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FRONTIER

Endoscopic foregut surgery and interventions: The future is now. The state-of-the-art and my personal journey

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Abstract

In this paper, I reviewed the emerging field of endoscopic surgery and present data supporting the contention that endoscopy can now be used to treat many foregut diseases that have been traditionally treated surgically. Within each topic, the content will progress as follows: "lessons learned", "technical considerations" and "future opportunities". Lessons learned will provide a brief background and update on the most current literature. Technical considerations will include my personal experience, including tips and tricks that I have learned over the years. Finally, future opportunities will address current unmet needs and potential new areas of development. The foregut is defined as "the upper part of the embryonic alimentary canal from which the pharynx, esophagus, lung, stomach, liver, pancreas, and part of the duodenum develop". Foregut surgery is well established in treating conditions such as gastroesophageal reflux disease (GERD), achalasia, esophageal diverticula, Barrett's esophagus (BE) and esophageal cancer, stomach cancer, gastric-outlet obstruction, and obesity. Over the past decade, remarkable progress in interventional endoscopy has culminated in the conceptualization and practice of endoscopic foregut surgery for various clinical conditions summarized in this paper. Regarding GERD, there are now several technologies available to effectively treat it and potentially eliminate symptoms, and the need for long-term treatment with proton pump inhibitors. For the first time, fundoplication can be performed without the need for open or laparoscopic surgery. Long-term data going out 5-10 years are now emerging showing extended durability. In respect to achalasia, per-oral endoscopic myotomy (POEM) which was developed in Japan, has become an alternative to the traditional Heller's myotomy. Recent meta-analysis show that POEM may have better results than Heller, but the issue of post-POEM GERD still needs to be addressed. There is now a resurgence of endoscopic treatment of Zenker's diverticula with improved technique (Z-POEM) and equipment; thus, patients are choosing flexible endoscopic treatment as opposed to open or rigid

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endoscopy options. In regard to BE, endoscopic submucosal dissection (ESD) which is well established in Asia, is now becoming more mainstream in the West for the treatment of BE with high grade dysplasia, as well as early esophageal cancer. In combination with all the ablation technologies (radiofrequency ablation, cryotherapy, hybrid argon plasma coagulation), the entire spectrum of Barrett's and related dysplasia and early cancer can be managed predominantly by endoscopy.

Importantly, in regard to early gastric cancer and submucosal tumors (SMTs) of the stomach, ESD and full thickness resection (FTR) can excise these lesions *en-bloc* and endoscopic suturing is now used to close large defects and perforations. For treatment of patients with malignant gastric outlet obstruction (GOO), endoscopic gastro-jejunostomy is now showing better results than enteral stenting. G-POEM is also emerging as a treatment option for patients with gastroparesis. Obesity has become an epidemic in many western countries and is becoming also prevalent in Asia. Endoscopic sleeve gastroplasty (ESG) is now becoming an established treatment option, especially for obese patients with body mass index between 30 and 35. Data show an average weight loss of 16 kg after ESG with long-term data confirming sustainability. Finally, in respect to endo-hepatology, there are many new endoscopic interventions that have been developed for patients with liver disease. Endoscopic ultrasound (EUS)-guided liver biopsy and EUS-guided portal pressure measurement are exciting new frontiers for the endo-hepatologists.

Key words: Endoscopy; Foregut diseases; Gastroesophageal reflux disease; Endoscopic sleeve gastroplasty; Endoscopic submucosal dissection; Per-oral endoscopic myotomy; Endo-hepatology

Core tip: In this paper, we demonstrate how foregut diseases, such as gastroesophageal reflux disease, achalasia, Barrett's esophagus with dysplasia and early cancer of the esophagus and stomach, Zenker's diverticulum, obesity, gastric outlet obstruction, and gastroparesis, which have been traditionally treated surgically are now being diagnosed and treated endoscopically. For each section, I will review "lessons learned", then discuss some "technical considerations" and consider "future opportunities".

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BIOGRAPHY

Kenneth J Chang (Figure 1), MD, FACG, FASGE is Professor of Medicine and Chief, Division of Gastroenterology at the University of California, Irvine, School of Medicine. He is also Executive Director of the H.H. Chao Comprehensive Digestive Disease Center and Vincent and Anna Kong Endowed Chair in GI Endoscopic Oncology.

Dr. Chang received his MD degree from the Brown University Medical School, Providence, Rhode Island in 1985. He completed internship and residency in General Internal Medicine at the Rhode Island Hospital, Providence, Rhode Island in 1988, and an academic fellowship in gastroenterology and hepatology at the University of California, Irvine, 1988-1991. From 1991 to the present, he had academic appointments as an assistant, associate and full professor of medicine, respectively, at the University of California, Irvine, in the Department of Medicine; and as a Professor in Clinical Radiological Sciences, Department of Radiological Sciences. He is board certified in Medicine and Gastroenterology; is a Fellow of the American College of Gastroenterology and a Fellow of the American Society of Gastrointestinal Endoscopy. He served 2009-2015 as Governor of the American College of

Figure 1 Kenneth J Chang, MD, FACG, FASGE.

Gastroenterology, Southern California B, and served in various capacities in the American Society of Gastrointestinal Endoscopy (EUS Committee, Postgraduate Education Committee Member, Scientific Program Committee Member and Chair, and the Technology Assessment Committee Member). He was a Founding Member of the American Endosonography Club, and recently was invited to be a founding Board Member of the newly formed American Foregut Society.

Dr. Chang is internationally recognized for his advanced expertise in gastrointestinal endoscopy and new technologies. He developed and pioneered endoscopic ultrasound (EUS)-guided fine needle aspiration (FNA), which broke the critical technology barrier and enabled a world-wide application of this technique. He then developed novel techniques for EUS-guided fine needle injection (FNI), including delivery of anti-tumor agents, paving the way to interventional endoscopy and interventional EUS. More recently, he has made major contributions to in vivo identification and diagnosing of pancreatic cystic neoplasms with needle-based confocal laser endomicroscopy, and developed a new method of EUS-guided portal pressure gradient measurement in humans with a simple novel device. Dr. Chang's career passion has been to expand the use of endoscopy within the field of oncology. This has stemmed the entire spectrum of early and precise detection of cancer and pre-cancerous lesions in the esophagus and pancreas, accurate local staging of gastrointestinal (GI) malignancies, complete eradication of pre-cancerous Barrett's esophagus (BE), and the delivery of novel therapeutics. One of the most widely used endoscopic device world-wide is the Barrx90 radiofrequency (RF) device, which bears the name "Chang Cap". More recently, his focus has included the endoscopic treatment of conditions that predispose patients to cancer, namely gastroesophageal reflux disease (GERD) and obesity.

He has authored over 170 scientific papers, including papers in *The New England Journal of Medicine*, *Nature Genetics*, *Gastroenterology*, *Nature Clinical Practice Gastroenterology and Hepatology*, *Cancer*, *Endoscopy*, *American Journal of Gastroenterology*, *Gastrointestinal Endoscopy*, *Clinical Cancer Research*, *Digestive Diseases and Sciences*, *Digestive Endoscopy*, *Gastrointestinal Endoscopic Clinics of North America*, *Journal of Clinical Imaging Science*, *Journal of Clinical Oncology*, *Journal of Gastroenterology and Hepatology*, *Journal of Physiology and Pharmacology*, *Journal of Hepatobiliary and Pancreatic Science*, *Journal of Hepatobiliary Pancreatic Surgery*, *Annals of Surgery*, *Annals of Surgical Oncology*, *Pancreas*, *United European Gastroenterology Journal*, *World Journal of Gastroenterology* and others. He authored 3 books, 3 CD-ROM teaching programs distributed world-wide, 200 abstracts and had over 480 presentations at local and national meetings (DDW/AGA/ASGE/ACG) including numerous papers at plenary sessions. He has 5 United States patents: (1) Methods of Using Nitroxides in Conjunction with Photosensitizers and Sonosenitizers; (2) Fluoroscopy-Free Guide Wire System and Methods; (3) Percutaneous Transgastric Gastroplication and Transgastric Minimally Invasive Surgery; (4) Ring Magnets for Surgical Procedures; and (5) Hood Method and Device for Endoscopic Submucosal Dissection. Dr. Chang was the key-note speaker at prestigious international meetings in Australia, Austria, Brazil, China, France, Hong Kong, Hungary, India, Japan, South Korea, Taiwan, Thailand and the United European Gastroenterology Week meetings. He received numerous awards including American College of Gastroenterology Senior Governor Award and the National Cancer Institute Clinician award. He has consistently been included in Best Doctors in America, which recognizes the top 5% of physicians in the country. He has mentored 42 Advanced Endoscopy Fellows, of whom 23 currently

hold prominent academic positions in the United States, and 11 are respected faculty in Japan, South Korea, and Australia.

INTRODUCTION

In this paper, I reviewed the emerging field of endoscopic surgery and present data supporting the contention that endoscopy can now be used to treat many foregut diseases that have been traditionally treated surgically. Within each topic, the content will progress as follows: "lessons learned", "technical considerations" and "future opportunities". Lessons learned will provide a brief background and update on the most current literature. Technical considerations discuss procedural issues including my personal experience - tips and tricks that I have learned over the years. Finally, future opportunities will address current unmet needs and potential new areas of development.

The foregut is defined as "the upper part of the embryonic alimentary canal from which the pharynx, esophagus, lung, stomach, liver, pancreas, and part of the duodenum develop". Foregut surgery is well established in treating conditions such as gastroesophageal reflux disease (GERD), achalasia, esophageal diverticula, Barrett's esophagus (BE) and esophageal cancer, stomach cancer, gastric-outlet obstruction, and obesity. Over the past decade, remarkable progress in interventional endoscopy has culminated in the conceptualization and practice of endoscopic foregut surgery for various clinical conditions summarized in this paper.

GERD

Lessons learned

GERD is the most prevalent gastrointestinal (GI) disorder in the United States[1] and the extent of anatomical alterations underlying the mechanism of GERD can be viewed as a spectrum from normal to a single anatomic alteration [*e.g.*, weak lower esophageal sphincter (LES)] to multiple anatomic alterations such as weak LES, open diaphragmatic hiatus, hiatal hernia (Figure 2)[2,3]. The degree of anatomical alterations also appear to correlate with the complications of GERD, namely degree of esophagitis, the presence of Barrett's metaplasia[4], dysplasia and its progression to esophageal adenocarcinoma. Thus, as GERD is a spectrum disorder, then treatment should be individualized to the anatomic alterations of each patient. While medical and surgical therapy have been the mainstay of treatment for GERD, there are currently several Food and Drug Administration (FDA)-approved devices available for endoscopic treatment of GERD, thus filling in the therapeutic gap between medications and surgery. Endoscopic treatment options are now considered appropriate treatment in patients early in the GERD spectrum.

The first group in the spectrum would include patients with a normal LES tone, no hiatal hernia, and a closed diaphragmatic hiatus. This has been called "Dynamic Failure" (Hill Grade I). These patients have the phenotype of daytime reflux, no esophagitis or Barrett's, and on ambulatory pH monitoring will have predominantly upright reflux. The main mechanism for GERD in these patients is inappropriate transient LES relaxation (tLESRs). The major stimulus for tLESRs is distension of the proximal stomach[3]. Interventions that decrease the distensibility of the proximal stomach have been shown to decrease tLESRs. This is one of the predominant mechanisms for endoscopic radiofrequency (RF) treatment for GERD (Stretta; Figure 3)[5]. With this device, RF energy is delivered endoscopically to the muscle of the LES and the gastric cardia. The hypothesis that Stretta alters esophageal gastric junction (EGJ) resistance dynamically was established in a double-blind randomized crossover study of Stretta and sham treatment in 22 patients with GERD[6]. Stretta decreased EGJ compliance, while administration of sildenafil normalized EGJ compliance back to pre-Stretta level, arguing against EGJ fibrosis as the underlying mechanism. The authors concluded that decreased EGJ compliance, which reflects altered LES neuromuscular function, may contribute to symptomatic benefit by decreasing refluxate volume. A number of clinical trials have confirmed that Stretta effectively improves GERD symptoms and reduces, but does not normalize esophageal acid exposure[7,8]. There have also been several meta-analysis papers similarly showing that Stretta is effective for symptom relief, is safe and well tolerated, and allows patients to decrease intake of proton pump inhibitors (PPI) medications[9,10]. Interestingly, the durability of Stretta's effects may reach beyond 10 years[11,12]. Some of the Stretta related issues and pathophysiology were elegantly elaborated upon by Triadafilopoulos[13].

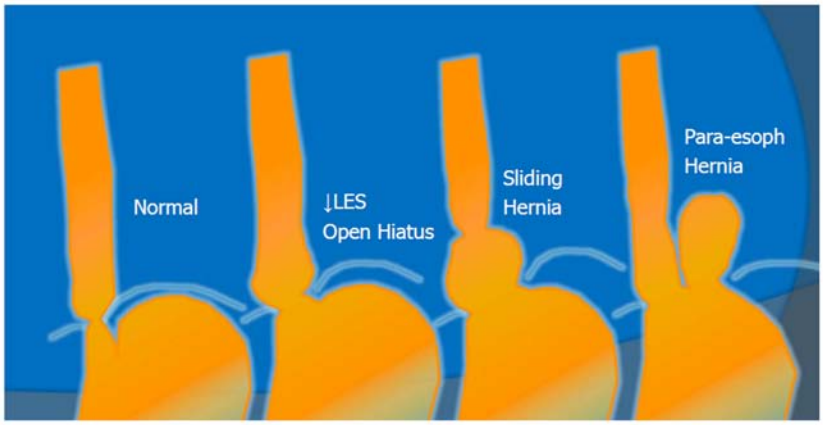

Figure 2 Anatomic spectrum of gastroesophageal reflux disease. LES: Lower esophageal sphincter.

Lessons learned for Stretta

I have been performing Stretta procedures for over 15 years. In my opinion, it is the least invasive procedure for GERD, takes about 25 min to perform, and if performed on the appropriate patient, has high effectiveness in symptom relief with the majority of patients able to come off PPIs. The main caveat is that the patients in this first group (dynamic failure) are the most suitable candidates for Stretta. These are the upright refluxers with minimal esophagitis (non-erosive reflux disease, or NERD).

Technical considerations for Stretta

The procedural goal of Stretta is to precisely deliver RF energy to the LES and the cardia. Other than placement, practically every other parameter is automated and self-regulated - including needle electrode temperature, mucosal temperature, 60 second application, and impedance. After the first 2 levels in the esophagus, I routinely re-insert the endoscope to see if the RF marks are properly placed starting at 1 cm proximal to the EGJ. If the marks are too distal, I will adjust the next 2 levels accordingly. However, it is my strong belief that the most important RF energy delivery is to the cardia. The 2 treatment levels at the cardia are done with the balloon inflated in the stomach and then the catheter is gently pulled back so the balloon fits snug up against the cardia. During the 3 applications at each level in the cardia, I find it useful to unhook the suction from the device, allowing the balloon to slide freely into position. The suction is applied just prior to advancing the needle electrodes. At the end of a successful procedure, a retroflexed view of the EGJ should demonstrate a cluster of RF marks around the cardia, creating a "swollen lip" appearance, with little to no "stray" RF marks (Figure 4A and B).

Lessons learned for TIF

The second group of the spectrum (Figure 2) would include patients with a low LES basal pressure, with no hiatal hernia, but the presence of a loose diaphragmatic hiatus (Hill Grade II). These patients have the phenotype of both daytime and nighttime reflux, with possible grade A or B esophagitis, and on ambulatory pH monitoring will have both upright and supine reflux. The mechanisms for GERD in these patients are an incompetent LES and/or an open diaphragmatic hiatus. These patients would be ideally suited for trans-oral incisionless fundoplication (TIF), using the Esophyx device (Figure 5). There have been 3 recent randomized control clinical trials using TIF 2.0, which employs the most advanced technique, similar to laparoscopic anti-reflux surgery. The first, known as the TEMPO trial, consisted of 63 patients randomized to TIF (40 patients) *vs* high dose PPI (23 patients)[14]. The primary outcome was elimination of daily troublesome regurgitation or extraesophageal symptoms. Secondary outcomes were normalization of esophageal acid exposure, PPI usage, and healing of esophagitis. At the 6-mo follow-up, troublesome regurgitation was eliminated in 97% of TIF patients versus 50% of PPI patients (RR = 1.9, 95%CI, P = 0.006). Globally, 62% of TIF patients experienced elimination of regurgitation and extraesophageal symptoms versus 5% of PPI patients (RR = 12.9, CI: 1.9-88.9, P = 0.009). Esophageal acid exposure was normalized in 54% of TIF patients versus 52% of PPI patients (RR = 1.0, 95%CI: 0.6-1.7, P = 0.914). 90% of TIF patients were off PPIs. The authors concluded that at the 6-mo follow-up, TIF was more effective than maximum standard dose PPI therapy in eliminating troublesome regurgitation and extraesophageal symptoms of GERD. The second clinical trial studying TIF 2.0 against

Figure 3 Stretta radiofrequency catheter.

PPIs was the RESPECT trial[15], which was a prospective, sham-controlled trial to determine if TIF reduced troublesome regurgitation to a greater extent than PPIs in patients with GERD. 696 patients with troublesome regurgitation despite daily PPI with 3 validated GERD-specific symptom scales, on and off PPIs, were initially screened. 87 patients with GERD and hiatal hernias ≤ 2 cm were randomly assigned to groups that underwent TIF and then received 6 mo of placebo, or sham surgery and 6 mo of once- or twice-daily omeprazole (controls, n = 42). Patients were blinded to therapy during the follow-up period and reassessed at 2, 12, and 26 wk. At 6 mo, patients underwent 48-h esophageal pH monitoring and esophagoduodenoscopy. By intention-to-treat analysis, TIF eliminated troublesome regurgitation in a larger proportion of patients (67%) than PPIs (45%) (P = 0.023). A larger proportion of controls had no response at 3 mo (36%) than patients who received TIF (11%) (P = 0.004). Control of esophageal pH improved after TIF (mean 9.3% before and 6.3% after; P < 0.001), but not after sham surgery (mean 8.6% before and 8.9% after). Patients from both groups who completed the protocol had similar reduction in GERD symptom scores. The authors concluded that TIF was an effective treatment for patients with GERD symptoms, particularly in those with persistent regurgitation despite PPI therapy, based on evaluation 6 mo after the procedure.

The third clinical trial performed in a European study[16] was a double-blind sham-controlled study in GERD patients who were chronic PPI users. Forty-four patients were randomized equally to 22 patients in each group. The primary effectiveness endpoint was the proportion of patients in clinical remission after 6-mo follow-up. Secondary outcomes were: PPI consumption, esophageal acid exposure, reduction in Quality of Life in Reflux and Dyspepsia and Gastrointestinal Symptom Rating Scale scores and healing of reflux esophagitis. Results showed that the time in remission after TIF procedure (197 d) was significantly longer compared to those submitted to the sham intervention (107 d), P < 0.001. After 6 mo, 13/22 (59%) of the chronic GERD patients remained in clinical remission after TIF. A recent meta-analysis[17] was conducted using data only from these 3 randomized studies that assessed the TIF 2.0 procedure compared to a control. The purpose of the meta-analysis was to determine the efficacy and long-term outcomes associated with performance of the TIF 2.0 procedure in patients with chronic long-term refractory GERD on optimized PPI therapy, including esophageal pH, PPI utilization and quality of life. Results from this meta-analysis, including data from 233 patients, demonstrated that TIF subjects at 3 years had improved esophageal pH, a decrease in PPI utilization, and improved quality of life. Recent publications are also showing favorable durability with long-term outcomes at 5 years[18,19] and even preliminary data at 10 years[20]. The FDA has recently expanded the device label to include concomitant laparoscopic hernia repair with TIF in patients with hiatal hernias greater than 2 cm in height.

Thus, with both Stretta and TIF showing level 1 data with durability, being FDA approved and commercially available, with category 1 CPT codes and reimbursement by many payors, endoscopic foregut surgery for GERD patients is now a reality. With the emergence of effective endoscopic treatment for GERD, and the simultaneous public concern over long-term PPI use, we have seen a dramatic growth in our GERD referrals. To meet this growing demand, we recently established a Comprehensive Heartburn Center at the University of California Irvine Medical Center, which allows patients easy access to medical, endoscopic and surgical options under a one-stop-

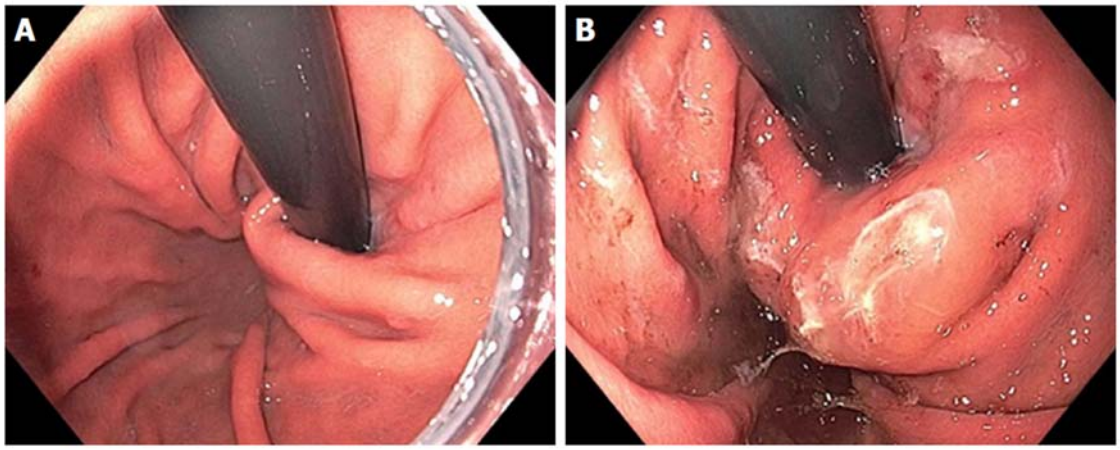

Figure 4 Endoscopic retroflex image of esophageal gastric junction prior to Stretta (A) and after Stretta treatment (B). The esophageal gastric junction and cardia have a typical "swollen lip" appearance.

shop individualized approach to patients with GERD.

Technical considerations for TIF

The EsophyX™ device (Figure 5) is composed of the following: a handle with controls; an 18 mm diameter frame through which control channels can run and a standard front-view 9 mm diameter endoscope can be introduced; the tissue invaginator, which consists of side holes stationed on the distal part of the frame, and to which external suction can be applied; the tissue mold, which pushes tissue against the shaft of the device; a helical screw, which is advanced into the tissue and allows for the retraction of the tissue between the tissue mold and the shaft; two stylets, which puncture the plicated tissue and tissue mold, and over which polypropylene H-shaped fasteners can be deployed; and a cartridge, which holds 20 fasteners. In the TIF2 technique, the device is introduced over the endoscope and into the stomach, and CO_2 is used to insufflate the gastric cavity (Figure 6A-G). The endoscope is positioned in retroflexion, and the lesser curve and the greater curve are located at 12 and 6 o'clock positions, respectively. The tissue mold is retroflexed, closed against the device, rotated to 11 (posterior, Figure 6D), and withdrawn such that the tip may be located at the EGJ. Once this is accomplished, the helical screw is advanced to engage tissue just below the squamocolumnar junction. Traction is then applied to allow the gastric cardia and distal esophagus to slide downward into the tissue mold. Plication is then achieved by deploying multiple H-shaped fasteners while rotating the tissue mold such that it slides the stomach over the esophagus. This results in a circumferential tightening of the newly-created valve ≥ 270 degrees. Twenty fasteners over ten plications are necessary to construct an adequate gastroesophageal valve (3 plications in each of the posterior and anterior corners, and 4 plications on the greater curve). The mechanism of action of the TIF procedure in many ways mirrors that of the Laparoscopic Anti-reflux surgery Nissen (LARS)[21]. One paper, published by Rinsma *et al*[22] characterizes such mechanisms. In their study involving fifteen patients, they performed 90-min postprandial combined with high-resolution manometry and impedance-pH monitoring followed by an ambulatory 24-h pH-impedance monitoring. EGJ distensibility was evaluated using an endoscopic functional luminal imaging probe using the endoscopic functional lumen imaging probe (EndoFLIP) before and directly after the procedures. The patients were followed for 6 mo. With regards to the stationary esophageal manometry and impedance-pH monitoring performed directly after the procedure, TIF resulted in a marked reduction of both the number of transient LES relaxation (tLESRs) (16.8 ± 1.5 *vs* 9.2 ± 1.3; $P < 0.01$) and the number of tLESRs associated with liquid-containing reflux after the procedure (from 11.1 ± 1.6 *vs* 5.6 ± 0.6; $P < 0.01$). TIF also led to a decrease in the number and proximal extent of reflux episodes and an improvement of acid exposure in the upright position; conversely, TIF had no effect on the number of gas reflux episodes, corroborating the low incidence of post-TIF gas-bloat symptoms. EGJ distensibility was reduced after the procedure (2.4 ± 0.3 mm^2/mmHg *vs* 1.6 ± 0.2 mm^2/mmHg; $P < 0.05$). Also of note, the basal LES pressure in the fasted state was increased after TIF (from 13.9 ± 1.0 to 20.5 ± 1.8 mmHg; $P < 0.01$). Thus, TIF reduces EGJ distensibility, thereby decreasing tLESRs, which is the main mechanism for upright refluxers. It also creates a 3-cm high pressure zone at the distal esophagus in the configuration of a flap

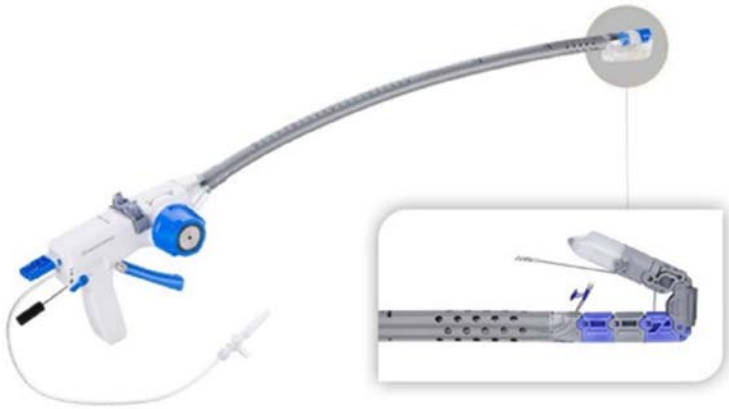

Figure 5 Esophyx® Z device used for transoral incisionless fundoplication.

valve, which should decrease both upright and supine reflux. However, since it is a 270° partial fundoplication, and the flap valve luminal diameter is controlled by the diameter of the device (prevents over-tightening), gas can still escape from the stomach into the esophagus, minimizing the side-effect of gas-bloat.

Future opportunities for endoscopic treatment of GERD

We are now exploring the role of TIF among patients with BE - including non-dysplastic Barrett's, and those patients with previous history of Barrett's dysplasia, who have now reached complete remission of intestinal metaplasia (CRIM) by endoscopic resection and/or ablation, but are destined to life-long PPIs. In addition, TIF after per-oral endoscopic myotomy (POEM) is a very exciting area of exploration - as the benefits of POEM over laparoscopic Heller myotomy (LHM) with partial fundoplication for patients with achalasia may be outweighed by the incidence of post-POEM GERD. If, however, post-POEM GERD can be controlled either by PPI or TIF, then it would tip the balance strongly to POEM as the procedure of choice for achalasia patients. This will be discussed more extensively in the next section.

We are also developing new techniques and technologies for endoscopic treatment of GERD among patients with altered anatomy (esophagectomy, sleeve gastrectomy, gastric bypass, failed Nissen fundoplication) using endoscopic suturing alone (Apollo Overstitch) and in combination with mucosal ablation and endoscopic resection. We reported the first case series of 10 patients who underwent endoscopic augmentation of the EGJ using the Apollo OverStitch endoscopic suturing system in patients with GERD[23]. Using a double-channel gastroscope affixed to the endoscopic suturing platform, interrupted (individual) sutures were placed on the gastric side of the EGJ in 2 layers in order to create a narrowed and elongated EGJ (Figure 7A-F). Technical success was achieved in all patients, including those with a history of previous anti-reflux procedures (n = 7) and those with a hiatal hernia (n = 6). Patients with prior esophagectomy and sleeve gastrectomy were also included. The median pre-procedure GERD-Health Related Quality of Life Questionnaire improved from 20 (range: 11-45) to a post-procedure score of 6 (range: 3-25) (P = 0.001). The median duration of GERD symptom improvement after the procedure, however, was only 1 month (range: 0.5-4). Adverse events were limited to one patient who developed self-limited nausea and vomiting. From this preliminary pilot study, we concluded that the technique using a commercially available device for suturing to create a gastro-gastric plication was feasible and appeared safe, especially among patients with altered anatomy which would have precluded all other available surgical or endoscopic options. However, it became apparent that the durability was inadequate. The sutures were not predictably placed through the muscularis propria (MP) and over time the sutures would easily cut through the mucosa and cause loosening of the plication. In addition, since this was a mucosa to mucosa approximation, adhesion would not be expected to occur, thus leading to a shortened durability. A potential solution was to ablate the mucosa with argon plasma coagulation (APC), a technique we use for gastric bypass revisions where we ablate the gastric mucosa prior to placing a purse-string suture around the pouch anastomosis to narrow the outlet. We termed this technique mucosal ablation and suturing of the EGJ (MASE), and conducted a pilot trial in 27 patients with a typical procedural length of 25 min[24] (Figure 8A-F). The mean follow-up time was 124 days. The indications for the procedure included either poorly controlled symptoms (48%) or a desire to

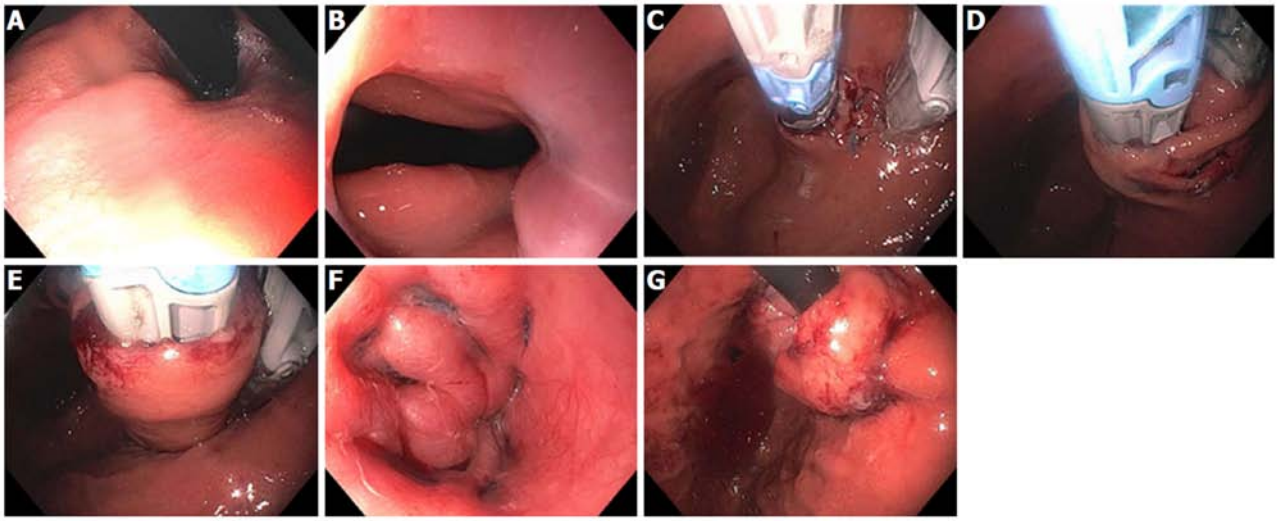

Figure 6 Transoral incisionless fundoplication 2 technique. A: Baseline Hill Grade 2 Valve; B: Baseline patulous lower esophageal sphincter; C: Building short lip valve along lesser curve at 12 o'clock; D: Rotating the device clockwise to create a partial fundoplication on the posterior side; E: Rotating the device counter-clockwise to create a partial fundoplication on the anterior side; F: After placement of 36 fasteners, a 3-4 cm length flap valve is created; G: Fasteners can be seen extending 3-4 cm in the distal esophagus.

discontinue their medication (52%). Seven patients (26%) had altered anatomy from prior surgery: Fundoplication (n = 4), Billroth II (n = 1), Roux-en-Y (n = 1), and Sleeve gastrectomy (n = 1). Pre-procedure, 22 patients (82%) were on once or twice daily PPI therapy and 5 patients were on H2-receptor antagonists/topical antacids. Of the 22 patients on daily PPI, 13 patients (59%) were able to discontinue their medication, and 3 patients (14%) were able to reduce their dose. Of the 7 patients with altered anatomy, 4 patients (57%) were able to discontinue or reduce their PPI after the procedure. With regards to tolerance, the most common side effect was self-limited epigastric pain post-procedure (22%). One patient required an overnight stay in hospital for intravenous pain control. There were no other early or late complications. Our experience thus far suggests that mucosal ablation prior to suturing helps to increase its durability. The other variation of this method is to perform endoscopic mucosal resection (EMR) prior to suturing. EMR or endoscopic submucosal dissection (ESD) has been shown in a small series to create scarring at the EGJ, which then decreases EGJ distensibility and improve reflux. This has been named anti-reflux mucosectomy (ARM) and in a small series of ten patients showed improvement of esophageal acid exposure[25]. Combining EMR and suturing was the next step and this was done in a series of 10 patients with GERD[26] with over-all improvement of GERD symptoms and 8 of 10 patients were able to stop their PPI medications. This is called the resection and plication (RAP) procedure. We recently performed a combination gastric-bypass revision plus RAP in an obese patient who gained weight after previous gastric-bypass and also had GERD, which was only partially responsive to PPI (Figure 9A-D). We are currently performing both the MASE and RAP procedures to examine the clinical benefits and nuances of each procedure. Since both can be done in an antegrade fashion, and may be the only option for GERD patients with altered anatomy, I anticipate that both of these techniques or a variation thereof, will be helpful in these special situations. They also may become reasonable options in patients who are in group 1 of the GERD spectrum, i.e. the upright-predominant phenotype without significant esophagitis.

