfired stapler, technically difficult to use stapler due to anastomotic considerations, or if there is not enough gastric conduit to overlap the anastomosis sufficiently.[15] The stapled anastomosis has the advantages of shortening the operation time and validity of anastomosis, especially anastomosis at the apex of thorax because of poor exposure for hand-sewn anastomosis. However, some non-randomized comparison of hand-sewn and stapled esophagogastric anastomosis suggested a higher stricture rate when the stapled technique was used.[16,17] The reasons why stricture rate was more common with the stapled method may include: (1) lacking of accurate mucosa-to-mucosa apposition when doing anastomosis; (2) tissue necrosis beyond the stapled line, inflammation, and delayed epithelialization may then predispose to excessive fibrosis and stricture formation; (3) the circumferentially placed unabsorbable mental staples do not allow the lumen to dilate beyond the size obtained originally.[18] Moreover, the stapled anastomotic technique has other shortcomings, such as circular stapler anastomosis in the neck is not convenient, and the balloon used to dilate postoperative anastomotic

Table 4 Multivariate regression analysis on factors that influence the anastomotic stricture

Variables	Regression coefficient	Statistic[a]		Odds ratio (OR)	95% CI of OR
		Wald	P		
Sex (1 male, 2 female)	−0.811	1.116	0.291	0.444	0.099~2.001
Site of anastomosis (0 above aortic arch, 1 below aortic arch)	0.704	2.608	0.106	2.021	0.860~4.746)
Anastomotic technique(0 LS, 1 HS, 2 CS)	1.698	13.074	0.000	5.462	2.176~13.711
Residual (0 negative, 1 positive)	1.048	1.391	0.238	2.852	0.500~16.280
Leakage (0 no, 1 yes)	−19.377	0.000	0.999	0.000	0.000~0.000
Age (40–4, 50–5, 60–6, 70–7…)	−4.277	45.719	0.000	0.014	0.004~0.048

[a] Model chi-square test: x^2 =22.226, P=0.001

stricture was easily torn by metal staples, resulting in dilatation failure.

Therefore, it is necessary to refine a new anastomotic technique. A new partially stapled anastomosis was described by Collard and modified by Orringer et al.[3,19] It has the privileges of reducing the incidence of leaks and stenosis. Orringer et al. performed a side-to-side stapled cervical esophagogastric anastomosis in 114 patients with esophageal carcinoma, the rate of anastomotic leakage was 2.7%, the rate of anastomotic stricture was 12%, significantly lower than Dewar's hand-sewn anastomosis (leakage 17%, stricture, 31%).[20] Jo et al. retrospectively reviewed 13 patients who underwent total esophagectomy by performing side-to-side cervical gastroesophageal anastomosis. There were no complications of anastomotic leakage or conduit necrosis. A mild anastomotic stricture was noted in one patient and required two endoscopic bourgination procedures at fourth postoperative month.[4] Raz et al. reported 33 consecutive patients with distal esophageal cancer or Barrett's esophagus with high-grade dysplasia underwent a transthoracic esophagectomy with a side-to-side stapled intrathoracic anastomosis. The overall morbidity was 27% with no anastomotic leakage and stricture.[21]

Our study revealed that the linear stapled anastomosis decreased the formation of anastomotic stricture and gastroesophageal reflux, and increased the anastomotic diameter on barium swallow compared with hand-sewn and circular stapler anastomosis. The stricture rate in the LS group was 1.9%, significantly lower than that in the HS group (9.3%) and the CS group (20.9%). The reason why linear stapled anastomosis could decrease stricture formation may be is that the anastomotic orifice was enlarged by extending the anastomosis along the posterior wall of the esophagus. Of course, absorbable Vicryl antibacterial suture used to close the gastrotomy and esophageal opening perhaps played a role in decreasing stricture formation. It was reported that Vicryl plus antibacterial suture (an absorbable suture) might inhibit bacterial growth and prevent postoperative infection,[22] while the anatomotic infection is related to the stricture formation.[23] For intrathoracic linear stapled anastomosis, the rate of stricture in our study was lower than other study performing this kind of anastomosis in cervical (Orringer 35% vs Kondra 31.3%).[3,24] A cervical anastomosis is associated with a higher risk for anastomotic tension and ischemia, a higher stricture rate would be expected when compared to an intrathoracic anastomosis.[21] For hand-sewn and stapled intrathoracic anastomosis, the rate of stricture formation was similar with a randomized trial (hand-sewn 10% vs stapled 16.7%).[25]

The rate of dysphagia in the LS group was 6.2%, which was lower than that in HS group (25.9%) and CS group (29.9%). Obviously, the linear stapled anastomosis could decrease postoperative dysphagia.

There were two (1.2%) patients in LS group with anastomotic leakage, similar to HS group (two patients, 3.4%) and CS group (one patient, 1.5%). The rate of intrathoracic anastomotic leakage for hand-sewn and circular stapled anastomosis was similar with other reports (hand-sewn 1.6~4.7%; circular stapler 0–3.4%).[18,25–27] So, we consider that the linear stapled anastomosis is a safety anastomotic technique.

In LS group, the gastroesophageal reflux was documented in 8.6% patients, while in HS group and CS group the gastroesophageal reflux rates were 16.7% and 20.9%, respectively. The grade of reflux in LS group was lower than other groups. In addition, this study found that age was the protective factor for stricture formation. Older patients were less prone to develop anastomotic stricture. The reasons were unknown and warrant further investigation.

The linear stapled anastomosis also has some drawbacks. First, the linear stapled anastomotic method needs a longer esophageal reminant in order to perform the posterior extension. Therefore, patients with tumor located at higher upper third of the esophagus might not be appropriate for this technique. Second, when the esophageal lumen is already obviously dilated at operation, it is not necessary to apply this technique to enlarge the anastomotic orifice further.

The study also had some shortcomings because the follow-up was limited to only 3 months. We recognized that the strength of the study would be improved with longer follow-up. However, we considered that most dysphagia and anastomotic stricture would occur within the initial 3 months after the operation. By that time, we might obtain an effective comparison. After 3 months, other influencing factors might be involved and impair the comparison: more patients lost to follow-up, some patients would have postoperative radio/chemotherapy, and some patients would die. Therefore, and unbiased long-term comparison is difficult. Currently, we are still following these patients and some of them undertook esophageal manometric study, 24 h pH monitoring, and impedence test for objective evaluation of the operative effect.

In conclusion, the linear stapled esophagogastric anastomosis is a safe and effective anastomotic technique, which can decrease the rate of postoperative dysphagia and anastomotic stricture. The procedure deserves more attention and further application.

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ORIGINAL ARTICLE

Effect of the Informed Consent Process on Anxiety and Comprehension of Patients Undergoing Esophageal and Gastrointestinal Surgery

Simonetta Betti · Andrea Sironi · Greta Saino · Cristian Ricci · Luigi Bonavina

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Abstract
Objective This study seeks to evaluate the level of anxiety, recall, and comprehension of the provided information in patients undergoing esophageal and gastrointestinal surgery.
Methods Sixty-one patients without cognitive disorders entered a prospective study designed to assess the effect of a surgical informed consent process. The written informed consent was administered to all patients and was supported by a verbal explanation and a schematic drawing of the operation. The State Trait Anxiety Inventory test was used to assess state anxiety and tract anxiety. The test was repeated after the informed consent process. A disease-specific feedback questionnaire was subsequently administered to assess the actual comprehension of the provided information.
Results A significant decrease of the state anxiety scores was documented in most patients ($p<0.001$). This effect was more evident in the elderly ($p=0.021$) and in those who used Internet as a previous source of information ($p=0.032$). The mean correct exact answer rate on the disease-specific questionnaire was 76% (IQ range 66.7–85%). No statistically significant relationship was found between the rate of correct answers and the state anxiety scores.
Conclusions An exhaustive surgical informed consent process was effective in providing comprehension and decreasing anxiety in patients who are candidates to minimally invasive esophageal and gastrointestinal surgical procedures.

Keywords Informed consent · Anxiety · Comprehension · State trait anxiety inventory · Esophageal surgery · Gastrointestinal surgery · Minimally invasive surgery

S. Betti
Clinical Psychology Unit IRCCS Policlinico San Donato, School of Medicine, University of Milano, Milan, Italy

A. Sironi · G. Saino · L. Bonavina (✉)
Department of Surgery IRCCS Policlinico San Donato, School of Medicine, University of Milano, Piazza Malan 2, 20097 San Donato Milanese, Milan, Italy
e-mail: luigi.bonavina@unimi.it

C. Ricci
Clinical Epidemiology and Biometry Unit IRCCS Policlinico San Donato, School of Medicine, University of Milano, Milan, Italy

Introduction

Surgical procedures have a profound impact on the psychological profile of patients and adversely affect their ability to cope with disease and hospitalization. When the information regarding the disease and the planned surgical operation is not adequate, the patient may become emotionally overwhelmed. In such circumstances, perceived understanding of the presented information may not correspond to the actual comprehension due to fear and anxiety for the risk associated with the surgical procedure. On the other hand, a very detailed information unsupported by a patient–surgeon trust relationship might increase anxiety and reduce patient's autonomy and decision-making ability.[1–3]

When properly performed, the surgical informed consent (SIC) should provide the patient with the basic information that promotes his autonomy and improves his capability to make decisions without increasing anxiety. However, an

adequate understanding of the information by the patients is reported in less than one third of the studies.[4]

The effects of the SIC process on the level of anxiety and comprehension in patients undergoing major thoraco-abdominal surgical procedures have not been investigated. The aim of this study was threefold:

1. To assess the degree of anxiety in patients scheduled to undergo elective esophageal and gastrointestinal surgery
2. To evaluate recall and comprehension of the provided information
3. To explore the effects of sociodemographic and clinical variables on the anxiety profile and on the comprehension of the surgical procedure

Patients and Methods

A prospective clinical study on the effects of the SIC process was conducted on consecutive patients admitted to the Department of Surgery of our University Hospital between January and May 2010. The study protocol was approved by the Internal Review Board of the hospital.

The day before surgery, all patients were asked to fill the Mini-Mental Scale[5] and, if eligible, the State Trait Anxiety Inventory (STAI) test.[6] The Mini-Mental test includes 30 questions and is used to select patients with adequate cognitive process. Patients who could not suitably fulfill the Mini-Mental test were excluded from the study.

The STAI test evaluates two different components of anxiety: anxiety of state (Y1) and anxiety of tract (Y2). The STAI test has proven to be a sensitive indicator of changes in transient anxiety experienced by patients in counseling, psychotherapy, and behavior-modification programs; it is widely used to assess the anxiety level induced by stressful experimental procedures and by unavoidable real-life stressors such as imminent surgery.[7,8] The state scale (Y1) of the questionnaire describes the level of anxiety generated by the specific context of hospitalization and surgical intervention. The tract scale (Y2) of the questionnaire describes the level of anxiety for each patient regardless of type of disease and surgical treatment. Each scale consists of 20 items, scoring the anxiety in four grades: 1, not at all; 2, somewhat; 3, moderately; and 4, very much. The subjects chose one response to each item, generating a total score between 20 (low anxiety) to 80 (very high anxiety). To evaluate the modifications of the baseline state anxiety, the STAI Y1 questionnaire was administered before and immediately after the informed consent discussion process.

The informed consent consisted of the standard written format, 600 to 800 words, endorsed by the Italian Society of Surgery.[9] For each surgical procedure, benefits and risks were described in detail. The SIC was administered by a trained junior staff surgeon (A.S.). In order to facilitate the comprehension of the surgical procedure, the patient was provided with an oral explanation and a schematic drawing of the operation. He/she was then allowed to read the informed consent sheet and sign it. Finally, a disease-specific questionnaire including 20 items (Appendix I) was administered to the patient to evaluate the degree of actual understanding of the type of operation and of the possible complications.

Statistical Methods

Location (mean, median, and first and third quartiles) and dispersion indexes (standard deviation) were calculated for continuous variables such as age, STAI scores, and STAI scores differences before and after the SIC procedure. Categorical variables (gender, disease, activity, and education) were described by percentage; patients' recall and comprehension were described by mean and first and third quartiles of the percentage of correct answers on the disease-specific questionnaire.

The linear regression analysis was applied to investigate the relation between the STAI Y1 scores after and before the SIC procedure; to account for the variables' skewness, the van der Waerten transformation[10] (vW) was applied using the Proc Rank of SAS software package.[11]

The overall Y1 addictive score reduction (differences of STAI Y1 addictive scores after the SIC procedure) and single item reductions were investigated by the non-parametric Wilcoxon signed rank test[12] and validated by a Generalized Linear Model (GLM) analysis. The least squares means estimates of item differences performed by the GLM were calculated. The GLM analysis of variance (GLM-ANOVA) was applied to investigate the effects of sociodemographic and clinical variables on the vW STAI Y1 score differences. The same analysis was performed for vW STAI Y2 scores. The patients comprehension was investigated by a GLM-ANOVA with the rate of correct answers given as response variables; the sociodemographic and clinical variables, and the STAI scores were given as covariates (Y2 score and Y1 differences after the SIC procedure); the arc sin inverse transformation was applied to sample means estimate of correct answer percentages. An α level of 0.05 was considered statistically significant. The statistical analysis was performed by SAS statistical software package version 9.1.3.

Results

Sixty-one patients successfully passed the Mini-Mental test and consented to surgery. The sociodemographic data of the patient population are reported in Table 1. The most common diagnosis was esophageal carcinoma (n=24, 39.3%), fol-

Table 1 Demographic data in the overall patient population stratified by type of disease

Categories	Overall	Benign	Malignant
n	61	31	30
Gender			
Male	36	15	21
Female	25	16	9
Age			
Median	63.5	57	65
Range	54–72	41–72	58–72
Education			
Primary school	41	18	23
High school	11	10	1
University degree	9	3	6
Employment status			
Workers	30	18	12
Retired	29	12	17
Students	1	1	0
Unemployed	1	0	1

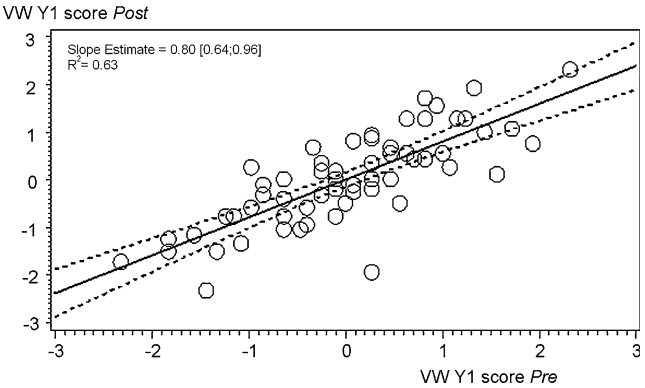

Fig. 1 Dispersion plot of the van der Waerten STAI Y1 scores. After informed consent administration, Y1 scores were plotted against baseline values (*Pre*); the *solid line* represents the interpolating linear equation while the *dotted curves* portray its 95% confidence limits

lowed by achalasia (n=16, 26.2%), gastroesophageal reflux disease (n=15, 24.6%), and colon carcinoma (n=6, 9.8%). In all patients, a minimally invasive surgical approach was planned. The mean time required for the SIC process, including the STAI Y1 test, the informed consent, and the disease-specific questionnaire, was 46 min (37–55 min).

Descriptive statistics of STAI scores and age were reported on Table 2. Most patients experienced a reduction of STAI Y1 scores after the SIC process (p<0.001, Wilcoxon rank signed test). This result is confirmed by the regression approach applied to the van der Waerten normit transformation of the STAI Y1 scores where the slope estimate was 0.80 [0.64–0.96] (R^2=0.63), which corresponds to a general reduction of the scores after the SIC process (Fig. 1). Both the GLM analysis and Wilcoxon rank signed test applied to the STAI questionnaire identified three sensitive items: number 4 "I feel under pressure," number 7 "I feel worry about accidents could happen," and number 14 "I feel hesitant" (Fig. 2).

Interestingly, about half of the patients declared to have been previously informed by Internet (42%) regarding the disease and the related treatment; all patients stated that they would have indeed preferred a minimally invasive surgical procedure. The statistically significant covariates were employment status, Internet use, and age class. Retired patients (p=0.026), patients who got previous information from Internet (p=0.032), and older patients (p=0.021) experienced a higher reduction of STAI Y1 score after SIC administration. The same analysis performed on vW STAI Y2 score did not find any statistically significant effect.

The mean correct answer rate on the disease-specific questionnaire was 76% (IQ range 66.7–85%). The patient's comprehension of the disease and of the proposed surgical intervention was influenced by age (p=0.008), where older patients had a lower rate of correct answers, and by employment status (p=0.017), where workers gave a higher rate of correct answers; also the interaction between age class and employment status was statistically significant (p=0.0081; Fig. 3). No statistically significant effects were found between the rate of correct answers and STAI scores (both vW STAI Y2 scores and vW STAI Y1 score reduction).

Furthermore, both the baseline STAI Y1 and Y2 scores in the study population indicate a moderate anxiety level, with a median value about the expected median value between the lower and the maximum measurable scores. Sample composition by gender, disease, education, and employment status was well balanced. Interestingly, there was no statistically significant difference between patients

Table 2 Descriptive statistics of STAI scores and patients age

Variable	Mean	Median	Lower Quartile	Upper Quartile	Standard Deviation
Y1 Baseline	44.46	43.00	36.00	50.00	12.62
Y2 Baseline	38.02	37.00	29.00	45.00	11.23
Y1 Post	43.15	42.00	34.00	50.00	11.02
Delta Y1	−0.016	0	−1.00	1.00	0.96
Age	60.95	63.52	53.91	72.41	14.30

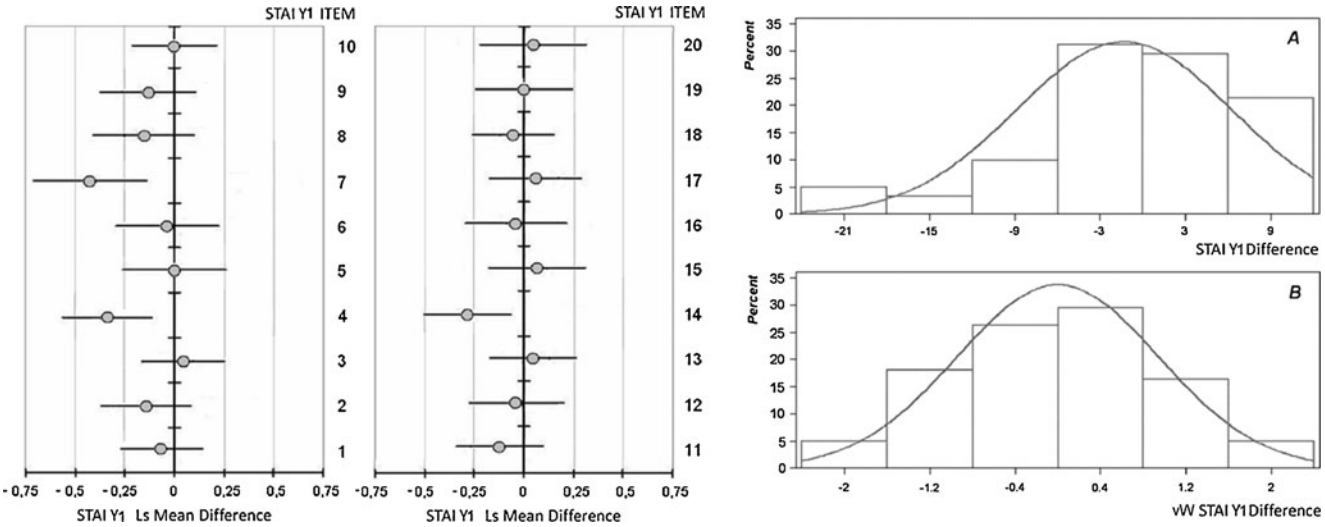

Fig. 2 The least squares means (Ls) of van der Waerten STAI Y1 score differences derived from the GLM analysis applied to single items were reported on the *left side*; statistically significant reduction of items 4, 7, and 14 was confirmed by the non-parametric Wilcoxon signed rank test. The effect of van der Waerten transformation is depicted on the *right side*; Histogram plot of STAI Y1 score differences before (*panel* **a**) and after vW transformation (*panel* **b**)

with cancer and those with a benign disease in terms of baseline STAY Y1 score.

Discussion

The present study showed that older and retired patients experienced a reduction of anxiety level after the SIC process was completed. This effect could be related not only to an improved understanding of the disease and of the surgical procedure, but also to a patient–surgeon trust relationship which developed during the informed consent discussion.

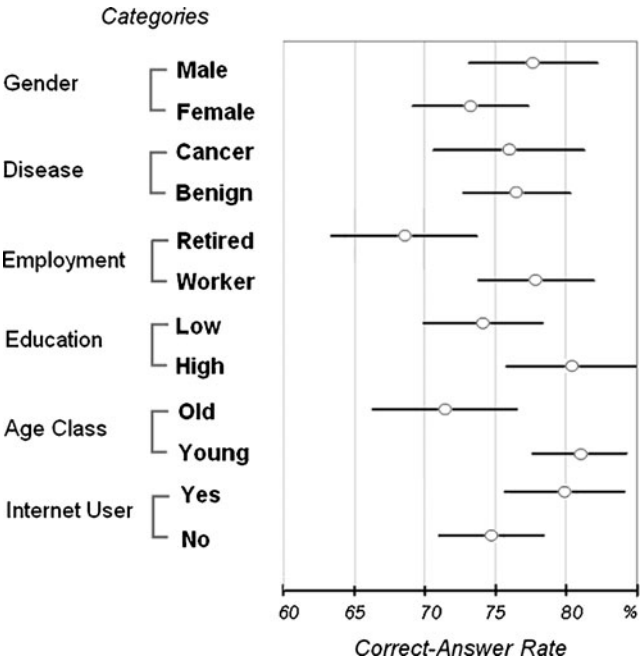

Fig. 3 Sample means rate of correct answers (*percentage*) after arc sin inverse transformation. *Dots* represent means, *whiskers* represent 95% confidence limits

The effect of the SIC process on preoperative anxiety has previously been investigated by Kerrigan et al. in patients undergoing elective inguinal hernia repair under general anesthesia.[13] They found that a very detailed account of potential complications did not increase state anxiety. Our aim was to test whether or not the same hypothesis could apply to patients undergoing elective esophageal and gastrointestinal surgical procedures.

We spent about 1 h with the patient to complete the SIC process and establish an adequate surgeon–patient relationship. Fink et al.[14] tested patient comprehension after the informed consent discussion using procedure-specific questionnaires. They found that patient comprehension was maximized when informed consent took between 15 and 30 min. To investigate whether standard methods of informed consent can be improved, Bollschweiler et al.[15] reported on a multicenter randomized trial in whom a multimedia-based program was tested in addition to the standard procedure in patients undergoing cholecystectomy. The program was positively evaluated by patients and significantly improved perceived understanding of the disease and treatment without increasing anxiety.

The detailed information provided during the informed consent process in our elderly patients significantly reduced state anxiety compared to baseline levels. The reduction was smaller in younger patients, probably because their anxiety level had already been decreased by information previously acquired on web sites.

The improvement of the perceived understanding of the disease and the surgical therapy by the patients is an important goal to be achieved before undertaking major thoraco-abdominal procedures such as an esophagectomy. Spending more time for the informed consent discussion and combining written and oral information as we did may definitely enhance actual patient's comprehension and decrease anxiety related to awareness of the diagnosis and magnitude of surgery. Patients' recall of the informed consent on our disease-specific questionnaire was excellent, with a 76% rate of correct answers. This may be important also from the legal standpoint because it has been shown that patients frequently forget or mistakenly attribute information to the consent interview.[2]

The SIC discussion should also focus on potential advantages (decrease of pain and respiratory complications) and disadvantages (longer operative time) of the minimally invasive approach, also explaining to the patient that the oncologic outcomes appear equivalent to open surgery in specialized centers.[16–18] Prognostic information should be realistic without deprive patients of hope.[19] Since most patients will experience eating problems after esophagectomy, they should also be reassured and provided with a diet they perceive as socially acceptable.

In conclusion, the patient–surgeon relationship remains crucial in the current era of information technology. In fact, most litigations are due to failure in communication rather than failures in treatment. Can multimedia-based presentations and/or web information further enhance the patient–surgeon relationship? We believe that integration of these tools with professional, not paternalistic physician counseling, can improve the quality of information and contribute to the lowering of stress levels in patients undergoing esophageal and gastrointestinal surgery.

Appendix I

Disease-Specific Questionnaire (Esophageal Cancer)

1. Is a surgical operation necessary to restore your ability to swallow and to remove your cancer? (YES–NO–DO NOT KNOW)
2. Are you aware that an endoscopic stent would be the only alternative to make you eating again? (YES–NO)
3. Is the operation performed with a few short incisions in your chest, abdomen, and/or neck? (YES–NO–DO NOT KNOW)
4. Do you like this minimally invasive operation as opposed to the traditional open approach? (YES–NO)
5. Could a long abdominal and/or chest incision become anyway necessary during the operation? (YES–NO–DO NOT KNOW)
6. Is the operation carried out under general anesthesia? (YES–NO–DO NOT KNOW)
7. Will part of your stomach and the esophagus be removed? (YES–NO–DO NOT KNOW)
8. Is your esophagus going to be replaced by a sort of tube made with the stomach? (YES–NO–DO NOT KNOW)
9. Will the operation take between 3 and 6 h? (YES–NO–DO NOT KNOW)
10. May blood transfusions be needed during or after the operation? (YES–NO–DO NOT KNOW)
11. Will you be transferred to the Intensive Care Unit (ICU) at the end of the operation? (YES–NO–DO NOT KNOW)
12. Will you have tubes placed through your nose abdomen and/or chest after the operation? (YES–NO–DO NOT KNOW)
13. Will it take at least 1 week before you can start eating again? (YES–NO–DO NOT KNOW)
14. Could this surgical procedure, even if correctly performed, cause serious complications or even death? (YES–NO–DO NOT KNOW)
15. Are you aware that the risks of this operation may be increased if you already received chemotherapy or chemoradiation therapy? (YES–NO)
16. Are you aware that in the event complications occur your postoperative course will be longer than expected? (YES–NO)
17. Do you know that your eating capacity can be markedly reduced by the operation and that you may need to eat small meals more frequently during the day? (YES–NO)
18. Do you know that you may need to take antisecretory drugs (as Omeprazole) to control postoperative heartburn and acid regurgitation? (YES–NO)
19. Have you been told that the disease can recur despite radical surgery and that you may need further treatments including chemotherapy? (YES–NO)
20. Did you check on Internet about your esophageal disease and the way to treat it? (YES–NO)

Please answer each question by marking a slash on YES, NO, or DO NOT KNOW when appropriate.

Thank you for your cooperation!

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ORIGINAL ARTICLE

Sleeve Gastrectomy with Ileal Transposition (SGIT) Induces a Significant Weight Loss and Diabetes Improvement Without Exclusion of the Proximal Intestine

Camilo Boza · Rodrigo Muñoz · Elliot Yung · Luca Milone · Michel Gagner

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Abstract
Introduction Current effective bariatric procedures such as gastric bypass generate a duodenal–jejunal exclusion, which has been implicated in the resolution of type 2 diabetes. The aim of this study was to test the hypothesis that sleeve gastrectomy with ileal transposition (SGIT), a new procedure, is as effective as Roux-en-Y gastric bypass (RYGB) to induce glucose control on an obese rat model of type 2 diabetes mellitus.
Methods Twenty eight obese diabetic Zucker rats, weighing 571±151 g were assigned into three procedures: SGIT (n=11), RYGB (n=7), and sham operation (n=10). Animals were followed, evaluating weekly weight increase and food intake. We performed an insulin tolerance test after 8 weeks and measured serum peptide tyrosine–tyrosine (PYY 3-36) and ghrelin levels.
Results Nine weeks after surgery, sham-operated animals increased their body weight by 24%. In far contrast, SGIT and RYGB rats weighed 21% and 18% less than sham animals, respectively (sham, 884±15 g; SGIT, 720±19 g; RYGB, 754±14 g; p<0.001). No significant differences were found between SGIT and RYGB. Cumulative food intake in SGIT and RYGB procedures decreased by 29.6% and 32.9%, respectively (sham, 576.3±33 g; SGIT, 405.8±10 g; RYGB, 386.4±21 g; p<0.001). No differences were found between SGIT and RYGB rats. Sixty minutes after oral gavage, PYY levels were increased by 185% and 74% in SGIT and RYGB, respectively (sham, 63.4±2.1 pg/ml; SGIT, 192.7±17 pg/ml; RYGB, 117.7±4.8 pg/ml; p<0.001). Glucose tolerance was improved after SGIT and RYGB surgery demonstrated by area under the curve analysis (sham, 27,090±1,424; SGIT, 17,704±1,288 mg/dl; p<0.018; RYGB, 16,212±2,522; p<0.01).
Conclusion SGIT proved to be as effective as RYGB on obese diabetic rats as a weight loss procedure. Also, glucose homeostasis improved in SGIT, similar to RYGB, in spite of the absence of duodenal–jejunal exclusion. This observation does not support the theory that RYGB reversal of diabetes is due to duodenal–jejunal exclusion.

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C. Boza · E. Yung · L. Milone · M. Gagner
Division of Laparoscopic and Bariatric Surgery,
Department of Surgery, NewYork-Presbyterian Hospital,
Weill Medical College of Cornell University,
New York, NY, USA

C. Boza · R. Muñoz
Department of Digestive Surgery, Division of Surgery,
Pontificia Universidad Católica de Chile,
Santiago, Chile

C. Boza (✉)
Departamento Cirugía Digestiva, Facultad de Medicina,
Pontificia Universidad Católica de Chile,
Marcoleta 350, Santiago, Chile
e-mail: bozauc@med.puc.cl

Keywords Sleeve gastrectomy · Ileal transposition · Diabetes · Bariatric surgery

Introduction

Surgical procedures for weight loss have shown to be the most effective treatment for severe obesity and also have demonstrated to effectively induce diabetes remission in up to 78% of severely obese type 2 diabetic patients.[1] Remission of type 2 diabetes mellitus (T2DM)—normal blood glucose levels and glycated hemoglobin levels (HbA1c) without anti-diabetic drugs—occurs early after surgery before a significant weight loss has occurs,[2–4] specifically in surgical procedures with exclusion of the proximal small intestine (duodenal–jejunal exclusion, DJE). Accordingly, Roux-en-Y gastric bypass (RYGBP) and

duodenal switch (DS)—both with DJE—are the most effective procedures to induce diabetes remission.[5–8] Although, different anti-diabetic mechanisms (weight loss, decreased caloric intake, exclusion of the proximal intestine, increased release of gut hormones involved in glucose homeostasis regulation) have been associated with diabetes remission, the specific surgical components and their underlying physiological mechanisms involved in diabetes remission remain to be elucidated.[9] Particulary, the exclusion of the proximal small intestine from nutrients flow has been considered necessary to induce diabetes remission. In support of this hypothesis, DJE in Goto–Kakizaki (GK) rats—a non-obese animal model of T2DM—improves glycemic control as demonstrated by lower fasting glycemia and improved glucose tolerance compared to control rats,[10] and restoration of nutrient flow in DJE-operated rats reversed these effects.[11] However, as a consequence of the exclusion of the proximal small intestine and the creation of the Roux limb, after RYGB, partially digested nutrients contact distal segments of the small intestine—distal ileum—which promotes the secretion of gut-derived peptides involved in glucose homeostasis and food intake regulation. To study the effect of early nutrient contact with the distal ileum, Strader et al.[12] performed ileal transposition (IT) in adult rats. IT-operated rats lost more weight, consumed less food, and improved insulin sensitivity compared to control rats. These results were associated with higher secretion of glucagon like peptide-1 (GLP–1) and peptide tyrosine–tyrosine (PYY).[12] Other studies have confirmed these results.[13–15] Therefore, early nutrient stimulation of the distal small intestine might be an important surgical component of procedures like RYGB and DS.

In a pig model, we demonstrated that sleeve gastrectomy with ileal transposition (SGIT)—a new metabolic and bariatric procedure—was effective as RYGB to induce a significant and sustained weight loss and to reduce food intake;[16] however, its efficacy to induce diabetes remission has not been assessed. The aim of this study was to test the hypothesis that S.G.I.T is as effective as RYGB to improve glucose homeostasis in an obese rat model of T2DM.

Materials and Methods

Animals and Diet

Twenty eight age–weight-matched male Zucker diabetic fatty (ZDF) rats (Charles River Laboratories, Wilmington, MA, USA) were individually housed in temperature/humidity controlled room with 12-h light/dark cycle. Animals had free access to tap water and were fed with a special diet to maintain the phenotype (Purina Diet, Formulab diet, irradiated 5008). Animals were acclimatized for 2 weeks before surgery. All procedures were approved by the IACUC of Weill Medical College of Cornell University. Animal care was in accordance with National Institute of Health guidelines.

Surgical Procedures

Ten-week-old male ZDF rats were randomized into four groups: (1) sham, (2) RYGB, (3) SGIT, and (4) IT. Prior to surgery, animals were fasted overnight (10–12 h) with access only to water. Rats were anesthetized using isoflurane followed by an intraperitoneal injection of ketamine (60 mg/ml)–xylazine (8 mg/ml) that was adjusted by body weight. For sham surgery, a 4-cm midline laparotomy was performed. Transection and anastomosis of the distal ileum and proximal jejunum in association with gastrostomy and repair were performed. For RYGBP surgery, a gastric pouch was created by firing 30-mm linear stapler (Endo GIA 30 mm, Autosuture, Covidien, Norwalk, CT, USA) in the proximal stomach. The Roux limb was created by a jejunal section 10 cm below Treitz ligament. Then, a gastro-jejunal (GJ) anastomosis was created using interrupted sutures. The biliopancreatic limb was anastomosed 15–18 cm below GJ anastomosis. Finally, a sleeve gastrectomy (SG) was performed by creating a gastric tube cutting from antrum towards angle of his firing a 30-mm stapler (Endo GIA 30 mm, Autosuture, Covidien. Norwalk, USA) through longitudinal axis of the stomach. A running suture was performed over the stapler line to prevent gastric leakage. IT was performed as previously described.[12] A 10-cm ileal segment was proximally interposed 4 cm below the Treitz ligament. This group was excluded from further analysis because only two animals were available for follow-up due to surgical complications during the learning curve of the technique.

Experimental Protocol

Weight Progression and Food Intake Assessment

Body weight was measured weekly until the end of the study. Weight progression is reported as percentage of preoperative body weight ($\%\text{preop} - \text{weight} = [(\text{current } \textit{weight} - \text{preoperative body weight})/\text{preoperative body}]) \times 100$. Total food intake was measure weekly after surgery and is expressed as cumulative food intake (grams) over the length of the study.

Glucose Homeostasis

Fasting glucose levels and oral glucose tolerance test (OGTT) were assessed 8 weeks after surgery. After

overnight fasting (10–12 h), glucose levels were measured using a blood glucometer (One Touch® Ultra®, Lifescan, Johnson & Johnson, Cincinnati, Ohio, USA) in conscious rats. After oral administration of glucose (3 g/kg), OGTT was evaluated by measuring glucose levels at 5, 15, 30, and 45 min.

An insulin tolerance test was performed at 8–9 weeks after surgery. Human insulin (0.5 UI/kg) (Actrarapid®, Novo Nordisk, Princeton, NJ, USA) was intraperitoneally injected in conscious rat, after 10–12 h of fasting. Blood glucose levels were measure as described earlier at baseline, 15, 30, 45 and 60 min after insulin injection.

Meal Test

Ten weeks after surgery, after an overnight fasting, a meal test was performed with 2 ml of a liquid diet (Ensure Plus, 14.8% protein, 57% carbohydrate, 28.2% fat). Blood samples for determination of ghrelin and PYY were obtained from a jugular central catheter. Blood was collected in EDTA tubes. Plasma was stored at −80°C until further analysis. Radioimmunoassay kits for Ghrelin (total ghrelin, LINCO research, cat no. GHRT-89HK, St. Charles, M, USA) and PYY (PYY, cat no. RK-059-03, Phoenix Pharmaceuticals, Inc, Belmont, CA, USA) were used according to manufacturer's protocols. Total ghrelin levels were determined at baseline and 30 min after oral gavage, and PYY levels were measured at baseline and 60 min after oral gavage.

Statistical Analysis

Continuous variables are presented as mean±s.e.m. Continuous variables were compared using one-way ANOVA for independent measures. Glucose tolerance was evaluated by area under the curve (AUC) analysis. A two-tailed p value <0.05 was considered statistically significant. Statistical analyses were performed using a commercially available software package MINITAB® version 14.1 for Windows.

Results

SGIT as Effective as RYGB to Induce a Significant Weight Loss

To evaluate the effect of surgery on weight loss, body weight progression was weekly assessed until 9 weeks after surgery. As expected, sham-operated animals exhibited a progressive weight gain (Fig. 1). At the end of the study, SGIT- and RYGB-operated rats weighed 21% and 18% less than sham-operated rats, respectively (sham, 884±15 g; SGIT, 720±19 g; RYGB, 754±14 g; $p<0.001$). No significant differences were found between SGIT- and RYGB-operated rats at the end of the study.

SGIT Induced a Significant Decrease on Ad Libitum Food Intake

To study the effect of surgery on food intake, cumulative food intake was measured until week 12 after surgery. SGIT- and RYGB-operated rats decreased cumulative food intake by 29.6% and 32.9%, respectively (sham, 576.3±33 g; SGIT, 405.8±10 g; RYGB, 386.4±21 g; $p<0.001$). Again, no differences were found between SGIT- and RYGB-operated rats (Fig. 2).

Meal Test

To evaluate the effect of gastrointestinal manipulation on fasting levels and nutrient-stimulated release of gut-derived peptides involved in food intake regulation, a meal test was performed 10 weeks after surgery. Fasting ghrelin levels were dramatically decreased by 46% and by 32% after RYGB and SGIT surgery, respectively (Fig. 3) (sham, 2,905±400 pg/

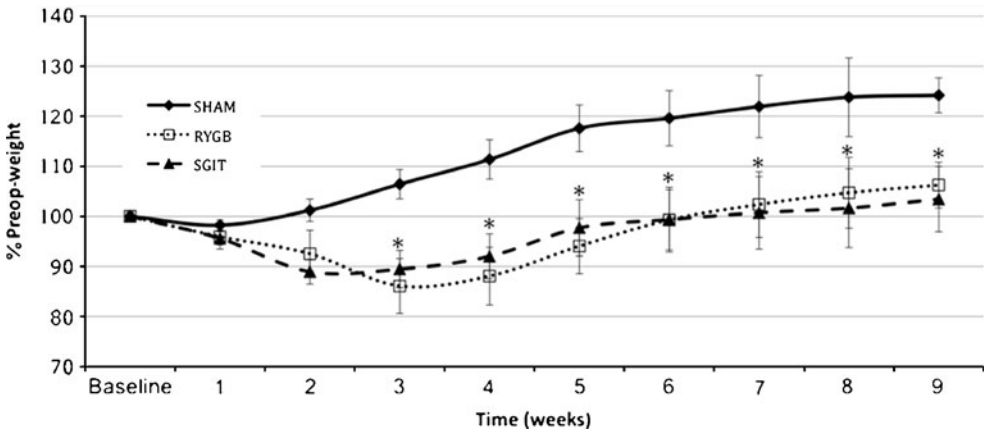

Fig. 1 Weight progression per week according to each surgical procedure

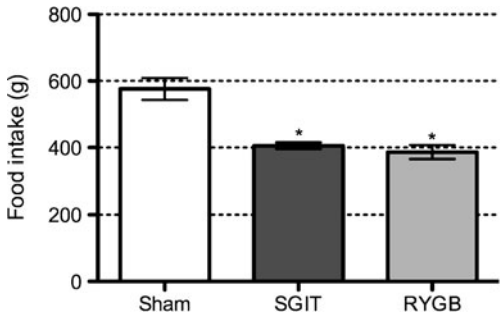

Fig. 2 Effects of each surgical procedure on food intake

ml; RYGB, 1,555±145 pg/ml; SGIT, 1,964±183 pg/ml; $p<0.001$). However, only RYGB-operated rats maintained decreased ghrelin levels 30 min after the meal gavage.

Another peptide involved in food intake regulation, specifically on meal termination is PYY. Basal and nutrient-stimulated PYY levels were increased after SGIT and RYGB. In addition, SGIT-operated rats had higher levels than RYGB-treated rats (Fig. 4). Fasting serum PYY levels were increased by 123% and 25.6% after SGIT and RYGB, respectively (sham, 67.5±3 pg/ml; SGIT, 150.9±2.7 pg/ml; RYGB, 84.8±7 pg/ml; $p<0.001$). Sixty minutes after oral gavage, PYY levels were increased by 185% and 74% in SGIT and RYGB, respectively (sham, 63.4±2.1 pg/ml; SGIT, 192.7±17 pg/ml; RYGB, 117.7±4.8 pg/ml; $p<0.001$).

Glucose Control

Fasting serum glucose levels were substantially improved after SGIT and RYGB (Fig. 5). At postoperative week 8, fasting glucose levels were reduced by 36% and 27% in SGIT- and RYGB-operated rats respectively, compared to sham-operated rats (sham, 261±41 mg/dl; SGIT, 167±16 mg/dl; RYGB, 190±30; $p<0.02$). The AUC demonstrated improved glucose tolerance after SGIT and RYGB surgery (sham, 27,090±1,424 AU; SGIT, 17,704±1,288 mg/dl; $p<0.018$; RYGB, 16,212±2,522; $p<0.01$).

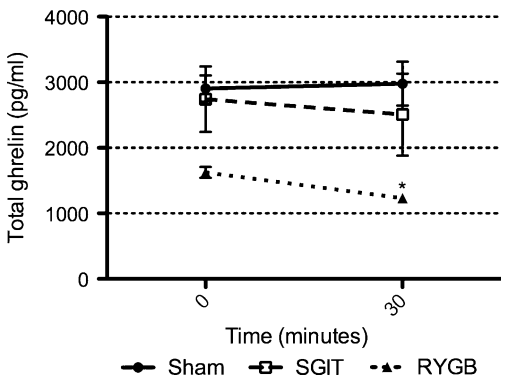

Fig. 3 Serum ghrelin levels after a meal test for each surgical procedure

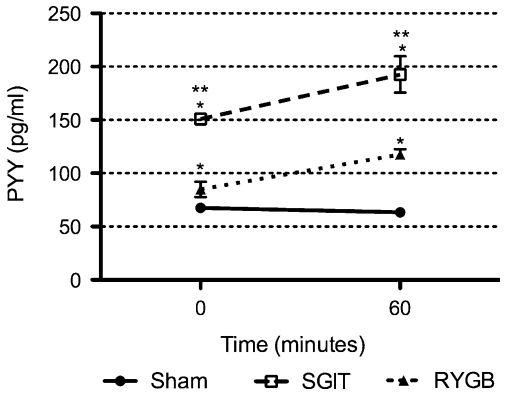

Fig. 4 Serum PYY levels after a meal test

Insulin Tolerance Test

Administration of exogenous insulin improved peripheral glucose disposal in SGIT- and RYGB-operated rats as shown in Fig. 6. A significant drop of fasting glucose levels were observed after insulin administration (Fig. 6). Again, no differences were observed between SGIT and RYGB groups.

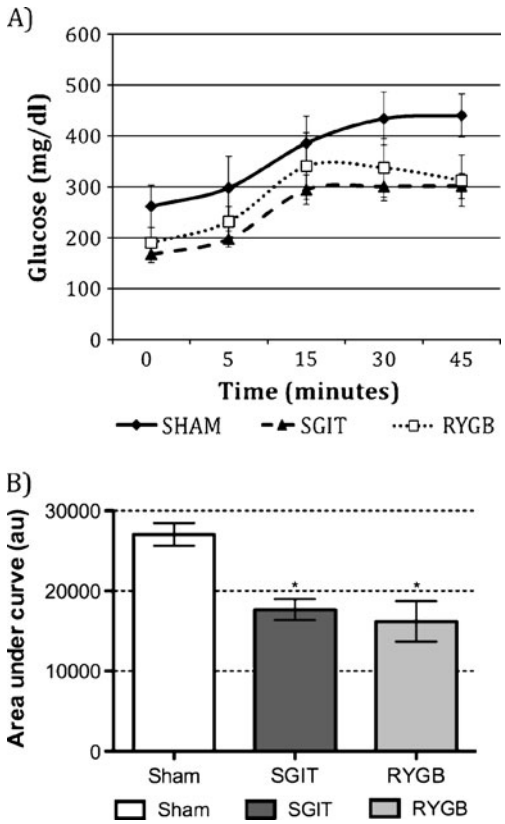

Fig. 5 **a** Oral glucose tolerance test **b** AUC for serum glucose levels over time

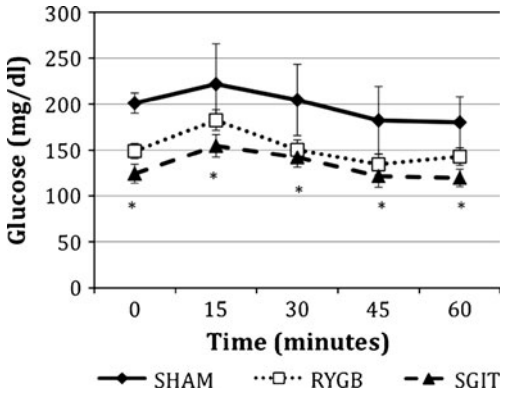

Fig. 6 Insulin tolerance test

Discussion

This study shows that by combining a moderate gastric restriction plus early contact of nutrients with distal ileum, without exclusion of the proximal intestine maintaining the length of the GI tract, it is possible to induce weight loss and improve glucose homeostasis. The degree of induced weight loss, reduced food intake, and improved glycemic control was similar between SGIT and RYGB, despite differences in the surgical procedures. These results suggest that nutrient exclusion from the proximal small intestine is not necessary to promote weight loss and to improve glycemic control after SGIT, since nutrient passage along the duodenum and proximal jejunum was possible.

To study the effect of surgery on body weight progression, weekly body weight and cumulative food intake were assessed until the end of the study. SGIT exhibited a significant weight loss, comparable to the weight loss induced by RYGB. We found a similar reduction in cumulative food intake between SGIT and RYGB that can explain weight loss. However, reduced caloric intake partially accounts for the induced weight loss after RYGB. In pair-feeding experiments, when a group of rats receives the same amount of food that the RYGB-operated rats eat ad libitum, the induced weight loss is lower compared to weight loss obtained after RYGB.[17] Thus, other mechanisms should explain the observed difference in body weight loss. It has been estimated that RYGB decreases nutrient absorption by 4%, a reduction that cannot explain the total observed weight loss of 25% induced by the RYGB.[18] Although, we did not evaluated nutrient absorption, it is likely that reduced nutrient absorption was absent in SGIT since this procedure maintains the length of the gastrointestinal tract. To study the contribution of a major regulator of body energy balance, Stylopoulos et al.[18] studied the effect of RYGBP on energy expenditure. Surprisingly, despite a significant weight loss, RYGB exhibited a significant increase of total energy expenditure and resting energy expenditure. To control for the effect of weight loss, a control group of weight-matched rats was included in the analysis, and as expected, the underfed rats activated a counter regulatory mechanisms to prevent more weight loss by decreasing energy expenditure, a response that was absent in RYGB. Since it is unlikely that SGIT induced a significant reduction in nutrients absorption, the observed weight loss could be explained by changes in energy expenditure; however, further experiments are needed to confirm this hypothesis.

Despite differences in the anatomical components of SGIT (lesser gastric restrictive component and no exclusion of the proximal intestine) compared to RYGBP, weight loss and reduction in food intake were similar in both groups. How SGIT procedure can elicit these effects despite the anatomical differences? It is possible to argue that the effects might be a consequence of an increased nutrient-stimulated ileal secretion of hormones involved in food intake regulation. It has been demonstrated that after SG, there is a significant increase in gastric emptying.[19] Thus, by increasing gastric emptying recently ingested and partially digested nutrients contact the transposed ileum. As demonstrated in animal models of IT, early contact of nutrients with the transposed ileum increases nutrient-stimulated secretion of key gut-derived hormones such as PYY and GLP-1.[12] PYY is a powerful hormone involved in food intake suppression, and evidences from animal and human studies have demonstrated its efficacy to reduce food intake.[20] Accordingly, SGIT exhibited increased fasting and nutrient-stimulated levels of PYY compared to RYGB and sham (Fig. 4).

The increase in PYY levels is responsible for a negative feedback mechanism known as “ileal brake”, which delays gastric emptying and gastrointestinal transit and generates satiety,[21] which has been argued as responsible for the observed weight loss after IT.[22] Consequently, enhanced release of PYY after SGIT and RYGBP could explain reduced food intake. Decreased food intake can also be explained by changes in the levels of the orexigenic hormone ghrelin. It has been demonstrated that postoperative weight loss after RYGB is correlated with the magnitude of the decrease in circulating ghrelin levels in rats[23] and human patients patient,[24,25] although some have reported increases levels after RYGB.[26,27] After RYGB, ghrelin fasting levels and nutrient-stimulated levels were substantially reduced compared to sham animals (Fig. 3). These results suggest that lower ghrelin levels are not necessary to decrease food intake after SGIT, and therefore other mechanisms not assessed in this study could explain the reduced food intake, along with the increased PYY levels and gastric restriction.

In terms of glycemic control, previous studies on non-obese animal models of T2DM have demonstrated that DJE

is associated with a significant improvement in glucose levels.[10,11] These observations led to hypothesize that the proximal small intestine has role in T2DM pathogenesis by releasing a yet unknown anti-incretin factor,[11] and therefore surgical procedures with DJE will block the secretion of this anti-incretin by preventing its nutrient-stimulated secretion. Based on this hypothesis, nutrient passage within the proximal small intestine should impair the effect of surgery on glucose restoration. However, SGIT induced a significant improvement in glucose homeostasis as demonstrated by significant reduction in fasting glucose levels and glucose tolerance, compared to sham-operated rats. In addition, these changes were comparable to those observed after RYGB. There results suggest that exclusion of the proximal intestine is not necessary to improve glucose homeostasis after SGIT; however, its role after RYGB remains to be elucidated. The observed weight loss along with the changes in the secretion profile of gastrointestinal hormones described above may also help to explain improvement of glucose homoeostasis (Figs. 5 and 6). After RYGBP, in addition to the nutrients exclusion from the proximal small intestine—foregut hypothesis—argued as a primary mechanism involved in diabetes remission, there is also an enhanced early delivery and exposure of partially digested nutrients into the distal small intestine (*hindgut hypothesis*). The latter has been associated with increased nutrient-stimulated incretin secretion (GLP-1). Thus, after SGIT, a similar increase in these hormones can explain improved glucose levels. In support of this hypothesis, recently, Peterli et al.[28] demonstrated that insulin, GLP-1, and PYY were similarly increased after RYGBP and SG in human patients, an effect that could be enhanced even further by the ileal intestine transposed.

These findings confirm our previous results obtained in a porcine model of SGIT,[16] and the findings also extend the efficacy of SGIT to glucose control. The effect on glycemic control is further supported by the efficacy of SGIT to induce diabetes remission in human diabetic patients.[29] All together, our results support the idea that exclusion of the proximal intestine is not necessary to induce diabetes remission; however, the role of the excluded distal stomach and proximal small intestine after RYGB remains to be elucidated.

The application of this procedure on humans has to be carefully assessed. There are a number of technical issues that need to be clarified. The most important concern would be the need of three intestinal anastomoses with the creation of three mesenteric defects. Although there have been reports in humans, no detailed analysis on postoperative complications has been addressed.

In conclusion, SGIT has proven to induce a significant weight loss and glucose homeostasis improvement, comparable to the effect obtained after RYGBP, without exclusion of the proximal intestine. These benefits have been confirmed in human studies, which highlight the potential role of SGIT as a new surgical procedure to treat obesity and T2DM.

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ORIGINAL ARTICLE

Improving the AJCC/TNM Classification for Use in Early Gastric Cancer

Paolo G. Gobbi · Lara Villano · Donatella Pozzoli · Manuela Bergonzi · Alessandro Vanoli · Francesca Tava · Paolo Dionigi · Gino Roberto Corazza

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Abstract
Purpose The current TNM classification is still unsatisfactory for collecting all the prognostic information from the clinical presentation of early gastric cancer: "T" is limited to two levels, the classes of "N" are still wide and "M" is generally absent.
Patients and Methods This study involved 99 patients who underwent radical gastric resection for early gastric cancer. Clinical and histological parameters were prognostically analyzed for both observed and relative survival. Univariate and multivariate analyses were applied to the proportional hazards model.
Results Number of metastatic lymph nodes and measure of the largest diameter of the tumor were the only independent prognosticators of observed and relative survival. Their similar relative hazards allowed an additive use of them in the N class. Two cut-off values of this composite clinical parameter are proposed for a good discrimination of the relative survival.
Discussion The number of metastatic lymph nodes is the cornerstone of the current TNM system and was confirmed as adequate. The possibility of adding tumor size to the number of the involved lymph nodes improves and amplifies the prognostic ability, which is presently limited by the rarity of lymph node involvement and the small number of the lymph nodes usually involved.

Keywords Early gastric cancer · Relative survival · TNM classification

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P. G. Gobbi (✉) · L. Villano · D. Pozzoli · M. Bergonzi · G. R. Corazza
Medicina Interna e Gastroenterologia, Università di Pavia,
Fondazione IRCCS Policlinico S. Matteo,
P.le Golgi n° 2,
27100 Pavia, Italy
e-mail: gobbipg@smatteo.pv.it

A. Vanoli · F. Tava
Dipartimento di Patologia Umana ed Ereditaria,
Sezione di Anatomia Patologica,
Università di Pavia, Fondazione IRCCS Policlinico S. Matteo,
Pavia, Italy

P. Dionigi
Dipartimento di Scienze Chirurgiche,
Sezione di Chirurgia Generale, Università di Pavia,
Fondazione IRCCS Policlinico S. Matteo,
Pavia, Italy

Introduction

Early gastric cancer (EGC) has been defined as a neoplastic invasion confined to mucosa or submucosa irrespective of the presence of lymph node metastasis.[1] It accounts for 10–20% of all resected gastric cancers in Western countries and about 40% in Japan.[2] Five-year survival rates of 90% or more have been documented for EGC, particularly in Japan[3,4] compared with 15–25% recorded for advanced gastric cancers (AGC).[5] Despite clinical differences, the same TNM prognostic classification is used for both presentations even according to the last AJCC Cancer Staging Manual,[6] which made some modifications to the staging criteria for gastric cancers described in the preceding 2002 edition.[7] This classification includes an identical evaluation of metastatic lymph nodes in spite of their different prevalence in EGC (12–15%)[8,9] and AGC (64–96%).[2,10] In fact, the large majority of patients with EGC are destined to have an allocation in N_0 (i.e., without metastatic lymph nodes) even according to the latest classification.

It is likely that the historical development of the TNM classification—in its present undifferentiated form for all gastric cancers—has been conditioned by the higher frequency and the severe characteristics of AGC and it appears less adequate for EGC. A refinement of the TNM classification specifically devised for EGC is necessary to increase its prognostic accuracy in this context and offer a better guide to the choice of treatment. Paradoxically, the low disease-specific mortality and wide age range at diagnosis complicate prognostic investigations on EGC because of the need to discriminate deaths due to cancer from those due to other causes, a particularly important matter during a very long follow-up. In this study, we tried to overcome these obstacles by evaluating prognostic factors of patients with EGC in relation not only to observed survival, but also to the relative survival as a reliable estimate of disease-specific mortality.

Table 1 Main clinical and pathological characteristics of the 99 patients included in the study (ranges between round brackets)

Sex	
M	57
F	42
Age mean±SD	60.1±13.5 years (24–86)
Tumor site	
Upper	2
Middle	24
Lower	73
Depth of involvement	
Mucosa	20
Submucosa	79
Tumor shape	
Ulcerated	40
Non-ulcerated	59
Tumor size	Largest Ø 2.4±1.3 cm (0.3–6)
Histological subtypes	
Adenocarcinoma	20
Papillary adenocarcinoma	4
Tubular adenocarcinoma	32
Mucinous adenocarcinoma	9
Signet ring cell adenocarcinoma	34
Histological grading	
G1 (well differentiated)	16
G2 (moderately differentiated)	48
G3 (poorly differentiated)	35
Lymphoinvasion	
Yes	52
No	47
Lymph node involvement	
Yes	26
No	73
No. of involved nodes	2.5±1.6 (1–6)
Hemoglobin (g/dL)	12.9±1.7 (7.6–16.0)
Serum albumin (g/dL)	3.9±0.5 (2.9–5.2)
Tis	20
T1	79
N0	73
N1	16
N2	10

Patients and Methods

Patients

Between 1 January 1984 and 31 December 2006, 99 patients with EGC underwent total or subtotal gastrectomy in the Surgical Department of the Fondazione IRCCS Policlinico S. Matteo of Pavia and were then followed up in the Department of Internal Medicine of the same Hospital until 31 December 2009.

The following information was collected for each patient: presenting signs and symptoms, location of the tumor, description of the surgical operation, radicalness of the resection performed, macroscopic features at presentation, diameters of the tumor mass, number of regional metastatic lymph nodes, microscopic subtype of the tumor, Lauren's histological type (intestinal or diffuse), depth of penetration into the gastric wall, cell differentiation (well, moderately, or poorly differentiated adenocarcinoma), grade of lymphatic invasion, and main laboratory data at presentation before surgery (blood cell count, serum protein electrophoresis, and liver and kidney function tests).

Macroscopic evaluation of the whole resected material and histological examination of the sampled specimens were performed centrally. The diameters of the tumor lesions were measured on fixed tissues. Vascular and lymphatic invasion was evaluated on paraffin sections

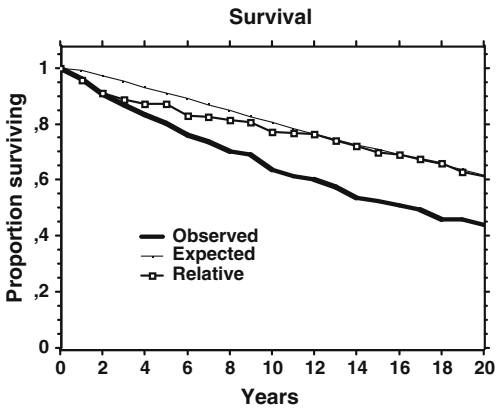

Fig. 1 Observed, expected, and relative survival of the 99 patients investigated

Table 2 Results of univariate analysis for overall survival and relative survival

Variables	Overall survival		Relative survival	
	Chi-square	*P* value	Chi-square	*P* value
Sex	0.940	0.3322	0.001	0.9809
Age	28.334	<0.0001	0.288	0.5914
Tumor site	0.017	0.9967	0.123	0.7257
Tumor size	4.360	0.0368	3.953	0.0468
Histological grading	1.420	0.2335	2.408	0.1207
Depth of involvement (T1 or T2)	1.201	0.2731	1.067	0.3017
No. of lymph nodes involved	2.907	0.0927	4.797	0.0285
Lymphatic invasion	1.310	0.2537	2.211	0.1425
Lauren classification	0.278	0.5981	0.080	0.7773
Subtotal/total gastrectomy	1.515	0.2184	0.243	0.6223

stained with hematoxylin–eosin; cases in which recognition of endothelial structures was uncertain underwent immunohistochemical studies for CD34 and CD31 markers. In fact, both the anti-CD31 antibody, which identifies the antigen ER-MP12, identical to the vascular endothelial adhesion molecule PECAM-1, and the anti-CD34 antibody, which stains normal and endothelial cells, make the identification of vascular and lymphatic vessels easier. Systematic re-examination of the specimens was carried out to verify the correct diagnostic allocation into the categories of the WHO Histological Classification.[11] Subclassification of growth patterns according to the histological appearance at the cut surface of the tumor was disregarded.[12]

For the purposes of this study, patients alive in 2009 who had not had a medical examination within the preceding 6 months were recalled for a new clinical and instrumental control. The vital status of those patients who did not respond to this recall was ascertained by telephone (information from relatives and/or from physician) or investigated in the General Registry Offices of their last known municipality of residence. All the patients were staged according to the TNM classification.[6]

Adjuvant chemotherapy and/or radiotherapy were not planned because of the largely radical excision of the tumor in all cases. Various chemotherapy regimens were administered to 21 patients at relapse, which occurred from 6 to 64 months after surgery. Forty-eight out of the 99 patients were dead at the time of this study. The median follow-up was 151 months (range, 9–309 months) for the whole population and 215 months for the patients alive.

Statistics

The time parameters taken into account were overall survival and relative survival. The latter was calculated as the ratio of the overall survival rate observed in the patients to the expected survival rate drawn from the general reference population for subjects similar to the patients with respect to age, sex, calendar year of initial observation, and duration of observation.[13] The age-, gender-, and calendar year-specific death rates available from national Italian mortality tables (ISTAT, Istituto Nazionale di Statistica) were used to calculate the expected deaths—and, thus, the expected survival. Age changes according to individual birthdays in every year of the follow-up were

Table 3 Final results of multivariate analysis for observed survival and relative survival

Observed survival	Coefficient	Chi-square	*P* value	Relative hazard
Age (years)	0.074	29.219	<0.0001	1.077
Tumor size (cm)	0.226	3.802	0.0461	1.253
No. of involved lymph nodes	0.186	4.408	0.0358	1.205
Relative survival (1st step)				
Age (years)	–0.008	0.261	0.6087	0.992
Tumor size (cm)	0.257	6.556	0.0105	1.293
No. of involved lymph nodes	0.234	6.775	0.0092	1.263
Relative survival (2nd step)				
Tumor size (cm)	0.242	5.753	0.0165	1.274
No. of involved lymph nodes	0.228	6.515	0.0107	1.256

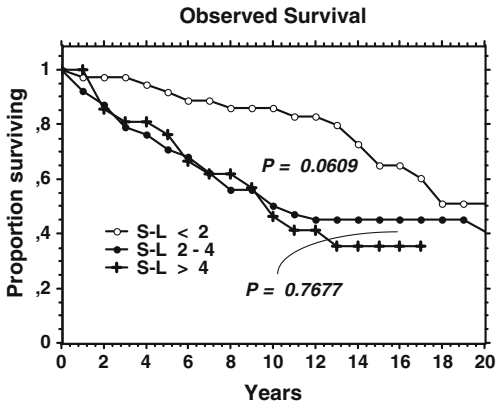

Fig. 2 Prognostic discrimination of observed survival provided by the combined consideration of tumor size and metastatic lymph nodes (S–L): <2 (37 pts), 2–4 (40 pts), >4 (22 pts)

taken into account. In this way, each patient was considered to have a large control group from the general population with corresponding personal characteristics with a well-defined probability of dying (or surviving). Consequently, the relative survival, obtained by adjusting observed survival for normal life expectancy, is considered a satisfactory estimate of the chance of surviving the effects of cancer. A detailed example of the calculations needed for each patient was given elsewhere in a study of a population with colorectal cancer.[14] The observed deaths recorded in the population of patients at the end of the follow-up period and the difference between the observed deaths and the cumulative expected probability of death during the corresponding period (i.e., excess mortality, which has to be taken into account for relative survival) are the variables that can be used in both survival calculations and multivariate analyses. When evaluating the curves of relative survival, we have to remember that if observed and expected deaths are equal (i.e., there is not excess mortality) their ratio is 1, and the curve shows a plateaux of 100%.

The Kaplan–Meier method[15] was used to evaluate survival and differences were analyzed by the log-rank test.[16] The clinical and pathological features that showed statistically significant prognostic values in univariate analyses were selected for multivariate analyses. These were performed by multiple regressions applied to a Cox proportional hazards model.[17] A stepwise selection of factors was applied to the multiple regressions (with enter/remove p value of 0.05).

Results

The main characteristics of the study population are reported in Table 1. The causes of death were the following: recurrence of EGC or complications directly related to it in 21 patients, diseases other than EGC in 27 subjects (myocardial infarction or congestive heart failure in nine, cerebrovascular disease in five, pneumonia in five, renal failure in four, second neoplasia in two, and pulmonary embolism in two). Figure 1 illustrates the three curves of observed, expected, and relative survival. The rates of observed overall survival of the whole series were 80% at 5 years and 64% at 10 years, while the corresponding rates of expected survival from the reference population were 91% and 82%. Thus, adjusting the observed survival for the normal life expectancy of the general population with the same age, sex and period of observation, the relative survival rates, corresponding to cancer-specific survival rates, were 87% and 80% at 5 and 10 years, respectively. It is clear from the figure that most of the deaths due to EGC occur within the first 10 years and that after this period the curves of expected and relative survival are very similar.

The results of the univariate analysis of the clinical and pathological parameters scrutinized in relation to observed and relative survival are reported in Table 2, which shows that age and tumor size have statistically significant roles in relation to observed survival while tumor size and number of involved lymph nodes seem to be important with regard to the relative survival.

Thus, age, tumor size, and number of involved lymph nodes were the only parameters that were entered into the multivariate analysis (Table 3). This analysis was performed for both observed and relative survival. The study of relative survival removed age from and included the number of involved lymph nodes and tumor size into the list of the statistically important prognostic determinants. Moreover, the evaluation of the relative hazards showed that the two significant parameters have very similar relative hazards for both observed and relative survival, with a nearly identical risk associated with an increase of either 1 cm in tumor size or one metastatic lymph node. In other words, the prognostic role of 1 cm of tumor size roughly corresponds to that of one involved lymph node, and for this reason the parameters can be used additively.

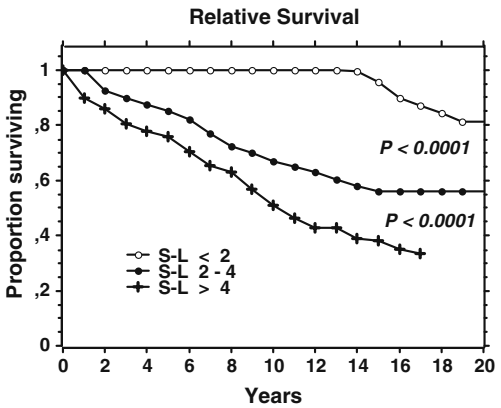

Fig. 3 Good prognostic discrimination of relative survival provided by the combined S–L parameter: <2 (37 pts), 2–4 (40 pts), >4 (22 pts)

An example of the combined computation of both tumor size and number of involved lymph nodes (a parameter hereinafter called S–L) is illustrated in Fig. 2 (observed survival) and Fig. 3 (relative survival). Both figures show the survival of three subgroups of patients identified by an exploratory and empirical categorization of the variability range of S–L (i.e., <2, 2–4, and >4). A comparison of the figures demonstrates the advantage of excluding causes of death other than those from cancer when evaluating tumor factors that directly influence survival. Table 4 reports the observed and relative survival at 5, 10, and 15 years, respectively, of the N classes of the TNM staging and of the new S–L categories: the S–L groups include more balanced numbers of patients and their relative survival is more clearly differentiated. In particular, it can be noted that the N_1 patients have a paradoxically better prognosis than the N_0 ones related to both observed and relative survival, whereas the analysis of the relative survival demonstrates the clearer prognostic discrimination allowed by the S–L grouping (as already shown in the Figs. 2 and 3). So, the possibility of a joint computation of tumor size (in cm) together with any single metastatic lymph node can lead to more flexible and adequate integration of the "N" class.