ACHALASIA - POEM

Lessons learned

The LHM has been the standard of care for definitive treatment of achalasia for many years[27,28]. POEM was first described by Inoue in 2010[29] and is now considered the procedure of choice for treating achalasia in most tertiary centers[30-35]. POEM has also been shown to be an effective rescue procedure in patients who have previously failed LHM[30,36,37]. Recent meta-analysis show that POEM may have better results than LHM[38-41], but the issue of post-POEM GERD being higher than post-LHM still needs to be addressed[42]. We need to keep in mind that LHM alone has a incidence of post-operative GERD of approximately 50%, while LHM in combination with a partial

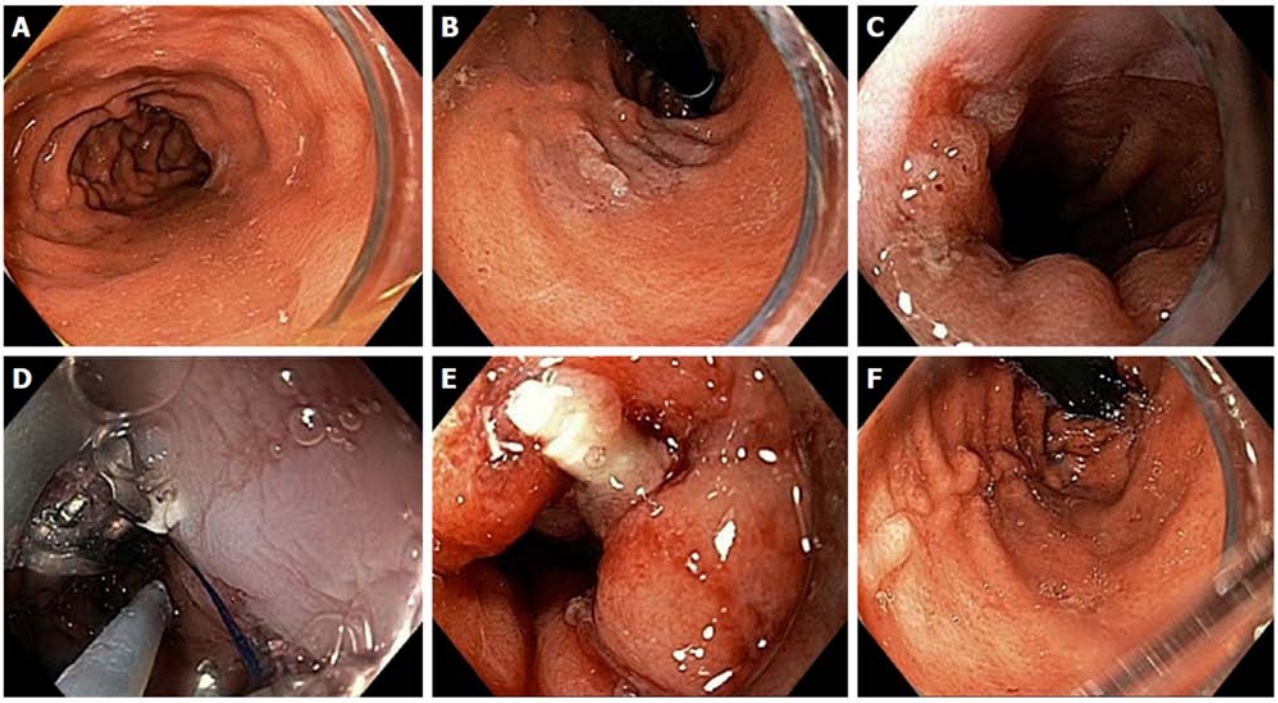

Figure 7 Endoscopic Suturing for gastroesophageal reflux disease in a patient post-Sleeve gastrectomy. A: Narrowed gastric body post gastrectomy; B: Retroflex view of loose esophageal gastric junction (EGJ); C: Patulous lower esophageal sphincter (LES); D: Suturing at EGJ; E: Post-suturing with tightened LES; F: Retroflex view of tightened EGJ post-suturing.

fundoplication reduces post-operative GERD to approximately 10%[43]. Therefore, most surgeons will automatically perform both operations together. If a substantial number of patients require anti-reflux surgery after POEM, then it could tip the balance back towards LHM plus partial fundoplication as the preferred first line option. Fortunately, there may be an endoscopic solution to post-POEM GERD - namely the TIF procedure[44]. In our experience of over 60 consecutive POEM procedures, only 3 patients were refractory to PPI medications and the TIF procedure was able to control GERD symptoms and esophagitis in all 3 patients. Further studies examining both efficacy and durability of TIF post POEM are underway. The other consideration between POEM versus LHM plus fundoplication is - while the durability of the myotomy (with both POEM and LHM) should be very long, perhaps several decades, the durability of the partial fundoplication may be more limited, probably less than 10 years. At the point of fundoplication loosening, these patients would require either chronic PPI, a revision fundoplication, or the TIF procedure. Ideally, a POEM with possible TIF among those patients refractory to PPI will prove effective, as a repeat TIF is much easier to perform than the revision of a fundoplication.

The POEM procedure has also now extended to other motility disorders such as Jackhammer esophagus[45-48] and distal esophageal spasm[49,50], which are categorized under spastic esophageal disorders (SED)[51].

Technical considerations

We are currently performing, on average 4-6 POEM procedures per month. On average, it takes 45-90 min to perform the procedure. We also include physiologic compliance measurements at the EGJ (EndoFlip) immediately pre- and post-POEM procedure. We also routinely perform a pre-procedure timed barium esophagram, and repeat it 4-6 wk post procedure. We are currently examining whether the timed barium swallow and/or the EGJ physiologic measurements will predict both response to treatment of dysphagia (Eckart Score), as well as the risk of post-POEM GERD. Based on current literature and personal experience, our current protocol for type 1 achalasia (aperistalsis and failure of LES relaxation) and type 2 achalasia (aperistalsis, pan-esophageal pressurization and failure of LES relaxation) is to perform an 8 cm myotomy in the esophagus (5 cm circular only, then 3 cm full thickness) and only 1.5-2 cm in the stomach (full thickness) (Figure 10). This protocol seems to strike a good balance between maximal relief of dysphagia while minimizing GERD. For type 3 achalasia (aperistalsis, failure of LES relaxation, and simultaneous contractions in the esophageal body), a much longer myotomy is usually required, based on high

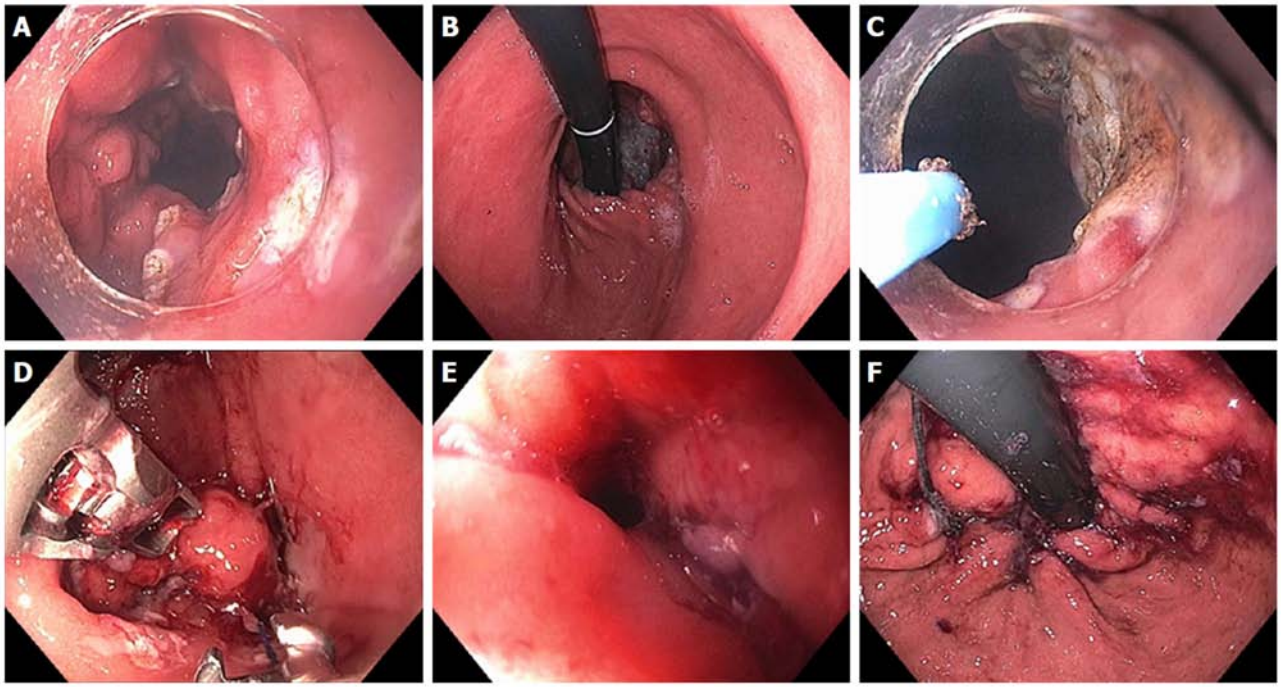

Figure 8 Mucosal ablation and suturing of the esophageal gastric junction in a patient with gastroesophageal reflux disease post-esophagectomy. A: Wide open anastomosis along with unchecked reflux; B: Retroflex view showing open anastomosis; C: Ablation of gastric mucosa at the anastomosis using argon plasma coagulation; D: Suturing at the anastomosis; E: Post-mucosal ablation and suturing of the esophageal gastric junction (MASE) tightening of anastomosis; F: Post-MASE retroflex view showing tightened anastomosis.

resolution esophageal manometry (MREM), barium esophagram, and endoscopic observance of spasm. We routinely use the Erbe i-knife for mucosal incision, tunneling, and circular myotomy (Figure 10A-E). However, for full thickness myotomy at the EGJ, we prefer using the Olympus stag beetle (SB) knife (Figure 10F and G)[52] as in my experience it decreases the rate of spontaneous bleeding (from vessels arising from the MP) and therefore is more expedient. I also use the SB knife for submucosal tunneling if I encounter large or abundant blood vessels in the submucosa (Figure 10H and I). We have published our initial experience using the SB knife for a small series of POEM cases[52].

As for closure of the submucosal tunnel, we have performed almost equal numbers of clip closure (Figure 10H and I) *vs* suturing (Figure 11C and D). The cost differential depends somewhat on the number of clips vs the number of sutures used, with a trend towards lower cost for using clips[53]. The best configuration for suturing is to perform the initial mucosal incision in a horizontal direction (Figure 11B-D). However, the best configuration for clipping is a vertical incision. The issue with a vertical incision is that traction of the scope pushing inside the tunnel can actual cause unintended elongation of the mucosotomy. This may result is difficulty maintaining CO_2 within the tunnel for optimal visualization as well a longer closure process. We have found that a horizontal incision actually is optimal for either closure method, while decreasing the risk of elongating the mucosotomy. The best technique for clipping in this scenario is to identify the most distal midpoint of the incision (Figure 10H) and place the first clip 1mm distal to that point. This creates an elevated crease and all subsequent clips can be placed adjacently (Figure 10I). Confirmation of adequate extension into the stomach can sometimes be challenging, and mere fluid injection into the tunnel with subsequent endoscopic evaluation in retroflexed position in the stomach may not be adequate. In our center, we are routinely placing a slim scope (4.9 mm) alongside the gastroscope and advancing into the stomach in retroflexed position. Once in the stomach, the light can be turned off on the slim scope and the light from the standard gastroscope within the submucosal tunnel can be confirmed easily (Figure 10I).

Future opportunities

Technical and device refinements are still evolving with POEM. A single center, prospective randomized trial in 63 patients suggested no difference between an anterior versus a posterior myotomy[54], although larger studies with longer follow-up are needed to validate this. Similarly, partial full-thickness myotomy versus circular

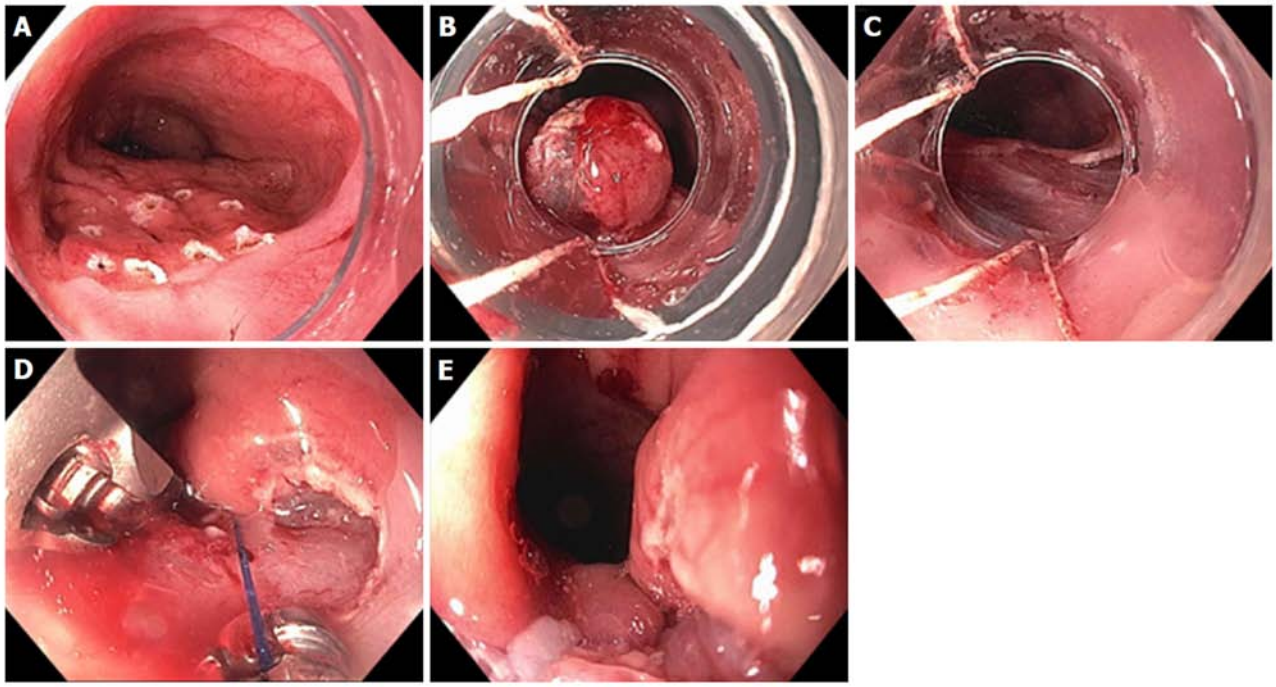

Figure 9 Resection and plication technique for gastroesophageal reflux disease in a patient after gastric by-pass. A: Endoscopic revision of anastomosis was performed followed by marking area for endoscopic mucosal resection (EMR) at the esophageal gastric junction (EGJ); B: EMR performed using band ligation technique; C: Exposure of muscularis propria accomplished; D: Sutures now deeply placed through muscle layer; E: Post-resection and plication appearance of tightened EGJ.

myotomy only has been compared in a small single center study and appears not to affect clinical outcomes, although the a partial full-thickness myotomy took less time[55]. The extent of myotomy in the proximal stomach is also being evaluated, with a tendency towards 1.5-2 cm as compared to the initial 3 cm. The issue of post-POEM GERD has already been addressed, and this will continue to fuel both technique refinements to minimize GERD as well as endoscopic solutions, such as TIF, in cases that require further anti-reflux intervention.

ZENKER'S DIVERTICULUM - Z-POEM

Lessons learned

Zenker's diverticulum has historically been treated by head and neck surgeons using either an open or rigid endoscopic approach with a stapler. A recent meta-analysis compared open to rigid endoscopic stapling approaches and found that failure of open and endoscopic approaches was 4.2% and 18.4%, respectively, and corresponding complication rates were 11% and 7%[56]. Similarly, another meta-analysis concluded that compared with the open surgical approach, the rigid endoscopic treatment appeared to result in a shorter length of procedure and hospitalization, earlier diet introduction, and lower rates of complications, but in higher rates of symptom recurrence[57]. Flexible endoscopy, largely in the hands of interventional gastroenterologists has emerged as a viable alternative for open or rigid endoscopic approaches. A recent meta-analysis which included twenty studies with a total of 813 patients showed that the pooled success, adverse events, and recurrence rates were 91%, 11.3%, and 11%, respectively[58]. A more recent meta-analysis examining thirteen studies including 589 patients showed a response rate, overall complication, bleeding and perforation of 88%, 13%, 5% and 7%, respectively[59]. However, the pooled data still demonstrated an overall recurrence rate of 14% (95%CI: 9%-21%). Diverticulum size of ≥ 4 cm and < 4 cm demonstrated pooled adverse event rates of 17% and 7%, respectively. Starting with a basic needle knife, and progressing to more specialized knives (such as the hook knife)[60,61] and endoscopic scissors[62-66], have led to some improvement in clinical outcomes. The challenge is to perform a complete myotomy of the cricopharyngeus muscle, without increasing the risk of mediastinitis or perforation. The issue surrounds the fact that most of these techniques involve cutting the entire septum (mucosa and muscle) from proximal to distal with no precise visual cue as to when the myotomy is complete.

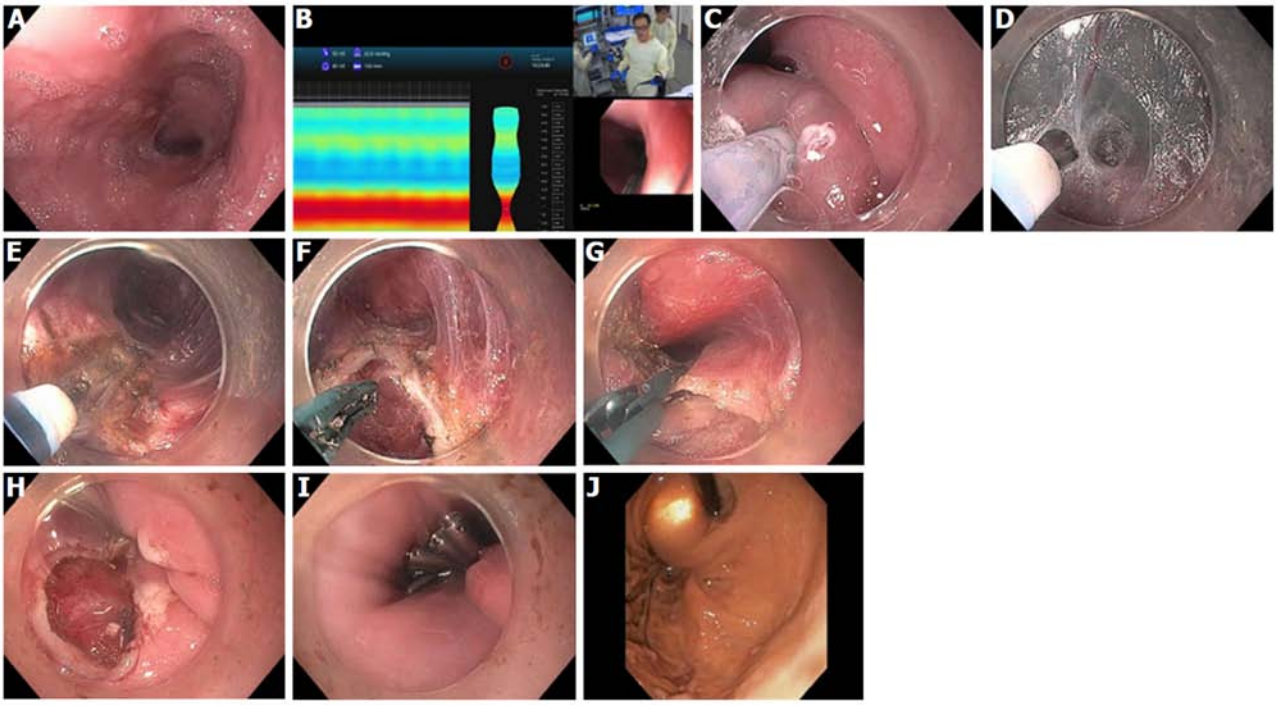

Figure 10 Per-oral endoscopic myotomy procedure on 40-year-old female with type 1 achalasia, s/p pneumatic dilation 20-years prior, now with dysphagia and chest pain/spasms (Eckardt Score 8). A: Dilated esophageal body; B: Endoscopic functional lumen imaging probe (Endo-Flip) 2.0 showing distensibility of 1.5; C: Initial mucosal incision; D: Submucosal tunnel with coagulation of vessel using Hybrid i-knife; E: Circular myotomy using Hybrid i-knife; F and G: Full thickness myotomy with scissor-type stag beetle (SB) knife; H and I: Closure of mucosotomy using 5 endoscopic clips; J: Thin scope retroflex view of esophageal gastric junction showing light from standard gastroscope within the per-oral endoscopic myotomy tunnel.

The most recent advances include various tunneling methods, similar to POEM, where the mucosal incision is either proximal or superficial to the myotomy[67-70].

Technical considerations

This Z-POEM technique allows for complete excision of the muscle, while minimizing the risk of mediastinitis or perforation due to the tunneling mechanism. We currently employ a modified tunneling approach, with mucosal incision at the apex of the septum (Figure 12B-D), followed by submucosal tunneling on both the esophageal and diverticular sides of the muscle (Figure 12E). This allows for optimal visualization, precise control of complete myotomy (Figure 12F), and closure of the short tunnel with mucosal clips (Figure 12G).

Future opportunities

With these technical and device improvements, flexible endoscopy may become the treatment of choice for most patients with Zenker's diverticulum. Further prospective trials are necessary to compare this most recent technique compared with standard flexible and rigid instrument techniques.

BE AND EARLY ESOPHAGEAL CANCER

Lessons learned

It was not that long ago that the standard of care for treating BE with high grade dysplasia (HGD) was esophagectomy[71-74]. Initial endoscopic approaches to treat Barrett's included photodynamic therapy (PDT), multipolar electrocoagulation (MPEC) and argon-plasma coagulation (APC). While these sparked initial enthusiasm, they were not able to achieve high rates of complete eradication while minimizing complications[75]. The emergence of RF ablation (RFA) completely changed the landscape and transformed our approach to Barrett's, with the expectation that Barrett's can be completely eliminated by endoscopic treatment in the vast majority of cases. I have been using RFA technology since 2004. At that time, there was only a 360-degree balloon device available for treatment. Given my experience with PDT (Figure 13) and using ND:YAG laser delivered through a sapphire contact probe (Figure 14A and B), I was able to "touch-up" any small areas

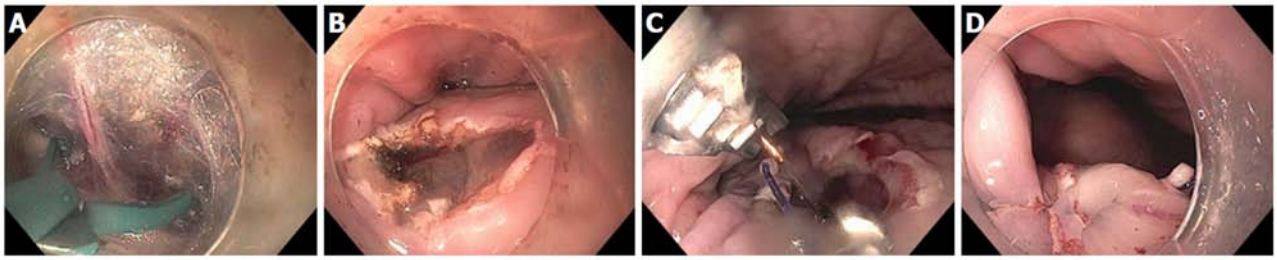

Figure 11 Per-oral endoscopic myotomy procedure on 43-year-old female with type 2 achalasia for 25 years, s/p prior failed Heller Myotomy and Botox injection. A: Abundant submucosal vessels coagulated with stag beetle knife; B: Mucosal incision made in horizontal orientation facilitates closure with endoscopic suturing; C: Double layer suturing, starting with initial bite just left of incision, then middle and right; D: Second running suture from right to left for completion of double layer closure.

of residual Barrett's post PDT. With this conceptual construct of devices for wide field treatment as well as devices for focal treatment, the Barrx Halo 90 device was conceived. My contributions were acknowledged by having the product "nick named" the Chang Cap (Figure 15). With the combination of the 360 device and the focal device (Figure 16), published clinical trials mounted to over 100 articles establishing the efficacy and safety of this technology with the over-all rate of CRIM of 77%, complete response of low grade dysplasia of 90%, and complete response of HGD of 81%[76]. In this seminal NEJM study, patients who received RFA had less disease progression (3.6% *vs* 16.3%, $P = 0.03$) and fewer cancers (1.2% *vs* 9.3%, $P = 0.045$). There are now multiple different RFA probes available for circumferential, focal, long and short segment, as well as through the scope applications (Figure 17).

More recently, cryotherapy has emerged as an alternative means of treating Barrett's. There are now two delivery systems available: a spray catheter[77-86] and a balloon based device[87-89]. While the number of published trials using cryotherapy currently pales in comparison to RFA, the potential theoretical advantages of cryotherapy may be better patient tolerance[80], slightly less stricture rate[90] and deeper penetration of cold energy[91] . Spray cryotherapy appears to be able to salvage approximately 50% of dysplastic Barrett's cases refractory to RFA[92] and provide a reasonable complete remission of dysplasia (CR-D) in 76% of naïve patients[77]. However, the CRIM of 46% in non-dysplastic BE is considerably lower than the established rate with RFA. Therefore, RFA still remains the standard of care for treatment of flat dysplastic Barrett's. The more recent development of a balloon-based cryotherapy, using nitrous oxide to cool the temperature within the balloon (Figure 18), is quite intriguing with preliminary data suggesting very respectable treatment and safety outcomes (CR-D of 95% and CRIM of 88%)[87].

The newest emerging ablation technology is argon plasma coagulation (APC) preceded by high pressure needleless submucosal injection of saline *via* a built-in water jet within the same catheter (called Hybrid APC). An initial *ex-vivo* study showed that submucosal saline injection decreased depth of coagulation by half[93]. This was followed by an initial series of 50 Barrett's patients in whom 78% achieved complete remission after a median of 3.5 APC sessions with a stricture rate of 2%[94]. We recently presented our initial Hybrid APC series of 17 patients with biopsy proven non-dysplastic BE (NDBE) 59%, low grade dysplasia 18% and HGD 24%[95]. Of these patients, 59% had undergone prior RFA, 18% prior EMR, 12% prior cryotherapy and 35% were naive. The average procedure time was 21 min. The pain scores were low (2.39 at day 1, and 0.42 on day 7) with only one treatment-related stricture (4.2%). Our protocol has been modified from the European reports, in that we utilize an EMR cap in order to provide stable visibility and precise focal distance for application of APC (Figure 19). After submucosal injection of saline (Figure 19B), APC is applied evenly to all visible Barrett's areas (Figure 19C and D). Then after removing the coagulated tissue using the ESD cap and washing (with scope water jet), this is followed by a second application of APC at a lower energy setting (Figure 19F and G). The final visual result should be a tan colored, dry appearance similar to that achieved after RFA. Long-term outcomes and larger studies are underway. Therefore, while RFA remains "king of the hill" as standard of care for flat dysplastic Barrett's, cryotherapy and Hybrid APC may eventually contend for a subset of Barrett's patients. I predict that spray cryotherapy may be useful in patients with nodular dysplasia or early cancer that are not suitable candidates for resection techniques (severe scarring, *etc.*), while balloon cryotherapy and Hybrid APC may be viable options for patients with short segment Barrett's and for focal "touch-up" treatments. Capital equipment and disposable accessory costs may become a deciding factor if outcomes are equivalent.

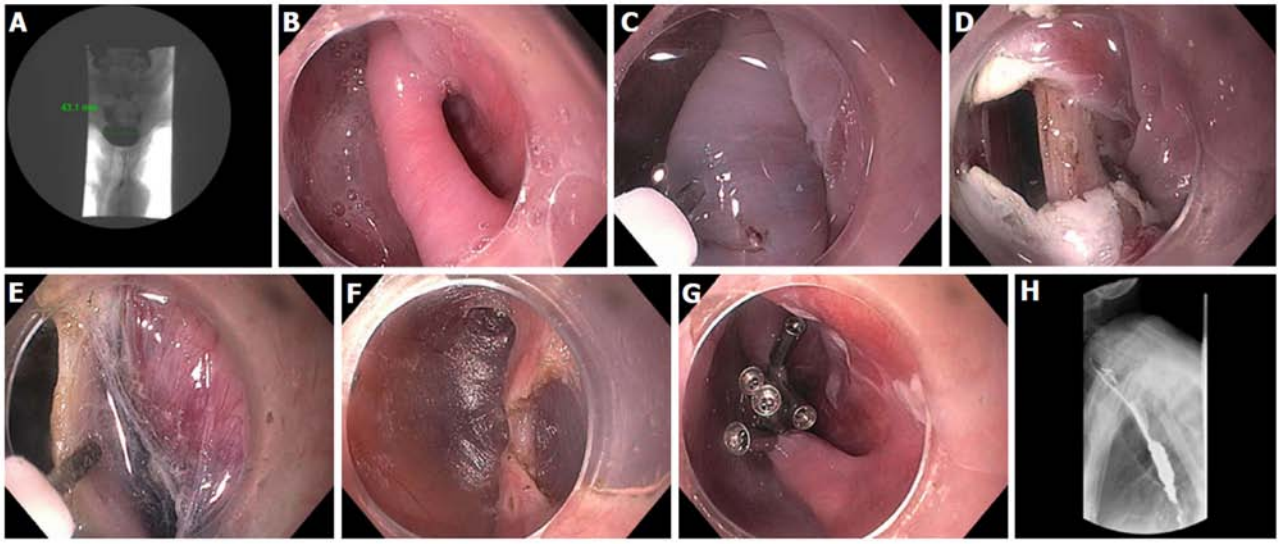

Figure 12 Zenker's - per-oral endoscopic myotomy procedure in a 91-year-old female with 4-cm Zenker's diverticulum and dysphagia, including solids, pills, and liquids. A: Pre- Zenker's - per-oral endoscopic myotomy procedure (Z-POEM) esophagram showing 4.3 cm Zenker's; B: Septum identified, with diverticulum on left, and esophageal lumen on right; C: Start of mucosal incision (horizontal) over the septum; D: Mucosal incision completed with exposure of cricopharyngeus muscle (CPM); E: Submucosal tunnel on each side of the CPM; F: Near completion of myotomy; G: Mucosal closure with 5 clips; H: Follow-up esophagram 2 mo post Z-POEM, contrast flows freely, and patient has complete resolution of dysphagia.