Discussion

Many studies have reported on the prognostic importance of several clinicopathological factors besides tumor size and number of involved lymph nodes, such as age, histological differentiation, Lauren's histological type, depth of gastric wall involvement, lymphatic invasion, and extent of nodal dissection.[18–26] The role of lymph node involvement is, however, considered to be primary, and many studies have investigated the risk factors for recording positive lymph nodes in order to identify patients who can be treated less invasively.[8,9]

Unfortunately, in patients with EGC little prognostic information can be derived from the current TNM classification, apart from the discriminatory level of wall involvement (T_{is} and T_1). Even the modified categorization of the "N" classes proposed in the Seventh AJCC Cancer Staging Manual[6] (with N_1 signifying 1 to 2 metastatic lymph nodes, N_2 from 3 to 6 and N_3 more than 6) was likely chosen as best fitting the much more frequent and severe AGC. Furthermore, as regards the "M" category, by definition no distant metastases can be expected in patients with EGC. Given these weaknesses in the prognostic power of the current TNM classification, it seems time to conceptually focus research on a prognostic classification specifically adapted to EGC.

To this aim, the choice of analyzing relative survival, as the best estimate of specific survival (i.e., related to the excess mortality due to the disease), could be particularly suitable for patients with EGC, who have a wide age range at diagnosis, elderly subjects included, and a long survival, and for these reasons are exposed to a number of co-morbidities, accidents or complications that can make the observed survival misleading for a prognostic study of the factors strictly related to the presentation of the disease. The first advantage of relative survival is that it is drawn from the expected survival from nationwide population life tables, stratified by age, sex, and calendar time. Generally, a direct consequence of this technique is the disappearance of age from most of the investigations on prognostic factors of cancer, because it correctly weights the different incidence of deaths due to co-morbidity at various ages. Moreover, the computation of relative survival frees the investigators from consideration of coded information—often unreliable—on causes of death and from the difficulty of assessing whether or not cancer has been the primary cause of death.

Our sample of patients is rather small (although very homogeneously treated) and precludes definitive conclusions, but it suggests that the prognostic significance of metastatic lymph nodes is well distributed over and shared by each individual, metastatic lymph node and, most importantly, can be additively computed with tumor size. Although the most recent modification of the TNM classification reduced the number of metastatic lymph nodes in every N class, its application to EGC is still rather inadequate. In our series, 74% of the patients

Table 4 Observed and relative survival according to the N classes of the AJCC/TNM Classification (which categorizes the number of positive lymph nodes) and to the proposed integration of the number of positive lymph nodes with the tumor size (S–L groups)

	Observed survival			Relative survival		
	5 years	10 years	15 years	5 years	10 years	15 years
N_0 (73 pts)	0.818	0.654	0.527	0.915	0.721	0.610
N_1 (16 pts)	0.867	0.700	0.700	0.921	0.817	0.801
N_2 (10 pts)	0.600	0.400	0.267	0.717	0.603	0.407
S–L<2 (37 pts)	0.917	0.840	0.651	0.995	0.995	0.907
S–L 2–4 (40 pts)	0.688	0.481	0.451	0.871	0.656	0.552
S–L>4 (22 pts)	0.762	0.464	0.354	0.780	0.510	0.381

would have been assigned to class N_0, 16% to N_1, and 10% to N_2. Folli et al. [22] first realized the need to reduce the number of lymph nodes in the prognostic classes and reported that patients with ≤3 metastatic lymph nodes had a significantly better 5-year survival (83%) than those with >3 positive lymph nodes (48%). Huang et al. [9] recently proposed new N classes with further reductions in the number of lymph nodes in each (N_1, 1–3; N_2, 4–6; N_3, >6). The application of these new categories to their 344 patients produced a clearly better survival discrimination. Our results can be considered the extreme step in splitting the N categories because of the demonstration of a distinct and individual prognostic value carried by each involved lymph node. From this point of view, the equivalent prognostic importance of number of lymph nodes involved and centimeters of tumor size, with the possibility of adding the centimeters of the largest diameter of the lesion to the number of metastatic lymph nodes, helps to amplify the predictive ability at diagnosis (otherwise strongly limited by the rarity of lymphatic involvement and by the small number of lymph nodes actually involved). A further, though secondary advantage, is the numerically more homogenous subdivision of the population of patients into the distinct prognostic groups (the two cut-offs of the new composite S–L variable subdivided our patients into groups with 37, 40, and 22 subjects, respectively). Our results need confirmation from other clinical studies, but we believe that other prognostic factors—apart from those so far identified as important in EGC—should be evaluated for a possible integration with number of lymph nodes and tumor size in order to develop a precise clinico-prognostic classification specifically suitable for EGC.

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ORIGINAL ARTICLE

Phosphorylated Insulin-Like Growth Factor 1 Receptor is Implicated in Resistance to the Cytostatic Effect of Gefitinib in Colorectal Cancer Cells

Li Yang · Jianjun Li · Li Ran · Feng Pan · Xiaoxin Zhao · Zhenyu Ding · Yuying Chen · Qiuping Peng · Houjie Liang

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Abstract
Introduction The ability of certain cancer cells to maintain signaling via the phosphoinositide-3-kinase/Akt and/or Ras/mitogen-activated protein kinase (MAPK) pathways has been repeatedly involved in resistance to epidermal growth factor receptor (EGFR) inhibition.
Discussion We investigated the potential mechanisms of the uncoupling of EGFR from its downstream signals in colorectal cancer (CRC) cells. Alternative growth factor receptors and regulation of downstream pathways in different gefitinib-responsive cell lines were determined. Basal insulin-like growth factor receptor-1β (IGFR-1β) phosphorylation was undetectable or present at very low levels in highly gefitinib-responsive cell lines and was present at strikingly high levels in less responsive cell lines. Further analysis of cell lines representing the most sensitive (Lovo), moderately sensitive (HT29), and most resistant (HCT116) strains was treated with an IGFR-1 inhibitor (AG1024), gefitinib, or both, revealing that elevated IGFR-1β phosphorylation can compensate for the loss of EGFR signaling function. Increased insulin-like growth factor II expression induced by gefitinib or heterodimerization of EGFR and IGFR-1β may trigger IGFR-1β signal transduction via activation of Akt and MAPK. In addition, high levels of EGFR and IGFR-1β phosphorylation were detected in CRC tumor tissue. We also showed that gefitinib- and/or AG1024-induced cytostatic effects could be mediated by glycogen synthase kinase-3β (GSK-3β) activation. Our data suggest that the crosstalk between EGFR and IGFR-1β signaling are likely to contribute to resistance of CRC cells to gefitinib and that measurement of GSK-3β activation may present a potential biomarker for evaluating the antitumor efficacy of receptor tyrosine kinase inhibition.

Keywords Colorectal cancer · Epidermal growth factor receptor · Gefitinib · Insulin-like growth factor receptor-1β · Phosphorylation

Li Yang and Jianjun Li contributed equally to this study.

L. Yang · J. Li · F. Pan · X. Zhao · Z. Ding · Y. Chen · H. Liang (✉)
Department of Oncology, Southwest Hospital,
Third Military Medical University,
30 Gaotanyan Street, Shapingba District,
Chongqing 400038, People's Republic of China
e-mail: lianghoujie@sina.com

L. Yang · L. Ran
Department of Oncology, Guizhou Cancer Hospital,
Guiyang Medical College,
Guiyang, Guizhou, People's Republic of China

Q. Peng
Department of Oncology, 94th Hospital,
Nanchang, Jiangxi, People's Republic of China

Introduction

Colorectal cancer (CRC) is one of the most common causes of cancer-related death in both sexes throughout the world. Adjuvant chemotherapy has made incremental improvements on the outcomes of patients with locally advanced and metastatic disease over the past decade; however, the overall clinical effect remains disappointing in the majority of patients with late stage tumors, accounting for 5-year survival rates of less than 10%.[1]

It is now evident that epidermal growth factor receptor (EGFR) is frequently overexpressed in a wide range of human epithelial malignancies, including CRC,[2] and aberrant EGFR expression has been associated with resistance to conventional therapies and has been found to act as an indicator of poor prognosis in CRC patients in some studies.[3,4] This resulted in considerable interest in the development of EGFR antagonist development for anti-tumor therapies. Of these agents, the two most promising and clinically advanced strategies are the administration of EGFR blocking monoclonal antibodies (e.g., cetuximab and panitumumab) and small molecule tyrosine kinase inhibitors (TKIs; e.g., gefitinib and erlotinib[5]).

Gefitinib (ZD1839, Iressa®) is an orally active and selective EGFR-TKI that competitively inhibits binding of ATP to the receptor tyrosine kinase (RTK) domain.[6] It has been demonstrated to have antiproliferative activity in various human tumors, including CRC, both in vitro and in vivo.[7,8] Furthermore, gefitinib could potentiate synergistic interactions with cytotoxic agents[9] and radiotherapy in xenograft models of CRC.[10] However, phase I/II clinical trials have produced the disappointing results that gefitinib has negligible activity in advanced CRC patients as a monotherapeutic agent,[11,12] and more recent studies showed that gefitinib did not appear to add substantial efficacy to irinotecan or capecitabine[13,14] or sensitize patients with fluoropyrimidine refractory CRC to 5-Fu chemotherapy.[15] These data support the notion of existing intrinsic or de novo resistance to gefitinib treatment in CRC, and the resistance mechanism of this inhibitor has yet to be determined.

The major downstream signaling routes of EGFR are via the phosphoinositide-3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathways, which mediate responses from cell growth and proliferation to survival and motility in both normal and malignant epithelial cells.[4] Blocking EGFR activation with specific inhibitors results in cell growth inhibition through a shutdown of similar downstream signal transduction processes[7]; however, a number of studies have indicated that this does not occur in a variety of cancer cells.[16,17] The ability of certain tumor cells to maintain signaling through Akt and/or ERK under EGFR inhibition might represent a potential mechanism of resistance by which cancer cells can escape the antiproliferative activity of gefitinib, and the uncoupling of EGFR autophosphorylation and its downstream signaling is not dependent on EGFR overexpression.[16,17] The presence of somatic mutations in the tyrosine kinase domain of the EGFR gene or an increased copy number of the EGFR gene have been well documented to correlate with responses to treatment with gefitinib of non-small cell lung cancer (NSCLC[18–20]). However, it has been proven that EGFR activating mutations occur at a very low frequency in CRC cell lines and tumor specimens[21–23] and that the effects of known EGFR mutations do not seem responsible for susceptibility of this tumor to gefitinib treatment. It is, therefore, a major challenge to find reliable, predictive factors for sensitivity or resistance of human CRC cells to treatment with this promising agent.

To date, most studies have demonstrated that responses to gefitinib, used alone or in combination with standard cytotoxic agents in vitro and in vivo, are not clearly associated with the levels of tumor EGFR expression,[24–27] which suggests that aberrant transduction of downstream signals in cancer cells under gefitinib treatment is likely to be EGFR-independent. It has been shown that cancer cells may be resistant to anti-EGFR therapies without altering EGFR expression by enhancing proliferative activities and/or inactivating apoptotic activities: the mutation of K-ras gene, loss of phosphatase and tensin homologue function, and maintenance of phosphorylated Akt and ERK.[28–30] In addition, EGFR family members like HER-2 and HER-3 were involved into counteracting the antitumor effects of EGFR inhibitors.[31,32] More recently, other cell membrane growth factor receptors such as insulin-like growth factor receptor-1 (IGFR-1[33]), hepatocyte growth factor receptor (MET[34]) and platelet-derived growth factor receptor[17] were shown to be involved in the development of gefitinib resistance in various types of cancers.

Those results suggest to us that the intact EGFR-mediated signaling pathway is required for the ability of EGFR inhibition to suppress cell proliferation, and specific signals or growth factor receptors may be responsible for maintaining activated signaling transduction. In the present study, we show that high IGFR-1β phosphorylation can compensate for loss of EGFR signaling function and constitutively activates the common downstream pathways and that glycogen synthase kinase-3β (GSK-3β) activation plays a critical role in the induction of receptor tyrosine kinase inhibitor-induced cytostatic effects on CRC cells.

Materials and Methods

Cell Culture and Reagents

The Department of Oncology of Southwest Hospital preserved the human CRC cell lines Lovo, HCT116 and HT29, while LS174T, SW480, and SW620 were purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. All cell lines were maintained as a monolayer in high-glucose Dulbecco's modified Eagle's medium (Gibco, USA) except for SW620 in Leibovitz's

L-15 (Hyclone, USA), supplemented with 10% fetal bovine serum (FBS; Gibco, USA), vitamins, sodium pyruvate, L-glutamine, penicillin, streptomycin, and nonessential amino acids.

Gefitinib (AstraZeneca) was prepared as a 200-mmol/L stock solution in DMSO (Sigma) and preserved at −20°C, and this stock was diluted in fresh medium to the indicated concentrations just before use so that the concentration of DMSO never exceeded 0.1%. AG1024, an IGFR tyrosine kinase inhibitor, and lithium chloride (LiCl) were purchased from Calbiochem Immunochemicals, and EGF was purchased from Sigma. A 10-mg/mL stock solution of AG1024 in DMSO was preserved at −20°C. Antibodies for immuoblotting and immunofluorescence were purchased from the following suppliers: EGFR, phospho-EGFR specific for Tyr1173, Akt, phospho-Akt specific for Ser473, p44/42 MAPK, phospho-p44/42 MAPK specific for Thr202/Tyr204, IGFR-1β, phospho-IGFR-1β specific for Tyr1131, MET, and phospho-MET specific for Tyr1234/1235 (Cell Signaling, Beverly, MA, USA) and EGFR for coimmunoprecipitation, phospho-GSK-3β specific for Tyr216, and β-actin (Santa Cruz Biotech, Santa Cruz, CA, USA)

Western Blot Analysis

CRC cells were serum-starved for 16 to 24 h and treated with the indicated concentrations of gefitinib for 3 h before exposure to 50 ng/mL EGF for 30 min. For EGF non-stimulated conditions, cells were incubated in medium supplemented with 10% FBS until growth to 80% confluence, then treated with indicated concentrations of gefitinib, AG1024, or both for 3 h. Cells were harvested and lysed, and the protein concentration was assayed. Samples containing 50 μg of protein per lane were electrophoretically separated on 10% sodium dodecyl sulfate (SDS)–polyacrylamide gels and transferred onto polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). After blocking in 5% nonfat milk, the membrane was probed with the indicated primary antibodies at 4°C overnight, followed by horseradish peroxidase conjugated secondary antibody adjunction for 2 h at room temperature. The bands were visualized by Super-Signal® West Femto Maximum Sensitivity Substrate (Pierce, USA). BandScan 5.0 software was used for gray scale scans to evaluate the relative levels of protein expression.

Eighteen tumor tissue and paired healthy adjacent tissue specimens were obtain from patients with CRC who had undergone surgery at the Southwest Hospital, Third Military Medical University. All tissue specimens were frozen in liquid nitrogen immediately after being resected and rinsed in phosphate-buffered saline (PBS).

Semi-quantitative Reverse Transcription-PCR

Total RNA of cells was extracted with Trizol reagent (Invitrogen), and first-strand cDNA was synthesized using 2 μg of total RNA. The IGF-II gene was amplified by PCR with the following primer sets: sense, 5′-TGGGAAT CCCAATGGGGAAG-3′; antisense, 5′-TCTATGGGGCA CCCGTTC-3′. The PCR cycling conditions used were as follows: initial denaturation at 94°C for 3 min, denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 90 s (35 cycles), and a final extension step at 72°C for 10 min in the presence of Taq polymerase (TaKaRa Biotechnology Co., Ltd.). The amplified fragment was 346 bp in length. GAPDH was used as an internal standard and its mRNA was amplified with the primer sets: sense, 5′-CAAATTCCATGGCACCGTCA-3′; antisense, 5′-GGAG TGGGTGTCGCTGTTGA-3′, with similar conditions except that the annealing temperature was 59°C. The predicted product was 715 bp in length. The PCR products were subjected to 1.8% agarose gel electrophoresis with ethidium bromide. Densitometric analysis was performed using the Scion Image software.

Cell Proliferation Assay

To determine the effects of gefitinib, AG1024, or both on CRC cell proliferation, 5×10^3 cells per well were plated in 96-well plates. The next day, cells were treated with either 0.1% DMSO as a diluent control or with indicated concentrations of drugs in medium for 3 days. At the end of the treatment period, cell proliferation was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. A 20-μL volume of MTT solution (5 mg/mL) was added to each well, and the cells were incubated for 2 h. The media were removed and 200 μL of DMSO was added to lyse the cells and solubilize the formazan. The absorbance of each well was measured at 490 nm using a standard microplate reader. The drug concentrations required to inhibit cell growth by 50% (IC_{50}) were determined by interpolation from the dose–response curves. Four replicate wells were used for each analysis, and at least three independent experiments were done. To evaluate the cytostatic effect of GSK-3β activity, cells were also treated with LiCl at concentrations of 10 and 20 μmol/L together with gefitinib and/or AG1024.

Cell Cycle and Apoptosis Assays

CRC cells were seeded in six-well plates in medium supplemented with 10% FBS and grown to 70% confluence. Cells were then exposed to the indicated concentrations of gefitinib, AG1024, or both for 72 h. Both adherent and floating cells were harvested and pelleted by

centrifugation. To determine the percentages of cells in different phases of the cell cycle (G1, S, and G2), the pellets were fixed with 70% ethanol at 4°C overnight, and then cells were stained with 50 μg/mL propidium iodide and analyzed them with a flow cytometer (Epics Profile II; Beckman Coulter Inc.) equipped with a 488-nm argon laser. To assess apoptosis, the pellets were resuspended in binding buffer, stained according to the manufacturer's instructions using the Annexin V-FITC kit (Jingmei Biotechnology, Shenzhen, China), and then measured by flow cytometry with a 488-nm argon laser. Results from at least three experiments are presented as means with 95% confidence intervals.

Coimmunoprecipitation

Immunoprecipitations were performed using 200 μL of cell lysate with 4 μL of mouse anti-EGFR antibody and incubated with gentle rocking overnight at 4°C, with healthy proimmune serum anti-mouse antibody as the negative control. The immunocomplexes were precipitated with Protein A+G Agarose beads (Upstate Cell Signaling Solution) and incubated with gentle rocking for 4 h at 4°C. The immunoprecipitates were collected by microcentrifugation for 30 s at 4°C and washed five times with ice-cold PBS. The pellets were resuspended with 20 μL of 3× SDS sample buffer, vortexed, and microcentrifuged for 30 s. Coimmunoprecipitated samples were heated to 95–100°C for 3–5 min and then subjected to Western blot analysis.

Immunofluorescence Confocal Analysis

CRC cells grown on glass coverslips were exposed to the indicated concentrations of gefitinib, AG1024, or both. After incubation for 3 h, covered cells were rinsed with PBS, fixed in 4% paraformaldehyde at room temperature for 20 min, and then permeabilized with ice-cold 2.5% Triton for 3–5 min. After blocking in 5% normal serum for 30 min, cells were incubated with a primary antibody against phospho-GSK-3β specific for Tyr216 overnight at 4°C. Cells were next treated with TRITC-labeled goat anti-rabbit antibody for 40 min and then with Hoechst33258 for 15 min at 37°C. Confocal analysis was carried out using a Zeiss laser-scanning confocal microscope and established methods, involving processing of the same section for each detector (two excitations corresponding to 546 and 488) and comparing images pixel by pixel.

Statistical Analysis

Bands in gels were scanned in gray scale to evaluate the relative levels of protein expression. All quantitative data are represented as means±SD. The one-factor ANOVA, Student–Newman–Keuls, and Pearson correlation tests were used in statistical analysis, with $P<0.05$ considered statistically significant, using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Comparisons of protein expression levels between the tumor and normal tissue groups were made using the Fisher's exact test.

Results

IGFR-1β Phosphorylation Correlates with Responses of CRC Cells to Gefitinib Treatment

We investigated the effects of gefitinib on cell proliferation in the Lovo, HCT116, HT29, LS174T, SW480, and SW620 cell lines and found that Lovo cells were the most sensitive to gefitinib with an IC_{50} value less than 10 μmol/L; HT29 and SW480 cells were moderately sensitive, with IC_{50} values ranging from 20 to 60 μmol/L; and HCT116, LS174T, and SW620 cells were the most resistant, with IC_{50} values greater than 100 μmol/L (not shown). We also determined the activation status of EGFR and its downstream signals in Lovo, HT29, and HCT116 cell lines with EGF stimulation and following gefitinib treatment. We showed that HT29 and HCT116 cells could maintain high activation of Akt and MAPK under EGFR inhibition and that HCT116 cells were barely stimulated by EGF for activation of EGFR signaling pathways (not shown). Therefore, we hypothesized that specific alternative growth factor receptors associated with EGFR and downstream kinases might be responsible for sustaining the proliferation and survival of CRC cells that would bypass the EGFR blockade of gefitinib. It is well-known that other growth factor receptors, such as MET and IGFR-1, are overexpressed and related to tumorigenicity and progression of human tumors, including CRC.[35–38]

The basal levels of MET, phosphorylated MET (p-MET), IGFR-1β, and phosphorylated IGFR-1β (p-IGFR-1β) were measured in the panel of CRC cell lines by Western blot analysis (Fig. 1a). All tested cell lines exhibited similar amounts of MET expression except for LS174T, and the level of p-MET in Lovo cells did not substantially differ from that in HCT116, indicating that gefitinib responses do not correlate with MET expression or its phosphorylation. The level of IGFR-1β expression also failed to predict gefitinib sensitivity, as Lovo, HT29, SW480, and SW620 cells expressed similar amounts of IGFR-1β but displayed differences in gefitinib sensitivity. However, the level of p-IGFR-1β correlated with the responses to gefitinib of these CRC cell lines. As shown (Fig. 1a), p-IGFR-1β was undetectable or expressed at very low levels in the most and moderately sensitive cell lines (Lovo, HT29, and SW480) and appeared to have much lower expression in

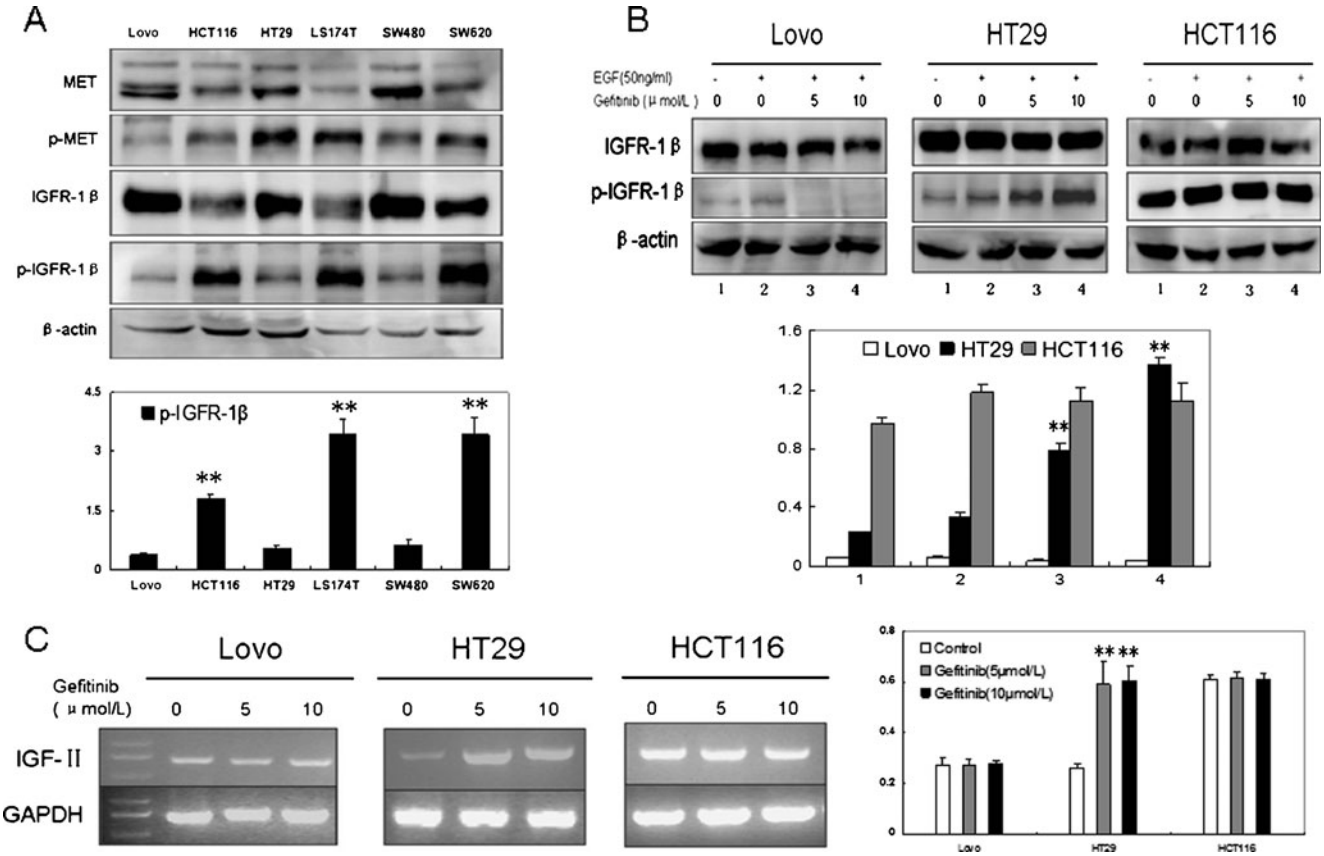

Fig. 1 **a** Western blot analysis showing basal expression of MET and IGFR-1β and their phosphorylated forms in Lovo, HCT116, HT29, LS174T, SW480, and SW620 CRC cell lines cultured in medium supplemented 10% FBS. **b** Western blot analysis showing levels of IGFR-1β expression and phosphorylation in Lovo, HT29, and HCT116 cells that displayed differences in gefitinib sensitivity. Exponentially growing cancer cells in medium containing 10% FBS were serum-starved for 24 h and stimulated with EGF for 30 min, with or without pretreatment with the indicated gefitinib concentrations for 3 h. **a**, **b** β-Actin was included as a loading control. *Bottom*, phosphorylated IGFR-1β was quantified by densitometry and expressed as a ratio of the β-actin protein, respectively. **c** IGF-II mRNA levels were detected by semi-quantitative reverse transcription-PCR in the three CRC cell lines treated with or without gefitinib. GAPDH was included as a loading control. *Right*, IGF-II mRNA was quantified by densitometry and expressed as a ratio of the GAPDH mRNA, respectively. **a**–**c**. *Columns*, averages of triplicate values; *bars*, SD. **a** $^{**}P<0.01$ for comparisons with Lovo, HT29 or SW480 cells. **b**, **c** $^{**}P<0.01$ for comparisons with control

the most sensitive cell line (Lovo) than in the moderately sensitive ones (HT29 and SW480). This protein was present at strikingly high levels in all resistant cell lines (HCT116, LS174T, and SW620).

We next selected three representative cancer cell lines, one sensitive (Lovo), one moderately sensitive (HT29), and one resistant (HCT116) to gefitinib, to see if IGFR-1β expression or phosphorylation would correlate with the antiproliferative effects when the cells were treated with gefitinib. As shown by Western blot analysis (Fig. 1b), compared with the basal levels, no obvious changes were detected in IGFR-1β expression in all three cell lines, and the level of p-IGFR-1β remained undetectable in Lovo cells and remarkably high in HCT116 cells. Surprisingly, we observed a gefitinib-induced and dose-dependent increase in p-IGFR-1β expression in HT29 cells. Together, the findings further confirmed that IGFR-1β phosphorylation correlated with the responses of CRC cells to gefitinib.

Because IGFR-1 activation can occur through binding of its ligands IGF-I/II and plays an important role in many courses of tumor progression,[39,40] we measured the levels of IGF-II mRNA expression in Lovo, HT29, and HCT116 cells using semi-quantitative RT-PCR (Fig. 1c). As expected, a marked increase in IGF-II mRNA expression of HT29 cells was seen after challenge with gefitinib, whereas IGF-II expression in Lovo and HCT116 cells remained at basal levels. This result seems to give us a reasonable explanation for the ability of HT29 cells to enhance IGFR-1β phosphorylation without increasing its expression following gefitinib treatment. In brief, the gefitinib-resistant CRC cells possessed much higher basal levels of phosphorylated IGFR-1β than sensitive cell lines did, and the moderately sensitive cells displayed elevated

IGFR-1β phosphorylation that may result from an IGF-II increase induced by gefitinib, while the most sensitive cells maintained low phosphorylation regardless of EGFR inhibition. Overall, we conclude that the antiproliferative effects on CRC cells of gefitinib may correlate with IGFR-1β phosphorylation.

Cytostatic Effects of the Blockade of IGFR-1 Activation on CRC Cells

To determine whether IGFR-1β inhibition could efficiently block the activation of Akt or MAPK, we treated the representative cancer cell lines with AG1024, an IGFR-1 TKI,[41] with or without gefitinib, and assayed the EGFR and IGFR-1 signaling pathways using Western blot analysis (Fig. 2a). Both Akt and MAPK phosphorylation were effectively decreased in Lovo cells following gefitinib addition but maintained following AG1024 alone (Fig. 2a, left). Either of the two agents alone could not significantly reduce the phosphorylation of Akt or MAPK in HT29 cells, while the combination of both resulted in substantial inhibition of the downstream pathways (Fig. 2a, middle). Interestingly, addition of AG1024 alone could induce a positive effect on HCT116 cells, and the combination treatment did not seem to produce synergistic inhibition (Fig. 2a, right). This suggests that maintenance of activated Akt or MAPK in gefitinib-unresponsive CRC cells could be inhibited by blocking IGFR-1 activation in combination with or even substituting for gefitinib.

We next observed cell proliferation levels in the three cancer cell lines treated with AG1024 alone or in combination with gefitinib by the MTT assay (Fig. 2b). Addition of AG1024 did not make any significant changes in the proliferation of Lovo cells, the most gefitinib-sensitive cell line (control versus AG1024 group or gefitinib versus combination group, $P>0.05$). AG1024 addition alone only slightly decreased the proliferation of HT29 cells (control versus AG1024 group), the moderately gefitinib-sensitive line, but combined treatment for the same duration led to a significant decrease (combination versus the other groups, $P<0.05$). AG1024 addition alone, however, efficiently suppressed the proliferation of HCT116 cells (control versus AG1024 group, $P<0.05$), the most gefitinib-resistant line, and the combination did not result in a synergistic effect (AG1024 versus combination group, $P>0.05$).

Flow cytometry analysis was used to evaluate effects on cell cycle distribution and apoptosis for the three cancer cell lines treated with AG1024, gefitinib, or both for 3 days. Consistent with the results of the cell proliferation assay above, no obvious changes were found in cell cycle progression or apoptosis of Lovo cells with addition of AG1024 (Fig. 3a, left). As expected, AG1024 combined with gefitinib treatment exerted a cytostatic effect on HT29 cells with the percentage of G1 phase population increasing from (62.40±5.15%) to (81.39±1.66%; $P<0.05$). In contrast, no significant changes occurred using either of the two agents alone (Fig. 3b, left). A marked cytostatic effect was also found in HCT116 cells treated with AG1024, whose percentage of G1 phase population was (70.28±5.78%) compared with the control (51.33±4.02%; $P<0.05$); however, a further increase was not observed for the combination treatment compared with AG1024 alone (Fig. 3c, left). Moreover, AG1024 treatment had similar effects on apoptosis as measured by flow cytometry analysis via FITC–PI double staining (Fig. 3, right). Taken together, these observations indicate that AG1024 can lead to cytostatic effects on CRC cells by inducing cell cycle G1 phase arrest and increasing apoptosis, so administration of AG1024 to block IGFR-1β activation might be an alternative avenue to overcome resistance to gefitinib.

Heterodimerization of EGFR and IGFR-1β Constitutively Activates the IGFR-1β Signaling Pathway That Mediates Resistance of CRC Cells to Gefitinib

Based on evidence demonstrating formation of heterologous receptors by physical association,[42] we examined whether the heterodimerization of EGFR and IGFR-1β could be induced in the three cell lines following treatment with gefitinib using coimmunoprecipitation with an EGFR antibody (Fig. 4a). All control groups of the three cell lines were immunoprecipitated with preimmune serum and showed no IGFR-1β immunoreactive bands, controlling for false positive results. EGFR immunoprecipitates from Lovo cells failed to display an IGFR-1β band with or without gefitinib treatment, whereas HCT116 cells displayed a band without treatment that became more obvious with the treatment. An IGFR-1β band was apparent in gefitinib-treated HT29 cells but not in untreated cells. These findings suggest that the interaction of EGFR and IGFR-1β can enhance IGFR-1β phosphorylation and then constitutively activate their common downstream pathways.

To further test whether IGFR-1β transactivation maintains Akt and MAPK activation to protect cells from EGFR inhibition, we next determined Akt and MAPK immunoreactive bands in the EGFR immunoprecipitates from the three cell lines treated with gefitinib, AG1024, or both (Fig. 4b). As expected, decreases in Akt and MAPK binding were found in Lovo cells treated with gefitinib, in HT29 cells treated with both molecules, and in HCT116 cells treated with AG1024. Therefore, we conclude that the resulting crosstalk from EGFR/IGFR-1β heterodimerization and constitutively activating IGFR-1β signaling via the Akt and MAPK pathways may contribute to gefitinib resistance of CRC cells.

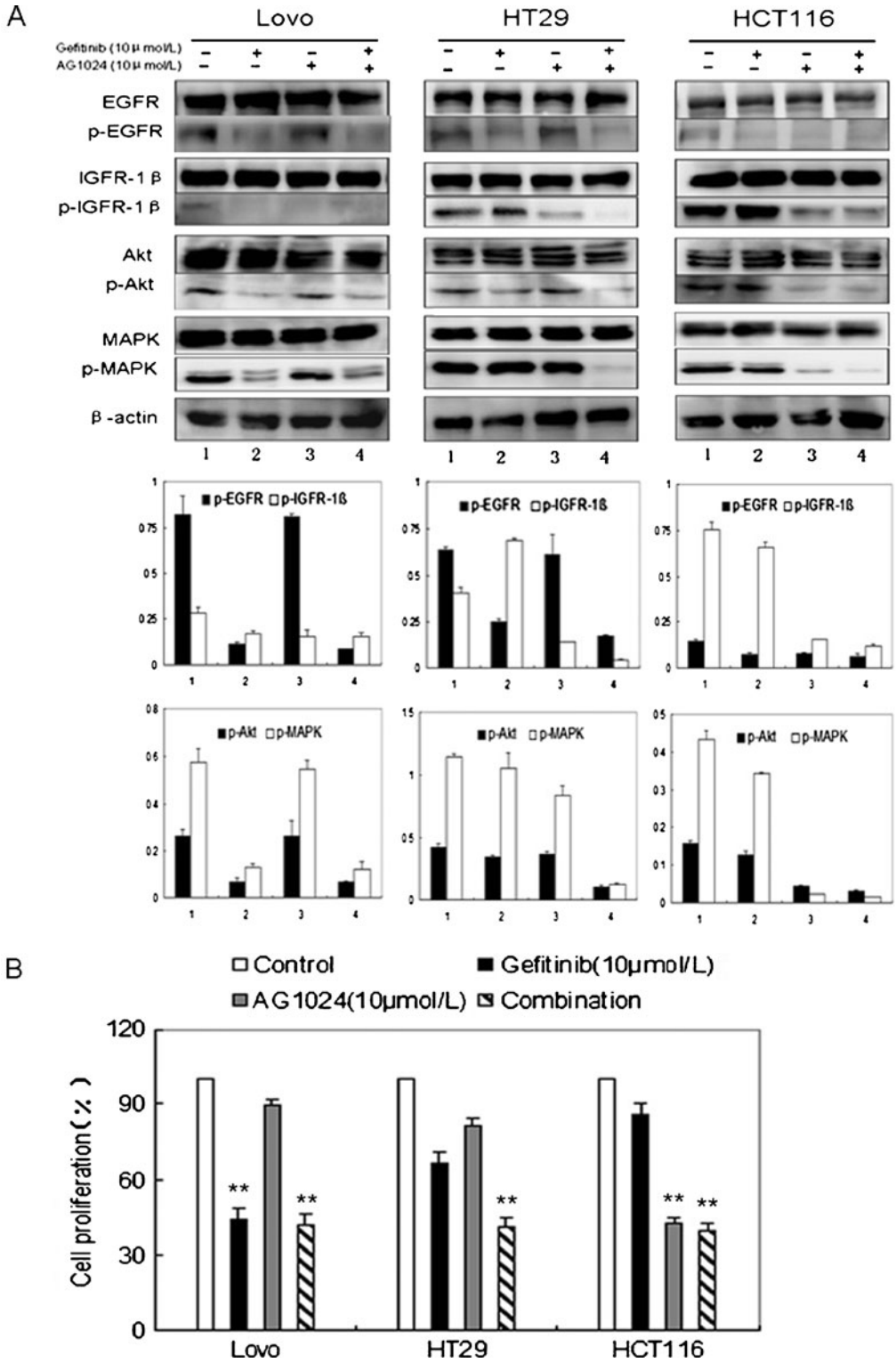

Fig. 2 **a** Western blot analysis showing levels of EGFR, IGFR-1β, Akt, and MAPK and their phosphorylated forms in Lovo, HT29, and HCT116 cells treated with 10 μmol/L gefitinib, 10 μmol/L AG1024, or both for 3 h. β-Actin was included as a loading control. *Bottom*, graphic representation of gray scale densities for the phosphorylated proteins after normalization using β-actin levels: *left*, Lovo; *middle*, HT29; *right*, HCT116. *Columns*, averages of triplicate values; *bars*, SD. **b** Effect of targeting EGFR and IGFR-1 on cell proliferation detected by MTT assay. The three cell lines were treated with gefitinib, AG1024, or both in medium supplemented with 10% FBS for 72 h, and equivalent DMSO addition but no drug as control. The results of one representative experiment are shown (independent experiments were repeated thrice for each cell line). *Columns*, mean value of four replicates; *bars*, upper 95% CIs. $^{**}P<0.01$ for comparisons with control

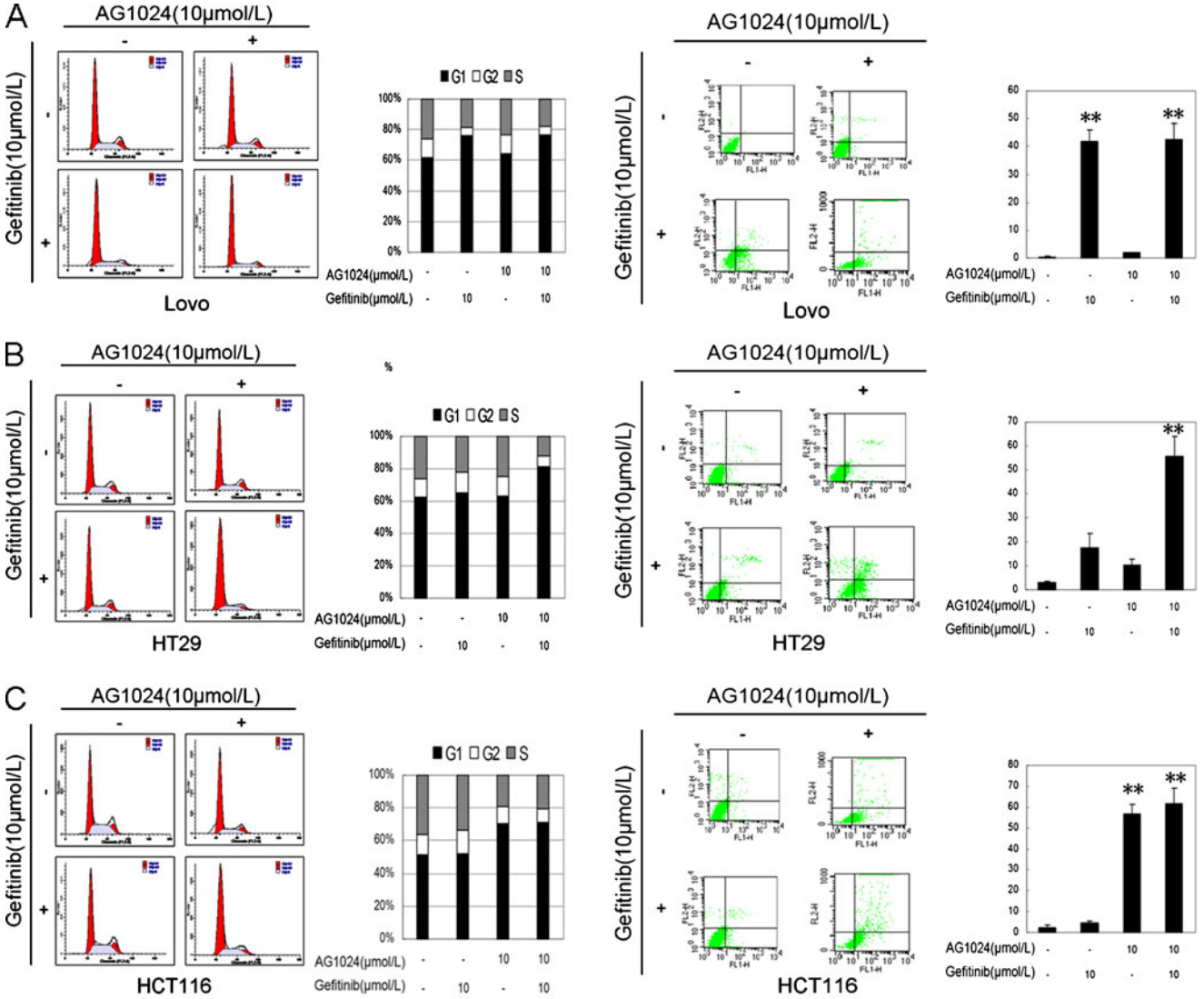

Fig. 3 Cytostatic effects of treatment with 10 μmol/L gefitinib, 10 μmol/L AG1024, or both for 72 h on cell cycle (*left*) and apoptosis (*right*) using flow cytometric analysis: **a** Lovo cells, **b** HT29 cells, **c** HCT116 cells. *Left*, fluorescence-based cell cycle sort analysis using propidium iodide staining. One representative experiment is shown; next to it is a graphic representation of the percentage of cell cycle phases G1, G2, and S. *Columns*, mean values of three independent experiments. *Right*, Annexin V-FITC/PI double-labeled apoptosis assay. A single representative experiment of flow cytometric histograms is shown; next to it is a graphic representation of the percentage of apoptosis. *Columns*, mean values of three independent experiments; *bars*, SD. $^{**}P<0.01$ for comparisons with control

Role of GSK-3β Activation in Gefitinib and/or AG1024 Induced Cytostasis

The data above demonstrated that effective inhibition of Akt and MAPK phosphorylation play a crucial role in the induction of cytostatic effects, suggesting that a common downstream signaling mediator of Akt and MAPK could reflect the activation status, such as GSK-3β. GSK-3β is a multifunctional serine/threonine kinase that regulates various cellular signaling pathways that depend on its substrates for phosphorylation.[43,44] GSK-3β inactivation has been implicated in growth factor-stimulated PI3K/Akt or Ras/MAPK signal transduction.[45,46] Because phosphorylation on the Y216 residue of GSK-3β is responsible for increased activity of the kinase, we measured the levels of its activated form in the CRC cell lines treated with or without gefitinib using Western blot analysis. As shown in Fig. 5a, the level of activated GSK-3β markedly increased in Lovo cells after treatment with gefitinib; in contrast, no obvious changes were detected in either HT29 or HCT116 cells. We also obtained negative results in SW480, LS174T, and SW620 cells treated with even higher doses of gefitinib (Fig. 5b). We next investigated whether weakly gefitinib-responsive cells (HT29 and HCT116 cells) have similar effects on the activation of GSK-3β after the inhibition of IGFR-1β (Fig. 5c). As expected, HT29 cells showed a

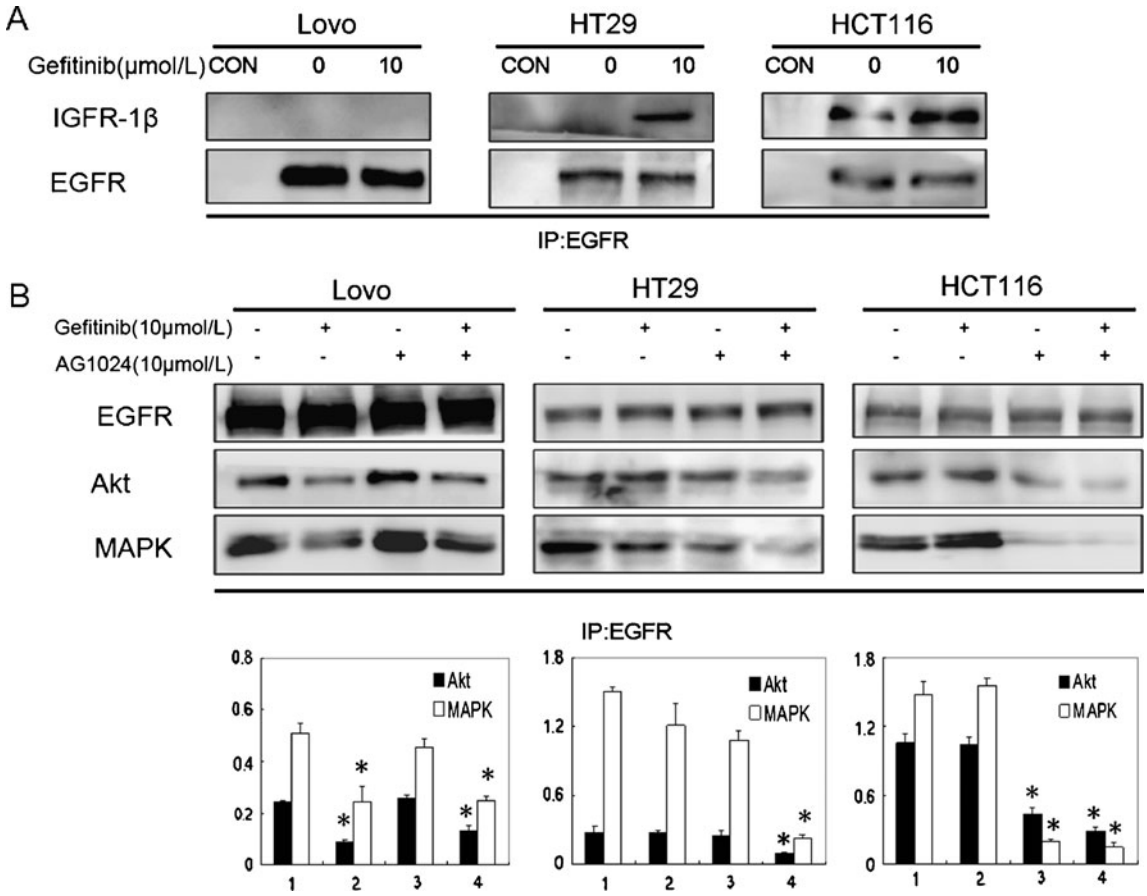

Fig. 4 **a** The anti-EGFR immunoprecipitates subjected to Western blot analysis showing heterodimerization between EGFR and IGFR-1β in Lovo, HT29, and HCT116 cells treated with or without 10 μmol/L gefitinib for 3 h. Cell lysate immunoprecipitated with preimmune serum is included as control. **b** The anti-EGFR immunoprecipitates subjected to Western blot analysis showing phosphorylation of Akt and MAPK in Lovo, HT29, and HCT116 cells treated with 10 μmol/L gefitinib, 10 μmol/L AG1024, or both for 3 h. *Bottom*, phosphorylated Akt or MAPK was quantified by densitometry and expressed as a ratio of the EGFR protein, respectively. *Left*, Lovo; *middle*, HT29; *right*, HCT116. *Columns*, averages of triplicate values; *bars*, SD. *$P<0.05$ for comparisons with control

marked increase in GSK-3β activation after treatment with the combination of gefitinib and AG1024, as did HCT116 cells after AG1024 treatment. These findings further suggest that GSK-3β is one of the downstream signals associated with Akt and MAPK and suggest that GSK-3β activation might reflect the cytostasis induced by gefitinib and/or AG1024 in the CRC cells.

We then determined the levels of cell proliferation in different gefitinib-sensitive cell lines in the presence or absence of LiCl, a potent GSK-3β inhibitor, which results in the loss of GSK-3β function.[47] As shown by the MTT assay, cell proliferation in gefitinib-treated Lovo cells was rescued by the addition of LiCl in a dose-dependent manner (Fig. 5d, left), and similar effects were also observed with the combination-treated HT29 cells (Fig. 5d, middle) and the AG1024-treated HCT116 cells (Fig. 5d, right). Overall, we conclude that the modulation of GSK-3β activation can affect the cytostasis induced by gefitinib and/or AG1024.

Because GSK-3β activation is linked with specific target recognition in well-defined cellular locations,[17,48] laser-scanning microscopy based on immunofluorescence staining for phosphorylated GSK-3β on residue Y216 was done in the CRC cells treat with gefitinib, AG1024, or both. As shown by confocal analysis, a significant increase in GSK-3β activation was found in the Lovo cells treated with gefitinib (Fig. 6a), HT29 cells treated with the combination (Fig. 6b), and HCT116 cells treated with AG1024 (Fig. 6c), compared to their non-treated control groups. Remarkably, the activated GSK-3β displayed intranuclear accumulation when the cells were treated with the corresponding agents, implying that the redistribution of activated GSK-3β may trigger certain nuclear protein kinases to regulate the cell cycle and apoptosis. Together, these findings demonstrate that gefitinib and/or AG1024 induced cytostasis could be mediated by GSK-3β activation.

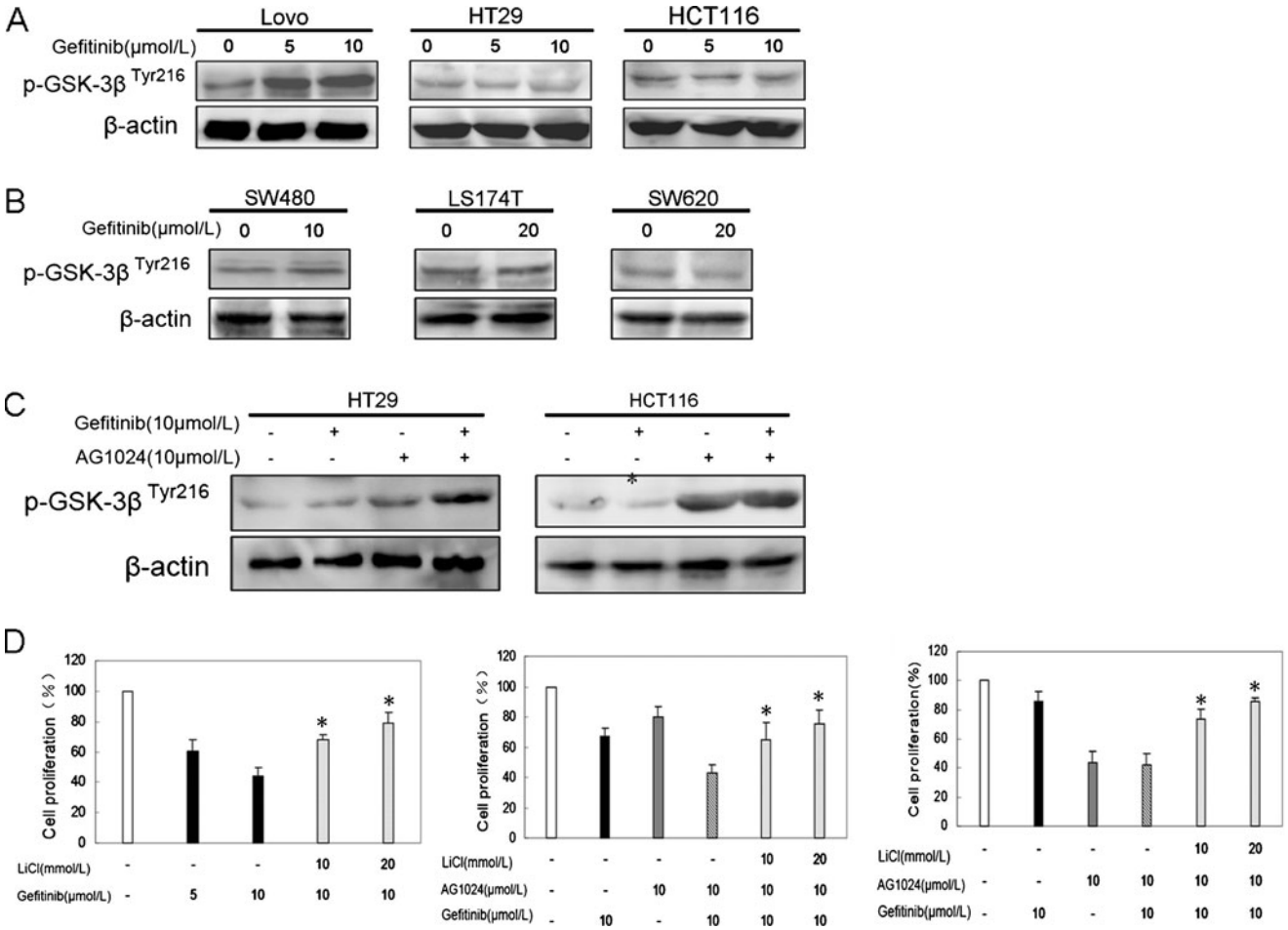

Fig. 5 **a** Western blot analysis showing levels of GSK-3β phosphorylation on residue Y216 (activated site) in Lovo, HT29, and HCT116 cells treated with or without the indicated concentrations of gefitinib for 3 h. β-actin was used as a loading control. **b** Western blot analysis showing levels of GSK-3β phosphorylation on residue Y216 in SW480, LS174T, and SW620 cells treated with or without the indicated concentrations of gefitinib for 3 h. β-Actin levels confirm equal protein loading. **c** Western blot analysis showing levels of GSK-3β phosphorylation on residue Y216 in HT29 and HCT116 cells treated with gefitinib, AG1024, or both for 3 h. β-Actin levels confirm equal protein loading. **d** Effects of GSK-3β inhibition on cell proliferation detected by MTT assay. Lovo (*left*), HT29 (*middle*), and HCT116 (*right*) cells were treated with gefitinib, AG1024, or both for 72 h with or without pretreatment with the indicated LiCl concentrations. The results of one representative experiment are shown (independent experiments were repeated thrice for each cell line). *Columns*, mean value of four replicates; *bars*, upper 95% CIs. Lovo cells, $*P<0.05$ for comparisons with gefitinib group. HT29 cells, $*P<0.05$ for comparisons with combination group. HCT116 cells, $*P<0.05$ for comparisons with AG1024 group

Expression of Phosphorylated EGFR or IGFR-1β Are Significantly Increased in CRC Tumor Tissue

Our data have shown that IGFR-1β phosphorylation correlates with the sensitivity of CRC cells to gefitinib; therefore, we determined the levels of EGFR and IGFR-1β phosphorylation in 18 CRC specimens by Western blot analysis (Fig. 7, top). The level of protein expression in the tumor tissue (T) was considered significant if it was statistically higher than that of paired normal tissue (N), which was shown as a ratio of T/N for EGFR, p-EGFR, and p-IGFR-1β expression (Fig. 7, bottom). As the data shown in Table 1, 33.3% (6 of 18) of the tumor specimens had higher levels of p-EGFR expression than did paired normal tissue ($P<0.05$), and 44.4% (8 of 18) of those specimens also had higher levels of p-IGFR-1β than the normal tissue ($P<0.05$). EGFR expression was not correlated with p-EGFR ($r=0.228$, $P>0.05$), nor did p-EGFR and p-IGFR-1β expression correlate ($r=-0.127$, $P>0.05$). These results suggest that the levels of phosphorylated EGFR and IGFR-1β are significantly increased in tumor tissue, which might be independently involved in tumor formation and progression of CRC.

Discussion

It is well-known that cancer cells are extremely adaptable or plastic in their ability to utilize multiple signaling pathways and to activate alternative ones to maintain cell survival and

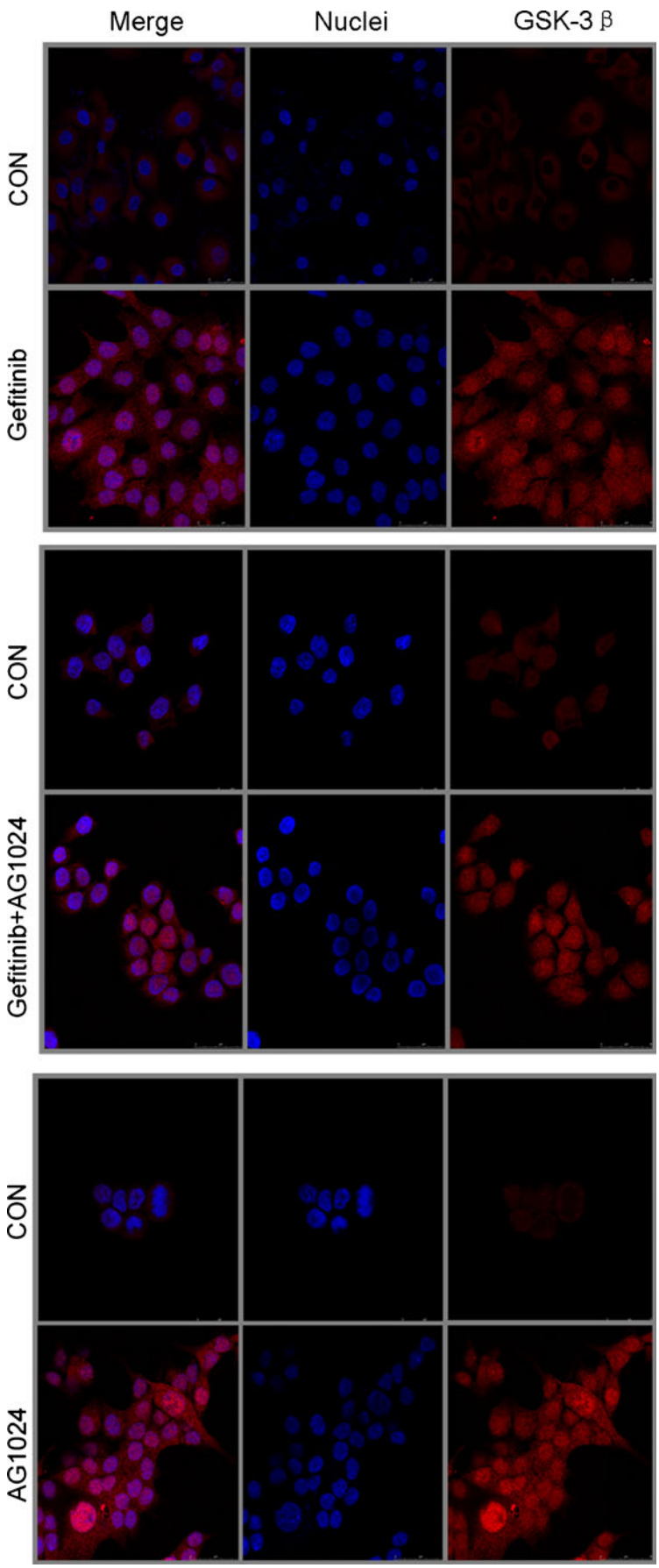

Fig. 6 Immunofluorescence staining for GSK-3β phosphorylation on residue Y216 (*red*) and nuclei (*blue*) of CRC cells treated with 10 μmol/L gefitinib, 10 μmol/L AG1024, or both for 3 h, compared with untreated controls. *Top*, Lovo. *Middle*, HT29. *Bottom*, HCT116. Two independent experiments were done; the confocal images shown are from a single representative experiment

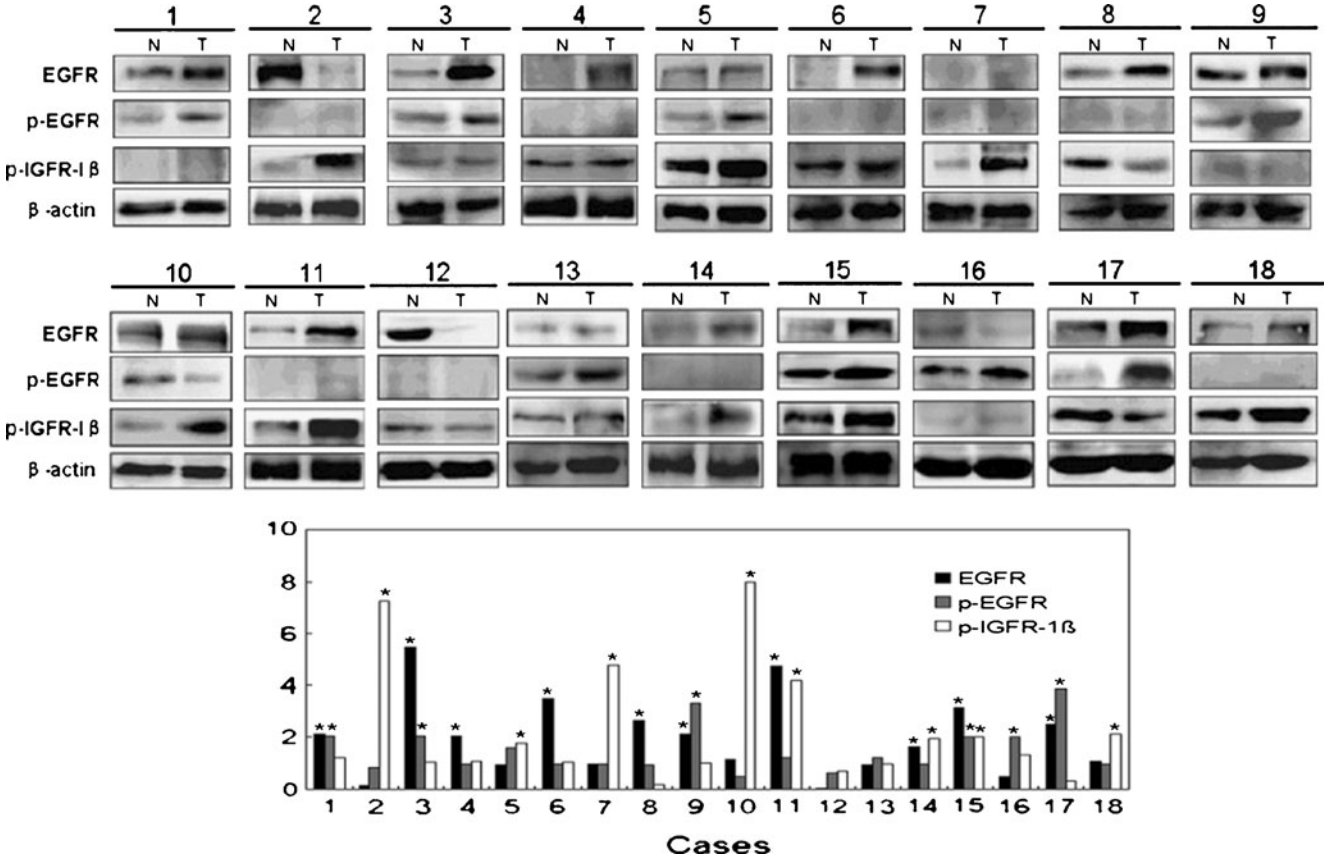

Fig. 7 Western blot analysis showing levels of EGFR, p-EGFR, and p-IGFR-1β expression in CRC tumor (*T*) and paired normal tissue (*N*) specimens. β-Actin was used as a loading control. *Bottom*, graphic representation of gray scale densities for the three proteins after normalization using β-actin levels, and the data are shown as a ratio of T/N. **P*<0.05 is considered higher expression for comparison with paired normal tissue

proliferation when the dominant pathway is blocked.[42] We have found that the response of CRC cells to EGFR inhibitory treatment (gefitinib) is defined by the dependence of cell growth on EGFR signaling and also identified that expression or activation of EGFR is not entirely predictive of sensitivity. In this study, we demonstrate that phosphorylated IGFR-1β can compensate for the loss of EGFR function by maintaining activation of common downstream signaling mediators in CRC cells (Fig. 8).

Initially, we characterized the association of gefitinib responses of a panel of CRC cell lines with basal expression or phosphorylation of MET and IGFR-1β, which have been reported to be involved in resistance to gefitinib in NSCLC.[33,34] We found that the high basal levels of IGFR-1β phosphorylation, but not its expression, correlated with the resistance to gefitinib and that neither MET expression nor its phosphorylation did. Further analysis of one representative highly sensitive (Lovo), moderately sensitive (HT29), and most resistant (HCT116) cell line treated with gefitinib revealed that a high level of IGFR-1β phosphorylation was maintained in the HCT116 cells and was undetectable in the Lovo cells and that elevated IGFR-1β phosphorylation was induced by gefitinib in the HT29 cells, which might make the cells less

Table 1 The expression levels of EGFR, p-EGFR, and p-IGFR-1β in CRC tumor and normal tissue

Type	Case	EGFR expression		p-EGFR expression*		p-IGFR-1β expression*	
		Higher	Lower	Higher	Lower	Higher	Lower
Tumor tissue	18	10 (55.6%)	8 (44.4%)	6 (33.3%)	12 (66.7%)	8 (44.4%)	10 (55.6%)
Normal tissue	18	3 (37.5%)	15 (62.5%)	1 (5.6%)	17 (94.4%)	2 (11.1%)	16 (88.9%)

**P*<0.05 is considered higher expression for comparison with normal tissue

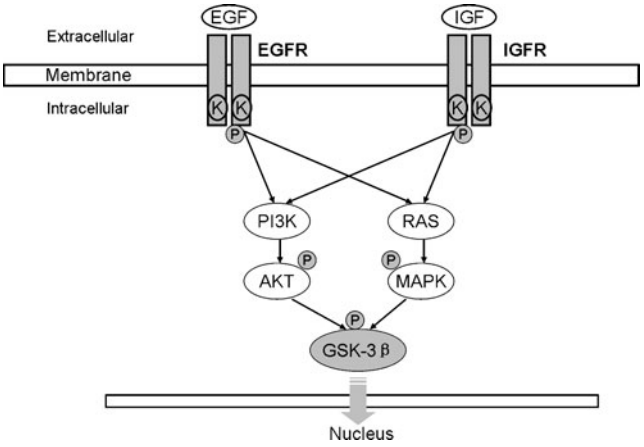

Fig. 8 Signal transduction pathways controlled by EGFR and IGFR. IGFR phosphorylation can compensate for loss of EGFR signaling function and constitutively activates the common downstream pathways. *K* kinase domains, *P* phosphate

sensitive than Lovo cells. Interestingly, we also identified increased IGF-II mRNA expression that was consistent with IGFR-1β phosphorylation in the HT29 cells, suggesting the presence of an autocrine growth factor loop for its activity.

As a member of the type II RTK family, IGFR-1 is a heterotetramer with tyrosine kinase activity and is composed of two extracellular α subunits containing the ligand-binding site and two transmembrane β subunits harboring the intracellular tyrosine kinase activity and has been reported to play a role in cancer development and progression, including CRC.[37–40,49–51] Interestingly, although a major high molecular mass receptor corresponding to unprocessed α/β pro-IGFR-1 was expressed in some cancer cells, the receptor was unable to induce intracellular signals, such as β-subunit tyrosine autophosphorylation; however, a small amount of successfully processed α/β heterodimers could produced the signaling, indicating that residual endoproteolytic processing of IGFR-1 as a posttranslational event might be crucial for its ligand binding and signaling activities.[52] Associated with our observation, it seems that the measurement of the IGFR-1β phosphorylation status is much more promising than its expression for the assessment of this receptor as a fundamental biomarker for response to anti-EGFR therapy. Similarly, another study has demonstrated that a further reduction in IGFR-1 expression but an elevation in phosphorylation was induced in a derived dually tamoxifen-/gefitinib-resistant breast cancer variant that showed an enhanced dependence on IGFR-1 signaling.[53]

It is established that IGFR-1 can activate many of the same downstream pathways as EGFR by regulating two major signaling routes: the PI3K/Akt pathway and the Ras/MAPK pathway.[33,54] To support our model, we observed regulation of the two major downstream pathway activities and cytostatic effects in different gefitinib-responsive CRC cell lines upon administration of the IGFR-1 tyrosine kinase inhibitor AG1024 alone or together with gefitinib. AG1024 released the sustained Akt and MAPK activation in the HCT116 cells, indicating that the IGFR-1-triggered signaling, but not EGFR kinase activity, dominates in cell proliferation and survival. Cytostasis emerged in the HT29 cells only when co-targeting EGFR with IGFR-1, suggesting that both of these receptors contributed to maintaining the proliferative state. Most strikingly, we found that neither Lovo nor HCT116 cells displayed synergistic effects of co-targeting treatment on cell proliferation, cell cycle G1 phase arrest, or apoptotic activity. Other recent publications also showed that the resistance or sensitivity of certain cancer cells to gefitinib might be determined, at least in part, by their ability to activate IGFR-1-mediated signaling.[33,53,55]

It would be interesting to find a series of mechanisms by which cancer cells switch to the IGFR-1 pathway when following inhibition of EGFR signaling, which ultimately converges on the machinery of cell cycle and apoptosis regulation. Adding another layer of intricacy to an already complicated system of growth factor receptor pathway switching is the fact that, although it is well established that members of the type I RTK EGFR family (erbB/HER 1–4) readily form heterodimers with each other and members of the type II RTK family, such as the IGFR-1, it is now becoming apparent that receptors from these heterologous families can also form physical associations with each other.[42] Furthermore, such interactions between different families of receptors may be integral to the regulation of their signaling in normal and cancer cells, as well as for drug-induced receptor crosstalk.[17,33,42,56] To address this issue, we determined the interactions of EGFR and IGFR-1β using coimmunoprecipitation and found that crosstalk between the two receptors is implicated in the development of gefitinib resistance in CRC cells. At first, the heterodimerization between IGFR-1β and EGFR was found in the gefitinib-treated or untreated HCT116 cells and treated HT29 cells, but not in the Lovo cells. Further investigation revealed that the association between the growth factor receptors and Akt or MAPK could be disrupted in the HCT116 cells by addition of AG1024 and in the HT29 cells by the co-targeting treatment. Therefore, we suggest that coactivation of EGFR containing heterodimeric receptors, in our case, IGFR-1β, may elevate the ability of IGFR-1β to mediate resistance to gefitinib through the constitutive activation of Akt and MAPK signaling that is necessary for cell proliferation and survival.

GSK-3β was initially described as an enzyme for glycogen metabolism, but now it is known to be involved in diverse biologic processes including regulating the cell cycle[17,57] and apoptosis[58,59] through the phosphorylation of a broad range of substrates.[60] Indeed, accumulation of active GSK-3β in nucleus was induced by several apoptotic

stimuli[59] or anti-tumorigenic agents,[17,61] contributed to p53-mediated p21 induction and caspase-3 activation[62] or associated with degradation of nuclear cyclin D1.[17,57] In our study, the amount of active GSK-3β and its intranuclear localization were substantially increased in the Lovo cells but not in the other cell lines following gefitinib treatment; these effects could be also induced in the HCT116 cells following AG1024 and HT29 cells following co-targeting treatment. Moreover, the inhibition of GSK-3β activity accelerated cellular proliferation even when coupled with inhibition of EGFR and/or IGFR-1, supporting the notion that GSK-3β activation is a necessary event for cytostatic effects on CRC cells.

Collectively, our results reveal that the crosstalk between EGFR and IGFR-1β signaling may be a mechanism by which CRC cells can evade the antiproliferative activity of gefitinib. Elevated IGFR-1β phosphorylation would be triggered by increased IGF-II expression induced by gefitinib or heterodimerization of EGFR and IGFR-1β, which constitutively activate downstream signals, including the Akt and MAPK pathways. Although both EGFR and IGFR-1β phosphorylation have been implicated in CRC formation and progression, the least gefitinib-responsive cells that can proliferate in an EGFR-independent manner are sensitive to a chemical IGFR-1 inhibitor, and the moderately gefitinib-responsive cells that can proliferate relying on both EGFR and IGFR-1 signaling are more sensitive to the combined treatment than either alone. It seems that effective therapeutic avenues for CRC might be obtained by determining the pattern of mitogens promoting cell proliferation through the receptor-mediated signaling of EGFR, IGFR-1, or both. In addition, measurement of GSK-3β activation may provide a further marker to evaluate the antitumor effect induced by receptor tyrosine kinase inhibition. However, as our in vitro study is limited to a small number of CRC cell lines, further investigation is needed to confirm IGFR-1β phosphorylation as a reliable pharmacodynamic marker of response to gefitinib in vivo or even in clinical trials.

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ORIGINAL ARTICLE

Initial Experience with Transanal Endoscopic Microsurgery: the Need for Understanding the Limitations

Emily Steinhagen · Gerard Chang · José G. Guillem

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Abstract
Introduction Transanal endoscopic microsurgery is an alternative to transanal excision or radical surgery for benign and carefully selected malignant rectal tumors. Advantages over transanal excision include better visualization, access to more proximal lesions, higher likelihood of negative margins, and lower recurrence rates. Compared to radical resection, patients experience lower rates of morbidity and mortality but may have higher rates of local recurrence.
Methods A review of a prospectively maintained database of patients scheduled for transanal endoscopic microsurgery was performed.
Results Ninety-three patients underwent 96 procedures for 13 carcinoid tumors, 1 submucosal mass, 46 adenomas, 12 in situ adenocarcinomas, and 21 invasive adenocarcinomas. Of these cases, 81.2% was successfully completed. There were nine complications (11.5%). Final pathology demonstrated 33 in situ and invasive adenocarcinomas. The mean follow-up was 25.9 months. The four recurrences (12.1%) occurred in: one tubulovillous adenoma, two in situ carcinomas, and one T2 lesion.
Conclusions Transanal endoscopic microsurgery is appropriate for benign lesions such as carcinoid tumors and adenomas and can also be curative in carefully selected patients with early-stage invasive rectal cancer. In cases of invasive adenocarcinoma, it should be reserved for low-risk cancers in patients who accept the possible increased risk of recurrence.

Keywords Transanal endoscopic microsurgery · Rectal cancer · Rectal adenoma · Carcinoma in situ · Carbon dioxide insufflation

Introduction

Transanal endoscopic microsurgery (TEM) offers a minimally invasive alternative to radical resection that overcomes many of the limitations associated with other local excision techniques such as poor visualization, inadequate access to the entire rectum, specimen fragmentation, and a high rate of positive margins. Indications for TEM include carcinoid tumors, adenomas, and in situ carcinomas that are proximally located and beyond the reach of a standard transanal excision. TEM also plays a role in palliating symptoms of locally advanced rectal cancer lesions with or without neoadjuvant therapy in an individual unable or unwilling to undergo a curative resection. Although technically feasible, the curative role of TEM in early-stage (T1) rectal cancer has been questioned given the high failure rates noted with any local procedure.[1] In this manuscript, we report our first 96 TEM cases and comment on indications for TEM.

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E. Steinhagen · G. Chang · J. G. Guillem (✉)
Department of Surgery, Colorectal Service,
Memorial Sloan-Kettering Cancer Center,
1275 York Avenue, C-1077, New York, NY 10065, USA
e-mail: guillemj@mskcc.org

Methods

A retrospective review of the prospectively maintained database of patients scheduled for TEM from August 2002

to May 2010 at Memorial Sloan-Kettering Cancer Center was performed. The study was approved by the Institutional Review Board. All TEM procedures were performed by a single colorectal surgeon (JGG). The database, supplemented by comprehensive chart review, was queried to determine patient and tumor characteristics, surgical treatment, pathology, neoadjuvant and adjuvant therapy, and oncologic outcome. When TEM was planned but not completed, the reason was documented.

Patients were prepared with a standard bowel preparation consisting of mechanical cleansing and oral antibiotics. Following general endotracheal or spinal epidural anesthesia, the patient was positioned in the lithotomy, lateral decubitus, or prone position so that the lesion would be in the inferior aspect of the operating field. After rectal washout, the anus was gently digitally dilated to allow a 4-fingerbreadth insertion. The TEM operating proctoscope was then gently inserted to the level of the lesion and secured in place with a mechanical arm fixed to the operating table. The airtight cap was applied to the proctoscope, and the rectum was distended with constant carbon dioxide insufflation to a pressure of 10 to 15 mmHg. The procedure was performed with the Richard Wolf (Knittlingen, Germany) TEM equipment.

With the use of a monopolar fine-tip cautery, a 1-cm margin around the lesion was demarcated. This circumferential demarcation was continued to the level of the perirectal fat. Then, the harmonic scalpel was used to excise the lesion. In our experience, dissection with the harmonic scalpel provides for more effective hemostasis while limiting smoke production that can impair the operative field of view as occurs with conventional monopolar electrocautery.[2] After the tumor was excised, the specimen was pinned to a corkboard and oriented for the pathologist. The rectal defect was closed with a running 3-0 polydioxanone suture approximately 6 cm in length. The stitch was secured in place with silver shots at the beginning and end of the closure. In most cases, the patient resumed intake of clear liquids the evening of the procedure. Pain control was achieved with oral analgesics. Patients were seen in the office for proctoscopic examination approximately 4 weeks after the procedure. For malignant cases, prospective follow-up continued every 3 months for the first 2 years, every 6 months for years 3 and 4, and yearly thereafter. Proctoscopy was performed at each follow-up visit. CEA was drawn, and CT scan of the chest, abdomen, and pelvis was performed on an annual basis. An endorectal ultrasound was performed only if an abnormality was noted on physical exam or proctoscopy.

Results

Ninety-three patients who underwent 96 procedures were included in this study. There were 53 men. The mean age was 63.3 years (range, 21.1–90.6 years). The mean follow-up was 25.9 months (adenoma, 20.4 months; carcinoid, 20.2 months; and adenocarcinoma, 32.8 months).

The average tumor size was 2.87 cm (0.7–10.0 cm), and the mean distance from the anal verge was 8.58 cm (3.0–15.0 cm). The indications for surgery included 13 carcinoids, 1 submucosal mass, 46 adenomas, 12 in situ adenocarcinomas, and 21 invasive adenocarcinomas. Thirty-three patients had an endorectal ultrasound before the procedure. TEM was completed in 78 cases (81.2%; Fig. 1). Five cases were aborted for the following reasons: two equipment malfunctions, one fecal impaction, and two anatomic limitations precluding the safe completion of TEM in patients who were unfit for major abdominal surgery. Thirteen cases were converted to a different procedure. Five of these cases were converted to transanal excision because of distal lesions and the inability to maintain insufflation with the TEM scope, and eight were converted to low anterior resection due to anatomic limitations prohibiting proximal passage of the TEM scope such as a narrow rectal lumen or pelvis or a large prostate.

There were nine complications in patients who underwent TEM (11.5%). Postoperative rectal bleeding occurred in four cases. All were treated conservatively, and none required transfusion or surgical intervention. Two patients experienced self-limited fevers. One patient experienced asymptomatic heart block that resolved without intervention. There was one case of urinary retention treated with foley catheter and one urinary tract infection treated with antibiotics.

The operative pathology in the 78 completed TEM cases demonstrated 19 instances of no residual lesion, 5 carcinoids, 31 adenomas, 5 in situ adenocarcinomas, and 18 invasive adenocarcinomas (12 T1, 5 T2, 1 T3; Table 1). The pre-TEM diagnoses for the 19 cases without residual pathology on TEM were: 2 adenomas, 8 carcinoids, 3 in situ adenocarcinomas, and 6 invasive adenocarcinomas. Two patients with

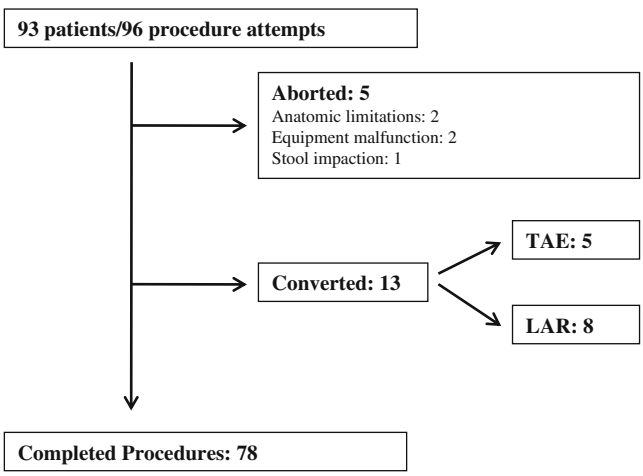

Fig. 1 TEM procedures attempted

Table 1 Biopsy and operative pathology from completed TEM cases

Biopsy	Pathology from TEM
Adenoma, 37	No residual, 2
	Adenoma, 30
	Tis, 2
	T1, 0
	T2, 3
Adenocarcinoma in situ, 12	No residual, 3
	Adenoma, 1
	Tis, 3
	T1, 4
	T2, 1
Adenocarcinoma, 16	No residual, 6
	Tis, 0
	T1, 8
	T2, 1
	T3, 1
Carcinoid, 13	No residual, 8
	Carcinoid, 5

T1 lesions underwent immediate radical surgery for high-risk histologic features, and the other ten T1 cases received no further treatment after TEM. Tumors were considered to be high risk if they demonstrated blood vessel invasion, lymphatic vessel invasion, or poor differentiation on histologic examination.[3] Of the six patients with T2 or T3 tumors, two had no further treatment due to patient preference, one had radiotherapy, two had chemoradiotherapy, and one patient underwent radical resection. Both patients who had no further treatment experienced a local recurrence, and one of the two who had chemoradiotherapy was found to have a distant recurrence.

Overall, three of 78 (3.8%) TEM cases had a positive margin. One was an adenoma, and the patient received no further treatment and has not had a recurrence. One was a tubular adenoma with in situ adenocarcinoma that recurred at 4.2 months and was successfully treated with a transanal excision. The final pathology revealed a tubular adenoma with high-grade dysplasia and negative margins. The patient is alive with no evidence of disease. The third was a carcinoid tumor. The patient declined further treatment and died 3.2 years later of unknown causes.

A review of the pathological reports from the preoperative biopsy and excised specimen identified 33 in situ and invasive adenocarcinomas (9 Tis tumors, 18 T1 tumors, 5 T2 tumors, and 1 T3) treated by TEM. In these 33 cases, there were four local recurrences (12.1%) and one distant recurrence (3.1%; Table 2). The first local recurrence occurred in a tumor that was Tis on biopsy and then adenoma with no dysplasia and negative margins on final pathology. The second recurrence occurred in a Tis tumor that had dysplasia and adenoma at the excision margins. The third recurrence was in a patient with a T2 lesion who was unfit for major abdominal surgery, and the fourth was in a patient with a tubulovillous adenoma with high-grade dysplasia on preoperative biopsy who was found to have a T2 lesion on final pathology but refused further treatment at that time. The distant recurrence was in a patient with significant medical comorbidities who underwent neoadjuvant chemoradiation and TEM for a clinical T2 tumor. The pathology demonstrated a histologically low-risk T1 adenocarcinoma that was excised with negative margins. Of the patients with local recurrence, two are alive with no evidence of disease, one is dead of disease, and one dead of other causes. The 73-year-old patient with distant recurrence died of disease 4.3 years after recurrence.

Discussion

Local excision techniques for rectal cancer include transcoccygeal, transphincteric, and transanal approaches. However, in some instances, these options are limited by poor exposure and inadequate access to lesions or high complication rates. This has contributed to higher recurrence rates compared to radical resection techniques. While radical surgery for rectal cancer may provide excellent local control and long-term survival, it is associated with significant morbidity and mortality. TEM provides the surgeon access to the upper rectum and the ability to perform precise excisions with a higher rate of negative margins while gaining the benefit of extremely low mortality and low morbidity associated with local excision. Data from numerous studies have shown that TEM can be performed safely with few complications.[4,5] Our experience confirms the ability of TEM to facilitate negative margins, but also illustrates the limitations of any local excision, including TEM, in the management of early-stage rectal cancer.

TEM has lower rates of margin positivity (0–15%) when compared with other local excision techniques (16–31%) and should therefore have lower recurrence rates.[6–9] However, in several studies, the local failure rates are not uniformly low as anticipated.[7,9,10] In this study, the rate of positive margins and local recurrences were 3.8% and 12.1%, respectively. Of interest, only one of the recurrences in this study was

Table 2 Local recurrence rate by tumor stage

Stage	No. of recurrences	%
Tis	2	22
T1	0	–
T2	2	40
T3	0	–
Overall	4	12.5

associated with a positive margin, and in that case, the margin showed dysplasia and adenoma rather than carcinoma.

As with any local procedure, tumor implantation is a significant concern following TEM since the mesorectum is intentionally entered as part of the procedure. Investigations of total mesorectal excision (TME) have shown that wide circumferential margins and inclusion of intact mesorectal fascia in the specimen minimize local recurrence rates.[11–13] Although TEM can overcome some of the difficulties encountered with other local excision techniques, it nevertheless, like all other local procedures, violates the principle of maintaining fascial planes and theoretically may allow for inoculation of tumor cells into the mesorectum.

The success of TEM as a curative procedure relies on the assumption that we can properly select patients who do not have nodal involvement. Predictors of lymph node involvement include depth of tumor penetration, tumor differentiation, and the presence of lymphovascular invasion.[14] Additionally, tumor location seems to be a factor: tumors in the low rectum have been associated with a higher rate of metastatic lymph nodes and recurrences compared to tumors in the mid- and upper rectum.[15] This may account for the higher rate of recurrence after TAE compared to TEM. A previous study from our institution showed that the frequency of lymph node metastasis after radical resection of T1 lesions was 10%, though this decreased to 7% when only tumors with low-risk features were included.[3] Other studies have shown similar rates of lymphatic involvement.[15] However, low-risk tumors may also recur. A recent study from the Netherlands on 88 patients undergoing TEM with negative margins for T1 rectal cancer with close follow-up reported a local recurrence rate of 20.5%.[1] Half of these recurrences were in histopathologic low-risk tumors. Other studies of TEM for T1 lesions also report recurrence rates of 0 to 24%.[1,4,5,8,9,16,17] In this series, there were no recurrences of T1 lesions. This highlights that either the current selection criteria for TEM are not precise enough or that even the most histologically favorable tumors may recur following a TEM excision.

Despite improved imaging modalities including MRI and endorectal ultrasound (ERUS), the preoperative determination of depth of invasion and involvement of lymph nodes is less than 100% accurate. This problem is magnified in T1 lesions, which are more likely to have smaller foci of metastasis within small lymph nodes and are therefore more likely to be missed on ERUS.[18] In several studies, approximately 10% to 34% of T1 lesions treated by radical resection were found to have positive lymph nodes, and these were often small and not detectable on preoperative endorectal ultrasound or MRI.[15,19] The sensitivity and accuracy for detecting nodal metastasis by ERUS in T1 disease are 67% and 48%, respectively, with 13% of cases understaged as a result.[18] Other studies show that lesions may be understaged by preoperative evaluation in up to 44.3% of cases.[8]

Predictors of local recurrence following TEM include maximum tumor diameter, depth of invasion (T stage and submucosal depth), and the presence of lymphovascular invasion.[8,20] Nevertheless, even patients at lowest risk for recurrence can recur, as has been demonstrated in this study and others.[20] The chance of recurrence may have been significantly lower if they had undergone a radical resection initially. Comparison of TEM and TME for T1 adenocarcinoma of the rectum has shown that while TEM had lower complication rates, the local recurrence rate was 24% for TEM compared with 0% recurrences following TME.[16] However, the overall survival for the two procedures was similar, at 75% after TEM and 77% after TME, with cancer-specific survival at 90% and 87%, respectively. The survival following salvage surgery for recurrence in that study was significantly higher than reported by others following traditional local excision, in which the estimated disease-free survival following salvage surgery for recurrence ranges from 30% to 58%.[21,22] It was also higher than reported in one study evaluating outcomes for recurrences after TEM for T1 rectal cancer; namely, overall survival of 31% and cancer-related survival of 58%. In another study that included T1, T2, and T3 lesions, 47% of the patients with recurrences underwent salvage procedures with curative intent, and 62% of those patients were alive and free of disease at last follow-up.[1,5] Therefore, since most studies examining this issue are confounded by bias in patient and tumor selection, low numbers of patients, and variable follow-up, the outcome following salvage surgery for recurrence after TEM for T1 rectal cancer is not fully known at this time.

The optimal method and frequency of surveillance following TEM are unknown at this time. Despite frequent follow-up, four patients in our study had a local recurrence. Though more intensive follow-up regimens following resection of rectal cancer have been examined, the impact of such regimens on long-term survival in patients who experience recurrences varies by report.[23–26] Some authors suggest that patients who undergo more frequent surveillance and have a recurrence are more likely to undergo salvage surgery. However, this does not uniformly improve overall survival.[26,27] Studies on this issue may have limited applicability because most compare surveillance rates following radical resection. To our knowledge, there is no study that examines the optimal surveillance regimen following TEM.

Conclusion

TEM may be used as a curative procedure in patients with carcinoid tumor, adenomas, and in situ carcinomas. In carefully selected patients with early-stage invasive rectal cancer, TEM may also be curative. However, it is clear that both practitioner and patient awareness of the limitations of

TEM are essential. While most patients will be cured with a less invasive and lower-risk procedure such as TEM, it appears that the risk of recurrence is substantially higher than it is with radical resection. Some of these recurrences may be related to inoculation of tumor cells into the perirectal space. Others may be related to undiagnosed, and therefore, untreated, lymph node involvement. Therefore, since radical resection is a more definitive treatment for T1 rectal cancer, local excision procedures including TEM should be reserved for low-risk cancers in patients who accept the possibly increased risk of recurrence, the need for prolonged surveillance, and the possibility of aggressive salvage surgery.

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ORIGINAL ARTICLE

Effects of Postoperative Adjuvant Radiotherapy on Recurrence and Survival in Stage III Rectal Cancer

Jung Wook Huh · Sang Woo Lim · Hyeong Rok Kim · Young Jin Kim

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Abstract
Background The aim of this study is to evaluate whether the addition of radiotherapy to chemotherapy would enhance the benefits obtained with chemotherapy alone, in stage III rectal cancer in the era of preoperative chemoradiotherapy and total mesorectal excision (TME).
Methods From January 1999 to January 2008, 300 stage III rectal cancer patients who underwent TME were prospectively identified; a total of 46 patients who received preoperative chemoradiotherapy or did not receive adjuvant therapy were excluded. Patients who received postoperative chemotherapy alone (n = 190) and those who received postoperative chemoradiotherapy (n = 64) were compared.
Results The median follow-up period was 52 (range, 4–129) months. Patients receiving radiotherapy were younger and had a higher percentage of advanced pT category or perineural invasion than patients who did not. The estimated 5-year local recurrence-free survival rate was radiotherapy 92% with radiotherapy and 86% without. The disease-free survival rate was 55% with radiotherapy compared to 57% without and the overall survival rates were similar (63% vs. 68%, all $P > 0.05$). In patients with a positive circumferential resection margin or insufficient distal resection margin, the local recurrence-free, disease-free, and overall survival rates were unaffected by radiotherapy.
Conclusions The addition of postoperative radiotherapy to chemotherapy did not reduce the recurrence or mortality in node-positive rectal cancer when compared with chemotherapy alone. Moreover, this approach may not compensate for a positive circumferential resection margin or insufficient distal resection margin following rectal cancer surgery.

Keywords Adjuvant chemoradiotherapy · Stage III · Rectal cancer

Introduction

Postoperative chemoradiotherapy was recommended for all patients with completely resected stages II and III rectal cancer by the US National Institutes of Health Consensus Development Conference in 1990.[1,2] Preoperative chemoradiotherapy is the current, more preferred treatment modality in locally advanced rectal cancer, and this approach is associated with improved local control and disease-free survival.[3–5] Total mesorectal excision (TME) is considered as standard surgery for rectal cancer because it is associated with low local recurrence rates.[6,7] Some recent reports have examined the role of postoperative radiotherapy in patients with stages II or III rectal cancer, raising questions regarding the necessity of postoperative radiotherapy in certain conditions.[8–10] Moreover, postoperative adjuvant radiotherapy is associated with an increased risk of late complications due to its accompanying adverse reactions.[11] In the era of preoperative chemoradiotherapy and TME, the role of postoperative radiotherapy should be reevaluated.

The aim of this study was to evaluate whether the addition of radiotherapy to chemotherapy enhances the benefits obtained when compared chemotherapy alone in patients with stage III rectal cancer following TME. In

J. W. Huh · S. W. Lim · H. R. Kim · Y. J. Kim (✉)
Department of Surgery, Chonnam National University Hwasun Hospital and Medical School,
160 Ilsimri, Hwasun-eup, Hwasun-gun,
Gwangju, Jeonnam, South Korea
e-mail: jwhuh@chonnam.ac.kr

addition, we aimed to determine whether postoperative radiotherapy compensated for a positive circumferential resection margin or insufficient distal resection margin following curative surgery.

Methods

From January 1999 to January 2008, we identified 300 patients who underwent curative surgery for stage III rectal cancer in a prospectively collected database. Patients with preoperative chemoradiation ($n = 29$) and those who did not receive postoperative adjuvant therapy ($n = 17$) were excluded. Ultimately, 254 patients were eligible for this study. Patients who received postoperative adjuvant chemotherapy alone ($n = 190$) and those who received postoperative chemoradiotherapy ($n = 64$) were compared. This study was approved by the institutional review board of our hospital.

The diagnostic evaluation included a physical examination, rigid proctoscopy, colonoscopy or double-contrast barium enema, endorectal ultrasound (ERUS), abdominopelvic computed tomography (CT), pelvic magnetic resonance imaging (MRI), chest CT or X-ray, a complete blood cell count, liver function tests, and assessing the serum carcinoembryonic antigen (CEA) level. The location of the tumor was defined as the distance between the caudal margin of the tumor and the anal verge, and this was measured by digital examination and rigid proctoscopy. The carcinomas were grouped as lower rectal cancer (0–7 cm) and upper rectal cancer (8–15 cm). The cancer was staged using ERUS and/or pelvic MRI to determine the extent of local disease. Abdominopelvic CT, chest CT, or X-ray was used to determine the extent of extrapelvic disease.

Excluding those with upper rectal cancers, all patients underwent TME.[12–15] Disease staging was determined by the final pathological features according to the 6th International Union against Cancer (UICC) TNM staging system. Postoperative adjuvant treatment was dependent on the patient's general condition or compliance and the preference of the physician. Radiotherapy consisted of 45–50.4 Gy in 25–28 fractions delivered to the pelvis using a four-field box technique and concurrent chemotherapy consisting of 425 mg/m^2 of 5-fluorouracil plus 30 mg leucovorin for 5 days was given intravenously every 28 days for 6 cycles.[12,13] Of the 190 patients without radiotherapy, five chemotherapeutic regimens were used: (1) 5-FU + leucovorin (6 cycles of monthly 5-FU 425 mg/m^2 and leucovorin 30 mg/m^2 for 5 days; $n = 115$); (2) tegafur + uracil (UFT; 6 cycles of UFT 300 mg/m^2 for 28 days; $n = 47$); (3) doxifluridine (6 cycles of 600 mg/m^2 for 28 days; $n = 17$); (4) capecitabine (8 cycles of 1,250 mg/m^2 twice daily for 14 days, followed by 7 days rest at the conclusion of each cycle; $n = 7$), and (5) oxaliplatin + 5-FU + leucovorin (12 cycles of oxaliplatin 85 mg/m^2 on day 1 and leucovorin 200 mg/m^2 as a 2-h infusion on day 1, 5-FU 400 mg/m^2 as a bolus and a 600-mg/m^2 22-h infusion on days 1 and 2 bimonthly; $n = 4$).

The patients were followed up at 3-month intervals for 2 years, at 6-month intervals for the next 3 years, and annually thereafter. For patients who did not return for observation after 1 year, information was obtained via a letter or telephone call. On a semiannual basis or when a suspicion of recurrence arose, the follow-up examination included a clinical history, physical examination, serum CEA level, chest X-ray or CT, abdominopelvic CT or MRI, colonoscopy, and positron emission tomography scanning if available. The determination of recurrence was made by clinical and radiological examinations or by histological confirmation. Local recurrence was defined as recurrence within the pelvis; distant metastasis was defined as disease outside of the pelvis. The main patterns of recurrence were recorded as the first site of detectable failure during the follow-up period.

Statistical evaluation was carried out using the statistical package SPSS for Windows (Version 14.0; SPSS Inc., Chicago, IL). Differences between the two groups were tested using a Student's t test and the chi-squared test, where appropriate. Local recurrence-free survival, disease-free survival, and overall survival curves were calculated using the Kaplan–Meier method. Differences between curves were evaluated using the log-rank test. Variables with a statistical P value <0.20 in a univariate analysis were entered into a Cox model multivariate analysis. A value of $P \leq 0.05$ was deemed to be statistically significant.

Results

The 254 patients included 143 males and 111 females with a median age of 61 (range, 33–83) years. Of these, 147 patients (57.9%) had upper rectal cancers, and 107 patients (42.1%) had lower rectal cancers. The mean preoperative serum CEA level was 4.5 (0.2–167.0) ng/mL. Of the 254 patients, 218 patients (85.8%) underwent low anterior resection, 32 (12.6%) underwent abdominoperineal resection, and four (1.6%) underwent Hartmann's procedure. Sixty-seven of the 254 patients (26.4%) underwent laparoscopic-assisted rectal cancer surgery. Histologically, 134 tumors (52.8%) were moderately differentiated, 93 (36.6%) were well differentiated, 15 (5.9%) were poorly differentiated, and 12 (4.7%) were mucinous. The median numbers of resected and metastatic nodes were 13 (range, 3–45) and two (range, 1–20), respectively. Using the 6th UICC TNM staging system, 24 patients (9.4%) were classified as stage IIIA, 144 (56.7%) as stage IIIB, and 86 (33.9%) as stage IIIC. Of the 254 patients, 190 (74.8%)

Table 1 Correlation between clinicopathologic factors and the use of adjuvant radiotherapy

	No RT (n = 190)	RT (n = 64)	P value
Age, years			0.006
<60	75 (39.5)	38 (59.4)	
≥60	115 (60.5)	26 (40.6)	
Gender			0.993
Male	107 (56.3)	36 (56.2)	
Female	83 (43.7)	28 (43.8)	
Tumor size, cm			0.829
<4.5	95 (50.0)	31 (48.4)	
≥4.5	95 (50.0)	33 (51.6)	
Tumor location			0.374
Upper	113 (59.5)	34 (53.1)	
Lower	77 (40.5)	30 (46.9)	
Differentiation			0.575
Well + moderate	171 (90.0)	56 (87.5)	
Poor + mucinous	19 (10.0)	8 (12.5)	
Type of surgery			0.391
LAR	161 (84.7)	57 (89.1)	
APR + Hartmann's	29 (15.3)	7 (10.9)	
Operative method			0.001
Open	161 (84.7)	26 (40.6)	
Laparoscopic	29 (15.3)	38 (59.4)	
Distal resection margin, cm			0.052
≤1.0	12 (6.3)	9 (14.1)	
>1.0	178 (93.7)	55 (85.9)	
Circumferential resection margin, mm			0.878
≤1.0	16 (8.4)	5 (7.8)	
>1.0	174 (91.6)	59 (92.2)	
pT category			0.050
pT1	4 (2.1)	0	
pT2	24 (12.7)	3 (4.7)	
pT3	150 (78.9)	56 (87.5)	
pT4	12 (6.3)	5 (7.8)	
pN category			0.477
pN1	128 (67.4)	40 (62.5)	
pN2	62 (32.6)	24 (37.5)	
No. of lymph nodes harvested			0.426
<12	79 (41.6)	23 (35.9)	
≥12	111 (58.4)	41 (64.1)	
Lymphovascular invasion			0.056
Negative	145 (76.3)	41 (64.1)	
Positive	45 (23.7)	23 (35.9)	
Perineural invasion			<0.001
Negative	140 (73.7)	31 (48.4)	
Positive	50 (26.3)	33 (51.6)	
Preoperative CEA, ng/ml			0.058
<5	85 (44.7)	34 (53.1)	
≥5	74 (38.9)	27 (42.2)	
Not available	31 (16.3)	3 (4.7)	

LAR low anterior resection, *APR* abdominoperineal resection, *CEA* carcinoembryonic antigen, *RT* radiotherapy

Table 2 Postoperative complications

	No RT (n = 190)	RT (n = 64)	P value
Anastomotic leakage	15	7	
Anastomotic stenosis	3	2	
Small bowel ileus	4	1	
Radiation-induced proctitis	0	2	
Ureter leakage	1	1	
Wound infection	4	2	
Incisional hernia	1	0	
Pulmonary edema	1	1	
Voiding difficulty	7	1	
Urinary tract infection	1	0	
Sexual dysfunction	2	0	
Total	39 (20.5)	17 (26.6)	0.314

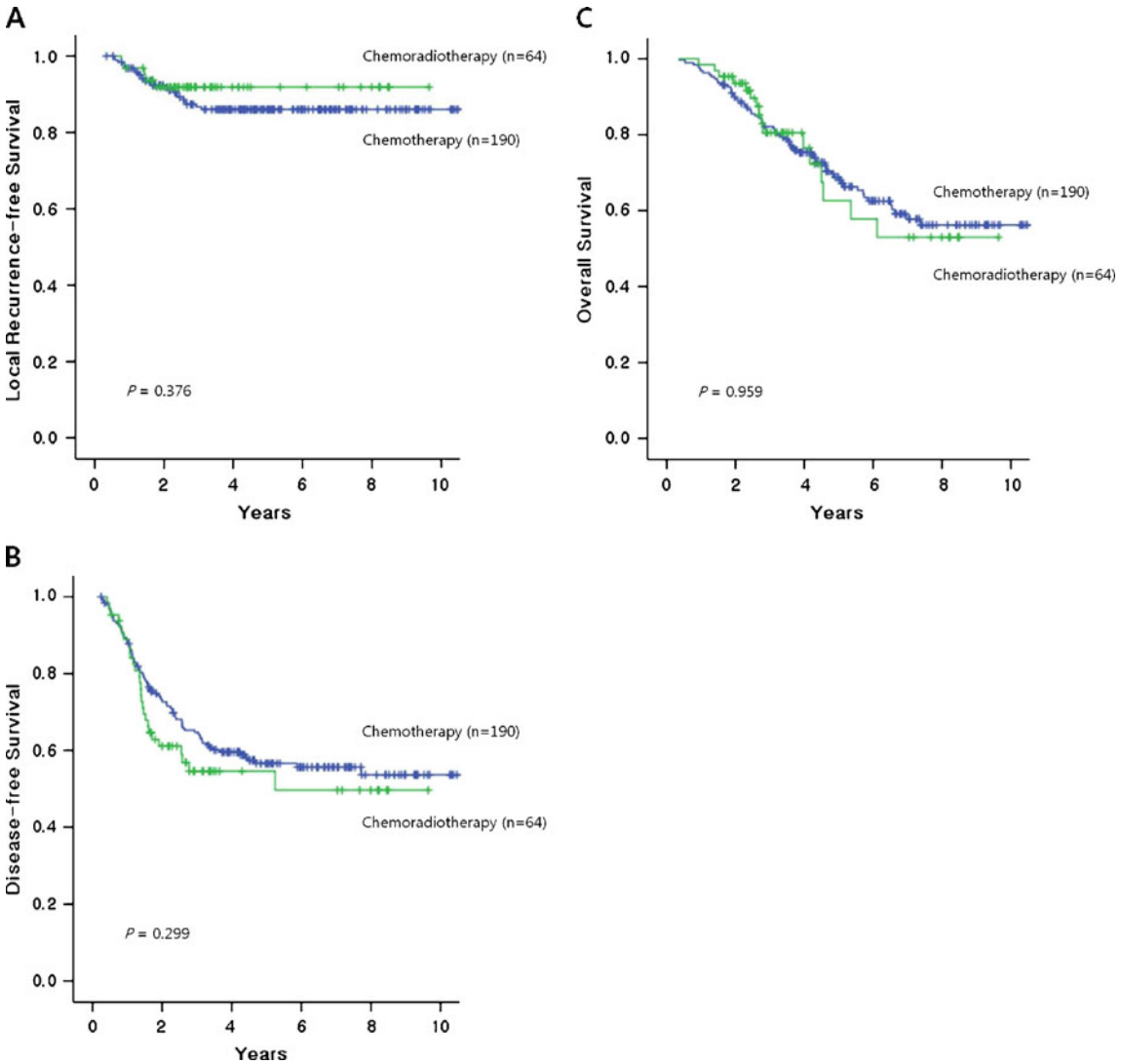

Fig. 1 Survival curves for all patients according to adjuvant therapy (n = 254). **a** Local recurrence-free survival. **b** Disease-free survival. **c** Overall survival

were treated with chemotherapy alone and 64 (25.2%) with combined chemoradiotherapy.

The patients who received radiotherapy were younger ($P = 0.006$), and they had a higher percentage of undergoing laparoscopic-assisted surgery ($P = 0.001$), advanced pT category ($P = 0.050$), or perineural invasion ($P < 0.001$) than those who did not receive radiotherapy. However, no significant differences were found in terms of age, gender, tumor size, location, differentiation, type of surgery, distal resection margin, circumferential resection margin, pN category, number of lymph nodes harvested, lymphovascular invasion, and preoperative CEA levels (Table 1). The postoperative complications related to the primary operation were similar between the patients who received radiotherapy and those who did not (26.6% vs. 20.5%, $P = 0.314$; Table 2).

During a median of 52 (range, 4–129) months of follow-up, the 5-year local recurrence-free survival rate was 92% with radiotherapy and 86% without ($P = 0.376$, Fig. 1a); disease-free survival rates were 55% with radiotherapy and 57% without ($P = 0.299$, Fig. 1b); and overall survival rates were similar at 63% vs. 68%, respectively ($P = 0.959$, Fig. 1c). When we performed a subset analysis to compare the two groups, among the pT3 patients, similar findings were also observed for the 5-year local recurrence-free survival, disease-free survival, and overall survival (Fig. 2a–c). Multivariate analyses revealed that the distal resection margin, the circumferential resection margin, and the preoperative CEA levels were the independent prognostic factors for local recurrence-free survival; the distal resection margin, the circumferential resection margin, the pN category, the perineural invasion, and the preoperative

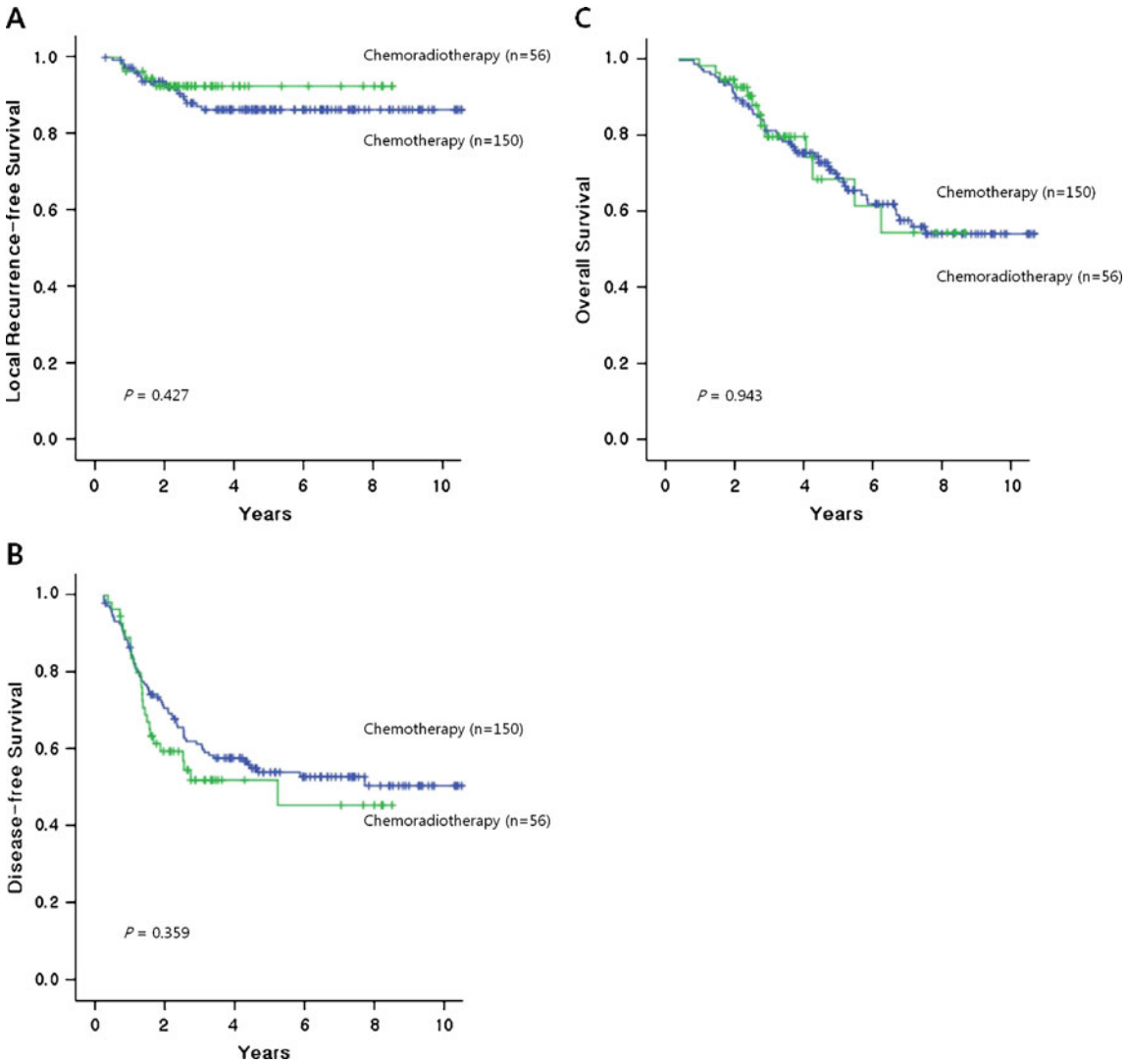

Fig. 2 Survival curves for pT3 patients according to adjuvant therapy ($n = 206$). **a** Local recurrence-free survival. **b** Disease-free survival. **c** Overall survival

CEA levels were the independent prognostic factors for disease-free survival. Additionally, the circumferential resection margin, the pN category, and the preoperative CEA levels were the independent prognostic factors for overall survival (Table 3). In the univariate and multivariate analysis, the use of adjuvant radiotherapy did not have a major prognostic value regarding the local recurrence-free survival, the disease-free survival, and the overall survival.

The prognostic significance on the survival of adjuvant radiotherapy according to the pN category and the status of the circumferential and the distal resection margins is shown in Tables 4 and 5. We found that the use of adjuvant radiotherapy had no effect on the 5-year local recurrence-free survival, disease-free survival, and overall survival rates in patients in the pN1 and the pN2 categories (Table 4). According to the status of circumferential and distal resection margins, radiotherapy had no prognostic impact on the 5-year local recurrence-free survival, disease-free survival, and overall survival rates in each subgroup (Table 5).

Discussion

The use of postoperative adjuvant radiotherapy in addition to chemotherapy is commonly believed to prolong the survival of patients with stages II and III rectal cancer, and this approach has been generally recommended.[1,2] However, the two sequential randomized studies using data from the National Surgical Adjuvant Breast and Bowel Project (NSABP) suggested that the addition of postoperative radiation therapy did not prolong disease-free and overall survival rates when compared with chemotherapy alone.[8,16] Those studies also raise the possibility that the survival benefit attributable to the combination of chemotherapy and radiotherapy from the previous Gastrointestinal Study Group 7175[2] was a consequence of the chemotherapy. We also confirmed this finding, with our results showing that the addition of radiotherapy to chemotherapy had no statistically significant advantage on the disease-free survival and overall survival rates as well as local recurrence-free survival.

Table 3 Multivariate analyses of factors for 5-year local recurrence-free survival (LRFS), disease-free survival (DFS), and overall survival (OS)

	P value	Hazards ratio (CI)
LRFS		
Tumor size (≥4.5 vs. <4.5 cm)	0.384	1.437 (0.635–3.253)
Distal resection margin (<1.0 vs. ≥1.0 cm)	0.002	4.856 (1.763–13.378)
Circumferential resection margin (<1.0 vs. ≥1.0 mm)	0.010	3.482 (1.347–9.001)
pT category (pT3 + pT4 vs. pT1 + pT2)	0.256	3.256 (0.424–24.979)
Preoperative CEA (≥5 vs. <5 ng/ml)	0.034	2.645 (1.074–6.511)
DFS		
Tumor size (≥4.5 vs. <4.5 cm)	0.122	1.390 (0.916–2.110)
Tumor location (upper vs. lower)	0.064	1.504 (0.977–2.315)
Differentiation (poor + mucious vs. well + moderate)	0.469	1.260 (0.675–2.351)
Distal resection margin (<1.0 vs. ≥1.0 cm)	<0.001	3.281 (1.723–6.248)
Circumferential resection margin (<1.0 vs. ≥1.0 mm)	<0.001	3.253 (1.742–6.072)
pT category (pT3 + pT4 vs. pT1 + pT2)	0.093	2.095 (0.885–4.958)
pN category (pN2 vs. pN1)	<0.001	2.508 (1.628–3.865)
No. of lymph nodes harvested (<12 vs. ≥12)	0.529	1.153 (0.740–1.798)
Lymphovascular invasion (positive vs. negative)	0.346	1.252 (0.784–1.997)
Perineural invasion (positive vs. negative)	0.032	1.560 (1.039–2.341)
Preoperative CEA (≥5 vs. <5 ng/ml)	0.023	1.664 (1.074–2.576)
OS		
Gender (male vs. female)	0.129	1.431 (0.901–2.273)
Tumor size (≥4.5 vs. <4.5 cm)	0.284	1.293 (0.808–2.068)
Differentiation (poor + mucious vs. well + moderate)	0.075	1.825 (0.942–3.538)
Type of surgery (APR + Hartmann's vs. LAR)	0.542	1.192 (0.678–2.096)
Operative method (open vs. laparoscopic)	0.167	1.736 (0.794–3.796)
Circumferential resection margin (<1.0 vs. ≥1.0 mm)	0.001	3.159 (1.634–6.104)
pT category (pT3 + pT4 vs. pT1 + pT2)	0.226	1.794 (0.696–4.623)
pN category (pN2 vs. pN1)	0.046	1.639 (1.009–2.663)
Lymphovascular invasion (positive vs. negative)	0.133	1.519 (0.880–2.624)
Perineural invasion (positive vs. negative)	0.265	1.325 (0.808–2.175)
Preoperative CEA (≥5 vs. <5 ng/ml)	0.026	1.774 (1.070–2.940)

LAR low anterior resection, *APR* abdominoperineal resection, *CEA* carcinoembryonic antigen

Table 4 Survival rates by the adjuvant therapy in patients with stage III tumor according to the N category (n = 254)

Factors	Number	5-year LFRS (%)	5-year DFS (%)	5-year OS (%)
pN1				
Chemotherapy	128	85	64	70
Chemoradiotherapy	40	95	70	65
P value		0.151	0.945	0.803
pN2				
Chemotherapy	62	89	41	67
Chemoradiotherapy	24	86	29	59
P value		0.643	0.131	0.843

LRFS local recurrence-free survival, *DFS* disease-free survival, *OS* overall survival

The interpretation of the benefits of postoperative radiotherapy in locoregional recurrence is still challenging. The results of the previous Gastrointestinal Tumor Study Group trial showed that the combined modality improved the local control in stages II and III rectal cancer.[17] Moreover, the data from NSABP R-02 indicated that the addition of postoperative radiotherapy to chemotherapy reduced the cumulative incidence of local relapse from 13% to 8% compared with chemotherapy alone.[8] In contrast to this, several recent reports have raised questions about the necessity of postoperative radiotherapy in selected patients with stages II or III rectal cancer.[9,10,18] Park and colleagues[9] reported that postoperative radiotherapy did not lower the local recurrence rate in selected patients with stage II rectal cancer. Kariv and coworkers[10] also showed that postoperative radiotherapy had an adverse effect on bowel function and that routine postoperative radiotherapy may not be necessary in stage IIIA rectal cancer if adequate resection is performed. However, these study populations did not include patients with stages IIIB or IIIC rectal cancer, who may be provided an additional benefit by postoperative radiotherapy. We found that radiotherapy had no impact on local recurrence in patients with stage III rectal cancer. Our patients underwent TME-based surgery, which has been shown to result in 5-year local recurrence-free survival rates of 87% in patients with node-positive rectal cancer, regardless of which radiotherapy was utilized. This finding suggests that the addition of radiotherapy to chemotherapy may be unnecessary if the surgery is optimized.

Among the several risk factors documented to be associated with local recurrence, the involvement of circumferential resection margin or inadequate distal resection margin is one of the most significant predisposing factors for the high risk of local recurrence.[19,20] Postoperative radiotherapy might theoretically benefit those patients with locally advanced rectal cancer who have an increased risk of disease recurrence. However, recent studies have not fully documented the beneficial effect of the addition of radiotherapy to chemotherapy in patients displaying these high-risk factors.[21,22] Marijnen and colleagues[22] reported that postoperative radiotherapy in patients with incomplete surgery with positive resection margins did not lead to a reduction in the incidence of local recurrences. Baik and coworkers[21] also suggested that adjuvant chemoradiotherapy is not a definite treatment option that can be used to compensate for a positive circumferential resection margin after TME in patients with stages II or III rectal cancer. Our data also agree with these findings that postoperative radiotherapy could not compen-

Table 5 Survival rates by the adjuvant therapy in patients with stage III tumor according to the status of the circumferential and distal resection margins (n = 254)

Factors	Number	5-year LFRS (%)	5-year DFS (%)	5-year OS (%)
Sufficient margins				
Chemotherapy	163	90	62	74
Chemoradiotherapy	50	94	58	75
P value		0.611	0.282	0.731
Insufficient margins				
Chemotherapy	27	60	27	38
Chemoradiotherapy	14	85	39	36
P value		0.178	0.887	0.706

Sufficient margins mean no involvement of the circumferential resection margin (≥1.0 mm) and sufficient distal resection margin (≥1.0 cm); insufficient margins mean an involvement of the circumferential resection margin (<1.0 mm) or insufficient distal resection margin (<1.0 cm)

LRFS local recurrence-free survival, *DFS* disease-free survival, *OS* overall survival

sate for positive resection margins in local recurrence, disease-free survival, and overall survival rates.

The current study was not without limitations, as the sample size was relatively small and may have been subject to various biases. The median follow-up period in our study was 52 months, and some patients could be expected to develop recurrences after this time. However, this possibility is considered to be low because most recurrences of patients with colorectal cancer and who undergo potentially curative surgery are reported to occur in the first 3 years after surgery.[23] In addition, our analysis was a retrospective study and not a properly randomized one. Therefore, our study should be understood as encouragement to raise appropriate questions about these issues. Further randomized trials are needed to reevaluate and confirm these observations, allowing a better identification of patients with a high risk of recurrence who may benefit from adjuvant therapy. In conclusion, the addition of postoperative radiotherapy to chemotherapy did not reduce local recurrence rates and improving survival rates in stage III rectal cancer beyond the benefit offered by chemotherapy alone. Moreover, this approach may not compensate for a positive circumferential resection margin or insufficient distal resection margin following rectal cancer surgery.

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ORIGINAL ARTICLE

Super-selection of a Subgroup of Hepatocellular Carcinoma Patients at Minimal Risk of Recurrence for Liver Transplantation

Shin Hwang · Chul-Soo Ahn · Ki-Hun Kim · Deok-Bog Moon · Tae-Yong Ha · Gi-Won Song · Dong-Hwan Jung · Gil-Chun Park · Young-Dong Yu · Pyoung-Jae Park · Young-Il Choi · Kyoung-Won Kim · Young-Suk Lim · Han Chu Lee · Eun-Sil Yu · Sung-Gyu Lee

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Abstract
Background A majority of patients with hepatocellular carcinoma (HCC) undergoing liver transplantation (LT) meet the Milan criteria, but these are still regarded as the narrowest criteria for transplantation. Prognostic analysis of incidentally detected HCC after LT suggests that a subgroup of HCC patients is at very low risk of recurrence. To determine the criteria defining this super-selection group, we retrospectively analyzed survival data of 593 adult living-donor LT recipients with HCC in the explanted liver
Discussion Tumor features of incidental HCC in 38 patients not showing recurrence were analyzed. Of these patients, 34 (89.5%) each had ≤2 tumors and tumors ≤2.0 cm in size. Applying these criteria to 555 patients with pretransplant known HCC (pkHCC) allowed us to identify 79 patients with untreated pkHCCs ≤2.0 cm in size. To date, only two of these patients have shown recurrence, making the conditions for super-selection the presence of tumors ≤2.0 cm in size, ≤2 tumors, alpha-fetoprotein ≤200 ng/mL, and no pretransplant treatment. In 87 patients satisfying these criteria, the 10-year recurrence and survival rates were 1.3% and 92.1%, respectively. After excluding patients meeting these criteria, the 5-year recurrence rates in patients satisfying the Milan, University of California at San Francisco, and Asan criteria were increased by 2.9–4.0%. In conclusion, this super-selection or super-Milan category may be used for validation assessment of various indication criteria and for the development of cost-effective post-transplantation HCC surveillance protocols. Further studies should be followed for deceased-donor LT and patients who have undergone pretransplant treatment.

Keywords Hepatocellular carcinoma · Liver transplantation · Recurrence · Indication criteria · Surveillance

Abbreviations

DDLT	Deceased-donor liver transplantation
HCC	Hepatocellular carcinoma

S. Hwang · C.-S. Ahn · K.-H. Kim · D.-B. Moon · T.-Y. Ha · G.-W. Song · D.-H. Jung · G.-C. Park · Y.-D. Yu · P.-J. Park · Y.-I. Choi · S.-G. Lee (✉)
Department of Surgery, Division of Hepatobiliary Surgery and Liver Transplantation, Asan Medical Center, University of Ulsan College of Medicine, Seoul 138-736, South Korea
e-mail: sglee2@amc.seoul.kr

K.-W. Kim
Department of Diagnostic Radiology, Asan Medical Center, University of Ulsan College of Medicine, Seoul, South Korea

Y.-S. Lim · H. C. Lee
Department of Internal Medicine, Asan Medical Center, University of Ulsan College of Medicine, Seoul, South Korea

E.-S. Yu
Department of Pathology, Asan Medical Center, University of Ulsan College of Medicine, Seoul, South Korea

iHCC	Incidentally found HCC
PkHCC	Pretransplantation known HCC
LDLT	Living-donor liver transplantation
LT	Liver transplantation

Introduction

Recent advances in diagnostic imaging have enhanced the rate of detection of hepatocellular carcinoma (HCC), but some small HCC lesions are still missed until they are detected during pathological evaluation of the resected liver specimens.[1] With liver transplantation (LT), the presence of concurrent HCC has a great prognostic impact on patient survival because HCC recurrence contributes to about one-half of patient deaths.[2] Most HCC lesions are diagnosed before LT, but HCC can be incidentally found in the explant livers, especially those that are severely cirrhotic. These lesions are usually small in size and often singular, having a low risk of HCC recurrence.[3–5] As this risk is, however, non-negligible, the presence of any HCC lesion indicates a necessity for long-term oncologic surveillance guarding against tumor recurrence.

A considerable proportion of patients undergoing LT have one or two small-sized HCC lesions, which are often left untreated because of marked impairment of liver function not permitting any treatment or prescheduled elective living-donor LT (LDLT) surgery. Some of these HCC lesions may have been missed preoperatively and may be regarded as incidental HCC (iHCC) if patients had undergone LT before the recent development of high-quality imaging techniques.[3–8] LT for patients with such HCC lesions also resulted in very low tumor recurrence rates. The clinical sequences of iHCC and small pretransplant known HCC (pkHCC) patients are usually very similar, with any clinical differences attributable only to whether the tumors are detected in recent trawl net-style screening processes using multiple diagnostic modalities.

The Milan criteria have long been accepted as the standard for the selection of HCC patients for LT.[9] Thus, nearly all newly proposed expanded criteria for LT in HCC patients have been compared with the Milan criteria to estimate their predictability. Worldwide, in every LT series reported to date, 70–90% of patients met the Milan criteria, with estimated overall 5-year patient survival rates of 70–80%.[2,10–13] The majority of patients meeting various expanded criteria also satisfy the Milan criteria, which may have contributed, at least in part, to the prognostic comparability between the expanded and Milan criteria. Therefore, any validation study of expanded criteria requires separate assessment of predictability in patient subgroups meeting the expanded criteria but exceeding the Milan criteria.

Although the majority of HCC patients in most LT series worldwide meet the Milan criteria, these criteria are still considered to be the narrowest, and patients meeting these criteria have not been divided into subgroups. Our clinical experience with LT for patients with iHCC and small pkHCC suggests that a subgroup of super-selectable patients fulfilling the Milan criteria may be associated with very low rates of HCC recurrence. Establishment of such a super-selection category would stratify the prognostic power of the Milan criteria into two portions, with one set of patients being naturally at very low risk of HCC recurrence and the other set being at limited risk artificially benefited from selection by the Milan criteria. Patients in the super-selection category could be examined for objective validation of the expanded criteria as well as in the development of cost-effective post-transplantation surveillance for HCC recurrence.

The aim of this study was to establish a super-selection category of HCC patients who would benefit most from LT. This study consisted of three parts. In the first part, we attempted to identify a minimal risk group through clinical analysis of iHCC patients encountered during our 10 years of experience with adult LDLT. In the second part, we sought to identify LT selection conditions for pkHCC patients and to make such criteria comparable to those for iHCC patients. In the third part, we assessed whether the creation of the super-selection category had any useful clinical impact on patient selection and post-transplantation surveillance.

Patients and Methods

Patient Selection

To make our results applicable to current clinical practice, we confined the study period to the past 10 years, to retain the diagnostic accuracy of current imaging modalities for HCC. The post-transplantation follow-up period was >1 year. In addition, the study population was confined to only LDLT recipients, to avoid the effects of variable waiting periods for deceased-donor LT (DDLT). In our institution, all adult LDLT recipients underwent complete imaging analysis once or repeatedly within 1 month before LT surgery. Our institutional eligibility criteria for HCC (the Asan criteria) include tumor size ≤5 cm, tumor number ≤6, and no gross vascular invasion;[2] patients with liver malignancies other than HCC are not indicated for LT in principle.

Our patient population thus consisted of 1,732 adults (≥18 years of age) who underwent primary LDLT at our institution between April 1999 and March 2009 (Fig. 1).[2,14] Of these, 564 (32.6%) had been diagnosed with or highly suspected of HCC during pretransplant assessment, and 555 were diagnosed with HCC or other types of liver

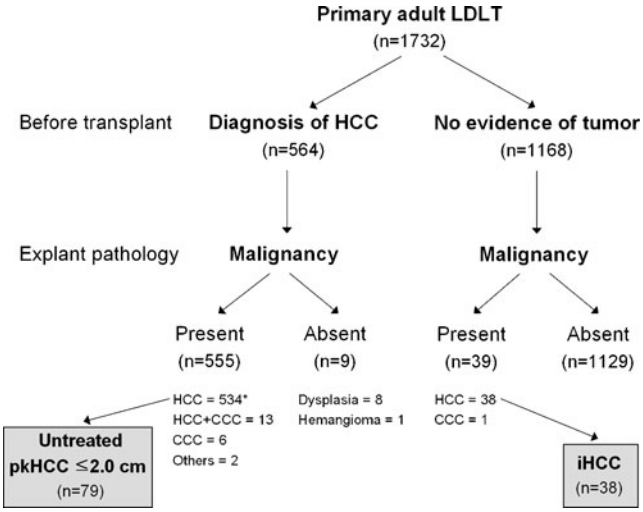

Fig. 1 Proportions of adult patients with hepatocellular carcinoma (*HCC*) undergoing living-donor liver transplantation (*LDLT*) between April 1999 and March 2009 at the Asan Medical Center. Patients showing loss of viable HCC after neoadjuvant therapy are also included in the HCC group. *CCC* cholangiocellular carcinoma, *iHCC* incidental HCC, *pkHCC* pretransplant known HCC

malignancies after pathologic evaluation of explanted livers. No definite malignant lesions were identified in the remaining nine patients. In addition, 39 patients, who were not suspected of liver malignancy before LDLT surgery, were diagnosed with HCC (n=38) or cholangiocellular carcinoma (n=1).

As liver malignancies other than HCC, including cholangiocellular carcinoma, HCC–cholangiocellular carcinoma mixed tumors, adenocarcinoma, and HCC with sarcomatous changes, have shown quite different patterns of tumor recurrence, usually being much more aggressive,[11–17] our study population was confined to patients with the usual form of HCC.

Medical records of study patients were retrospectively reviewed and followed up until April 2010. Our institutional review board approved this retrospective observational study.

Setting of Target Level for the Risk of HCC Recurrence in the Super-selection Category

As the cumulative 5-year HCC recurrence rates in patients satisfying the Milan criteria have been found to range from 10% to 30%, we arbitrarily set the target level of risk for patients in the super-selection category as a 5-year HCC recurrence rate <5%, and then searched for tumor characteristics that would allow patients to meet this target level.

Pretransplantation Screening and Assessment for HCC

During the study period, all LDLT recipients underwent routine triphasic dynamic computed tomography (CT) and magnetic resonance imaging for HCC screening and liver status evaluation. Until 2003, hepatic arteriography was routinely performed, and positron emission tomography (PET) using [^{18}F]-fluorodeoxyglucose was selectively chosen for patients suspected of HCC. Since early 2004, PET scans have been routinely performed on all recipient candidates,[18,19] whereas hepatic arteriography was no longer performed. The last pretransplant liver dynamic CT scan was performed on all patients 1 day to 2 weeks before LDLT surgery. Thus, the chronological discrepancy between HCC screening and LT surgery was quite short.[2]

In addition to imaging studies, HCC tumor markers including alpha-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist-II were also routinely measured.[20,21] Korean practice guidelines for the management of HCC suggest that serum AFP >200 ng/mL is a significant cutoff for the diagnosis of HCC.[22]

Post-transplantation Surveillance for HCC

As each HCC patient is at a different degree of risk for post-transplantation HCC recurrence, we established a three-step follow-up protocol for HCC surveillance according to risk level (Table 1). As patients with advanced HCC are usually at the highest risk of recurrence during the first year, such patients are more strictly monitored, especially during that period.[2,10,11,23] PET scans and chest CT scans were performed immediately in patients suspected of HCC recurrence by analysis of tumor markers or upon imaging.[24] Successful establishment of a super-selection category would simplify the surveillance protocol for such patients, without loss of surveillance reliability for patients meeting the Milan criteria (Table 1).

Retrospective Validity Assessment Using Currently Available Criteria

To analyze the clinical impact of the super-selection category on the three indication criteria for HCC (Milan, University of California at San Francisco—UCSF, and Asan),[2,9,25] a series of validity assessments were performed using a pkHCC dataset of 221 patients assessed between February 1997 and December 2004, which was published previously.[2] Patient follow-up period was extended to April 2010, resulting in a median follow-up time of 75 months (range, 1–158 months).

Statistics

Perioperative mortality was defined as patient death from any cause within 3 months after LT surgery. All numerical data are presented as means and standard deviations or as

Table 1 Post-transplant follow-up protocols for HCC surveillance and routine checkups according to risk levels of HCC recurrence at the Asan Medical Center

Risk of HCC recurrence at 5 years Selection criteria	<5% Super-selection category[a]	≤20% Within Asan criteria	20–80% Beyond Asan criteria	>80% Beyond Asan criteria + AFP >3,000 IU/L
1st year	TM every 2 months	TM every month	TM every month	TM every month
	CT every 6 months	CT every 3–4 months	CT every 2–3 months	CT every 1–2 months for the first 6 months
				CT every 2–3 months for the next 6 months
2nd and 3rd years	TM every 2 months	TM every 2 months	TM every 2 months	TM every 2 months
	CT every 12 months	CT every 4–6 months	CT every 3–4 months	CT every 3–4 months
4th and 5th years	TM every visit[b]	TM every visit[b]	TM every visit[b]	TM every visit[b]
	CT every 12 months	CT every 6 months	CT every 6 months	CT every 6 months
>5 years[c]	TM every 6 months	TM every 6 months	TM every 6 months	TM every 6 months

Computed tomography (CT) includes liver dynamic CT with less frequent chest CT

AFP alpha-fetoprotein, *TM* tumor markers including AFP and PIVKA-II

[a]Established after this study

[b]The intervals for routine outpatient clinic visit usually do not exceed 3 months

[c]Formal HCC surveillance finishes at 5-year follow-up. Chest X-rays are taken along with CT scans at every visit

medians with ranges. Incidences were analyzed by the chi-squared test and Fisher's exact test. Survival rates were determined using the Kaplan–Meier method and compared using the log-rank test. Statistical significance was set at $p<0.05$.

Results

Patient Selection Process to Define Super-selection Category

Patients diagnosed with HCC only from examination of explant livers ($n=38$) were classified as the iHCC group. As survival analysis revealed no incidence of HCC recurrence in this iHCC group during the study period, this group was regarded as a valid control group of patients at very low risk of HCC recurrence.

After reviewing the tumor features of the iHCC group, we found that 34 (89.5%) had pathological HCC lesions ≤2.0 cm in maximum diameter and 34 (89.5%) had only one to two tumors. Thus, to make the tumor features of the pkHCC group comparable to those of the iHCC group, we selected 79 patients by applying the following three criteria: diagnosis with or highly suspected of HCC by pretransplant imaging and/or tumor marker analysis; pathological HCC lesions of largest tumor diameter ≤2.0 cm regardless of tumor number; and no previous locoregional treatment for HCC, including transarterial chemoembolization, percutaneous ethanol injection therapy, radiofrequency ablation, radiotherapy, or surgical resection, to avoid bias associated with variable responses to treatment.[26–28] This group of 79 patients was defined as the small untreated pkHCC group and was used to determine the criteria for the super-selection category (Table 2).

Clinicopathological Features of iHCC

Profiles of the 38 patients in the iHCC group are summarized in Table 2. Of these, 34 (89.5%) had pathological HCC lesions ≤2.0 cm in maximal diameter, 30 (80.0%) had single tumors, and 28 (73.7%) had single tumors ≤2.0 cm in size. Distribution of HCC lesions according to size and number is depicted in Fig. 2.

Of these 38 patients, 3 died—1 from uncontrolled pulmonary hypertension 11 days after surgery; 1, who had advanced gastric cancer surgery at 30 months, from gastric cancer recurrence at 44 months; and 1 from progressive graft failure of unknown cause at 15 months. The other 35 patients remain alive to date (Fig. 3), with none showing HCC recurrence after a median follow-up time of 75.3 months (range 11 days–132 months) (Fig. 4).