Any nodular lesion should be treated, if possible, by endoscopic resection. This is mostly accomplished with EMR, often requiring multiple piecemeal resections. Some drawbacks of piecemeal EMR include depth of resection and inability to adequately determine resection margins. Endoscopic Submucosal Dissection (ESD) while initially developed for gastric and then colonic lesions, is now being used more frequently for early esophageal cancer. The indications for ESD in esophageal squamous cancer include: HGD to well (G1) to moderately (G2) differentiated, Paris 0-II lesions, with suspected superficial invasion (m1-m2) with two thirds or less of the esophageal circumference involved[96]. The expanded indications include m3 or sm < 200 micron involvement, any size, with clinically N0 staging. While the role for ESD in early esophageal squamous cell carcinoma (ESCC) is well established, the role of ESD for esophageal adenocarcinoma (EAC) arising from BE has been less studied until recently. This can be attributed to the low incidence of Barrett's associated cancer in Asia and the relatively recent adoption of ESD by endoscopists in western countries. In a recent German study ESD was performed on 111 early esophageal cancers (87 EACs and 24 ESCCs)[97]. *En bloc* resection rates were 95.4% for EAC and 100% for ESCC (P = 0.575), and R0 resection rates were 83.9% and 91.7 %, respectively (P = 0.515). The R0 resection rate was higher in Barrett's ≤ M3 *vs* > M3 (90% *vs* 70.4%; P = 0.029). The curative resection rate was 72.4% for EAC *vs* 45.8% for ESCC (P = 0.026). Endoluminal recurrence was observed in 2.4% of EACs (8% in Barrett's > M3, 0%in Barrett's ≤ M3), and 0% of ESCCs. Complications included strictures (11.7%) and bleedings (0.9%), but no perforation. Disease-specific survival was 97.7% (EAC) and 95.8% (ESCC), and overall survival was 96.6% (EAC) and 66.7% (ESCC) with over 2-year follow-up. These results are similar to an Asian single center experience with 91 patients with Barrett's associated EAC[98].

Therefore, within the confines of these guiding principles, endoscopic treatment is now the preferred approach for both BE and early esophageal cancer. In our center, we have been gravitating more towards endoscopic resection - certainly for nodular Barrett's, but also for flat Barrett's where based on narrow-band imaging (NBI) mucosal pattern or advanced imaging (endomicroscopy or optical coherence tomography), there is suspicion for HGD or intramucosal carcinoma. For nodular lesions larger than 1.5 cm, we are routinely performing ESD (Figure 20) instead of EMR, with the caveat of avoiding circumferential resection.

Technical considerations

Considerations for ablation: The goal for ablation, irrespective of modality, is to eliminate all Barrett's epithelium, including buried Barrett's, while minimizing the risk of stricture and patient discomfort. Ablation in the proximal esophagus and length of treatment area seem to correlate with more patient discomfort during the healing phase. In my experience, the addition of post-procedure sucralfate (as an oral

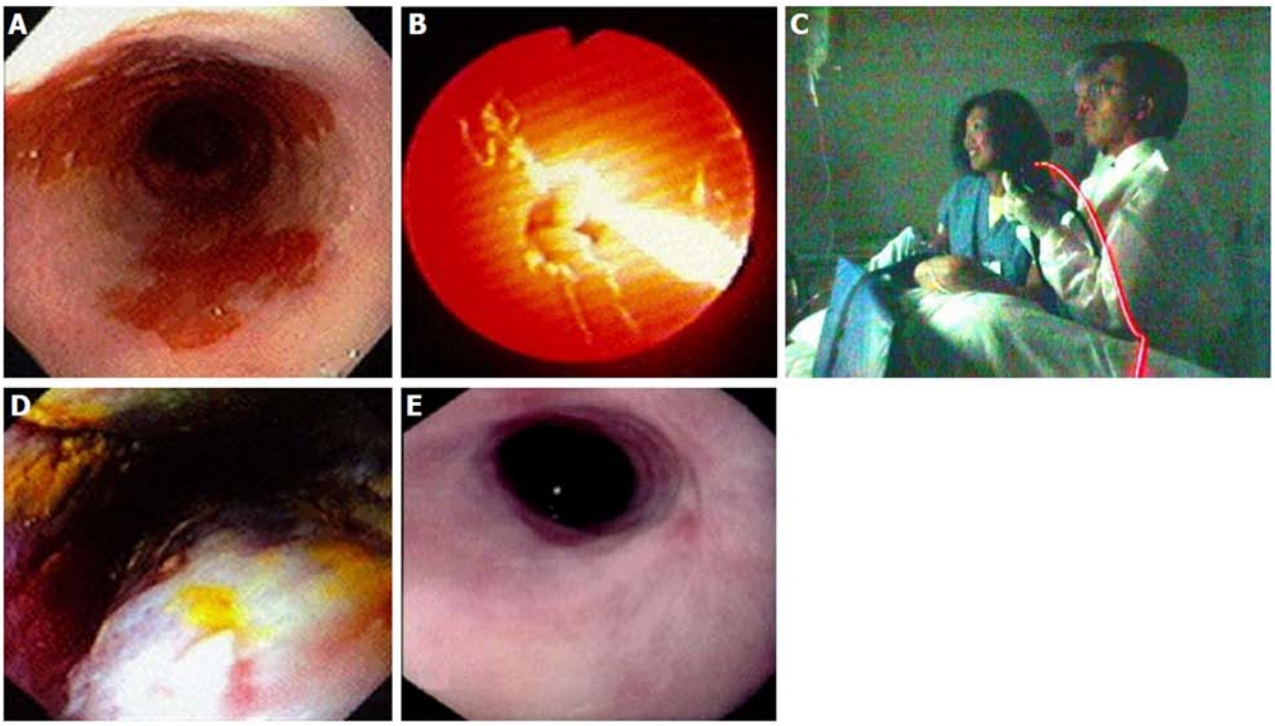

Figure 13 Photodynamic therapy in a patient with Barrett's esophagus and high grade dysplasia, performed by Dr. Chang and assistant (who consented to publishing this figure); photo taken in 1997. A: Endoscopic image of 4 cm Barrett's esophagus (BE) with high grade dysplasia (HGD); B: Photoactivation of photofrin with 630 nm diffuser fiber; C: Room view of diffuser fiber advanced through endoscope; D: Forty-eight hours post photodynamic therapy - endoscopic view of mucosal necrosis; E: Complete eradication of BE and HGD with mild stricture.

suspension taken 4 times a day for 4 wk) seems to reduce pain and promote quality healing. In patients with long segment BE, one could consider treating the proximal 50% of the lesion during the first session, in order to "test" the patient's tolerance to treatment and to potentially decrease risk of stricture. For RFA, excellent contact between the electrode surface and BE is critical. While recent studies have shown that the routine removal of the device to clean the electrode surface is not required, in my experience if the coagulation effect does not appear adequate on endoscopic viewing, I would remove and clear the device surface of undesirable coagulum. For both RFA and Hybrid APC, the final endoscopic appearance of a relatively "dry", tan colored surface, indicates an adequate ablation affect. For balloon-base cryotherapy, using the new foot pedal control gives the physician much more control of all aspects of balloon and spray nozzle movement and positioning. Since visualization is accomplished through the inflated balloon, good contact between the therapeutic endoscope lens and the balloon surface is important. When the balloon is inflated, sometimes focal areas of Barrett's can be difficult to visualize. Therefore, I routinely mark the focal areas using the tip of a snare to create coagulation spots on the mucosa which facilitates targeting through the balloon (Figure 18A).

Considerations for ESD

For ESD in the esophagus, our technique begins with marking the perimeter of the lesion. A submucosal injection of carboxymethylcellulose (artificial tears) is used for the initial lift. This is followed by mucosal incision using the hybrid i-knife (Erbe), which has the ability of high pressure saline injection through the center of the needle. This allows for sequential inject-cut-inject without the need for device exchange. Other devices for concurrent energy delivery with water injection (mostly through the catheter as opposed to the needle itself) are emerging. While the mucosal incision is usually straight-forward, the submucosal dissection can be more challenging. Changing the energy settings, creating traction of the lesion, and the tunneling method[99] can often make the dissection go more smoothly. While forced coagulation is the usual setting for dissection, I have found that spray coagulation, similar to my preferred POEM technique, can often speed up the dissection. Counter-traction can be achieved with a variety of methods involving clips and sutures[100,101]. In the esophagus I prefer the "yo-yo" technique[102,103]: place an endoscopic clip at the edge of the specimen, capturing the excised mucosa/submucosa, then remove scope; place a soft rubber nasal airway trumpet (Robertazzi Style) into the nasal cavity, guide a standard

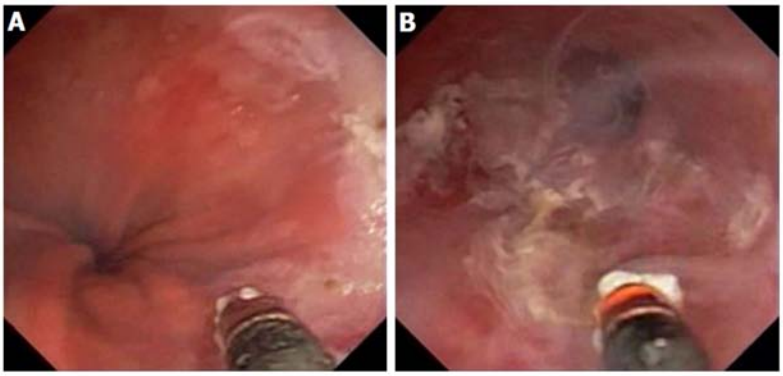

Figure 14 ND:YAG laser for treatment of Barrett's esophagus with low grade dysplasia. A: Endoscopy showing sapphire contact probe back-loaded into scope channel; B: Focal ablation of Barrett's esophagus using ND:YAG contact probe.

snare catheter with a plastic tape at the distal tip to create a "fin" for grasping - advanced through the nasal trumpet into the proximal esophagus, advance endoscope along the snare catheter, grab the fin with a grasping forceps and guide to proximal edge of specimen, where snare can capture the clip. This allows for traction in either forward or backward directions. Finally, if the lesion is long and wide, consider using the tunnel technique[99], similar to the POEM procedure, to start the submucosal dissection. Since tunneling does not require any traction for visibility, this can save quite a bit of time.

Future opportunities

Treatment of Barrett's and early esophageal cancer - this area has seen tremendous progress in the past decade, and future areas of refinement will include indications for treatment, the optimal modality for specific and individual situations, and pushing the envelope with endoscopic resection. These resection and tunneling techniques may also be employed to remove submucosal tumors (SMTs), such as leiomyoma, by submucosal tunneling endoscopic resection (STER) technique[104-107].

Detection and staging Barrett's - while endoscopic treatment of Barrett's and early esophageal cancer has progressed rapidly, the endoscopic detection and staging of Barrett's has not progressed to the same extent. We still live in the world of uncertainty regarding who and how to screen for Barrett's. There are 4 "buckets" of unmet needs: (1) in patients with possible ultra-short segment Barrett's, we need to be confident in either ruling in or ruling out Barrett's; (2) in patients with established Barrett's, we need to be able to detect the presence or absence of dysplasia with high accuracy; (3) in patients with known dysplasia, we need to predict which patients will respond to RFA or other ablative modalities and separate out those patient who would require deeper and more aggressive treatment, such as endoscopic resection; and (4) among patients undergoing endoscopic treatment for Barrett's, we need to detect residual Barrett's that is not obvious by white light or narrow band imaging (*e.g.*, Buried Barrett's, or residual neoplasm in the gastroesophageal junction) and risk stratify patients who are more likely to have early recurrence of Barrett's and dysplasia. Improved imaging with high resolution white light, narrow band imaging, acetic acid and chromoendoscopy, in addition to new technologies such as endomicroscopy (Cellvizio) and optical coherence tomography/volumetric laser endomicroscopy (VLE) show promise in addressing the needs within these 4 buckets - but we still have not fully addressed these important issues. The use of probe based confocal laser endomicroscopy (pCLE) (Figure 21) in the evaluation of BE has been recognized for a list of clinical situations, including its use in conjunction with narrow band imaging[108,109], the surveillance of BE dysplasia in patients undergoing follow-up[110-114] and the definition of the lateral extent of neoplasia prior to therapy[108,115]. The pCLE-targeted biopsies appear to reduce the number of physical biopsies and to increase the accuracy of the procedure, in real time[108,115-117]. As a result, the yield for neoplasia is higher than that of white light endoscopy and random biopsies. However, the additional cost, skill, and time for performing pCLE has limited its widespread use in clinical practice. I have been involved with pCLE clinical and translational research[108,118-122] and training since 2009 and in our practice, we use pCLE routinely for "buckets" 1 and 2.

VLE is a wide-field, second-generation optical coherence tomography endoscopic platform commercially available for advanced imaging in BE (Figure 22). We recently

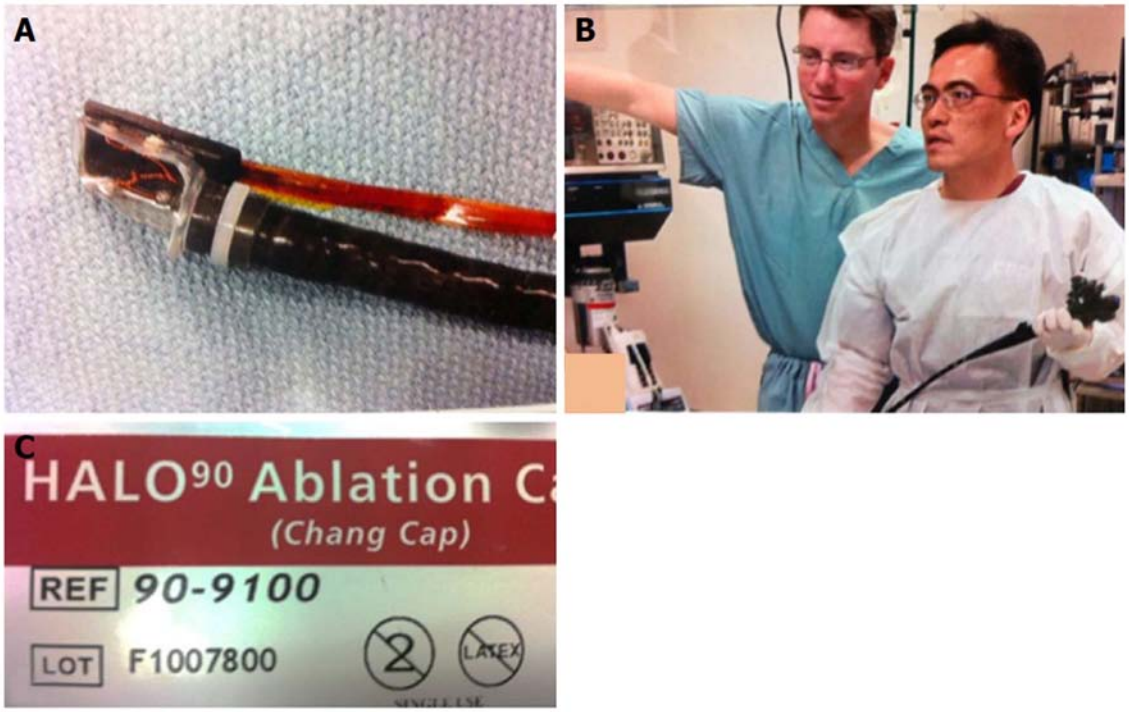

Figure 15 Radiofrequency ablation for Barrett's using focal device (Halo90), photo taken in 2004. A: Prototype focal ablation device; B: Dr. Chang working closely with Medical Director of Barrx Medical, Dr. David Utley, to refine technical aspects of focal device; C: Focal ablation device, Halo90, nicknamed "Chang Cap" from 2004 to present. (Dr. Utley gave consent to publish this Figure).

published a review article summarizing current clinical data and knowledge gaps[123]. Based on *ex-vivo* studies, criteria have been established for identifying BE-associated neoplasia. In addition, recent studies, case series, and case reports have demonstrated that VLE is well tolerated, efficacious, and can target neoplasia. The current system has the capability of placing laser markings in real-time in order to pin-point and biopsy areas of concern on VLE[124]. The following are needed to establish VLE's clinical role: studies showing incremental yield of dysplasia detection using VLE are emerging[125], studies to determine VLE's in-vivo diagnostic accuracy for identifying and classifying BE-associated neoplasia, and studies on the cost-efficacy of VLE. *In-vivo* diagnostic imaging criteria should be available soon, and may be augmented by the artificial intelligence (AI) analysis[126,127]. In my experience, the use of VLE can detect wide-field abnormalities very quickly, both within and below the surface epithelium. Figure 22 illustrates this nicely - a patient with BE and HGD where VLE showed atypical glands covered by normal squamous epithelium, ie buried Barrett's (Figure 22A and B). I performed endoscopic resection and the pathology showed moderately differentiated adenocarcinoma in the background of HGD and BE. The malignant glandular structures were buried beneath squamous epithelium.

EARLY GASTRIC CANCER AND SUBMUCOSAL TUMORS (SMTs) OF THE STOMACH

Lessons learned

ESD has become standard of care in Asia and is the most effective treatment for early gastric cancer when performed within established guidelines, including both absolute and expanded indications[128-132] . ESD is absolutely indicated in mucosal differentiated carcinomas without ulceration and a diameter of < 2 cm. Expanded indications include differentiated carcinomas limited to the mucosa without ulcer and > 2 cm in diameter or with ulceration but < 3 cm, as well as small undifferentiated carcinomas (< 2 cm). Lymphovascular infiltration must to be absent in all cases. ESD has been established in Asia to be superior to EMR for treating early gastric cancer. However, outcomes from centers in Asia may not be representative of the western experience[133]. In a recent article by Daoud *et al*[134], reviewed and compared outcomes of ESD between Eastern and Western countries. Their meta-analysis included 238 publications and 84318 patients who had ESD. The 90% of the studies were conducted in Eastern countries (Japan, China, South Korea, Taiwan) and only 10% of the studies

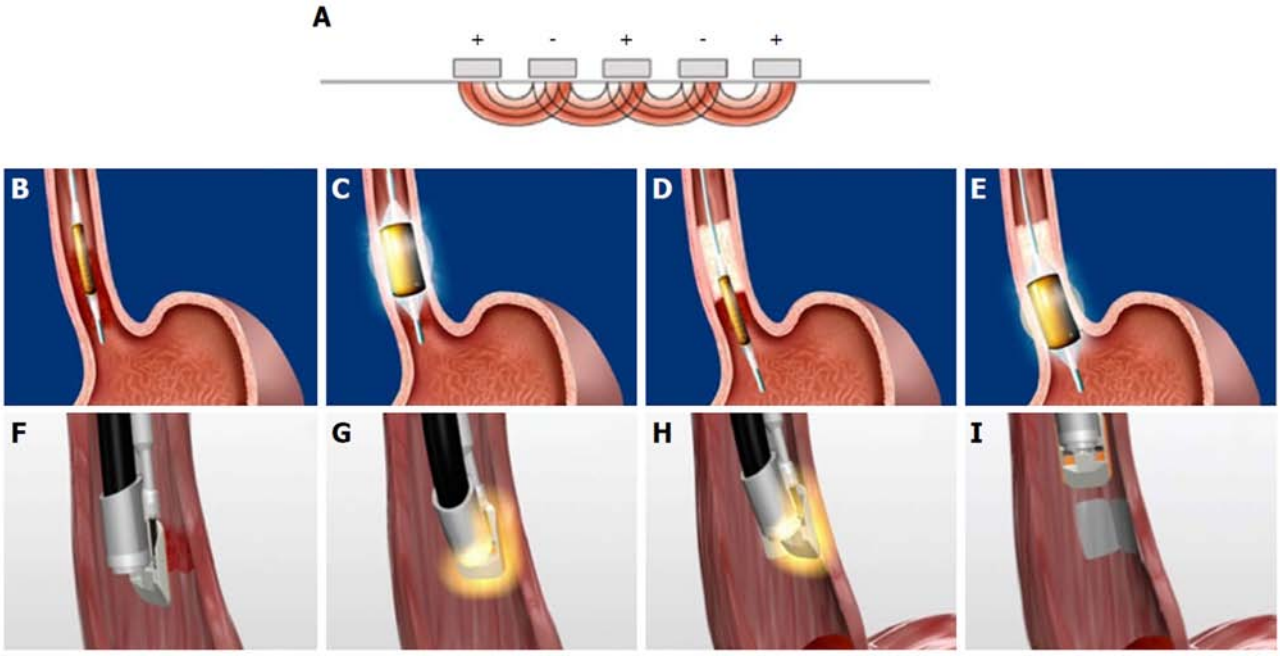

Figure 16 Radiofrequency ablation mechanism and technique. A: Tightly spaced electrodes (250 μm apart) with pre-set energy and power densities, generator turns off when a pre-determined resistance level in the ablated tissues is reached (mean of 0.3 s); B and C: Circumferential radiofrequency ablation (RFA) delivered by balloon electrode to treat 4 cm segment with single activation; D and E: The catheter is advanced to the next segment with slight overlap; F-I: Focal RFA delivered with Barrx90 device which is secured onto scope tip and endoscopically directed over Barrett's lesion.

reporting ESD outcomes in 2216 lesions were from Western countries (United States and Europe). The percentage of curative, *en bloc*, and R0 resection was higher "in the Eastern studies; 82% (CI: 81%-84%), 95% (CI: 94%-96%) and 89% (CI: 88%-91%) compared to Western studies; 71% (CI: 61%-81%), 85% (CI: 81%-89%) and 74% (CI: 67%-81%), respectively". Also, the percentage of perforation requiring surgery was significantly increased in the Western countries (0.53%; CI: 0.10-1.16) compared to Eastern countries (0.01%; CI: 0%-0.05%). ESD procedure times were longer in Western countries (110 min *vs* 77 min). They concluded that "ESD performed in Eastern countries is associated with better outcomes than studies reported from Western countries with regard to R0, *en bloc* and curative resection rates". Moreover, perforations requiring surgery are more common in Western studies. The clinical decision-making for or against ESD *vs* EMR should consider regional outcomes and locally available expertise as well as the necessity for resection according to oncologic standard based on the risk for cancer versus pre-cancerous lesions. The differences in outcomes may be partly related to the skills of the endoscopist, as case volume and ESD mentors are more limited, and training in ESD is still in its developmental stage. My learning curve was initially quite slow, but I had the great fortune of having advanced fellows from Japan stay in my unit for 2-3 years at a time continuously since 2005. This afforded me ongoing in-house training and refinement. In addition, our Digestive Disease Center at the University of California Irvine, Medical Center in Orange, CA is situated in a relatively high density Asian population in Southern California. Finally, having ready access to an animal lab, new techniques and devices can be adopted very quickly. Therefore, the training for ESD in western countries must take into consideration these factors and adopt a model that incorporates hands-on ex-vivo, live animal, and a regional mentor/coach as described well in an article by Draganov *et al*[135].

More recently, Submucosal Tunneling Endoscopic Resection (STER) and Full thickness resection (FTR) have emerged for the endoscopic treatment of SMTs. The STER technique has predominantly been used to treat esophageal SMT's, namely leiomyoma and GIST lesions. For gastric SMT's, techniques include either FTR or a combination of ESD and FTR. For example, a gastric SMT arising from the MP can be approached by initially performing an ESD until reaching the central attachment of the tumor to the MP. At this point, FTR can be performed, but with a smaller diameter than a de-novo FTR. For smaller lesions, FTR can be accomplished using the FTRD device (Ovesco), which integrates a large suction cap with both over-the-scope clip (OTSC) and snare (similar to some EMR devices). Using this device, FTRs in the stomach can potentially be accomplished with great efficiency. We recently used

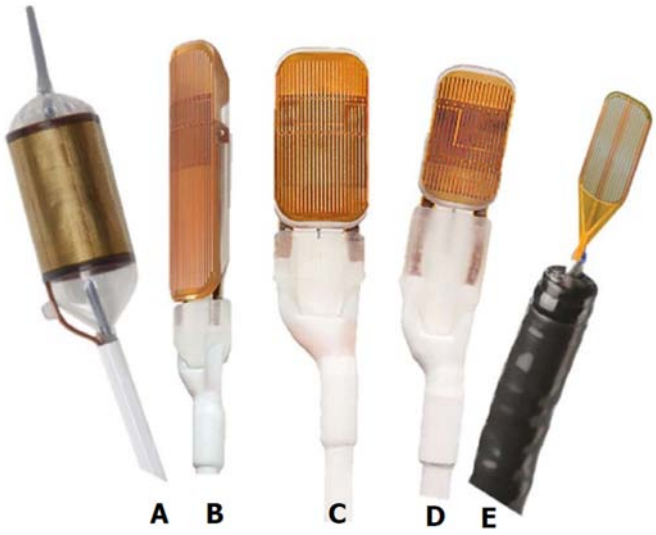

Figure 17 Radiofrequency ablation device "family". A: Barrx 360 express, 4 cm self-sizing balloon device; B: Barrx 90 Ultra - 40 mm × 13 mm platform; C: Barrx 90 (Chang Cap) - 20 x 13 mm platform; D: Barrx 60 - 15-10 mm platform; E: Barrx channel catheter - fits through biopsy channel, 15.7 mm × 7.5 mm platform when "wings" expanded.

FTRD to perform en block resection of a gastric SMT (Figure 23) which turned out to be a neuroendocrine tumor.

Technical considerations

For gastric ESD, lesions in the antrum and body along the greater curve tend to be easier, while lesions in the proximal third of the stomach and those along the lesser curve tend to be more difficult[136]. We tend to utilize traction techniques[137-140] especially in the more difficult areas. Our most common traction technique for the stomach and colon is what we call the multi-loop (M-loop) technique (Figure 24): create 3 loops using either suture or dental floss (can tie loops around a 3-mL syringe for sizing), grab one end of the loop with clip, pass the clip with M-loop through the scope channel (Figure 24C), open the clip and grasp the proximal edge of the specimen (Figure 24E) and release the clip and string, then using a second clip, grasp and secure the distal loop to the opposite wall (Figure 24F), if needed, to tighten or change angle of traction, use 3rd clip to grasp and secure middle loop to another spot (Figure 24G and H). This is a simple method that does not require scope removal nor any additional equipment other than clips and string.

Future opportunities

Although the instrumentation and energy sources have made tremendous progress, we are still quite far from the instrumentation available to the laparoscopic or robotic surgeon. There are a number of scissor-type cutting and coagulation devices emerging that may be helpful. For the stomach, we routinely use the i-knife and in some cases will also employ the SB knife[141]. This device can both coagulate and cut tissue using a scissor motion. It is useful for mucosal incision around "corners", but mostly for quick and efficient submucosal dissection. Scissor-type instruments that can cut and coagulate without using heat energy (*e.g.*, harmonic energy) would be a great advancement. Traction for the endoscopic surgeon is still at its infancy. New devices, such as magnets are emerging which may be useful, especially in areas where good traction is paramount[142]. And finally, closure of large defects and FTR will require robust endoscopic wound closure. ESD for duodenal lesions have recently been shown to benefit from complete closure[143] as the risk of post ESD delayed bleeding and more importantly, delayed perforation (possibly caused by the high concentration of bile and digestive enzymes) can be catastrophic. Complete closure for colonic ESD is also being employed by expert centers who have reported accelerated mucosal healing[144], decreased delayed bleeding[145], as well as decreased need for post-ESD hospitalization[146]. For many of these larger closures, suturing is emerging as an alternative to clip closure[147]. And for FTRs, suturing is becoming a must (Figure 25). In my ESD practice, closure, especially with sutures is to be avoided if possible if there is a possibility of residual neoplastic tissue. The mucosal healing and scar formation make subsequent endoscopic resections quite difficult. Small defects in the muscle layer are best treated with clips. Otherwise, large defects in the

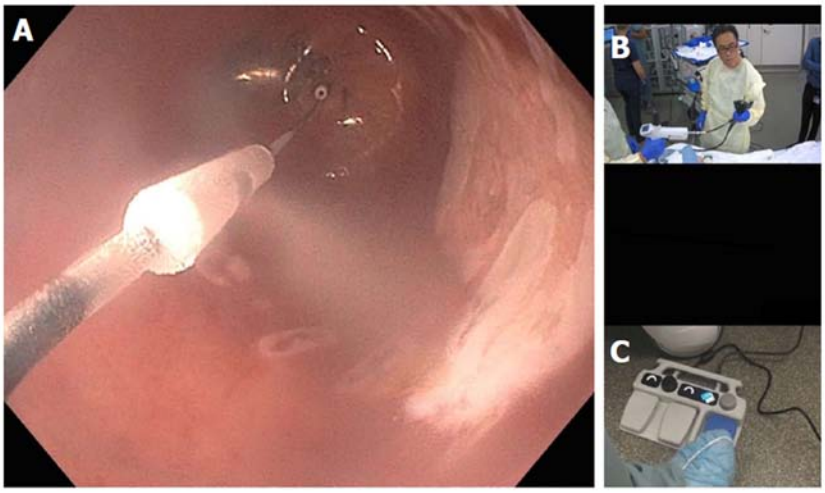

Figure 18 Cryotherapy using nitrous oxide within balloon. A: Endoscopic view of focal cryoablation of multiple islands of Barrett's epithelium; marking around the lesion with a snare tip helps with targeting; B: In-room view of hand-held control (by Dr. Chang) with nitrous oxide cartridges; C: New foot pedal console which controls nitrous oxide flow as well as vertical and left-right rotational movement of the spray orifice.

stomach, duodenum, and colon, where-ever technically possible, should be closed with suturing. In Figure 25, I share a case of a patient with a gastric neuroendocrine tumor who have previous attempts at EMR. The scarring was so severe I had to proceed with a full thickness strategy. Using the Overstitch device, closure in the posterior aspect of the stomach is quite suitable. However, closure along the lesser curve or anterior wall is much more difficult due to the scope stiffness. The technique for suturing adheres to surgical principles - such as the distance from the suture to the wound edge should be approximately equal to the thickness of the tissue, *etc*. If there is concern for possible uneven edge approximation after the initial suture is placed, I will place a second layer of suture. For POEM closure, I sometimes use sutures if the mucosal incision is uneven. And in these cases, I routinely place double sutures. Technologies are also emerging for more flexible suturing devices and those that do not require a double channel therapeutic scope.

GASTRIC OUTLET OBSTRUCTION AND GASTRIC POEM

Lessons learned

For malignant gastric outlet obstruction (GOO), endoscopic gastro-jejunostomy is now showing better results than enteral stenting in treating these patients[148] and may have similar results to laparoscopic gastro-jejunostomy[149]. In patients with benign GOO, namely gastroparesis contributed by a tight pylorus, gastric POEM (G-POEM) of the pylorus is now emerging as a treatment option. After the initial case report of G-POEM in a patient with gastroparesis[150], there have been a number of small series[151-160] showing safety, feasibility, and good clinical outcomes with approximately 70% patients showing improvement in their quality of life scores at 1 year[152]. One non-randomized study compared laparoscopic pyloroplasty (LP) *vs* G-POEM and found that patients who underwent LP had a longer average length of stay (4.6 d *vs* 1.4 d, $P = 0.003$), operative time (99.3 min *vs* 33.9 min, $P < 0.001$), and estimated blood loss (12.9 mL *vs* 0.4 mL, $P < 0.001$). There were also more complications in the LP cohort (16.7% *vs* 3.3%, $P = 0.086$), which included surgical site infection (6.7% *vs* 0%, $P = 0.153$), pneumonia (6.7% *vs* 0.0%, $P = 0.153$), and unplanned intensive care unit (ICU) admission (10.0% *vs* 0.0%, $P = 0.078$). LP and G-POEM both resulted in similar, significant improvements in both in GCSI scores and objective gastric emptying.

Technical considerations

The procedure begins with a mucosal incision approximately 5 cm from the pylorus along the greater curve (Figure 26A-C). The submucosal tunnel is then extended until the pylorus is reached (Figure 26D and E). A hook knife is used to carefully excise the pylorus muscle (Figure 26F) and then extended proximally into the antrum (Figure 26G). Closure can be accomplished with either clips or suturing (Figure 26H), although in this situation I prefer suturing, as positioning of the device is easy and the sutures can be placed deep into the gastric wall.

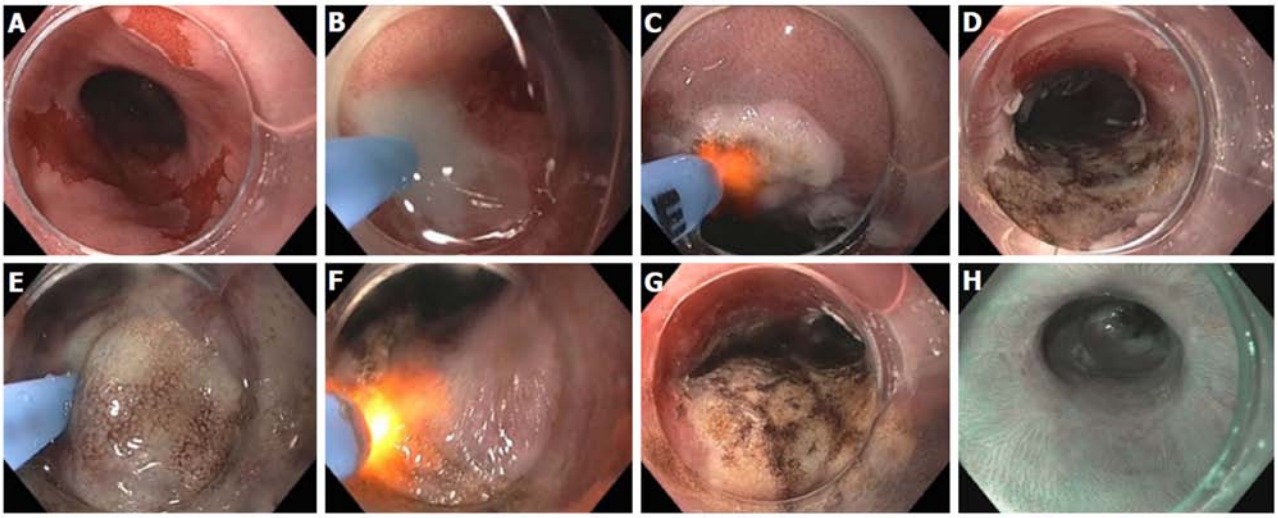

Figure 19 Hybrid argon plasma coagulation to treat residual diffuse, multi-focal Barrett's after endoscopic submucosal dissection for early esophageal cancer. A: Endoscopic view of multiple residual Barrett's islands; B: Needle-free high pressure water jet to create saline cushion in the submucosal space; C: Pass 1 of argon plasma coagulation (APC) ablation with energy settings of Pulsed APC flow rate 0.8 L/min 60 W; endoscopic submucosal dissection (ESD) cap used for visibility, traction and maintaining precise focal distance for even application; D: Completion of Pass 1; E: Repeat water jet into submucosa; F: Pass 2 of APC ablation with energy settings of pulsed APC flow rate 0.8 L/min 40 W; G: Completion of Pass 2 with desired tan colored surface; H: Follow-up endoscopy 6 months post hybrid APC treatment, with biopsy and WATS brushing confirming complete response of intestinal metaplasia.

Future opportunities

While the procedure is quite similar to POEM for achalasia, the patient selection and prediction of response to treatment is much less developed. The mechanisms leading to symptoms in patients with gastroparesis is still unclear and can be variable. The amount of delay in gastric emptying does not seem to predict symptoms or relief of symptoms post treatment. While "pylorospasm" may be one possible mechanism for patients with gastroparesis[161], other factors may include fundic accommodation as well as central nervous system factors. Although we routinely perform EndoFLIP to measure luminal geometry and pressure before and after each G-POEM, the parameters to predict clinical response have not yet been established. If a patient has had a response, even if temporary, to botox injection to the pylorus, we consider this somewhat predictive of positive response to G-POEM.

OBESITY

Lessons learned

Obesity has become an epidemic is many western countries and is becoming more prevalent in Asia as well. Laparoscopic bariatric surgery with sleeve gastrectomy and Roux-n-y gastric by-pass are the standard of care options for patients with BMI > 40 alone, or in patients with BMI > 35 and concurrent co-morbidity. However, for obese patients with BMI between 30 and 35 who don't qualify for bariatric surgery, a growing number of endoscopic treatment options are now available, including intra-gastric balloons[162-165]. Among the endoscopic bariatric devices and procedures that I offer my patients, most will choose the endoscopic sleeve gastroplasty (ESG), which I believe is currently the most effective endoscopic option. This procedure falls squarely in the paradigm of endoscopic foregut surgery. A recent large international multicenter study of 112 consecutive patients with baseline BMI 37.9 ± 6.7 kg/m^2 underwent ESG[166]. At 1, 3, and 6 mo, Δweight was 9.0 ± 4.6 kg (Total Body Weight Loss - TBWL 8.4% ± 4.1%), 12.9 ± 6.4 kg (TBWL 11.9% ± 4.5%), and 16.4 ± 10.7 kg (TBWL 14.9% ± 6.1%), respectively. Three (2.7%) severe adverse events were observed. Another multicenter study with 248 patients showed robust clinical outcomes with a 24-month follow-up[167]. Baseline BMI was 37.8 ± 5.6 kg/m^2. At 6 and 24 mo, %TBWL was 15.2 (95%CI: 14.2-16.3) and 18.6 (15.7-21.5), respectively. At 24 mo, % of patients achieving ≥ 10% TBWL was 84.2 and 53% with PP and ITT analyses, respectively. On multivariable linear regression analysis, only %TBWL at 6 mo strongly predicted %TBWL at 24 mo (adjusted for age, gender, and baseline BMI, β = 1.21, P < 0.001). This is in contrast to %TBWL of 20-30 in patients who undergo bariatric surgery and about 10% TBWL for intra-gastric balloons[167]. Moreover, intra-gastric balloons are all removed after 6 mo, and only 2/3 of the weight loss is

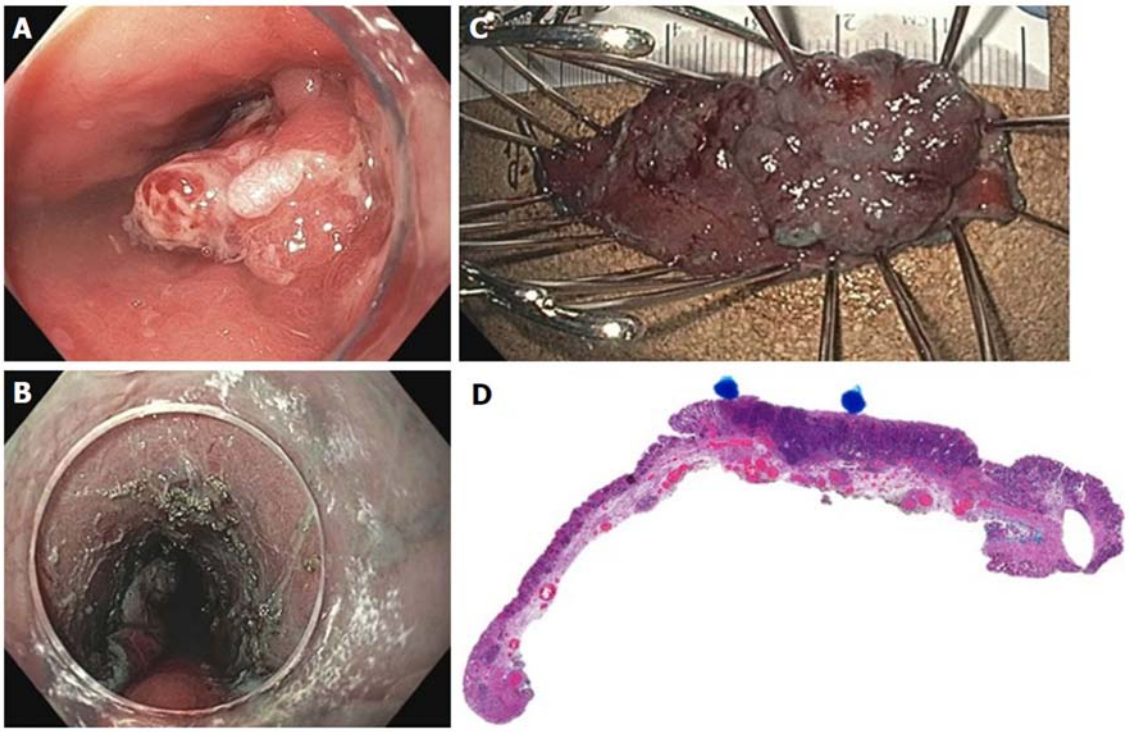

Figure 20 Endoscopic submucosal dissection of early esophageal adenocarcinoma arising from Barrett's esophagus. A: Nodular, polypoid (Paris IIa) lesion within long segment Barrett's esophagus; B: Endoscopic submucosal dissection completed; C: *En-bloc* resected specimen; D: Histology showing poorly differentiated adenocarcinoma (blue dots), T1b pathologic staging, lateral and deep margins negative (> 300 microns), no lymphovascular invasion.

sustained at 1 year[168]. Thus, I explain to my patients that they can expect to lose approximately 35-50 pounds with the procedure, and if they are successful at 6 mo, they can also expect to maintain or lose more weight out to 2 years. In some select cases, I have performed ESG "touch-up" or revisions after 6 mo in successful patients in order to achieve further weight reduction. These are technically even easier than the initial ESG.

Technical considerations

There are some slight differences in techniques among experts performing ESG. My technique (Figure 27) starts with marking the anterior and posterior wall of the stomach using APC starting from the level of the incisura all the way back to the proximal fundus, approximately 3-4 cm from the EG junction (Figure 27A). The first running suture starts by taking a bite at the anterior marking adjacent to the incisura, followed by a bite at the greater curve, then over the posterior marking (Figure 27B). Next, I move about 2 cm proximally and cross over to take the 4th bite along the anterior wall, then greater curve, and posterior wall again. The suture anchor is then released and the suture is tightened, crimped and cut with the accessory device. The second suture is then placed in a similar "Z" pattern, starting approximately 2 cm more proximally and taking between 6 to 8 bites. Usually a total of 6 to 8 sutures are placed to create an adequate length sleeve (Figure 27C and E). I have modified this technique to include a tight entrance to the sleeve portion. This is accomplished by placing a final suture in a purse-string fashion around an inflated 8mm balloon to prevent complete closure (Figure 27D). I have even added mucosal ablation in the cuff just prior to suture placement to maximize narrowing at the neck of the sleeve. This modification results in a proximal "pouch" (Figure 27F) which typically will hold about 50-60 mL of water. The rationale behind this technique is as follows: the pouch functions similar to a gastric by-pass, causing restriction of oral intake with a rapid sense of satiety after a very small portion; the pouch then empties through a very small opening at the neck of the sleeve, again similar to a gastric bypass or a laparoscopy band placement, which then causes severely delayed gastric emptying (Figure 27H). This helps patients feel a prolonged period of satiety. Then food passes through a long, narrow sleeve. The tight sleeve neck prevents reflux back into the pouch. Then finally, food lands in the antrum where grinding function is intact. In select patients who also suffer from GERD, I may perform an anti-reflux procedure at the same time - either MASE, RAP or even TIF can be performed on the same session. For ESG plus TIF combination, I start with the TIF first as it requires the device and

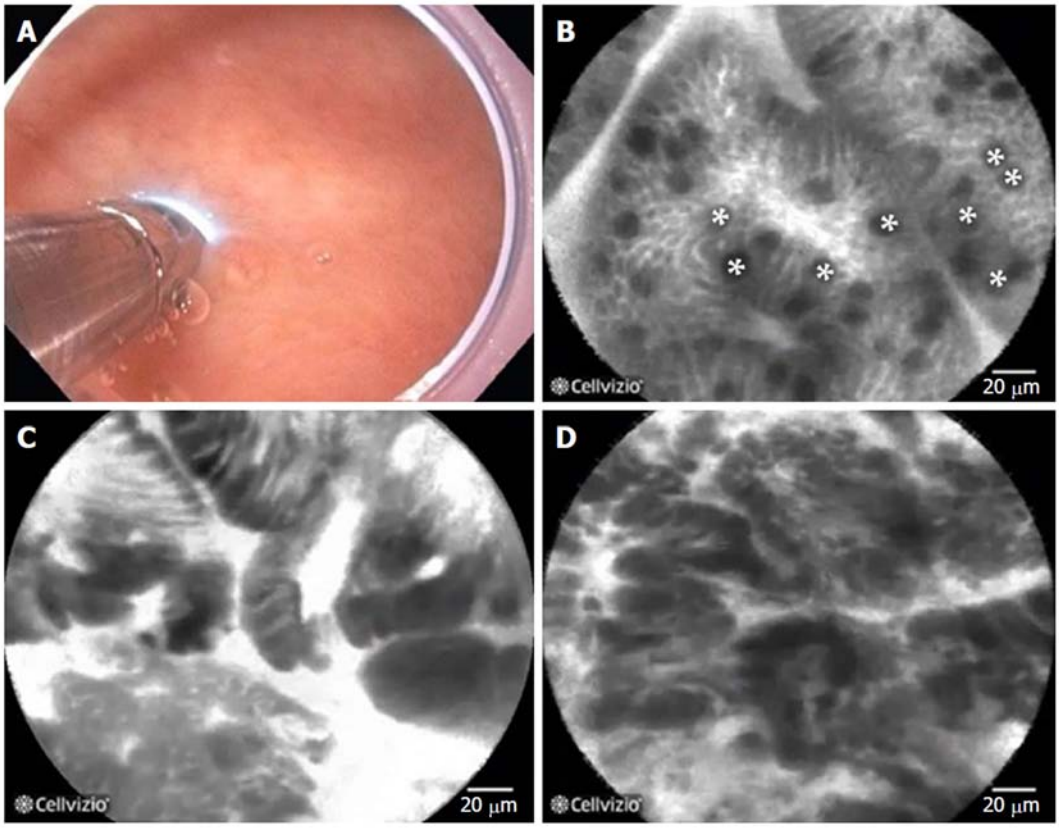

Figure 21 Probe-based confocal laser endomicroscopy in patients with Barrett's esophagus. A: the probe is advanced through the biopsy channel, while stabilizing the scope against the mucosa, the probe makes gentle contact with the surface epithelium; B: Probe-based confocal laser endomicroscopy (pCLE) image showing non-dysplastic Barrett's esophagus (BE). *goblet cells; C: pCLE image showing BE with high grade dysplasia; D: pCLE image showing early adenocarcinoma arising from BE.

scope to maneuver into a retroflexed position, which is difficult after ESG. The MASE and RAP procedures can be accomplished immediately before or after ESG, although I prefer the latter. These patients usually stay overnight, in our own short-stay unit for 23-hours observation, intravenous hydration, and as-needed anti-emetics and analgesics. The patient shown in Figure 27 started with a weight of 327 and has lost 100 pounds over a 1 year period, has no reflux or gastroparesis symptoms and remains off PPI medications.

Future opportunities

Endoscopic bariatrics is still early in its development. There are ample opportunities to explore innovative techniques and devices in this space, including those that target gastric restriction, bypassing the duodenum, as well as duodenal resurfacing[162-165,169,170].

ENDO-HEPATOLOGY

Lessons learned

Endo-Hepatology is a term that I coined in a published paper where I described many of the emerging endoscopic techniques and devices which may be useful in the diagnosis and treatment of liver disease (Figure 28)[171]. Among these, EUS-guided liver biopsy and EUS-guided portal pressure measurement are exciting new frontiers for the endo-hepatologists. I wish to share my personal perspective on how these techniques and devices evolved over the past 30 years. My faculty career began in the early 1990's with the development of EUS-guided FNA[172-182] having reported the first case of EUS-guided FNA to diagnose a small pancreatic cancer in 2004[172], followed the same year by a series of 38 patients who underwent EUS-guided FNA (Figure 29)[173]. Many papers followed[174-182], with application of EUS-guided FNA to diagnosing and staging pancreatic tumors[174,176], aspiration of pleural and ascitic fluid[175], left adrenal gland[178], mediastinal tumors and lymph nodes[181] and liver lesions[182]. I subsequently joined forces with the other early pioneers of EUS-guided

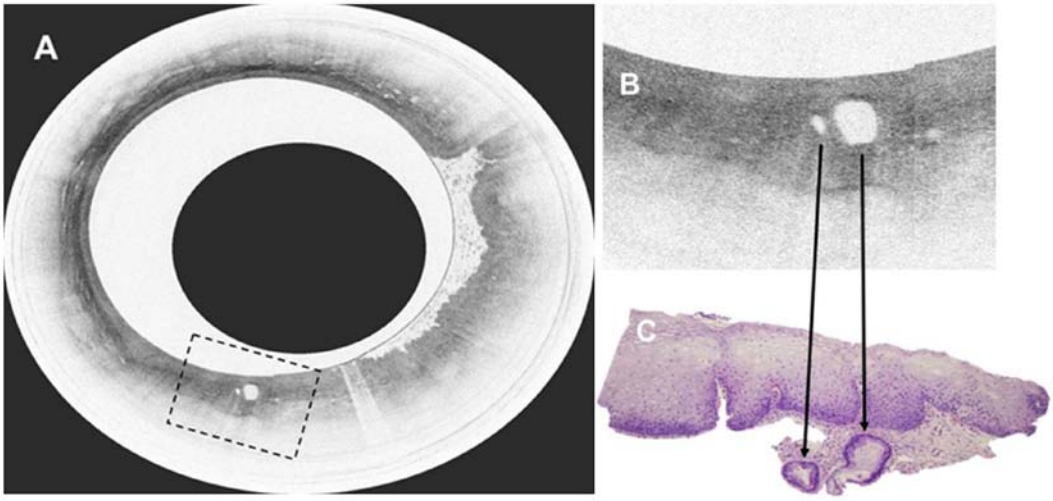

Figure 22 Volumetric laser endomicroscopy in patient with Barrett's esophagus and high grade dysplasia. A: Volumetric laser endomicroscopy showing an area of atypical glands covered by normal squamous epithelium; B: Magnification of region of interest (dotted box in image A) showing presence of 2 atypical glands; C: This area underwent endoscopic resection with pathology showing moderately differentiated adenocarcinoma in the background of high grade dysplasia and Barrett's esophagus. The malignant glandular structures (arrows) were buried beneath squamous epithelium.

FNA in the United States and Europe[183] to publish the first multicenter study on EUS-guided FNA. After establishing EUS-guided FNA, I started developing EUS-guided FNI (fine needle injection), including the delivery of anti-tumor agents and EUS-guided brachytherapy[184-197]. This then led to EUS-guided fine needle imaging with through-the-needle fiber optic probe combined with endomicroscopy in the evaluation of pancreatic cystic neoplasia[118,119,198-203]. EUS-guided FNA eventually become the mainstream modality for the tissue diagnosis of pancreatic cancer[185,204-209]. At this point, I coined a new term "Interventional EUS" in 1997[210] since we had moved away from mere diagnostic imaging to tissue acquisition and intervention. The next iteration for tissue acquisition was to move from EUS-FNA to EUS-FNB (fine needle biopsy). Larger gauge needles and needles designed to obtain histologically intact core specimen began to emerge[211-220]. EUS-guided FNA in the liver began with targeting local lesions - those suspicious of metastatic or primary tumors[182]. However, with the development of specialized histology needles, we started to explore the possibility of EUS-guided liver biopsy for benign liver disease[221-223]. We published the first case report of autoimmune hepatitis diagnosed using a new 19G histology needle[211] in 2012. Over the past few years, many studies[224-232] have supported the current notion that EUS-guided liver biopsy is as good as percutaneous biopsy[233] and may be better than trans-jugular biopsy[227].

Moving from EUS-guided liver biopsy, I innovated the possibility of performing EUS-guided porto-systemic pressure gradient (EUS-PPG) measurement. Working together with the engineers at Cook Medical, I was able to help develop (through bench and animal testing) a simple compact hand-held manometer that attaches to a 25G FNA needle. The background and rationale for this development is as follows. Portal hypertension (PH) is a serious adverse event of liver cirrhosis. The hepatic venous pressure gradient or portosystemic pressure gradient (PPG) accurately reflects the degree of PH and is the single best prognostic factor in liver disease. This is usually obtained by interventional radiology (IR) *via* a transjugular approach requiring radiation and intravenous contrast exposure. The transjugular PPG measurement is not routinely performed as a stand-alone diagnostic test, and in most instances is done only in conjunction with transjugular intrahepatic portosystemic shunt (TIPS). In the animal study, we tested this novel EUS-guided system using a 25G FNA needle and compact manometer to directly measure PPG and evaluated its performance against the current gold standard - transjugular hepatic venous pressure gradient[234]. Manometry was performed in venous (baseline and PH) and arterial (aorta) systems. The PH model was created by rapid Dextran-40 infusion peripherally. Under EUS guidance a 25G FNA needle with attached compact manometer was used to puncture (transgastric-transhepatic approach) and measure pressures in the portal vein, right hepatic vein (RHV), inferior vena cava (IVC), and aorta. With the IR approach, RHV (free and wedged), IVC, and aorta pressure were measured with an occlusion balloon. Pressure correlation was divided into 3 groups; low pressure (baseline), medium pressure (noncirrhotic portal hypertensive model), and high pressure (arterial). Correlation between the 2 methods of measurement was charted in

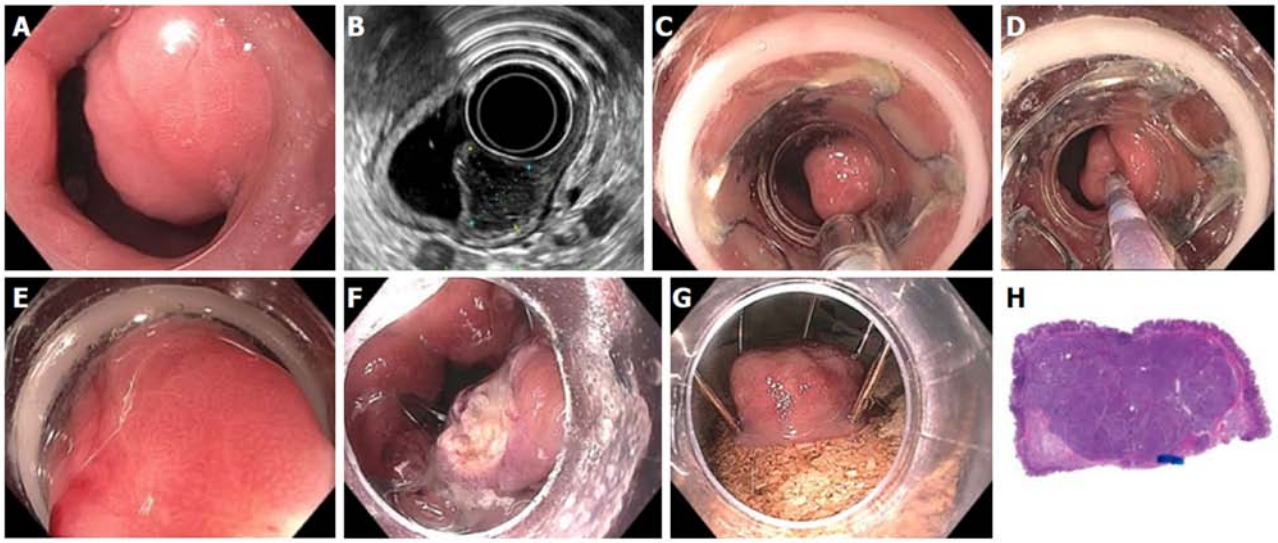

Figure 23 Full thickness resection using FTRD Device for submucosal tumor in the gastric pylorus. A: One point five centimeters submucosal tumor at the level of pylorus; B: Endoscopic ultrasound showing 1.3 cm × 1.1 cm submucosal tumor concerning for neuroendocrine tumor; C: Lesion viewed through double channel therapeutic scope with 21-mm transparent cap; D: Helical retractor was used to secure the lesion and pull it into the cap; E: Entire lesion is now within the cap; F: The entire wall was captured in the large clip and resection was completed above the closed clip; G: Lesion resected *en bloc*; H: Histologic specimen showing complete specimen with negative deep and lateral margins.

scatter plots, and the Pearson's correlation coefficient (*R*) was calculated. Our results showed that EUS identification, access, and manometry was successful in all targeted vessels. There was excellent correlation (*R*: 0.985-0.99) between EUS and IR methods in all pressure ranges. No adverse event occurred. We then went on to conduct a human pilot study performing EUS-PPG measurement on 28 patients with suspected cirrhosis[235]. The portal vein and hepatic vein (or inferior vena cava) were targeted using a transgastric-transduodenal approach. Clinical parameters of PH were evaluated in each patient. Feasibility was defined as successful PPG measurement in each patient. Our results showed 100% technical success and no adverse events. EUS-PPG values ranged from 1.5 to 19 mmHg and had excellent correlation with clinical parameters of PH including the presence of varices (P = 0.0002), PH gastropathy (P = 0.007), and thrombocytopenia (P = 0.036). EUS-PPG was increased in patients with high clinical evidence of cirrhosis (P = 0.005). We concluded that "This novel technique of EUS-PPGM is feasible and safe. Given the availability of EUS and the simplicity of the manometry setup, EUS-guided PPG may represent a promising breakthrough for procuring indispensable information in the management of patients with liver disease".

Technical considerations

We recently published a comprehensive video article which includes animal and human data as well as equipment set-up and an illustrative case[236]. The procedure itself takes only about 20 min (Figure 30). Identifying the vascular structures within the liver is key[237]. The hepatic veins all converge into the inferior vena cava (IVC). The middle hepatic vein is usually best suited for EUS-PPG given its size and angle to the FNA needle (Figure 30A and B). The 25G needle (primed with heparin) is advanced through liver parenchyma which stabilizes the needle and also tamponades the needle tract upon withdrawal (Figure 30C). Once in the vessel, the needle is flushed (few drops) and the pressure is measured by the compact hand-held manometer (Figure 30C). This measurement is repeated for a total of 3 consecutive measurements. The needle is then slowly withdrawn into the liver parenchyma, and Doppler flow is applied to make sure there is no blood flow within the needle tract. Once this is established, the needle is withdrawn. Next, the portal vein is identified. The umbilical portion of the left portal vein is the most common target (Figure 30D and E), with its characteristic pulse wave showing a continuous venous hum. The needle is then advanced through liver parenchyma into the left portal vein (Figure 30F), where 3 consecutive manometry pressure measurements are taken in a similar fashion. The EUS-PPG is then calculated by calculating the difference between the mean pressures of each vessel.

Future opportunities

Both EUS-guided liver biopsy and EUS-guided PPG measurements are novel and

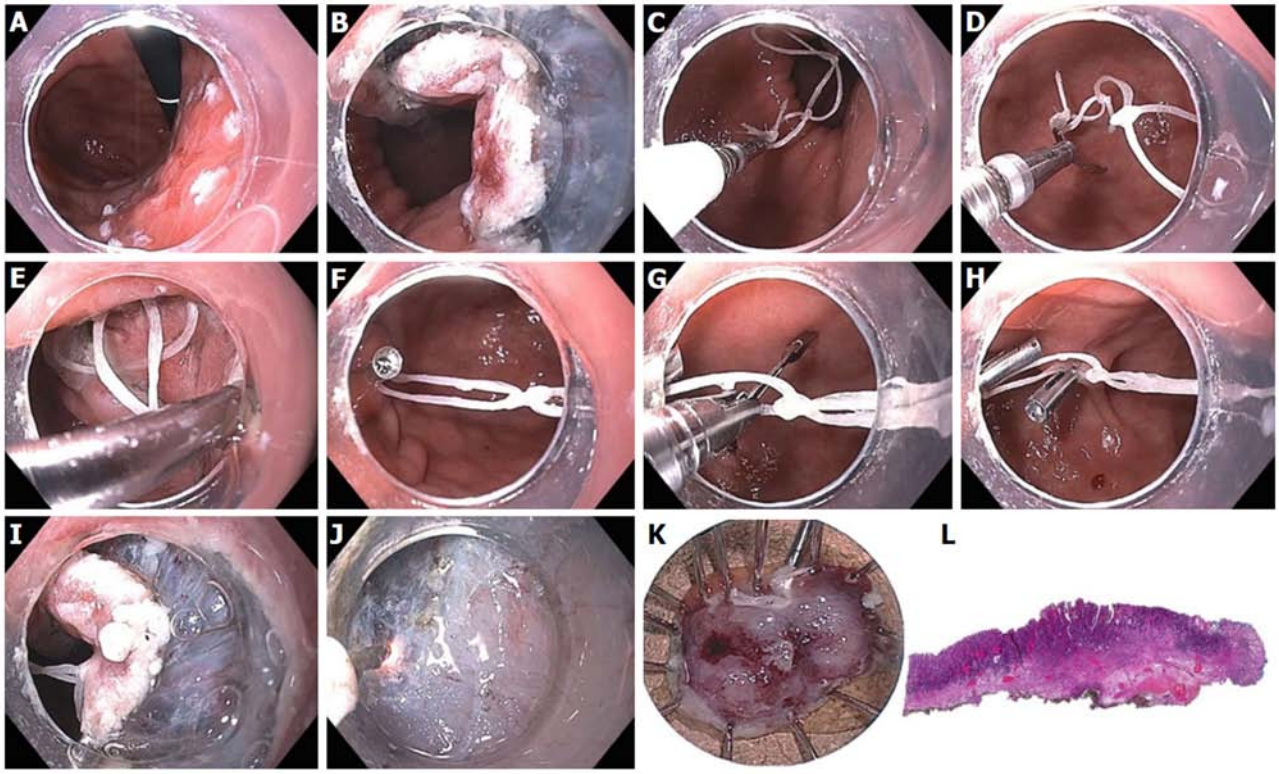

Figure 24 Endoscopic submucosal dissection of 15-mm IIa+c intramucosal carcinoma of stomach along lesser curve body using multi-loop technique. A: The lesion is marked using spot coagulation; B: Circumferential incision is performed using the i-knife, however the angle of approach on the proximal side is difficult for submucosal entry and dissection; C: Three loops are created using dental floss, captured with a clip passed through the biopsy channel (no need for scope removal or additional equipment); D: The clip is opened (the string can be pre-tied to one leg of the clip) and positioned to grasp the proximal edge of the specimen; E: The 1st clip is released, anchoring the string to the specimen; F: A 2nd clip is used to catch the distal loop and anchor to opposite wall of stomach; G: If necessary, a 3rd clip can be used to grasp the middle loop; H: This can be helpful to further tighten or re-direct the traction angle; I: the submucosal space is much easier to enter with multi-loop traction; J: submucosal dissection in the antegrade approach is greatly facilitated; K: The specimen is resected *en bloc*; L: Histology confirmed a well differentiated grade 1 adenocarcinoma with invasion to the muscularis mucosa with negative deep and lateral margins, no lymphovascular invasion.

exciting new technologies within endo-hepatology. The fact that both of these procedures can be combined in the same session makes it very appealing[238]. The potential application of these techniques is substantial. In an editorial to our EUS-PPG publication, Adler *et al*[239] mention some of these possible applications: pre-operative risk stratification, variceal management, and primary hemorrhage prevention are on their list. Patients who are started on non-selective beta blockers as primary prevention would have a more precise and liver-specific paradigm for titrating medication dosages. New therapeutic drugs for liver disease, including anti-viral, anti-fibrosis, and anti-inflammatory medications, perhaps may employ EUS-PPG as an objective outcome measure in clinical trials. Patients with hepatocellular carcinoma and cirrhosis may benefit from EUS-PPG in determining liver function prior making the decision between lobectomy versus liver transplantation.