Clinicopathological Features of Untreated pkHCC ≤2.0 cm in Tumor Diameter

Profiles of the 79 patients in the untreated pkHCC group are also summarized in Table 2. After a median follow-up period of 61.2 months (range 6 days–127 months), 6 have died to date. There were two perioperative mortalities, a subdural hemorrhage at 6 days and a graft dysfunction at

Table 2 Clinicopathological profiles of patients with incidental HCC (iHCC) and small untreated pretransplant known HCC (pkHCC)≤2.0 cm in diameter

Group	iHCC (n=38)	pkHCC (n=79)	p value
Age (years)	50.1±8.5	52.1±5.9	0.156
Male gender	29 (76.3%)	62 (78.5%)	0.792
Underlying liver disease			0.494
HBV-LC	35	71	
HCV-LC	1	6	
Alcoholic LC	1	2	
Unknown etiology	1	–	
MELD score	23.4±7.1	17.9±7.2	<0.001
Pretransplant serum tumor marker			
AFP (ng/mL)			
Mean ± SD	37.7±82.4	199.7±574.9	0.087
Median (range)	12.2 (1.5–457)	17.7 (1–3,630)	
PIVKA-II (mAU/mL)			
Mean ± SD	41.2±29.8	70.2±109.3	0.576
Median (range)	31 (18–91)	22 (9–354)	
Urgent LDLT (n, %)	11 (28.9%)	7 (8.9%)	0.0048
Graft type			
Right liver	24	59	
Left liver	7	7	
Dual graft	7	13	
Explant pathology			
Maximal tumor size			
Mean (cm)	1.4±0.7	1.5±0.5	0.389
Median (cm)	1.25 (0.3–3.2)	1.5 (0.2–2.0)	
≤1 cm (n)	13	17	
1.1–2.0 cm (n)	21	62	
>2 cm (n)	4	0	
Tumor number			
1 (n)	30	55	
2 (n)	4	11	
3 (n)	2	4	
≥4 (n)	2	9	
Bilateral distribution of multiple tumors (n, %)	6 of 8 (75%)	16 of 24 (66.7%)	
Microvascular invasion (n, %)	0 (0%)	1 (1.3%)	
Edmondson–Steiner grade			
Most: 1/2/3/4 (n)	13/18/6/1	24/43/11/1	
Worst: 1/2/3/4 (n)	2/12/2/22	6/20/21/32	
Within Milan criteria (n, %)	35 (92.1%)	70 (88.6%)	
Within Asan criteria (n, %)	36 (94.7%)	74 (93.7%)	
Peritransplantation mortality (n, %) (<3 months)	1 (2.6%)	2 (2.5%)	
5-year HCC recurrence rate	0%	1.3%	
Overall 5-year patient survival rate	91.2%	93.7%	

HBV hepatitis B virus, *LC* liver cirrhosis, *HCV* hepatitis C virus, *MELD* model for end-stage liver disease, *AFP* alpha-fetoprotein, *PIVKA-II* protein induced by vitamin K absence or antagonist-II, *LDLT* living-donor liver transplantation

40 days. Two patients died from HCC recurrence and their detailed profiles are summarized in Table 3. One patient died from hepatitis B virus recurrence at 10 months and one died of progressive graft failure at 14 months. The remaining 73 patients remain alive to date (Fig. 3), including 1 patient who underwent deceased-donor re-transplantation at 19 months because of chronic rejection, 1 who underwent thyroid cancer resection at 12 months, and 1 who underwent a pancreatoduodenectomy for ampulla of Vater cancer at 23 months.

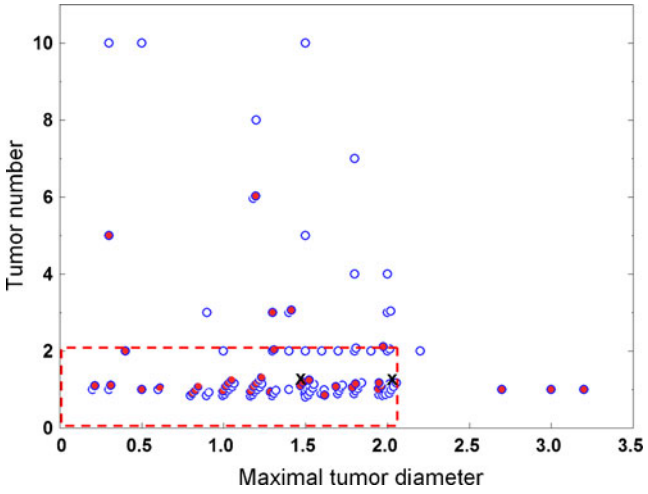

Fig. 2 Distribution of HCC according to tumor size and number in patients with incidental HCC (*red-colored circles*) and untreated small pkHCC ≤2.0 cm in diameter (*empty circles*). The two-by-two territory is defined by a *dotted box* and includes 92 of 117 patients. Each "X" indicates a patient showing HCC recurrence

Eligibility Conditions for Super-selection

After combining the iHCC and pkHCC groups, we assessed the overall distribution of tumor size and number (Fig. 2). From an analysis of tumor features in the iHCC group, we set the tumor size limit at 2 cm, met by 113 of 117 patients (96.6%). A tumor number ≤3 is included within the Milan criteria. However, the two patients with tumor recurrence each had two HCC lesions, resulting in a slight difference in recurrence rates for tumor number limits of 2 versus 3. As the super-selection category was designed to be more restrictive than the Milan criteria, we intentionally set the tumor number limit to 2.

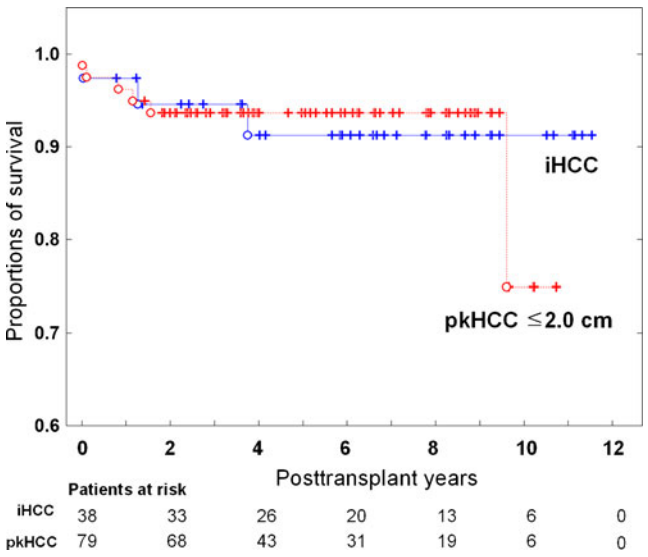

Fig. 3 Overall survival of patients with iHCC and untreated small pkHCC ≤2.0 cm in size (p=0.922)

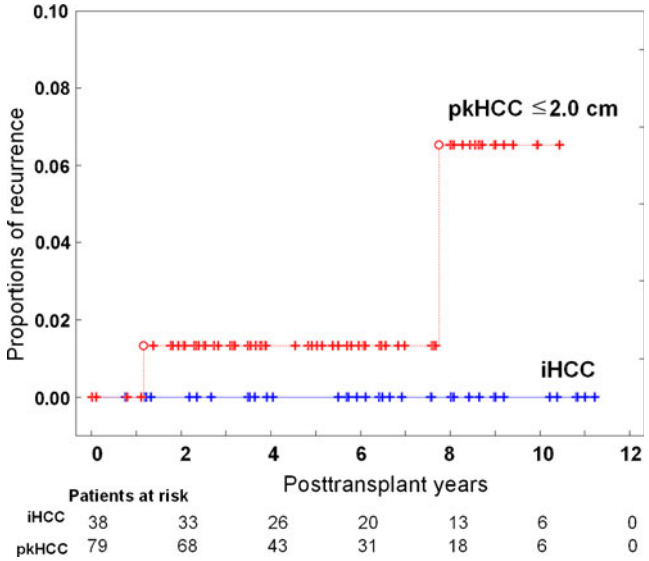

Fig. 4 Cumulative HCC recurrence rates in patients with iHCC and untreated small pkHCC ≤2.0 cm in size (p=0.267)

Of the 113 patients with untreated HCC ≤2 cm in diameter, 96 (85.0%) had ≤2 lesions (Fig. 2). Two of them had HCC recurrence. Thus, in these 96 patients, the cumulative 5- and 10-year HCC recurrence rates were 1.1% and 5.4%, respectively, and the overall 5- and 10-year survival rates were 91.9% and 78.7%, respectively.

In these 96 patients with untreated one or two HCC ≤2 cm, the distribution of pretransplant AFP levels was depicted at Fig. 5. There was no HCC recurrence in 62 patients with AFP ≤20 ng/mL, one recurrence in 25 patients with AFP between 20–200 ng/mL, and one recurrence in 9 patients with AFP>200 ng/mL. Only two iHCC patients showed AFP>200 ng/mL. When the condition of AFP ≤200 ng/mL is added to the super-selection category, the 10-year HCC recurrence rate and 10-year overall patient survival rate in 87 patients improved to 1.3% and 92.1%, respectively. Thus, the super-selection category was finally defined as four components of no pretransplant HCC treatment, tumor number of one or two, tumor size ≤2 cm, and AFP ≤200 ng/mL.

Retrospective Application of Super-selection Criteria to the Current Indication Criteria for Validity Assessment

To assess the validity of the super-selection category relative to the Milan, UCSF, and Asan criteria, we assessed HCC recurrence and survival in 221 pkHCC patients who underwent LDLT from February 1997 to December 2004.[2] Of these, 40 patients (18.1%) belonged to the super-selection category, and only two showed recurrence after a median follow-up time of 66.5 months. After excluding patients belonging to the super-selection category, we found that the cumulative 5-year recurrence rates in patients

Table 3 Profiles of two patients showing HCC recurrence

	Patient 1	Patient 2
Gender/age (years)	Male/60	Male/61
Underlying liver disease	Hepatitis C virus infection	Hepatitis B virus infection
MELD score	18	14
Pretransplantat serum AFP (ng/mL)	46.9	2670
Graft type	Dual graft	Right liver graft
Explant pathology		
Tumor size (cm)	2	1.5
Tumor number	1	1
Microvascular invasion	Absent	Present
Edmondson–Steiner grade (most/worst)	3/4	3/3
HCC recurrence timing (months)	14	93
Initial recurrence site	Liver	Vertebral bone
Overall patient survival (months)	19	111

MELD model for end-stage liver disease, *AFP* alpha-fetoprotein

meeting the explant pathology-based eligibility criteria were increased by 2.9–4.0% (Table 4). Survival analysis of patients partially meeting the super-selection category (due to with inclusion of patients who have undergone pretransplant treatment) revealed improvement of the overall patient survival rates solely due to a significant decrease in the HCC recurrence rates (Fig. 6).

Retrospective Application of Super-selection Criteria to Develop a Cost-Effective Surveillance Protocol for Post-transplantation HCC Recurrence

In the current series of 534 pkHCC patients, 59 belonged to the super-selection category of 4 conditions, with only 1 of the latter (1.7%) showing HCC recurrence. Thus, membership of the super-selection category would be associated with a very low risk of HCC recurrence. Thus, any HCC surveillance other than routine checkups may be unnecessary for patients in this category. We therefore revised the surveillance protocol for these patients to be simpler than that of the Milan criteria (Table 1). Nearly all patients meeting the super-selection criteria would benefit from such simplified surveillance.

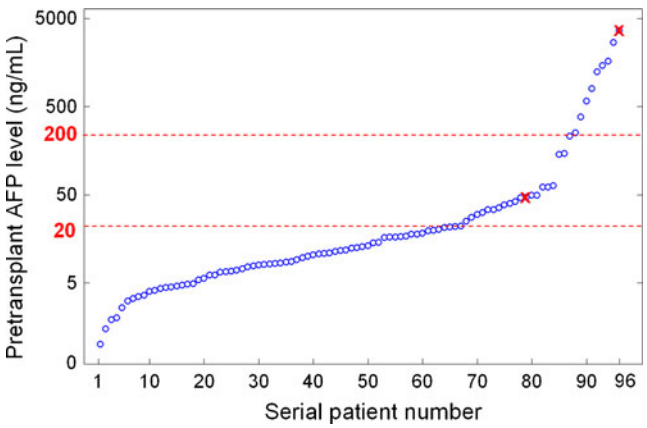

Fig. 5 Logarithmic distribution of pretransplant serum alpha-fetoprotein (*AFP*) levels in 96 patients with untreated one or two HCC ≤2 cm. The level of 20 ng/mL is an institutional reference value and that of 200 ng/mL is a cut-off of diagnostic importance. Each "X" indicates a patient showing HCC recurrence

Comparison of Super-selection Category Versus the Lowest Stages in Current HCC Staging Systems

To evaluate the validity of the super-selection category, it was compared with the lowest stages of the currently available seven HCC staging systems developed for resection. The super-selection category was nearly comparable to stage 0 or 1 of the seven HCC staging systems (6th AJCC/UICC staging system, TNM staging system of the Liver Cancer Study Group of Japan, Japan Integrated Scoring system, UNOS-modified TNM staging system, Pittsburgh scoring system, Cancer of the Liver Italian Program scoring system, and the Barcelona Clinic Liver Cancer staging classification).[29]

Discussion

The risk of post-transplantation HCC recurrence is a principal concern of adult LT patients because the overall number of recipients at higher risk of HCC recurrence would be increased as indication criteria are expanded. However, few effective treatment modalities have been developed to date to cope with HCC recurrence.[2,12,25,30] Patients with recurrent HCC after LT have a high probability of tumor metastasis, ultimately leading to fatality despite the application of available treatments.[31,32] Thus, the unexpected detection of iHCC in the explant liver increases patient concern about the risk of HCC recurrence.

Table 4 Proportions and prognostic outcomes according to three different indication criteria with inclusion/exclusion of the super-selection category in 221 pkHCC patients undergoing LDLT[2]

Indication criteria	Within criteria		Beyond criteria
Patients of super-selection category	Included *n* (%)	Excluded *n* (%)	*n* (%)
Super-selection category	40 (18.1%)	–	181 (81.9%)
5-year recurrence rate	2.9%	–	27.2%
5-year patient survival rate	85.0%	–	66.3%
Milan criteria	164 (74.2%)	144 (62.4)	57 (25.8%)
5-year recurrence rate	14.2%	17.8%	47.7%
5-year patient survival rate	76.2%	73.4%	50.9%
UCSF criteria	174 (78.7%)	134 (60.6%)	47 (21.3%)
5-year recurrence rate	17.3%	21.3%	46.6%
5-year patient survival rate	74.7%	71.7%	48.8%
Asan criteria	186 (84.2%)	146 (66.1%)	35 (15.8%)
5-year recurrence rate	13.5%	16.4%	76.8%
5-year patient survival rate	77.4%	75.3%	28.6%

Explant pathology was used for criteria fulfillment

UCSF University of California at San Francisco

Although the potential risk of HCC recurrence in patients with iHCC has been assessed, the explanatory power of the work is now not so convincing because of the higher number of HCC missed by now obsolete diagnostic modalities and the fact that small sample numbers do not permit reliable analysis.[5] After carefully reviewing iHCC profiles in the literature, we recognized that some of these HCC lesions might have been detected before LT if more advanced imaging techniques had been applied or if imaging had been taken just before performing DDLT. As the iHCC category indicates that the HCC remained undetected by pretransplant imaging modalities of limited diagnostic accuracy,[5,33,34] there was a need to objectively assess the actual risk of HCC recurrence in patients with iHCC.

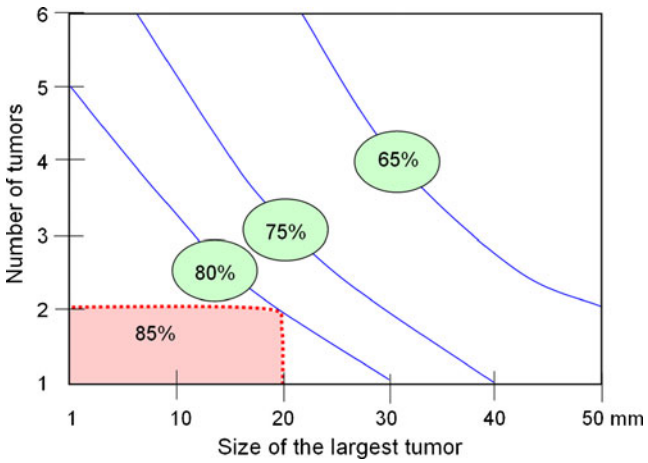

Fig. 6 Five-year patient survival rates in LDLT recipients regardless of performance of pretransplant treatment. The *dotted line* indicates the boundary of the super-selection category. Patient profiles have been presented elsewhere[2]

The absence of HCC recurrence in our iHCC group indicates that such patients have a better prognosis than earlier thought. That is, our results indicate that there is almost no potential for post-transplantation HCC recurrence when current diagnostic modalities cannot detect occult HCC buried in a cirrhotic liver. This low risk of HCC recurrence may be attributable, at least in part, to favorable tumor biology and small tumor volume load, preventing the early hematogenous propagation of tumor cells.[31] Conversely, these oncologic features may also contribute to difficulties in detecting iHCCs using current diagnostic modalities.

Questions have also been raised about the post-transplantation outcomes of patients with small untreated pkHCC, which are comparable to iHCC in size. To objectively analyze patient prognosis, we strictly selected a cohort of HCC patients to form a study group valid in comparisons. First, we chose only patients with untreated HCC because any pretransplant treatment can influence tumor recurrence rate.[26,27] Downstaging is a form of natural selection of HCC responsive to neoadjuvant treatment, but such artificial reduction in tumor size and/or number is not always related to a drop in HCC recurrence.[35,36] In a preliminary analysis during this study, we found that patients with viable HCC ≤2.0 cm in diameter, after various pretransplant treatments, showed significantly higher recurrence rates than those of patients with untreated HCC of comparable size. Thus, we had to exclude the patients with pretransplant treatment in order to obtain more consistent results. In contrast, we also found that the pathological profiles of small untreated pkHCC ≤2.0 cm in diameter were very similar to those of iHCC, with the only clinical difference being whether they were concealed on pretransplant screening.

Of 117 patients with iHCC or untreated pkHCC ≤2.0 cm in diameter, only 2 experienced HCC recurrence, resulting in recurrence rates of 0.9% at 5 years and 4.0% at 10 years. HCC recurrence in one patient was unexpected because that patient had no known risk factor except for poor tumor cell differentiation. In contrast, the second patient had definite risk factors, including a very high AFP concentration and microvascular invasion, but recurrence occurred unusually late, after 93 months. This patient survived only 18 months after diagnosis of recurrence, indicating that the recurrent HCC lesion was far more aggressive than was the original tumor. The very late recurrence in this patient suggests that there is some possibility of de novo HCC occurrence which is associated with occult hepatitis B virus infection despite strong prophylactic antiviral treatment.[37] In contrast to the previous finding, we also found that poor tumor differentiation was not associated with HCC recurrence when the tumor was ≤2.0 cm in diameter.[38]

The low recurrence rate in patients with iHCC or untreated pkHCC ≤2.0 cm in diameter distinguishes these patients from those satisfying the Milan criteria. The Milan criteria for pretransplant selection include patients with untreated pkHCC ≤2.0 cm in diameter, but not those with iHCC, whereas both subgroups are included if prognostic analysis using explant pathology is planned. That is, the prognostic power of the Milan criteria is based on the integration of one subgroup at a naturally low risk of HCC recurrence and another subgroup at limited risk. We have termed the former subgroup the super-selection category.

Before defining the super-selection category, we compared seven currently available staging systems for HCC.[29] The requirements for stage 1 or minimal scores included single tumor <2 cm in diameter in four of the seven staging systems. As these staging systems were designed to select patients for surgical resection, whereas LT surgery removes all intrahepatic lesions, two or three multicentric nodules <2 cm in diameter would be the minimal tumor extent for best prognosis. These definitions are comparable to features of iHCC as well as untreated pkHCC ≤2.0 cm in diameter. As the super-selection category should be precisely placed within the boundaries of the Milan criteria, we set the tumor number limit to two.

In addition to tumor size and number, some clinical factors reflecting the tumor biology should be included to ensure the low incidence of HCC recurrence.[23,24] We analyzed the distribution of pretransplant AFP levels, but no significant cut-off value was identified due to very low HCC recurrence rate. However, to reduce the risk of recurrence, a condition of serum AFP <200 ng/mL was added, which finally resulted in nearly ideal outcomes as like 10-year HCC recurrence rate of 1.3% and 10-year overall survival rate of 92.1%.

We primarily set the 5-year recurrence rate after LT for patients in the super-selection category to be <5%, identical to stage 0 or 1 of any HCC staging system developed for LT. The next stage would take patients into the Milan criteria; thus, patients within the Milan criteria but beyond the super-selection category would be stage 2. Nearly all patients in the various expanded criteria, including those of UCSF, up-to-seven, Tokyo, Kyoto, and Asan, would be compatible with stage 3.[2,39–43] As the majority of patients satisfying these criteria also satisfy the Milan criteria, their overall outcomes would not differ from those who meet Milan criteria. Patients who do not fall within any of these selection conditions would be classified as stage 4. These stratified concepts have been integrated into the idea of a metro ticket. Furthermore, a recently proposed prognostic model, including tumor size, number, and microvascular invasion status, enabling a more precise estimation of survival according to individual tumor characteristics, seeks to replace the various expanded criteria.[43] However, this prognostic model was based on the results of patients exceeding the Milan criteria, with the highest 5-year survival rate being set at 75%. Survival analysis of patients in the super-selection category provides further detailed information, allowing us to add one more stratum within the boundaries of the Milan criteria (Fig. 6). The boundaries of the super-selection category are 2 cm in tumor diameter and 2 in tumor number; thus, the category could be also described as “two-by-two category” or the “super-Milan category”.

Super-selection category definition based on the explant pathology enables us to quantify the risk of HCC recurrence in patients within the Milan criteria, which can be used to establish cost-effective post-transplantation surveillance.[23,44,45] Strict tumor surveillance is not necessary for such patients because their 5-year tumor recurrence rate is much lower than that of patients meeting the Milan criteria. A first 2-year follow-up of patients whose explant pathology exceeds the Milan criteria has been reported to be relatively cost-effective, depending on the survival benefit of recurrence treatment.[23] Before the present study, patients meeting the Milan criteria were allowed to follow a relatively relaxed protocol of HCC surveillance in our institution. Adoption of the concept of super-selection category can permit surveillance of such patients less frequently than before, with imaging assays being performed every 6–12 months and tumor marker assessment at every outpatient clinic visit for the first 5 years. These intervals of follow-up imaging indicate that no additional assessment, other than routine checkup, is needed for such LDLT recipients. In contrast, elimination of the super-selection category from the Milan criteria increased the 5-year incidence of HCC recurrence by only 2.9–4.0%; thus, follow-up for patients of these criteria need not be stricter than before. As a result, of the 164 patients meeting the

Milan criteria, 40 patients (24.4%) who met the super-selection category would benefit from the newly revised surveillance protocol.

This study was performed only in patients undergoing LDLT to avoid bias from the variable waiting period occurring between the last imaging analysis and actual DDLT operation. The prognostic outcome of DDLT for HCC has been reported to be superior to or at least compatible with that of LDLT. Thus, our concept of super-selection category in patients undergoing LDLT may be worthy of applying to those undergoing DDLT.[13] However, it is essential to validate again its clinical roles through further studies with DDLT series since waiting period changes everything which are related to this narrow category.

The limitation of this study should be clarified that the study population was highly selected since most of the patients were hepatitis B virus associated and neoadjuvant therapy was not permitted. If a higher number of hepatitis C virus-associated patients had been involved, the survival outcome would be worsened.[46,47] Considering that many patients meeting the Milan criteria have undergone neoadjuvant therapy during waiting for DDLT, even before LDLT, and a majority of them showed favorable short-term outcomes,[2,28,48,49] further study should be followed for patients who underwent pretransplant HCC treatment before LT.

In conclusion, the super-selection category, derived from our LDLT experience with patients with iHCC and small untreated pkHCC, can be defined as patients with one or two untreated HCC ≤2.0 cm and AFP <200 ng/mL, with an expectation of 5-year recurrence rate <5%. This category may be used for validation assessment of various indication criteria, including the Milan criteria, as well as for the development of cost-effective post-transplantation surveillance protocols for HCC recurrence. Further studies should be followed for DDLT and patients who have undergone pretransplant treatment.

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ORIGINAL ARTICLE

Outcome Comparison of Right Hepatectomy for Living Liver Donation Versus for Hepatic Patients Without Cirrhosis

Chuan Li · Kai Mi · Tian-fu Wen · Lu-nan Yan · Bo Li

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Abstract
Background Hepatectomy is one of the primary methods used in the treatment of hepatic lesions and liver graft harvesting. However, few studies have evaluated the postoperative recovery process of donor patients and that of normal liver hepatic patients following a right hepatectomy procedure.
Methods For this current study, the clinical data from the most recent 60 cases of each patient type receiving treatment at West China Hospital (group A [donors] and group B [normal liver hepatic patients]) were retrospectively analysed. Preoperative parameters, intraoperative variables, postoperative complications classified by the Clavien–Dindo classification system, and liver function changes were all statistically analysed and compared.
Results The preoperative parameters of the two groups were comparable. Group A experienced more intraoperative bleeding; however, the average amount of blood transfusion products was similar between the two groups. The overall postoperative surgical morbidity incidence for group A was 31.7% and for group B was 35%, with a p value of 0.699. The total bilirubin level and coagulation functions of group A were worse than what was observed in group B during the early postoperative period.
Conclusion Live liver donation via right hepatectomy results in similar complication rates and average blood product use to non-cirrhotic hepatic diseases. However, following the procedure, donor liver function showed greater dysfunction during the early postoperative period.

Keywords Right hepatectomy · Liver donor · Hepatic disease

Introduction

Living donor liver transplantation (LDLT) has emerged as an accepted therapy for patients with end-stage liver disease, and the use of LDLT is increasing at many transplant centres worldwide. LDLT represents an efficient solution to minimising the gap between organ availability and need.[1,2] Hepatectomy is the current standard method for treating many hepatic lesions and liver graft harvesting.[3] Despite advances in surgical procedures, hepatectomy still poses significant morbidity and mortality risks for the donors.[4–6] Morbidity and mortality rates for right hepatic lobectomy have been reported to be as high as 20% to 60% and 0.4% to 0.6%, respectively.[7]

The use of LDLT was initiated at our centre in 2001 and has accounted for >50% of our total adult liver transplantation activity in the past 3 years due to a lack of cadaver grafts.[8] Not unlike other centres, our principal concern is donor safety,[9] and we have done our best to examine the lessons learned from previous surgeries. However, few reports exist in the literature regarding the outcome differences between right hepatectomy for living donor donation for donors and liver disease patients. Some investigators have hypothesised that living donor hepatectomy may be as safe as hepatectomy for hepatic diseases,[1] but the conclusion still has insufficient clinical evidence supporting it. The aim of this study was to determine whether living

C. Li · K. Mi · T.-f. Wen (✉) · L.-n. Yan · B. Li
Division of Liver Transplantation Center,
West China Hospital of Sichuan University,
Chengdu 610041, Sichuan Province, China
e-mail: aderwe@yahoo.cn

donor hepatectomy was as safe as hepatectomy for patients suffering hepatic diseases and whether the postoperative recovery process of the donor patients and hepatic disease patients was similar.

Methods and Patients

Study Design

The clinical data for the most recent 60 patients at our centre who donated the right hepatic lobe without the middle hepatic vein (MHV) and the 60 most recent hepatic patients with normal livers were retrospectively analysed. Cases were divided into two groups based on the surgical purpose and the primary disease: group A (liver donors) and group B (liver disease without cirrhosis). All LDLTs were approved by the ethics committee of West China Hospital, Sichuan University.

Donor Selection and Surgical Procedure

Donors were required to be healthy relatives with compatible ABO blood types. Serological testing for viral hepatitis and human immunodeficiency virus antibodies as well as testing for other acute or chronic diseases was negative. Volumetric computed tomography with contrast was performed to assess the right lobe of the donor's liver. The right lobectomy without MHV needed to be at least 0.8% of the recipient's standard weight and involves no more than 60% of the donor's liver volume.[9]

The surgery was performed through a right subcostal incision with an extension to the upper midline. After a thorough abdominal exploration, a liver biopsy was performed to evaluate the amount of steatosis present. An intraoperative cholangiogram via the cystic duct was used to evaluate the biliary anatomy after cholecystectomy. The first hepatic hilum was then dissected, which was followed by dissection of the right hepatic artery to the bifurcation of the proper hepatic artery. The right portal vein was identified under the artery and isolated from the bifurcation of the main portal vein. At the end of the liver parenchymal transaction, the right bile duct, artery and portal vein divisions were performed. The right lobe of the liver was mobilised, and the vena cava was dissected. All tiny inferior right hepatic and caudate venous tributaries to the vena cava were divided. Any short hepatic veins larger than 0.5 cm were preserved until the time of harvesting for potential anastomosis in the recipient. The liver parenchyma was divided to the right of the middle hepatic vein with an ultrasonic dissector. Liver transection was performed using a CUSA Excel™ device without inflow occlusion. The falciform ligament was reconstructed, and the stumps of the right hepatic vein and portal vein were closed with continuous non-absorbable sutures. A drain was inserted into the right subphrenic cavity before closure.[10] Intraoperative autotransfusion was conventionally used during the donor liver surgery.

Surgical Procedure for Liver Patients

After general anaesthesia, the liver was exposed via a right subcostal incision with an extension to the upper midline. After a thorough abdominal exploration, cholecystectomy was performed, followed by isolation of the right hepatic artery and right portal vein, along with mobilisation of the right lobe. Hemihepatic vascular occlusion was the primary technique utilised to reduce intraoperative bleeding. The Pringle manoeuvre was considered as a back-up strategy. A CUSA Excel™ device was used for liver transection. Drainage was routinely placed in the subphrenic cavity before closure. Intraoperative autotransfusion was considered in the case of benign diseases such as hepatic cavernous hemangioma.

Data Collection and Statistical Analysis

Preoperative liver functions and the general characteristics of the two groups were subjected to retrospective analysis. Intraoperative data, including blood loss and transfusion requirements, were collected. Postoperative complications were classified using the Clavien–Dindo classification system.[11,12]

The results of the analysis of all continuous variables are presented as the mean±SD. A chi-square test or Fisher's exact test was performed for categorical variables. One-way analysis of variance was used to determine the significance of the differences between the experimental group means. All statistical analyses were performed using SPSS 13.0 for Windows. A p value less than 0.05 was considered significant.[13]

Results

Preoperative Clinical Data from the Two Groups

The primary liver diseases affecting group B patients were as follows: cavernous hemangioma (n=32), metastatic colorectal diseases (n=21), hydatid disease (n=4), adenoma (n=2) and inflammatory pseudotumour (n=1). As shown in Table 1, groups A and B had similar preoperative characteristics, including age in years (40.7±9.9 for A and 41.3±10.1 for B; p=0.321), total bilirubin (TB [mg/dL]; 0.78±0.28 for A and 0.78±0.31 for B; p=0.981), albumin (ALB [g/dL]; 4.29±0.37 for A and 4.39±0.67 for B; p=0.334), international

Table 1 Diagnoses and preoperative parameters of the two groups

Parameters	Group A (n=60)	Group B (n=60)	P value
Age (years)	40.7±9.9	41.3±10.1	0.321
Sex (male)	45	39	0.166
BMI	23.5±2.5	23.0±2.0	0.722
TB (mg/dL)	0.78±0.28	0.78±0.31	0.981
ALT (IU/L)	23.1±8.9	25.3±15.1	0.345
AST (IU/L)	23.9±8.0	27.6±14.1	0.080
ALB (g/dL)	4.29±0.37	4.39±0.67	0.334
INR	0.99±0.06	0.97±0.07	0.121
HGB (g/dL)	13.6±1.2	13.5±1.6	0.504

normalised ratio (INR; 0.99±0.06 for A and 0.97±0.07 for B; p=0.121), haemoglobin (HGB [g/dL]; 13.6±1.2 for A and 13.5±1.6 for B; p=0.504), alanine aminotransferase (ALT [IU/L]; 23.1±8.9 for A and 25.3±15.1 for B; p=0.345), aspartate aminotransferase (AST [IU/L]; 23.9±8.0 for A and 27.6±14.1 for B; p=0.080), and sex (p=0.166).

Intraoperative Variables and Complications

The average intraoperative blood loss of group A was 751±361 mL, which was greater than what was observed in group B (595±230 mL; p=0.006). However, there was no significant difference observed for the average intraoperative allogeneic transfusion of the two groups (0.66±1.16 U for A, 0.42±0.92 U for B; p=0.224). The removed liver volume, which was measured by water replacement, did not differ between the two groups (541±49 mL for A and 523±60 mL for B; p=0.083).

All postoperative surgical complications, classified according to the Clavien–Dindo system, are summarised in Table 2. The total complication rates of groups A and B were 31.7% and 35%, respectively, and the rates of complications were not statistically significant (p=0.699). No cardiac events, organ failure or deaths occurred in either group. One patient in group B was returned to the operating room for intraabdominal bleeding. Four patients in group A and three patients in group B underwent thoracentesis with local anaesthesia for moderate or serious hydrothorax. A serious bile leak required one patient in group B to undergo ultrasound-guided abdominal paracentesis. Stress ulceration was only observed in group B. These two patients received proton pump inhibitor therapy. Pleural effusion was the most common complication for both donors and hepatic patients.

Postoperative Liver Functions in the Two Groups

The postoperative biochemical parameter changes are summarised in Table 3 and Fig. 1. The bilirubin levels and the INR of group A significantly increased on the first and third postoperative days and were much higher than those observed in group B. However, there was no significant difference between the two groups by the seventh postoperative day. ALB decreased significantly in both groups after hepatectomy. By the seventh postopera-

Table 2 Surgical complications by Clavien–Dindo classification

Complications	Group A (n=60)	Group B (n=60)	P value
Grade I			
Hyperbilirubinemia*	1	0	
Transient bile leak	3	4	
Pleural effusion, mild	5	4	
Total	9 (15%)	8 (13.3%)	
Grade II			
Pneumonia	3	2	
Wound infection/abscess	3	4	
Stress ulcer	0	2	
Total	6 (10%)	8 (13.3%)	
Grade IIIa			
Pleural effusion (thoracentesis)	4	3	
Bile leak (ultrasound-guided drainage)	0	1	
Total	4 (6.7%)	4 (6.7%)	
Grade IIIb			
Re-operation for bleeding	0	1	
Total	0 (0%)	1 (1.7%)	
Grade IV	0	0	
Grade V	0	0	
Total number of complications	19 (31.7%)	21 (35%)	0.699

Table 3 Biochemical parameter changes at 1st, 3rd and 7th postoperative day

Parameters	Group A	Group B	*P* value
1st postoperative day			
TB (mg/dL)	2.50±0.91	1.47±0.71	0.000
ALT (IU/L)	305.6±212.9	329.4±264.9	0.589
AST (IU/L)	293.7±224.1	301.8±256.6	0.854
ALB (g/dL)	2.99±0.37	3.12±0.49	0.110
INR	1.49±0.22	1.27±0.16	0.000
3rd postoperative day			
TB (mg/dL)	2.33±1.33	1.56±0.95	0.000
ALT (IU/L)	201.6±156.2	269.6±246.2	0.074
AST (IU/L)	127.4±86.0	172.8±210.8	0.125
ALB (g/dL)	3.2±0.24	3.14±0.29	0.236
INR	1.31±0.20	1.18±0.07	0.000
7th postoperative day			
TB (mg/dL)	1.22±0.65	1.19±0.72	0.797
ALT (IU/L)	90.5±42.7	104.8±71.9	0.188
AST (IU/L)	59.1±32.6	60.8±40.4	0.808
ALB (g/dL)	3.53±0.32	3.51±0.42	0.743
INR	1.09±0.13	1.06±0.08	0.082

tive day, ALB for groups A and B had returned to normal levels (3.5–5.5 g/dL). The ALT and AST level changes were similar between the two groups.

Discussion

In our study, we found that the overall morbidity for a healthy donor, as classified by Clavien–Dindo classification, was the same as that for the hepatic patients with a normal liver; however, donors may suffer from more intense liver dysfunction during the early postoperative period. Compared to previous studies, this study analysed the outcome of the donor compared to a comparable group with normal liver. In addition, previous studies on donor safety concentrated on postoperative morbidity and mortality, with little regard for any postoperative biochemical parameter changes. We analysed the biochemical variables of the two groups and found that the donors (group A) experienced more serious liver dysfunction during the early postoperative period than the patients with hepatic lesions. In addition, group A patients demonstrated higher bilirubin and INR levels.

The morbidity rate associated with live liver donation varies widely. No uniform definition of complications exists, and different complication classifications are utilised.[1] A systematic review performed by Middleton et al.[14] revealed that the morbidity rate ranges from 0% to 100%, with a median of 16%. A report of the Vancouver Forum suggested that the complication rate associated with live right liver donation ranges from 20% to 60%, with a median of 35%.[7] With an overall morbidity rate for live liver donation of 31.7% and a complication rate for hepatic lesions with normal liver function of 35%, our results are comparable to those reported in some previous studies.[1,15] In our study, the different complication rates between donors and patients with hepatic lesions did not reach statistical significance. Pleural effusion was the most frequent complication for both donors and hepatic patients, although most of them did not require surgical treatment. Intraoperative manipulations such as right hepatic lobe mobilisation, which may stimulate the diaphragm, may be associated with this complication. However, many previous studies have suggested that biliary complication is the most common cause for liver donor morbidity, ranging from 5% to 18.6%.[16–20] In our study, 5% (n=3) of donors and 8.3% (n=5) of hepatic patients suffered from biliary complications. We believe that careful preoperative evaluation of the anatomy of the hepatic biliary tree could minimise the incidence of postoperative biliary complications. Overall, the total number and type of complications were distributed similarly across the two groups.

The major intraoperative risk from right hepatectomy is massive bleeding, resulting in intraoperative hypotension, ischemic injury and the need for transfusion.[21] Previous studies have confirmed that low central venous pressure and hepatic or hemihepatic blood inflow occlusion may greatly reduce intraoperative bleeding.[22,23] These techniques should be routinely utilised for patients with hepatic lesions to ensure surgical safety. Some investigators have reported that hepatic venous back-perfusion can maintain viability of hepatocytes during hepatic inflow occlusion; however, considering the risk of ischemic reperfusion injury, the safety of this vascular occlusion technique for use in live liver donation has not been well established.[21,24] Our study demonstrates that right hepatectomy for healthy donors results in more intraoperative bleeding than it does for non-cirrhotic hepatic patients; however, there was no observed significant difference in intraoperative blood product usage. The lack of blood inflow occlusion used in donor group patients may be associated with the increased intraoperative bleeding. Intraoperative autotransfusion for donors and patients suffering from some benign liver diseases, such as hepatic cavernous hemangioma, greatly minimised the need for allogeneic blood product transfusion.

Restoration of normal liver function is a significant concern following liver resection. Despite preoperative liver function tests confirming that patients could undergo right hepatectomy, postoperative liver dysfunction was not totally eliminated.[25] Fortunately, there were no liver dysfunction-related deaths in our study. However, significant differences in liver function were observed between

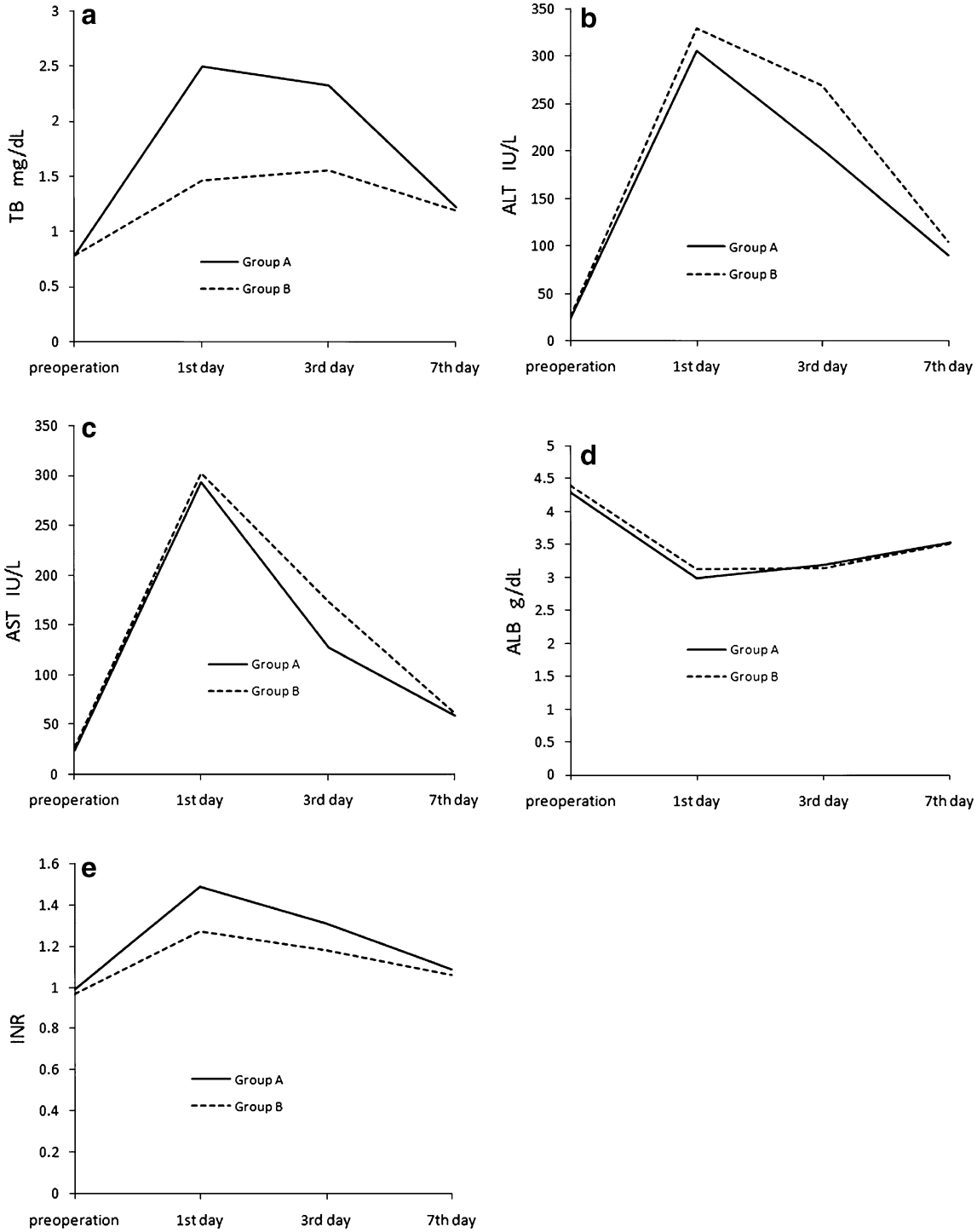

Fig. 1 Mean value of the total TB (**a**), ALT (**b**), AST (**c**), ALB (**d**), and INR (**e**) of the two groups before surgery and during the first postoperative week

the two groups. Early postoperative plasma TB and INR levels of the donors were much higher than the levels of hepatic patients with normal livers. Furthermore, a donor with hyperbilirubinemia (TB >5 mg/dL[26]) was observed in the donor group. Overall, liver dysfunction in the donors was more serious during the early postoperative period. Donor patients experienced removal of normal hepatic tissue. The hepatic group patients underwent removal of tumours or damaged hepatic tissues that had impaired liver function. The hepatic patients had time to compensate before surgery.[25] Thus, donors lost more normal hepatic tissue than hepatic patients.

There were some limitations in our study. We performed our analysis using 60 cases in each group. The surgical period of the two groups was non-synchronous; however, both of sets of surgeries were performed between 2009 and 2010. Thus, the non-synchronicity should have little influence on the final conclusions. In addition, the surgical techniques used on the patients in the two groups had some differences which may have resulted in different complications. We believe a larger number of patients and a multicentre study design would minimise these limitations.

In conclusion, similar complication rates and average blood product use can be achieved between donors and non-cirrhotic hepatic patients following right hepatectomy without MHV. However, the donors may experience intense liver dysfunction during the early postoperative period.

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ORIGINAL ARTICLE

Significance of Liver Hanging Maneuvers for Invasive Liver Lesions in No-Routine Anterior Approach Policy

Junichi Shindoh · Kiyoshi Hasegawa · Taku Aoki · Yoshifumi Beck · Yasuhiko Sugawara · Masatoshi Makuuchi · Norihiro Kokudo

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Abstract
Background Liver hanging maneuver is a widely used novel suspending technique to facilitate liver resection using the "anterior approach" where hepatic mobilization is preceded by parenchymal transection. However, its true indication and surgical advantages in the conventional "liver mobilization approach" are still controversial.
Study Design The medical records of 1,451 consecutive patients who underwent hepatectomy at a single institute, where conventional liver mobilization technique is routinely adopted, were retrospectively reviewed. Surgical situations in which the hanging maneuvers could actually be expected to be advantageous and the clinical outcomes of the tape-assist techniques were investigated.
Results Of the 1,451 hepatectomies, 1,446 (99.6%) were successfully performed using the conventional approach. Of the 1,446 patients, 42 (2.9%) required tape-assist techniques to secure safe surgical manipulation of bulky lesions (61.9%), tumor infiltration (16.7%), massive tumor thrombi (9.5%), vascular protection (7.1%), and other technical reasons (4.8%). The perioperative morbidity/mortality rates were 19.0/0% in these 42 tape-assisted cases, and 21.9/0.14% in the remaining 1,404 cases (p=0.82 and 0.06, respectively), with no significant difference in either the overall or the recurrence-free survival between the two groups.
Conclusions Although liver resection may be accomplished safely by the conventional approach in most cases, its safety may be enhanced by the use of valid tape-assist techniques in selected situations.

Keywords Liver hanging maneuver · Liver resection · Hepatectomy · Anterior approach

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J. Shindoh · K. Hasegawa · T. Aoki · Y. Beck · Y. Sugawara · N. Kokudo (✉)
Hepato-biliary-pancreatic Surgery Division,
Department of Surgery Graduate School of Medicine,
University of Tokyo,
7-3-1 Hongo, Bunkyo-ku,
Tokyo 113-8655, Japan
e-mail: KOKUDO-2SU@h.u-tokyo.ac.jp

M. Makuuchi
Department of Hepato-biliary-pancreatic Surgery,
Japan Red Cross Medical Center,
Tokyo, Japan

Introduction

Since the introduction of Belghiti's liver hanging maneuver[1] (LHM), a variety of sling suspension techniques[2–6] have been reported to facilitate safe, faster, and bloodless hepatic parenchymal transection, especially for massive and/or invasive liver lesions. These techniques are usually applied in combination with the so-called anterior approach,[7,8] in which the parenchymal transection is preceded by full mobilization of the part of the liver to be resected. However, the anterior approach is not always required during hepatectomies because mobilization and lifting of the liver with the operators' left hand, a basic technique in liver surgery, can be performed using the conventional approach, as long as a good surgical field can be obtained.[9]

Because the oncological benefit of the anterior approach still remains controversial, indications for this approach should be based purely on technical reasons. Therefore, the

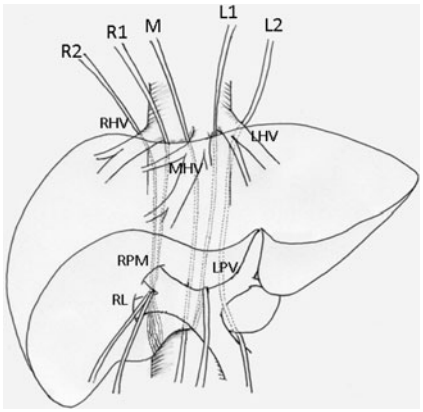

Fig. 1 Various positions of the tape for simple sling suspension. The original position of the tape is indicated as "Position M". In the tape switching technique, the position of the tape is modified to a position appropriate for each surgical procedure. *M* original position for right hepatectomy or extended left hepatectomy. *L1* position for left hepatectomy or extended right hepatectomy. *L2* position for isolated resection of the Spiegel lobe. *R1* position for left trisectionectomy or extended right lateral sectorectomy. *R2* position for right lateral sectorectomy. *LPV* left portal vein, *RPM* right paramedian portal pedicle, *RL* right lateral portal pedicle

authors have been routinely trying the conventional approach (i.e., full mobilization of the part of the liver to be resected prior to parenchymal transection), and applied a sling suspension with or without using the anterior approach only for selected cases, in which sling suspension would truly be expected to facilitate surgical manipulation.

In the present study, we reviewed the surgical situations in which the use of the hanging maneuvers could actually be expected to be advantageous and facilitate surgical manipulation under a no-routine anterior approach policy, and also present several tape-assist techniques to facilitate hepatectomies for invasive lesions using the conventional approach.

Material and Methods

Subjects

The subjects comprised 1,451 patients who underwent hepatectomy at the University of Tokyo Hospital between January 2000 and October 2008. Of these, five patients who were operated by the anterior approach due to difficulty in liver mobilization were excluded from this study, while the remaining 1,446 patients who were successfully treated using the conventional approach were included for the analysis.

The 1,446 patients were divided into two groups according to whether or not any tape-assist techniques were employed during the liver resection: the "tape-assist (+) group" and the "tape-assist (−) group"; the outcomes in the two groups were compared to clarify the surgical situations in which tape-assistance would confer true advantage and facilitate liver resection under the no-routine anterior approach policy.

Basic Surgical Techniques

The liver is exposed through either a J-shaped or inverted T-shaped abdominal incision. If necessary, a thoracotomy is

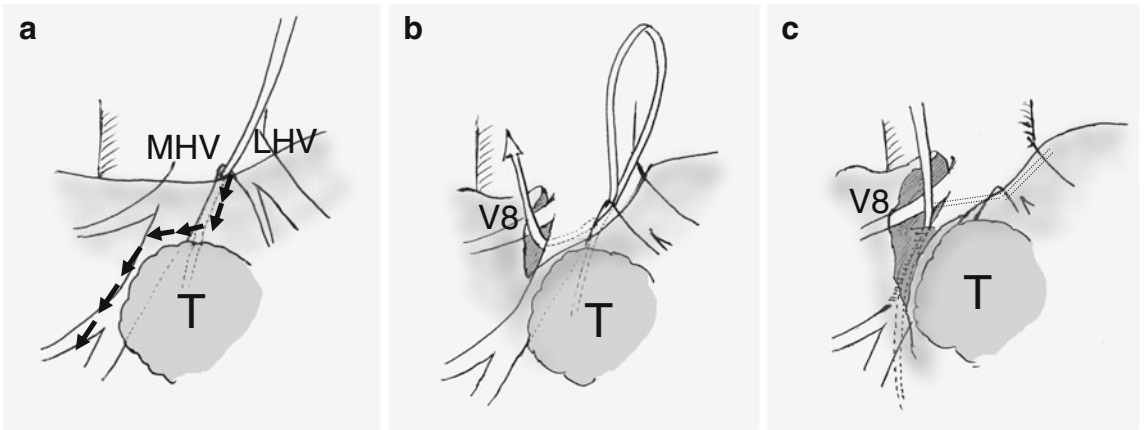

Fig. 2 Tape-repositioning technique for the selective hanging maneuver with vascular protection. The schema demonstrates the case of an extended left hepatectomy in a patient where the MHV was invaded at its peripheral part by a tumor. *Arrows* indicate the planned transection line (**a**). Because the thick drainage vein from segment VIII (*V8*) usually flows into the MHV near its root, the tributary was preserved through the tape-repositioning in this case (**b**). The tape served as a guide for transection and the cutting surface was designed for en-bloc excision of the peripheral part of the MHV (**c**). *T* tumor

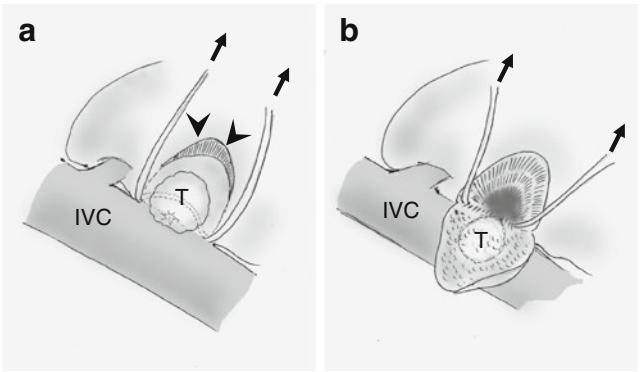

Fig. 3 Limited resection of a juxtacaval tumor using a regional sling suspension. Possible invasion of the IVC was suspected in this case and mobilization was impossible. After careful dissection around the tumor attachment site, the affected part was encircled and isolated by the tape. Parenchymal transection (*arrow heads*) was started with pulling up of the sling (**a**). Adequate hemostasis could be expected and the tumor infiltrative point was left as shown in (**b**). Thereafter, the part of the IVC infiltrated by the tumor could be treated safely

performed at the ninth intercostal space to obtain an adequate surgical field. Then, the location of the tumor and its relation to the vascular structures are evaluated by intraoperative ulitrasonography. The following surgical procedures are tailored to individual surgical situations.

Selection of the Transection Approach

In principle, we adopt the so-called conventional approach[9] for liver resection. After hilar dissection and isolation of the principal arterial/portal branches, the part of the liver to be resected is fully mobilized prior to parenchymal transection, because control of bleeding during liver transection is of utmost importance for safe liver surgery. In the case of a bulky tumor invading the IVC and/or the retroperitoneum, mobilization is difficult and sometimes dangerous with the use of the conventional approach. These situations are absolute indications for use of the anterior approach.[7,8] The hepatic parenchymal transection should be performed prior to the mobilization in such circumstances.

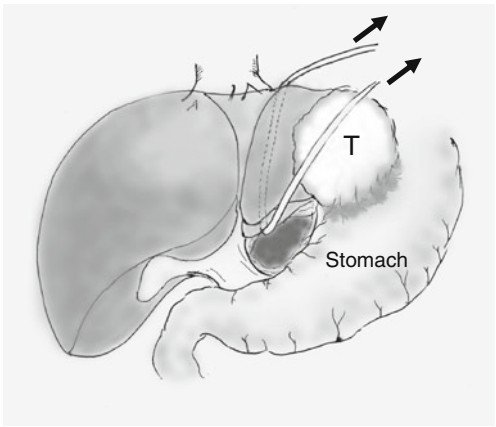

Fig. 4 Sling suspension of the left lobe for a bulky lesion showing severe adhesion to the stomach. In this case, the left lobe could not be mobilized due to the bulky lesion infiltrating the stomach. To obtain better exposure of the suprahepatic region, the left lobe was encircled and retracted using the tape. *T* tumor

Retrohepatic Tape Placement for the Hanging Maneuver

The LHM is applicable to both anatomic and non-anatomic liver resections in which the transection plane is oriented to the IVC. It is indicated for all patients treated by the anterior approach and for selected cases in which the conventional approach is used. Retrohepatic tape placement is basically performed in accordance with the original technique described by Belghiti[1] (i.e., typical LHM). It would be desirable to include real-time ultrasonographic confirmation[10] of the position of the significant short hepatic veins and of the dissection route for tape placement during this process, in order to decrease the risk of incidental injury to the short hepatic veins and to the retrohepatic liver capsule. If some resistance is felt at the tip of the Kelly's clamp during the retrohepatic dissection, where significant vascular structures cannot be visualized by US, forceful manipulation of the clamp is dangerous and should be avoided to prevent incidental injury. Under such instances, the tape should be placed after the side of the liver to be resected is partially or completely mobilized (modified LHM[6]).

Simple Sling Suspension Using the Retrohepatically Placed Tape (Fig. 1)

The original position of the tape for the hanging maneuver is indicated as "position M" in Fig. 1. To secure an adequate squeezing pressure on the cutting plane, the tape should encircle the plane to be transected. Accordingly, both tips of the tape should be adjusted from their original positions to the required appropriate positions using the tape adjustment methods reported by Kim et al.[5]

Selective Regional Hanging Maneuver Through a Tape-Repositioning Technique (Fig. 2)

During parenchymal transection, several significant venous tributaries derived from bigger venous trunks will be encountered. Because it has been reported that venous-deprived areas of the liver show poor regenerative potential,[11] significant venous tributaries should be preserved in accordance with the estimated congestive area in the future remnant liver. To apply adequate squeezing

Table 1 The demographics of 1,446 patients operated using the conventional liver mobilization approach

	Tape-assist (−)	Tape-assist (+)	*p* value
n	1,404 (97.1%)	42 (2.9%)	
Age	62 (19–84)[c]	65 (21–91)	0.161
Gender			
Male	1,028	27	0.199
Female	376	15	
Diagnosis			
Hepatocellular carcinoma	880 (98.3%)	15 (1.7%)	0.003
Metastatic tumor	415 (95.8%)	18 (4.2%)	
Intrahepatic cholangiocarcinoma	45 (88.2%)	6 (11.8%)	
Klatskin tumor	45 (95.7%)	2 (4.3%)	
Hemangioma	12 (100%)	0 (0%)	
Other diseases	7 (87.5%)	1 (12.5%)	
Procedure			
Hemihepatectomy	252 (92.6%)	20 (7.4%)	<0.001
Right hepatectomy	78 (94.0%)	5 (6.0%)	
Extended right hepatectomy	70 (93.3%)	5 (6.7%)	
Left hepatectomy	59 (92.2%)	5 (7.8%)	
Extended left hepatectomy	45 (90.0%)	5 (10.0%)	
Right trisectionectomy	3 (100%)	0 (0%)	
Left trisectionectomy	1 (33.3%)	2 (66.7%)	
Central bisectionectomy[a]	8 (100%)	0 (0%)	
Sectorectomy/sectionectomy	111 (96.5%)	4 (3.5%)	
Left lobectomy	45 (100%)	0 (0%)	
Extended left lobectomy[b]	4 (80.0%)	1 (20.0%)	
Right paramedian sectorectomy	24 (100%)	0 (0%)	
Right lateral sectorectomy	38 (92.7%)	3 (7.3%)	
Segmentectomy	281 (100%)	0 (0%)	
Limited resection	748 (97.9%)	16 (2.1%)	
Spiegel lobe resection	30 (71.5%)	12 (28.5%)	

[a] Resection of segments 4, 5, and 8

[b] Resection of segments 2, 3, and Spiegel lobe

[c] Figures represent median (range)

pressure on the regional plane between the veins to be preserved, the tape-repositioning technique[2] introduced by our group in living donor surgery may be useful. By switching the tips of the tape selectively behind venous tributaries, regional hanging of the liver parenchyma becomes possible and step-by-step parenchymal transection can be conducted safely.

Mini-hanging Maneuver for Juxtacaval Invasive Lesion (Fig. 3)

Sling suspension is also applicable in the other special surgical situations in which mobilization of the liver is difficult or impossible due to tumor infiltration of the surrounding tissues. Figure 3 represents a case of limited resection of a juxtacaval tumor that had invaded the IVC. In such a case, dissection of the anterior surface of the IVC should be performed from the side opposite to that of the liver part to be resected, leaving behind the tumor-infiltrated part. After the dissection, the remaining tumor-infiltrated part is encircled and isolated with the tape and parenchymal transection is started from the periphery toward the tape. During the parenchymal transection, the tape is used not only as a guide for transection, but also as a sling to hang the transection plane, like a mini-hanging maneuver. When the parenchymal transection is completed, only the tumor-infiltrated part remains and en-bloc resection of the wall of the IVC becomes safer and easier with the availability of a better surgical field.

Left Lobe Retraction Using a Tape (Fig. 4)

Figure 4 demonstrates another case with a bulky lesion in the left lobe infiltrating the stomach. In this case, neither insertion of the surgeon's hand behind the left lobe nor mobilization was possible. To obtain a better surgical field and safer access to the suprahepatic region for dissecting

Table 2 Reasons for adopting the tape-assist techniques according to diagnosis and surgical procedure

	No.	Tape-assist cases	Maximum diameter of tumor (cm)	Reasons for tape-assist techniques				
				Bulky lesion	Tumor invasion	Bleeding control	Tumor thrombus	Vascular protection
Diagnoses								
HCC	895	15 (1.7%)	7.5 (2–18)[a]	11	2	0	2	0
Metastatic tumor	433	18 (4.2%)	4.0 (2–24)	11	3	0	1	3
ICC	51	6 (11.8%)	4.8 (3–8.5)	3	2	1	0	0
Klatskin tumor	47	2 (4.3%)	Not evaluated	0	0	1	1	0
Other disease	20	1 (5.0%)	7.0	1	0	0	0	0
Procedures								
Right hepatectomy	83	5 (6.0%)	13.2 (10–24)	3	0	0	2	0
Extended right hepatectomy	75	5 (6.7%)	13.5 (2.6–18)	4	0	0	0	1
Left hepatectomy	64	5 (7.8%)	5 (3–9)	3	0	1	0	1
Extended left hepatectomy	50	5 (10.0%)	7.5 (4.5–17)	3	1	0	1	0
Left trisectionectomy	3	2 (66.7%)	8.5	1	0	1	0	0
Extended left lobectomy	5	1 (20.0%)	7.3	1	0	0	0	0
Right lateral sectorectomy	41	3 (7.3%)	4.6 (3.8–5)	1	0	0	1	1
Limited resection	764	16 (2.1%)	2.8 (1.1–7)	9	7	0	0	0
(Spiegel lobe resection)	(42)	12 (28.6%)	2.8 (1.1–7)	9	3	0	0	0

HCC hepatocellular carcinoma, *ICC* intrahepatic cholangiocarcinoma

[a] Figures represent median (range)

the ligaments and hepatic veins, a tape was placed behind the left lobe and utilized for traction.

Statistical Analysis

Clinical data were maintained on an Excel 2007 (Microsoft) spreadsheet and analyzed using the statistical software, JMP 8 (SAS institute Japan, Tokyo, Japan). For the statistical analysis, a Wilcoxon's rank sum test was used for continuous data and a chi-squared test or Fischer's exact test was used for categorical data, as appropriate. All analyses in this study were performed in accordance with the ethical guidelines for clinical studies at the University of Tokyo Hospital.

Table 3 Surgical outcomes

	Tape-assist (−) (n=1,404)	Tape-assist (+) (n=42)	p value
Operation time (min)	360 (280–456)	454 (330–589)	0.0004
Blood loss (ml)	690 (380–1,130)	1,060 (588–1,675)	0.002
Transfusion	57/850 (6.7%)[a]	6/42 (13.0%)	0.12
Thoracotomy	612/850 (72.0%)[a]	25/42 (59.5%)	0.08
Morbidity	185/850 (21.9%)[a]	8/42 (19.0%)	0.82
Bile leakage	79 (9.3%)	3 (7.1%)	
Surgical site infection	38 (4.5%)	3 (7.1%)	
Postoperative bleeding	7 (0.8%)	1 (2.4%)	
Refractory ascites	37 (4.4%)	0 (0%)	
Pulmonary embolism	0 (0%)	1 (2.4%)	
Hepatic failure	2 (0.2%)	0 (0%)	
Peptic ulcer	3 (0.4%)	0 (0%)	
Others	19 (2.2%)	0 (0%)	
Mortality	2/1,404 (0.14%)	0/42 (0%)	0.06

Figures represent median (inter-quartile range)

[a] Reference data from HCC database only

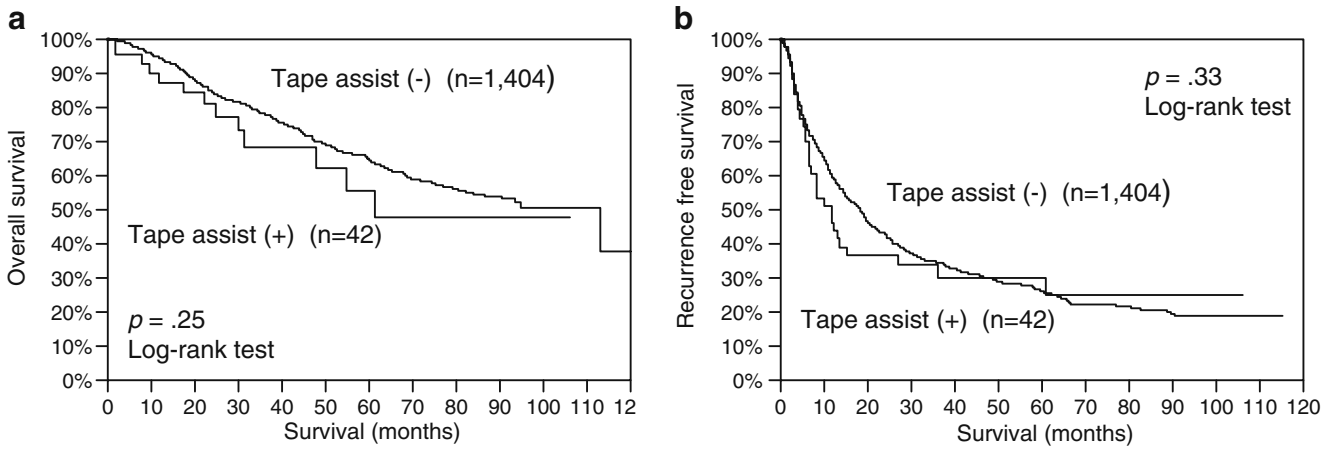

Fig. 5 Comparison of the postoperative survival curves between the cases with and without tape-assistance under the conventional liver mobilization approach. **a** Overall survival, **b** recurrence-free survival

Results

Among the 1,446 liver resections performed using the conventional approach, 42 patients (2.9%) required the use of the tape-assist techniques reported in this article: simple sling suspension (n=34; Fig. 1), selective regional hanging maneuver (n=3; Fig. 2), mini-hanging maneuver (n=4; Fig. 3), and/or left lobe retraction (n=1; Fig. 4). The demographics of the study population are summarized in Table 1. All patients were successfully operated using the conventional approach and no specific complication associated with the use of tape manipulation was recorded in the present series.

In Table 2, the indications for the use of the tape-assist techniques in the 42 patients are summarized. As expected, large tumor size was the most common indication, followed by tumor infiltration of the surrounding tissues. When stratified by the diagnosis, the frequency of use of tape-assist techniques was higher and the maximum diameter of the tumor was smaller in metastatic tumors and intrahepatic cholangiocarcinoma (ICC) as compared with those in hepatocellular carcinoma (HCC). In regard to the surgical procedures, the frequency of use of the tape-assist techniques ranged from 6.0% to 66.7% in major hepatectomy, while it was only 2.1% in limited resection. Among the several types of limited resections performed, tape-assistance was most frequently needed during isolated resection of the Spiegel lobe[5,12] (12/42, 28.6%).

The clinical outcomes of the 42 challenging cases in which tape-assistance was required and of the remaining 1,404 cases are compared in Table 3. Naturally, the operation time and blood loss in these challenging cases were significantly higher than those in the remaining typical hepatotectomies. However, there were no significant differences in the morbidity/mortality rates between the two groups. Regarding the long-term outcome, there was no significant difference between the two groups in either overall or the disease-free survival (Fig. 5).