CONCLUSION

In conclusion, I have been truly blessed with a career in GI endoscopy that spans 3 decades of discovery, teaching, healing, and collaboration. I have been an eye witness to the evolution of endoscopy from diagnosis to tissue acquisition and staging, to advanced imaging, and finally, endoscopic surgery. As we consider where we've been and the current acceleration of advancements, the future is full of promise and opportunities for new developments that will impact many patients and their families. The future for endoscopic foregut surgery is now!

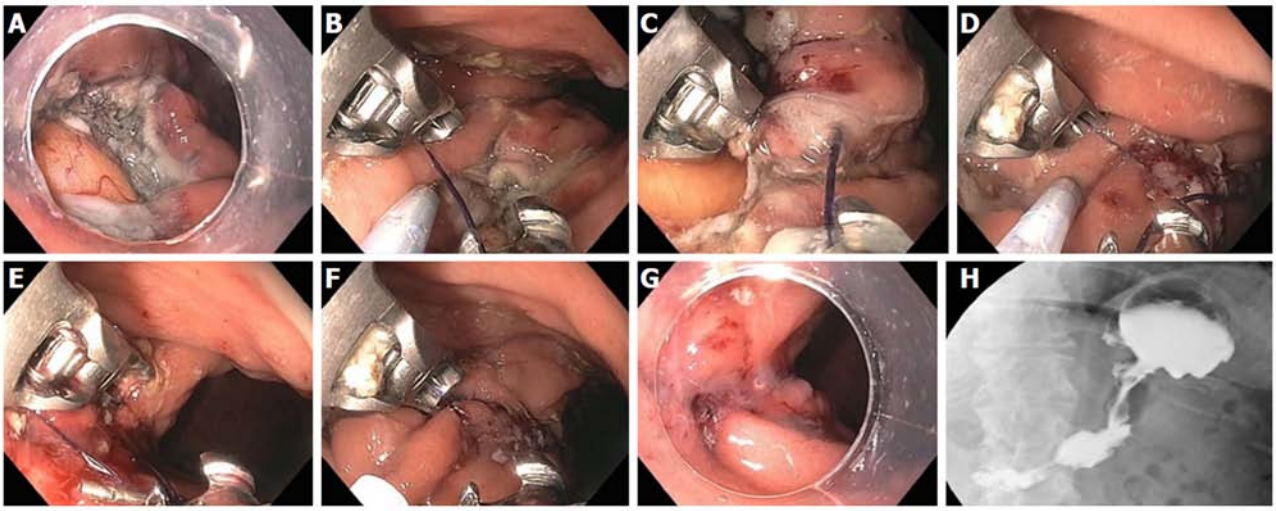

Figure 25 Large gastric defect after full thickness resection of neuroendocrine tumor closed with endoscopic suturing. A: Four-centimeter full thickness wound in posterior body of stomach, omental fat seen at base of defect; B: First suture - use helical tissue retractor to grab left edge of distal defect; C: After taking first bite on distal left (needle going from mucosa to serosa), second bite on distal right (needle going from serosa to mucosa); D: Approximately 8 bites are taken, alternating from left to right, with the last bite having the needle from mucosa-serosa-mucosa in single throw; E: First continuous running suture completed and needle anchor released for tightening and suture release; F: Second row of running suture placed for double reinforcement; G: Double suture closure completed; H: Contrast study shows luminal narrowing with no leak.

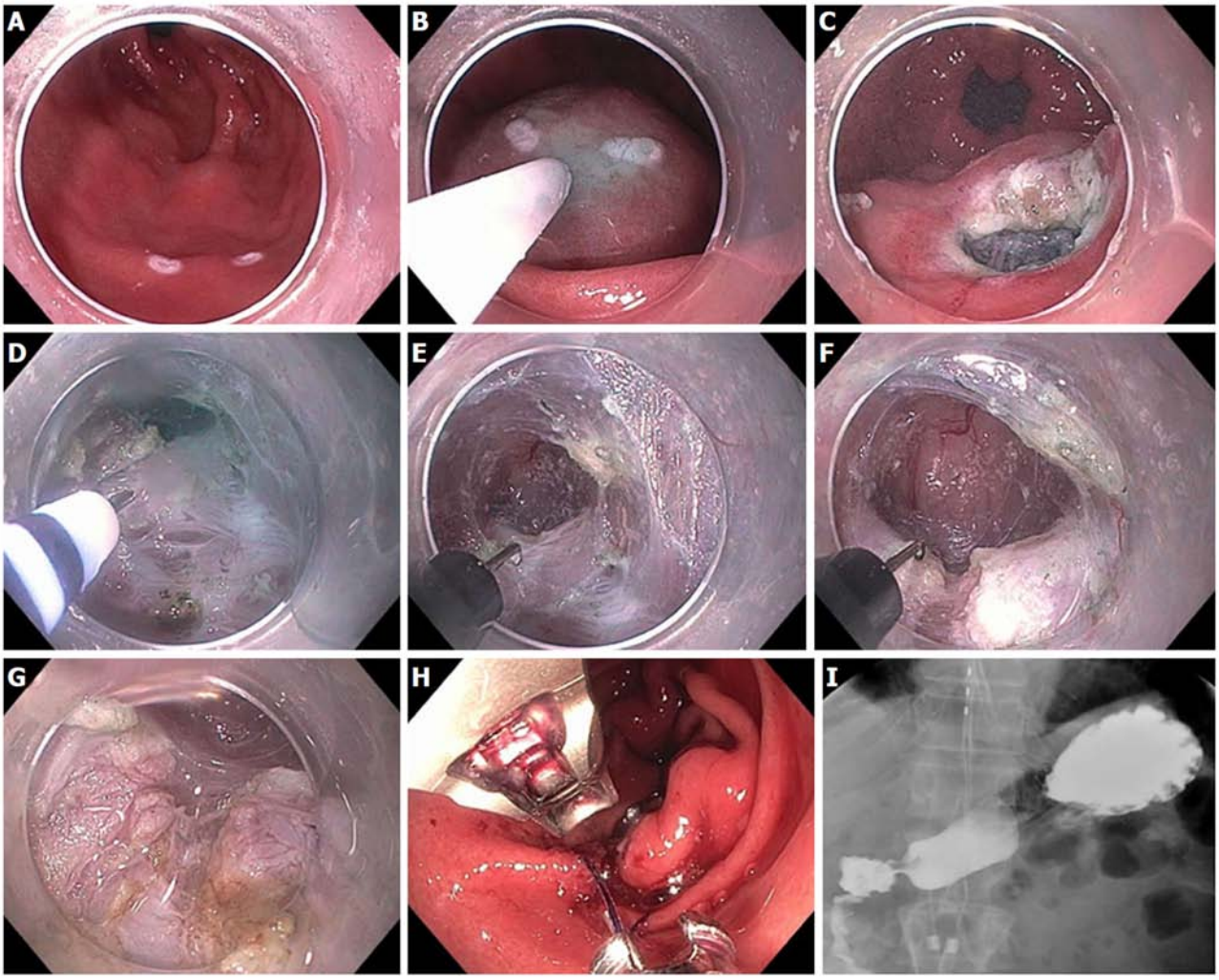

Figure 26 Gastric per-oral endoscopic pyloromyotomy in a patient with severe gastroparesis. A: Markings made for mucosal incision on the antrum greater curve, 4-5 cm proximal to the pylorus; B: Submucosal injection; C: Initial mucosal incision; D: Submucosal tunnel extended with i-knife until pylorus muscle is identified; E: The hook knife is then used to carefully cut the pylorus muscle; F: Myotomy of the pylorus; G: The myotomy is extended proximally for approximately 2

cm along the antrum; H: Closure of the mucosotomy is accomplished with Overstitch suturing device; I: Upper gastrointestinal contrast X-ray study shows no leak, with free flow of contrast through the pylorus.

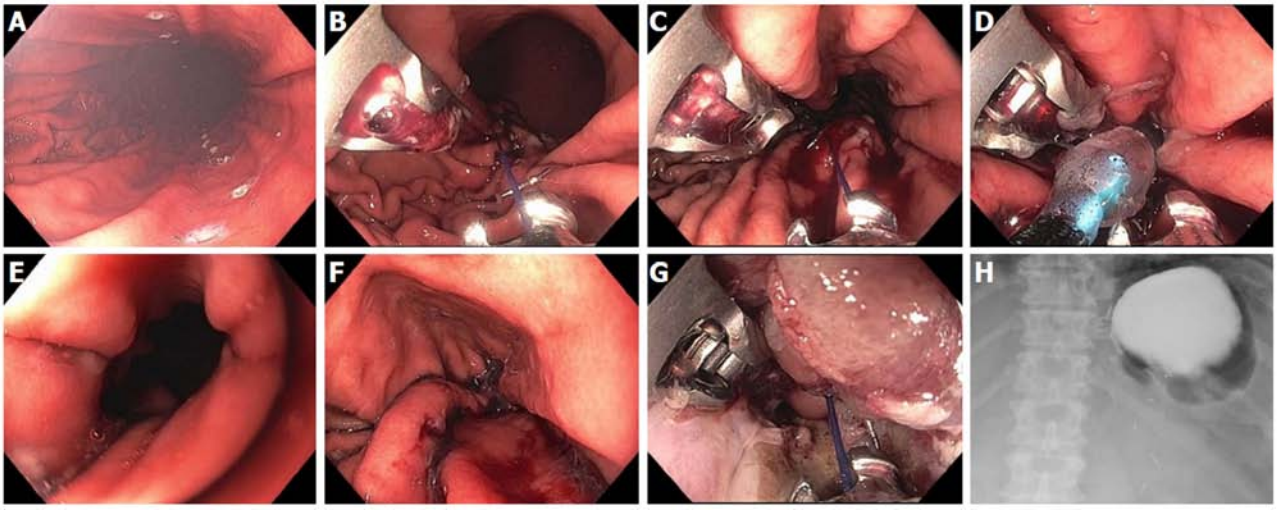

Figure 27 Endoscopic sleeve gastroplasty plus mucosal ablation and suturing of the esophageal gastric junction in a 45-year-old female with super morbid obesity, gastroparesis and gastroesophageal reflux disease. Her body mass index was 51 (327 lb) and she declined bariatric surgery. A: Markings created along anterior and posterior gastric wall to guide suture placement; B: First suture placed by taking bites at anterior, greater curve, posterior wall × 2; C: Total of 8 running sutures placed to complete the "sleeve"; D: At the very proximal aspect of the sleeve, a final suture is placed in a purse-string fashion around an 8 mm balloon to prevent complete closure; E: Endoscopic appearance within the completed sleeve; F: Endoscopic appearance of the proximal "pouch" which filled up with approximately 60 mL of water; G: Mucosal ablation and suturing of the esophageal gastric junction (MASE) procedure was performed as well, using 3 additional sutures, to treat her gastroesophageal reflux disease; H: UGI X-ray with contrast 1 d post endoscopic sleeve gastroplasty and MASE shows contrast filling the small gastric pouch with little to no passage through the sleeve at 1 h.

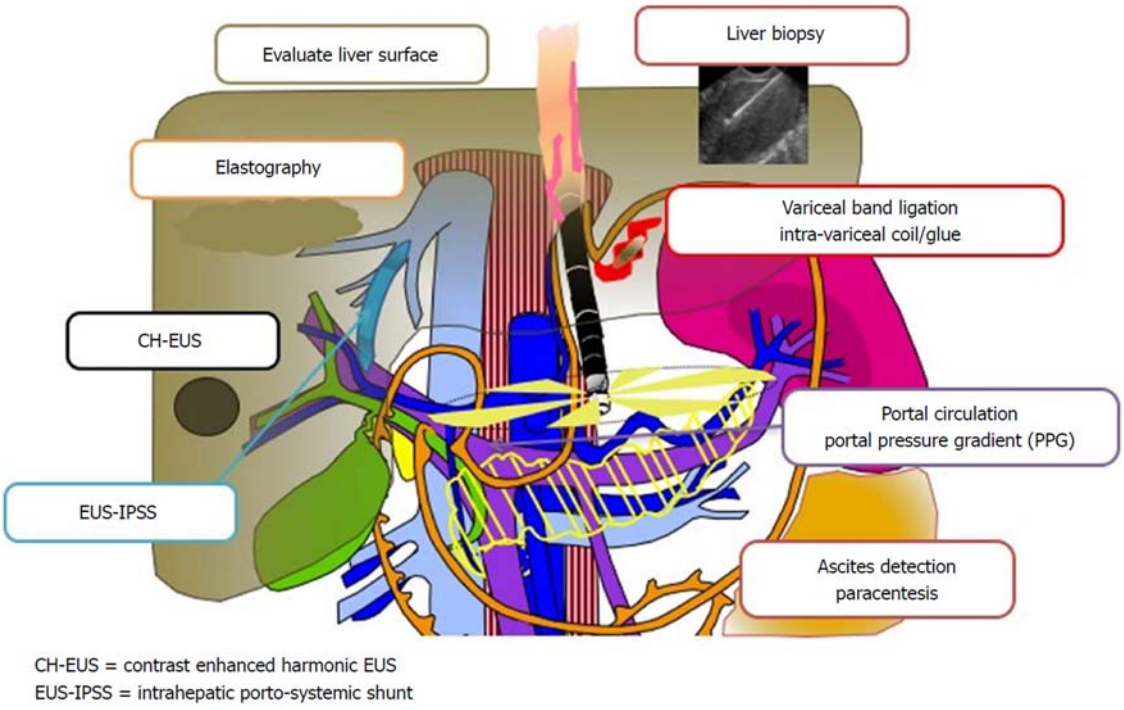

Figure 28 A promising new paradigm "Endo-Hepatology" intersects and integrates endoscopy in the diagnosis and treatment of liver disease. This includes: evaluation of the liver surface [by endoscopic ultrasound (EUS)], elastography to determine liver stiffness and fibrosis, contrast enhanced harmonic EUS to detect focal lesions, EUS-guided intrahepatic porto-systemic shunt, EUS-guided liver biopsy, endoscopic variceal band ligation and intra-variceal injection with glue +/- coil (by EUS), evaluation of the portal circulation and EUS-guided portosystemic pressure gradient, and detection of ascites with EUS-guided paracentesis. EUS: Endoscopic ultrasound; PPG: Portosystemic pressure gradient; IPSS: Intrahepatic porto-systemic shunt.

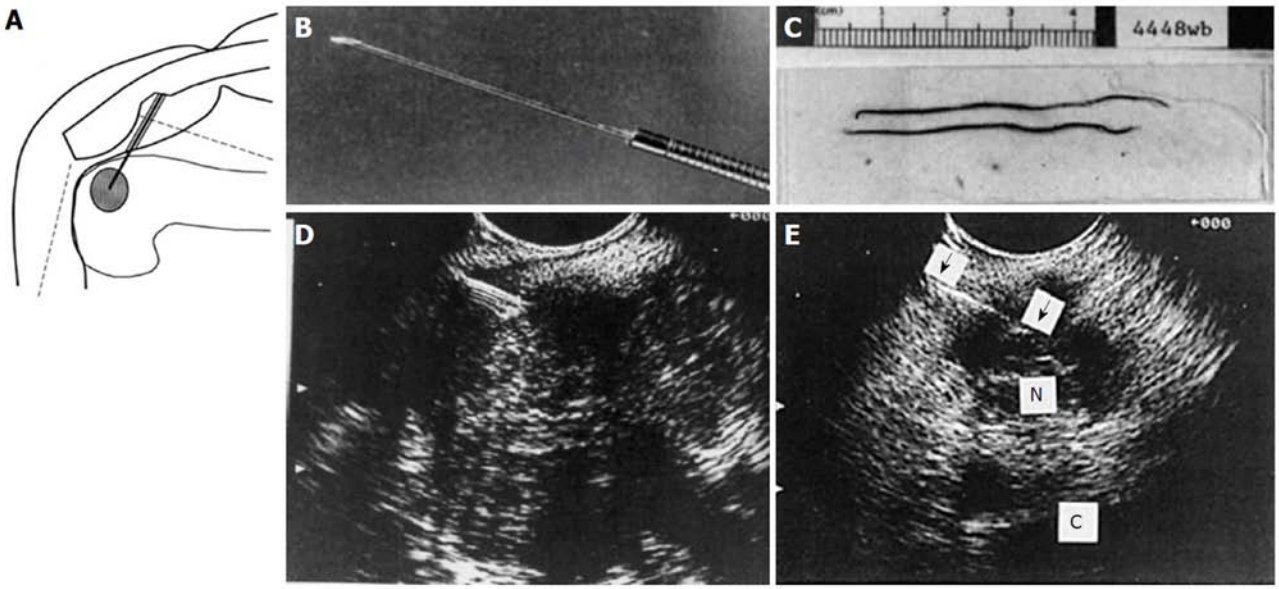

Figure 29 Endoscopic ultrasound guided fine needle aspiration initial series of 38 patients reported in 1994. A: Diagram of endoscopic ultrasound (EUS) guided fine needle aspiration (FNA) created on first generation Apple Macintosh computer; B: Black and white photo of prototype 23G 4-cm FNA needle attached to Teflon tubing; C: Photo of needle specimen, much of which was probably blood clot; D: Early linear array EUS image showing needle coming from left side, as established by ultrasound convention at the time, into a pancreatic tumor; E: EUS image of FNA needle into a 1.5-cm celiac lymph node. (Reprinted with permission from reference 173).

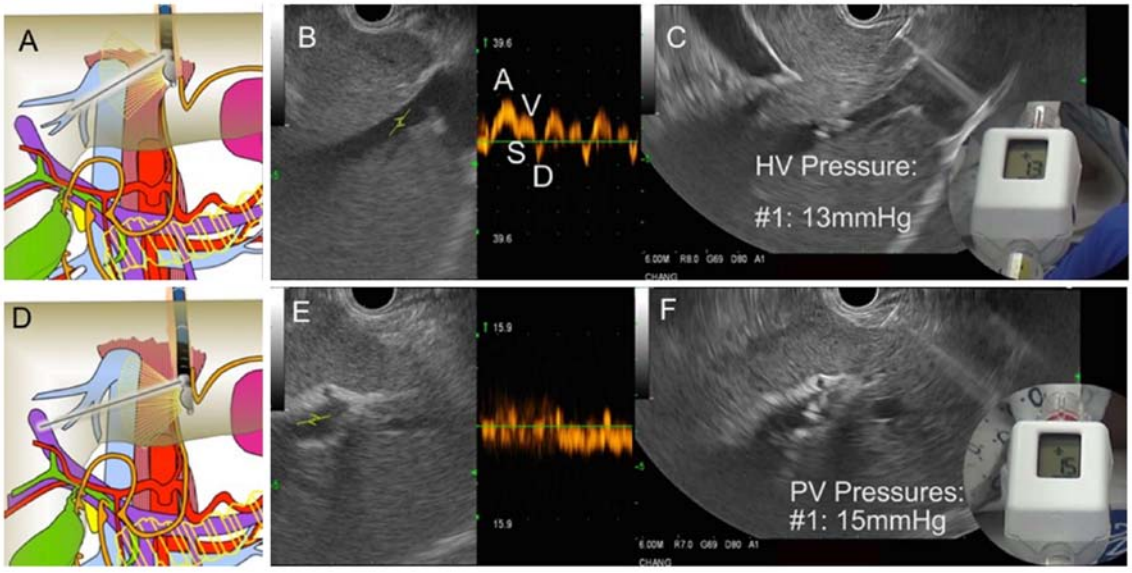

Figure 30 Endoscopic aultrasound guided portosystemic pressure gradient measurement in a patient with suspected cirrhosis. A: Diagram showing fine needle aspiration (FNA) needle within the middle hepatic vein; B: Endoscopic ultrasound (EUS) image of middle hepatic vein with Doppler wave form demonstrating 4 phases (ASVD); C: 25G needle placed directly through liver parenchyma into the middle hepatic vein; compact hand-held manometer showing a pressure of 13 mmHg; D: Diagram FNA needle within the left portal vein; E: EUS image of left portal vein (umbilical portion); typical Doppler waveform showing venous hum; F: 25G needle placed directly through liver parenchyma into the left portal vein; compact hand-held manometer showing a pressure of 15 mmHg. Thus, the EUS-portosystemic pressure gradient measurement is 2 mmHg, which is within normal range.

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I dedicate this article to my awesome wife, Chrissie, and our amazing 3 children: Kristen, Kelsey and Justin - who have been my inspiration and the wind beneath my wings. - Chang KJ.

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REVIEW

Hepatitis C virus core protein modulates several signaling pathways involved in hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the fifth most common cancer, and hepatitis C virus (HCV) infection plays a major role in HCC development. The molecular mechanisms by which HCV infection leads to HCC are varied. HCV core protein is an important risk factor in HCV-associated liver pathogenesis and can modulate several signaling pathways involved in cell cycle regulation, cell growth promotion, cell proliferation, apoptosis, oxidative stress and lipid metabolism. The dysregulation of signaling pathways such as transforming growth factor β (TGF-β), vascular endothelial growth factor (VEGF), Wnt/β-catenin (WNT), cyclooxygenase-2 (COX-2) and peroxisome proliferator-activated receptor α (PPARα) by HCV core protein is implicated in the development of HCC. Therefore, it has been suggested that this protein be considered a favorable target for further studies in the development of HCC. In addition, considering the axial role of these signaling pathways in HCC, they are considered druggable targets for cancer therapy. Therefore, using strategies to limit the dysregulation effects of core protein on these signaling pathways seems necessary to prevent HCV-related HCC.

Key words: Hepatitis C virus; Core protein; Transforming growth factor β; Vascular endothelial growth factor; Wnt/β-catenin; Cyclooxygenase-2; Peroxisome proliferator-activated receptor α; Hepatocellular carcinoma

Core tip: Hepatitis C virus (HCV) core protein can modulate several signaling pathways involved in cell cycle regulation, cell growth promotion, cell proliferation, apoptosis,

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oxidative stress and lipid metabolism. The dysregulation of these signaling pathways by HCV core protein is implicated in the development of hepatocellular carcinoma (HCC). Considering the axial role of these signaling pathways in HCC, they are considered druggable targets for cancer therapy. Therefore, using strategies to limit the dysregulation effects of core protein on these signaling pathways seems necessary to prevent HCV-related HCC.

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INTRODUCTION

At the beginning of the new century, it became clear that infectious agents have an undeniable role in the development of some cancers in humans. It is currently estimated that nearly 16%-18% of all human cancers are attributed to oncogenic viruses[1]. To date, several viruses are linked to cancer in humans, including Epstein-Barr virus (EBV), human papillomavirus (HPV), hepatitis B virus (HBV), human T-cell lymphotropic virus (HTLV), Kaposi's sarcoma herpesvirus (KSHV), Merkel cell polyomavirus (MCV) and hepatitis C virus (HCV)[2]. HCV is classified as a member of the *Flaviviridae* family and the *Hepacivirus* genus. HCV primarily affects the liver and causes chronic HCV infection. Chronic HCV infection inevitably causes additional liver damage, such as hepatitis, cirrhosis and hepatocellular carcinoma (HCC)[3]. Globally, an estimated 185 million people, equating to about 2.8% of the world population, have been infected with HCV[4]. Although the prevalence of HCV is declining, the burden of HCV-related mortality due to advanced liver disease is on the rise[4,5]. Two major forms of HCV infection are acute and chronic infection. Acute HCV infection can be seen in nearly 20%-25% of infected individuals, and approximately 15% of these acute infections develop recognizable symptomatic disease[6]. Chronic HCV infection develops in 75%-85% of acute HCV infections, and 10%-20% of all cases with chronic HCV infection slowly progress to liver cirrhosis, of which 1%-5% lead to HCC annually[7]. HCC is a significant health burden worldwide, and it is interesting to note that HCC is the fifth common malignant tumor in men (554000 cases) and the ninth common tumor in women (228000 cases). HCC is the second leading cause of cancer deaths worldwide and was responsible for about 746000 deaths in 2012[8]. Interestingly, 27% and 25% of cases with cirrhosis and HCC, respectively, are associated with HCV infection worldwide[9].

Globally, approximately 399000 deaths per year occur due to HCV-related liver diseases. According to the World Health Organization (WHO) treatment guidelines, more than 95% of HCV-infected patients can be cured by antiviral medicines. Therefore, the use of appropriate antiviral therapy can reduce the risk of death from HCC. The current standard of care for patients with HCV infection is therapy with a novel class of direct-acting antivirals (DAAs) in combination with pegylated-interferon α (Peg-IFNα) plus ribavirin. To date, the sustained virologic response (SVR) is the best indicator of successful therapy for chronic HCV infection. SVR is defined as having no detectable HCV RNA at 12-24 wk after completion of antiviral therapy, and increasing the chances of achieving SVR is the main goal of treatment[10]. In the treatment course of HCV infection, the rate of SVR has improved to over 95%[11]. Several studies showed that the risk of HCC is significantly lower in patients who achieved SVR following antiviral therapy compared to untreated patients[12-14]. Overall, more studies are needed to determine whether HCC is reduced among hepatitis C patients after achieving SVR. However, the achievement of SVR is important for HCC prevention. There is currently no prophylactic vaccine for HCV; however, research is ongoing to generate an efficient vaccine[15].

HCV is an enveloped positive-stranded RNA virus that exhibits significant variations across the viral genome. Accordingly, HCV is currently classified into seven genotypes and 67 confirmed subtypes[16]. The HCV genome is approximately 9600 nucleotides in length and encodes a single polyprotein of ~3000 amino acids (aa). The polyprotein is cleaved into ten different structural and nonstructural proteins by viral and cellular proteases. Structural proteins, including core, E1, E2 and p7, are

located near the 5′ end of the genome, and nonstructural proteins, including NS1, NS2, NS3, NS4A, NS4B, NS5A and NS5B, are located near the 3′ end of the genome[17] (Figure 1). These proteins make numerous interactions with host cell factors involved in important activities such as cell cycle regulation, cell proliferation, cell growth promotion, transcriptional regulation, apoptosis, oxidative stress and lipid metabolism[18,19]. Many lines of evidence clearly indicate that HCV proteins such as core, NS3, NS5A and NS5B can modulate several potentially oncogenic pathways. These proteins also potentiate oncogenic transformation through direct and indirect interactions with various transcription factors and their induction[20-24]. The core protein is an important HCV protein and is responsible for packaging viral RNA and virion budding. This protein (191 aa) is organized into three main domains that include an N-terminal two-thirds hydrophilic domain (D1, approximately 120 aa), a C-terminal one-third hydrophobic domain (D2, approximately 50 aa), and approximately the last 20 aa that serves as a signal sequence for targeting E1 (D3)[25] (Figure 1). Kunkel *et al*[26] showed that the residues 76-113 (tryptophan-rich region) are largely solvent exposed, suggesting that it may interact with cellular proteins. It has been shown that core protein has multi-functional activity and can interact with cellular proto-oncogenes and change their expression patterns, thereby leading to hepatocarcinogenesis[27]. Several lines of investigation have demonstrated that core protein plays a pivotal role in the modulation of several key signaling pathways involved in HCC, such as transforming growth factor β (TGF-β), nuclear factor κB (NF-κB), tumor necrosis factor α (TNF-α), cyclooxygenase-2 (COX-2), Wnt/β-catenin (WNT), vascular endothelial growth factor (VEGF), and peroxisome proliferator-activated receptor α (PPARα)[22,28-32]. The mechanisms by which core protein modulates these signaling pathways are extremely complicated. To prevent HCV-related HCC, the molecular events underlying the interactions between HCV core protein and the signaling pathways need to be well understood. In this review, we investigate how the interaction of HCV core protein with several signaling pathways contributes to the development of HCC in HCV-infected patients.

TGF-BETA SIGNALING PATHWAY

TGF-β is a multifunctional profibrotic cytokine that is found in three isoforms (TGF-β1-3). Of them, TGF-β1 plays a key role in the pathogenesis of liver inflammation, fibrosis, cirrhosis and HCC[33]. It is interesting to note that TGF-β is considered a central mediator of fibrogenesis and plays an important role in the regulation of tumorigenesis, as it controls numerous cellular functions, including apoptosis, differentiation, proliferation, extracellular matrix production, embryonic development, epithelial-mesenchymal transition (commonly known as EMT), and immune response[34,35]. Fibrosis is one of the most important consequences of TGF-β dysregulation, which is characterized by excessive accumulation of extracellular matrix (commonly known as ECM). TGF-β activity is mediated through activation and proliferation of hepatic stellate cells and connective tissue growth factor. Eventually, progressive fibrosis leads to the development of cirrhosis and HCC[36]. TGF-β acts as a double-edged sword depending on the cellular context; in the early stages of cancer development, it exhibits anti-tumor effects, while in the late stages, it has tumor-promoting activities[37]. TGF-β is implicated in several human diseases such as cardiovascular diseases, connective tissue diseases, skeletal and muscular disorders, reproductive disorders, autoimmune disorders, fibrotic disease, atherosclerosis and carcinogenesis[38,39]. Studies have shown that the downstream signaling pathways for TGF-β involve both canonical (Smad-dependent) and non-canonical (Smad-independent) pathways. TGF-β can induce fibrosis *via* activation of these two pathways, which results in activation of myofibroblasts and excessive production of ECM[40-43]. Smads mediate intracellular responses to TGF-β and have three classes, including receptor-regulated Smads (named R-Smads, including Smad1, 2, 3 ,5 and 8), comediator Smads (named Co-Smad, including Smad4) and inhibitory Smads (named I-Smads, including Smad6 and 7)[44]. Smads are regulated *via* direct phosphorylation by kinase activities of TGF-β receptors (TβRI and TβRII). The model of TGF-β-induced Smad activation is as follows: TGF-β binds to its receptor TβRII, which further interacts and activates TβRI. Activated TβRI then activates Smad2 and Smad3 *via* phosphorylation. Subsequently, the activated Smad2/3 forms a heterotrimer with Smad4 that translocates into the cell nucleus. Ultimately, the complex associates with other transcription factors and regulates the expression of target genes by binding to promoters containing the minimal Smad binding element[45,46]. Furthermore, TGF-β can activate non-canonical pathways such as JAK, Erk, JNK, p38 MAPK kinase, Ras and RhoA[40,47] (Figure 2). It has also been shown that

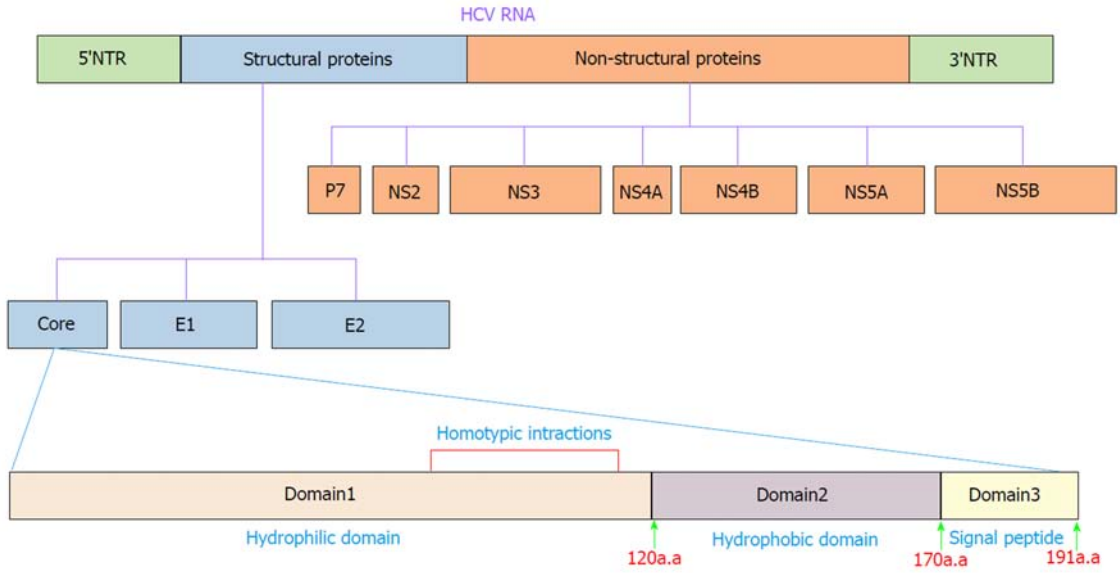

Figure 1 Genome organization of HCV. Scheme of HCV core protein domains and functional residues. HCV: Hepatitis C virus.

HCV-induced transcription factors such as AP-1, Sp1, NF-κB, EGR-1, USF and STAT-3 can active the TGF-β1 promoter[48]. Taken together, it should be noted that the dysregulation of TGF-β signaling pathway could result in cancer development through either direct or indirect effects on other intracellular signaling pathways involved in carcinogenesis.

It has been indicated that oncogenic viruses such as HCV, HBV, HPV, EBV, KSHV and HTLV-1 can modulate TGF-β signaling pathway through various direct or indirect mechanisms, suggesting that this pathway is a desirable target for connecting viral proteins[37]. Several studies support the contention that HCV induces TGF-β1 secretion in HCV patients, as TGF-β1 levels are extremely high in these patients[49-51]. The results of the study performed by Jee *et al*[52] indicated that TGF-β1 protein in HCV-infected cells and in neighboring cells was more than 20-fold higher than in uninfected cells, and this increased production was observed 2 d after HCV infection. Several studies have demonstrated that HCV core protein can induce TGF-β1 promoter activity and directly and indirectly upregulate its gene expression. These findings suggest that HCV infection is one mechanism by which liver fibrosis can be exacerbated[28,53-56]. Taniguchi *et al*[28] showed that TGF-β1 mRNA expression is increased by HCV core protein, whereas TGF-β2 and TGF-β3 mRNA levels do not change upon core protein expression. The results of their study suggested that bases 331 to 376 in the TGF-β1 promoter are upregulated by HCV core protein[28]. In studies conducted by Battaglia *et al*[57] and Pavio *et al*[58], it was revealed that core protein is able to switch TGF-β from a tumor suppressor to tumor promoter by decreasing hepatocyte apoptosis and increasing EMT by decreasing Smad3 activation. The Smad proteins consist of two principal domains, DNA-binding domain (N-terminal Mad homology 1, MH1 domain) and protein-protein interacting module (C-terminal Mad homology 2, MH2 domain). Pavio *et al*[58,59] indicated that the central domain (59-126 aa) of core protein binds to the MH1 domain of Smad3, leading to TGF-β inhibition. Cheng *et al*[60] explained that HCV core protein utilizes various mechanisms to regulate TGF-β, including the following: (1) suppression of TGF-β/Smad3-mediated transcriptional activation through interference with the DNA-binding ability of Smad3; (2) block of TGF-β-induced G1 phase arrest *via* downregulation of TGF-β-induced p21 promoter activation; and (3) resistance to TGF-β/Smad3-mediated apoptosis. Notably, over-expression of HCV core protein indirectly induces TGF-β production by increased reactive oxygen species production, which in turn increases JNK, Erk and p38 MAP kinase activity in a NF-κB-dependent manner[56,61,62] (Figure 2). In addition, Shin *et al*[54] revealed that HCV core protein can regulate other factors associated with fibrosis, such as connective tissue growth factor, TβRII and TGF-β1. Nevertheless, considering the pivotal role of TGF-β in the development of fibrosis and tumor progression, this pathway is an important pharmaceutical target to prevent cancer progression. Furthermore, treatment with antiviral drugs such as DAAs in combination with Peg-IFNα and ribavirin increases the chances of achieving SVR. On the other hand, there are some but not conclusive data that TGF-β1 serum levels in chronic hepatitis C patients under antiviral therapy significantly decreases, especially

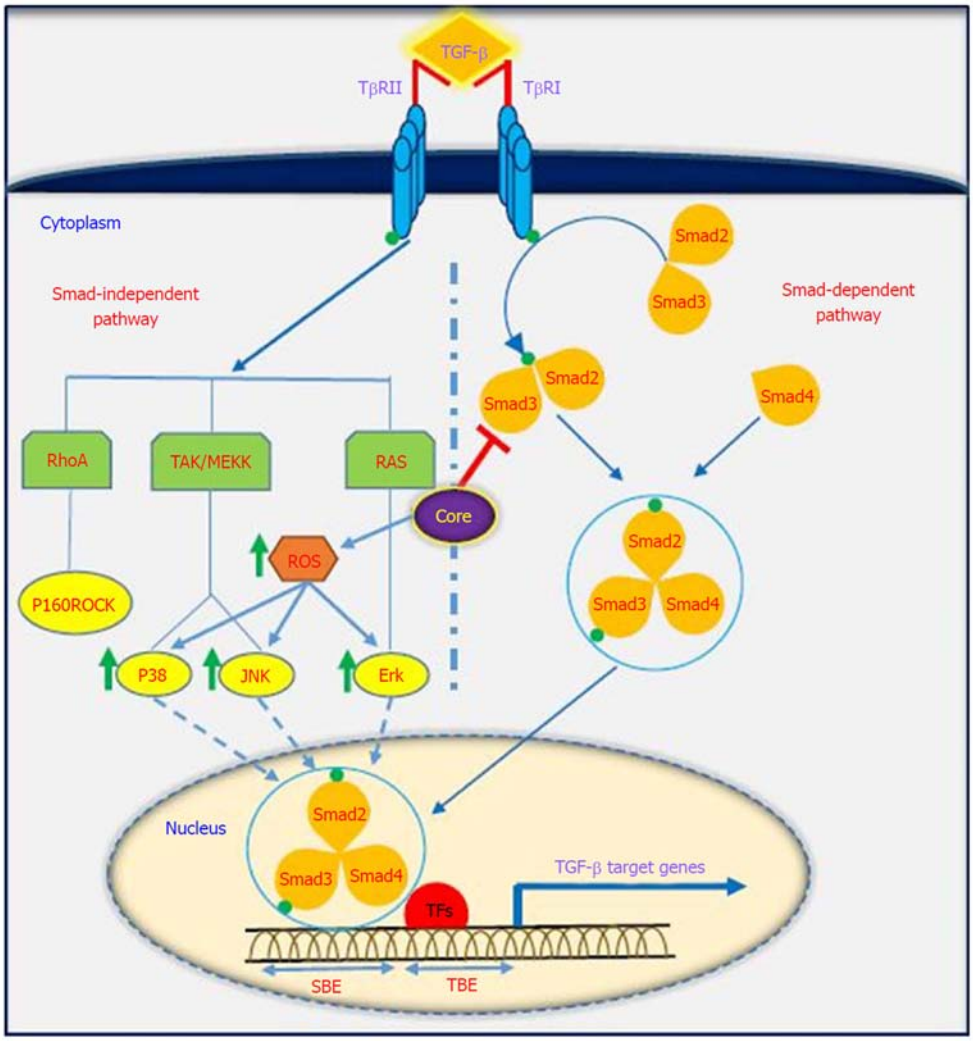

Figure 2 Interaction between the TGF-β signaling pathway and HCV core protein. TGF-β regulates target gene transcription *via* canonical (Smad-dependent) and non-canonical (Smad-independent) pathways, and HCV core protein modulates TGF-β signaling pathway in various ways. For detailed information, see text. TGF-β: Transforming growth factor β; HCV: Hepatitis C virus; TβR: TGF-β receptor; ROS: Receptor tyrosine kinase c-ros oncogene 1; TFs: Transcription factors; Smad: Small mothers against decapentaplegic; RhoA: Ras homolog gene family member A; MEKK: Mitogen-activated protein kinase kinase; RAS: Rat sarcoma; ERK: Extracellular signal-regulated kinase; JNKs: Jun N-terminal kinases; P160ROCK: Rho-associated coiled-coil containing protein kinase 1; SBEs: SMAD-binding elements; TBE: T-box binding element.

in patients achieving SVR[63-66]. In this condition, HCV proteins are unable to upregulate TGF-β1 expression, which in turn leads to reduced fibrogenesis and prevention of cancer progression.

VEGF SIGNALING PATHWAY

VEGF is a signal protein that is produced by most parenchymal cells and stimulates angiogenesis. In cancer progression, VEGF is the key mediator of angiogenesis and vasculogenesis, which leads to the formation of new blood vessels from pre-existing vessels. In turn, this allows tumors to access oxygen and nutrients[67]. VEGFA, also known as VEGF, is a protein with vascular permeability activity that is a member of a family of growth factors. In addition, VEGFB, VEGFC, VEGFD and placental growth factor are also in this family. These growth factors play an important role in angiogenesis and differ in their biological functions and expression patterns[68]. Several factors play roles in the upregulation of VEGF. It has been demonstrated that a number of growth factors such as PDGF, epidermal growth factor, fibroblast growth factor, TNF, TGF-β and interleukin-1 can induce VEGF gene expression[69]. VEGF applies its effects by binding to VEGF receptors, which are expressed on vascular endothelial cells. The VEGF receptors include VEGF receptor-1 (Flt-1), VEGF receptor-2 (KDR) and VEGF receptor-3 (Flt-4)[70]. VEGF has several roles, including the following: (1) inducing angiogenesis through a direct impact on endothelial cells; (2)

inducing cells to invade the underlying matrix and to form capillary-like tubules; (3) elicitation of non-mitogenic responses by vascular endothelial cells; (4) instrumental in maintaining the viability of immature vasculature and inducing hemotaxis; and (5) the expression of plasminogen activators and collagenases in endothelial cells[71]. It should be noted that the induction and activation of hypoxia-inducible factor-1 alpha (HIF-1α) is a major inducer of VEGF expression in tumors. It is one of the first transcription factors in response to hypoxia and is a closely related angiogenesis factor. HIF-1α responds to hypoxia through binding to hypoxic response element, which in turn leads to an increase in VEGF protein expression because it plays a regulatory role in VEGF expression[72,73]. Given the importance of the angiogenic effects of VEGF, dysregulation of its expression is important in disease processes and progression toward cancer. A large body of evidence exists that suggests a role for VEGF in tumorigenesis in human cancers[74].

Oncogenic viruses such as HCV, EBV, HPV, KSHV and HBV can upregulate VEGF with the use of cellular signaling machinery, which leads to angiogenesis[75]. Several reports have shown upregulation of VEGF in patients with HCV-related HCC[76-80]. Mukozu *et al*[77] demonstrated that VEGF serum levels of patients with HCV-related HCC are significantly higher than those in the control group. HCV core protein can upregulate cellular VEGF expression through HIF-1α transcription factors and activator protein 1 (AP-1). Core protein induces over-expression and stabilization of HIF-1α, which in turn induces VEGF expression[81-84]. Abe *et al*[82] showed that core protein increases HIF-1α expression level by activating the NF-κB signaling pathway, which leads to an increase in VEGF expression under hypoxia followed by HIF-1α upregulation. They also observed that when cells were incubated with HIF-1α inhibitor, VEGF expression clearly decreased[82]. In a study performed by Zhu *et al*[83] in Huh7.5.1 cells, it was demonstrated that core protein contributes to VEGF biosynthesis by inducing VEGF expression and secretion. They indicated that using HIF-1α siRNA in Huh7.5.1 cells, which results in reducing expression of HIF-1α, significantly reduces VEGF expression. Shao *et al*[85] indicated that VEGF expression increased due to activation of AP-1 transcription factor, because the promoter region of VEGF contains binding sites for AP-1 transcription factors, which lead to enhanced VEGF expression *via* promoter binding. This observation is in line with previous studies of AP-1 activity associated with VEGF expression and HCV core protein-induced AP-1 activity[86-89]. HCV core protein affects androgen receptor (AR) transcriptional activity by activating several signaling pathways such as phosphatidylinositol 3-kinase (PI3K)/AKT and JAK/STAT3. Since VEGF is a target gene for AR in the liver, HCV core protein increases VEGF expression through increased activity of the AR signaling pathway[90]. Hassan *et al*[31] recently reported that HCV core protein induces VEGF expression mediated by JNK, p38 and ERK signaling pathways (Figure 3). Given the results of various studies, HCV core protein can upregulate the VEGF signaling pathway. These data increase our insights into the molecular mechanisms by which HCV core protein mediates angiogenesis in HCV-infected patients.

WNT SIGNALING PATHWAY

The WNT signaling pathways are a group of signal transduction pathways that regulate different cellular processes such as cell polarity, organogenesis, cell migration and neural patterning during embryonic development[91]. Alteration of WNT activity has been linked to the development of HCC and other liver diseases[92]. β-catenin has a crucial role in Wnt signaling and also tightly binds to the cytoplasmic domain of type I cadherins and is implicated in the structural organization and function of cadherins[93]. Wnts are secreted cysteine-rich lipid-modified glycoproteins[94,95] that bind to the N-terminal extra-cellular cysteine-rich domain of the Frizzled (Fz or Fzd) receptor family and low-density-lipoprotein-related protein 5/6 (LRP5/6) as co-receptors[96,97]. When WNT signaling is inactive, cytoplasmic β-catenin interacts with a multiprotein degradation complex comprised of casein kinase I (CK1), adenomatous polyposis coli gene product (APC), glycogen synthase kinase 3β (GSK3β) and Axin[98]. Axin binds to newly synthesized β-catenin, which is subsequently phosphorylated by CKI and GSK3 on conserved Ser and Thr residues in the amino terminus[99]. Following phosphorylation, β-catenin is targeted for proteasome-dependent degradation, including an interaction with β-transducin repeat-containing protein (β-TrCP), a part of the E3 ubiquitin ligase complex, leading to β-catenin ubiquitination and degradation[100,101]. WNT signaling is regulated by secreted proteins, including secreted Frizzled-related proteins (sFRPs) and Wnt inhibitory protein (WIF) that can bind to Wnts and prevent interactions between Wnt

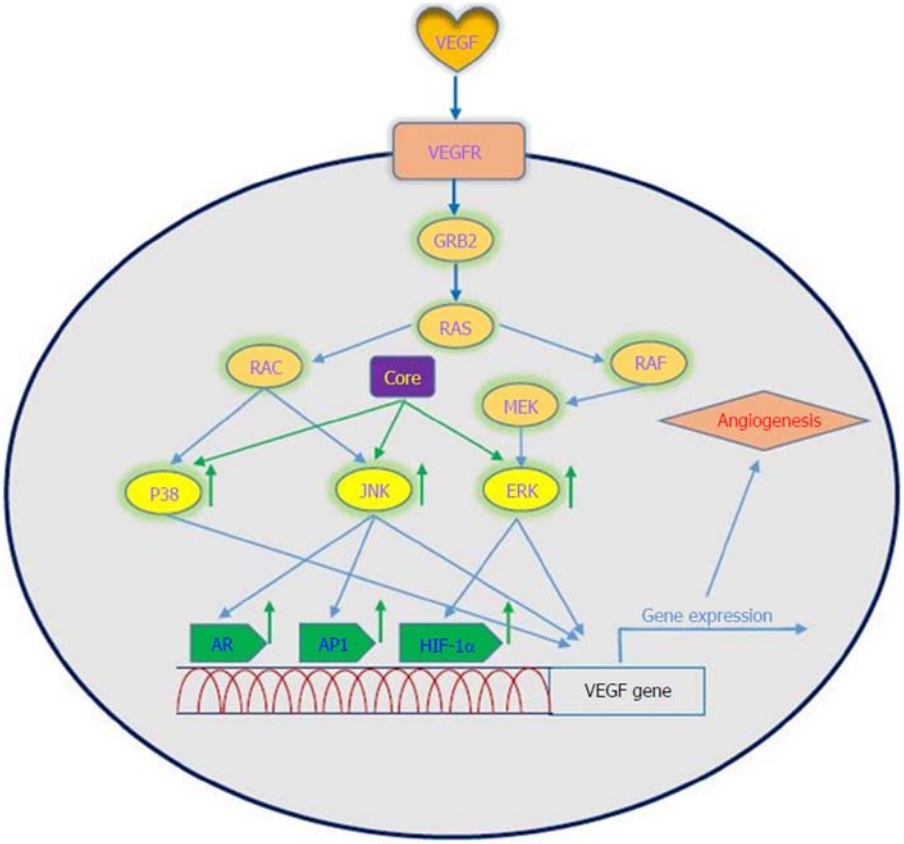

Figure 3 Interaction between VEGF signaling pathway and HCV core protein. HCV core protein upregulates VEGF expression mediated by AR, AP1 and HIF-1α. VEGF: Vascular endothelial growth factor; VEGFRs: Vascular endothelial growth factor receptors; GRB2: Growth factor receptor bound protein 2; RAS: Rat sarcoma; RAF: Rapidly accelerated fibrosarcoma; MEK: Mitogen-activated protein kinase kinase; ERK: Extracellular signal-regulated kinase; JNKs: Jun N-terminal kinases; AR: Androgen receptor; AP1: Activator protein 1; HIF-1α: Hypoxia-inducible factor-1 alpha.

and Wnt receptors. Other Wnt inhibitors include Dickkopf (DKK) proteins, which inhibit Wnt signaling by binding to LRP5/6[95]. If the concentration of Wnts increases, Wnts interact with receptors to activate Dishevelled (Dsh or Dvl) protein. Dvl, a modular phosphoprotein, is phosphorylated by several kinases such as CK1. Activated Dvl recruits the destruction complex to the plasma membrane and binds to Axin and Fz[102,103]. Intriguingly, Axin binds to the cytoplasmic domain of LRP5/6. When Dvl is activated, it leads to the inhibition of GSK3 activity, which activates a complex series of events that decreases β-catenin phosphorylation and degradation[104]. Therefore, β-catenin accumulates in the nucleus and activates the transcription of target genes through interaction with DNA-bound T-cell factor (TCF) and lymphoid enhancer-binding factor 1 (LEF) family members[105]. WNT signaling has been implicated in the modulation of innate immunity by stimulating invariant natural killer T cell (iNKT) responses and production of chemokine-like chemotactic factor leukocyte cell-derived chemotaxin 2 (LECT2) or by decreasing the release of tumor necrosis factor[106-108]. LECT2 is involved in inflammation, chemotaxis, immunomodulation, cell proliferation and carcinogenesis. In addition, LECT2 signaling induces inflammatory responses by activating the proinflammatory NF-κB pathway[109]. Abnormal Wnt and Fz expression in hepatocytes, stellate and Kupffer cells might play important roles in liver pathobiology[110,111].

Khanizadeh *et al*[112] indicated that oncogenic viruses can interact with and modulate the Wnt signaling pathway. Because the WNT signaling pathway is a key pathway in HCV-positive HCC, it is important to understand how HCV core protein induces liver carcinogenesis through Wnt signaling[113] (Figure 4). DNA methylation also contributes to the development of HCC. Quan *et al*[114] showed that HCV core protein can increase Wnt signaling *via* down-regulation of the sFRP1 promoter in HCC cells through methylation and histone deacetylation. In another study, Umer *et al*[115] demonstrated that sFRP2 and DKK1 promoters of Wnt inhibitor genes increase DNA methylation in HCC patients compared to normal controls. In addition, HCV core protein is involved in hypermethylation of the CDH1 (E-cadherin) gene

promoter. Decreased production of E-cadherin induces Wnt signaling activation[116]. HCV core protein enhances β-catenin expression and nuclear stabilization by inactivating GSK-3β. Nuclear accumulation of β-catenin forms a transcriptional complex with TCF and activates downstream target genes, such as c-Myc, Cyclin D1 and WNT1 inducible signaling pathway (WISP-2), which regulate cell growth and cell cycle progression[22,113]. Additionally, HCV core protein elevates the expression of LRP5/6 co-receptors and FZD receptors and releases β-catenin from the β-catenin-E-cadherin complexes[108]. Taken together, the direct involvement of HCV core protein in the Wnt pathway is an attractive candidate to mediate liver pathogenesis.

COX-2 SIGNALING PATHWAY

The cyclooxygenase (COX) family consists of constitutive COX-1, inducible COX-2 and COX-3, which are involved in prostaglandin synthesis by conversion of arachidonic acid to prostanoids, including thromboxanes and prostaglandins[117,118]. COX-2 can be induced by tumor promoters, cytokines and growth factors *via* the cis-acting elements within the 5′ UTR of the COX-2 gene and is known as a pathogenic factor involved in cellular proliferation, anti-apoptosis activity, inflammation, fibrogenesis and tumorigenesis. Moreover, increased levels of prostaglandin E_2 and COX-2 contribute to various biological processes, including oxidative stress, liver damage, bacterial and viral infection, acute and chronic inflammation and cancer[117,119,120].

Studies have shown that there is a close relationship between oncogenic viruses such as HCV, HBV, EBV and HPV and COX-2[121-124]. Previous reports demonstrated that increased production of COX-2 is observed in response to HCV infection[125-127]. Overexpression of COX-2 supports HCV replication and has a potential role in hepatocarcinogenesis in HCC and human hepatoma cell lines[121,128,129]. Several studies have previously documented that HCV stimulates COX-2 expression *via* oxidative stress[130,131]. Oxidative stress is a key contributor in liver fibrosis and carcinogenesis related to HCV infection[132]. HCV core protein upregulates COX-2 levels in hepatocytes and has carcinogenic effects that lead to HCC[62,121,133]. Jahan *et al*[30] demonstrated that core protein of HCV genotype 3a induces COX-2 expression in Huh-7 cells compared to the core protein of HCV genotype 1a. Conversely, several studies have shown that HCV core protein downregulates COX-2 expression. HCV might avoid the inflammatory responses of host by downregulating this signaling pathway[29,117,134]. COX-2 plays a crucial role in the production of matrix metalloproteinases (MMPs), particularly MMP-2 and MMP-9, by liver cells, which are associated with cell migration *via* degradation of cellular and extracellular components during organ development, morphogenesis, tissue damage and cancer invasion[135,136]. COX-2 is involved in the regulation of superoxide anion expression, namely NADPH oxidase 1 (NOX1) and NADPH oxidase 4 (NOX4), in hepatocytes[137]. Lan *et al*[138] demonstrated that NOX1 and NOX4 are increased in cirrhotic patients and play an important role in liver fibrosis *via* regulating proliferation, inflammation and fibrogenesis in hepatic stellate cells. COX-2 stimulates β-catenin activation, which leads to WNT pathway activation and subsequent cell growth and proliferation through its interaction with TCF in the nucleus[139]. COX-2 induces the production of VEGF and the anti-apoptotic proteins of the Bcl-2 family, which are associated with an increase in angiogenesis, invasiveness, and resistance to apoptosis[140]. Leng *et al*[141] showed a positive correlation between COX-2 and phosphatidylinositol 3-kinase-Protein Kinase B (PI3K-Akt/PKB) pathway activation in human HCC tissue. PI3K-Akt/PKB plays a major role in cancer development and progression by inhibiting apoptosis and stimulating cell proliferation. A recent study showed that long non-coding RNA (lncRNA) COX-2 inhibits immune evasion, migration and invasion of HCC cells and may provide a novel theoretical basis for HCC treatment[142]. In view of these facts, there is a direct relationship between HCV core protein and COX-2 overexpression, which in turn leads to alterations in cell signaling pathways (Figure 5). Therefore, the COX-2 signaling pathway should be considered a target to prevent HCV replication and HCV-associated diseases.

PPAR ALPHA SIGNALING PATHWAY

PPARs are a family of nuclear receptor proteins that belong to the steroid/thyroid hormone receptor superfamily and function as transcription factors. This family has three members, including PPARα, PPARγ and PPARβ/δ[143], which play regulatory roles in cellular processes such as differentiation, proliferation, inflammation,

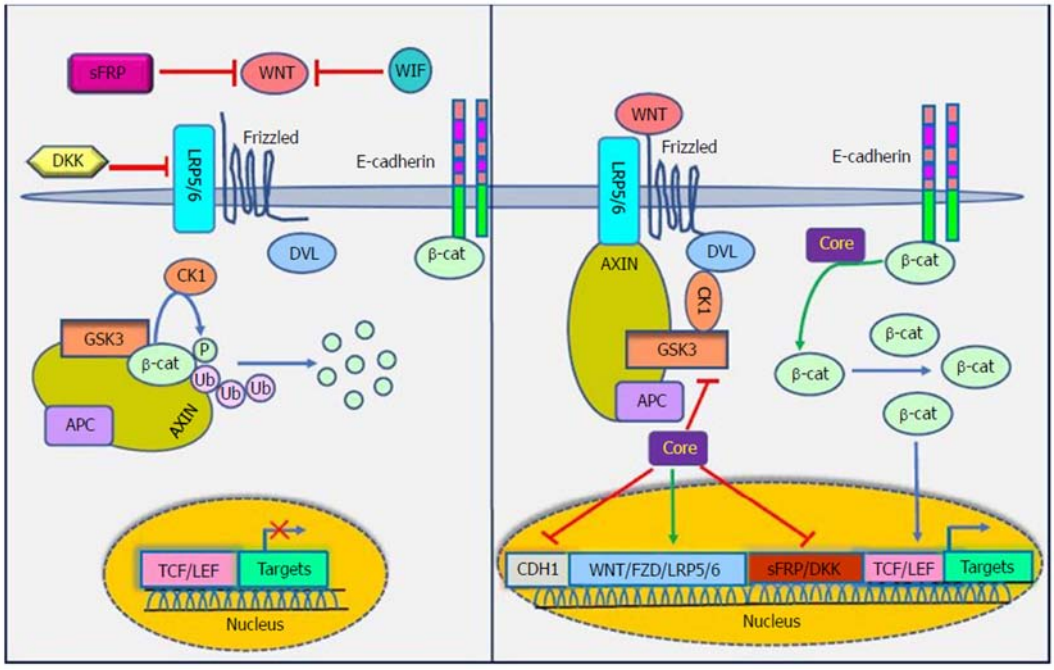

Figure 4 Interaction between Wnt/β-catenin signaling pathway and HCV core protein. HCV core protein increases the expression of Fzd and LRP5/6, decreases the expression of sFRPs and Dickkopf and suppresses the E-cadherin gene promoter. The components shown are explained in more detail in the text. sFRPs: Secreted Frizzled-related proteins; WIF: Wnt inhibitory factor; DKK: Dickkopf WNT signaling pathway inhibitor; LRP5/6: Low-density-lipoprotein-related protein 5/6; Fzd: Frizzled; CDH1: E-cadherin; DVL: Dishevelled segment polarity protein; GSK3: Glycogen synthase kinase 3; CK1: Casein kinase 1; UB: Ubiquitin protein; APC: Adenomatous polyposis coli; TCF/LEF: Transcription factor/lymphoid enhancer-binding factor.

oxidative stress, tumorigenesis and metabolism (carbohydrate, lipid, protein)[144]. In order to function, all PPARs initially heterodimerize with the retinoid X receptor (RXR) and then bind to peroxisome proliferator hormone-response elements (PPREs), which are located in the promoter region of PPAR target genes[145]. PPARα is one of the most abundant nuclear receptors expressed in hepatocytes, and it acts as an important and vital regulator in lipid and lipoprotein metabolism. It becomes activated by several molecules such as long-chain unsaturated fatty acids, and once activated, it promotes lipid clearance through β-oxidation upregulation[146]. The relationship between PPARα and cancer development is very complicated. Epidemiological studies have shown that PPARα has carcinogenic consequences in the liver of humans and rodents[147-150]. It is also noted that long-term administration of PPARα ligands can lead to increased ROS generation, accelerated hepatocyte proliferation and development of HCC[151]. In other words, persistent PPARα activation induces hepatic steatosis through increased liver triglyceride synthesis. On the other hand, hepatic steatosis promotes HCC development and acts as an important accelerating factor of HCC in HCV-infected patients[152-154]. Therefore, the hepatic steatosis induced by alteration of fatty acid metabolism in hepatocytes may act as a mediator in causing HCC in HCV-infected patients. Thus, it is important to understand the interaction between HCV infection and PPARα signaling pathway.

On the basis of several lines of evidence, PPARα activity is impaired in patients with chronic hepatitis C infection, which may contribute to hepatocarcinogenesis[32,155]. Dharancy *et al*[32] showed that PPARα expression is significantly decreased in HCV-infected patients compared to the control group. In this study, HCV core protein expression in HepG2 cells led to disruption of PPARα transcriptional activity, demonstrated by decreased expression of the PPARα target gene CPT1A. Shen *et al*[156] indicated that PPARα has an inhibitory effect on NF-κB. On the other hand, HCV core protein can disrupt PPARα activity. From this point of view, HCV core protein negatively regulates the repressive effect of PPARα on nuclear NF-κB, which in turn leads to NF-κB activation and HCC development. Studies have shown that HCV core protein can indirectly activate PPARα through activation and phosphorylation of ERK1/2 and P38 MAPK[155,157] (Figure 6). HCV core protein also binds to RXR and enhances RXR transcriptional activity, leading to the upregulation of some lipid metabolism enzymes. This result suggests that the dysregulation of RXR by HCV core protein may contribute to HCC development[158]. These findings demonstrate that HCV core protein disrupts PPARα activity, leading to HCC in HCV-infected patients. Thus, PPARα can be considered a new therapeutic target for preventing HCC.