Discussion

The LHM was first introduced by Belghiti et al.[1] in 2000 as an aid for major hepatectomy using the anterior approach.[7,8] To date, after several meticulous studies on the anatomical basis for the LHM,[13–15] the clinical indications of LHM have been expanded to include various anatomic resections[5] and even donor hepatectomies,[2] coupled with refinements of the tape manipulation techniques. However, the true indications of the anterior approach using/not using sling suspension techniques in liver surgery are still debatable.[9]

As we have demonstrated in the present study, the conventional approach could be applied safely in most cases (1,446/1,451, 99.7%), as long as an adequate surgical field was obtained. Indeed, thoracotomy was required in approximately 70% of the cases (Table 3). However, the morbidity/mortality rates in 1,446 cases were 21.6% and 0.14%, respectively. These results appeared to be comparable with those of the anterior approach reported elsewhere.[16,17] Despite the safety of the conventional approach, there still remain some difficult cases, such as patients with massive and/or invasive lesions, or tumor thrombi (42/1,446, 2.9%). To enhance the safety of the surgical manipulations in such cases, we have presented several tape-assist techniques based on the concept of the LHM.

In general, the LHM is utilized mainly in hepatectomy performed using the anterior approach; it is rarely required when the conventional approach is used, because the part of

the liver to be resected can easily be retracted with the retrohepatically inserted hand of the surgeon and therefore, bleeding control is relatively easy in the conventional approach. In challenging cases such as in patients with bulky and/or invasive lesions, however, the size of the tumor or tumor infiltration of the surrounding tissues may interfere with the standard surgical manipulations, including mobilization or retraction of the part of the liver to be resected. Under conditions when elevation or compression of the transection plane is impossible, blood loss during parenchymal transection may increase, because hemorrhage from the cut surface of the liver is attributed for the most part to the backflow from the hepatic veins under total vascular occlusion at the hepatic hilum.

The techniques reported herein were originally devised as aids for surgical manipulations in different surgical situations during resection of invasive liver lesions. The mini-hanging maneuver (Fig. 3) and left lobe retraction (Fig. 4) have been suggested to be useful for invasive tumors in the juxtacaval portion and tumors in the left lobe, respectively, for accessing the part of the vena cava infiltrated by the tumor or the root of the hepatic veins, both of which are difficult to expose in the conventional approach. The selective regional hanging maneuver (Fig. 2), which is a modification of the tape-repositioning technique,[2] enables simultaneous fine manipulation of the vessels to be preserved and curative resection of the lesion. By flexibly switching the tips of the retrohepatically inserted tape, adequate squeezing pressure can be applied on the "regional" liver parenchyma to be transected in a step-by-step fashion.

Using the reported techniques, curative resections were accomplished safely in all of the 42 challenging cases, and no specific complications associated with the use of the tape manipulations were recorded. Both the short-term and long-term outcomes appeared to be comparable with those in the remaining 1,404 cases (Table 3, Fig. 5). Although the reported tape-assist techniques were not required so frequently, the combined use of the respective taping methods was apparently helpful for fine surgical manipulation.

In liver surgery, because the functional reserve is often limited due to chronic hepatitis or cirrhosis, extended resection is not always safe in the treatment of invasive lesions. Also, because the recurrence rate is generally high for liver tumors, drainage veins for the remnant liver should be preserved as much as possible to allow surgical options for future repeat liver resection. In such a context, delicate manipulation of the blood vessels to be preserved around the tumor or complex limited resection of the liver is sometimes required to balance the safety and curativeness of the surgery.

Our results suggest that most liver resections can be accomplished using the conventional approach without any tape-assist techniques, as long as an adequate surgical field can be obtained. However, the tape-assist techniques demonstrated herein may facilitate safe and curative liver resections in selected cases in which conventional mobilization or vascular preservation is difficult.

Conclusion

Liver resection may be accomplished through the conventional mobilization approach in most cases, and rarely requires sling suspension as long as an adequate surgical field can be obtained. However, the safety of the conventional approach may be enhanced by the use of valid tape-assist techniques, which may facilitate surgical manipulations, especially in the resection of invasive liver lesions.

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ORIGINAL ARTICLE

Changes of Organic Anion Transporter MRP4 and Related Nuclear Receptors in Human Obstructive Cholestasis

Jin Chai · Donglin Luo · Xiaoping Wu · Huaizhi Wang · Yu He · Qiong Li · Yanmei Zhang · Lei Chen · Zhi-Hong Peng · Tianli Xiao · Rongquan Wang · Wensheng Chen

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Abstract
Background Hepatic multidrug resistance-associated protein 4 (Mrp4) levels are low, but increase markedly in rodent cholestatic liver. Nuclear receptors (NRs) are essential for regulating Mrp4 expression in cholestasis models. However, information about MRP4 and related NRs, including constitutive androstane receptor (CAR), pregnane X receptor (PXR), and retinoic X receptor-α (RXRα), is relatively lacking in human obstructive cholestasis. We collected liver samples from patients with obstructive cholestasis or without liver disease and investigated the expression of MRP4 and NRs CAR, PXR, and RXRα by semi-quantitative RT-PCR, Western blot and immunostaining assays.
Results MRP4 mRNA/protein levels were markedly increased in obstructive cholestasis. Concentration of serum total bile acids (TBA) was significantly correlated with MRP4 protein in cholestasis samples ($P<0.01$). PXR and RXRα mRNA/protein levels were significantly increased in obstructive cholestasis. CAR mRNA levels were unchanged while protein levels were markedly induced in obstructive cholestasis. There was a statistically positive correlation between MRP4 mRNA and CAR protein ($P<0.05$), suggesting that CAR may activate transcription of MRP4 genes by its nuclear translocation.
Conclusion Hepatic MRP4 levels were dramatically induced in human obstructive cholestasis, which may reduce liver injury by increasing efflux of toxic bile acids from hepatocytes into blood.

Jin Chai and Donglin Luo contributed equally to this study.

J. Chai · X. Wu · Y. Zhang · L. Chen · Z.-H. Peng · T. Xiao · R. Wang · W. Chen (✉)
Institute of Gastroenterology, Southwest Hospital,
Third Military Medical University,
Chongqing 400038, People's Republic of China
e-mail: wenshengchen@hotmail.com

J. Chai
e-mail: chaijinsw@yahoo.com

X. Wu
e-mail: wxpwxh_2008@163.com

Y. Zhang
e-mail: zyanmei1983@163.com

L. Chen
e-mail: chenlei1977603@126.com

Z.-H. Peng
e-mail: penzhh@yahoo.com.cn

T. Xiao
e-mail: miker_x2003@yahoo.com

R. Wang
e-mail: Rongquanw@hotmail.com

D. Luo
Department of Abdominal Surgery, Daping Hospital,
Third Military Medical University,
Chongqing 400038, People's Republic of China
e-mail: ldl1967@sina.com

H. Wang · Y. He
Institute of Hepatobiliary Surgery, Southwest Hospital,
Third Military Medical University,
Chongqing 400038, People's Republic of China

H. Wang
e-mail: whuaizhi@gmail.com

Y. He
e-mail: heruidi505@sina.com

Q. Li
Laboratory and Education Center,
College of Basic Medical Science,
Third Military Medical University,
Chongqing 400038, People's Republic of China
e-mail: liqiong@hotmail.com

Keywords Hepatic multidrug resistance-associated protein 4 · Nuclear receptors · Cholestasis

Introduction

Multidrug resistance-associated protein 4 (MRP4/Mrp4; *ABCC4*/*Abcc4*) is an ATP-dependent organic anion transporter belonging to the ABC transporter superfamily.[1–4] It is widely expressed in a variety of tissues, including the liver, kidneys, erythrocytes, adrenal glands, platelets, brain, and pancreas in humans and rodents.[2,4–11] A unique feature of MRP4/Mrp4 is its dual localization in polarized cells; it can be present in both basolateral membranes of the liver as well as apical membranes of kidneys, depending on the cell type.[5,6] MRP4/Mrp4 functions as an efflux pump for bile acids, folic acid, 6-mercaptopurine, conjugated steroids, methotrexate, dehydroepiandrosterone-3-sulphate, among others.[1–3,5–7,10–15] Hepatic Mrp4 levels are normally low in rodents, but are elevated in obstructive cholestasis.[16–18] Similar to elevations in expression of multidrug resistance-associated protein 3 (Mrp3; *Abcc3*) and organic solute transporter α/β (Ostα/β), induction of hepatic Mrp4 expression is also considered to be an adaptive response to obstructive cholestasis in rodents, which may play a role in counteracting intracellular bile acid toxicity by increasing efflux of bile acids from hepatocytes into blood.[17–22] Recent research suggests that Mrp4 rather than Mrp3 represents the major hepatic basolateral bile acid export pump in obstructive cholestasis.[23,24] However, the role of hepatic MRP4 in humans is not well understood in obstructive cholestasis diseases.

Nuclear receptors (NRs), including constitutive androstane receptor (CAR), pregnane X receptor (PXR), retinoic X receptor-α (RXRα), and farnesoid X-activated receptor (FXR), play essential roles in the transcriptional regulation of hepatobiliary transport systems involved in bile acid metabolism in cell lines and animal cholestasis models.[18,25–30] CAR and PXR have been reported to participate in regulation of transcription of drug transporter genes (e.g., Mrp2-4) and transcriptional activity of NRs correlates well with their concentration in the nucleus.[27–31] Bilirubin can modulate CAR activity by indirect activation, promoting its subsequent nuclear translocation and heterodimerization with RXRα to regulate target genes such as Mrp2-4.[26–33] The pregnane X receptor PXR is activated by ligands of bile acids and also dimerizes with RXRα in the nucleus to induce expression of Mrp2-4.[29–31,33] The importance of NRs in orchestrating the adaptive response to toxic bile acids has been demonstrated in various NR knockout mouse models.[29,33–35] To date, however, there is little published information about localization and expression of altered NRs CAR, PXR, and RXRα in human obstructive cholestasis.

Therefore, more insight is needed on the role of hepatic MRP4 expression and altered NRs in human obstructive cholestasis diseases. In the present study, we collected liver samples from ten cases each of obstructive cholestasis and controls without liver disease. Expression and localization of MRP4 and NRs CAR, PXR and RXRα in liver samples were then determined by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR), Western-blot and immunofluorescence assays. Finally, we statistically analyzed the relationship between MRP4 mRNA and protein levels of NRs PXR, CAR, or serum total bile acids (TBA) by the Pearson correlation test. Consistent with results from rodent models, our results indicate that expression of MRP4 was markedly elevated in human obstructive cholestasis diseases, which may reduce liver injury by increasing efflux of toxic bile acids from hepatocytes into blood. The transcriptional activation of MRP4 gene may be mainly regulated by the nuclear translocation of CAR.

Materials and Methods

Patient and Sample Collection

Liver specimens comprising samples from patients with obstructive cholestasis (n=10) and controls without liver disease (n=10) were analyzed. All liver samples were collected from Southwest Hospital, Third Military Medical University, Chongqing, China. Control liver samples were acquired by liver biopsy and analyzed for exclusion of liver disease (n=4); in addition, normal liver tissue was also obtained during resections for colorectal metastases without cholestasis (n=6). The resected cholestasis liver tissues were from patients with obstructive jaundice caused by stones originating from common bile duct. Surgery for those patients was carried out within 3 days of admission due to severe symptoms of biliary obstruction and jaundice; no ursodeoxycholic acid choleretic drugs or other preoperative therapy was administered. The isolated liver samples were immediately cut into small pieces and frozen in liquid nitrogen until used. Biochemical characteristics of patients are listed in Table 1. This study was approved by the hospital institutional ethics review board, and informed consent was obtained from all participants.

RNA Extraction and RT-PCR

Samples of liver tissue frozen in liquid nitrogen (100 mg) were ground and subsequently used for extraction of total RNA with Trizol according to instructions of the vendor (Invitrogen, San Diego, CA, USA). cDNA was synthesized from 1 μg of total RNA using a reverse transcription kit

Table 1 Clinical features of patients[a]

Total samples (males/females)	Controls 10 (7/3)	Obstructive cholestasis samples 10 (4/6)
Age (years)	51±3	52±3
TBA (μ mol/L)	3±1	53±16**
Bilirubin (μ mol/L)	13±1	156±46*
ALT (IU/L)	27±3	277±101**
ALP (IU/L)	76±12	207±45**

TBA total bile salts, *ALT* alanine aminotransferase, *ALP* alkaline phosphatase

*$p<0.01$; **$p<0.05$ versus controls

[a] Note: values are means ± SEM

(Fermentas GmbH, St. Loen-Rot, Germany), and PCR was subsequently performed with 2×Taq PCR master mix (TIANGEN, Beijing, China). The PCR mixtures were initially denatured at 94°C for 4 min, followed by denaturation for 45 s at 94°C, primer annealing for 30 s at 62–66°C, and extension for 1 min at 72°C for 26–32 cycles with the iCycler thermal cycler (Bio-Rad Laboratories Inc., Hercules, CA, USA). A final extension for 7 min at 72°C ensured complete extension of the PCR products. Detailed parameters for PCR are summarized in Table 2. PCR products were separated on 2% agarose gels and band intensity was quantified using QuantityOne 4.2.0 software (Bio-Rad Laboratories Inc., Hercules, CA, USA) after background subtraction from each band. glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization.

Western Blot Analysis

Total membranes and nuclear extracts were prepared as described previously[21,36,37] and protein concentrations were determined using a Bradford kit (Thermo Scientific, Rockford, IL, USA). For Western immunoblot analysis, 50 μg of nuclear extract or 100 μg of membrane protein were loaded on to 12% or 7.5% SDS polyacrylamide gels. Following electrophoresis, gels were subjected to electroblot onto PVDF membranes, and uniformity of loading and transfer was confirmed by Ponceau staining. Membranes were blocked in TS complete solution (20 mM Tris–HCl, 150 mM NaCl, 5% non-fat dry milk, and 0.1% Tween 20) for 2 h at 37°C. Blots were incubated overnight at 4°C with anti-MRP4 1:1,000 (Abcam, Cambridge, MA, USA), anti-RXRα 1:1,600, anti-CAR1/2 1:1,000, and anti-PXR 1:1,600 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were washed and then incubated with anti-rat, anti-rabbit, or anti-mouse horseradish peroxidase-conjugated antibody (1:50,000; Thermo Scientific) for 1 h at room temperature. Immune complexes were detected with the SuperSignal Femot reagent kit (Thermo Scientific). Membranes were then stripped and used for detection of cytoplasmic protein (anti-GAPDH, 1:8,000; Abcam) or nuclear protein (anti-SH-PTP1, 1:2,500; Santa Cruz) to calibrate the target protein signals.

Immunofluorescence Analysis

Small blocks of liver tissues embedded in Tissue-Tek O.C.T. Compound (Sakura Finetechnical Co. Ltd., Tokyo, Japan) were snap frozen in liquid nitrogen. The frozen block was then used to prepare 6-μm thick sections at −25°C, which were then fixed in acetone at room temperature for 10 min. Sections on the slides were hydrated twice in phosphate-buffered saline (PBS) for 15 min, blocked for 1 h with 5% BSA, and then incubated with anti-MRP4 (1:50), anti-RXRα (1:100), anti-CAR1/2 (1:50), or anti-PXR (1:50) diluted in PBS overnight at 4°C. After rinsing with PBS, slides were incubated with FITC-labeled secondary antibodies for 1 h at 37°C. Samples were mounted in VECTASHIELD with propidium iodide (Vector Laboratories, Burlingame, CA, USA) for counter-staining of nuclei. Sections were analyzed with a confocal fluorescence microscope.

Statistical Analysis

Statistical analysis was performed using the SPSS 10.0 software package (SPSS, Chicago, IL, USA). Values were expressed as mean ± SD of at least three experiments. The difference between two groups was analyzed by paired-samples *t* test or independent-samples *t* test (two-tailed). The correlation among CAR, PXR, and MRP4 mRNA and protein or serum TBA was assessed by the Pearson's correlation test. A value of $P<0.05$ was considered as statistically different.

Table 2 Sense and antisense primers used for RT-PCR

Gene	Sense primers(5′→3′)	Antisense primers(5′→3′)	Annealing temperature (C)	Cycles	Products (bp)
MRP4	TGCCTTTGGGTCCCGATTC	TGGTGGTGGGCGTTTCTGAT	66	32	208
CAR	ACTGAGTAAGGAGCAAGAA	CAGGGAACGGAAGACG	56	30	241
PXR	AGAGCGGCATGAAGAAGGAGATG	GAAATGGGAGAAGGTAGTGTCAAAGG	62	30	207
RXRα	CGGAACGAGAATGAGGTGG	GCTTGGCAAATGTTGGTGA	62	30	170
GAPDH	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTTGCTGTA	63	28	452

Results

Expression of the Basolateral Bile Acid Transporter Protein MRP4 in Hepatocytes Is Induced in Obstructive Cholestasis

We performed semi-quantitative RT-PCR and Western blot analyses using total RNA and membrane protein from liver of obstructive cholestasis samples and controls. MRP4 mRNA (Fig. 1a, c) and protein (Fig. 1b, d) levels were significantly increased in liver samples from patients with obstructive cholestasis versus controls (2.0- and 3.1-fold, respectively). Immunofluorescence assay also demonstrated that MRP4 staining at the basolateral membrane in cholestatic hepatocytes was significantly higher than that in controls (Fig. 2a, b). These data suggested that the basolateral membrane transporter MRP4 was markedly induced under cholestatic conditions, which may be an adaptive response to cholestasis in humans.

Correlation of Serum TBA/Bilirubin and MRP4 Protein Levels in Obstructive Cholestasis Patients

We next analyzed the correlation between serum TBA/bilirubin and MRP4 protein levels in cholestatic human liver samples by Pearson's correlation analysis. Concentration of serum TBA was positively correlated with MRP4 protein level in cholestasis samples (r=0.81 and P<0.01; Fig. 3). There was no correlation, however, between serum bilirubin and MRP4 protein in cholestasis samples (data not shown). These data indicate that the marked elevation of serum TBA may be mainly associated with the hepatic MRP4 levels that are dramatically induced in obstructive cholestasis in humans.

Changes of Nuclear Receptors CAR, PXR, and RXRα in Human Obstructive Cholestasis

We next determined whether or not expression of CAR, PXR, and RXRα was also induced in human obstructive cholestasis. In the present study, CAR mRNA (Fig. 4a, b) levels were unchanged while protein levels were markedly induced (2.0-fold; Fig. 4c, d) in obstructive cholestasis as compared with the controls. Both PXR mRNA and protein were significantly up-regulated in obstructive cholestasis (1.7- and 3.1-fold, respectively) compared with the controls (Fig. 4a–d). RXRα mRNA and protein were also dramatically elevated in obstructive cholestasis (1.9- and 3.6-fold, respectively; Fig. 4a–d). CAR, PXR, and RXRα staining was also more prominent in the nucleus of obstructive cholestasis liver cells compared with the controls (Fig. 5). Similar to results in rodent models, these data indicate that CAR may undergo nuclear translocation and CAR or PXR may also dimerize with RXRα to regulate the expression of the basolateral membrane transporter MRP4.

Significant Correlation of MRP4 mRNA and CAR Protein in Obstructive Cholestasis Liver Samples

Inasmuch as CAR and PXR are known to participate in regulation of transcription of drug transporter genes such as

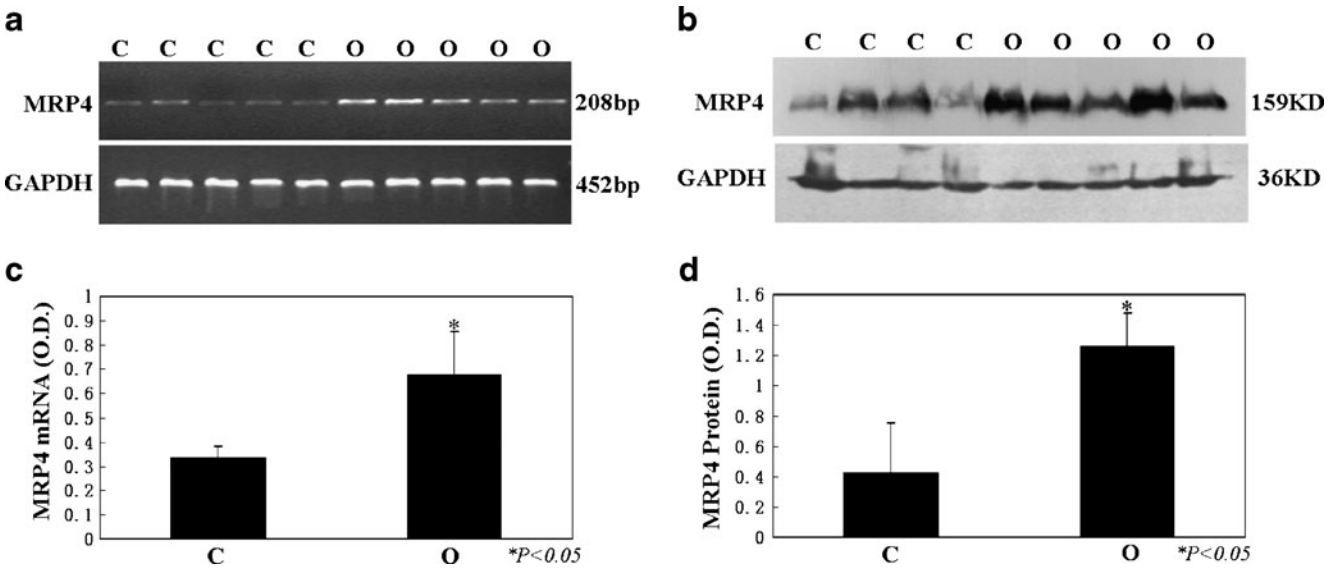

Fig. 1 RT-PCR and Western blot analysis of MRP4 expression in liver tissue of patients with obstructive cholestasis. **a** RT-PCR analysis of MRP4 in liver tissue of patients with obstructive cholestasis and controls. **b** Western blot analysis of MRP4 protein in liver tissue of patients with obstructive cholestasis and controls. **c**, **d** Quantitations of A and B, respectively. Data were analyzed using paired samples t test. *P<0.05 vs. controls. *C* controls, *O* obstructive cholestasis liver samples

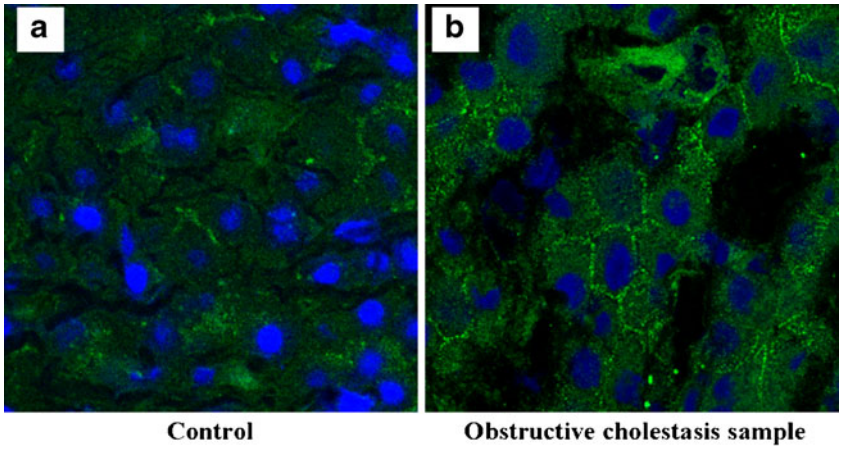

Fig. 2 Immunostaining analysis of MRP4 expression in liver tissue of patients with obstructive cholestasis and controls. **a** Membrane localization of MRP4 staining in liver tissues of controls; **b** membrane localization of MRP4 staining in liver tissues of patients with obstructive cholestasis

MRP2-4[25–33] and transcriptional activity of NRs correlates well with their concentrations in the nucleus,[26–33] we analyzed the correlation between CAR or PXR protein and MRP4 mRNA levels in tissue samples by Pearson's correlation analysis. MRP4 mRNA levels were positively correlated with CAR protein levels in obstructive cholestasis samples (r=0.68 and P<0.05; Fig. 6a). A statistically positive, although less prominent, correlation was also found between CAR and MRP4 protein levels (data not shown). However, there was no correlation between MRP4 mRNA and PXR protein (r=0.42 and P=0.23; Fig. 6b). Statistical analysis suggested that expression of MRP4 may be mainly induced by nuclear receptor CAR and not by nuclear receptor PXR.

Discussion

MRP4/Mrp4 is an important organic anion transporter at the basolateral membrane in hepatocytes.[6,16,24] Substrates of MRP4/Mrp4 include bile acids, folic acid, steroids and several anti-cancer drugs.[3,6,7,10,11] Levels of the basolateral membrane transporter Mrp4 in hepatocytes under physiological conditions are relatively low, but increase markedly as an adaptive response in rodent cholestatic liver.[1–3,5–7,10–16] It has been suggested that alterations in NRs such as CAR, PXR, RXRα and FXR are essential for mediating this response in cholestasis models.[16–18] Little information is available, however, regarding MRP4 and related NRs in human obstructive cholestasis liver disease. To address this issue, we explored the role of hepatic MRP4 expression and the changes of related NRs CAR, PXR, and RXRα in human obstructive cholestasis diseases, which may have major clinical and therapeutic implications.

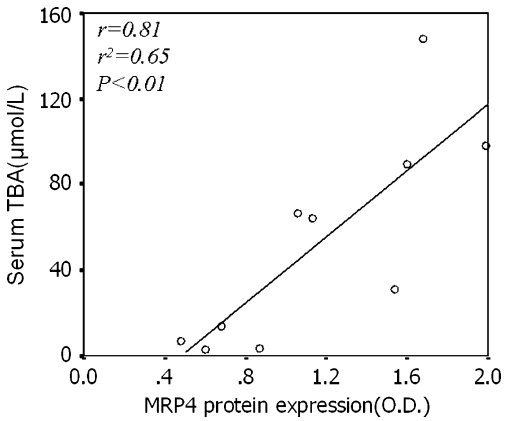

Fig. 3 Correlation between serum TBA and MRP4 protein levels in liver tissue samples from patients with obstructive cholestasis. Data were analyzed by the Pearson's correlation test. *TBA* total bile acids, *r* Pearson's correlation coefficient; n=10

Recent research suggests that Mrp4 represents the major hepatic basolateral bile acid transporter in obstructive cholestasis animal models.[23,24] Although Mrp3 and Ostα/β expression were markedly increased, Mrp4/mice also exhibit liver injury in cholestasis models.[24] In this study, we found that patients with obstructive cholestasis diseases caused by stones originating from common bile duct had significantly higher levels of hepatic MRP4 expression than controls. MRP4 staining at the basolateral membrane domain in cholestatic hepatocytes was also more prominent than in controls. These data indicate that hepatic MRP4 levels were dramatically induced in human obstructive cholestasis, which may be an adaptive response to cholestasis. Previous studies have demonstrated that hepatic Mrp4 expression level was up-regulated markedly in obstructive cholestatic rodent liver.[16–18] MRP4 mRNA expression tended to be elevated in patients with extrahepatic cholestasis caused by pancreatic malignancy.[38] Hepatic MRP4 protein expression increased significantly in liver tissues from patients with PBC.[39] In addition, Mrp4 expression both at mRNA and protein levels was also induced in animal models with non-obstructive cholestasis, such as sepsis-associated cholestasis and carbon tetrachloride-induced liver injury.[40,41] Those studies implied that bile acids and/or other factors

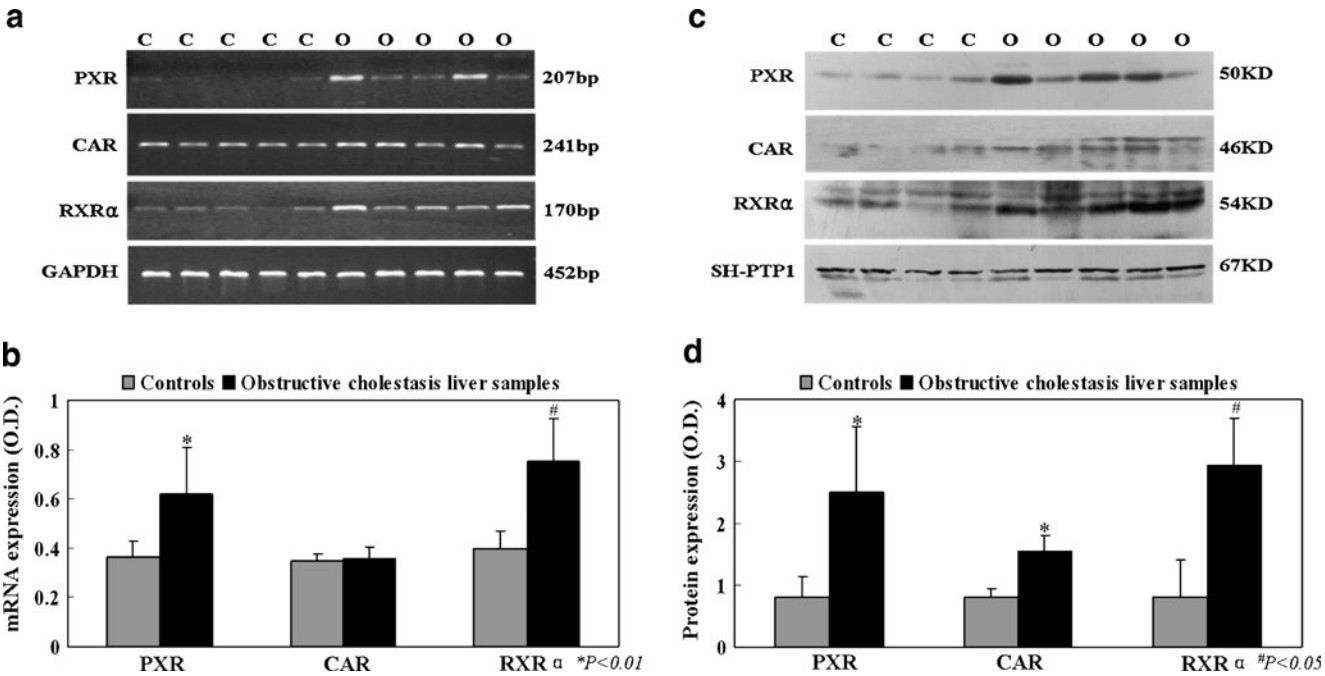

Fig. 4 RT-PCR and Western blot analysis of the expression of PXR, CAR, and RXRα in liver of patients with obstructive cholestasis. **a** RT-PCR analysis of PXR, CAR, and RXRα in liver tissue of patients with obstructive cholestasis and liver tissue of controls. **b** Quantitation of mRNA levels of PXR, CAR, and RXRα; **c** Western blot analysis of PXR, CAR, and RXRα in liver tissue of patients with obstructive cholestasis and liver tissue of normal controls. GAPDH and SH-PTP1 were used as internal references for RT-PCR and Western blot, respectively. **d** Quantitation of protein levels of PXR, CAR, and RXRα. Data were compared using paired samples *t* test; **P*<0.01; #*P*<0.05 vs. controls. *C* controls, *O* obstructive cholestasis liver samples

(e.g., cytokines) were involved in the up-regulation of MRP4/Mrp4 expression in both obstructive and non-obstructive cholestasis, rather than the increased intraductal pressure.

Because hepatic MRP4/Mrp4 transports bile acids with high affinity[33] and concentrations of serum TBA are dramatically increased in blood of obstructive cholestasis patients, we analyzed the correlation between serum TBA and hepatic MRP4 protein levels by Pearson's correlation analysis. There was a statistically positive correlation between serum TBA and MRP4 protein levels in cholestasis patients. This suggests that the dramatic increase in concentrations of TBA in the blood of patients may be closely associated with the induction levels of hepatic MRP4 under obstructive cholestasis conditions. The present study also concludes that a possible protective mechanism in liver cholestasis patients due to induction of hepatic MRP4 may be that this induction counteracts intracellular bile acid toxicity by increasing efflux of bile acids from hepatocytes into blood.

The mechanism of regulation of MRP4 gene expression is presently unclear. It has been reported that NRs, including CAR, PXR, RXRα, and FXR, participate in the regulation of transcription of drug transporter genes (e.g., MRP2, MRP3, MRP4, and OSTα/β), and transcriptional activity of NRs correlates well with their concentration in the nucleus.[18,25–35] CAR and PXR have been reported to be activators of Mrp4 expression in rodents.[29,32] Bilirubin can modulate CAR activity by indirect activation. CAR activation can occur by direct binding of an agonist, subsequent nuclear translocation, and heterodimerization with RXRα, prior to DNA binding and induction of drug transporter gene expression.[26–33] PXR is activated by bile acid ligands and also dimerizes with RXRα in the nucleus, inducing expression of MRP2-4.[29–31,33] The importance of NRs in the adaptive response to toxic bile acids has been demonstrated in various NR knockout models.[29,33–35] In the present study, we found that CAR mRNA levels were unchanged but protein levels increased markedly in obstructive cholestasis samples. Meanwhile, CAR staining was also more prominent in the nucleus of obstructive cholestasis hepatocytes. The expression of nuclear PXR and RXRα, as determined by semi-quantitative RT-PCR, Western blot, and immunofluorescence, was also dramatically induced in obstructive cholestasis. These data indicate that CAR may undergo nuclear translocation and heterodimerization with RXRα in the nucleus of hepatocytes in obstructive cholestasis. There was a statistically positive correlation, not between PXR protein levels and MRP4 mRNA levels, but between CAR protein levels and MRP4 mRNA levels. This suggests that expression of MRP4 at the hepatocellular basolateral membrane may be mainly

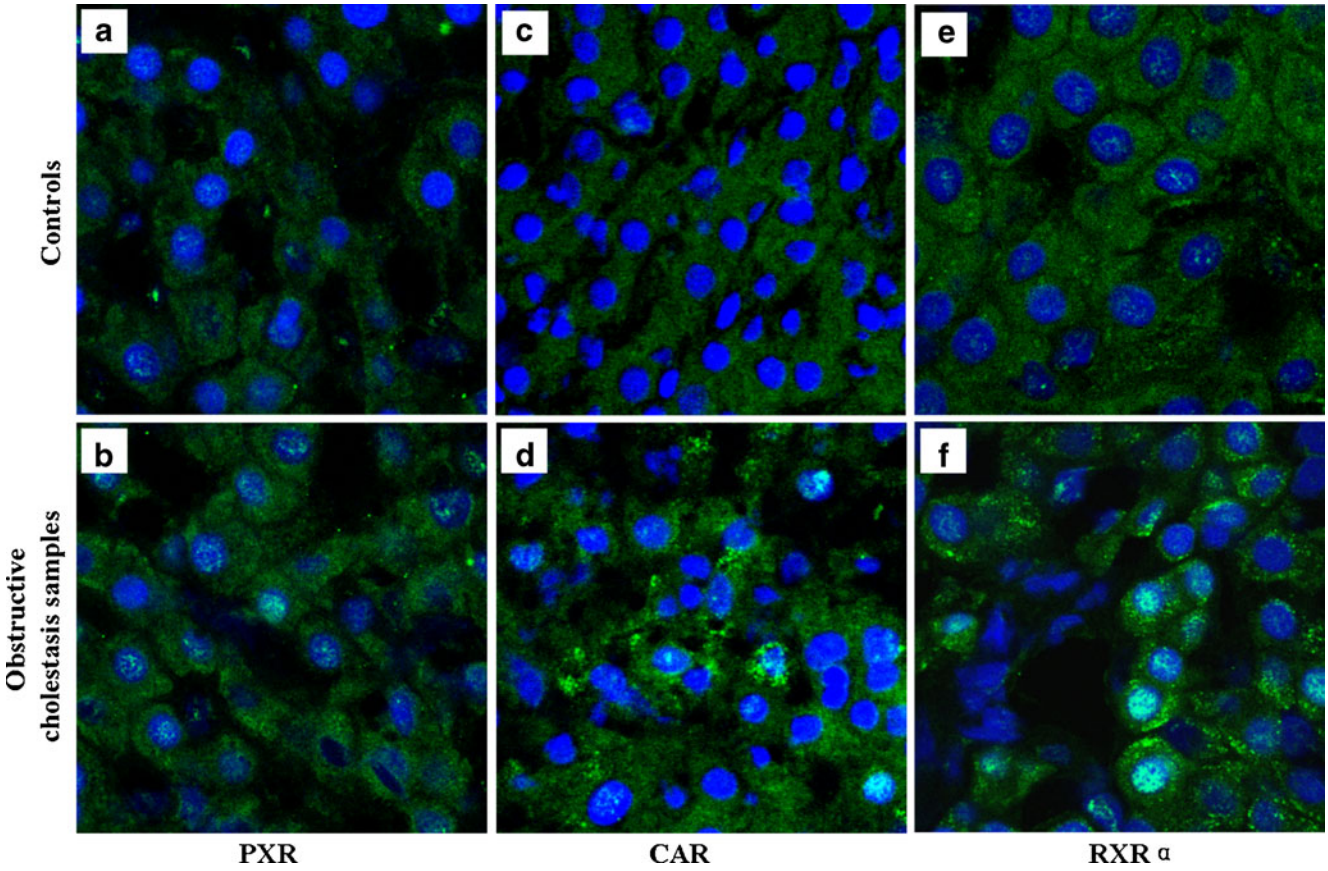

Fig. 5 Immunofluorescence analysis of the expression of PXR, CAR, and RXRα in liver tissue of patients with obstructive cholestasis and controls. **a**, **b** PXR staining in liver tissue from normal control and obstructive cholestasis, respectively; **c**, **d** Staining of nuclear receptor CAR in liver tissue from normal control and obstructive cholestasis, respectively; **e**, **f** staining of RXRα in liver tissue from normal control and obstructive cholestasis, respectively

induced not by nuclear receptor PXR, but by the nuclear translocation of nuclear receptor CAR in human obstructive cholestasis. In addition, CAR and PXR heterodimerization with RXRα/SXR in the hepatocellular nucleus may also play an essential role for regulation of phase I and II bile acid detoxification enzymes in obstructive cholestasis.[31,32,42] However, additional studies are needed to fully demonstrate this in human obstructive cholestasis diseases.

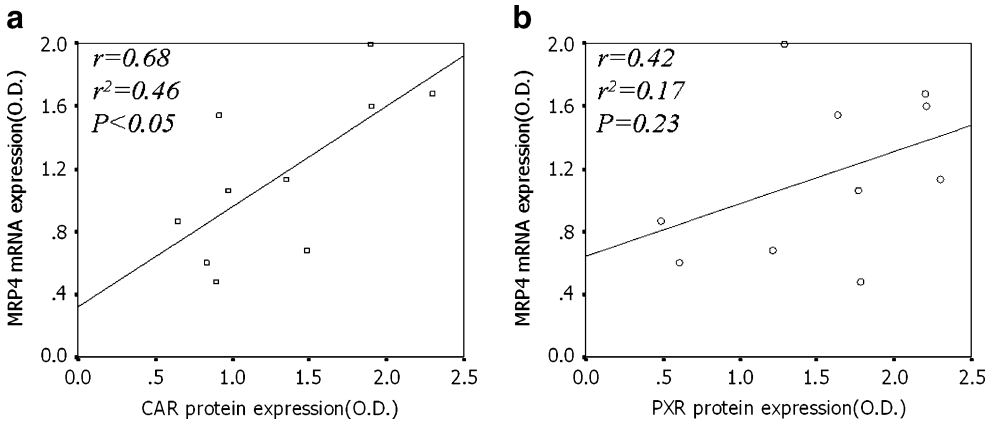

Fig. 6 Correlation between CAR or PXR protein levels and MRP4 mRNA levels in obstructive cholestasis tissue samples. **a** Significant correlation between CAR protein and MRP4 mRNA in obstructive cholestasis tissue samples; **b** lack of correlation between PXR and MRP4 mRNA in obstructive cholestasis tissue samples. Data were analyzed by the Pearson's correlation test. *r* Pearson's correlation coefficient, $n=10$

Conclusions

In summary, our study demonstrated that hepatic MRP4 levels were significantly elevated at the basolateral membrane in human obstructive cholestasis, which may be an adaptive response to cholestasis to reduce liver injury by increasing efflux of toxic bile acids from hepatocytes into blood. Nuclear receptor CAR may activate transcription of MRP4 by nuclear translocation in cholestatic conditions. Because of the important roles of MRP4 and CAR in obstructive cholestasis, there may be therapeutic benefits for cholestasis patients in the future.

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ORIGINAL ARTICLE

Prevention of Biliary Leaks

Nathaniel J. Soper

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Abstract
Introduction Since the introduction of laparoscopic cholecystectomy more than two decades ago, the incidence of bile duct injury has remained greater than that established during the era of open cholecystectomy.
Discussion This article reviews the common causes of bile duct injury during laparoscopic cholecystectomy and makes recommendations that should help prevent these serious injuries from occurring.
Conclusions The incidence of bile duct injury during laparoscopic cholecystectomy, although greater than during open cholecystectomy, can be minimized using specific operative strategies and dissection principles.

Keywords Laparoscopic cholecystectomy · Bile duct injury

In the two decades since its introduction into clinical practice in the USA, laparoscopic cholecystectomy (LC) has become the primary technique for gallbladder removal. Despite a large experience with this operation, the rate of bile duct injury (BDI) remains approximately twice that for open cholecystectomy. There are many potential causes for this increased risk of BDI with LC. These include (1) less sensory information as a result of the two-dimensional view and diminished haptic sensation, (2) inappropriate dissection principles and techniques, (3) lack of a precise plan for conclusively identifying the cystic structures, (4) resistance or inability to perform imaging of the common bile duct and, 5) hesitancy to convert to open cholecystectomy. There is also a natural tendency for surgeons to create a heuristic image of the operative anatomy, making atypical anatomy seem normal.

Several factors increase the risk of BDI during a LC. Inexperience of the surgeon, severe acute or chronic inflammation of the gallbladder, aberrant or unusual anatomy, and inadequate surgical technique may play a role. The most common direct cause of ductal injury is misidentification of the common hepatic or common bile ducts as the cystic duct, with subsequent deliberate clipping or division of a major duct. Less common causes of biliary injury or bile leak include inadequate closure of the cystic duct, tenting injuries of the common duct, dissection deep into the liver bed with unroofing of superficial intrahepatic ducts, and injudicious use of electrocautery.

Surgeons must overcome the technical and visuospatial shortcomings of laparoscopy to minimize the risk of BDI during LC. An angled laparoscope will allow alternative views of the operative field. Dissection should begin on, and stay on the wall of the gallbladder, working toward the cystic structures. Dissection then proceeds similarly to a modified "top-down" approach, albeit starting on the gallbladder body rather than its fundus. Views of the gallbladder from both its medial and lateral sides can be obtained by appropriate traction. When dissecting the region of the infundibulum, it is helpful to retract the gallbladder laterally to dis-align the cystic and common ducts. The entire hepatocystic triangle should be cleared of

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N. J. Soper (✉)
Department of Surgery, Northwestern University,
251 E Huron St, Galter 3–150,
Chicago, IL 60611, USA
e-mail: nsoper@nmh.org

tissue, such that the liver bed can be seen through the "window" below the gallbladder and only two structures (cystic duct and artery) are seen traversing this window—this is the so-called critical view of safety. Techniques to image the common bile duct, either by cholangiography or ultrasonography, should be used liberally. Cholangiography itself does not prevent BDI, but it likely decreases the overall rate of BDI when assessed by administrative databases. The proviso, though, is that the cholangiograms should be interpreted correctly and that the entire biliary tree is imaged. Laparoscopic ultrasound may show the location of the common bile duct in relation to the plane of dissection and be useful in staying out of harm's way. The presence of "aberrant" anatomy (e.g., multiple cystic ducts) should alert the surgeon to the likelihood of a dangerous situation.

If in doubt about the anatomy, the surgeon must gain more anatomical information that can be used to guide subsequent steps. This may mean inserting another port to allow more vigorous traction, or changing the operative strategy. Performing the entire dissection "top-down" starting at the fundus may help display the infundibular anatomy more safely. Occasionally, a subtotal cholecystectomy may be required—leaving a small amount of infundibulum in situ, while removing all gallstones. This maneuver may be especially useful in the presence of severe scarring near the bile duct, which may tether the infundibulum to the common hepatic duct. Another tactic that can be particularly helpful to avoid injury is to ask for another experienced surgeon to assess the operative field. This will allow a "new set of eyes" to view the anatomical dissection, which can either confirm or deny the surgeon's perceptions and minimize any heuristic bias.

Finally, if there is any doubt in the surgeon's mind as to the anatomy that cannot be resolved by further dissection or ductal imaging, conversion to an open procedure—returning the surgeon's 3-dimensional view and sense of touch—should be undertaken.

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ORIGINAL ARTICLE

Prognostic Factors of Patients with Advanced Gallbladder Carcinoma Following Aggressive Surgical Resection

Yoshiaki Murakami · Kenichiro Uemura · Takeshi Sudo · Yasushi Hashimoto · Akira Nakashima · Naru Kondo · Ryutaro Sakabe · Hironori Kobayashi · Taijiro Sueda

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Abstract
Background The prognosis for patients with advanced gallbladder carcinoma is dismal despite aggressive surgical resection. The aim of this study is to determine useful prognostic factors for patients with gallbladder carcinoma following aggressive surgical resection.
Methods Medical records of 62 patients with gallbladder carcinoma who underwent surgical resection were retrospectively reviewed. Univariate and multivariate models were used to analyze the effect of clinicopathological factors on long-term survival.
Results According to the UICC staging system, ten (16%), 11 (18%), eight (13%), 16 (25%), nine (15%), and eight patients (13%) were diagnosed with stages I, II, IIIA, IIIB, IVA, and IVB disease, respectively. Partial hepatectomy and pancreatoduodenectomy were performed for 43 (69%) and 11 (18%) patients, respectively. Overall survival rates of all 62 and 41 patients with UICC stages III and IV disease were 71% and 56% at 1 year, 48% and 23% at 3 years, and 48% and 23% at 5 years, respectively (median survival time, 15.8 and 12.7 months, respectively). Multivariate analysis revealed that independent prognostic factors included tumor differentiation (p=0.006), hepatic invasion (p=0.002), lymph node metastasis (p=0.009), and surgical margin status (p=0.002) for all patients, and adjuvant chemotherapy (p=0.005), tumor differentiation (p=0.008), hepatic invasion (p=0.001), and surgical margin status (p=0.022) for patients with UICC stages III and IV disease.
Conclusions R0 resection and adjuvant chemotherapy are significant prognostic factors in advanced gallbladder carcinoma and should be performed to improve survival.

Keywords Gallbladder carcinoma · Prognostic factor · R0 resection · Adjuvant chemotherapy

Introduction

Gallbladder carcinoma is a relatively uncommon malignancy with an estimated 9,760 new cases of gallbladder or bile duct carcinoma diagnosed per year in the USA.[1] Higher incidence rates have been reported in Chile, Peru, Korea, and Japan while the USA and Europe have lower incidence rates.[2,3] However, according to the reports from these countries, the prognosis of this disease is often unfavorable, with reported 5-year survival rates at less than 15%, including unresectable and resected cases.[4,5] It was common to find invasion into the surrounding organs, including the duodenum, pancreas, and colon, and metastasis to lymph nodes, peritoneum, and liver at the time of diagnosis. Therefore, despite the fact that complete surgical resection is the only potentially curative treatment option, only a small proportion of patients are candidates for resection.[6–8] In order to improve survival of patients with this disease, aggressive surgical procedures including major hepatectomy,

Y. Murakami (✉) · K. Uemura · T. Sudo · Y. Hashimoto · A. Nakashima · N. Kondo · R. Sakabe · H. Kobayashi · T. Sueda
Department of Surgery, Division of Clinical Medical Science, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan
e-mail: mura777@hiroshima-u.ac.jp

pancreatoduodenectomy,[9–13] and extended lymphadenectomy, as well as adjuvant therapeutic modalities including chemotherapy and radiotherapy,[14–22] have been advocated by several investigators. However, despite the use of aggressive surgery and adjuvant therapy, the 5-year survival following surgical resection remains low, especially in advanced gallbladder carcinoma.[7,9,10,14,15,23–35] Therefore, evaluation of prognostic factors and establishment of new therapeutic strategies are necessary to improve long-term survival of patients with gallbladder carcinoma.

Several investigators have described prognostic factors of patients with gallbladder carcinoma following surgical resection.[7,10,15,19–35] However, these reports are based on patients with many stages of gallbladder carcinoma. The aim of this study is to utilize univariate and multivariate survival analysis to determine useful prognostic factors for patients with advanced gallbladder carcinoma who have undergone aggressive surgical resection in a single institution.

Patients and Methods

Study Design

Medical records of 62 patients with gallbladder carcinoma treated at the Department of Surgery, Hiroshima University Hospital between January 1990 and December 2010 were retrospectively reviewed. All patients underwent tumor resection with curative intent and had a confirmed pathological diagnosis. Factors analyzed included patient demographics, perioperative factors, tumor characteristics, and patient survival. Univariate and multivariate survival analysis was performed on 11 factors (gender, age, hepatic resection, use of adjuvant chemotherapy, tumor differentiation, hepatic invasion, choledochal invasion, lymph node metastasis, surgical margin status, the International Union Against Cancer (UICC)[36] pT factor, and UICC stage) to determine useful prognostic factors of patients with gallbladder carcinoma following surgical resection. Written informed consent was obtained from all patients for surgical treatment and pathological examinations according to the institutional guidelines.

Preoperative Workup and Surgical Procedures

Preoperative workup included ultrasonography, computed tomography, endoscopic ultrasonography, endoscopic retrograde cholangiography, and percutaneous transhepatic cholangiography to evaluate the local or distant extension of the tumors. If jaundice (serum bilirubin level more than 3 mg/mL) was identified preoperatively, percutaneous transhepatic biliary drainage (PTBD) or endoscopic retrograde biliary drainage (ERBD) was performed to reduce the cholestatic liver damage. Radical surgery was performed when serum bilirubin level decreased to 3 mg/dL. Preoperative percutaneous transhepatic portal embolization (PTPE) was performed on the liver segment to be resected to induce compensatory hypertrophy of the future remnant liver. This was done if the estimated liver resection volume exceeded 60% of the whole liver as calculated by computed tomography. Two or three weeks after PTPE, the future remnant liver volume was reevaluated by computed tomography. Hepatic resection was performed if the future remnant liver volume exceeded 40% of the whole liver.

All surgical resections included cholecystectomy with or without resection of liver or extrahepatic bile duct. If the tumor invaded the pancreatic head, duodenum, or colon, then, pancreatoduodenectomy or partial resection of the duodenum or colon was also performed. All patients underwent dissection of the regional lymph nodes, which included the nodes along the common hepatic artery, nodes in the hepatoduodenal ligament, and posterior pancreaticoduodenal nodes. Intraoperative pathological assessment of the proximal or distal bile duct transection lines was performed with frozen tissue sections. If the bile duct transection line was positive for malignant cells, further resection of the bile duct was performed to the maximum extent possible. Biliary continuity was restored by a Roux-en-Y biliary-enteric anastomosis when extrahepatic bile duct was resected.

Pathological Investigations

Following tumor resection, permanent sections with hematoxylin and eosin staining were prepared. All specimens were examined pathologically, and each tumor was classified as well-differentiated, moderately differentiated, or poorly differentiated adenocarcinoma according to the predominant pathological grading of differentiation. Hepatic invasion, choledochal invasion, and lymph node metastasis were all examined pathologically. Surgical margins were considered positive if infiltrating adenocarcinoma was present at the hepatic transection line, the proximal or distal bile duct transection line, or dissected periductal soft tissue margins. The final stage of gallbladder carcinoma was determined pathologically according to the TNM classification system of malignant tumors published by the UICC, 7th edition.[36]

Postoperative Adjuvant Chemotherapy

Postoperative adjuvant chemotherapy was administered to patients with UICC stages III and IV disease, beginning in 2002. The regimen of adjuvant chemotherapy has been

described previously.[16,37–39] In summary, patients were treated with ten cycles of gemcitabine plus S-1 every 2 weeks. Each chemotherapy cycle consisted of intravenous gemcitabine at a dose of 700 mg/m^2 on day 1 and orally administered S-1 at a dose of 50 mg/m^2 for seven consecutive days, followed by a 1-week break from chemotherapy. Neither external beam radiation nor intraoperative irradiation was administered to any patients during the study period.

Survival

Patients were followed up regularly in outpatient clinics at 3-month intervals by undergoing a blood test, ultrasonography, and computed tomography for up to 5 years after surgery. Information after 5 years was collected by telephone or personal interview. For patients who died, survival time after surgery and the cause of death were recorded. For surviving patients, postoperative survival time and status of recurrence were recorded. The median follow-up time after operation was 157 months (range, 2–255 months).

Statistical Analysis

Survival curves were constructed using the Kaplan–Meier method, and differences in survival curves were compared by univariate log-rank (Mantel–Cox) test. Factors found to be $p<0.05$ on univariate analysis were subjected to multivariate analysis using a Cox proportional hazards model. $p<0.05$ was considered statistically significant. Statistical analysis was performed with the Macintosh version of StatView (version 5.0; SAS Institute, Cary, NC).

Results

Patient Demographics and Operative Procedures Performed

The 60 eligible patients included 32 men and 30 women (median age, 69 years; range, 33–92 years), and 31 patients (50%) were more than 70 years old. Preoperative jaundice was identified in 16 patients (26%). For reduction of serum bilirubin levels or preoperative workup, PTBD and ERBD were performed for 15 and one patients, respectively. Percutaneous transhepatic portal embolization was performed in two patients who underwent right hemihepatectomy. Depending on the local extension of the tumor, a wide variety of surgical procedures were performed. Partial hepatectomy and pancreatoduodenectomy were performed for 43 (69%) and 11 (18%) patients, respectively. In addition to surgical procedures listed in Table 1, four patients each underwent partial resection of the duodenum or colon. Surgical procedures performed for patients with UICC stages III and IV disease are also listed in Table 1. There were no perioperative deaths within 30 days of operation.

Tumor Characteristics

Pathologically, tumors were identified as well-differentiated adenocarcinoma in 25 patients (40%), moderately differentiated adenocarcinoma in 16 patients (26%), and poorly differentiated adenocarcinoma in 21 patients (34%). Hepat-

Table 1 Operative procedures of patients with gallbladder carcinoma

	No. of patients	
	All patients (n=62)	Patients with UICC stages III and IV disease (n=41)
Right trisectionectomy+CHx+BDR	1	1
Right hepatectomy+CHx+BDR	5	5
Right hepatectomy+CHx+PD	1	1
Right hepatectomy+CHx+PPPD	1	1
(S4a+S5) hepatectomy	1	
(S4a+S5) hepatectomy+BDR	3	3
(S4a+S5) hepatectomy+PD	2	2
(S4a+S5) hepatectomy+PPPD	1	1
Gallbladder bed resection	7	3
Gallbladder bed resection+BDR	13	7
Gallbladder bed resection+PPPD	5	4
Gallbladder bed resection+PD	1	1
Cholecystectomy+BDR	3	3
Cholecystectomy	18	9

In addition to surgical procedures above, each four patients underwent partial resection of the duodenum or colon

CHx caudate lobectomy, *BDR* bile duct resection, *PPPD* pylorus-preserving pancreatoduodenectomy, *PD* conventional pancreatoduodenectomy

ic invasion and choledochal invasion were identified in 21 (34%) and 15 patients (24%), respectively. There were 30 tumors (48%) with lymph node metastasis and 32 (52%) without lymph node metastasis. Forty-nine patients (79%) had negative surgical margins. According to the UICC staging system, eight (13%), three (5%), 21 (34%), 21 (34%), and nine patients (15%) had pT1a, pT1b, pT2, pT3, and pT4 tumors, respectively, and ten (16%), 11 (18%), eight (13%), 16 (25%), nine (15%), and eight patients (13%) were diagnosed with stages I, II, IIIA, IIIB, IVA, and IVB disease, respectively.

Survival

Actuarial overall survival rates for the 62 patients were 71% at 1 year, 48% at 3 years, and 48% at 5 years (median survival, 15.8 months; range, 2–243 months). Actuarial overall survival rates for 21 patients with UICC stages I and II disease were 100% at 1 year, 94% at 3 years, and 94% at 5 years while actuarial overall survival rates for 41 patients with UICC stages III and IV disease were 56% at 1 year, 23% at 3 years, and 23% at 5 years (median survival, 12.7 months; range, 2–244 months; Fig. 1). Tumor recurrence occurred in 30 patients. The sites and nature of recurrence in these patients included peritoneal dissemination (n=17), liver metastases (n=6), and local disease (n=7). Twenty-nine patients died of recurrent disease. One patient with peritoneal recurrence was still alive at the time of this writing.

Eleven clinicopathological factors were investigated in all patients to determine their prognostic significance. The results of the log-rank test are shown in Table 2. Gender, age, hepatic resection, and adjuvant chemotherapy did not influence postoperative survival by univariate survival analysis. In contrast, univariate analysis revealed that tumor differentiation (p<0.001), hepatic invasion (p<0.001), choledochal invasion (p=0.002), lymph node metastasis (p<0.001), surgical margin status (p<0.001), UICC pT factor (p<0.001), and UICC stage (p<0.001) were significantly associated with survival. These factors were entered into multivariate analysis with a Cox proportional hazards model, and tumor differentiation (p=0.006), hepatic invasion (p=0.002), lymph node metastasis (p=0.009), and surgical margin status (p=0.002) remained independently associated with survival (Table 2). Table 3 indicates prognostic factors of 41 patients with UICC stages III and IV disease. Tumor differentiation (p=0.015; Fig. 2a), hepatic invasion (p=0.036; Fig. 2b), lymph node status (p=0.016), surgical margin status (p=0.012; Fig. 3a), and adjuvant chemotherapy (p=0.005; Fig. 3b) were found to be significant survival prognostic factors. These five factors were entered into a Cox proportional hazards model. Multivariate analysis showed that adjuvant chemotherapy (p=0.005), tumor differentiation (p=0.008), hepatic invasion (p=0.001), and surgical margin status (p=0.022) were independent prognostic factors of patients with UICC stages III and IV disease. Lymph node status did not reach significance by multivariate analysis (p=0.076).

There were six 5-year survivors with UICC stages III and IV disease. Of the six patients, five patients underwent hepatic resection or pancreatoduodenectomy. Nodal involvement and hepatic invasion were found in two and three patients, respectively. All six patients underwent R0 resection and three patients received adjuvant chemotherapy.

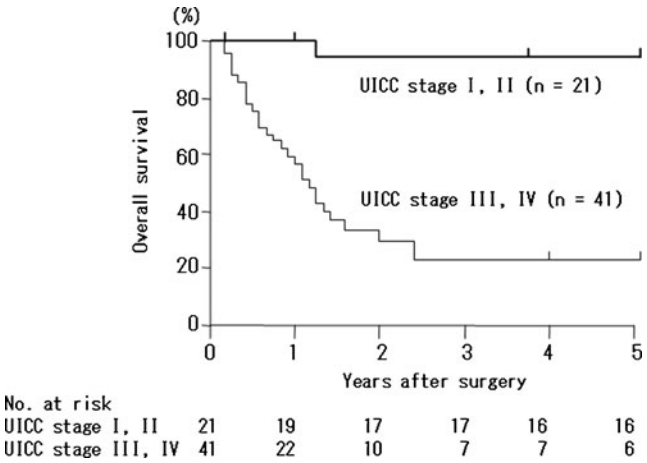

Fig. 1 Comparison of postoperative survival in patients with gallbladder carcinoma who underwent surgical resection, stratified by UICC stage (p<0.001)

Discussion

Survival after surgical resection for patients with gallbladder carcinoma depends heavily on stage of disease. According to previous reports, 5-year survival rates of patients with resected gallbladder carcinoma are 33–63%[26,28,32,34,35] for patients at all disease stages and 9–20%[9,10,25] for patients with UICC stages III and IV disease (Table 4). In the current study, 5-year survival rates for all patients and patients with UICC stages III and IV disease were 48% and 23%, respectively. These results are comparable to previous reports. Most patients with UICC stages I and II disease, who have mucosal carcinoma or subserosal carcinoma without nodal involvement, have survived for more than 5 year without recurrence if complete tumor resection is performed. However, the prognosis is extremely poor once the tumor invades the surrounding organs including liver, common bile duct, duodenum and colon or metastasizes to regional lymph nodes. It is mandatory to evaluate prognostic factors and to establish optimal therapeutic strategies especially for patients with UICC stages III and IV gallbladder carcinoma.

Table 2 Univariate and multivariate survival analysis of prognostic factors for patients with gallbladder carcinoma (n=62)

Factors	Univariate analysis			Multivariate analysis	
	No. of patients	5-year survival rate (%)	p Value	Hazard ratio (95% CI)	p Value
Gender					
Male	32	51	0.547		
Female	30	45			
Age (years)					
<70	31	49	0.928		
≧70	31	48			
Hepatic resection					
Yes	43	40	0.133		
No	19	67			
Adjuvant chemotherapy					
Yes	11	48	0.579		
No	51	47			
Tumor differentiation					
Well	25	83	<0.001	4.50 (1.53–13.3)	0.006
Moderate, poor	37	23			
Hepatic invasion					
Yes	21	12	<0.001	6.08 (1.93–19.1)	0.002
No	41	69			
Choledochal invasion					
Yes	15	13	0.002	2.87 (0.98–8.43)	0.055
No	47	62			
Lymph node metastasis					
Yes	30	13	<0.001	4.14 (1.42–12.0)	0.009
No	32	82			
Surgical margin					
Positive	13	0	<0.001	3.86 (1.63–9.14)	0.002
Negative	49	61			
UICC pT factor					
pT1 and 2	32	78	<0.001		
pT3 and 4	30	17			
UICC stage					
I and II	21	94	<0.001		
III and IV	41	23			

CI confidence interval, *UICC* International Union Against Cancer

Many investigators have used multivariate analysis to determine useful prognostic factors for gallbladder carcinoma after surgical resection (Table 4).[7,10,15,23–25,27–34] According to these reports, potentially significant factors include nodal involvement,[15,23,24,29–34] hepatic invasion,[23,31] choledochal invasion,[23,31] pathological grading of differentiation,[24,32] perineural invasion,[24,33], and pathologically curative resection.[7,24,25,27,29] In the current study, multivariate analysis revealed that adjuvant chemotherapy, tumor differentiation, hepatic invasion, and surgical margin status were independent prognostic factors for patients with UICC stages III and IV disease. To our knowledge, there have been no multivariate analysis reports of survival benefits of adjuvant chemotherapy on patients with advanced gallbladder carcinoma (Table 4). We believe the new adjuvant chemotherapy regimen of gemcitabine plus S-1 may contribute to long-term survival of patients with UICC stages III and IV disease.

There have been a few studies on adjuvant therapy for patients with gallbladder carcinoma in the previous literature. With regard to adjuvant chemoradiotherapy, Cho et al. reported a retrospective study in which radiotherapy with concurrent 5-fluorouracil (5-Fu) improved overall survival for patients with gallbladder carcinoma, especially for

Table 3 Univariate and multivariate survival analysis of prognostic factors for UICC stages III or IV patients with gallbladder carcinoma (n=41)

Factors	Univariate analysis			Multivariate analysis	
	No. of patients	5-year survival rate (%)	p Value	Hazard ratio (95% CI)	p Value
Gender					
Male	21	29	0.382		
Female	20	18			
Age (years)					
<70	19	21	0.770		
≧70	22	27			
Hepatic resection					
Yes	32	20	0.740		
No	9	40			
Adjuvant chemotherapy					
Yes	10	48	0.036	5.72 (1.71–19.1)	0.005
No	31	16			
Tumor differentiation					
Well	10	57	0.015	4.61 (1.50–14.1)	0.008
Moderate, poor	31	10			
Hepatic invasion					
Yes	21	11	0.036	4.72 (1.86–12.0)	0.001
No	20	38			
Choledochal invasion					
Yes	15	13	0.479		
No	26	33			
Lymph node metastasis					
Yes	30	13	0.016	2.86 (0.90–9.14)	0.076
No	11	56			
Surgical margin					
Positive	13	0	0.012	2.55 (1.15–5.69)	0.022
Negative	28	36			
UICC pT factor					
pT1 and 2	11	45	0.195		
pT3 and 4	30	17			

CI Confidence interval, *UICC* International Union Against Cancer

node-positive patients.[14] In addition, other investigators reported survival benefits from adjuvant chemoradiotherapy for patients with gallbladder carcinoma.[16–18] However, these analyses were based on a small number of patients, and no beneficial effects of chemoradiotherapy have been reported by other investigators.[15] Survival benefits of chemoradiotherapy on patients with resected gallbladder carcinoma are controversial. Furthermore, Jarnagin et al. reviewed 97 patients with resected gallbladder carcinoma and reported that initial recurrence involving a distant site occurred in 85% of patients. They concluded that an adjuvant therapeutic strategy targeting locoregional disease, such as radiotherapy, is unlikely to have a significant impact on the overall survival of gallbladder carcinoma.[40] In the current study, distant recurrence including peritoneal dissemination or liver metastasis occurred in 77% of patients. Based on these results, we believe that systemic chemotherapy, but not radiotherapy, is the preferred strategy for adjuvant therapy of gallbladder carcinoma.

Reports concerning adjuvant chemotherapy for gallbladder carcinoma are scarce. To our knowledge, there has been only one randomized controlled study of survival effects of adjuvant chemotherapy for patients with gallbladder carcinoma. Takada et al. compared therapy with mitomycin C and 5-Fu to surgery alone after radical resection of gallbladder carcinoma; they reported that the 5-year survival rate was significantly better in the chemotherapy group (26%) compared with the surgery alone group (14%).[21] In addition, Kayahara et al. reported survival benefits of adjuvant chemotherapy for patients with

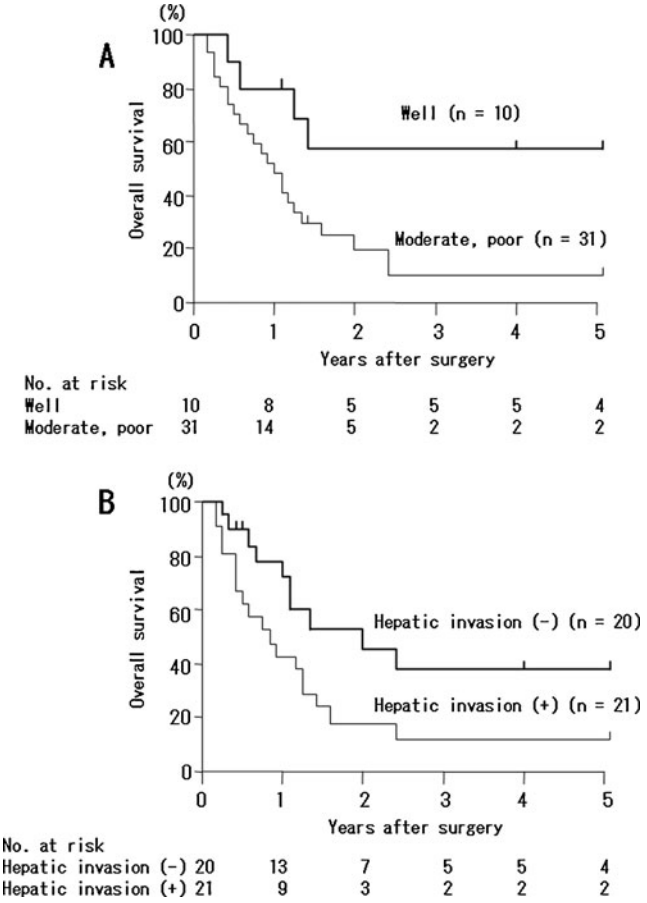

Fig. 2 Comparison of postoperative survival in UICC stages III and IV patients with gallbladder carcinoma who underwent surgical resection. **a** Stratified by tumor differentiation (p=0.015). **b** Stratified by the presence or absence of hepatic invasion (p=0.036)

advanced gallbladder carcinoma based on data from the Japanese Biliary Tract Cancer Registry.[28] Recently, new anticancer drugs including gemcitabine,[41–43] cisplatin,[42], and S-1[43,44] have been reported to have favorable anticancer effects on patients with unresectable biliary tract carcinoma. We have already reported that adjuvant gemcitabine plus S-1 chemotherapy improves survival significantly after surgery for biliary carcinoma.[19] In the current study, patients with UICC stages III and IV disease, who underwent resection after 2002, received adjuvant gemcitabine plus S-1 chemotherapy. As a result, three patients have survived for more than 5 years and adjuvant chemotherapy was an independent prognostic factor among patients with UICC stages III and IV disease. These results suggest that adjuvant gemcitabine plus S-1 chemotherapy may improve survival of patients with advanced gallbladder carcinoma.

Previous reports showed that curative (R0) resection was performed in 66–100% of patients with gallbladder carcinoma undergoing surgical resection (Table 4).[7,10,14,15,23–27,29] In the current study, the rate of patients resected with negative margin was 79% for all patients and 68% for patients with UICC stages III and IV disease, and surgical margin status was also identified as an independent prognostic factor. All patients with positive surgical margins died of recurrent disease within 3 years and six 5-year survivors with UICC stages III and IV disease had negative surgical margins. Kai et al. reported that in an analysis of 39 patients with advanced gallbladder carcinoma, survival of patients with negative surgical margins was significantly better than that of patients with positive surgical margins and concluded that long-term survival can be expected by an operation with tumor-free surgical margins even in patients with advanced gallbladder carcinoma.[29] Other investigators also have presented that pathologically curative resection (R0 resection) can improve patient survival.[24,25,27] Surgeons should carefully pursue R0 resection whenever possible, even in advanced gallbladder carcinoma, by performing extended surgical resection including hepatectomy or pancreatoduodenectomy.

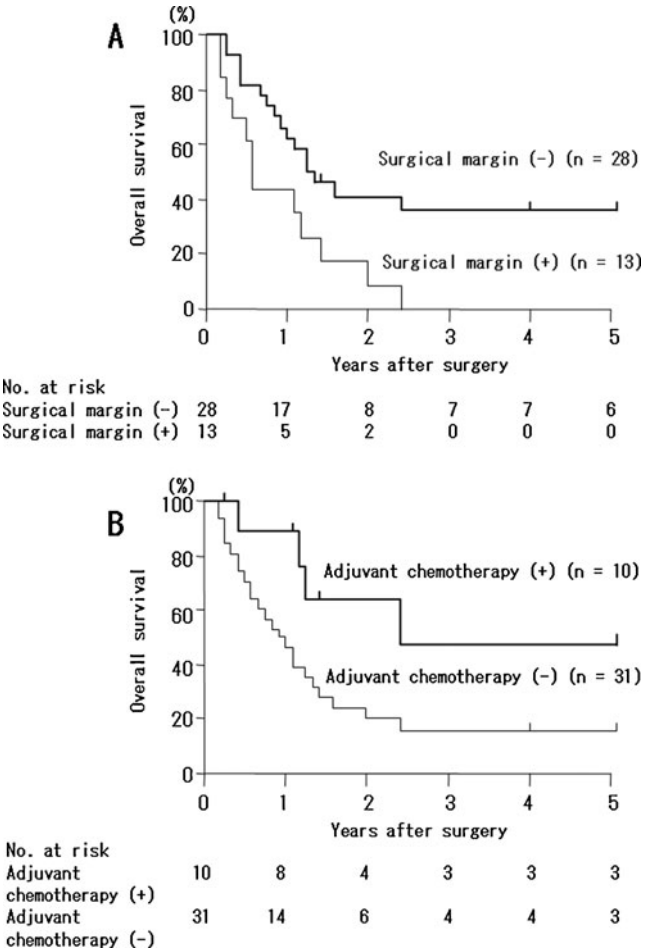

Fig. 3 Comparison of postoperative survival in UICC stages III and IV patients with gallbladder carcinoma who underwent surgical resection. **a** Stratified by the presence or absence of positive surgical margins (p=0.012). **b** Stratified by use of adjuvant chemotherapy (p=0.036)

Table 4 Recent reports on resectional treatment of gallbladder carcinoma

Author	Year	No. of patients	UICC stage	Mortality (%)	R0 resection (%)	Nodal involvement (%)	Median survival (months)	5-year survival rate (%)	Prognostic factors by multivariate analysis
Present study	2010	62	I–IV	0	79	48	16	48	Hinf, R, N, and G
		41	III and IV	0	68	73	13	23	Hinf, R, G, and AC
Kim[15]	2010	166	I–IV	–	100	39	–	–	N
Cho[14]	2010	68	II–III	–	91	59	–	59	–
Miura[23]	2010	149	II–IV	7	47	55	–	35	Hinf, N, and Binf
Choi[24]	2010	83	II and III (pT2)	1	77	22	33	29	R, N, G, PN, and VI
Wakai[25]	2010	42	III and IV	–	79	71	11	20	R and MHS
Miyakawa[26]	2009	1094	I–IV	1	69	39	–	42	–
Shibata[27]	2009	72	I–III	–	75	33	–	–	pT, LI, and R
Kayahara[28]	2008	3023	I–IV	–	–	–	–	40	Age, sex, stage, and OP
Kai[29]	2007	90	I–IV	0	80	46	–	–	PV, N, and R
Yokomizo[30]	2007	94	II and III (pT2)	–	–	30	–	80	N
Shimizu[10]	2007	79	III and IV	11	66	65	–	9	None
Yagi[31]	2006	50	II–IV	0	–	46	–	–	Hinf, N, and Binf
Kokudo[32]	2003	152	I–IV	–	–	38	–	63	N, G, and pT
Kondo[9]	2002	29	IV	21	–	–	12	17	–
Yamaguchi[33]	2002	68	I–IV	10	–	44	–	–	N and PN
Puhalla[7]	2002	60	I–IV	11	75	32	–	–	R
Fong[34]	2000	100	I–IV	10	–	36	26	38	N and pT
Shimada[35]	1997	41	I–IV	2	–	63	–	33	–

UICC International Union Against Cancer, *Hinf* hepatic invasion, *R* pathologically curative resection, *N* nodal involvement, *Binf* choledochal invasion, *G* pathological grading of differentiation, *AC* adjuvant chemotherapy, *PN* perineural invasion, *VI* vascular invasion, *MHS* mode of hepatic spread, *pT* UICC pT factor, *OP* operative procedures, *PV* portal vein invasion

Hepatic invasion occurred in 51% of patients with UICC stages III and IV disease and it was also an independent prognostic factor in this series. Prognostic impact of hepatic invasion has been reported by other investigators.[23,31] In order to resect invaded areas of liver or prevent hepatic recurrence, several surgeons have advocated gallbladder bed resection or S4a+S5 hepatectomy. However, the optimal procedure for gallbladder carcinoma with hepatic invasion is inconclusive. Araida et al. analyzed 485 cases of pT2 and pT3 stage gallbladder carcinoma treated at 112 institutions belonging to the Japanese Society of Biliary Surgery, and found no significant differences in survival rates or hepatic recurrence rates between patients undergoing gallbladder bed resection and patients undergoing S4a+S5 hepatectomy, and ultimately concluded that gallbladder bed resection is more preferable for surgical hepatic procedure.[45] In the current study, three of six 5-year survivors with UICC stages III and IV disease underwent gallbladder bed resection.

The frequency of nodal involvement has been reported to range from 33–71% in patients with gallbladder carcinoma who underwent surgical resection,[7,14,15,23–27,29–35] although it depended on disease stage. In this series, nodal involvement developed in 48% of all patients and 73% of patients with UICC stages III and IV disease. The rate of nodal involvement in patients with advanced gallbladder carcinoma was somewhat high compared with that of previous reports. Many authors have described a significant correlation between nodal involvement and patient survival.[15,23,24,29–34] In the present study, multivariate analysis revealed that statistical significance in lymph node status was not obtained among patients with UICC stages III and IV disease although patients with nodal involvement had significantly worse survival by univariate analysis. Five-year survival rate in patients with nodal involvement was 13%, and two patients with nodal involvement have been alive for more than 5 years. Dissection of regional lymph nodes, which is routinely performed in our institution, may contribute to improved survival of patients with nodal involvement.

In conclusion, adjuvant chemotherapy, tumor differentiation, hepatic invasion, and surgical margin status are independent prognostic factors in patients with UICC stages III and IV gallbladder carcinoma following surgical

resection. Curative resection and adjuvant chemotherapy are recommended for long-term survival of patients with advanced gallbladder carcinoma. However, the limitations of this study are its retrospective design and the small number of patients studied. Further studies on larger numbers of patients, including prospective studies, are required to confirm the results of this study.

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ORIGINAL ARTICLE

Lymph Nodal Involvement as Prognostic Factor in Gallbladder Cancer: Location, Count or Ratio?

Sanjay Singh Negi · Amanjeet Singh · Adarsh Chaudhary

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Abstract
Background Lymph nodal involvement is a critical prognostic factor in patients with gallbladder cancer (GBC). Controversy exists regarding optimal categorization of nodal status, and no study has investigated the relevance of metastatic to examined nodes ratio (LNR) in these patients.
Methods Demographic, operative and pathologic data including total lymph node count (TLNC), positive lymph node count (PLNC), LNR and involved nodal location was recorded in 57 patients with GBC who underwent curative intent resection. Disease-free survival (DFS) and predictors of outcome were analyzed.
Results At a median follow-up of 19 (i.q.r: 11–39.5) months, median DFS was 28.25±3.62 months and 35 (61%) patients had developed recurrence. Thirty-three (58%) patients had nodal involvement, and a linear correlation was observed between TLNC and PLNC (r^2=0.249, p<0.001). Optimal TLNC and LNR were determined to be 6 and 0.50, respectively. Patients with negative nodes (N0) were better sub-stratified based on TLNC (median DFS, TLNC≥6 vs. TLNC<6: not reached vs. 32.00± 4.80 months, p=0.012). Amongst patients with involved nodes, LNR was significantly associated with DFS (median DFS, 0< LNR≤0.50 vs. LNR>0.50: 14.00±2.46 vs. 9.00±1.55 months, p<0.001). Prognosis was not related to location of involved nodes. Multivariable analysis revealed T stage, tumor differentiation and LNR to be independent predictors of DFS.
Conclusions LNR is a strong predictor of outcome after curative resection for GBC. The retrieval and examination of at least 6 nodes can influence staging quality and DFS in node-negative patients.

Keywords Gall bladder cancer · Lymph node · Metastasis · Survival

Background

Gallbladder cancer is an aggressive disease and has traditionally been associated with poor prognosis.[1] Complete surgical resection with tumor-free surgical margins, especially in the early stages of the disease, offers the only hope for long-term survival and is considered a standard of care.[2] Unfortunately, even with recent advances in imaging techniques, majority of patients are diagnosed with advanced disease not amenable to curative resection. Moreover, despite the adoption of aggressive surgical approach, most of the patients who undergo a potentially curative resection develop recurrent disease. Hence, it is pertinent to identify factors that influence recurrence free survival, which is critical for prognostication and planning of postoperative adjuvant therapy.

The presence or absence of lymph node metastasis is an important prognostic factor in patients with curatively resected gallbladder cancer. Studies have demonstrated that patients with lymph nodal metastasis have significantly worse survival than those with negative nodes.[3] It is increasingly being recognized that an inadequate number

S. S. Negi · A. Singh · A. Chaudhary
Department of Surgical Gastroenterology
and Liver Transplantation, Sir Ganga Ram Hospital,
Rajinder Nagar,
New Delhi 110060, India

A. Chaudhary (✉)
Room No. 122001, Medanta-The Medicity Sector-38,
Gurgaon, Haryana 122001, India
e-mail: adarsh_chaudhary@yahoo.com

of lymph nodes examined may adversely influence survival and lead to understaging of various gastrointestinal cancers.[4] Recent studies have also demonstrated the influence of involved lymph node count and metastatic to examined nodes ratio (LNR) on survival of patients with pancreatic and biliary cancers.[5,6]

However, the method of optimally categorizing lymph nodal involvement in gallbladder cancer remains controversial. Some investigators have highlighted the importance of metastatic lymph node count as a means of stratification while others rely on the location of involved nodes.[7] This lack of clarity has led to frequent revision of nodal staging in the various staging systems. For instance, the 6th edition of the American Joint Commission on Cancer (AJCC) manual on staging of gallbladder cancer categorized lymph nodal staging in gallbladder cancer based on presence or absence of lymph node metastasis irrespective of absolute count or location.[8] However, in its subsequent edition, AJCC has revised the nodal staging and based the classification on location of involved lymph nodes regardless of absolute count.[9] To the best of our knowledge, no study has addressed the association between the metastatic lymph node ratio and survival in patients with gallbladder cancer. The present study evaluates the prognostic impact of number, location and ratio of involved lymph nodes, in addition to well-described prognostic parameters, in patients with curatively resected gallbladder cancer.

Patients and Methods

Between September 2003 and September 2009, 98 patients with gallbladder cancer were referred to our unit at Sir Ganga Ram Hospital, New Delhi (India) for surgical management. The details of these patients are provided in Fig. 1. Fifty seven of these patients, with gallbladder adenocarcinoma, had complete surgical resection with tumor-free surgical margins (R0 resection) and comprise the study group. Patients who were explored but did not undergo R0 resection were excluded.

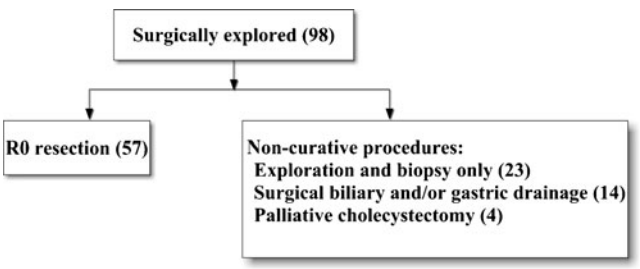

Fig. 1 Flow chart detailing surgical management of all the patients with gallbladder cancer

Operative Details

The operative procedure consisted of staging laparoscopy as an initial step, prior to laparotomy, to exclude metastatic disease. During laparotomy, frozen section biopsy of interaortocaval lymph nodes was performed, and radical resection proceeded only if this was reported as negative for malignancy. Radical cholecystectomy consisted of en-bloc cholecystectomy with a portion of surrounding liver and systematic lymph node clearance. Lymphadenectomy included en-bloc clearance of cystic duct, pericholedochal, hepatic artery, portal vein, periduodenal and peripancreatic lymph nodes. Para-aortic, superior mesenteric artery and coeliac nodal clearance was not performed in any patient. The extent of liver resection was guided by the extent of liver infiltration by the tumor on preoperative cross sectional imaging, the guiding principle being attainment of negative surgical margin while at the same time preserving the maximal amount of liver parenchyma. A 2-cm non-anatomical wedge of gallbladder fossa was performed if the tumor was confined to gallbladder and formal resection of segment 5 and caudal part of segment 4 undertaken if there was gross liver involvement. None of the patients had extended liver resection. In all the patients, frozen section analysis of cystic duct remnant was obtained to ensure negative margin. Resection of extrahepatic bile duct was selectively undertaken in patients with positive frozen section biopsy of cystic duct stump, invasion of bile duct by tumor, previous cholecystectomy where it was not possible to reliably differentiate malignancy from fibrosis, multiple large lymph nodes adherent to bile duct, papillary cancer and choledochal cyst. In these patients, bile duct margin negativity was confirmed on frozen section biopsy. Port site excision was performed in patients who have had previous laparoscopic cholecystectomy. Postoperative complications were graded according to the Clavien–Dindo classification.[10]

Pathologic Examination

Immediately after resection, fibrofatty tissue containing lymph nodes was separated from the specimen, and nodes were meticulously separated by the surgeon into containers marked as cystic duct, pericholedochal, hepatic artery, portal vein and peripancreatic lymph nodes. Chemical fat dissolution methods were not employed. The specimen was then fixed in 10% buffered formaldehyde solution. Primary tumor was examined to determine the histologic type, tumor grade, depth of infiltration, lympho-vascular invasion, tumor involvement of excised contiguous viscera and resection margins. Histologic grade was determined based on the areas of tumor with highest grade and was classified as well differentiated or not well differentiated (moderate or

poor). Lymph node (LN) metastasis was defined as tumor cells detected on histopathologic examination using hematoxylin and eosin stain. Immuno-histochemical analysis was not performed routinely to detect occult or micrometastasis. Total lymph node count (TLNC) and positive lymph node count (PLNC) were recorded for each patient. Lymph node ratio (LNR) was estimated by dividing the total number of positive lymph nodes (PLNC) by the total number of lymph nodes examined (TLNC). The location of positive lymph node was classified into three categories according to the 7th edition of AJCC classification as N0 (no nodal metastasis), N1 (cystic duct, pericholedochal, hepatic artery and portal vein) or N2 (peripancreatic).[9] Although the disease was staged using the 6th edition of the AJCC manual during the period of study,[8] final restaging was done in accordance with the 7th edition of AJCC classification.[9]

Follow Up

Adjuvant chemotherapy was offered to all the patients with positive lymph nodes. The patients were followed up at three monthly intervals or earlier if clinically symptomatic. The standardized follow-up protocol consisted of three monthly clinical examination, abdominal sonography, hematology and biochemical parameters including liver function tests. Contrast-enhanced computed tomography was performed at six monthly intervals. Disease-free survival (DFS) was calculated from the day of surgery to the time when recurrent disease was first detected. The site of recurrence was classified as loco-regional (liver resectional margin, hepaticojejunostomy, regional, or retroperitoneal lymph nodes) or distant (hepatic other than resectional margin, peritoneum, lung, or other distant organs).

Statistical Analysis

Continuous data are presented as median (interquartile range (i.q.r)). Student's t test was used for analysis of normally distributed and Wilcoxon test for non-normally distributed continuous data. The χ^2 test or Fisher's exact test was used, as appropriate, for categorical data. The primary outcome parameter of interest was DFS and is expressed as median±SE. Based on the magnitude of the log-rank test χ^2 statistic, cut-point analysis was performed to identify the numeric lymph node value that determined the greatest DFS difference between resulting groups.[4] Actuarial DFS was calculated with the Kaplan–Meier method, and univariable comparison between groups was performed using the log-rank test. Variables with $p<0.100$ in univariable analysis were included in a multivariable logistic regression model using Cox proportional hazard model; an odds ratio (OR) with 95% confidence interval was estimated using the final regression model. $P\leq 0.050$ was considered statistically significant. All reported P values are two tailed and were not adjusted for multiple testing. Statistical analysis was performed using SPSS version 15.0 (SPSS Chicago, Illinois, USA).

Results

Demographic and Treatment Related Characteristics

The study group consisted of 44 (77%) women and 13 (23%) men with a median age of 46 (i.q.r: 42–58) years. At the time of presentation, 41 (72%) patients had abdominal pain and 40 (70%) had weight loss. Seven (12%) patients presented with jaundice and three of these patients had undergone endoscopic biliary stenting on account of cholangitis. None of the patients had vascular involvement or bowel obstruction. Eleven (19%) patients had associated co-morbid conditions and 33 (58%) had gallstones. Prior to referral for definitive surgery, 21 (37%) patients had undergone cholecystectomy.

All the patients underwent conservative liver resection, and none of the patients had extended hepatectomy. Concomitant bile duct excision was undertaken in 33 (58%) patients. Bile duct resection did not significantly increase the yield of lymph nodes (six (i.q.r: 5–7) vs. five (i.q.r: 4–6), $p=0.166$) but was associated with increased operative time (290.00 (i.q.r: 280.00–340.00) vs. 260.00 (i.q.r: 235.00–310.00) minutes; $p=0.002$), higher incidence of postoperative complications (18 (54%) vs. 5 (21%); $p=0.014$) especially bile leak (10(30%) vs. 1 (4%); $p=0.017$), delayed resumption of oral diet (3.00 (i.q.r: 3.00–4.00) vs. 2.00 (i.q.r: 1.00–2.00) days; $p<0.001$) and prolonged length of stay (7.00 (i.q.r: 6.00–8.00) vs. 4.00 (i.q.r: 4.00–5.00) days; $p<0.001$). Adjacent organ resection was performed in five (9%) patients and consisted of duodenal sleeve resection in three and segmental colectomy in two patients. The median operative time was 280 (i.q.r: 250–320) minutes and perioperative blood transfusion was required in seven (12%) patients. Postoperative complications developed in 23 (40%) patients with five patients developing more than one complication. The specific complications included bile leak in 11 (19%), surgical site infection in 11 (19%), gastroparesis in 4 (7%) upper gastrointestinal bleed in 1 (2%) and incisional hernia in 1 (2%) patients. Of 23 patients with postoperative complications, 15 (65%) had grade I, 5 (22%) grade II and 3 (13%) grade III complications. There was no postoperative mortality and median hospital stay was 6 (i.q.r: 4–7) days.

Although all the 33 patients with nodal involvement were offered postoperative adjuvant chemotherapy, due to

logistic or financial limitations only 12 (36%) finally received treatment. The chemotherapy regimen consisted of 5-flurouracil, cisplatin, gemcitabine or oxaliplatin administered either as single agent or combination therapy. None of the patients received radiation therapy. On subgroup analysis of patients with lymph node involvement, administration of adjuvant chemotherapy did not significantly alter the DFS (38.00±14.40 vs. 14.00±2.13 months; p=0.213).

Pathologic Characteristics

On final pathologic analysis, 30 (53%) patients had well-differentiated cancer, 25 (44%) lympho-vascular invasion and 7 (12%) had associated xanthogranulomatous inflammation. With regard to final pathologic staging based on the 7th edition of AJCC classification, 5 (9%) had T1, 17 (30%) had T2 and 35 (61%) had T3 cancer. Five (9%) patients had stage I, 13 (23%) stage II, 5 (9%) stage IIIa, 16 (28%) stage IIIb and 18 (31%) stage IVb disease. Of 33 patients who underwent concomitant bile duct resection, pathological involvement of bile duct by cancer was observed in only five (15%) patients. All the patients with adjacent organ resection had histologic involvement by cancer.

Total Lymph Node Count (TLNC) Thirty-three (58%) patients had LN metastasis and 24 (42%) negative nodes. The median TLNC in node-negative group was 5.00 (i.q.r: 4.00–6.00) compared with median TLNC of 6.00 (i.q.r: 5.00–7.00) in node positive group (p=0.009). Curvilinear regression revealed a linear correlation between TLNC and positive LN count (r^2=0.249, p<0.001) (Fig. 2). Based on the magnitude of the log-rank test χ^2 statistic, cut-off value for optimal TLNC for the entire cohort was determined to be 6. When the patients were categorized as N0 based on TLNC≥6 (Group A, n=7), N0 based on TLNC<6 (Group B, n=17) and all node positive patients (Group C, n=33), Group B patients had significantly worse DFS than Group A (Group A vs. B: not reached vs. 32.00±4.80 months, p=0.012) but significantly better than Group C patients (Group B vs. C: 32.00±4.80 vs. 14.00±2.33 months, p=0.039) (Fig. 3). Amongst 33 patients with node positive disease, TLNC was not associated with prognosis (median DFS, TLNC≥6 vs. TLNC<6: 19.00±3.24 vs. 14.00±1.22 months, p=0.108).

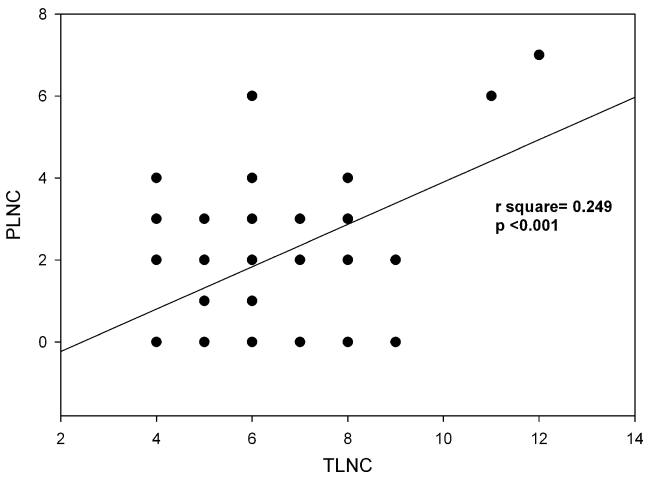

Fig. 2 Scatterplot with regression line showing relationship between total lymph node count (*TLNC*) and positive lymph node count (*PLNC*)

Positive Lymph Node Count (PLNC) The median PLNC for the entire cohort was 2 (i.q.r: 0–3). DFS progressively worsened with increasing PLNC and no definite cut-off value could be identified. Patients with single involved LN (n=3) had similar DFS as those with N0 disease (38.00±16.26 vs. 38.00±3.52 months, p=0.982). Based on the quartile values of PLNC, when all the patients were grouped into those with PLNC<2 (Group I, n=27), PLNC 2–3 (Group II, n=19) and PLNC>3 (Group III, n=11); DFS differed significantly amongst the groups (Group I vs. II vs. III: 38.00±1.29 vs. 17.00±1.89 vs. 9.00±1.42 months, p<0.001) (Fig. 4).

Lymph Node Ratio (LNR) Median LNR for the entire study group was 0.25 (i.q.r: 0–0.50). The cut-off value for

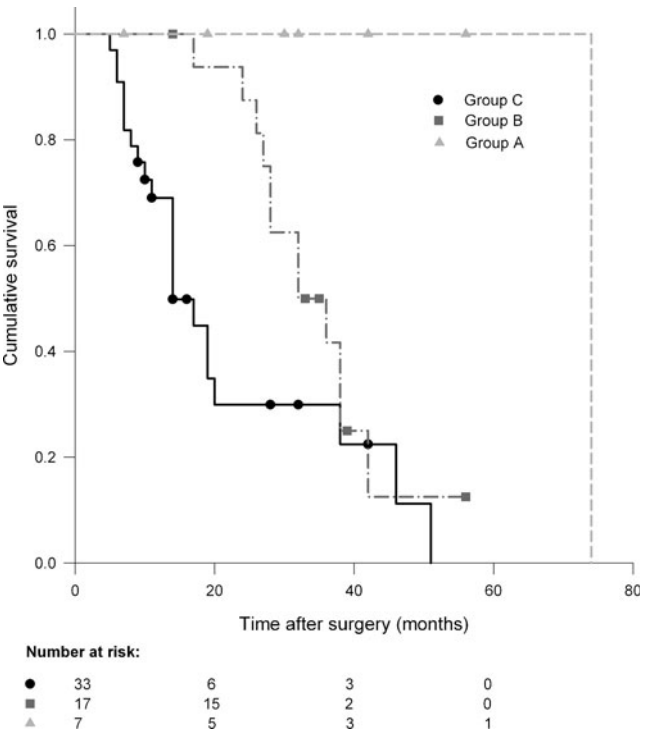

Fig. 3 Disease-free survival (*DFS*) of the entire cohort categorized into three groups according to nodal status. *Group A* N0 patients based on total lymph node count (*TLNC*)≥6, *Group B* N0 patients based on TLNC<6 and *Group C* all node-positive patients

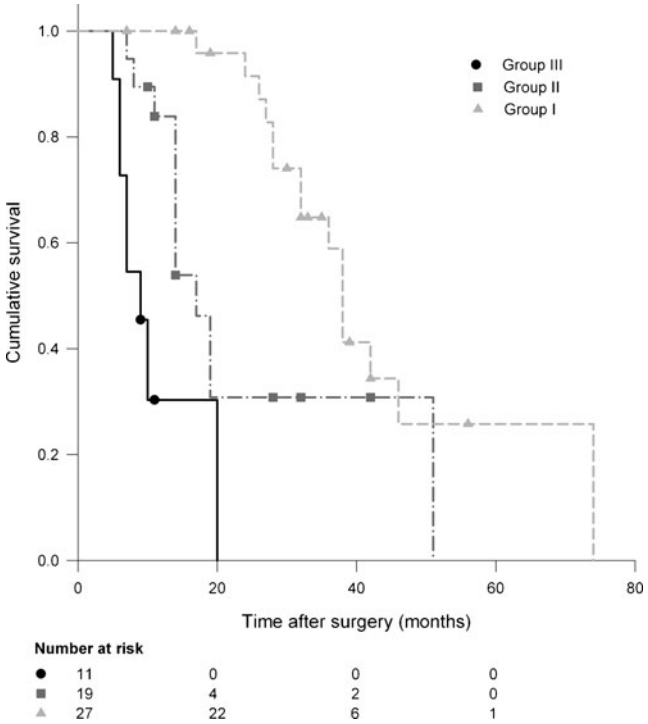

Fig. 4 Disease-free survival (*DFS*) of the entire cohort stratified into three groups according to positive lymph node count (*PLNC*). *Group I* PLNC<2, *Group II* PLNC 2–3, *Group III* PLNC>3

optimal LNR for the entire cohort, based on the magnitude of the log-rank test χ^2 statistic, was determined to be 0.50. When the patients were grouped as those with LNR=0 (Group 1, n=24), 0<LNR≤0.50 (Group 2, n=22) and LNR>0.50 (Group 3, n=11), DFS progressively worsened with increasing LNR (Group 1 vs. 2 vs. 3: 38.00±3.52 vs. 14.00±2.46 vs. 9.00±1.55 months, p<0.001) (Fig. 5). Moreover, LNR>0.50 was associated with an increased probability of adverse tumor variables like higher incidence of T3 tumors (11 (100%) vs. 24 (52%), p=0.004) and pathologic adjacent organ involvement (3 (27%) vs. 2 (4%), p=0.045). TLNC≥6 provided further prognostic stratification in patients with LNR≤0.50 but not in patients with LNR>0.50. In fact, TLNC≥6 was associated with worse, though statistically insignificant, DFS in patients with LNR>0.50 (Table 1).

Lymph Node Location The relative frequency of positive LN location included cystic duct in 24%, pericholedochal in 25%, periportal in 19%, hepatic artery in 19% and peripancreatic or periduodenal in 13% patients. Based on the 7th edition of AJCC classification, 23 (40%) patients had N0, 21 (37%) had N1 and 13 (23%) had N2 disease. All the patients in the present cohort were classified as N2 based on involvement of periduodenal and peripancreatic lymph nodes alone. The DFS was not significantly different between patients categorized as N1 or N2 (N1 vs. N2: 17.00±2.15 vs. 10.00±2.54 months, p=0.186) (Fig. 6).

Pattern of Recurrence and Risk Factor Analysis

At a median follow-up of 19 (i.q.r: 11–39.5) months, median DFS for the entire cohort was 28.25±3.62 months. This corresponded to a 1-, 3- and 5-year actuarial DFS of 82.07%, 44.46% and 16.28% respectively. Until last follow up, 35 (61%) patients had developed recurrence which was classified as distant in 22 (39%) and loco-regional in 17 (30%). Combined (loco-regional and distant) recurrence occurred in 4 (7%) patients. The relative frequency of recurrence site was liver in 33%, retroperitoneal nodes 30%, lungs 7% and peritoneal in 4% patients. The only demographic, therapeutic, or pathologic factor associated with recurrence pattern was the significantly higher incidence of distant recurrence in patients with LNR>0.50 (8 (73%) vs. 14 (30%), p=0.015).

On univariable analysis, histological involvement of bile duct by cancer, moderately or poorly differentiated cancer, lymphovascular invasion, T3 tumors, LN positivity, N2 disease and LNR>0.50 was associated with significantly worse DFS after curative resection of gallbladder cancer (Table 2). However subsequent multivariable analysis

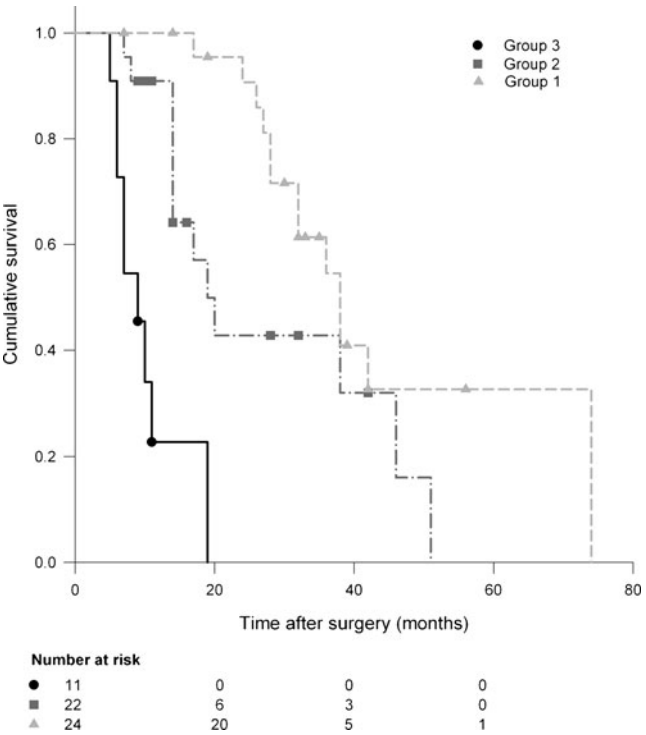

Fig. 5 Disease-free survival (*DFS*) of the entire cohort categorized into three groups according to lymph node ratio (*LNR*). *Group 1* LNR=0, *Group 2* 0<LNR≤0.50, *Group 3* LNR>0.50

Table 1 Disease-free survival in different categories of lymph node ratio stratified according to number of lymph nodes examined

Variable	Patients, no. (%)	Disease-free survival, months, median±SE	*p* value
Lymph node ratio=0			
Lymph nodes examined≥6	7 (12)	Not reached	0.012
Lymph nodes examined<6	17 (30)	32.00±4.80	
0<Lymph node ratio≤0.50			
Lymph nodes examined≥6	16 (28)	20.00±11.77	0.037
Lymph nodes examined<6	6 (11)	14.00±1.77	
Lymph node ratio>0.50			
Lymph nodes examined≥6	6 (10)	6.00±0.82	0.174
Lymph nodes examined<6	5 (9)	11.00±1.69	

revealed only T stage, degree of tumor differentiation and LNR to be independent predictors of DFS after surgical resection (Table 3).

Discussion

Complete surgical resection is the mainstay of treatment in patients with localized gallbladder cancer. While considering the best surgical approach, two pertinent issues that need to be addressed are the extent of hepatectomy and limits of lymphadenectomy. Lymph nodal dissection is a key component of surgery in these patients.[11] In fact, it has been observed that hepatic resection without concomitant lymphadenectomy does not improve survival in early stage gallbladder cancer.[12] Moreover, as compared to limited non-anatomical liver resections, performance of extended or anatomical hepatectomy does not translate into survival benefit but is associated with increased risk of postoperative complications.[13,14] Hence, we believe that the thrust should be on ensuring negative resection margins while preserving maximal hepatic parenchyma.

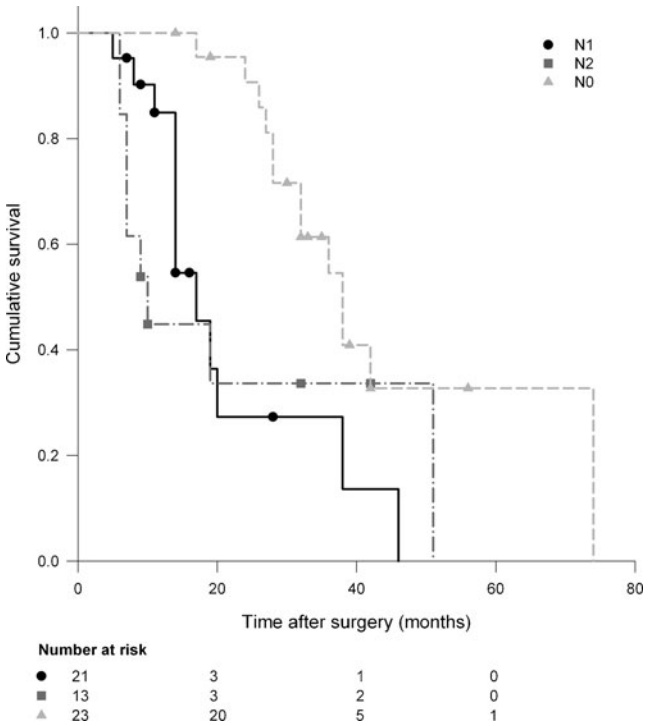

Fig. 6 Disease-free survival (*DFS*) of the entire cohort stratified according to lymph node status based on the 7th edition of AJCC classification

Lymph node dissection, on the other hand, carries the potential to provide more accurate pathologic staging, better regional disease control and possibly some survival advantage. The optimal extent of lymphadenectomy, however, remains unresolved and there are no uniform evidence-based guidelines on the issue. This is further compounded by the rarity of disease and low resectability rates, which limit the ability to perform large cohort studies or prospective randomized trials. Investigators from western centres rely on limited nodal dissection involving hepato-duodenal ligament,[13] while those from the east recommend extended lymphadenectomy including para-aortic lymph node dissection.[15] This difference in surgical approach has led to higher lymph nodal yield reported in eastern than western studies. The number of lymph nodes retrieved also depends on performance of concomitant pancreaticoduodenectomy.[7] Similar to a recent report,[14] our study demonstrates that routine extrahepatic bile duct resection does not increase the nodal yield or survival, but is associated with increased postoperative morbidity. Hence, it should be selectively performed to ensure negative surgical margins.

Accuracy of nodal staging depends on a critical number of lymph nodes analyzed; insufficient number of nodes retrieved during surgery or examined pathologically leads to underestimation of disease stage. Although, the 6th edition of AJCC suggests a minimum of three lymph nodes to be assessed for appropriate pathologic nodal staging of gallbladder cancer, the basis of recommendation is not clear, and there are no established standards.[8] A large population-based study on SEER database demonstrated that 75% of pathologic specimens of gallbladder cancer had two or fewer nodes, and threshold for minimum number of lymph nodes could therefore not be determined.[16] Another

Table 2 Univariable analysis of factors associated with disease-free survival after curative intent resection for gallbladder cancer

Variable	Patients, no. (%)	Disease-free survival, months, median±SE	*p* value
Age			
>45 years	35 (61)	32.00±3.99	0.879
≤45 years	22 (39)	27.00±4.15	
Sex			
Male	13 (23)	28.00±0.78	0.795
Female	44 (77)	32.00±5.12	
Pain			
Yes	41 (72)	32.00±7.61	0.771
No	16 (28)	28.00±1.65	
Weight loss			
Yes	40 (70)	27.00±5.28	0.854
No	17 (30)	32.00±6.69	
Jaundice			
Yes	7 (12)	19.00±3.90	0.651
No	50 (88)	32.00±4.84	
Gallstones			
Yes	33 (58)	32.00±3.93	0.783
No	24 (42)	28.00±8.45	
Associated comorbidity			
Yes	11 (19)	26.00±7.67	0.528
No	46 (81)	32.00±4.01	
Previous cholecystectomy			
Yes	21 (37)	36.00±6.74	0.404
No	36 (63)	27.00±6.34	
Bile duct excision			
Yes	33 (58)	28.00±6.75	0.827
No	24 (42)	32.00±8.91	
Histological bile duct involvement			
Yes	5 (9)	14.00±3.29	0.014
No	52 (91)	32.00±4.91	
Contiguous visceral resection			
Yes	5 (9)	19.00±8.90	0.251
No	52 (91)	32.00±5.48	
Perioperative blood transfusion			
Yes	7 (12)	19.00±5.23	0.986
No	50 (88)	32.00±3.53	
Postoperative complications			
Yes	23 (40)	27.00±5.85	0.207
No	34 (60)	32.00±4.89	
Well differentiated			
Yes	30 (53)	38.00±1.87	0.001
No	27 (47)	17.00±3.47	
Lympho-vascular invasion			
Yes	25 (44)	24.00±6.40	0.086
No	32 (56)	32.00±5.58	
T-stage			
T 1–2	22 (39)	42.00±5.85	<0.001
T 3–4	35 (61)	17.00±2.60	
Lymph node involved			
Yes	33 (58)	14.00±2.33	0.001
No	32 (56)	38.00±3.53	
N2 involvement			
Yes	13 (23)	10.00±2.54	0.067
No	44 (77)	32.00±4.54	
Lymph nodes examined ≥ 6			
Yes	29 (51)	46.00±26.98	0.216
No	28 (49)	28.00±1.62	
Lymph node ratio ≤ 0.50			
Yes	46 (81)	36.00±3.83	<0.001
No	11 (19)	9.00±1.55	

study in patients with extrahepatic cholangiocarcinoma, ampullary and gallbladder cancer suggested that a minimum of ten lymph nodes are required for adequate staging.[17] Since tumors with diverse biology and outcome were grouped together, such an analysis is perhaps methodologically inappropriate. Although a greater number of examined nodes might stage the disease better, the result of our study suggests that minimum TLNC for accurate nodal staging of gallbladder cancer is perhaps six. Furthermore, TLNC significantly correlated with DFS in node-negative patients and allowed better prognostic sub-stratification of these patients. Since the total lymph node count–survival relationship was observed only in node-negative patients and not in those with involved nodes, we believe that a higher count only helps in stage purification and therapeutic benefit, if any, is likely to be small. These findings should heighten awareness about the importance of TLNC not only amongst surgeons performing lymphadenectomy but also pathologists retrieving and examining lymph nodes.

The burden of nodal disease (PLNC) also had impact on prognosis, with significantly reduced DFS observed in our patients with involved nodes. The DFS progressively worsened with increasing PLNC, and we were not able to identify any specific cut-off value. We also observed that patients with single involved node had similar survival as those with negative nodes. Though the number of patients with single involved node was limited, our results are

Table 3 Regression analysis of factors associated with disease-free survival after curative intent resection for gallbladder cancer

Variable	Odds ratio (95% CI)	*p* value
Well differentiated (yes versus no)	2.26 (1.05, 4.85)	0.037
T 1–2 stage (yes versus no)	4.70 (1.76, 12.56)	0.002
Lymph node ratio≤0.50 (yes versus no)	6.55 (2.49, 17.21)	<0.001

similar to an earlier reported study.[15] The use of PLNC as a prognostic factor is limited by inherent bias of inadequate number of lymph nodes retrieved or histologically examined which leads to the phenomenon of 'stage migration'. LNR is a better and reproducible method of stratifying nodal status which incorporates not only the burden and biology of disease (PLNC) but also the quality of lymphadenectomy and pathologic examination (TLNC). LNR has been shown to be an important predictor of survival after surgery for pancreatic and bile duct cancer.[5,18] To the best of our knowledge, no study has assessed the association between LNR and survival in patients with gallbladder cancer. Our study suggests that, along with tumor stage and differentiation, LNR is an independent prognostic factor and the single most important lymph nodal variable in patients undergoing curative resection for gallbladder cancer. The prognostic impact of LNR was observed in the entire cohort and subgroup of patients with positive nodes.

Based on detailed anatomical studies, it has been suggested that lymphatic metastasis from gallbladder cancer spreads widely through hepatoduodenal ligament towards peripancreatic region and beyond.[19,20] Cystic and pericholedochal regions were most commonly involved in our patients followed by periportal and hepatic artery nodes. Retropancreatic nodes were involved in 23% patients and were classified as N2 disease, and hence metastatic, according to the 7th edition of AJCC classification. However, we observed that categorization of patients as having N2 disease based on involvement of retropancreatic lymph nodes alone did not adversely influence DFS as compared to those with N1 disease. Similarly, studies from Japan have also reported long-term survivors in patients with involved pancreatioduodenal and hepatic artery lymph nodes but none in those with involved paraaortic, celiac or superior mesenteric nodes.[19,21] Hence, we believe that involvement of retropancreatic nodes should perhaps be categorized as N1 rather than N2 disease. Moreover, our data suggests that location of involved nodes has lesser impact on prognosis than bulk of nodal involvement (LNR).

The median DFS of 28 months in the present study compares favorably with previous reports.[13,14] The present study confirms earlier observations that most of the recurrences are intra-abdominal and chiefly involves liver or retroperitoneal lymph nodes.[22,23] Besides, we also observed that patients with LNR>0.50 had significantly higher probability of developing distant recurrence. The role of postoperative adjuvant therapy in the management of patients with gallbladder cancer remains undefined. Recent studies have shown conflicting results with some showing benefit of chemo-radiotherapy especially in those with involved nodes,[24] while others failed to demonstrate survival advantage.[23] Our data demonstrates better but statistically insignificant difference in DFS amongst lymph node positive patients receiving postoperative chemotherapy. Unfortunately, the number of patients was small, and the treatment regimen was not standardized. The strengths of our study include the reasonable-sized cohort of patients managed in a single institution using a standardized treatment approach. However, the study is limited by its retrospective nature and relatively short follow-up period. Focusing on the discrete outcome measure of DFS and the fact that over 60% of patients had developed recurrence during the follow-up period perhaps minimized the limitation of follow up. Nonetheless, these observations need to be confirmed in larger, especially population-based, cohort.

The results of the present study demonstrate that, rather than categorizing patients with gallbladder cancer based on PLNC or location of involved nodes, LNR is a more appropriate tool to stratify patients with regards to prognosis and risk of recurrence. Our data also suggests that removal and pathological examination of at least six lymph nodes can influence staging quality and disease-free survival especially in node-negative patients. This knowledge should heighten awareness amongst surgeons and pathologists, about the importance of retrieving and examining an adequate number of lymph nodes.

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ORIGINAL ARTICLE

Improvement in Treatment and Outcome of Pancreatic Ductal Adenocarcinoma in North China

Yong Chen · JiHui Hao · WeiDong Ma · Yong Tang · ChunTao Gao · XiShan Hao

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Abstract
Background The incidence of pancreatic cancer has increased in China in the last decade, though efforts have been made in early detection and multimodality treatment. The aim of this study is to describe the decade-based development in early diagnosis and treatment modalities, as well as outcome for patients with pancreatic ductal adenocarcinoma (PDAC) in a high-volume facility.
Methods All the PDAC patients underwent surgery between 1991 and 2009 and were selected from the database of TianJin Cancer Institute and Hospital. Decade-based changes in early diagnosis, treatment modalities, and outcome of the patients were retrospectively analyzed.
Results Of the 565 patients with PDAC, patients in this decade (n=460) had better overall survival than those in the last decade (n=105), median survival was 10 months and 3 months, respectively. Patients in this decade had significantly improved in (P<0.001) 2-year (14.7%) and 5-year survival rates (3.5%) as compared to those in the last decade (6.7% and 3.4%, respectively). Patients with metastasis at diagnosis in the last decade and this decade were 54% and 26% (P<0.001), respectively. More patients in this decade had underwent R0/R1 resection (33% vs 20%, P=0.010), chemotherapy (37% vs 12%, P<0.001), and radical resection (34% vs 21%, P=0.014) than those in the last decade.
Conclusion Patients operated on for PDAC in this decade had a better outcome than those in the last decade. Early detection, improved resection margin, and development in multimodality treatment contribute to this improvement.

Keywords PDAC · Decade · Surgery type · Outcome

Introduction

Pancreatic cancer ranked 13th in the list of most commonly diagnosed cancers and was the fifth most common cause of cancer death in China in 2006.[1] The 5-year survival rate for all pancreatic cancer patients is below 5%.[2] Among the various subtypes of pancreatic cancer, pancreatic ductal adenocarcinoma (PDAC) is by far the most common and most important tumor type, accounting for more than 85% of pancreatic tumors.[3] Surgical removal of the tumor with negative resection margins remains to be the most potentially curative therapy.[3,4] Late presentation and fear of perioperative mortality[5] have left only a minority of patients amenable to curative resection[6] in the last decade. Efforts have been made towards early detection with biomarkers and advanced imaging techniques such that perioperative outcome has been markedly improved within the past two decades due to advances in both surgical technique and perioperative care.[7,8] Today, pancreas resection can be performed in more than 20% of pancreas cancer patients with localized disease resulting in a very low mortality rate (below 5%)[7–9] and producing a 5-year survival rate that exceeds 20% with adjuvant therapy.[10,11]

JiHui Hao contributed equally with Yong Chen.

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Y. Chen · X. Hao (✉)
Key Laboratory of Cancer Prevention and Therapy,
Huanhu Xi road, Hexi district, Tianjin 300060, China
e-mail: Haoxishan1950@gmail.com

J. Hao · W. Ma · Y. Tang · C. Gao
Department of Pancreatic Cancer,
Tianjin Medical University Cancer Institute and Hospital,
Tianjin 300060, China

With the development of diagnostic capacity and environmental influence, the incidence of PDAC in China has increased in the past several decades.[1,12] Though highly specialized centers for pancreatic cancers were built up with skilled surgical oncologists and experienced multimodality physicians, the improvement in treatment modalities and patients' outcomes have not yet been delineated. The purpose of this study is to investigate the decade-based improvement in diagnosis modalities and surgical treatment, and to compare the different outcomes in the same cancer center between two decades.

Materials and Methods

Retrospectively collected patient data from Tianjin Medical University Cancer Institute and Hospital was used to identify all patients with PDAC that underwent surgical treatment (n=565). Clinical variables include age at diagnosis, gender, serum CA-199, preoperative bilirium, site, American Joint Committee on Cancer (AJCC) stage,[13] perioperative blood transfusion, and hospital stay. Pathologic characteristics included tumor size, French Federation of Cancer Centres (FNCLCC) grade,[14] Union Internationale Contre Cancer (UICC) margin,[15] lymph nodes invasiveness (LNI), vascular involvement (VI), and perineural involvement (PNI).

Treatment modalities included the surgical procedures, radiation therapy, and neoadjuvant/adjuvant chemotherapy. All patients underwent surgical treatment, the surgical type included radical resection (n=179) and palliative surgery (n=386). Radical resection is an intraoperative evaluation which refers to an extensive en bloc resection of the tumor and surrounding tissues, as well as lymph drainage and vascular structures. Patients with operable diseases (n=132) were assigned to radical resection, patients with inoperable lesions were assigned either to neoadjuvant chemotherapy (n=156) or palliative surgery (n=277). Neoadjuvant chemotherapy was based on 5-Fu in the last decade and gemcitabine in this decade, including CF, CDDP, and paclitaxel, single dose or combined for 2 to 4 cycles. After neoadjuvant chemotherapy, the lesions were reassessed for resectability by surgical oncologists (JH H and XS H) with comparable pre- and post-neoadjuvant chemotherapy, computed tomography, or magnetic resonance imaging. After the reassessment, patients with operable lesions were assigned to radical resection (n=47) with postoperative chemotherapy of same regimen; patients with inoperable lesions (n=109) were treated with palliative procedures. All the pre- and post-adjuvant chemotherapy images were reviewed to identify borderline resectable lesions; their actual treatments depending on patients' preference were recorded.

SPSS 13.0 statistical software (SPSS, Chicago, IL, USA) was used for statistical analysis, the following variables were considered for their prognostic value: age at diagnosis, sex, tumor site, tumor size, AJCC stage, surgical type, blood transfusion, hospital stay, UICC margin, FNCLCC grade, chemotherapy, PNI, and vascular involvement. Univariate and multivariate analyses were carried out for overall survival (OS). Survival curves were computed by the Kaplan–Meier[16] method and compared by the log-rank test. Multivariate analyses based on the stepwise Cox[17] proportional hazards model were used to identify the most significant factors related to outcome. A stepwise forward selection procedure was used and a significance level of 5% was chosen as the criterion for entering factors in the multivariate model.

Results

Clinical, Pathologic, and Treatment Variable

All 565 patients underwent surgical treatment for radical or palliative purpose, clinical, pathologic, and treatment variables and their distribution for decades are listed in Table 1. The median follow-up for survivors was 6 months (range, 0–156 months). There were 359 (63.5%) males and 206 (36.5%) females with a median age of 61.0 years (range, 24–88 years). The median tumor size was 4.0 cm (range, 0.8–18 cm). At the time of diagnosis, the tumor stages according to AJCC staging system were as follows: I, 77 patients; II, 172 patients; III, 136 patients, and IV, 176 patients. Local invasiveness of tumor were confirmed by intraoperative exploration or postoperative pathological examination, 166 patients had vascular involvement, either in the artery or vein or both. Ninety-three patients had lymph node involvement and 34 patients had PNI.

Carbohydrate cantigen 19-9 (CA19-9) was investigated with a cutoff level of 30 U/L in 463 patients. Two hundred sixty-four patients had a positive CA19-9 with a sensitivity of 57.0%. In those with both CA19-9 and tumor size records (n=289), CA19-9 was positive in 170 patients, and most of them (163/170, 95.9%) had a primary tumor of over 2 cm. Of 552 patients that had a preoperative bilirium test, patients with preoperative bilirium of less than 200 U/L, between 200 and 300 U/L, and over 300 U/L were 372 (372/552, 67.4%), 85 (85/552, 15.4%), and 95 (95/552, 17.2%), respectively.

Palliative resection and exploratory laparotomy were assigned to be R2 resections. Of the 179 patients submitted to radical resection, resection was grossly complete (R0/R1) in 171 patients (94.4%), seven patients (10%) had a tumor rupture intraoperatively (R2), and one patient had missing data. Forty-eight of the 179 patients had undergone a

Table 1 Relationship between decades and clinical, pathologic, treatment parameters

Issues	Categories	All	Between 1990 and 2000	Between 2000 and 2009	χ^2	*P*
N		565	105	460		
Age (*n*=565)	≥50	458	70	388	17.409	<0.001
	<50	107	35	72		
Sex (*n*=565)	Male	359	70	289	0.544	0.461
	Female	206	35	171		
Site (*n*=565)	Head	502	97	405	1.623	0.203
	Body or tail	63	8	55		
Size (cm) (*n*=399)	≥2	387	77	310	2.959	0.085
	<2	12	0	12		
FNCLCC grade (*n*=30,918)	G1	54	19	35	0.147	0.701
	G2	68	3	65		
	G3	67	14	53		
	Gx	129	23	97		
UICC margin (*n*=5,567)	R0+R1	171	21	150	6.637	0.010
	R2	386	83	303		
Radiotherapy (*n*=565)	Yes	14	1	13	1.242	0.265
	No	551	104	447		
Chemotherapy (*n*=565)	Yes	184	13	171	23.928	<0.001
	No	381	92	289		
AJCC stage (*n*=565)	I–II	252	44	208	0.322	0.570
	III–IV	313	61	252		
Surgery type (*n*=565)	Radical	179	22	157	10.679	0.014
	Palliative	303	61	242		
	Exploratory	83	22	61		
PB (*n*=552)	<200	372	79	293	4.288	0.117
	200–300	85	12	73		
	>300	95	13	82		
CA199 (*n*=436)	Positive	264	4	260	113.465	<0.001
	Negative	172	70	102		
PNI (*n*=358)	Yes	34	6	28	0.247	0.619
	No	324	69	255		
VI (*n*=400)	Yes	166	31	135	0.207	0.649
	No	234	48	186		
LNI (*n*=398)	Yes	93	22	71	0.955	0.328
	No	305	58	247		
MAD (*n*=561)	Yes	176	57	119	31.500	<0.001
	No	385	48	337		
Blood tranfusion	Mean[a]	1.4 U	2.4 U	1.2 U	11.740	0.001
	Yes	164	46	118	13.657	<0.001
	No	401	59	342		
Hospital stay	Mean[b]	29.6	32.5	29.0	9.909	0.002
	<4 weeks	287	41	246	7.110	0.008
	>4 weeks	278	64	214		

FNCLCC Fédération Nationale des Centres de Lutte Contre le Cancer, *UICC* International Union Against Cancer, *AJCC* American Joint Committee on Cancer, *PB* preoperative bilirium, *PNI* perineural involvement, *VI* vascular involvement, *LNI* lymph node involvement, *MAD* metastasis at diagnosis

[a] Blood transfusion expressed as mean, F=11.740, P=0.001

[b] Hospital stay expressed as mean, F=9.909, P=0.002 (ANOVA test)

vascular resection and reconstruction; 28 patients had extended lymphadenectomy.

Neoadjuvant chemotherapy was performed in 156 patients with locally advanced or disseminated diseases,

after neoadjuvant treatment, 30.1% (47/156) of them underwent radical resection. One hundred eight-four (184/565, 32.6%) patients were treated with adjuvant chemotherapy. Adjuvant chemotherapy was administered after radical resection, palliative resection, and exploratory laparotomy in 82, 73, and 29 patients, respectively. Chemotherapy agents included 5-Fu, CF, CDDP, gemcitabine, and HCPT, single or combined for at least 2 cycles. Fourteen patients underwent postoperative radiotherapy.

Of the 565 patients, 164 underwent perioperative blood transfusion of a mean amount of 1.4 U. Blood transfusion was required in 45.3% (81/179) of patients who underwent radical resection, while this number decreased to 31.5% in patients with other procedures. All the 565 patients had a mean hospital stay of 29.6 days, and expectantly, patients with radical resections had a significantly longer hospital stay than those with non-radical resection (P=0.002, mean 31.6 days vs 28.7 days).

A Decade-Based Analysis of Early Diagnosis and its Impact on Outcomes

Of all 565 patients, 2-year and 5-year overall survival was 13.5% and 3.6%, respectively. AJCC stage was associated with OS, 2-year and 5-year OS was 38.0% and 8.8% for stage I, 13.2% and 2.6% for stage II, 8.1% and 1.6% for stage III, and 6.7% and 0% for stage IV, respectively (P< 0.001). More patients (208/460, 45.2%) operated on for PDAC in this decade had a stage I/II disease at diagnosis than those (44/105, 40.3%) in the last decade. Furthermore, fewer patients (119/460, 26.1%) operated on for PDAC in this decade were at their terminal stage compared to those (57/105, 54.3%) in the last decade (P<0.001) (Table 1). Patients with stage I/II PDAC in this decade had a significantly longer median survival of 14 months compared to those of 4 months in last decade (P<0.001). Accordingly, patients with stage III/IV PDAC in this decade had a significantly longer median survival of 6 months compared to those of 3 months in last decade (P<0.001) (Fig. 1a).

A Decade-Based Analysis of Clinical and Pathological Variables Predictive of Outcome

Patients of the two decades had similar tumor sites (P= 0.203), sizes (P=0.085), grades (P=0.701), stages (P= 0.570), PNIs (P=0.619), VIs (P=0.649), and LNIs (P= 0.328). Tumor site was an important prognostic factor (Fig. 1b). The 2-year and 5-year OS was 31.4% and 12.6% in patients with a tumor in pancreas body and tail, which was significantly different from 11.2% and 2.0% in those with a tumor in the pancreas head (P<0.001) (Table 2). Of all 565 patients, FNCLCC grade was a strong prognostic factor for OS (Fig. 1c), 2-year and 5-year OS was 24.3% and 4.0% for grade 1, 19.4% and 0% for grade 2, and 10.8% and 3.6% for grade 3, respectively (P=0.003) (Table 2). PDAC patients with normal CA19-9 had a poorer prognosis. Two-year and 5-year OS was 18.5% and 4.4% in patients with positive CA19-9 (n=264) compared to 9.7% and 2.2% in those with normal CA19-9 (n=172), respectively (P=0.001). But in this decade, CA19-9 did not seem to be significantly associated with prognosis. Median survival in 260 patients with positive CA19-9 was 10 months, which was similar (P=0.694) to 11 months in 102 patients with normal CA19-9, indicating that a more sensitive cutoff value should be investigated for prognostic purpose.

More patients underwent operation for PDAC in this decade (150/453, 33.1%) had a R0/R1 resection than those in the last decade (21/104, 20.2%, P=0.010). R0/R1 resection was significantly associated with improved OS compared to R2 resection, 2-year and 5-year OS in patients with R0/R1 margin was 29.6% and 6.1%, while in patients with R2 resection, they were 6.7% and 2.6%, respectively (P<0.001) (Fig. 1d).

We further identified that LNI was also associated with poorer prognosis in PDAC patients (Fig. 1e), as patients without LNI had a median survival of 11 months, which was significantly longer than LNI patients of 5 months (P= 0.005). PNI was not associated with OS (P=0.803) as indicated in Table 2, but VI was significantly associated with poor prognosis in PDAC patients (Fig. 1f). In 400 patients who had detailed information of vascular status, median OS in patients with VI (n=166) was 9 months, compared to 12 months in those without (n=234, P=0.013).

Blood transfusion was recognized as a prognostic factor for PDAC,[18] though not universally accepted.[19,20] We next investigated blood transfusion in the 565 patients. For all surgical procedures, blood transfusion was required in 43.8% (46/105) of patients in the last decade with a mean amount of 2.4 U, but it fell to 25.7% (118/420) and 1.2 U in this decade (Table 1). Though blood transfusion was not prognostic for survival in all patients (Table 2), it was of prognostic value in the subset of patients who underwent radical resection (P=0.032). Decade-based analysis indicated that requirement of blood transfusion for radical resection was significantly higher (P=0.001) in the last decade (77.3%, 17/22) than that in this decade (40.7%, 64/157).

Hospital stay reflects the capacity of multidisciplinary care in specialized high-volume centers. The mean hospital stay in the last decade was significantly longer than that in this decade (mean 32.5 days vs 29.0 days, P=0.002). Patients with radical resection had a significantly longer hospital stay than those with other procedures, indicating reasonably that more aggressive surgeries necessitate longer postoperative recovery.

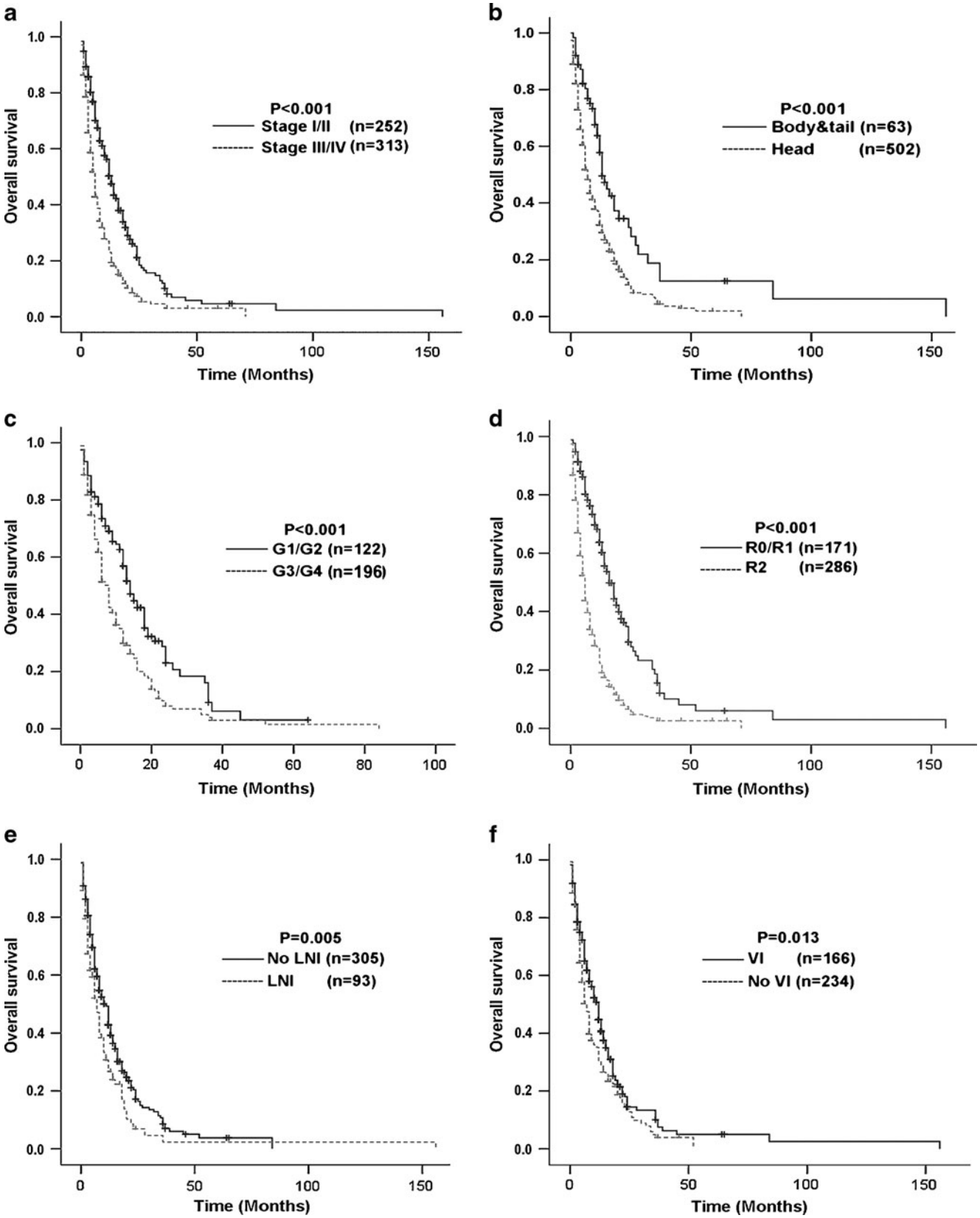

Fig. 1 The curves of overall survival in patients according to clinical and pathological parameters. **a** tumor site; **b** AJCC stage; **c** FNCLCC grade; **d** UICC margin; **e** lymph node involvement; **f** vascular involvement

Table 2 Univariate and multivariate analysis of variable factors for OS

Factors		Survival rate		Univariate analysis			Multivariate analysis		
		2 years	5 years	HR	95% CI	*P*	HR	95% CI	*P*
Age	≥50 (*n*=458)	12.9	1.8	1.252	0.981–1.597	0.059			
	<50 (*n*=107)	15.5	9.8	1					
Sex	Male (*n*=359)	10.6	3.1	1		0.197			
	Female (*n*=206)	18.2	4.7	0.884	0.726–1.075				
Site	Head (*n*=502)	11.2	2.0	1.915	1.384–2.649	<0.001	1.501	1.057–2.134	0.023
	Body/tail (*n*=63)	31.4	12.6	1			1		
FNCLCC grade	G1/G2 (*n*=122)	23.0	3.1	1		0.001	1		0.012
	G3/Gx (*n*=18,796)	9.7	1.5	1.723	1.318–2.252		1.414	1.081–1.851	
UICC margin	R0+R1 (*n*=171)	29.6	6.1	1		<0.001	1		<0.001
	R2 (*n*=386)	6.7	2.6	1.604	1.432–1.795		1.555	1.322–1.828	
Chemotherapy	Yes (*n*=184)	24.8	4.7	1		<0.001	1		<0.001
	No (*n*=381)	7.7	3.1	2.116	1.715–2.610		2.176	1.700–2.786	
AJCC stage	I–II (*n*=252)	21.2	4.6	1		<0.001	1		<0.001
	III–IV (*n*=313)	7.4	3.1	1.907	1.572–2.314		1.810	1.407–2.329	
Surgery type	Radical (*n*=179)	29.8	5.5	1		<0.001	1		<0.001
	Nonradical (*n*=386)	6.4	2.7	2.512	2.016–3.129		2.674	2.101–3.403	
VI	Yes (*n*=166)	13.8	0	1.315	1.049–1.648	0.013	1.002	0.777–1.292	0.988
	No (*n*=234)	14.5	5.0	1			1		
VR	Yes (*n*=48)	34.9	0	1		<0.001	1		<0.001
	No (*n*=118)	6.6	2.6	2.710	1.786–4.114		2.788	1.811–4.292	
LNI	Yes (*n*=93)	6.9	2.3	1.425	1.099–1.847	0.005	1.325	1.015–1.731	0.038
	No (*n*=305)	17.1	3.7	1			1		
EL	Yes (*n*=28)	20.9	10.4	1		<0.001	1		<0.001
	No (*n*=65)	1.9	0	2.926	1.690–5.066		2.868	1.648–4.991	
MAD	Yes (*n*=176)	6.7	0	1.827	1.500–2.226	<0.001	1.448	1.097–1.912	0.009
	No (*n*=389)	16.6	3.5	1			1		
Blood transfusion	Yes (*n*=164)	13.8	2.6	0.977	0.797–1.198				
	No (*n*=401)	13.4	4.5	1					

FNCLCC Fédération Nationale des Centres de Lutte Contre le Cancer, *UICC* International Union Against Cancer, *AJCC* American Joint Committee on Cancer, *PB* preoperative bilirium, *PNI* peri-neural involvement, *VI* vascular involvement, *VR* vascular reconstruction, *LNI* lymph node involvement, *EL* extended lymphadenectomy, *MAD* metastasis at diagnosis

A Decade-Based Analysis of Treatment Variables Predictive of Outcome

Of the 565 patients, more patients (157/460, 34.1%) operated for PDAC in this decade underwent a radical resection than those in last decade (22/105, 21.0%, $P=0.014$). Surgery type was significantly associated with OS (Fig. 2a); 2-year and 5-year OS in patients with radical resection was 29.8% and 5.5%, while in patients with non-radical resection, they were 6.4% and 2.7%, respectively ($P<0.001$). Neoadjuvant chemotherapy increased the rate of radical resection in patients with inoperable diseases (47/156 vs 8/277, $P<0.001$), and this increase was even more dominant in this decade, 36.2% (46/127) of the patients who underwent neoadjuvant chemotherapy for their inoperable diseases were able to have radical resection, indicating that the emergency of first-line gemcitabine for PDAC might have contributed to this improvement.