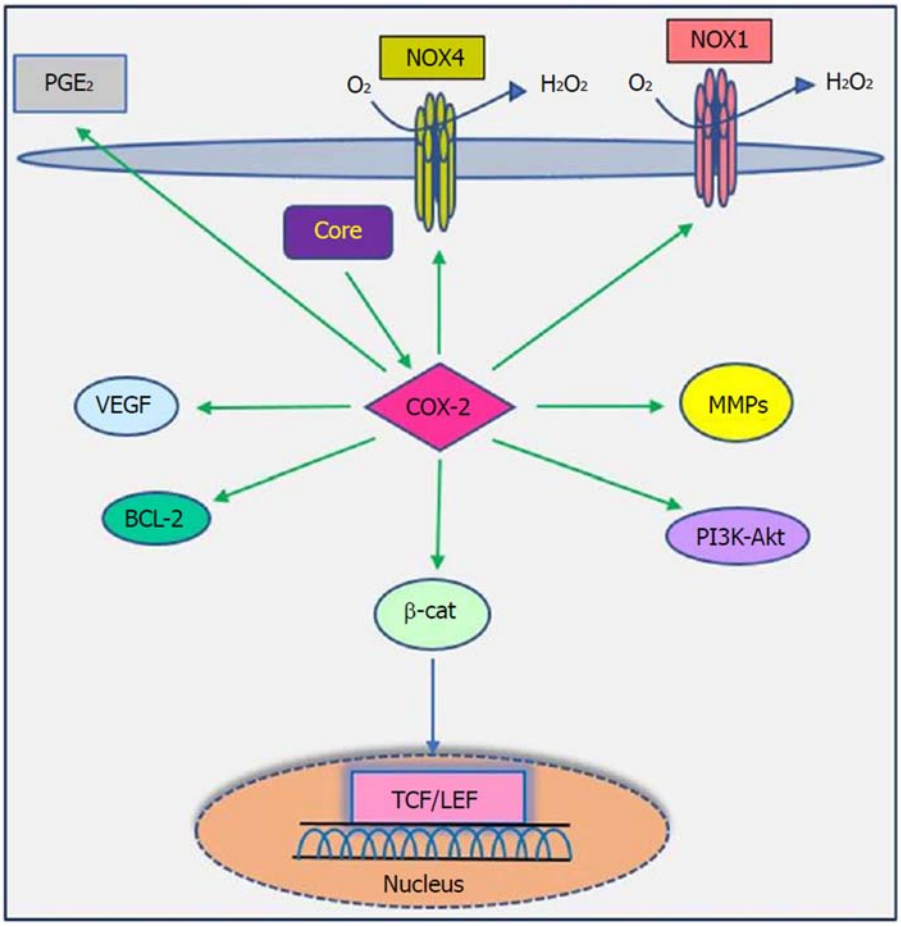

Figure 5 Interaction between COX-2 signaling pathway and HCV core protein. HCV core protein enhances the expression of COX-2 and increases synthesis of PGs, which in turn lead to hepatocarcinogenesis. For detailed information, see text. COX-2: Cyclooxygenase-2; HCV: Hepatitis C virus; PGs: Prostaglandins; PGE_2: Prostaglandins E2; NOX: NADPH oxidase; VEGF: Vascular endothelial growth factor; MMPs: Matrix metalloproteinases; BCL-2: B-cell lymphoma 2; PI3K/AKT: Phosphatidylinositide 3-kinases/serine/threonine-protein kinase; TCF/LEF: Transcription factor/lymphoid enhancer-binding factor.

CONCLUSION

HCV can cause liver diseases, especially HCC, through the modulation of various signaling pathways. TGF-β, VEGF, WNT, COX-2 and PPARα signaling pathways play important roles in the regulation of fibrogenesis, angiogenesis and tumorigenesis by controlling cell proliferation, apoptosis, transcriptional regulation and cell growth promotion. Therefore, their dysregulation is associated with HCC. As previously mentioned, HCV core protein uses various mechanisms to dysregulate these pathways. Hence, it seems necessary to undertake major research efforts for a better understanding of how HCV core protein leads to the dysregulation of these signaling pathways, which in turn helps in designing effective therapeutic methods. Furthermore, these signaling pathways should be considered therapeutic targets for cancer therapy, and prevention programs should be implemented to prevent their overexpression. Given the commercially available inhibitors against these pathways, HCV-related HCC development can be prevented in the near future. Moreover, the use of antiviral drugs such as DAAs in combination with Peg-IFNα plus ribavirin leads to SVR in chronic hepatitis C patients, which in turn can help reduce some of the factors involved in cancer development, such as TGF-β1. However, these findings require more research.

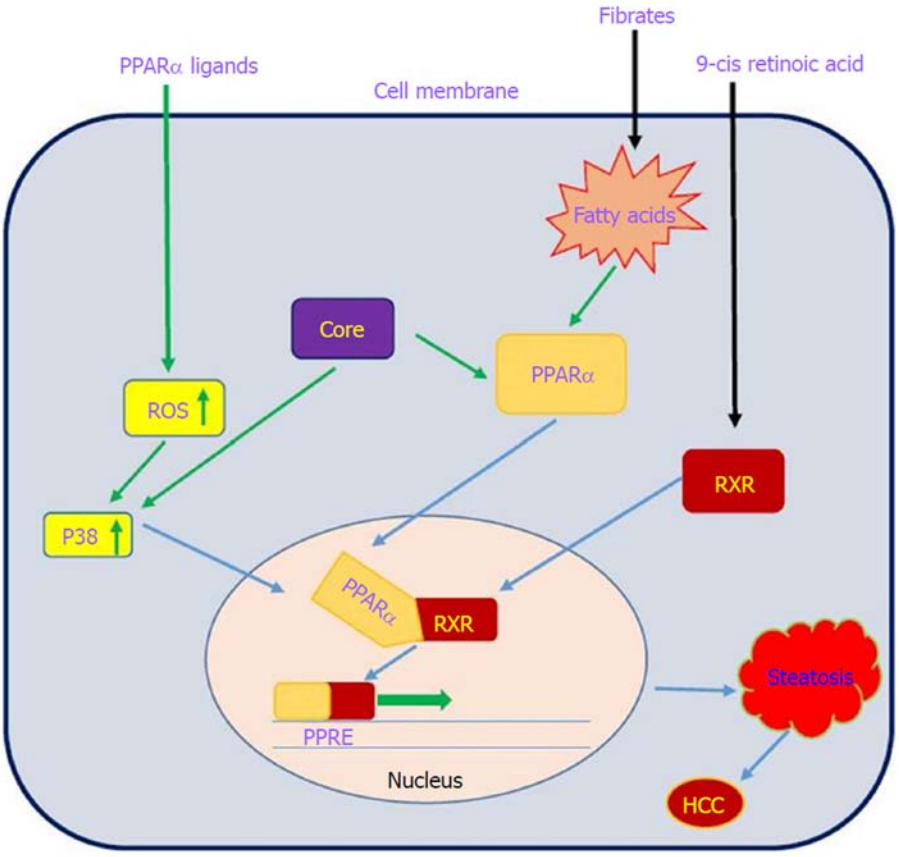

Figure 6 Interaction between the PPARα signaling pathway and HCV core protein. HCV core protein can directly and indirectly activate PPARα, and PPARα activation increases liver triglyceride accumulation, leading to hepatic steatosis. See the text for more details. PPARα: Peroxisome proliferator-activated receptor α; ROS: Receptor tyrosine kinase c-ros oncogene 1; RXR: Retinoid X receptor; PPRE: Peroxisome proliferator hormone response elements.

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MINIREVIEWS

Role of surveillance imaging and endoscopy in colorectal cancer follow-up: Quality over quantity?

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Abstract

Colorectal cancer (CRC) is a prevalent disease and represents a major cause of morbidity and mortality in the developed world. Intensive post-treatment surveillance is routinely recommended by major expert groups for early stage (II and III) CRC survivors because previous meta-analyses showed a modest, but significant survival benefit. This practice has been recently challenged based on data emerging from several large phase III randomized trials that demonstrated a lack of survival benefit from intensive surveillance strategies. In addition, findings from cost-effectiveness analyses of such an approach are inconsistent. Data on real-world practice, specifically adherence to these follow-up guidelines, are also limited. The debate is especially controversial in resected stage IV patients where there are currently no clear guidelines for follow-up. In an era of personalized medicine, there may be a shift towards a more risk-adapted approach to better define the optimal follow-up strategy. In this article, we review the evidence and highlight the role of surveillance in CRC survivors.

Key words: Surveillance; Imaging; Endoscopy; Colorectal cancer; Follow-up

Core tip: Although several reviews in the literature have analyzed the different surveillance strategies for colorectal cancer, this is the most updated review that includes the current state of surveillance approaches with endoscopy and imaging, the most recently completed clinical trials and meta-analysis that failed to demonstrate survival benefit from traditional intensive surveillance strategies recommended by professional guidelines, real-world data, and recommendations for special populations.

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INTRODUCTION

Colorectal cancer (CRC) remains one of the most common cancers in Western countries, and ranks second in North America[1]. Over one million cases of CRC are diagnosed annually in the world[2]. It is also one of the most common causes of cancer-related mortality. Approximately 80% of all CRC cases are diagnosed at an early stage at which point curative intent surgery is the standard of care. Despite improvements in surgical techniques and adjuvant treatments, including chemotherapy and radiation, approximately 40% of patients with localized disease will experience disease recurrence after completing potentially curative treatment. Half of these recurrences are locoregional, and 90% occur within the first 3 to 5 years of treatment[3,4]. Median survival following recurrence is estimated at one year, based on findings from the ACCENT database in 2008[5]. There have been improvements in survival over the last decade due to an increase in the rates of metastatic resections. For example, 5-year survival rates can be over 40% for select patients who undergo hepatectomy for liver-limited metastasis[6]. In addition, locoregional recurrences may also be considered for resection, with some being potentially cured.

The goal of postoperative surveillance in CRC is to identify potentially resectable recurrences because this may improve survival outcomes. Surveillance may also lead to the early identification and removal of precancerous polyps, thereby preventing secondary metachronous CRC[7]. Standard CRC postoperative surveillance guidelines have been published by various cancer societies and expert groups, and they are continuously being updated[8-10]. The majority of these guidelines incorporate a combination of clinic visits with history and physical examinations, carcinoembryonic antigen (CEA), computed tomography (CT) scans, and endoscopies at regular intervals. The frequency of these different modalities has been subject to debate. Recently, several studies showed that less intensive approaches to surveillance may not be inferior to more intensive strategies[11,12]. Conversely, there is also some degree of evidence from the literature that demonstrates improvements in survival with an intensive follow-up approach[13]. It is important to recognize that postoperative surveillance is not without clinical and financial risks, which are important to consider in the era of personalized medicine as well as within the context of healthcare systems that are facing increasing fiscal constraints. In this review, we describe the CRC surveillance guidelines published by major societies and highlight findings from several large clinical trials that question the value of intensive follow-up.

CURRENT SURVEILLANCE GUIDELINES

Several recommendations regarding post-treatment surveillance for resected CRC have been published and endorsed by professional societies. The most recent ones are shown in Table 1. In general, a history and physical examination along with a CEA measurement is recommended every 3-6 mo for 5 years, a CT scan of the chest/abdomen/pelvis is recommended every 6-12 mo for 3 to 5 years, and a colonoscopy is recommended at 1 and 3 years. Subsequent endoscopies are guided by findings in the initial colonoscopy. For example, if the first colonoscopy is normal, a repeat endoscopy would not be needed until 5 years later. The frequency of these investigations appears to be the major difference across guidelines. For CEA and clinic visits, for instance, a 3-6 mo frequency is recommended for 5 years by American society of clinical oncology (ASCO), whereas European society for medical oncology (ESMO) only recommends this for the first 3 years followed by a frequency of every 6-12 mo for the last 2 years. Similarly, for CT scans, while national comprehensive cancer network (NCCN) recommends CT imaging every 6-12 mo for up to 5 years, ASCO recommends CT imaging only annually for 3 to 5 years[7,14-17].

For surveillance colonoscopy, however, the recommendations are more variable and largely guided by pathologic findings. In 2016, the United States Multi-Society

Table 1 Summary of postoperative surveillance recommendations for colorectal cancer by different professional societies

Organization	History/physical	CEA	CT scan	Endoscopy
ASCO 2013[14] (stage II-III)	Every 3-6 mo for 5 yr	Every 3-6 mo for 5 yr	Chest/abdomen +/-pelvis (if rectal) annually for 3-5 yr	Colonoscopy at 1 yr; if negative, every 5 yr. Rectal cancer: proctosigmoidoscopy every 6 mo for 2-5 yr if no pelvic RT
ESMO colon 2013[15] (Stage I, II, III)	Every 3-6 mo for 3 yr, then every 6-12 mo for 2 yr	Every 3-6 mo for 3 yr, then every 6-12 mo for 2 yr	Chest and abdomen every 6-12 mo for 3 yr; transabdominal ultrasound can be used instead of CT abdomen	Colonoscopy at 1 yr; of negative, every 3-5 yr subsequently.
ESMO rectal 2013[16] (Stage II, III)	Every 6 mo for 2 yr	Every 6 mo for 3 yr	At least 2 chest/abdomen/pelvis in the first 3 yr	Colonoscopy every 5 yr up to age 75
NCCN 2018[17] (Stage II, III, resected IV)	Every 3-6 mo for 2 yr, then every 6 mo for 3 yr	Every 3 to 6 mo for 2 yr for ≥ T2 disease, then every 6 mo for 3 yr (up to 5 if resected metastatic)	Colon: Chest/abdomen/pelvis every 6-12 mo for up to 5 yr. For rectal cancer, CT chest/abdomen and pelvis every 3-6 mo for 2 yr, then every 6-12 mo for up to 5 yr	Colonoscopy at 1 yr; if negative, repeat at 3 yr, then every 5 yr subsequently. If adenoma found, repeat at 1 yr.
USMSTF 2016[7] (only for endoscopic surveillance)				Colonoscopy at 1 yr; if negative, repeat at 3 yr, then every 5 yr. For rectal cancer, flexible sigmoidoscopy or EUS every 3-6 mo for the first 2 to 3 yr after surgery for patients at high risk for local recurrence

CEA: Carcinoembryonic antigen; CT: Computed tomography; ASCO: American society of clinical oncology; ESMO: European society for medical oncology; NCCN: National comprehensive cancer network; USMSTF: United States Multi-Society Task Force.

Task Force (USMSTF) published updated recommendations on the role of surveillance endoscopies after resected CRC[7]. Compared to the previous set of recommendations published in 2006[18], the frequency of colonoscopies after surgical resection has not changed, with the need for a colonoscopy within the first year after surgery, followed by a repeat procedure in three years if negative, and another repeat procedure in five years (or nine years after initial resection) if negative. However, if polyps are found, endoscopies would be more frequently performed, as per polypectomy surveillance guidelines. This more intensive approach is based on evidence and is cost-effective[19].

What's new in the literature?

Over the past two decades, several literature reviews suggested a survival benefit from intensive surveillance strategies. A recent systematic review published by Pita-Fernandez *et al*[13] evaluated 11 studies (n = 4055 patients) and showed a modest but significant improvement in overall survival (HR: 0.75, 95%CI: 0.66-0.86), a higher probability of detection of asymptomatic recurrences (RR: 2.59, 95%CI: 1.66-4.06), a higher rate of curative surgeries attempted at recurrences (RR: 1.98, 95%CI: 1.51-2.60), and better overall survival after recurrences (RR: 2.59, 95%CI: 1.24-3.69) that favored the use of intensive follow-up strategies. However, there was no significant difference in cancer-specific survival when compared to less intensive strategies. Prior to this, two additional meta-analyses by Tjandra *et al*[20] and Jeffery *et al*[21] from 2007 showed similar results regarding intensive surveillance, with an improvement in overall survival (HR: 0.74, 95%CI: 0.59-0.93 and HR: 0.73, 95%CI: 0.59-0.91), but no improvement in cancer-specific survival. Taken together, this body of evidence appears to support the notion that earlier detection of asymptomatic recurrences may improve survival, and likely contributed to many of the recommendations adopted by professional guidelines.

Since that time, new randomized trials and prospective studies have not consistently offered support towards the findings from these prior meta-analyses. The most recently completed studies are shown in Table 2. In the COLOFOL study published in JAMA in 2018[11], investigators examined 2509 patients with stage II or III CRC treated at 24 centers in Sweden, Denmark, and Uruguay from 2006 to 2010. Patients were followed until 2015. Specifically, patients were randomized to either follow-up testing with CT and CEA every 6 mo after surgery for 3 years (high

frequency group) or follow-up testing with CT and CEA at 12 mo and 36 mo after surgery (low frequency group). At the end of the follow-up period, they found no statistically significant differences in 5-year overall mortality (risk difference 1.1%, 95%CI: -1.6% to 3.8%, P = 0.43), 5-year CRC-specific mortality (risk difference 0.8%, 95%CI: -1.7% to 3.3%, P = 0.52), and CRC-specific recurrence (risk difference 2.2%, 95%CI -1.0% to 5.4%, P = 0.15) between the high frequency and low frequency groups. Likewise, another randomized trial performed by the GILDA group in Italy published updated results in 2016[22]. Authors randomized 1228 patients with resected Duke B2-C CRC from 1998 to 2006 to either intensive or minimal surveillance. They found no statistically significant difference in overall survival between the two strategies and no difference in health-related quality of life scores. Finally, the FACS trial published in 2014 evaluated the effect of 3 to 5 years of scheduled CEA and CT follow-up in detecting CRC recurrences[23]. A total of 1202 patients from the United Kingdom participated between 2003 and 2009. These subjects had undergone curative surgery for primary CRC and they were subsequently assigned to 4 different follow-up groups: CEA only, CT only, CEA + CT, or minimal follow-up where patients received follow-up only if symptoms occurred. The results showed that the use of imaging or CEA measurements resulted in an increased rate of curative resection at the time of recurrence when compared to minimal follow-up. However, there was no additional benefit seen by combining CEA and CT (adjusted OR: 3.1, 95%CI: 1.1-8.71). Furthermore, the number of deaths was not significantly different between the group that underwent CEA and CT and the group that underwent minimal, symptom-based follow-up (difference 2.3%, 95%CT: -2.6% to 7.1%). These three large clinical trials showed highly consistent results. Collectively, they seem to indicate that intensive surveillance strategies are not associated with a survival advantage. There is one ongoing phase III trial called the PRODIGE 13 study, which is based in France. In this particular trial, investigators randomized 1750 patients with stage II or III resected CRC to an intensive group consisting of clinic visits, CEA measurements, colonoscopies, and CT imaging studies, or to a control group consisting of only abdominal ultrasounds and chest X-rays. Results from this study is anticipated in 2021 and may provide further clarity regarding the role of intensive follow-up[24].

An updated systematic review published in 2016 appears to corroborate the findings from the recent clinical trials and seems to suggest that there is no overall survival benefit for intensive post-operative follow-up[25]. This systematic review represents a second update from the Cochrane Collaboration Group, which contrasts the first one published in 2007 that demonstrated a survival advantage[21]. With 5403 participants enrolled in 15 studies, a statistically significant advantage with intensive follow-up was not detected for overall survival (HR: 0.90, 95%CI: 0.78-1.02, high quality evidence), cancer-specific survival (HR: 0.93, 95%CI: 0.78-1.12, moderate quality evidence), or relapse-free survival (HR: 1.03, 95%CI: 1.53-2.56, high quality evidence). Harms from colonoscopies also did not differ with intensive follow-up (RR: 2.08, 95%CI: 0.11-40.17). Finally, a large retrospective cohort study of patient data from the National Cancer Database was published recently in 2018[12]. With a random sample of 8529 patients with resected stage I, II, or III CRC from 1175 facilities who underwent follow-up, there was no significant association between imaging and CEA surveillance intensity and detection of cancer recurrence.

Continuing controversy

There is increasing evidence that intensive surveillance strategies, whether they pertain to the type and number of tests or their frequency interval, are not associated with improved cancer survival. However, most guidelines still recommend relatively intensive approaches. Because many of the endpoints in the clinical trials that examined surveillance were different and the control groups for comparison were also not consistent, consensus regarding the best approach has been difficult to reach. From an endoscopy perspective, there appears to be relatively good quality evidence to support colonoscopy at 1 year after surgery, which is then followed by the same procedure at 3- and 5-years if findings are benign. This is currently recommended by the updated USMSTF. While there is no cancer specific survival advantage demonstrated in any of the studies, the standard use of post-operative endoscopic surveillance is endorsed by all major societies. Evidence from cost-effectiveness data also supports this practice. Similarly, data from the most recent meta-analysis did not reveal a significant harm from such an approach.

The use of CEA and CT imaging is more controversial. From a CEA perspective, there have been a number of studies evaluating its utility as both a screening and a surveillance test. Due to its low sensitivity and specificity[26], CEA is not viewed as a useful screening tool. However, it has a more established role in informing prognosis and disease burden. For example, an elevated preoperative CEA should normalize after surgery such that a persistently high level following resection may represent the

Table 2 Summary of recent randomized control trials evaluating intensive *vs* less intensive surveillance strategies

Trial	Setting	Enrollment period	Patient population	Intensive group	Control group	Results
FACS (JAMA 2014)[23]	United Kingdom	2003-2009	1201 stage I-III	Either: CEA every 3 mo for 2 yr, then every 6 mo for 3 yr, with a single chest, abdomen, and pelvis CT scan at 12-18 mo if requested; CT of the chest, abdomen, and pelvis every 6 mo for 2 yr, then annually for 3 yr; Both blood CEA measurement and CT imaging as above	No scheduled follow-up except a single CT scan of the chest, abdomen, and pelvis at 12-18 mo if requested	No difference in overall mortality for combined CEA and CT compared to minimal follow-up
GILDA (Ann Oncol 2016)[22]	Italy	1998-2006	1228 Dukes B2-C (high risk stage II and III)	Office visit, blood tests (CEA, CBC, liver tests, CA19-9) every 4 mo for 2 yr, then every 6 months for 2 yr then at 5 yr; Colonoscopy and chest X-ray every year for 5 yr; Liver ultrasound at 4, 8, 12, 16, 24, 36, 48, and 60 mo	Office visit, CEA, every 4 mo for 2 yr, then every 6 mo for 2 yr then at 5 yr; Colonoscopy at 1 yr and at 4 yr; Liver ultrasound at 8 and 20 mo	No difference in overall survival or health-related quality of life scores
COLFOL (JAMA 2018)[11]	24 centers in Sweden, Denmark, and Uruguay	2006-2010	2509 stage II and III	CEA and CT thorax/abdomen at 6, 12, 18, 24, and 36 mo	CEA and CT thorax/abdomen at 12 mo and 36 mo	No difference in overall mortality, cancer-specific mortality, and cancer recurrence
PRODIGE-13[24]	96 centers in France and Belgium	2009-2015	1997 stage II and III	Clinical assessments every 3 mo until year 3 and every 6 mo until year 5, then at least yearly thereafter; Alternating assessments every 3 mo of CT thorax/abdomen/pelvis or abdominal ultrasound until year 3 and then every 6 mo until year 5; Colonoscopy at 3 yr after surgery then every 3 to 6 yr thereafter	Clinical assessments every 3 mo until year 3 and every 6 mo until year 5, then at least yearly thereafter; Abdominal ultrasound every 3 mo until year 3 and then every 6 mo until year 5; chest X-ray every; 6 mo until year 3 and then annually until year 5; Colonoscopy at 3 yr after surgery then every 3 to 6 yr thereafter	Pending for 2021

CEA: Carcinoembryonic antigen; CT: Computed tomography.

presence of residual disease[27]. Based on a pooled analysis in a Cochrane review[26], an elevation in postoperative CEA was associated with a high probability of disease recurrence, but a normal postoperative CEA was associated with a high false negative rate since this alone is not always useful for excluding disease recurrence. In fact, 30 to 40% of all CRC recurrences do not have an accompanying elevation in tumor markers, such as CEA[28]. There are also no clear data that confirm a consistent survival benefit with the use of CEA testing[29], and its cost-effectiveness continues to be unclear. Of interest, one meta-analysis[20] showed that CEA testing was the only investigation that was associated with a higher probability of detecting asymptomatic recurrences. In addition, a cost-analysis from the Eastern Collaborative Oncology Group database showed that CEA represented the most cost-effective method for detecting potentially curable recurrences[30]. For CT imaging, there is also evidence to support its value in detecting asymptomatic distant recurrences which may still be resected curatively[31,32]. CT scans are particularly helpful given the high false negative rate of CEA assays along with the inability of endoscopies to detect asymptomatic distant recurrences. However, the optimal frequency of CT imaging is not well established. With the most recent clinical trials, we have new evidence that the frequent use of CEA and CT does

not seem to be superior to less frequent use (COLOFOL), and that the combination of CEA and CT does not seem to be superior to either alone (FACS). These recent findings have not yet been incorporated into the expert guidelines from ASCO, ESMO, or NCCN.

Very few studies have evaluated the cost-effectiveness of different post-operative surveillance strategies. One study used decision analysis to assess the cost-effectiveness of surveillance colonoscopy at 1 year after cancer resection[19]. This study compared a 1-year endoscopic surveillance strategy with a "no early" endoscopy approach. They found the incremental cost-effectiveness ratio to be $40313 per life-year gained. The number needed to treat to detect one CRC and to prevent one CRC-related death was 143 and 926, respectively. They concluded that conducting a colonoscopy at 1 year following CRC resection is cost-effective and clinically effective for both cancer detection and cancer-specific death prevention. An older study from 2004 published in the British Medical Journal[33] compared cost-effectiveness of intensive *vs* conventional follow-up after curative resection for CRC; this also concluded that intensive follow-up using CT and CEA was economically justified based on an adjusted cost of life saved of $5884 USD. A more recent study published in Cancer in 2016[34] analyzed cost-effectiveness of the USMSTF guideline regarding colonoscopy surveillance postoperatively. The results showed that the US guideline is not cost-effective, with an incremental cost-effectiveness ratio as high as $140000 per life year gained. Given the paucity and inconsistency of data, along with variability in the cost impact under different health care systems, more research is warranted to explore the benefits, harms, and economic implications of different practices.

Risks with surveillance

Importantly, any extra investigations pose associated risks. Although rare, endoscopy is associated with the potential for bleeding and perforation, and the risk increases with more frequent use[35]. In addition, lack of a proper bowel cleaning regimen prior to endoscopy may result in an inadequate procedure that would lead to repeat testing. As with most procedures, the use of local anesthetics may also be associated with side effects. For CEA assays, the levels may be falsely elevated in the context of cigarette smoking[36] and adjuvant 5-FU treatment[37], which can lead to unnecessary imaging and anxiety. Finally, routine CT imaging is associated with radiation exposure and a small but real risk of second malignancies, which is of particular concern in younger individuals undergoing surveillance. As such, alternative imaging modalities such as chest X-rays and liver ultrasound may be employed, although the evidence to support the use of these modalities is poor, and none of the prior meta-analyses addressed thoracic imaging specifically.

Real-world practice

Few population-based studies have evaluated real-world practice patterns with respect to CRC follow-up. One Canadian population-based study evaluated adherence to guidelines on CRC surveillance and outcomes for patients enrolled in an innovative and intensive follow-up program at the Cross Cancer Institute in Edmonton. With 408 patients, the investigators found 14%, 33%, and 24% non-adherent rates to annual CT imaging, colonoscopy, and CEA testing. Less than half had complete adherence to all 3 components. The recurrence rate after a median of 1.6 years was 17%, most of which were diagnosed *via* surveillance, and almost half were considered potentially resectable[38]. Another Canadian study assessed adherence to ASCO CRC surveillance guidelines and compared patterns between a community and an academic cancer center. The authors observed significant inconsistencies between practices, with an academic institution using more intensive surveillance strategies, consisting of more frequent imaging studies, than the community cancer center. There were no significant differences in the use of CEA monitoring and surveillance colonoscopies. Of note, the researchers also found that surveillance was associated with a higher proportion of resectable tumor recurrences[39]. In contrast, another large population-based cohort study from the National Cancer Database compared high *vs* low intensity imaging or CEA testing for CRC surveillance and detected no significant association between intensity of surveillance and survival outcomes[12].

Special considerations

While the guidelines for surveillance apply to most survivors of CRC, there are specific populations that are not explicitly addressed. Older patients, for example, form a significant proportion of the survivorship population, but the existing guidelines do not specifically indicate the age at which continued surveillance is unlikely to provide meaningful benefit. The updated 2016 USMSTF states that "postoperative colonoscopic surveillance in CRC patients is indicated long term, or until the benefit is outweighed by decreased life expectancy due to age and/or

competing comorbidity"[7]. Very few studies have evaluated outcomes of surveillance in the elderly population. One retrospective study evaluated 4834 elderly patients over the age of 75 years who were undergoing surveillance and found that the incidence of CRC among older adults was significantly lower than in younger individuals (0.24 *vs* 3.61 per 1000 person-years), and that advanced age was an independent factor associated with post-endoscopic hospitalization after adjusting for other factors (adjusted OR: 2.54, 95%CI: 2.06-3.14)[40]. It should be noted that these current guidelines also do not apply to patients with hereditary syndromes, such as Lynch syndrome, as these patients need more frequent endoscopic screening and surveillance, as per USMSTF consensus guidelines for Lynch syndrome patients[41].

Further, the expert guidelines do not consistently consider stage I and resected stage IV patients. There are significant variations in their recommendations due to a lack of robust data. Because over 95% of stage I patients are cured with surgery alone, adjuvant chemotherapy is not recommended, nor is intensive surveillance. The only exception is that postoperative colonoscopy is endorsed at the same frequency and interval as for stage II and III patients. Data from the COST trial[42] suggest, however, that stage I patients likely benefit equally from postoperative surveillance. The authors analyzed over 500 patients with stage I, II, and III resected colon cancer undergoing surveillance with CEA every 3-6 mo, chest X-ray every 6-12 months, and colonoscopy as per USMSTF guidelines. The investigators noted higher recurrence rates at 5 years with more advanced stages of disease, but there were similar salvage rates and sites of recurrences across all stages. Thus, they concluded that implementation of similar surveillance guidelines for all early stages of resected colon cancer patients is appropriate. This has not been routinely endorsed by expert guidelines from ASCO and NCCN. However, ESMO consensus guidelines on surveillance of early stage colon cancer includes stages I to III[15], while the same guidelines for rectal cancer are not clear whether these recommendations would apply to stage I patients[16].

Resected stage IV patients face similar uncertainty. There are no data for surveillance in this population, and decisions are often individualized based on patient factors and institutional practices. The rate of curative metastatic resections is increasing[43,44]. For liver limited metastasis, surgical resection is associated with the highest likelihood of cure, with 5-year survival rates of over 40%[45]. There is also medical advancement in many other domains, such as stereotactic radiotherapy, which can provide an alternative option for achieving potential cure in the setting of metastatic disease. Because of this, many have adopted the standard surveillance strategies for early stage CRC in otherwise fit stage IV patients who may be candidates for further curative treatments. Currently, the NCCN recommends routine surveillance for resected stage IV patients, including CEA every 3-6 mo for 2 years then every 6 mo for 3 years, CT of chest/abdomen/pelvis every 3-6 mo for 2 years then every 3-6 mo for up to 5 years, and colonoscopy at 1 year and then every 5 years subsequently, if normal[17]. In one study that evaluated outcomes of intensive surveillance after resection of hepatic metastases, 5-year survival rates were significantly higher in patients managed with hepatic resection compared to those managed palliatively[46]. In addition, intensive surveillance with 3-monthly CT for the first two years along with CEA at each clinic visit resulted in a relatively high rate of early detection of recurrences (444/705 patients). The authors also analyzed cost per life-year gained with this intensive strategy and found this to be reasonable within the British health care system. Therefore, they concluded that intensive 3-monthly surveillance CT after hepatic resection is reasonable, cost-effective, and can detect a considerable number of recurrent patients to improve outcomes.

Future directions

There is a heterogeneous group of patients that may benefit from CRC surveillance. Given this scenario, there is significant interest in a more risk-adapted surveillance strategy, where follow-up investigations and intervals are tailored based on the individual's risk profile for cancer recurrence. This risk would be based on pathological and molecular biomarkers. In an era of personalized medicine, such tools are increasingly needed, but few studies have evaluated risk-adapted surveillance strategies in CRC. An older study randomized patients to either a risk-adapted follow-up protocol or a minimal follow-up schedule based on their risk status (high *vs* low), which was predefined prior to randomization[47]. The research group observed significantly improved 5-year overall survival for the risk-adapted follow-up protocol group regardless of risk status. However, the choice and definition of the risk factors were not well validated. Other similar studies in this area have largely evaluated prognostic and predictive biomarkers for survival outcomes and responses to chemotherapy. With a better understanding of CRC through these molecular studies, we may eventually be able to implement these into a standardized recurrence risk

calculator where we can guide personalized planning of post-treatment surveillance. Such tools already exist to assist with decision regarding adjuvant chemotherapy for stage II patients, such as the Oncotype DX, but similar tools to guide surveillance is lacking.