Similarly, of all 565 patients, more patients in this decade (171/460, 37.2%) underwent adjuvant chemotherapy than those in the last decade (13/105, 12.4%, $P<0.001$). Adjuvant chemotherapy was significantly associated with improved OS in PDAC patients; 2-year and 5-year OS in patients with chemotherapy was 24.8% and 4.7%, while in those without, they were 7.7% and 3.1%, respectively ($P<0.001$, Fig. 2b). Vascular resection and reconstruction performed for the purpose of radical resection mostly occurred in this decade (1/31 vs 47/135, $P<0.001$), with a single artery reconstruction in five, single venous reconstruction in 17, and both in 26. Of the 176 patients with radical resection who had a record of VI status, vascular reconstruction had no significant impact on survival; 2-year

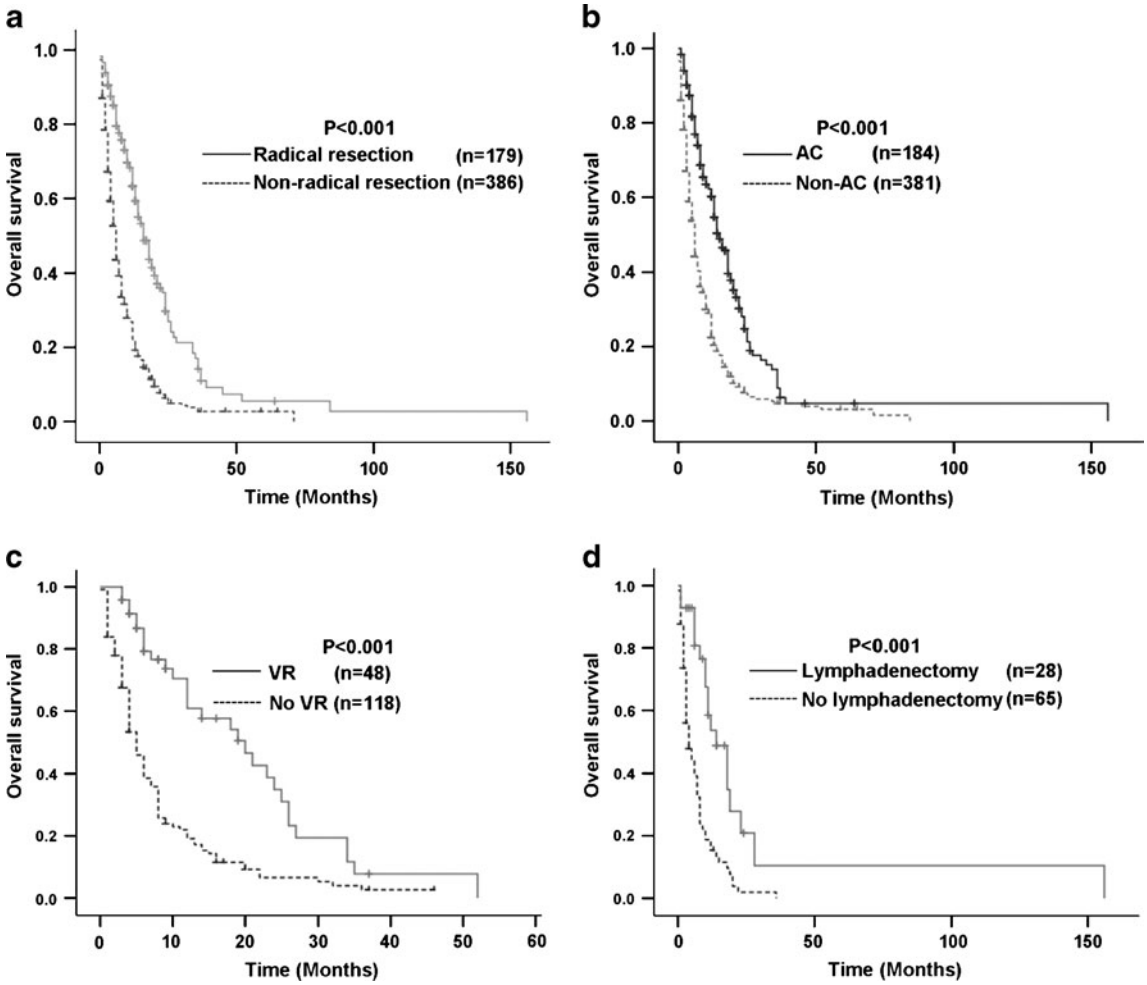

Fig. 2 The curves of overall survival in patients according to treatment variables. **a** surgery type; **b** adjuvant chemotherapy; **c** vascular reconstruction; **d** extended lymphadenectomy

and 5-year OS was 34.9% and 7.8% in patient with vascular reconstruction, which was similar to 27.0% and 8.4% in those without (P=0.686). But in all patients with VI, vascular reconstruction was significantly associated with improved OS, as patients with vascular reconstruction had a 2.7-fold decreased risk of death compared to those without (95% CI: 1.786–4.114, P<0.001, Fig. 2c). Twenty-eight patients underwent extended lymphadenectomy because of LNI, this was performed more in this decade (24/71, 33.8%) than in last decade (4/22, 18.2%). Of all 93 patients who had an LNI, extended lymphadenectomy was significantly associated with improved OS (Fig. 2d) and patients with extended lymphadenectomy had a 2.9-fold (95% CI: 1.690–5.066, P<0.001) of decreased risk of death compared to those without.

Discussion

Pancreatic adenocarcinoma is one of the leading causes of death due to cancer, with the lowest survival rate.[21] Difficulty in diagnosing the disease at an early stage is the major obstacle for improving the outcome in PDAC patients.[22] Current methods for early diagnosis are often ineffective and/or inaccurate, especially in identifying smaller, potentially curable lesions.[23] In our study, we detected that in patients with tumor size no more than 3 cm, the sensitivity of CA19-9 was only 57.0%, and its positive accuracy was also limited (63/93, 67.7%). Though it was a little higher than Steinberg's reports of 55% 20 years ago,[24] these unsatisfactory results indicated the limited predictive value of CA19-9 in detecting PDAC at early stage cancer, and necessitated other biomarkers and genetic markers to be used as a panel in combination with other modalities for early detection with high sensitivity and specificity in PDAC patients.[25]

Postoperative margin status in PDAC patients has been reported by various authors to be a prognostic factor associated with survival.[26–29] In our study, patients with R2 resection had a 1.60-fold of increased risk of death compared to those with R0/R1 resection (P<0.001). We

concluded here that the grossly total resection of tumor should be the primary goal in attempting a resection. R0/R1 resection was more common in this decade, indicating that more patients were resectable at diagnosis and that surgical techniques have improved in this high-volume center. Furthermore, patients with vascular involvement in our study benefited from vascular reconstruction, which was performed for the purpose of radical resection without extra morbidity. Some outcomes were satisfactorily extended, as vascular reconstruction had improved radical resection rate in patients with vascular involvement and subsequently improved survival. This is in accordance with previous reports that vascular reconstruction might provide a negative surgical margin and better survival in patients with locally advanced pancreatic cancer.[30,31] Similarly, more vascular reconstruction was performed in this decade, indicating that the procedures are complicated challenges to surgical oncologists, who should be surgically skilled in high-volume center with experience in perioperative care.

With awareness of surgical techniques and their impact on survival, surgical oncologists have improved survival in PDAC patients significantly in recent years,[32,33] but inoperable diseases remain to be the main cause of mortality. In our study, 47 patients were downstaged from inoperable PDAC to radical resections, and most of the downstage occurred to gemcitabine-based neoadjuvant chemotherapy in this decade. With increasing data supporting neoadjuvant chemotherapy in borderline resectable PDAC patients,[34,35] we now routinely recommend gemcitabine-based regimen for patients with inoperable PDACs.

Unfortunately, even an R0 resection will not guarantee long-term survival, and many patients will eventually have local and/or system failure and ultimately die of disease progression.[36,37] Therefore, the use of adjuvant therapy is a logical strategy for systemic disease and survival. Gemcitabine has proven to be essential in the management of advanced pancreatic cancer for clinical benefit and OS improvement in 1997.[38] Updated data from the CONKO-001 trial definitively ascertain the value of adjuvant chemotherapy and provide level 1 evidence supporting the use of adjuvant chemotherapy for patients with resected PDA.[11] In our study, median survival in patients with adjuvant chemotherapy was significantly longer than that in patients without (15 months vs 6 months, $P<0.001$). More patients underwent chemotherapy in this decade, reflecting that adjuvant chemotherapy has been recognized as an important component in treatment of PDA in this high-volume center.

The late presentation and poor response to chemotherapy has been the main obstacle in improving the outcome of PDAC patients. Sensitive and specific tumor markers are needed for patients to be diagnosed at an early, operable disease stage. To this point, CA glycoproteins are less satisfactory because of limited sensitivity and false-positives. Newly investigated genetic markers are still undergoing evaluation. Efficient evaluation of new markers of pancreatic neoplasia will benefit from recently completed pancreatic cancer genome[39] and require greater enrollment of patients with suspected pancreatic disease into clinical trials. These developments in early diagnosis of PDAC in this decade will undoubtedly increase the proportion of early disease and result in improved outcome in the coming decades. Also, with multiple genetic mutations being identified as the precursor to the development of pancreatic cancers and recognized as the target for therapeutic interventions,[40] with multiple immunotherapy and targeted therapy being investigated with encouraging results in different clinical trials,[41–44] and with high-volume centers being built worldwide, we believe that the results of treatment and outcome in PDAC patients in the next decade will be even more encouraging.

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CASE REPORT

Regeneration and Functional Recovery of Intrapelvic Nerves Removed During Extensive Surgery by a New Artificial Nerve Conduit: A Breakthrough to Radical Operation for Locally Advanced and Recurrent Rectal Cancers

Hiroyuki Tsujimoto · Tatsuo Nakamura · Tsuneharu Miki · Toshikazu Kubo · Eigo Otsuji · Hisakazu Yamagishi · Akeo Hagiwara

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Abstract
Purpose In the current strategy against locally advanced and recurrent rectal cancers possibly involving intrapelvic nerves, there has been a serious dilemma between extensive surgery and limited surgery. The former can attain high tumor curability by sacrificing the nerve functions while the latter prioritizes the patient quality of life by preserving the nerve functions but with a compromised curability. Here we present a new surgical strategy for locally advanced and recurrent rectal cancers, which realize both high tumor curability and good quality of life.
Methods A new artificial nerve conduit (polyglycolic acid collagen tube) developed by in site tissue engineering technology was applied to recovery the disturbed functions after removing the nerves from 11 patients undergoing extensive surgery for intrapelvic advanced or recurrent colorectal cancers. The reconstructed nerves included eight autonomic nerves which are essential for the genitourinary function and three somatic nerves which control the sensation and mobility of the legs.
Results Out of ten cases followed up more than 2 years and evaluated fully, eight including two report cases showed a functional recovery of the disturbed autonomic and somatic nerves clinically. The nerve function started to recover from 3 to 6 months after the operation and continued to improve with times. No specific complications associated with the nerve repair have been noted.
Conclusions The new strategy utilizing the nerve conduit can be a breakthrough in radical operations for locally advanced and recurrent rectal cancers to resolve the problems between tumor curability and the patient quality of life.

H. Tsujimoto (✉) · A. Hagiwara
Division of Medical Life System,
Department of Life and Medical Science, Doshisha University,
1-3 Tatara-Miyakodani,
Kyotanabe, Kyoto 610-0321, Japan
e-mail: htsujimobb@yahoo.co.jp

H. Tsujimoto · E. Otsuji · H. Yamagishi · A. Hagiwara
Department of Digestive Surgery,
Kyoto Prefectural University of Medicine,
645 Kajii-cho Kawaramachi-Hirokoji,
Kamigyo-ku Kyoto 602-0841, Japan

T. Nakamura
Department of Bio-Artificial Organs,
Institute for Frontier Medical Sciences, Kyoto University,
53 Kawahara-cho, Shogoin,
Sakyo-ku, Kyoto 606-8507, Japan

T. Miki
Department of Urology,
Kyoto Prefectural University of Medicine,
Kyoto, Kyoto, Japan

T. Kubo
Department of Orthopedics,
Kyoto Prefectural University of Medicine,
Kyoto, Kyoto, Japan

Keywords Nerve regeneration · Artificial nerve conduit · Intrapelvic nerves · Rectal cancer · Extensive surgery

Introduction

Surgery for locally advanced and recurrent intrapelvic tumors such as rectal cancers presents a serious dilemma between tumor curability and the patient quality of life. An extensive resection is likely to be more effective for the former, while a limited resection prioritizes the latter by preserving the nerve functions. To attain greater curability by extensive resection, surgeons are apt to remove not only the clearly affected region but also the surrounding region which may possibly be contaminated by tumor cells. The intrapelvic space contains autonomic nerves, which are essential for the genitourinary function, as well as somatic nerves, which control the mobility and sensation of the legs. When the affected regions contain those important intrapelvic nerves, an extensive resection results in a dysfunction of such nerves.[1,2] On the other hand, when the patient quality of life is emphasized, the nerves are often retained. However, this means a higher risk of cancer cells remaining. To resolve this dilemma, a new strategy is necessary, in which high tumor curability is achieved by an extensive resection including the possibly contaminated nerves and then the disturbed functions of the removed nerves are repaired.

To repair peripheral nerve gap injuries, the current standard clinical procedure is the transplantation of an autologous nerve graft as a conduit along which peripheral nerve fibers can regenerate.[3] However, autologous nerve grafts entail unavoidable difficulties due to their limited supply and the dysfunction of donor nerves at the sites where the graft nerves are harvested. It is therefore difficult to obtain autologous nerve grafts for repairing sizable or multiple nerve sites. These disadvantages make autologous nerve graft transplantation less suitable for the repair of nerves with longer and/or sizable gaps which occurred as a result of an extensive resection for intrapelvic malignancies such as locally advanced rectal cancer. Indeed, no successful clinical trials of autologous nerve graft transplantations following rectal cancer surgery have been reported.

A possible alternative to autologous nerve graft transplantation is the use of an artificial nerve conduit. Several types of artificial nerve conduits have so far been clinically applied. However, the uses of such conduits have been restricted to shorter gap measuring less than 30 mm in peripheral somatic nerves.[4–6] To overcome these disadvantages, we applied a new artificial nerve conduit. The polyglycolic acid (PGA) collagen tube was developed by recent advanced in site tissue engineering technology. This tube consists of a bio-absorbable outer layer of PGA mesh with optimal permeability and an inner layer of collagen sponge as a scaffold to fascinate regeneration of nerves with a longer gap.[7,8] Previous experiments using canine models have demonstrated that our artificial conduit resulted in the successful regeneration of the somatic nerve across an 80-mm gap[8,9], which is long enough to repair the nerve gaps incurred in extensive surgery for rectal cancers. Interestingly, the nerve conduit was found to be superior to autologous grafts for nerve regeneration both histologically and electrophysiologically.[10] Additionally, in another dog experiment, the nerve conduit has successfully regenerated hypogastric nerve, which is an important intrapelvic autonomic nerve for the genitourinary function and often has to be removed during extensive surgery for locally advanced rectal cancers.[11]

This report presents a new strategy to realize both high tumor curability by an extensive resection and the patient quality of life by repairing the nerve function using a newly developed nerve conduit. We describe actual clinical cases in which the use of a nerve conduit successfully regenerated and functionally recovered the intrapelvic nerves removed during extensive surgery for locally advanced rectal cancers in a pilot study. Especially, we show herein two cases with the detailed findings of subjectively and objectively functional improvement on the reconstructed somatic and autonomic intrapelvic nerves, respectively.

Patients and Methods

Nerve Conduit

The artificial nerve conduit consists of a braided cylindrical tube woven from fibrils of PGA, a synthetic bio-absorbable polymer. The interior of the tube is filled with a collagen sponge, and the wall features a micromesh structure (Fig. 1a). The conduit takes in nourishments through numerous micropores in the mesh wall and supplies them to the regenerating nerves growing inside. The inner collagen sponge functions as a scaffold to facilitate the regeneration of the nerve fibers and Schwann cells.[7,8]

The PGA fibers are constructed from a medical grade polymer used widely in surgical sutures (Mitsui Chemical Ltd., Japan). The woven PGA conduit is coated with amorphous collagen layers and then filled with the collagen sponge. The collagen (Nippon Meatpackers, Ibaraki, Japan) itself is made from 70% to 80% Type I collagen and 20–30% Type III collagen obtained enzymatically from atelocollagen extracted from young porcine skin. The collagen is carefully checked for viruses, while the enzymatic extraction removes antigenic telopeptides to minimize antigenicity. The collagen molecules are cross-linked dehydrothermally (140°C for 24 h) under a vacuum to control the rate of bioresorption, and the conduits are sterilized with ethylene oxide gas before use.

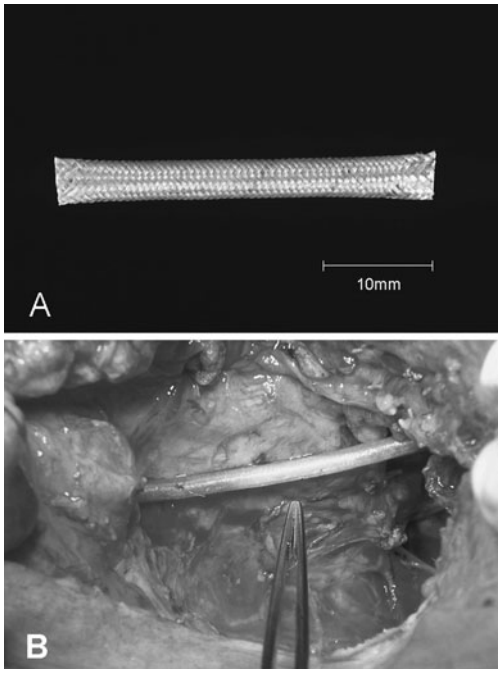

Fig. 1 **a** Outer view of the PGA–collagen tube. **b** Intraoperative view of reconstructed nerve using the PGA–collagen tube

To install the conduit, it was first interposed between the nerve gaps, and 5-mm sections of the ends of the nerve stumps were inserted into either end of the conduit. Finally, the end points were fixed with 4-0 to 6-0 polyprolene sutures (Prolene; Ethicon Inc., Somerville, NJ, USA; Fig. 1b).

Patients

All patients voluntarily participated in this project. They did not receive any benefits, including reduction or/and exemption of medical expenses, nor other monetary rewards for participation in the medical trials since such payments are not allowed in Japan. The patients were selected from extensive surgery cases where long-term survival was likely. In addition, they all met the criteria of the absence of hematogenous or peritoneal metastases during both the preoperative assessments (CT, MRI, and FDG-PET) and intraoperative observations.

While the nerves were preserved whenever possible, they were removed if the nerves were intraoperatively clearly observed or merely suspected to have been affected or contaminated by the tumor, whether clearly observed, and an excision of the nerves was considered necessary to ensure curability.

The clinical trials were performed with the approval of the Ethical Committee of the Hospital of Kyoto Prefectural University of Medicine and also with the informed consent of all patients.

Evaluation of the Nerve Function

Evaluations were mostly based on interviews concerning the status of kinetics (somatic nerves) or the status of urinary and/or sexual functions (autonomic nerves). In addition, a manual muscle test (MMT) and skin perception test were also conducted for the somatic nerves, and cystometry and neurometric assessment (current perceptive threshold (CPT) test by a neurometer) were used to evaluate for autonomic nerves.[12] Patient interview, manual muscle and skin perception tests were done every 1 to 3 months. Cystometry and neurometric assessment were performed at interval of 3 to 6 months except the first month after operation. These interviews and clinical tests were continued up to full recovery of the disturbed functions or more than 2 years after operation. However, penile tumescence test was not done since patients' consents were not obtained fully. While, stimulation tests were rarely performed, because these tests may potentially worsen the disease.[13]

Results

Nerve repair was performed using the conduits during extensive surgery for intrapelvic colorectal cancers in 11 patients, including 10 rectal cancers, between January 2002 and January 2007 (Table 1). The gap lengths of the reconstructed nerves ranged from 25 mm to 90 mm. They included 3 cases of peripheral somatic nerves (two obturator nerves and one femoral nerve branch), which are associated with mobility and sensation of leg partially. Others were 8 cases of autonomic nerves (8 hypogastric nerves).

The hypogastric nerve is a sympathetic nerve projecting from the lumbar splanchnic nerves to the bladder neck, spermatic duct and prostate which controls urinary and male sexual function such as the retention of urine, the erection and ejaculation. The nerve consists of hypogastric nerve trunk (hypogastric nerve plexus) and bilateral branches.[14] The 8 cases consisted of patients that underwent (a) the removal of the hypogastric nerve trunk and/or both of the bilateral nerve branches, with the repair with the conduit of the trunk and/or only one of the branches or (b) the removal of any one of the branches and the repair of the removed branch, but the opposite branch was so severely damaged over a distance of for more than 4 cm that its function could not be successfully regained. From our previous experiences, the situations described above as (a) and (b) interrupt completely the hypogastric nerve pathway to intrapelvic organs and result in severe permanent damages of urinary and male sexual functions such as urinary sensation, erection, and ejaculation, if the removed and/or damaged nerves are not repaired.

Ten out of the 11 patients were followed up more than 2 years and evaluated for the functional recovery of the

Table 1 Clinical trial of our PGA–collagen conduit

Patient			Disease	Surgical operation	Repaired nerve	Size of conduit (mm)		Result and comment	Survival
Number	Age	Gender				Length	Diameter		
Cases with somatic nerve regenerations									
1	67	F	Recurrent rectal cancer	Subtotal pelvic exenteration	Lt. obturator nerve	25	5	R: Perceptivity and kinesthesia in the left hip joint began to improve in 3 months. Slight sensory disturbance remained for the 24 months	24 m, D
2	69	F	Recurrent colon cancer	Resection of tumor, Lt. iliac artery and vein	Lt. Femoral nerve, branch	90	5	R: Perceptivity and kinesthesia in the left thigh began to improve in 3 months. Slight sensory disturbance still remained	24 m, A
3	39	M	Rectal cancer	Total pelvic exenteration	Lt. Obturator nerve	65	7	R: Presented as case 2	24 m, A
Cases with autonomic nerve regenerations									
4	63	M	Rectal cancer	Anterior resection	Lt. hypogastric nerve	60	2	R: sexual function was completely lost for the first 6 months after the operation. After another 6 months, the function came back a degree that he had erection	34 m, A
5	70	M	Rectal cancer	Rectal amputation	Lt. hypogastric nerve	50	4	R: no erection for the first 2 years. After 2 years, he was able to maintain a weak erection	34 m, A
6	70	F	Rectal cancer	Anterior resection	Rt. hypogastric nerve	40	3	R: urinary disturbance began to improve in 3 months. After 2 years, no perceptible difficulty in urinating.	34 m, A
7	67	M	Rectal cancer	Anterior resection	Lt. hypogastric nerve	80	4	R: urinary disturbance had been serious for the first 3 months After three, it began to improve.	35 m, A
8	74	M	Rectal cancer	Anterior resection	Lt. hypogastric nerve	50	5	N: not evaluated satisfactory because of early recurrence	9 m, D
9	59	F	Recto-sigmoid colon cancer	Anterior resection, resection of tumor	Hypogastric nerve trunk	45	4	U: subjective symptom is weak, while cystometry shows an atonic bladder yet.	24 m, A
10	51	F	Rectal cancer	Anterior resection	Hypogastric nerve trunk, Lt. branch	50	6	U: the complaint on urination was a little due to another postoperative complication. Then the complaint improved within the first 3 months.	24 m, A
11	49	M	Rectal cancer	Anterior resection	Lt. hypogastric nerve	45	4	R: presented as case 1	32 m, A

F female, *M* male, *Lt.* left, *Rt.* right, *R* recovered, *U* unclear, *N* not followed fully, *m* months, *D* dead, *A* alive

reconstructed nerves. Only one case was excluded from the evaluation because of early recurrent events. Eight of the ten cases showed a functional recovery of the disturbed autonomic or somatic nerves. The nerve function started to recover from 3 to 6 months after operation in most cases and improved with times. The functional recovery began 2 years postoperatively in one case. The levels of functional recovery varied, and some cases continued to experience partial nerve disturbance. The recovery appeared to be positive in the remaining two cases, but the results were unclear (Table 1). No specific complications associated with the nerve repair have been noted. The details of two cases with either autonomic or somatic nerve regeneration are herein described.

Table 2 Cystometric assessment of and subjective findings for the urination functions in case 1

Months		Preoperative	Postoperative (months after operation)		
			1	3	12
Cystometric assessment	FDV	49	236[a]	120	241
	NDV	189	239[a]	275	350
	SDV	?	239[a]	326	476
	Response pressure to voiding	+++ (Normal)	−(No response)	+	++
Subjective findings	Sense of urination	Normal	No[b]	Fair	Normal
	Difficulty in urination	−	+	−	−
	Incontinence of urine	−	+(?)	−	−

Cystometry results suggest that the bladder function was improving but not completely

FDV volume for first desire to void, *NDV* volume for normal desire to void, *SDV* volume for strong desire to void

[a] FDV, NDV, and SDV (in milliliters) are almost similar because of incontinence

[b] Sense of urination dull

Case Reports

Case 1

A 49-year-old male patient was diagnosed with rectal cancer. Intraoperatively the tumor was found to have contaminated the bilateral branches of the hypogastric nerve. The left branch was removed and then the section from the left branch to the left plexus was repaired with the conduit (length, 45 mm; diameter, 4 mm). The right branch was divided during the dissection.

The findings for urination and sexual intercourse functions are shown in Tables 2 and 3 and Fig. 2a, b. The patient's urinary and sexual functions were terribly poor both subjectively and objectively just after operation. However, the subjective findings of both the urinary and sexual function started to improve from 2 to 3 months after surgery and they eventually fully recovered within 12 months (Tables 2 and 3). The improvements were objectively confirmed by neurometric assessments. The CPTs of the urinary bladder and the urethra in the penis both returned to normal levels by 7 months postoperatively (Fig. 2a, b). The CPTs for 250 and 5 Hz currents correspond to the afferent responses through the Aδ and C nerve fibers, respectively.[12] Since afferent fibers of hypogastric nerve projecting urinary bladder and urethra consist of those Aδ and C nerve fibers[15], the recovery of the CPT data indicate the regeneration of the reconstructed hypogastric nerve. In contrast, the cystmetric results improved substantially by 12 months after the operation, however, had not yet completely recovered (Table 2).

Table 3 Conditions of sexual intercourse for case 1

Months	Preoperative	Postoperative (months after operation)			
		1	2–3	3−	12−
Erection	Yes	No	Yes	Yes	Yes
Ejaculation	Yes	No	?	Yes	Yes

Case 2

A 39-year-old male patient was diagnosed with locally advanced rectal cancer and underwent pelvic exenteration. Intra-operative findings showed that the tumor had contaminated the left obturator nerve with a diameter of 7 mm, which was larger than usual, thus suggesting that this nerve to be more dominant than in most patients. A 55-mm-long segment of the left obturator nerve was surgically removed, and then the gap in the nerve was repaired by the implantation of a conduit with a length of 65 mm and lumen diameter of 7 mm.

The MMT results immediately after surgery (Table 4) show paralysis of the left adductor muscle, which is controlled by the left obturator nerve. However, its function began to improve within 2–3 months after the operation and thereafter markedly improved with time. Improvements were also seen in the patient's rehabilitative status, daily life activities and the sensory function controlled by the nerve (Table 5).

Discussion

To develop and improve surgical treatment of locally advanced and recurrent rectal cancer, surgeons have pursued more extensive resection aimed at higher tumor curability. The extensive surgery can, indeed, attain a high curability rate.[1,2] However, it is apt to sacrifice the intra-

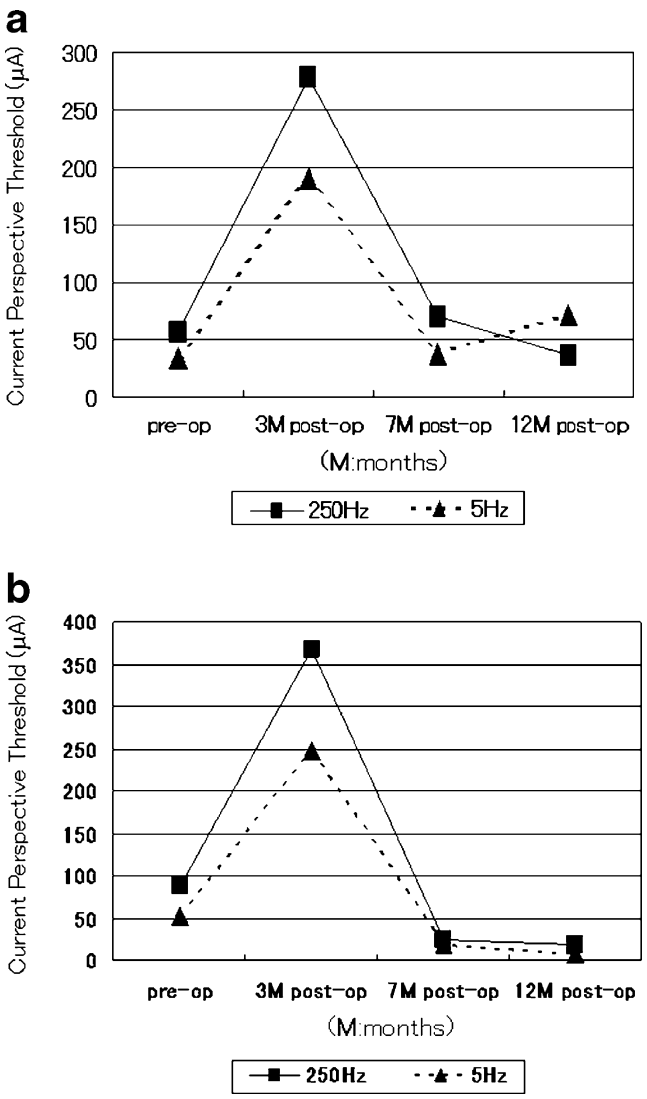

Fig. 2 Neurometric assessment (CPT by neurometer). The CPTs for 250 and 5 Hz currents correspond to the response through the Aδ and C nerve fibers, of which the regenerated hypogastric nerve consists. **a** The CPT of the urinary bladder. **b** The CPT of the urethra in penis

pelvic nerves, especially, the pelvic autonomic nerves, and results in a high incidence of genitourinary morbidity.[1,2] In addition, the urinary and male sexual dysfunction that follows extensive resection for more advanced rectal cancers tends to be permanent excluding few exceptions due to complete resection of the pelvic autonomic nerves. While, the dysfunction occasionally accompanied by conventional resection for rectal cancers is often transitory and resolves over 2–3 weeks due to incomplete division of the pelvic autonomic nerves.[2,16] Therefore, extensive surgery sacrificing the intrapelvic nerves causes patients to suffer a poor quality of life.

A straightforward alternative to the extensive approach is an autonomic nerve-preserving operation.[17,18] When the rectal cancers have not clearly infiltrated and/or contaminated the autonomic nerve system, autonomic nerve-preserving surgery is the obvious choice. However, such limited operation risks leaving tumor cells in rectal cancers that do not meet those prerequisites. Although removal of nerves during extensive resection to eradicate such locally advanced rectal cancers is necessary, surgeons are likely to hesitate to impair the patient's quality of life. A combination of extensive resection and recovery of nerve function is therefore highly appealing.

As mentioned in the introduction, transplantation of autologous nerve grafts appears to have been the only clinically practicable method to solve the dilemma between curability and quality of life in rectal cancer surgery. However, no successful clinical trials of autologous nerve graft transplantations have been reported for rectal cancer surgery because of inevitable difficulties due to the limited supply of suitable grafts and permanent dysfunction of donor nerves. Among other intrapelvic surgery, only modest results for prostatectomy have been shown in some clinical trials.[19–21]

As a possible alternative to autologous nerve graft transplantation, several types of artificial nerve conduits have been investigated, and some have been applied

Table 4 Manual muscle test of the left hip joint muscles of case 2

Months	Preoperative	Postoperative (months after operation)									
		Just after the operation	2	3	4	7	10	13	17	20	24
Adductor muscle[a]	5	0~1	1	2−~2	2^{+}~3−	3	3^{+}	3+	3~3+	3+	4
Abductor muscle[b]	5	5	4								
Quadriceps femoris muscle[b]	5	5	3^{+}~4	3^{+}~4	3^{+}~4	4	4	4	5	5	5
Gluteus muscle[b]	5		4	4	4	4	4	4	5	5	5

Grade of muscle strength during manual muscle test: 0, zero; 1, trace; 2, poor; 3, fair; 4, good; 5, normal

[a] Loss of muscle strength in the adductor muscle is considered to be due to paralysis of the obturator nerve

[b] Loss of muscle strength in the abductor muscle, quadriceps femoris muscle, and gluteus muscle is considered to be due to secondary disuse weakness rather than primary weakness from paralysis of the obturator nerve

Table 5 Rehabilitative status and daily life activities of case 2

Months	Preoperative	Postoperative (months after operation)									
		1	2	3	4	7	10	13	17	20	24
Indoor walking(physiotherapy)	Normal	With a walker[a]	With a walker[a]	With a walker[a]	Possible[b]						
Outdoor walking(physiotherapy)	Normal	Impossible	Impossible	Impossible	Possible[b]	Possible	Possible	Possible	Normal	Normal	Normal
Stair stepping exercise(physiotherapy)	Normal	Impossible	Impossible	Possible[c]	Possible[c]	Possible	Possible	Possible	Possible	Normal	Normal
Daily life activities (self-reported)	Normal for walking	Indoor walking	Indoor walking	Indoor walking		Normal[d]	Normal[d]	Normal[d]	Normal	Normal	Normal
Sensory function[e]	L = R	L < R	L < R	L < R	L < R		L = R	L = R		L = R	L = R

R right thigh, *L* left thigh

[a] Walking with the aid of a walker

[b] With the aid of a T-shaped stick

[c] With the aid of a handrail

[d] Normally walking for daily activities with the aid of a T-shaped stick only when going outdoors

[e] Sensory function on the left thighskin

clinically.[4–6] In particular, the PGA hollow tube is one commonly available. It has been used in clinical practice to repair peripheral somatic nerves with gap injury such as the medial, ulnar, and digital nerves.[22,23] The PGA hollow tube has produced superior results to those for autologous grafts in humans for gaps with a length ranging from 8 to 30 mm.[23] Thus, the tube is an excellent artificial conduit for the regeneration of nerve gaps. However, its use is restricted to gaps of less than 30 mm in peripheral somatic nerves. For the longer gaps encountered in the extensive surgery for rectal cancers, another type of artificial conduit is required.

To resolve these problems, we have developed the conduit, a PGA tube filled with collagen sponge. The conduit is designed so that nourishments can pass freely through the micromesh structure of the PGA wall into the interior, and that the nourishments can be supplied to the nerves regenerating inside the conduit. Thus, the nerve fibers can regenerate even at sites distant from the ends of the conduit. In addition, the tube is filled with a collagen sponge as a scaffold to help the nerves regenerate.[7,8] Previously, this conduit have successfully regenerated the removed nerves across an 80-mm gap in dog experiments, and the regeneration was confirmed both electrophysiologically and histologically.[8,10] The conduit also proved to be superior to autologous grafts in terms of nerve regeneration both histologically and electrophysiologically.[9] From these superior results, Inada et al. reported successful clinical use of this tube to repair peripheral somatic nerves with gaps up to 65 mm.[6,24] In addition, the hypogastric nerve, which often has to be removed to ensure curability during extensive surgery for locally advanced rectal cancer in human, was regenerated using the conduit in dogs and the regeneration was confirmed electrophysiologically.[11] Thus, the animal experiments have indicated that the conduit can be expected to be useful in rectal cancer surgery for regeneration of gaps, which are commonly more than 30 mm in length, in not only somatic but also autonomic nerves.

Table 1 summarizes the clinical trials of the conduits in extensive surgery for intrapelvic colorectal cancers. In ten of the cases listed, we used the conduit to repair the nerve gaps ranged from 40 to 90 mm, which are longer than the gaps successfully repaired with other artificial conduits.[4–6] Finally, seven of the cases demonstrated the function recovery of the damaged nerves, indicating that the nerve conduit can clinically support the regeneration of the nerves with such longer gaps. Of the two cases described in the previous section, case 1 presented an autonomic nerve, and case 2 a somatic nerve. The former is also of special importance since there has been no clinical report of the regeneration of an autonomic nerve using an artificial nerve conduit. In addition, we have successfully repaired pelvic

autonomic nerves with the conduits in another four patients. These results indicate that the nerve conduit is useful for the regeneration of autonomic as well as somatic nerves in human.

In case 2, the obturator nerve, which controls mobility of abductor muscle of leg and sensation of inner part of thigh, was reconstructed. The successful regeneration was observed following surgery for a highly advanced cancer in the intrapelvic space. For highly advanced or recurrent rectal cancers in the pelvic space, good curability is often likely to be achieved by a more extensive resection such as pelvic exenteration including intrapelvic nerves controlling sensation/mobility of the legs. There has been a tendency to avoid the more extensive operations with the nerve resection, since those formidable operations inevitably entail serious disability to the patients.[25,26] The nerve conduit presents a possibility to perform such extremely extensive surgery without causing the patients serious disability.

The results presented here with two case reports indicate that the artificial nerve conduit will help patients recover the functions of sacrificed nerves and enhance patient's quality of life. This approach thus offers a new strategy to realize both high tumor curability and good quality of life following extensive surgery for locally advanced or recurrent rectal cancers.

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MULTIMEDIA ARTICLE

A Protective Technique for Retraction of the Liver During Laparoscopic Gastrectomy for Gastric Adenocarcinoma: Using a Penrose Drain

Toshihiko Shinohara · Seiichiro Kanaya · Fumihiro Yoshimura · Yoshihiro Hiramatsu · Shusuke Haruta · Yuichiro Kawamura · Simone Giacopuzzi · Tetsuji Fujita · Ichiro Uyama

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Abstract
Background Retraction of the liver is necessary to ensure an adequate working space in laparoscopic surgery, but the retraction force applied may cause transient liver dysfunction. We have introduced the technique using a Penrose drain to suspend the liver with the performance of laparoscopic gastrectomy for gastric adenocarcinoma.
Methods 111 patients with gastric adenocarcinoma underwent laparoscopic gastrectomy using either a Penrose drain (n=47) or a Nathanson's retractor (n=64) for displacement of the liver. Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, alkaline phoshatase (ALP) and albumin were compared among the groups at baseline, immediately after operation, and on postoperative days (POD) 1, 2, 3, 5, and 7.
Results The levels of ALT on POD 2, 3, and 5 were significant higher in the Nathanson's retractor group than in the Penrose drain group. Levels of AST on POD 2 and 3 were also higher in the Nathanson's retractor group than in the Penrose drain group. There was no significant difference in total bilirubin, ALP, and serum albumin levels between groups.
Conclusions The use of the Penrose drain for retraction of the liver appears to attenuate postoperative liver dysfunction during laparoscopic gastrectomy for gastric adenocarcinoma.

Keywords Laparoscopic gastrectomy · Stomach neoplasm · Liver dysfunction · Liver retraction · Penrose drain

Introduction

Postoperative liver dysfunction is frequently encountered after laparoscopic surgery. In 1994, Halevy et al.[1] first pointed out transient derangement of liver function following laparoscopic cholecystectomy in the absence of bile duct injury. They showed the possibility that increased intra-abdominal pressure under the CO2 pneumoperitoneum could decrease hepatic perfusion and cause transient liver dysfunction. As to the hemodynamic state of the liver, the effect of the CO2 pneumoperitoneum on hepatic perfusion during laparoscopic surgery remains controversial.[2–7] Several authors have investigated other possible risk factors for liver dysfunction associated with laparoscopic surgery, such as inadequate patients position, specific type of procedure, adverse effects of anesthetic agents, division of the aberrant hepatic artery, inadvertent thermal injury during surgery, and liver contusion from a surgical liver retractor.[1,3,8–11]

The lateral segment of the liver often interferes with the extension of surgical field during surgery for patients with diseases of the upper abdomen. For displacement of the liver, various surgical liver retractors have been usually applied.[10–13] However, such mechanical retractors can cause focal hepatic injury that may result in hepatocellular damage, consequent postoperative transient rise in amino-

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T. Shinohara (✉) · S. Kanaya · F. Yoshimura · Y. Hiramatsu · S. Haruta · Y. Kawamura · S. Giacopuzzi · I. Uyama
Department of Surgery,
Fujita Health University School of Medicine,
1-98 Dengakugakubo, Kutsukakecho, Toyoake,
470-1192 Aichi, Japan
e-mail: shinohara@jikei.ac.jp

T. Shinohara · T. Fujita
Department of Surgery, The Jikei University School of Medicine,
Tokyo, Japan

transferases.[9,11] Therefore, to reduce postoperative liver dysfunction, a protective technique for retraction to enable visualization of the operative field is desirable. For this purpose, we have introduced a simple technique using a Penrose drain to suspend the liver with the successful performance of 42 laparoscopic gastrectomies for gastric adenocarcinoma.[13] To assess whether the liver dysfunction can be reduced by this technique, we performed a prospective nonrandomized observational study.

Methods

This study was conducted after Institutional Review Board approval of from Fujita Health University School of Medicine. Between October 2007 and March 2009, 111 patients who underwent laparoscopic gastrectomy with curative intent were included in the present study. Patients with chronic liver damage and a history of alcohol abuse or liver disease such as hepatitis B virus, hepatitis C virus, and acute viral hepatitis were excluded from the study. Patients who underwent palliative resection and in whom sacrifice of an aberrant left hepatic artery during the operation and patients who underwent concomitant cholecystectomy were excluded from the study.

The premedication and anesthesic techniques for laparoscopic gastric surgery were standardized during the study period. Briefly following, general anesthesia was assisted with an epidural block, CO2 pneumoperitoneum was maintained at 10 mmHg and each patient was placed in reverse Trendelenburg position with the legs held apart. After completion of the CO2 pneumoperitoneum, a Penrose drain was used in 47 patients, and a Nathanson's liver retractor was used in the remaining 64 patients to displace the lateral segment of the liver and provide a wide view of the operative field. The Penrose drain measuring 6 mm in width was threaded with three pieces of 2-0 nylon thread 5 cm apart (Fig. 1a). First, the end of the nylon thread at the center was placed in the space between the diaphragm and the liver below the small hole that has been prepared in the left triangular ligament of the liver, and then pulled out to the ventral side of the liver through the small hole. Next, the nylon thread is pulled out through the abdominal wall using an End Close™ (Covidien, Mansfield, MA, USA; Fig. 1b). The End Close™ was introduced from the position slightly caudal to the right costal arch into the peritoneal cavity so that it emerged through the abdominal wall at the right side of the falciform ligament of the liver. The nylon thread on the right side of the Penrose drain was grasped and directed outside the abdomen. Finally, the End Close™ was inserted into the peritoneal cavity from the area around the left costal arch: the nylon thread at the left was led out of the body (ESM 1). The lateral segment of the liver was suspended while being held at three points (Fig. 2). The Nathanson's liver retractor was introduced close to the xiphoid process and then placed on the lateral segment of the liver. Surgery was performed by the same surgical team with a standardized laparoscopic technique. All patients were selected for the Penrose drain or Nathanson's liver retractor at the discretion of the surgeon's preference, but different surgeons utilized one technique over another. Furthermore, there was not an inherent selection bias by the surgeons who used both techniques. Laparoscopic gastrectomy was completed using the methods originated by us as described previously.[12–14]

All relevant clinical data were prospectively collected and recorded including patient demographics, types of gastrectomy, operation time, estimated blood loss, incidence of concomitant splenectomy, extent of lymph node dissection, presence or absence of postoperative complications, length of postoperative hospital stay, depth of tumor invasion, patho-

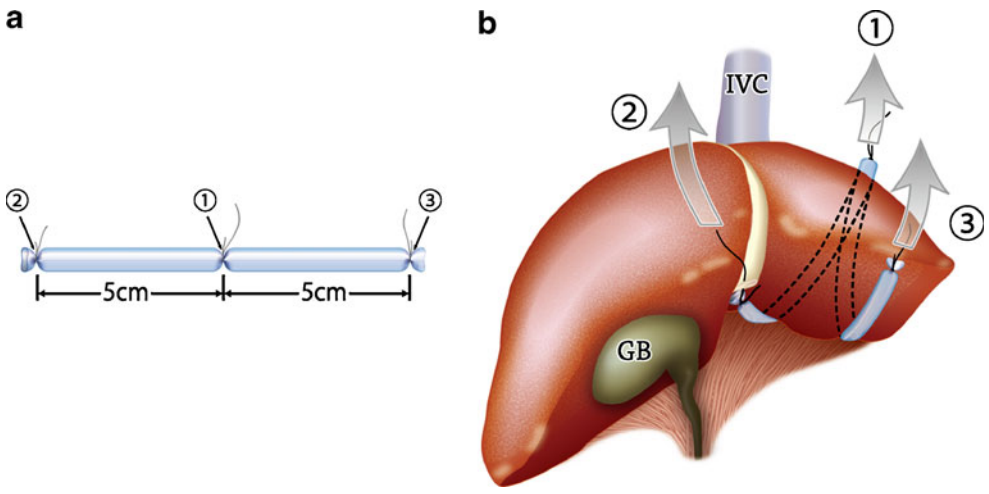

Fig. 1 Schematic illustration of suspension the lateral segment of the liver using a Penrose drain. **a** Formation of the Penrose drain, **b** the nylon thread at the right side of the Penrose drain (*2*) penetrated through the inferior margin of the round ligament of the liver

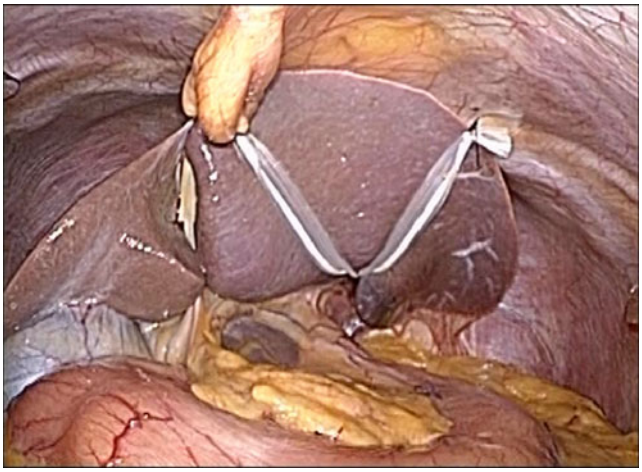

Fig. 2 Appearance of completed, suspending the liver by the Penrose drain. This technique provided satisfying view of the working fields during laparoscopic gastrectomy

logical type. All patients had routine hematological surveys of liver function assessed by alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, alkaline phoshatase (ALP), and albumin prior to and immediately after operation and on postoperative days (POD) 1, 2, 3, 5, and 7. The normal ranges for these parameters are 6–30 IU/L for ALT, 13–33 IU/L for AST, 0.3–1.2 mg/dl for total bilirubin, 115–359 IU/L for ALP, and 4.0–5.0 for albumin.

Results

As previously mentioned, 47 patients (42.3%) had laparoscopic gastrectomy using the Penrose drain for liver retraction that took less than 10 min in all cases, whereas the Nathanson's liver retractor was used in a group of 64 patients (57.7%). Both techniques provided a satisfactory view of the working fields during laparoscopic gastrectomy and we encountered no complications requiring any treatments during retraction of the liver. There was no significant difference between groups in age, sex ratio, preoperative body mass index, the depth of tumor invasion, and histological type (Table 1).

The operative outcomes are summarized in Table 2. All operations were accomplished using an entirely laparoscopic approach. The two groups were similar in types of gastrectomy, operation time, estimated blood loss, incidence of concomitant splenectomy, extent of lymph node dissection, presence or absence of postoperative complications, and length of postoperative hospital stay. No patients in either group received a blood transfusion during or after the operation.

There was no significant difference between groups in the patient's baseline levels of each liver function tests. Circulating ALT and AST levels increased significantly from baseline within 24 h following operations in each group. The levels of serum ALT on POD 2, 3, and 5 were statistically significant higher in the Nathanson's liver retractor group (mean ± SD, 213.8±30.9 IU, 146.6± 20.6 IU, and 98.0±13.4 IU) than in the Penrose drain group (124.0±11.5 IU, 90.2±7.8 IU, and 60.9±4.1 IU; P= 0.008, P=0.012, and P=0.010, respectively; Fig. 3a). Furthermore, levels of serum AST on POD 2 and 3 were significant higher in the Nathanson's liver retractor group (mean ± SD, 173.0±23.4 IU and 72.0±8.1 IU) than in the Penrose drain group (94.3±8.5 IU and 50.9±4.1 IU; P= 0.002 and P=0.022, respectively; Fig. 3b). Peak AST occurred on POD 1 and gradually returned to preoperative values after 7 days in each group. The ALT also peaked at 24–48 h following operation and gradual decreased but did not return to the normal range within 7 days in each group.

The total bilirubin levels peaked at POD 1 both in the Nathanson's liver retractor and the Penrose drain group. The levels did not differ between groups (mean ± SD, 1.26±0.43 and 1.78±1.26 mg/dl; P=0.302). The ALP levels decreased

Table 1 Comparison of patient characteristics

	Penrose drain (n=47)	Nathanson's retractor (n=64)	P value
Age, year	64.8±10.6	62.8±10.0	0.310
Sex, n (%)			0.683
Female	13 (27.7)	20 (31.2)	
Male	34 (72.3)	44 (68.8)	
Body mass index, kg/m^2	21.8±3.9	21.4±2.6	0.519
Depth of tumor invasion[a]; n (%)			0.740
Early (cT1)	22 (46.8)	32 (50.0)	
Advanced (cT2, T3, T4)	25 (53.2)	32 (50.0)	
Pathological type[a], n (%)			0.680
Intestinal	29 (61.7)	37 (57.8)	
Diffuse	18 (38.3)	27 (42.2)	

An unpaired t test was used to determine statistical significance between means

The chi-square test was used to test the equality of percentages between dichotomous groups

[a] According to the guidelines of the Japanese Research Society for Gastric Cancer[18]

Table 2 Comparison of operative outcomes

	Penrose drain (n=47)	Nathanson's retractor (n=64)	P value
Operation time, min	308.7±79.1	333.81±95.6	0.145
Blood loss, ml	129.9±36.4	88.3±12.2	0.283
Type of resection, n (%)			0.244
Distal gastrectomy	35 (74.5)	41 (64.1)	
Total gastrectomy	12 (25.5)	23 (35.9)	
Concomitant splenectomy, n (%)			0.463
Yes	7 (14.9)	13 (20.3)	
No	40 (85.1)	51 (79.7)	
Lymph node dissection[a], n (%)			0.695
D1	26 (55.3)	33 (51.6)	
D2	21 (44.7)	31 (48.4)	
Postoperative complications, n (%)			0.129
Yes	4 (8.5)	12 (18.7)	
No	43 (91.5)	52 (81.3)	
Postoperative hospital stay, days	17.0±2.4	21.1±2.2	0.218

An unpaired t test was used to determine statistical significance. The chi-square test was used to test the equality of percentages between dichotomous groups. The Fisher exact test was used when a table had a cell with an expected frequency of <5

[a] According to the guidelines of the Japanese Research Society for Gastric Cancer[18]

postoperatively and reached their lowest levels at POD 2 in the Nathanson's liver retractor group and the Penrose drain group. There was no significant difference in ALP levels between groups (mean ± SD, 172.6±50.3 and 183.8±53.8 IU/L; P=0.329). Serum albumin levels also decreased immediately and reached their lowest levels on POD 1 in the Nathanson's liver retractor and the Penrose drain group. The levels did not differ between groups (mean ± SD, 3.13±0.38 and 3.12±0.36 IU/L; P=0.917). There was no 30-day or in-hospital mortality or postoperative liver failure in either group.

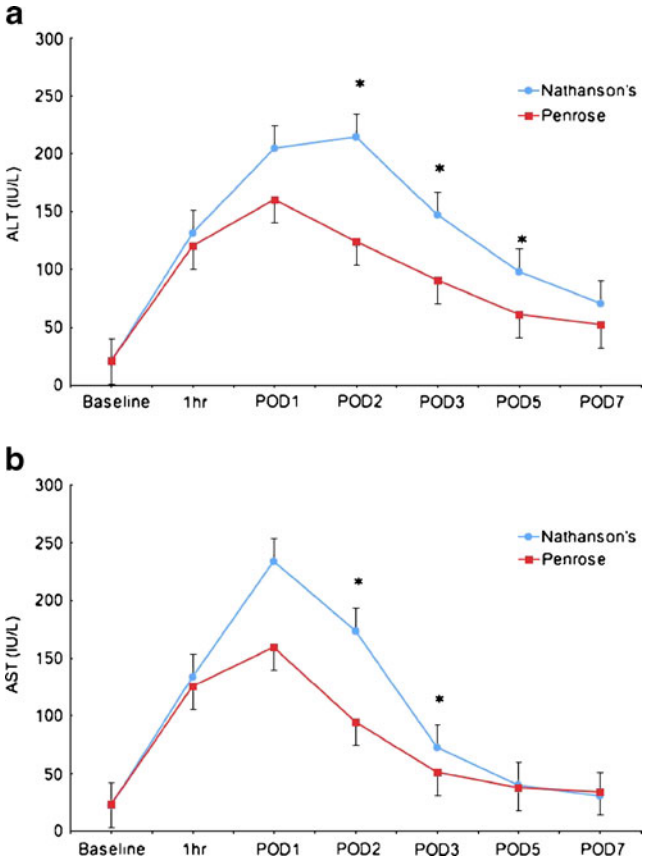

Fig. 3 Changes of ALT levels (**a**), AST levels (**b**) after laparoscopic gastrectomy. *Significant difference between groups

Discussion

Laparoscopic surgery has been established for the treatment of various types of diseases over the last decade. Despite its advantages over conventional open surgery in terms of less postoperative pain, shorter recovery time and improved cosmesis, a transient derangement in liver function has been recognized following various laparoscopic operations.[1,8–11,15,16] Our patients showed the evidence of a transient derangement in liver function after laparoscopic gastrectomy, as did patients with gastric adenocarcinoma in the reports by Etoh et al.[10] and Morris-Stiff et al.[11] Fortunately, serum AST and ALT levels gradually returned to the reference range in most patients with favorable clinical outcomes in all patients. However, minimizing liver dysfunction is important during the early postoperative period because the liver plays a crucial role in recovery from surgical stress and trauma. In the present study, we found that liver dysfunction could be decreased as evidenced by a reduction in serum ALT and AST levels by the use of the Penrose drain for liver retraction during laparoscopic gastric adenocarcinoma surgery.

The mechanisms of transient elevation of liver enzymes after laparoscopic resection may implicate several factors; including the presence, duration, and pressure of the CO2

pneumoperitoneum, type of surgery, the effects of anesthetic agents, and local hepatic injury from a surgical retractor. Morino et al.[15] in a series of 52 patients who underwent laparoscopic surgery, have reported that the increase of postoperative liver enzyme was higher in the group performed at 14 mmHg of the CO2 pneumoperitoneum than those performed at 10 mmHg, and higher in the group with the CO2 pneumoperitoneum for a duration of >60 min than those of <60 min. They concluded that the elevation of liver enzymes caused by cytolysis had a significant correlation with the pressure and duration of the CO2 pneumoperitoneum during laparoscopic surgery.

Several recent researchers have evaluated the changes in liver enzymes following several types of laparoscopic surgery, and found that among the various procedures anti-reflux surgery, wherein extensive retraction is required to obtain adequate exposure of the esophagogastric junction, was significantly associated with postoperative liver dysfunction.[11,15] Practically, for patients with diseases of the cardia or upper stomach, sufficient exposure of the esophagogastric junction is vital for safe laparoscopic surgery and such extensive retraction may results in an hepatic cell damage, consequent more serious liver dysfunction after surgery.

It has been suggested that rigid retraction of the lateral segment of the liver is one of the mechanism for elevation of the aminotransferases. Yassa and Peters[17] noted visual signs of ischemia of the distal retracted liver after surgery for upper gastrointestinal malignancy. We have utilized the elastic retraction offered by the Penrose drain for over 200 cases, including patients with liver cirrhosis or those with large and friable livers and encountered no complications requiring any treatments. In our prospective cohort study, we excluded patients at high risks for postoperative liver dysfunction to compare the effect on liver function test of two liver retraction techniques. Furthermore, in our series, all the operations were carried out in the same fashion and all the patients received similar anesthetic agents. Nevertheless, ALT and AST levels were statistically significant higher in the Nathanson's liver retractor group than in the Penrose drain group, suggesting that transient liver dysfunction during laparoscopic gastrectomy was influenced by the type of liver retractor.

From a clinical standpoint, these changes were transient and thought to clinically be insignificant. In addition, the long-term oncological significance of this for patients with gastric adenocarcinoma remains unclear. However, "hepatocytes" have vital roles in the synthesis and metabolism of essential body defense proteins and carrier proteins. In the current study, transient liver rises were identified, but were evidently alleviated by using the Penrose drain technique during laparoscopic gastrectomy for gastric adenocarcinoma.

Conclusion

This technique is easy and safe to perform and thus could be performed without requiring any specific training. Retraction of the liver is necessary to ensure adequate working space in laparoscopic surgery involving the upper abdominal organs, so we believe that this technique is useful for the treatment not only of laparoscopic gastrectomy, but also of laparoscopic anti-reflux surgery, vagotomy, and obesity surgery.

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HOW I DO IT

Management Strategies for Internal Hernia after Gastric Bypass

Robert W. O'Rourke

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Abstract
Background Internal hernia after gastric bypass is common, occurring with an incidence approaching 10% in some series. Operative management of internal hernia after gastric bypass presents significant conceptual and technical challenges.
Methods This manuscript reviews management of internal hernia after gastric bypass with a focus on operative strategy.

Keywords Gastric bypass · Internal hernia · Petersen's hernia · Retrocolic hernia · Mesenteric hernia

Introduction

The incidence of internal hernia after gastric bypass ranges from 0.5% to 9%.[1–8] Given the increasingly popularity of gastric bypass, surgeons will be faced more frequently with patients requiring exploration for suspected internal hernia. The differential diagnosis for abdominal pain after gastric bypass is large. This manuscript focuses on symptoms and signs specific to internal hernia and provides an overview of general management strategy with a focus on operative approach.

Diagnosis

Patients with internal hernia most often present within 2 years of gastric bypass and report severe chronic episodic diffuse abdominal pain lasting one or more hours. Risk factors may include extreme weight loss, which may lead to enlargement of mesenteric defects due to loss of mesenteric fat. Pregnancy has also been identified as a risk factor as a result of changes in intra-abdominal anatomy due to the enlarging uterus.[9] Pain may be post-prandial or spontaneous, and may persist for months, presumably due to episodic transient incarceration that spontaneously resolves. Nonetheless, the risk of strangulation remains present. The diagnostic workup should be guided by clinical presentation, and may include EGD, ultrasound, CT scan, and other tests as indicated. CT scan is the test of choice for internal hernia, but in the absence of pain, is often non-diagnostic. While experience and clinical judgment are of course necessary, and patients with chronic pain unrelated to internal hernia will be encountered, patients with acute onset severe pain in the absence of a clear alterative diagnosis should undergo surgical exploration, preferably through a laparoscopic approach to rule out internal hernia. Such patients should *not* undergo an initial trial of conservative management with nasogastric decompression, which incurs the risk of allowing incarcerated hernias to progress to strangulation. Rather, expedient surgical exploration is necessary. Closure of internal hernia defects, even if incarceration is not found at the time of operation, relieves symptoms in the majority of patients.[10–12] Less commonly, patients present with unrelenting pain. While the yield of CT is higher in such cases, and may demonstrate a mesenteric swirl sign [13–15] or other signs of bowel obstruction, strangulation may nonetheless be imminent even if CT is non-diagnostic. For this reason,

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R. W. O'Rourke (✉)
Department of Surgery, Oregon Health & Science University,
3181 SW Sam Jackson Park Road, L223A,
Portland, OR 97239-3098, USA
e-mail: orourkro@ohsu.edu

surgical exploration is warranted in patients with ongoing acute onset severe pain even in the face of non-diagnostic imaging.

Gastric Bypass and Internal Hernia Anatomy

An understanding of the anatomy of gastric bypass is required for effective clinical diagnosis and surgical management of internal hernia. From an anatomic standpoint, gastric bypass consists of a gastroplasty with a Roux-en-Y reconstruction. Standard terminology for intestinal limbs are made in reference to the distal intestinal anastomosis (jejunojejunostomy), and include Roux, afferent, and efferent limbs, also termed alimentary, biliopancreatic, and common channel limbs, respectively, in bariatric parlance. The majority of Roux limbs in gastric bypass are positioned antecolic, while a minority are retrocolic, retrogastric, and even fewer are retrocolic, antegastric.[16] While variations exist, a common configuration positions the biliopancreatic limb in the left upper quadrant, with the Roux limb positioned along the right side of the abdomen. The side-to-side jejunojejunostomy anastomosis is thus configured with the distal stapled end of the biliary limb directed cephalad along the Roux limb, pointing towards the gastrojejunal anastomosis (Fig. 1).

The three primary types of internal hernia are *mesenteric, Petersen's*, and *mesocolic* (Figs. 1 and 2). A *mesenteric hernia* traverses the space created by division of the mesentery of the jejunum adjacent to the jejunojenostomy and is present in all types of reconstruction. *Petersen's hernia*, in modern parlance,[17] may occur in an antecolic or retrocolic reconstruction, although it is thought to be more common in the former, and its borders are the Roux limb and its mesentery anteriorly/ventrally and the tranverse colon and its mesentery posteriorly/dorsally. Finally, a *mesocolic hernia* traverses the defect in the transverse colon mesentery and is specific to the retrocolic configuration.

Specific Technical Considerations at Primary Gastric Bypass

Technical considerations at primary gastric bypass may impact on the frequency of post-operative internal hernia. Roux limb anatomy is one of the most important of these considerations. A retrocolic limb reduces tension on the gastrojejunostomy and is thus an important tool in the repertoire of the bariatric surgeon, especially when faced with the patient with foreshortened mesentery or otherwise unfavorable anatomy. Proponents of retrocolic reconstruction argue that pouch emptying is improved as well, and contend

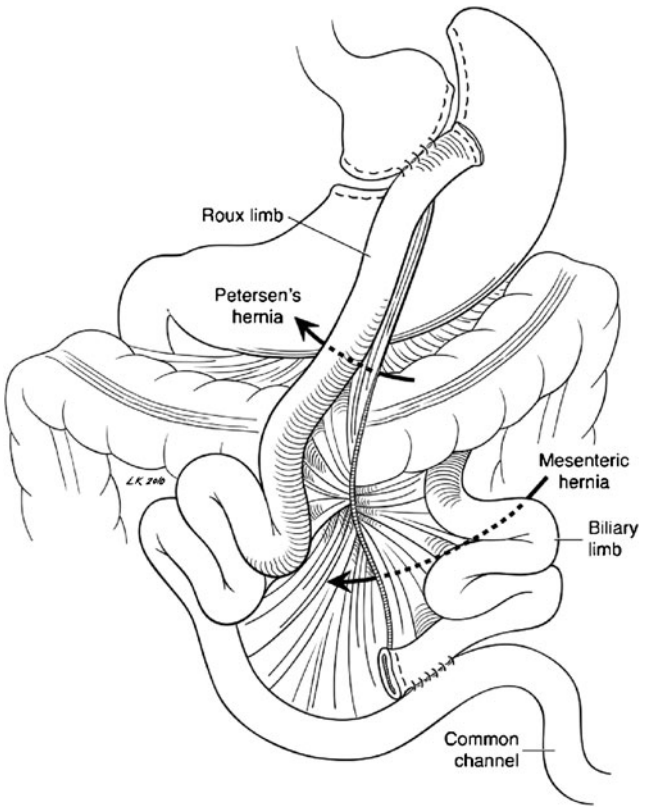

Fig. 1 Gastric bypass and internal hernia anatomy

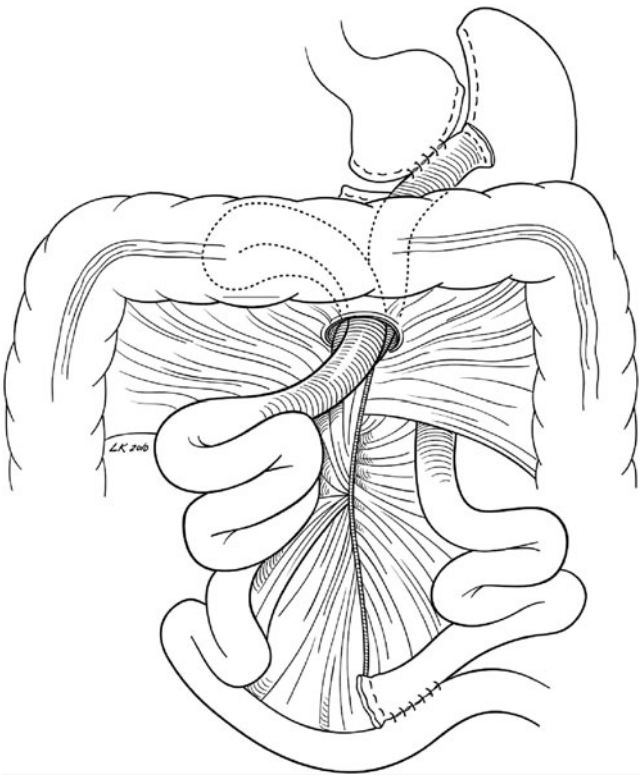

Fig. 2 Mesocolic hernia

that high rates of mesocolic hernias are due to learning curve issues related to closure of this defect, which is difficult in a laparoscopic environment in obese patients with large amounts of mesocolic fat. Proponents add that mesocolic hernia rates can be lowered with experience with defect closure.[1,18] Despite these arguments, the antecolic Roux limb configuration is more commonly utilized.[16] Proponents argue that no data demonstrate lower clinical anastomotic leak or ulcer rates with retrocolic anatomy despite reduced tension on the gastrojejunostomy. Furthermore, the antecolic approach eliminates mesocolic hernias, which in series of retrocolic bypasses, are the most common type of internal hernia.[1,6,19] Antecolic reconstruction may therefore be associated with a lower overall internal hernia rate.[20]

Much debate surrounds the issue of routine closure of internal hernia defects at the time of primary gastric bypass. While some authors advocate for routine closure,[20] the literature as a whole does not clearly demonstrate a lower incidence of internal hernia associated with this practice. Series with and without routine closure demonstrate similar incidences of internal hernia ranging from 0.2% to 9%.[5–8] Despite this debate, routine closure of defects at the time of gastric bypass is straightforward, and we therefore advocate this practice.

Debate exists regarding mesenteric defect length during primary gastric bypass. The mesentery of the small intestine is a primary source of downward (caudad) tension on the Roux limb, and extended division of its mesentery reduces tension on the Roux limb at the gastrojejunal anastomosis. Long mesenteric divisions may, however, create larger defects that are more likely to result in internal hernia. For this reason, some surgeons minimize the length of mesenteric division and, simply divide the jejunum but not the mesentery, accepting a higher degree of tension on the anastomosis.[8] With routine closure of the mesenteric defect, however, we feel that the benefit of reduced anastomotic tension associated with a long mesenteric split exceeds the risk of hernia.

Limb length may also affect internal hernia rates. Virtually any segment of small intestine can incarcerate in any type of internal hernia, but the biliary limb is often involved, and longer biliary limb lengths may be associated with an increased risk of internal hernia.[2] Similarly, a long redundant Roux limb may be at increased risk of herniation through a Petersen's defect by rotating on itself (Fig. 3).

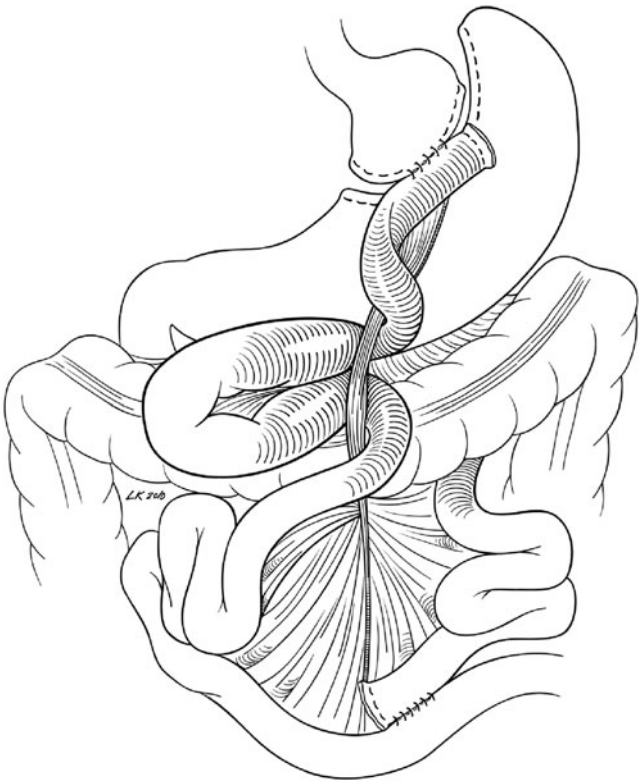

Fig. 3 Roux limb rotating on itself and herniating from left to right through Petersen's defect

Operative Management

This review focuses on laparoscopic exploration for internal hernia in patients who have formerly undergone laparoscopic gastric bypass, although less commonly, patients may present with internal hernia after open gastric bypass. While a laparoscopic approach may be considered in such patients, an open approach is often necessary. For laparoscopic exploration, the patient is positioned supine with the surgeon on the patient's right. Trocars are placed in right upper (surgeon's left hand), middle (camera), and lower (surgeon's right hand) quadrants. An assistant port is placed in the left upper quadrant. We use a hydraulic camera holder to the right of the surgeon, but a second assistant may be used in this position instead.

The bowel is run starting at the gastrojejunostomy, down the Roux limb to the jejunojejunostomy. From the jejunojejunostomy, the biliopancreatic limb is then run proximally to the ligament of Trietz, which is exposed by elevating the transverse colon with cephalad retraction of an appendix epiploicae. Next, the common channel is run from the jejunojejunostomy to the cecum. In the absence of an internal hernia, it should be possible to run the entire bowel in this manner without difficulty. If difficulty is encountered at any point in running the bowel, then an internal hernia is likely present. In some cases, a loop of bowel that is clearly incarcerated in an identifiable internal hernia defect is encountered. With larger hernias, however, the surgeon may encounter a loop of bowel that dives under other bowel or into the retroperitoneum and cannot be traced further. In such cases, deciphering the anatomy and type of internal hernia in a laparoscopic environment may be challenging.

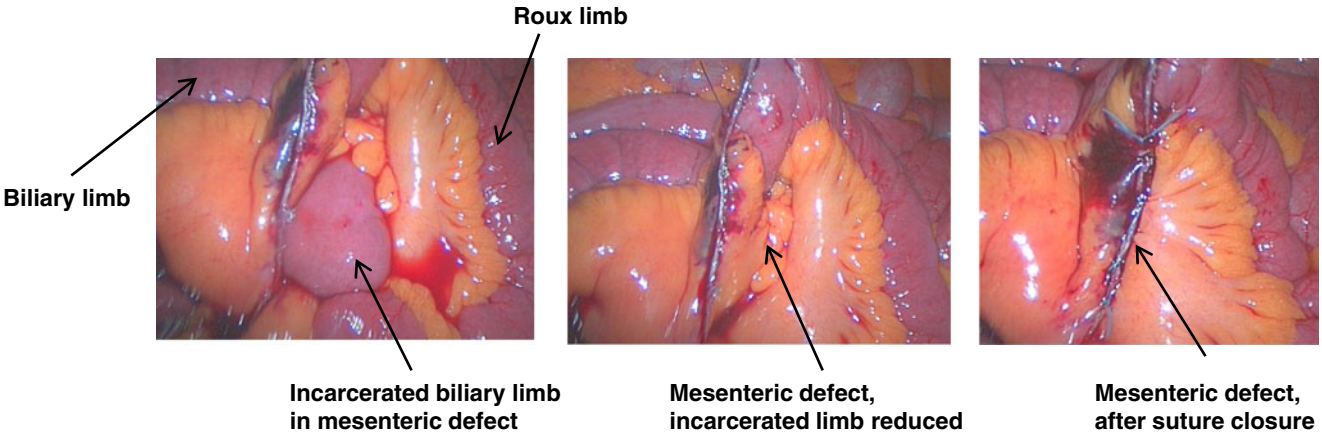

Fig. 4 Repair of mesenteric defect: view from the patient's right, with jejunojejunostomy elevated to expose mesenteric defect

A mesenteric hernia is identified by elevating the jejunojejunostomy, thus exposing the mesenteric defect. Mesenteric hernias often involve the biliopancreatic limb, which most often herniates through the mesenteric defect from its normal position in the left upper quadrant to the right. Less commonly, the Roux limb or the common channel may herniate through a mesenteric defect in either direction. Closure of the mesenteric defect is accomplished by elevating the jejunojejunostomy ventrally, reducing the hernia, and closing the defect with non-absorbable suture (Fig. 4).

Petersen's hernia usually involves the Roux limb or the biliary limb, and the incarcerated intestine often traverses the space between the mesentery of the Roux limb and the transverse colon from left to right, positioning incarcerated bowel in the right upper quadrant. Thus, the finding of small intestine in the right upper quadrant that cannot be clearly identified as unincarcerated Roux limb (i.e., if the Roux limb cannot be run from gastrojejunostomy to jejunojejunostomy without encountering incarceration) is suggestive of a Petersen's hernia (Fig. 3). Closure consists of suturing the Roux limb mesentery to the underlying transverse colon and its mesentery. This may be difficult in the obese patient, as the Roux limb is often tightly apposed to the underlying transverse colon, and completing the closure to the root of the small bowel mesentery can be challenging. Such closures are typically performed from the right side of the abdomen, with the Roux limb reflected to the patient's left, exposing the root of the small bowel and transverse colon mesenteries (Fig. 5a). The mesentery of the Roux limb is then sutured to the underlying transverse colon and its mesentery. Closure

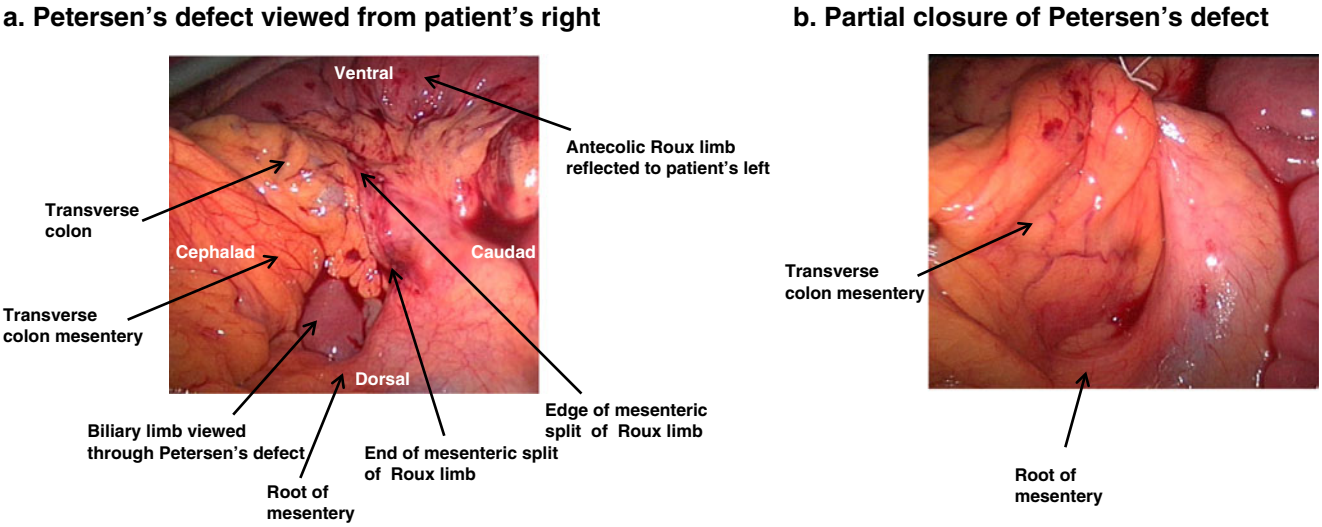

Fig. 5 Repair of Petersen's defect: **a**. View of Petersen's defect from the patient's right, with Roux limb reflected to patient's left. **b**. Partial closure of Petersen's defect—the edge of the Roux limb mesenteric split has been sutured to the transverse colon and its mesentery, and only the distal suture of this closure is seen in the upper photograph, which marks the end of the Roux limb mesenteric split. The remaining defect (which should be closed) is shown extending beyond the Roux limb mesenteric split to the root of the mesentery

should be carried dorsally down to the root of the mesentery, which extends beyond the end of the split in the mesentery of the Roux limb (Fig. 5b).

A mesocolic hernia is identified by elevating the transverse colon cephalad using an appendix epiploicae. While other intestine may be involved, the majority of mesocolic hernias involve a herniation of the Roux limb itself, with the jejunojejunostomy involved in some cases. Closure is accomplished with circumferential sutures around the Roux limb as it passes through the defect.

Large defects may lead to large hernias. Often, the jejunojejunostomy acts as a lead point, and carries large segments of biliopancreatic, Roux, and common channel limbs through a Petersen's or mesenteric defect. In such cases, a majority of the small intestine may be incarcerated, and most of the small bowel mesentery may be twisted on itself. Identification of anatomy and reduction of the hernia in a laparoscopic environment in such cases may be difficult. When faced with large hernias, returning to known anatomy is useful. The terminal ileum is often clearly identifiable in the presence of a large incarcerated hernia, and retrograde tracing of the terminal ileum is often the best place to start. This sometimes requires the surgeon to operate from the patient's left initially and transition to the patient's right as the bowel is run proximally. That said, we are usually able to run the entire small bowel in most cases from the patient's right side. Similarly, the proximal Roux limb is rarely involved in internal hernia, as it is tethered by the gastrojejunostomy proximally. Running the Roux limb distally from the gastrojejunostomy is therefore also a useful starting point. Finally, identification of the jejunojejunostomy, especially in cases where it is the lead point for herniation, is also useful. When an incarceration is encountered, gentle traction on the intestine may reduce the hernia. If not, it is often helpful to explore the “other side” of the internal hernia defect and “push” rather than pull the incarcerated intestine through the defect. What constitutes the “other side” of the hernia of course depends on the specific anatomy. In the case of a mesenteric hernia with biliopancreatic limb incarceration from left to right, the incarcerated hernia is found on the right “medial” side of the mesenteric defect, and pushing from right to left (rather than pulling from left to right) may reduce the hernia. In the case of a Petersen's hernia traversing from left to right, the incarcerated bowel may be found in the right upper quadrant, and pushing from right to left under the Roux limb mesentery may reduce the hernia. Once reduced, closure of internal hernia defects is usually straightforward. We close all defects with non-absorbable suture. Tacking the closure to non-fat fixed structures such as adjacent bowel or the ligament of Treitz in the case of a mesocolic hernia has been suggested and may enhance the strength of such repairs.

The surgeon may rarely encounter atypical internal hernias. One such hernia occurs between the limbs of jejunum distal to the end of the staple line and proximal to the distal stay suture of the side-to-side jejunojejunostomy.[21] Prevention consists of ensuring that the staple line fully traverses these stay sutures. The surgeon must of course be alert for alternative diagnoses as well as other types of hernia, including ventral hernias, and acquired or congenital diaphragmatic (e.g., hiatal, Bochdalek, Morgagni) and abdominal wall (e.g., trocar site, Spigelian) hernias.

Conclusion

The management of internal hernia after gastric bypass presents numerous challenges, and surgeons will be faced with such patients with increasing frequency. An understanding of the anatomy and technical aspects of primary gastric bypass will inform diagnostic and operative management of subsequent internal hernia. While this review is directed towards the general surgeon, management is expedited and optimized at centers with experience in bariatric surgery. All patients with symptoms suggestive of internal hernia or bowel obstruction should be treated by surgeons and centers with significant experience in bariatric surgery when possible.

Acknowledgements Many thanks to Lynn Kitagawa for illustrations.

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REVIEW ARTICLE

Systematic Review of Delayed Postoperative Hemorrhage after Pancreatic Resection

Didier Roulin · Yannick Cerantola · Nicolas Demartines · Markus Schäfer

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Abstract
Introduction This review assesses the presentation, management, and outcome of delayed postpancreatectomy hemorrhage (PPH) and suggests a novel algorithm as possible standard of care.
Methods An electronic search of Medline and Embase databases from January 1990 to February 2010 was undertaken. A random-effect meta-analysis for success rate and mortality of laparotomy vs. interventional radiology after delayed PPH was performed.
Results Fifteen studies comprising of 248 patients with delayed PPH were included. Its incidence was of 3.3%. A sentinel bleed heralding a delayed PPH was observed in 45% of cases. Pancreatic leaks or intraabdominal abscesses were found in 62%. Interventional radiology was attempted in 41%, and laparotomy was undertaken in 49%. On meta-analysis comparing laparotomy vs. interventional radiology, no significant difference could be found in terms of complete hemostasis (76% vs. 80%; P=0.35). A statistically significant difference favored interventional radiology vs. laparotomy in term of mortality (22% vs. 47%; P=0.02).
Conclusions Proper management of postoperative complications, such as pancreatic leak and intraabdominal abscess, minimizes the risk of delayed PPH. Sentinel bleeding needs to be thoroughly investigated. If a pseudoaneurysm is detected, it has to be treated by interventional angiography, in order to prevent a further delayed PPH. Early angiography and embolization or stenting is safe and should be the procedure of choice. Surgery remains a therapeutic option if no interventional radiology is available, or patients cannot be resuscitated for an interventional treatment.

Keywords Postpancreatectomy hemorrhage · Sentinel bleed · Pseudoaneurysm

Introduction

Despite the fact that modern pancreatic surgery has successfully evolved during recent years, postoperative hemorrhage still represents an important source of concern after major pancreatic resection. Together with delayed gastric emptying, pancreatic fistula and intraabdominal infections (i.e., abscess formation), bleeding complications mainly contribute to the high rate of postoperative morbidity after pancreatic surgery.[1–5]

Standardized definition and classification of postpancreatectomy hemorrhage (PPH) have been lacking until recently. In consequence incidences and mortality rates in the literature reveal a large range from 2% to 18% and 15% to 60%, respectively.[1,3–8] To overcome these shortcomings, the International Study Group of Pancreatic Surgery (ISGPS) proposed a new classification of PPH based on time of onset, location, and severity of hemorrhage.[9] Early PPH within 24 h postoperatively is generally caused by a technical failure of appropriate hemostasis at anastomotic sites, e.g., suture lines and resection area, or by an underlying perioperative coagulopathy.[6] Delayed PPH after the first postoperative day, is related to ulceration of

D. Roulin · Y. Cerantola · N. Demartines · M. Schäfer
Department of Visceral Surgery,
University Hospital of Lausanne (CHUV),
Lausanne, Switzerland

M. Schäfer (✉)
Service de Chirurgie Viscérale, CHUV,
Rue du Bugnon 46,
1011 Lausanne, Switzerland
e-mail: markus.schafer@chuv.ch

gastroenteral anastomosis (marginal ulcer), leakage of venous anastomosis after portal vein resection or, more importantly, to erosion of peripancreatic vessels. Stepwise erosion of the celiac trunk and the superior mesenteric artery induces pseudoaneurysm formation that may subsequently rupture.

While early bleeding can be treated rather easily, delayed PPH is more difficult to manage. Since typical clinical signs such as sentinel bleeding heralding pseudoaneurysm formation may be lacking, early diagnosis may not be possible. However, prompt diagnosis and treatment are crucial factors determining successful outcome. During recent years, interventional radiology and gastroenterology offered new approaches for the treatment of various postoperative complications after major pancreatic surgery, and have challenged surgery as rescue procedure.

The aim of the study was to assess the clinical presentation, management, and outcome of delayed PPH by systematically reviewing the current literature, and to provide a novel algorithm as a possible standard of care.

Methods

Literature Search Strategies

An electronic search of Medline and Embase databases was performed using different keywords: pancreatectomy, duodenopancreatectomy, pancreaticoduodenectomy, pancreatic resection, postoperative bleeding, postoperative hemorrhage delayed bleeding, delayed hemorrhage, arterial bleeding, arterial hemorrhage, risk factor, pseudoaneurysm, pancreatic fistula, sentinel bleed, angiography, transcatheter arterial embolization, coil embolization, covered stent, and stent graft. Terms were searched both in isolation and in combinations (Boolean operators). The search terms were identified in the title, abstract or medical subject heading. In addition, hand-searching of electronic links to related articles and of references of selected studies was performed.

Inclusion and Exclusion Criteria

Only original articles that evaluated the outcomes of delayed PPH were included. Delayed PPH was defined, in accordance to the recently published ISGPS classification, as a postoperative bleeding occurring more than 24 h after major pancreatic resection.[9] The term "pancreatic resection" includes all of the following procedures: pylorus-preserving or classical pancreaticoduodenectomy, pancreatic left (tail) resection, duodenum-preserving pancreatic head resection, pancreatic segment resection, or total pancreatectomy. Studies that evaluated outcomes after other pancreatic procedures such as necrosectomy and pancreatic transplantation were excluded.

We decided to exclude review articles, studies with less than ten patients, experimental studies, case reports, as well as studies that were only reported as abstracts or letters. Only articles published in English between January 1990 and February 2010 were included.

Data Extraction and Analysis

Summaries and abstracts of each identified publication were screened for exclusion criteria. Only publications, which fulfilled the inclusion criteria and addressed the clinical questions of this analysis, were further assessed. Each of these publications was independently and thoroughly reviewed by D.R. and M.S. Relevant data including authors, title, study design, methodology, main results, and conclusions were extracted and documented on a separate data sheet developed a priori for each publication.

Excel (Microsoft Corp.) was used for all data collection and tables. The meta-analysis was performed by using Review Manager, version 5.0 (The Cochrane Collaboration) according to its instructions. The Mantel–Haenszel method was used to combine the odds ratio by mean of a random-effect model. An odds ratio of less than 1 favored laparotomy, and the point estimate of the odds ratio was considered to be statistically significant at the level of $P<0.05$, if the 95% confidence interval did not include the value 1.

Results

The literature search retrieved 1,357 publications. Of these, 1,319 were primarily excluded because they were not relevant (1,303: no outcome data reported, duplicated data, letters and abstracts only), included less than ten patients (10), or were review articles (6). The remaining 38 publications were fully assessed. Another 23 articles were excluded due to incomplete data (11), insufficient number of patients (six), duplicated data (one), lack of relevance (four), or patient inclusion operated before 1980 (one). Finally, 15 studies with 7,400 patients were included for final analysis (Fig. 1). In total, there were 248 patients with delayed PPH (Table 1). All included articles were retrospective case series; eight of them presented prospectively collected data. No randomized controlled trial was found.

Onset of Bleeding

All studies, except two[10,11] which applied the 24-h cut-off defined by the ISGP definition, used different time points to determine delayed PPH. Six studies[2,12–16] used the fifth postoperative day, another five studies[17–21] used the seventh

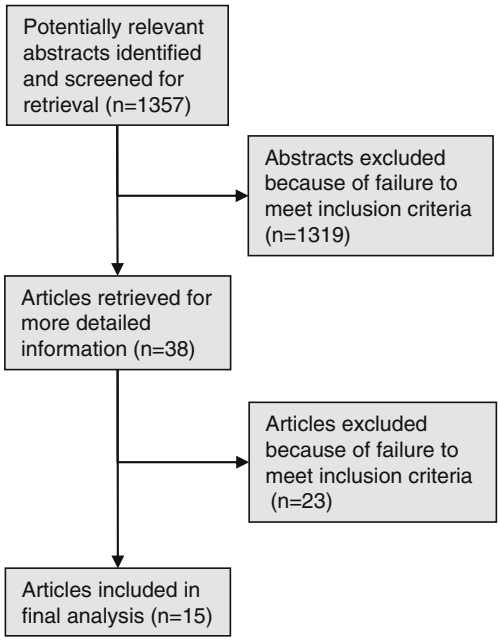

Fig. 1 Flow chart of systematic search

postoperative day, and the two remaining studies[22,23], in which all of their PPH occurred after the first postoperative week, did not specify any cut-off as time point.

Incidence, Clinical Presentation, and Risk Factors

There were 248 patients with delayed PPH out of an overall group of 7,400 patients who underwent pancreatic resection. However, five patients were excluded from the incidence calculation since pancreatic resection was performed outside the index hospital.[14,21] Therefore, the overall incidence of delayed PPH was 3.3% (243/7,400), ranging from 1.6% to 12.3% among the included studies. While the incidence was 4.5% in studies considering the ISGP definition as time point, studies using the fifth and seventh postoperative day revealed an overall incidence of 2.8% (range 2.0–4.9%) and 3.4% (range 1.9–8.7%), respectively.

Onset of delayed PPH revealed an overall median range of 13 to 27 days postoperatively. The most frequent localization of bleeding was the abdominal cavity in 58% (106/182) of cases, followed by the gastrointestinal tract in 35% (64/182). Seven percent of delayed PPH (12/182) occurred in both the abdominal cavity and the gastrointestinal tract. Sentinel bleeding heralding a PPH was observed in 45% (54/119) of cases. Only three studies[14,22,23] with a total of 23 sentinel bleedings described their location: 15 were coming from abdominal drains, and eight were originating from the gastrointestinal tract.

A pancreatic leak was found in 78 of 156 patients (50%) with delayed PPH. When cumulating both pancreatic and biliary leaks with intraabdominal abscess, the prevalence of intraabdominal complications reached 62% (96/156). Among five studies analyzing risk factors of PPH,[2,11,12,17,20] only pancreatic leak was always found to be an independent risk factor. Biliary leak and intraabdominal abscess were also significant prognostic factors in four of these publications.[2,11,12,17] Balachandran further identified male gender,

Table 1 Incidence and clinical presentation of delayed postpancreatectomy hemorrhage

Authors	Year	Pancreatectomy (*n*)	PPH (*n*)	Onset of PPH (median POD)	Sentinel bleed (*n*)	Type of PPH (*n*) (GI/IA/both)
Balachandran et al. [17]	2004	218	19	NR	NR	10/9/0
Blanc et al.[18]	2007	411	16	NR	3	5/10/1
Boggi et al.[19]	2007	818	19	NR	4	3/16/0
Buchler et al.[10]	2000	331	12	NR	NR	NR
Choi et al.[12]	2004	500	22	13	NR	10/12/0
Koukoutsis et al. [20]	2006	362	14	13	8	NR
Liu et al.[13]	2009	308	15	10	NR	4/9/2
Makowiec et al.[21]	2005	464	12	24	4	7/5/0
Miura et al.[22]	2009	708	11	11	5	0/11/0
Sato et al.[23]	1998	81	10	27	10	NR
Tien et al.[2]	2005	402	10	22	3	0/6/4
Treckmann et al.[14]	2008	189	11	17	8	6/5/0
Wei et al.[11]	2009	628	31	NR	NR	12/19/0
Yekebas et al.[15]	2007	1,524	30	NR	NR	NR
Yoon et al.[16]	2003	456	16	13	9	7/4/5

PPH postpancreatectomy hemorrhage, *POD* postoperative day, *GI* gastrointestinal, *IA* intraabdominal, *NR* not reported

longer duration of jaundice, and duct-to mucosa type of pancreaticojejunal anastomosis.[17] Neither the type of operation,[2,11,12] nor a duration of surgery of more than six hours,[17] nor a lymph node dissection,[2,12] could be identified as risk factors predicting bleeding after pancreatic resection.

Diagnostics and Site of Bleeding

The diagnostic procedures employed after the development of delayed PPH were endoscopy, angiography, and computed tomography (CT). The frequency and the sensitivity of the CT were not systematically reported in the included studies. When reported,[2,11–13,15,16,18–23] a diagnostic angiography was performed in 55% of the patients with delayed PPH (113/206). The main reason for not performing a diagnostic angiography was hemodynamic instability. The bleeding source could be localized in 88% (99/113) of cases. An endoscopic approach, when precisely numbered,[15,21,23] was used in ten out of 52 patients. The use of endoscopy was restricted to gastrointestinal bleeding. When documented,[11–14,16,18,21–23] the anatomical site of bleeding (Table 2) determined either after angiography, endoscopy, or relaparotomy, was as follows: eroded or ruptured visceral arteries in 66% (101/154), the pancreatic stump in 12% (18/154), and the entero-jejunostomy in 6% (9/154) of cases. The exact bleeding location could not be determined in sixteen out of 154 cases (10%). Among visceral arteries, the most frequent source was the gastroduodenal artery, representing 50% (50/101) of all arterial hemorrhage. The common hepatic artery was implicated in 21% (21/101) and the proper hepatic artery and its branches in 11% (11/101).

Treatment and Outcome (including mortality)

The first-line treatment of delayed PPH was either interventional radiology (coil embolization or covered stenting) or laparotomy. Among the 215 patients with reported first-line treatment,[2,11–16,18–23] interventional radiology was attempted in 39% (83/215) of cases, and the laparotomy approach was chosen in 53% (114/215) of cases. The 18 remaining patients were either treated conservatively or endoscopically. There was one study including 11 patients for whom no radiological treatment was performed.[14] Hemodynamic instability, which was quantified in four studies, occurred in 34 out of 51 patients.[2,14,18,20] This was the main criterion for surgical approach in these studies. When a radiological approach was taken, arterial coil embolization was most commonly used (95%). The implementation of a covered stent in case of bleeding from the hepatic or the superior mesenteric artery was described in four patients.[11,18,21] Regarding the surgical approach, a completion pancreatectomy was undertaken in 41% (20/49) as documented in seven studies.[2,14,18,20–23] Half of these completion pancreatectomies ($n=10$) were described in a single study.[2] Other interventions including hemostasis and arterial ligature were undertaken in 59% (29/49) of cases.

The documented outcomes after the first-line treatment[2,11–13,21–23] are shown in Figs. 2 and 3. The intervention was considered as successful when complete hemostasis was achieved at the end of the procedure. The success rate of laparatomy was 76% (34/45) and 80% (48/60) in interventional radiology. On statistical analysis, there was a tendency favoring laparotomy in term of success rate (Fig. 2), without reaching stastistical significance ($P=0.35$). The mortality rate of the patients having

Table 2 Bleeding source of delayed postpancreatectomy hemorrhage

	Visceral arteries (*n*)							PS (*n*)	EJ (*n*)	Other (*n*)	Unknown (*n*)
	GDA	CHA	PHA	SA	SMA	Other	Total				
Blanc et al.[18]	4	0	0	3	0	0	7	6	1	1	1
Choi et al.[12]	5	3	2	1	3	0	14	2	1	0	5
Liu et al.[13]	14	0	0	0	0	0	14	1	0	0	0
Makowiec et al.[21]	6	5	0	1	0	0	12	0	0	0	0
Miura et al.[22]	5	0	2	0	4	0	11	0	0	0	0
Sato et al.[23]	2	3	3	1	1	0	10	0	0	0	0
Tien et al.[2]	1	5	4	0	0	0	10	0	0	0	0
Treckmann et al.[14]	1	2	0	2	0	1	6	0	2	1	2
Wei et al.[11]	8	0	0	0	0	0	8	7	3	7	6
Yoon et al.[16]	4	3	0	0	0	2	9	2	2	1	2
Total							101	18	9	10	16
Relative amount							66%	12%	6%	6%	10%

PPH postpancreatectomy hemorrhage, *GDA* gastroduodenal artery, *CHA* common hepatic artery, *PHA* proper hepatic artery, *SA* splenic artery, *SMA* superior mesenteric artery, *PS* pancreatic stump, *EJ* enterostomy

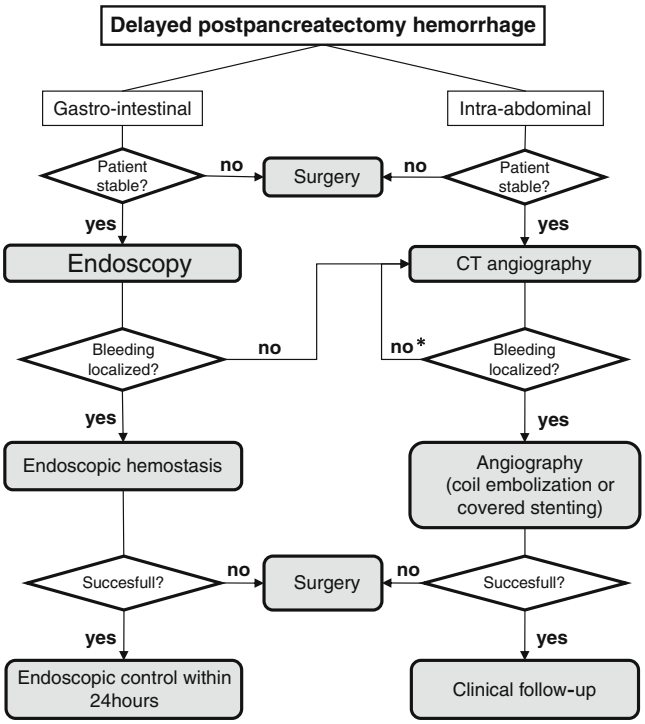

Fig. 2 Forest plots of success rate (i.e., complete hemostasis) for laparotomy vs. interventional radiology after delayed postpancreatectomy hemorrhage. *Squares* indicate the point estimate of the treatment effect (odds ratio), with 95% confidence intervals [CI] indicated by *horizontal bars*. The *diamond* represents the summary estimate from the pooled studies with 95% CI

initially undergone a laparotomy or a radiological intervention was 47%, and 22%, respectively. On meta-analysis, there was a statistically significant difference (P=0.02) in favor of interventional radiology in term of mortality after PPH (Fig. 3).

The overall mortality rate of delayed PPH was 35% (87/248). Reported causes of death were hemorrhagic shock, septic shock, disseminated intravascular coagulation, and multiple organ failure.

Discussion

Delayed PPH is a rare complication which occurred in 3.3% of the included patients, but it is associated with a high mortality rate as high as 35% in our review. Therefore, its timely identification and prompt management as proposed by the diagnostic and therapeutic algorithm (Fig. 4) is of critical importance to achieve a good outcome. The ideal cut-off for the definition of early versus late bleeding remains controversial and somewhat arbitrary. While the majority of the included studies used the fifth or the seventh postoperative day for the definition of late bleeding, the recent consensus statement of the ISGPS proposed a cut-off of 24 h.[9] This variability of definition induced a lack of homogeneity in the publications included in our review. The median onset of delayed PPH was reported to be within a range of 13 to 27 days postoperatively. Therefore, there remains a major risk of delayed PPH even after patient's discharge.

Different mechanisms inducing major bleeding from visceral arteries and veins are discussed. First, extensive skeletonization of the celiac axis and the superior mesenteric artery during lymphadenectomy or resection of the pancreas may injure the vessel wall. This may be due to thermal injuries by using electrocautery or to damages to the vascular outer layer during dissection.[24,25] Secondly, postoperative leak of the pancreaticojejunostomy or hepaticojejunostomy may induce digestion of vascular structures by the erosive pancreatic or biliary juice, respectively.[2,6,11,12,17] In addition, subsequent abscess formation can also erode the vessel wall or a vascular anastomosis, e.g., after portal vein resection. In some few cases, local tissue destruction may disrupt ligatures and sutures, typically at the stump of the gastroduodenal artery. Finally, local vessel wall necrosis could be induced by mechanical pressure of a drain lying on a vessel or an ascending infection along the drain.[26] Typically, the

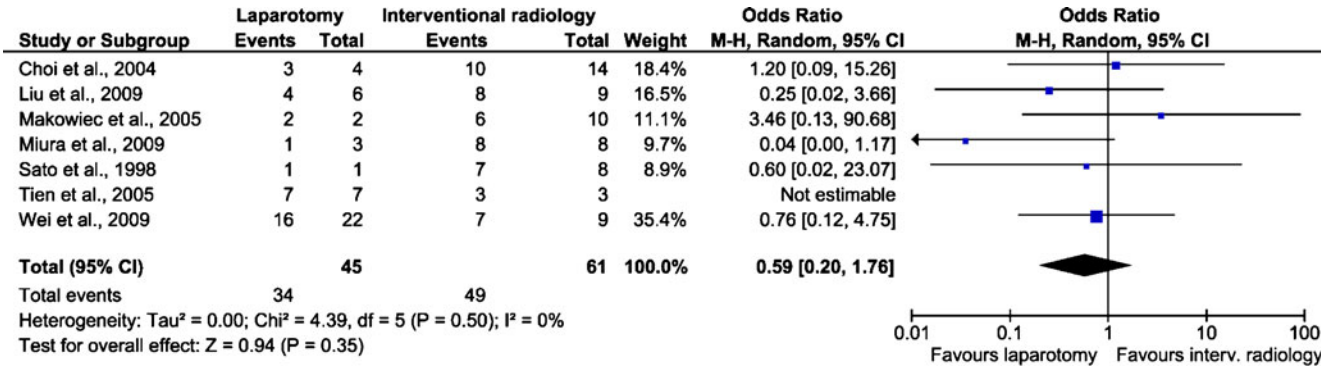

Fig. 3 Forest plots of mortality for laparotomy vs. interventional radiology after delayed postpancreatectomy hemorrhage. *Squares* indicate the point estimate of the treatment effect (odds ratio), with 95% confidence intervals [CI] indicated by *horizontal bars*. The *diamond* represents the summary estimate from the pooled studies with 95% CI

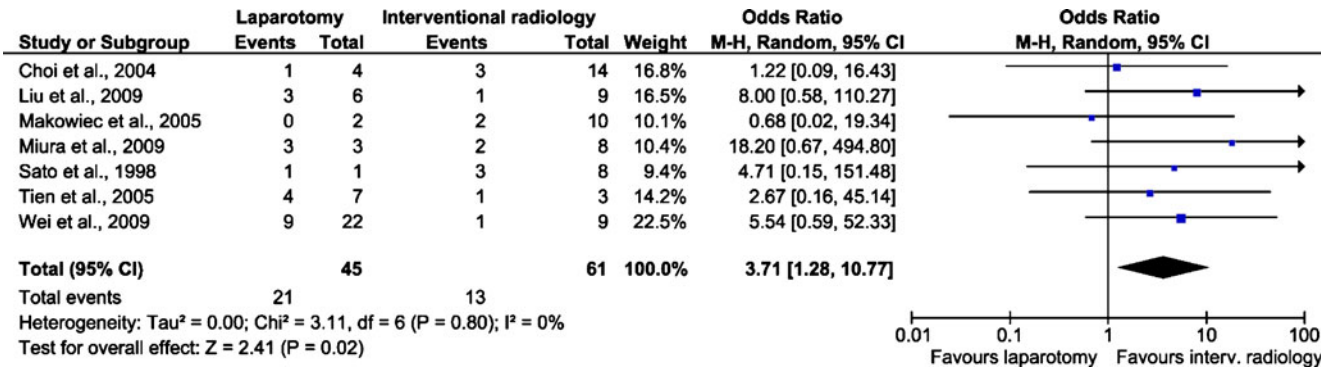

Fig. 4 Diagnostic and therapeutic algorithm for delayed postpancreatectomy hemorrhage. *Asterisk*, repeat CT angiography in case of persistent suspected bleeding

stepwise and rather slow destruction of the vessel wall causes a pseudoaneurysm of a major visceral artery. The presence of a local abscess, mainly resulting from an anastomotic leakage (either pancreatic or biliary), was the most common risk factor, and was identified in 62% of all delayed PPH in our review. We also found that the majority (66%) of all delayed PPH were coming from an eroded or ruptured visceral artery. Since pancreatic leak and intraabdominal sepsis have been demonstrated to be independent risk factors of subsequent massive bleeding,[2,11,12,17] their prompt recognition and management represents the mainstay of delayed PPH prevention. In case of conservatively treated pancreatic fistula, some authors advocated a strict surveillance including a weekly CT angiography to detect the development of a pseudonaneurysm.[12,14,27]

The term "sentinel bleed" was first introduced by Brodsky et al. in 1991[28] to describe intermittent minor bleeding (either intraabdominal or intraluminal) that sometimes precede delayed PPH. In our review, this preliminary event was identified in almost half of the patients before massive bleeding occurred. Yekebas et al.[15] showed that the coincidence of pancreatic fistula and a sentinel bleed, preceding delayed PPH, is associated with a mortality of 57%. If there was no sentinel bleed before PPH, the mortality was lowered down to 38%. On the contrary, Treckmann et al.[14] could not detect any mortality difference between patients with or without a preceding sentinel bleed. However, as delayed PPH is a highly lethal event, some authors suggested that any sentinel bleed after pancreatic surgery should lead to an emergency angiography.[11,14,15,23,29] Alternatively, the use of CT angiography, as proposed by Blanc et al.,[18] is less invasive and also identifies pseudoaneurysm and associated complications.

Angiography was able to localize the bleeding source in almost 90% of cases. The false-negative angiographies could be due to the intermittent character of bleeding episodes.[5] If the bleeding source could not be identified, Yekebas et al.[15] suggested proceeding to a novel angiography after 6–24 h as long as the patient remains hemodynamically stable. "Blind" coiling of the gastroduodenal artery could also provide bleeding control after a negative angiography. The success rate of angiographic hemostasis was 80% in our review, which was close to the 76% of successful hemostasis obtained after relaparotomy. There was no statistically significant difference between both treatments regarding their respective success rate. In contrast, the mortality rate of the patients having initially undergone a relaparotomy was 47% versus 22% for those with primary radiological intervention. This difference reached statistical significance, favoring interventional radiology in term of mortality. However, this difference of outcome may reflect a selection bias as stabilized patients can be transferred to angiography, but those who are hemodynamically unstable require a crush laparotomy. The mortality rate after a delayed PPH is higher than the failure rate of hemostasis, emphasizing the fact that even after a successful hemostasis the underlying complications such as pancreatic leak and intraabdominal abscess have to be considered and treated. For that reason, some authors still prefer surgery as first-line therapy.[6,30] On the other hand, patients with pancreatic leaks can be treated conservatively if no local or systemic inflammatory response occurs.[31] Intraabdominal abscess can be treated by a CT-guided percutaneous drainage,[32,33] to prevent recurrent bleeding.

Different surgical techniques have been reported to prevent pseudoaneurysm formation. Turrini et al.[34] suggested performing the pancreaticojejunostomy far on the left side, away from the celiac trunk and portal vein to avoid direct contact of pancreatic juice with adjacent vessels in case of pancreatic leak. Kurosaki et al.[35] proposed to wrap an omental flap around the pancreaticojejunostomy to minimize anastomotic leakage. Koukoutsis et al.[20] described the spreading of the round ligament around the common hepatic artery after pancreaticoduodenectomy. The real benefits of all these technical modifica-

tions are poorly supported by clinical data, and remain on the level of personal opinion and experience.

The traditional treatment of delayed PPH has so far been surgery. However, surgical access to the bleeding vessel is always difficult because of the overlying pancreaticoenteric and bilioenteric anastomosis as well as the presence of postsurgical adhesions. The eroded bleeding vessel is also difficult to repair due to peripancreatic inflammation and vessel wall friability.[17,26] With recent advances in interventional radiology, radiological hemostasis (e.g., coiling or stenting) was described as the preferred option in hemodynamically stabilized patients.[2,12,16,23,36–38] Bleeding arising from the gastroduodenal artery is the most frequently encountered in delayed PPH, representing half of all delayed arterial bleeding in our review. Bleeding coming from this artery may be difficult to control surgically, as reported by Balachandran et al.,[17] where all three patients re-bled following surgical ligation of the gastroduodenal stump. In contrast, the gastroduodenal artery can easily be embolized. The second most common site of arterial bleeding is the common and the proper hepatic artery, which accounted for almost a third of all delayed visceral arteries hemorrhage included in the present review. Complete occlusion of these arteries by angiographic embolization can lead to intra-hepatic abscess as a result of liver necrosis, biliary ischemia, as well as fatal hepatic failure.[16,23,30,39] To avoid these severe complications, the use of covered stent was described for the treatment of bleeding of hepatic arteries.[11,40–42] However, anatomical reasons, such as kinking, and anatomical variations may impede successful stent placement.[21] Bleeding from the superior mesenteric artery (SMA), which occurred in 7.9% in our review, also inherits the potential risk of intestinal infarction subsequently to coil embolization. In 1998, Mc Graw et al.[43] first reported the successful use of covered stents for the management of a SMA pseudoaneurysm occurring after pancreaticoduodenectomy. Thus, if technically feasible, covered stents may represent the best treatment option in order to preserve a superior mesenteric and hepatic arterial flow.

Conclusion

Delayed postoperative bleeding after a pancreatic resection is a rare but highly lethal complication. The prompt recognition and treatment of risk factors such as pancreatic leakage and intraabdominal abscess is essential to prevent its deleterious outcome. Sentinel bleeding occurring after a pancreatic surgery needs to be thoroughly investigated for a pseudoaneurysm formation, and if detected, interventional angiography provides optimal management by avoiding collateral damage after major revisional surgery. Early angiography with embolization or stenting should be the procedure of choice in case of delayed bleeding, whenever possible. Surgery remains a therapeutic option if no interventional radiology is available, or patients cannot be resuscitated for an interventional treatment.

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GI IMAGE

Giant Splenic Artery Pseudoaneurysm

Ross Frederick Goldberg · Warren Maley · Eugene P. Kennedy · Charles J. Yeo · Harish Lavu

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Abstract
Background Giant splenic artery pseudoaneurysms (≥5 cm in size) are rare entities. We document the successful operative management of one of the largest splenic artery pseudoaneurysms (18 cm) ever reported as well as review the world literature on the subject.
Method Our literature review identified 160 cases of splenic artery pseudoaneurysm in the last 43 years. These ranged in size from 0.3 to 17 cm, and of these, 18 (11%) were 5 cm or larger. The majority of patients underwent treatment, either endovascularly or with open surgery, and their outcomes were independent of presenting symptoms or size.
Results Giant splenic artery pseudoaneurysms are uncommon, most often caused by pancreatitis, trauma, or iatrogenic etiologies and typically present with vague constitutional symptoms, or occasionally with hemorrhage. Most can be treated endovascularly, though in our case this was not possible due to the presence of celiac artery occlusion with retrograde filling of the pseudoaneurysm from superior mesenteric artery collaterals. Ultimately, we opted for an open technique, with supraceliac aortic control prior to manipulation and resection of the pseudoaneurysm.
Conclusion Our recommendation is that splenic artery pseudoaneurysms should be repaired when encountered, regardless of aneurysm size at presentation.

Keywords Splenic artery pseudoaneurysm · Pancreatitis

Case Report

A 68-year-old man presented to an outside hospital complaining of nausea, vomiting, and diffuse abdominal pain. He also noted that for the past several months, he had been experiencing bilateral upper and lower extremity swelling. The patient had a complicated past medical history, which included coronary artery disease with three-vessel coronary artery bypass graft surgery, left ventricular ejection fraction of 35%, minimal change disease of the kidney, and a history of an unknown prior operation to his aorta. As part of his workup, a non-contrast CT scan of the abdomen/pelvis was performed, which revealed a large heterogeneous mass with calcifications in the upper abdomen, abutting the lesser curvature of the stomach and lying anteriorly and superiorly to the pancreas. The CT scan also showed high-density fluid in the pelvis, suggestive of recent hemorrhage. The patient was hemodynamically stable, with no drop in serial hemoglobin counts. He was transferred to our institution for further workup and intervention. A contrast-enhanced thin-section CT scan of the abdomen/pelvis was performed, revealing an 18-cm mass with large vascular channels running within it (Figs. 1 and 2).

After reviewing the CT scan, the patient was sent to interventional radiology in an attempt to embolize the arterial blood supply to the lesion. During the visceral angiography, the celiac axis was found to be occluded at its origin from the aorta. The hepatic and splenic arteries filled in a retrograde

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R. F. Goldberg · W. Maley · E. P. Kennedy · C. J. Yeo · H. Lavu (✉)
Department of Surgery, Jefferson Pancreas, Biliary and Related Cancer Center, Thomas Jefferson University,
1025 Walnut Street, College Bldg., Suite 605,
Philadelphia, PA 19107, USA
e-mail: harish.lavu@jefferson.edu

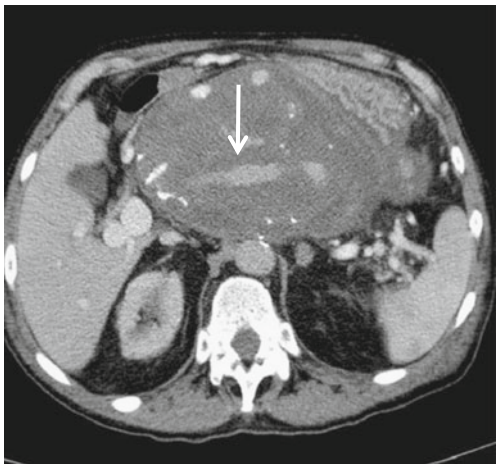

Fig. 1 CT angiogram: axial view of the 18-cm partially calcified giant splenic artery pseudoaneurysm lying posterior to the stomach and anterior to the aorta. Contrast enhancement of blood flow within the pseudoaneurysm is noted. The *arrow* points to the false channel

fashion through the pancreaticoduodenal arcade from branches off of the superior mesenteric artery (Fig. 3). These findings precluded successful angiographic embolization of the arterial inflow to this mass and helped identify it as a giant splenic artery pseudoaneurysm. The patient was therefore taken for exploration where after obtaining supraceliac aortic control, the splenic artery was ligated and the pseudoaneurysm resected. The patient tolerated surgery well, only losing 250 mL of blood, and his postoperative course was uneventful. He was discharged to home on postoperative day 5.

Discussion

Splenic artery pseudoaneurysms are rare entities, most often caused by pancreatitis, trauma, or by iatrogenic etiologies

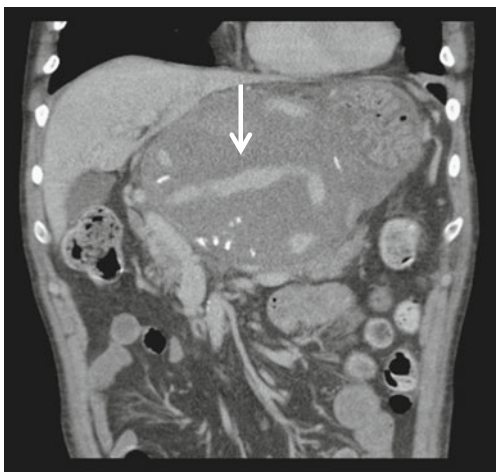

Fig. 2 CT angiogram: coronal view of the 18-cm giant splenic artery pseudoaneurysm. Contrast enhancement of blood flow within the pseudoaneurysm is noted. The *arrow* points to the false channel

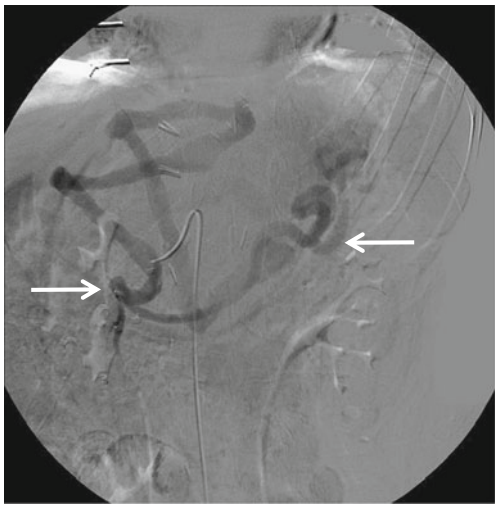

Fig. 3 Visceral arteriogram via the superior mesenteric artery: view of the large vascular channels flowing within the giant splenic artery pseudoaneurysm. *Arrows* show the proximal and distal segments of the splenic artery

such as prior open or endovascular surgery. They may present with vague constitutional symptoms or with acute hemorrhage. The most common cause of a splenic artery pseudoaneurysm is pancreatitis, in both its acute and chronic forms.[1] The mechanism behind pseudoaneurysm formation in pancreatitis is thought to occur via injury to the splenic artery wall by the digestive action of activated pancreatic enzymes, causing a necrotizing arteritis that leads to fragmentation of the elastic tissue and destruction of the vessel wall architecture.[2]

Patients with this disease can have a wide range of presentations, from being completely asymptomatic to presenting with acute hemodynamic collapse from sudden hemorrhage.[3] The most common presenting symptom is abdominal pain, occurring in approximately 30% of patients.[1] When hemorrhage does occur, it can lead to massive bleeding into any surrounding intra-abdominal organs or spaces, which include the peritoneal cavity, retroperitoneal space, or pancreatic duct (hemosuccus pancreaticus).[4,5] One posited theory suggests that a splenic artery rupture directly into a pseudocyst is the likely causative event in the formation of some larger pseudoaneurysms.[1] The risk of pseudoaneurysm rupture can be as high as 37%, with the mortality rate for free rupture approaching nearly 90%.[6,7] Interestingly, it has been reported that the size of splenic artery pseudoaneurysms is not predicative of their risk of rupture.[1]

Diagnosing splenic artery pseudoaneurysms can be difficult to accomplish even with the use of adjunctive radiographic techniques. The gold standard for diagnosis is via direct catheter visceral angiography,[8] which has the dual advantage of assisting in the diagnosis and allowing for therapeutic intervention. Radiographic alternatives to angi-

Table 1 Literature review of giant splenic artery pseudoaneurysms (>5 cm) and their treatments

Patients (n=18)	Gender	Size	Cause	Treatment
Median age: 51.7 (range 34–74 years)	12 M	5–17 cm[a] (mean 7.5 cm)	15 Pancreatitis	8 Embolization
				7 Distal pancreatectomy/splenectomy
	6 F		3 Iatrogenic[b]	2 Thrombin injection
				1 Pancreatectomy

[a] One patient did not have size reported in literature

[b] The three iatrogenic causes were: splenectomy for trauma 14 years earlier, splenectomy 5 years earlier for lymphoma, and cystgastrostomy for infected pancreatic pseudocyst

ography include ultrasound and cross-sectional imaging such as CT or MRI, which are able to assess the size and location of the pseudoaneurysm, but are unable to offer therapeutic interventions. These imaging modalities also run the risk of mistaking pseudoaneurysms for pseudocysts or other peripancreatic fluid collections.

There are a variety of interventions available to treat splenic artery pseudoaneurysms. Current therapeutic guidelines advocate for the treatment of all pseudoaneurysms to prevent sudden massive hemorrhage. Treatments available include (1) transcatheter embolization using a variety of techniques including coils, thrombin injections, or gelfoam; (2) ligation of the splenic artery with resection of the pseudoaneurysm; or (3) concomitant splenectomy with or without distal pancreatectomy along with resection of the pseudoaneurysm.[1,2,7–10]

A review of the English language literature identified 160 cases of splenic artery pseudoaneurysm reported in the last 43 years.[1–10] These ranged in size from 0.3 to 17 cm, and of these, 18 (11%) were 5 cm or larger (giant pseudoaneurysms; Table 1). The majority of patients underwent treatment, either via embolization or with open surgery. Patient outcomes were found to be independent of presenting symptoms or pseudoaneurysm size. Although many of these pseudoaneurysms can be treated via an endovascular approach, in our case this was not possible due to the presence of celiac artery occlusion, with retrograde filling of the pseudoaneurysm from superior mesenteric artery collaterals. We opted for an open technique, with supraceliac aortic control prior to manipulation and resection of the pseudoaneurysm. We were able to spare the spleen despite ligation of the splenic and short gastric arteries because of a large collateral artery feeding the splenic hilum arising from the left renal artery. In our case, we identified the causative factor in this patient's splenic artery pseudoaneurysm as being iatrogenic, likely related to a prior attempt at repairing a splenic artery aneurysm, but the prior operative records were unable to be recovered because the previous surgery had occurred in the distant past (18 years prior). At the time of the operation, we identified an occluded short segment of polytetrafluoroethylene graft at the level of the celiac axis which ended in the pseudoaneurysm cavity. Disruption of the distal end of this graft which was likely sewn to the downstream splenic artery was the likely cause of the formation of the pseudoaneurysm. Interestingly, pathologic examination of the resected pseudoaneurysm identified course fibrin-lined vascular channels, which can be clearly seen in the arteriogram (Fig. 3). Our recommendation is that via a multidisciplinary approach, all splenic artery pseudoaneurysms be treated when encountered, regardless of aneurysm size at presentation (Table 2).

Table 2 Details of the case report of our giant splenic artery pseudoaneurysm (>5 cm)

Patient (n=1)	Gender	Size	Cause	Treatment
68 years	M	18 cm	Iatrogenic	Vascular control, resection of giant splenic artery pseudoaneurysm

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LETTER TO EDITOR

Peng's Binding Pancreaticojejunostomy Plus External Drainage of the Pancreatic Duct After Pancreaticoduodenectomy: A Potential Choice

Sun Hu Yang · Ke Feng Dou · Wen Jie Song

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To the Editor:

We read with interest the paper published by Dr. Emmanuel and colleagues and congratulate them for a work well done.[1] I give the authors particular credit for controlling the many variables involved in the performance of a Peng's binding pancreaticojejunostomy (BPJ) and its postoperative management. In this study, they have shown the lower rate of pancreatic fistula following BPJ after pancreaticoduodenectomy (PD).

An ideal pancreaticojejunostomy (PJ) after PD should be safe and applicable to all kinds of pancreatic remnants. However, it is difficult to choose an ideal method of anastomosis for all patients. Patients with a "small" duct or a "soft" pancreas were at risk for mortality, postoperative complications, and pancreatic fistula after PD.[2]

In the author's study, 45 patients with soft pancreas and non-dilated main pancreatic duct underwent BPJ, 4 patients (8.9%) developed a pancreatic fistula, and 24 patients presented at least one postoperative complication (53.3%). The pancreatic fistula rate in this study is lower in patients with soft pancreas who underwent PJ in a randomized controlled trial,[3] but the reoperations and mortality were higher.

External drainage of the pancreatic duct should decrease the rate of pancreatic fistula following PJ reconstruction during PD procedure. Poon and colleagues, in their randomized controlled trial, performed a subgroup analysis that showed a reduced pancreatic fistula rate in patients with soft pancreas in whom an external drainage of the pancreatic duct was placed. The rate of pancreatic fistula was 12% among patients with external drainage of the pancreatic duct and 30% in the non-external drainage patients. For the patients with firm pancreas, the rate of pancreatic fistula was actually similar between the two groups. So, this may suggest that the external drainage of the pancreatic duct may be more beneficial in patients who have higher risk of leakage such as those with soft pancreas.[4]

Some retrospective studies reported that pancreatic fistula occurs in 0% to 4.2% of patients with PJ anastomosis using an external stent.[5, 6] As well in our institute, pancreatic fistulas were observed in about 5% of patients with BPJ using an external stent.

A stent may help divert the pancreatic secretion away from the anastomosis and prevents activation of pancreatic enzymes by bile.

BPJ cannot be performed in all patients such as in cases where the pancreas remnant was larger than the jejunum. So,

S. H. Yang · K. F. Dou (✉) · W. J. Song
Fourth Military Medical University,
Xi'an, Shaanxi, People's Republic of China
e-mail: doukefeng2010@126.com

selection of proper PJ techniques according to pancreatic texture and the main duct size is necessary.

The authors of the original article were given the opportunity to respond to the letter and chose not to do so.

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LETTER TO THE EDITOR

The Critical View of Safety and Routine Intraoperative Cholangiography Complement Each Other as Safety Measures During Cholecystectomy

K. Tim Buddingh · Vincent B. Nieuwenhuijs

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Dear Editor,

We read, with great interest, the study by Sanjay et al. in which they describe the outcome of 447 cholecystectomies using the critical view of safety (CVS) technique.[1] The authors are to be congratulated for performing a medium-sized series of cholecystectomies for acute pathology with no bile duct injuries or leaks. We, too, fully endorse the practice of careful dissection in the triangle of Calot and achievement of the CVS before clipping and dividing any tubular structures. However, rather than viewing the CVS as a replacement for routine intraoperative cholangiography (IOC), we feel that the two safety measures complement each other.

Sanjay and colleagues rightly argue that the large population-based studies often used to propagate routine IOC date from the pre-CVS era.[2,3] They continue to suggest that this protective effect is therefore not to be expected in modern surgical practice. In our point of view, this is an unlikely assertion.

The CVS has been standard practice in the Netherlands for several years. A recent nationwide survey by our group confirmed that 98% of the Dutch surgeons use this technique.[4] Nonetheless, common bile duct (CBD) injuries remain a substantial problem in the Netherlands with an incidence that is estimated to be higher than the 0.5% often quoted in literature.[5] Referrals to the largest tertiary referral center for bile duct injury (BDI) in the Netherlands show no decreasing trend in the course of the past decade.[6] In our own center, eight CBD injuries (1.9%) occurred between 2004 and 2006 despite the use of the CVS technique. In January 2007, routine IOC was implemented, and no CBD injuries were observed in the 3 years thereafter ($p=0.004$) (unpublished data).

IOC reduces the risk of BDI in several ways at different levels ranging from revealing which duct has been cannulated and demonstrating aberrant anatomy to increasing surgeon insight into the diversity of anatomical variations. These advantages cannot be replaced by CVS technique.

It may be argued that IOC could be performed selectively in case of uncertain anatomy. There are two arguments against this option. Firstly, it is unclear whether surgeons can reliably identify patients at higher risk for BDI. Secondly, the importance of IOC becoming a routine part of the procedure is that the whole team expects it, is ready for it, and can plan for it. Only then does it fit smoothly into the operative routine. Selectively performing IOC may lead to unfamiliarity with the technique and raise the threshold to perform it.

Sanjay et al. mention that opponents of IOC caution that it is a potentially hazardous procedure. However, there is virtually no evidence that IOC leads to complications rather than prevents them. The negligible amount of radiation received, too, is not a valid argument against its use in the adult population.[7]

Although we applaud the efforts of Sanjay et al. to further advocate the CVS technique as optimal surgical technique to prevent BDI, we feel that routine IOC should not be abandoned as an additional safety measure. Bile duct injury has serious, sometimes fatal, consequences.[8] It is a frequently performed "routine" operation and such complications are especially difficult for patients and surgeons to accept. We advocate, therefore, that both the CVS technique and routine IOC are used to complement each other for the safest way to remove the gallbladder.

K. T. Buddingh · V. B. Nieuwenhuijs (✉)
Division of Abdominal Surgery, Department of Surgery,
University Medical Centre Groningen,
Groningen, The Netherlands
e-mail: v.b.nieuwenhuijs@chir.umcg.nl

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LETTER TO THE EDITOR

'Critical View of Safety' (CVS) as an Alternative to Routine Intraoperative Cholangiography (IOC) During Laparoscopic Cholecystectomy for Acute Biliary Pathology

Pandanaboyana Sanjay · David J. Exon

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Dear Editor

We would like to thank Buddingh et al. for their interest and comments on our article. We adopted the policy of selective intraoperative cholangiography (IOC), with its use restricted to patients with increased risk of ductal stones. In our opinion, the indications for IOC are twofold—firstly, to clarify the anatomy on the Calot's triangle and, secondly, to exclude common bile duct (CBD) stones. In the presence of normal liver function tests and a non-dilated duct, the risk of harbouring CBD stones is extremely low, and a critical view of safety in the majority of the cases clarifies the anatomy, thereby excluding anatomical variations in the Calot's triangle. We do not advocate 'critical view of safety' (CVS) as an alternative to IOC in the presence of anatomical variations confirmed either on preoperative imaging or noted intraoperatively. The size of the current series precluded identification of such anatomical variations.

Advocates for routine IOC during laparoscopic cholecystectomy claim that IOC reduces biliary injury and allows for the diagnosis of asymptomatic choledocholithiasis. In order for IOC to satisfy these assertions, operators need to be able to both perform and correctly interpret the IOC which can be technical and interpretative.[1] In our experience, from a recent survey of surgeons in New Zealand (unpublished data), accuracy of identifying anatomical variations during IOC was poor irrespective of policy of IOC (routine versus selective). This raises questions regarding training in IOC and the appropriateness of routinely performing IOC. This is probably reflected in the current practice of majority of the surgeons both in the UK and New Zealand who practice a policy of selective IOC.[2]

We agree with Buddingh et al. that IOC is an important technique in the surgeons' armamentarium to minimise the risk of bile duct injury. Nevertheless, it carries the inherent risk of misinterpretation of anatomy and identification of asymptomatic choledocholithiasis necessitating further invasive investigations. We therefore recommend a selective policy of IOC in the presence of high risk of CBD stones or aberrant anatomy noted whilst obtaining CVS.

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P. Sanjay (✉) · D. J. Exon
Ninewells Hospital and Medical School,
Dundee, UK
e-mail: sanjay.pandanaboyana@nhs.net

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LETTER TO THE EDITOR

Equipoise of Outcome is Sufficient to Justify the Laparoscopic Approach for Colorectal Cancers

Parul J. Shukla · Jeffrey W. Milsom

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We read with interest the study on lymphadenectomy by laparoscopic resection in rectal cancer.[1] Over the past 10 years, laparoscopic surgery has been increasingly applied to resection of cancers. Level 1 evidence is now available providing strong support for similar oncological outcomes (both short-term surrogate end-points as well as long-term end-points like disease-free and overall survival) between laparoscopic surgery and open surgery for colon cancers.[2–5] The colorectal surgical community needs to be congratulated for producing this robust evidence, which now needs to be emulated for other GI sites also being approached by the laparoscopic technique (gastric, pancreatic, small intestine, etc.).

The present study reports a 51% adequate harvest of lymph nodes ($n \geq 12$, according to AJCC TNM staging requirements), which raises some concerns.[1] The authors have mentioned that their two groups were equally matched in terms of pre-operative radio-chemotherapy, but they need to report this proportion, in case that can explain the relatively low proportion of adequacy in number of nodes excised.

We have recently published our experience on long-term outcomes of patients undergoing curative laparoscopic surgery for mid and low rectal cancer, and we feel the excellent long-term survival (both overall and disease-free) was directly related to the adequacy of surgical resection (both total mesorectal excision and lymph nodal resection).[6]

Placing the above in context and recognizing the true current impact of laparoscopic surgery, i.e., superior patient satisfaction and short-term outcomes, equipoise of long-term outcomes is a sufficient argument *in favor* of the laparoscopic approach.

P. J. Shukla (✉) · J. W. Milsom
Weill Cornell Medical College
& New York Presbyterian Hospital,
New York, NY, USA
e-mail: pjshukla@doctors.org.uk

P. J. Shukla · J. W. Milsom
New York Presbyterian Hospital,
New York, NY, USA

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LETTER TO THE EDITOR

Response to JW Milsom et al. (Equipoise of Outcome is Sufficient to Justify the Laparoscopic Approach for Colorectal Cancers)

Oncological Outcomes Strongly Depend on the Quality of Lymphadenectomy in Rectal Cancer

Jean-Luc Faucheron

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Dear Sir:

I agree with the remarks made by JW Milsom et al. concerning our article recently published in *Journal of Gastrointestinal Surgery*[1]. Similar oncological outcomes are obtained by open and laparoscopic colonic resections for cancer, either for crude long-term survival or disease-free long-term survival.

We reported a 51% adequate harvest of lymph nodes (12 lymph nodes or more per patient following the American Joint Committee on Cancer recommendations), but nearly half of the patients received preoperative radiotherapy (48% and 49% of patients for the laparoscopic and the open group, respectively); moreover, about two third of them also received chemotherapy. JW Milsom and colleagues will find what they requested in Table 1. The number of patients receiving chemotherapy was not mentioned as type of regimen, duration of treatment, and dose of drugs were different from a patient to another. Indeed, chemoradiotherapy has been shown to downstage tumors, with more cases of NO stage.[2] That is probably why we recorded a rather low percentage of adequate harvest of nodes.

I congratulate JW Milsom et al. for their excellent article.[3] Concerning rectal cancer, the relationship between adequate lymphadenectomy and good long-term survival seems evident, and we participate to this demonstration as well.

But similar long-term outcome between open and laparoscopic approach might also be due to other factors. For instance, one of the two groups based on the surgical approach might have been associated with a higher rate of mortality, which could have been compensated by a better lymphadenectomy in the same group of patients. Oncological outcomes strongly depend on the quality of lymphadenectomy in rectal cancer.

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J.-L. Faucheron (✉)
Colorectal Unit, Department of Surgery, University Hospital,
BP 217, 38043, Grenoble Cedex, France
e-mail: JLFaucheron@chu-grenoble.fr