Another emerging instrument in the field of oncology is the use of circulating tumor DNA (ctDNA) to detect the presence of tumor cells in a more reliable and less invasive way. ctDNA is a portion of tumor DNA that is shed into the patient's bloodstream, which can be detected *via* blood analysis without imaging or biopsy. Many studies evaluating ctDNA have found it to be a sensitive test for assessing disease recurrence, often times much earlier than standard testing[48]. One study evaluated ctDNA postoperatively in 27 CRC patients who underwent surgery. Remarkably, the investigators detected ctDNA to be present in all 14 patients who relapsed but absent in the other patients. In addition, ctDNA detected recurrences much earlier than either CEA or CT scan[49]. Unfortunately, these data have not been consistently replicated. At present, the routine use of ctDNA for monitoring disease recurrence should not be widely implemented[50].

CONCLUSION

In summary, the current state of surveillance for resected stage II and III CRC is controversial. Although standard guidelines from professional societies recommend relatively intensive strategies for disease monitoring, new data suggest that less intensive approaches may not be inferior. With the emergence of precision medicine and a better understanding of CRC, the future of surveillance may be moving towards a more risk-adapted, personalized approach that accounts for both patient and disease factors, as well as cost. More research is needed to clarify the role of surveillance for the growing population of resected stage IV patients who have undergone successful metastatic resections.

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MINIREVIEWS

Initial management for acute lower gastrointestinal bleeding

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Abstract

Acute lower gastrointestinal bleeding (LGIB) is a common indication for hospital admission. Patients with LGIB often experience persistent or recurrent bleeding and require blood transfusions and interventions, such as colonoscopic, radiological, and surgical treatments. Appropriate decision-making is needed to initially manage acute LGIB, including emergency hospitalization, timing of colonoscopy, and medication use. In this literature review, we summarize the evidence for initial management of acute LGIB. Assessing various clinical factors, including comorbidities, medication use, presenting symptoms, vital signs, and laboratory data is useful for risk stratification of severe LGIB, and for discriminating upper gastrointestinal bleeding. Early timing of colonoscopy had the possibility of improving identification of the bleeding source, and the rate of endoscopic intervention, compared with elective colonoscopy. Contrast-enhanced computed tomography before colonoscopy may help identify stigmata of recent hemorrhage on colonoscopy, particularly in patients who can be examined immediately after the last hematochezia. How to deal with nonsteroidal anti-inflammatory drugs (NSAIDs) and antithrombotic agents after hemostasis should be carefully considered because of the risk of rebleeding and thromboembolic events. In general, aspirin as primary prophylaxis for cardiovascular events and NSAIDs were suggested to be discontinued after LGIB. Managing acute LGIB based on this information would improve clinical outcomes. Further investigations are needed to distinguish patients with LGIB who require early colonoscopy and hemostatic intervention.

Key words: Lower gastrointestinal bleeding; Predictive model; Colonoscopy; Computed tomography; Medication

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Core tip: Several concerns exist when managing acute lower gastrointestinal bleeding (LGIB). Fortunately in recent years, novel findings in the acute LGIB setting have accumulated with respect to predictive scores for severe bleeding, the clinical significance of contrast-enhanced computed tomography before colonoscopy, the utility of early colonoscopy, and the management of direct-acting oral anticoagulants. Here, we review evidence for the initial management of acute LGIB.

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INTRODUCTION

Traditionally, gastrointestinal bleeding (GIB) was classified into upper gastrointestinal bleeding (UGIB) and lower gastrointestinal bleeding (LGIB). LGIB was defined as bleeding from the lesion distal to the ligament of Treitz, including the small and large bowels. In the last decade, the availability of advanced diagnostic innovations such as capsule endoscopy and balloon-assisted enteroscopy has led to better understanding of the etiological profile of small bowel bleeding. Thus, some recent reports adopted three categories of GIB: Upper-, mid-, and lower GIB[1].

Acute LGIB has increased due to aging of the population, and with the increasing use of antithrombotic agents[2-4]. Although many UGIB events can be prevented by proton pump inhibitors (PPIs) and eradicating *Helicobacter pylori*, there are few effective methods for preventing LGIB recurrence. The estimated hospitalization rate for LGIB is 33-87 per 100000 population[2,5,6], with mortality rates of 2.5%-3.9% during hospitalization[7-9] and rebleeding rates of 13%-19% after 1 year[10,11].

Several concerns exist when managing acute-onset hematochezia suspected as acute LGIB. First, the causes of bleeding varied from many types of colonic diseases to UGIB and small-bowel bleeding. Most cases experience spontaneous cessation with conservative therapy. In contrast, patients with vascular diseases, such as diverticular bleeding and angioectasia, often suffer from continuous and/or recurrent bleeding, requiring hemostatic intervention and blood transfusion[9,12]. A few cases die during hospitalization. Therefore, risk stratification tools for severe LGIB would be useful for deciding on emergency hospitalization or an early intervention. However, unlike UGIB, predictive clinical scores for severe acute LGIB are not well defined.

Second, colonoscopy, which is essential for diagnosis and therapy of LGIB[13], requires bowel preparation to identify the bleeding source, unlike upper endoscopy. The timing of time-consuming and laborious colonoscopy should be optimized, but the utility of early colonoscopy remains controversial.

Third, there are numerous antithrombotic agents, including dual antiplatelet therapy and direct-acting oral anticoagulants (DOACs), and the management of antithrombotic agent use requires considering the conflicting risks of ongoing/recurrent bleeding and thromboembolic events.

Thus, appropriate decision-making is needed when managing acute LGIB. Fortunately, novel findings in the acute LGIB setting have accumulated in recent years, such as predictive scores for severe bleeding, the clinical significance of contrast-enhanced computed tomography (CE-CT) before colonoscopy, the utility of early colonoscopy, and the management of DOACs.

The purpose of this literature review is to summarize findings regarding the initial management of acute LGIB, in line with the 2016 American College of Gastroenterology guideline for acute LGIB and the 2016 American Society for Gastrointestinal Endoscopy guideline for the management of antithrombotic agents on gastrointestinal endoscopy[13,14].

INITIAL ASSESSMENT

History-taking, physical examination, and laboratory testing are important at the time of presentation of patients with presumed acute LGIB. The need for intravenous fluid resuscitation and blood transfusion should be determined by evaluating hemodynamic status according to history of syncope, level of consciousness, and vital

signs, including postural changes. Hematochezia with hemodynamic instability requires attention, as brisk UGIB can also result in that type of stool. A blood urea nitrogen/creatinine (BUN/Cr) ratio > 30 (likelihood ratio, 7.5) and nasogastric aspirate/lavage with blood or coffee grounds (likelihood ratio, 9.6) are the features of UGIB[15], being useful to distinguish UGIB from LGIB. In addition, in a report of patients with hematochezia, the systolic blood pressure was significantly lower in UGIB than in LGIB (mean pressure, 114 mmHg *vs* 133 mmHg)[16]. If the likelihood of UGIB is high based on these factors, upper endoscopy is recommended.

The presence of certain symptoms can suggest the source of LGIB[13]. Patients with colitis (ischemia, infection, or inflammatory bowel disease) often present with diarrhea and abdominal tenderness, whereas those with vascular diseases, such as diverticular bleeding, hemorrhoid, angioectasia, and rectal ulcers, usually do not present with lower gastrointestinal symptoms. Weight loss and altered bowel habits suggest malignancy.

Risk stratification

Although most patients with acute LGIB experience spontaneous hemostasis, some suffer from severe, persistent hemorrhage and rebleeding. The frequency of adverse outcomes varies by cause of LGIB. The causes of acute LGIB in the Western countries are as follows[17]: Diverticular bleeding (30%-65%), ischemic colitis (5%-20%), hemorrhoids (5%-20%), colorectal polyps/neoplasms (2%-15%), angioectasia (5%-10%), post-polypectomy bleeding (2%-7%), inflammatory bowel disease (3%-5%), infectious colitis (2%-5%), rectal ulcer (0-5%), colorectal varices (0-3%), radiation proctitis (0-2%), drug-induced colitis (0-2%), and Dieulafoy's lesion (rare). On the other hand, in the tropical countries, colorectal polyps/neoplasms (29%-53%) and colitis (23%-38%) are the common causes, and diverticular bleeding is less common (4%-19%)[16,18]. The rate of rebleeding is 22%-38% in patients with diverticular bleeding[12]. Ischemic colitis cases have significantly lower blood transfusion requirements (4%) compared with other forms of LGIB[19].

Physicians must understand the predictive factors for severe LGIB to improve triage of appropriate patients for emergency hospitalization or early intervention. Several studies have investigated risk factors for adverse outcomes (rebleeding, severe bleeding, need for emergent hospitalization, need for intervention, adverse events, or death) in patients with acute LGIB[7,8,20-28]. These include older age, presenting symptoms (no abdominal tenderness, no diarrhea, altered mental status, or blood on rectal examination), vital signs, comorbidities, medication use [nonsteroidal anti-inflammatory drugs (NSAIDs) and antithrombotic agents], and laboratory data [hemoglobin (Hb), hematocrit, albumin, BUN, Cr, and prothrombin time (PT)] (Table 1). We also previously reported a predictive model of severe LGIB (NOBLADS score), which included NSAID use, no diarrhea, no abdominal tenderness, systolic blood pressure ≤ 100 mmHg, albumin level < 3.0 g/dL non-aspirin antiplatelet drug use, Charlson comorbidity index score ≥ 2, and syncope[24]. Several predictive models have been validated in other settings (Table 2)[21,22,24,27,28]. Applying these models to manage LGIB could improve clinical outcomes and resource utilization. However, compared with established models of severe UGIB[29,30] such as the Blatchford score, predictive models of severe LGIB require further validation and improvements in accuracy.

INITIAL MANAGEMENT

Intravenous fluid resuscitation with crystalloids should be initiated, particularly in hemodynamically unstable patients[13,31]. In a review of fluid administration in bleeding patients, the best fluid resuscitation strategy was not determined on the basis of the timing, volume, and type of fluid[32]. Another review on critically ill patients indicated that colloids do not improve the mortality rate and are more expensive compared with crystalloids[33].

Although patients with LGIB often require a blood transfusion[9], transfusion strategies specific to LGIB have not been investigated. In a recent meta-analysis of five randomized controlled trials (RCTs) comparing restrictive and liberal transfusion strategies in the acute UGIB setting, restrictive transfusion of red blood cells (Hb threshold, 7-8 g/dL) was associated with a lower risk of all-cause mortality [relative risk (RR): 0.65, 95% confidence interval (CI) 0.44-0.97] and rebleeding (RR: 0.58, 95%CI: 0.40-0.84) than liberal transfusion (Hb threshold 9-10 g/dL)[34]. The LGIB guideline applied this result to recommendations for LGIB[13].

However, it should be noted that a previous RCT and a meta-analysis indicated that for patients with cardiovascular disease which limits myocardial oxygen delivery, rates of mortality and cardiovascular events were higher in a restrictive

Table 1 Risk factors and odds ratios for various outcomes according to 11 studies[7,8,20-28]

	Severe/recurrent bleeding	In-hospital complications[3]	Adverse outcomes[4]	Mortality
Patient characteristic				
Older age	2.3[1]	-	4.2[1]	4.9[2]
Male sex	[6]	-	-	1.5-1.6
Lower body mass index	-	-	-	2.0
Smoking	-	-	0.5	-
Comorbidities				
Charlson index > 2 or ≥ 2	1.7-1.9	-	-	3.0
Unstable comorbid diseases	-	2.9	-	-
Congestive heart failure	-	-	-	1.5
Cardiovascular disease	[6]	-	-	-
Dementia	[6]	-	-	5.2
Metastatic cancer	-	-	-	5.0
Chronic kidney disease	-	-	-	1.8-2.2
Liver disease	-	-	-	1.9
Chronic pulmonary disease	-	-	-	1.6
History of colonic diverticulosis and/or angiodysplasia	[6]	-	-	[6]
Presenting symptom				
Syncope / altered mental status	2.5-3.3	2.0	-	[6]
No diarrhea	2.2	-	-	-
No abdominal tenderness	2.4-3.0	-	-	-
Ongoing bleeding	-	3.1	-	-
Bleeding in the first 4 h	2.3	-	-	-
Medication				
NSAIDs (non-aspirin)[1]	2.5	-	-	1.5
Aspirin	1.9-2.1	-	-	-
Antiplatelet drugs (non-aspirin)	2.0	-	-	-
Anticoagulants	-	-	-	1.5
Physical examination				
Blood pressure ≤ 100 or ≤ 115 mmHg	2.3-3.5	3.0	-	[6]
Heart rate ≥ 100/min	3.7	-	-	-
Abnormal vital signs after 1 h	4.3	-	-	-
Abnormal hemodynamic parameters	-	-	2.1	-
Gross blood on rectal examination	3.5-3.9	-	-	[6]
Laboratory data				
Hemoglobin < 10 g/dL	3.6	-	-	-
Albumin < 3.0 or < 3.8 g/dL	2.0-2.9	-	-	2.9
Creatinine > 150 or > 133 μmol/L	[6]	-	10.3	[6]
Hematocrit < 35% or < 30%	4.7-6.3	-	-	[6]
Prothrombin time > 1.2 times control	-	2.0	-	-
Clinical course				
Rebleeding	-	-	1.9	-
Intestinal ischemia	-	-	-	3.5
Coagulation defects	-	-	-	2.3
Hypovolemia	-	-	-	2.2
Blood transfusion	-	-	-	1.6-2.8
Need for intervention[5]	-	-	-	2.3-2.4
In-hospital onset LGIB	-	-	-	2.4

[1]Age > 60 years;
[2]Age > 70 years;
[3]Either surgery, intensive care unit admission, or mortality;
[4]Either rebleeding, surgery, or mortality;
[5]Interventional radiology or surgery;

[6]The variables were identified as risk factors, but odds ratios of these were not described. NSAIDs: Non-steroidal anti-inflammatory drugs.

transfusion group than in a liberal transfusion group[35,36]. LGIB guideline recommended that liberal transfusion be considered in patients with massive bleeding or cardiovascular disease[13].

With respect to platelet transfusion, a systematic review concluded that there are no data to inform optimal therapeutic platelet count targets in the acute gastrointestinal bleeding (GIB) setting[37]. Based on expert opinion and the standard in the hematology literature, a platelet count of 50 × 10^9/L is proposed as the LGIB guideline threshold[13,38].

DIAGNOSIS AND TREATMENT

Colonoscopy

Colonoscopy is the initial procedure for nearly all patients presenting with acute LGIB, because it has both diagnostic and therapeutic utility[13]. Common causes of acute LGIB include diverticular bleeding, ischemic colitis, angioectasia, and post-polypectomy bleeding. Other, less common causes include rectal ulcers, infectious colitis, inflammatory bowel disease, colorectal polyps/neoplasms, radiation proctitis, and hemorrhoids[13]. One of the most important issues in diagnostic colonoscopy for acute LGIB is identifying stigmata of recent hemorrhage (SRH), including active bleeding, a non-bleeding visible vessel, and an adherent clot[39,40]. SRH is regarded as an indication for endoscopic hemostasis because a prospective study showed that the rebleeding rate within 30 d in patients with SRH was 66% when endoscopic therapy was not performed, whereas patients without SRH had no rebleeding[40].

When to perform colonoscopy

The optimal timing of colonoscopy remains controversial. The definition of early colonoscopy used in most studies was within 24 h of presentation, and the definition in a few prospective trials was within 6-12 h[41-45]. Two RCTs and three meta-analyses examined the utility of early colonoscopy compared with elective colonoscopy in the acute LGIB setting (Table 3). These studies showed that early colonoscopy had the possibility of improving identification of the bleeding source, and the rate of endoscopic intervention, compared with elective colonoscopy. However, there is no clear evidence that early colonoscopy reduces important clinical outcomes, such as rebleeding or mortality.

The limitations of past studies may have affected these results. Previous RCTs were single-center studies and were terminated during enrollment because of difficulties in achieving the originally planned sample size. To address these issues, we are now conducting a multicenter RCT to examine the superiority of early colonoscopy over elective colonoscopy in patients with acute LGIB[46]. The primary outcome measure is identification of SRH. Secondary outcomes include 30-d rebleeding, the need for transfusion, and 30 d mortality. This trial will provide high-quality evidence of the benefits of early colonoscopy.

The safety of early colonoscopy in the acute LGIB setting has been reported. The rate of complications associated with bowel preparation was not significantly different between early colonoscopy (1.8%) and elective colonoscopy (1.2%) in a study based on a propensity score-matching analysis[47]. A literature review showed that the rate of complications associated with colonoscopic procedures is low for both early colonoscopy (0.6%) and elective colonoscopy (0.3%)[48].

For whom and how to perform early colonoscopy

The LGIB guideline recommends that patients with high-risk clinical features and signs of ongoing bleeding should undergo early colonoscopy, *i.e.*, within 24 h of presentation[13]. One of the signs of ongoing bleeding is extravasation on a CT scan, which should lead to early colonoscopy. However, clinical factors remain uncertain which can be easily obtained at the presentation and can suggest the indication for early colonoscopy. Although the NOBLADS score, one of the predictive score for severe LGIB, indicated the need for intervention in a derivation cohort (P = 0.001 for trend)[24], the score was not a significant predictor of the need for intervention in an externally validated cohort (P = 0.060 for trend; area under the curve, 0.54)[49]. In a recent LGIB study, all seven previous models for predicting severe GIB were not useful for distinguishing patients who required therapeutic intervention[27]. No existing model directly predicts the need for intervention in the LGIB setting; thus, appropriate models and novel strategies are required.

Table 2 Risk scoring systems for severe acute lower gastrointestinal bleeding which have been validated

Derivation study	Outcomes	Risk factors	ROC-AUC	Validation study
Strate *et al*[21]	Severe bleeding	Syncope	0.76	Prospective cohort (n = 275)
(n = 252)	(continuous and/or recurrent bleeding)	No abdominal tenderness		ROC-AUC: 0.75
		Aspirin use		
		Heart rate ≥ 100/min		
		Systolic blood pressure ≤ 115 mmHg		
		Bleeding per rectum in the first 4 h		
		Charlson comorbidity index > 2		
Das *et al*[22]	Rebleeding	(19 factors)	0.92	Prospective cohort (n = 142)
(n = 120)	Need for intervention	Age	0.93	
Artificial neural network based model	In-hospital mortality	Comorbidity (5 factors)	0.95	
		History (4 factors)		
		Features at presentation (2 factors)		
		Features at initial assessment (2 factors)		
		Initial laboratory data (5 factors)		
Aoki *et al*[24]	Severe bleeding	(NOBLADS)	0.77	Prospective cohort (n = 161)
(n = 439)	(Continuous and/or recurrent bleeding)	NSAIDs use		ROC-AUC: 0.76
		No diarrhea		Retrospective cohort (n = 511)
		No abdominal tenderness		ROC-AUC: 0.74
		Blood pressure (systolic) ≤ 100 mmHg		
		Albumin level < 3.0 g/dL		
		Antiplatelet drugs use (non-aspirin)		
		Disease score ≥ 2[1]		
		Syncope		
Oakland *et al*[27]	Safe discharge	Age	0.84	Prospective cohort (n = 288)
(n = 2336)	(Absence of death, rebleeding, intervention, blood transfusion,	Male sex		ROC-AUC: 0.79
	or 28 d readmission)	Blood on rectal examination		
		Heart rate		
		Systolic blood pressure		
		Hemoglobin level		
		Previous LGIB admission		
Sengupta *et al*[28]	30 d mortality	Age	0.81	Retrospective cohort (n = 2060)
(n = 4044)		Dementia		ROC-AUC: 0.72
		Metastatic cancer		
		Chronic kidney disease		
		Chronic pulmonary disease		
		Anticoagulant use		
		Hematocrit level		
		Albumin level		

[1]Charlson comorbidity index. LGIB: Lower gastrointestinal bleeding; ROC-AUC: The area under the receiver operating characteristics curve.

Based on evidence to date, the primary purpose of early colonoscopy is to identify the bleeding site and perform endoscopic hemostatic therapy. In addition to earlier colonoscopy, adequate colon preparation, an expert colonoscopist, use of a cap, and use of a water-jet scope have been suggested to improve the rate of SRH identification

Table 3 Utility of early colonoscopy compared with elective colonoscopy according to randomized controlled trials and meta-analyses

Study	Study design	Sample size	Bleeding source localization	Endoscopic intervention	Surgery required	Rebleeding	Length of stay	Adverse events	Mortality
Green *et al*[41]	RCT[1]	100	2.6 (1.1-6.2)[4]	-	NS	NS	NS	NS	NS
Laine *et al*[42]	RCT[2]	72	NS	-	-	NS	NS	-	-
Sengupta *et al*[44]	Meta-analysis[3]	901	2.97 (2.11-4.19)[4]	3.99 (2.59-6.13)[4]	NS	NS	-	-	NS
Kouanda *et al*[43]	Meta-analysis[3]	24,396	NS	1.70 (1.08-2.67)[4]	-	NS	-	NS	NS
Seth *et al*[45]	Meta-analysis[3]	23,419	SRH detection 2.85 (1.90-4.28)[4]	NS	NS	NS	NS	-	NS

[1]Primary end point was rebleeding;
[2]Primary end point was further bleeding (continuous bleeding and/or rebleeding);
[3]Meta-analyses included 2 randomized controlled trials;
[4]Odds ratio (95% confidential interval). RCT: Randomized controlled trial; NS: Not significantly; SRH: Stigmata of recent hemorrhage.

in patients with diverticular bleeding[39,50]. Because more than half of SRH has been reported to locate in the right colon (71%)[51], cecal intubation with adequate colon preparation is required, even for early colonoscopy. A nasogastric tube can be placed for bowel preparation when patients with LGIB are unable to tolerate rapid colon preparation[39,41].

Computed tomography

A systematic review showed high sensitivity (85.2%) and high specificity (92.1%) of CT angiography for diagnosing acute GIB[52]. The American College of Gastroenterology guideline suggest that CT angiography should be considered to localize the bleeding site before angiography or surgery, when the hemodynamics do not permit endoscopic evaluation and/or when patients are unable to tolerate the bowel preparation[13].

The clinical significance of performing CE-CT before colonoscopy has been examined in recent years. Our retrospective study of acute LGIB reported that the detection rate for vascular lesions was higher for colonoscopy following CT than for colonoscopy alone (35.7% *vs* 20.6%, $P = 0.01$), leading to more endoscopic therapies (34.9% *vs* 13.4%, $P < 0.01$)[53].

Furthermore, several studies have focused on the association between extravasation on CT and definitive diverticular bleeding on colonoscopy (Table 4). The colonoscopic detection rate of bleeding diverticula is significantly higher in patients with extravasation on CT than in those without (60%-76% *vs* 18%-31%)[54-56], suggesting that extravasation on CT is a reasonable indication for urgent colonoscopy to detect SRH. However, CT is not recommended for all cases due to the low rate of positive extravasation (15%-25%) documented in prospective studies of diverticular bleeding[56,57]. The intermittent nature of diverticular bleeding can reduce the sensitivity of CT for diagnosing diverticular bleeding. A prospective multicenter study suggested that patients who can be examined within 4 h of the last hematochezia would be candidates for urgent CT, because sensitivity is higher in this group than in those examined after 4 h (64.7% *vs* 33.3%, $P < 0.01$)[56].

Angiography and embolization

The major advantage of angiography and embolization is that it can control severe bleeding without bowel preparation. A systematic review reported that super-selective angiographic embolization achieves immediate hemostasis in 40%-100% of diverticular bleeding with occasional rebleeding (15%)[58]. The disadvantages of angiography and embolization include the requirement for active bleeding and the risk of bowel ischemia and contrast-induced nephropathic complications. The rate of bowel ischemia following embolization was 1%-4% in recent studies[59,60]. The LGIB guideline recommends that this intervention should be reserved for patients with very brisk, ongoing bleeding who do not respond adequately to hemodynamic resuscitation efforts and are unlikely to tolerate bowel preparation and early colonoscopy[13].

Angiography localizes the LGIB source in 24%-70% of cases[59,61]. Angiography requires blood loss rates > 0.5 mL/min to localize a bleeding site[62].Transfusion of > 5

Table 4 Clinical significance of performing contrast-enhanced computed tomography before colonoscopy for colonic diverticular bleeding

Study	Study design	Sample size[1]	Detection rate of extravasation on CT (%)	SRH detection rate on CS after extravasation on CT (%)	SRH detection rate on CS after no extravasation on CT (%)	Predictors for extravasation on CT
Obana *et al*[57]	Prospective	52	15	50	36	History of diverticular bleeding Within 2 h of last hematochezia
Nakatsu *et al*[54]	Retrospective	346	30	68	20	-
Nagata *et al*[53]	Retrospective	77	31	63	38	History of diverticular bleeding
Sugiyama *et al*[55]	Retrospective	55	36	60	31	-
Wada *et al*[118]	Retrospective	100	23	70	-	-
Umezawa *et al*[56]	Prospective	202	25	76	18	Within 4 h of last hematochezia

[1]Patients with colonic diverticular bleeding who underwent contrast-enhanced computed tomography before colonoscopy. LGIB: Lower gastrointestinal bleeding; CE-CT: Contrast-enhanced computed tomography; SRH: Stigmata of recent hemorrhage; CS: Colonoscopy; UGIB: Upper gastrointestinal bleeding; PPI: Proton pomp inhibitor; DOAC: Direct-acting oral anticoagulant; BUN: Blood urea nitrogen; Cr: Creatinine; NSAID: Nonsteroidal anti-inflammatory drug; Hb: Hemoglobin; PT-INR: Prothrombin time-international normalized ratio; ROC-AUC: The area under the receiver operating characteristics curve; RR: Relative risk; CI: Confidence interval; GIB: Gastrointestinal bleeding; RCT: Randomized controlled trial; OR: Odds ratio; HR: Hazard ratio.

units of red blood cells or 4 units of fresh frozen plasma within 24 h, hemodynamic instability at the time of angiography, and older age are predictors of a positive angiography[63,64]. In addition, CT angiography may be useful as a noninvasive diagnostic tool before angiography, because it is more sensitive than transcatheter angiography and identifies bleeding at rates of 0.3 mL/min[65].

In a retrospective study of colonic diverticular bleeding with SRH on colonoscopy, the rate of interventional radiology and/or surgery due to failure of repeated colonoscopic hemostasis was higher for bleeding from the ascending colon (19%) than from other parts of the colon (0)[51]. Therefore, patients with bleeding from the ascending colon have a higher risk of being transferred for interventional radiology after colonoscopy.

Surgery

Studies on surgery for acute LGIB have recently decreased, probably because of advances in endoscopic hemostasis and interventional radiology. The complication and mortality rates of surgery for acute LGIB are as high as 60% and 16%, respectively[66]. Given these high rates, surgery should be reserved for patients with brisk, ongoing LGIB. Indications for emergency surgery for severe LGIB include (1) the bleeding source has been clearly identified but non-surgical interventions have failed, and ii) continued bleeding (6 units of red blood cells transfused) and the lack of a diagnosis despite a thorough work-up using endoscopic and radiographic modalities[48,67].

Localizing the bleeding lesion before surgical resection is important to prevent rebleeding after surgery from an unresected culprit lesion, and to prevent excess mortality after a blind total colectomy. In previous studies of surgical management for acute LGIB[68-71], the rebleeding rate was higher after a limited colonic resection (4%-18%) than after a total colonic resection (0-4%). In most of these studies[68-70], the mortality rate was lower after limited colonic resection (7%-22%) than after total colonic resection (20%-40%).

Therapeutic barium enema for diverticular bleeding

High-dose barium impaction therapy using concentrated (200%) barium sulphate for diverticular bleeding has been reported. Evidence of the effectiveness of initial hemostasis is poor, due to the studies being case reports or case series. Nevertheless, these reports suggest that this therapy may have advantages for hemostasis in patients with uncontrolled or recurrent presumptive diverticular bleeding[72-75]. Novel barium impaction therapy using an enteroscopic overtube with a balloon has been reported for diverticular bleeding in the right-sided colon, and can be used to apply sufficient barium pressure to the deep colon[76].

The effectiveness of barium impaction therapy with respect to long-term prevention of rebleeding was demonstrated in an RCT[77]. The hazard ratio (HR) of rebleeding in the barium group, comparing barium therapy to conservative therapy after spontaneous cessation of diverticular bleeding, was 0.34 (95%CI: 0.12-0.98).

MEDICATION MANAGEMENT

The management of medication use in the LGIB setting requires considering the risks of ongoing/recurrent bleeding and thromboembolic events (Figure 1). Cessation of these agents can be considered in patients on antithrombotic agents with life-threatening or serious bleeding. Although there are few data to guide the timing of the resumption of antithrombotic agents, current guidelines recommend resumption as soon as hemostasis is achieved[13,14]. A multidisciplinary approach involving cardiology, neurology, hematology, and gastroenterology is necessary, particularly for managing patients taking dual antiplatelet agents or anticoagulants.

Non-aspirin NSAIDs

Previous studies have indicated that NSAIDs increase the risk of both event and recurrence of LGIB[11,78-80]. In a retrospective cohort study of 342 patients with LGIB, the HR of NSAID use for recurrence was 2.0 (95%CI: 1.2-3.3)[11]. In a prospective study of 132 patients with diverticular bleeding, the recurrence rate at 12 mo was significantly higher in patients who continued NSAID use (77%) than in those who discontinued use (9%)[81]. Therefore, non-aspirin NSAIDs should be discontinued after acute LGIB, particularly in cases of diverticular bleeding. Unlike UGIB, changing from a non-selective NSAID to a cyclooxygenase-2 (COX-2) selective NSAID might be ineffective for preventing recurrence, because COX-2-selective and -non-selective agents increase the risk of LGIB[79,82].

Antiplatelet agents

Antiplatelet agents increase the risk of both event and recurrence of LGIB[11,79,80,83]. The risk of LGIB with antiplatelet agents use is approximately three times that for UGIB[84,85], probably because LGIB lacks prophylactic measures, such as *H. pylori* eradication and PPIs.

Available data on the influence of discontinuing aspirin in the GIB setting are as follows. A retrospective cohort study of patients with LGIB showed that the rate of cardiovascular events was significantly higher in those who discontinued aspirin (37%) than in those who continued the drug (23%), while the rate of recurrent LGIB was lower in the former cohort (7%) than in the latter cohort (19%) within 5 years[86]. In an RCT of peptic-ulcer bleeding, 60 d mortality was significantly higher in patients who discontinued aspirin after endoscopic therapy than in those who continued; the rate of rebleeding was not different between the groups[87]. Based on this evidence, aspirin for secondary prophylaxis in patients with established cardiovascular disease should not be interrupted to prevent thrombotic events in the LGIB setting. However, aspirin as the primary prophylaxis for patients who are not at high risk of cardiovascular events had little effect (0.07% absolute risk reduction)[88] and should be discontinued after LGIB.

The influence of short-term drug interruption in single antiplatelet users (aspirin or other antiplatelet agents) has not been determined. No difference in in-hospital rebleeding was observed in a retrospective study comparing patients who had their antiplatelet drug stopped for < 5 d with those who continued it throughout their admission[83]. In that study, cardiovascular events were too few to allow meaningful comparison.

There are some data that can guide the management of dual antiplatelet therapy. The risk of myocardial infarction and death after discontinuing dual antiplatelet therapy is high during the first 30 d following coronary stenting and during the first 90 d following acute coronary syndrome[89]. Such patients are advised to continue dual therapy. In contrast, discontinuing the second non-aspirin antiplatelet agent for up to 7 d is allowed for patients with more distant coronary stenting or coronary syndrome, because it seems to carry a relatively low risk as long as aspirin is continued[90].

Anticoagulants

Anticoagulants are classified into warfarin and DOACs. Two types of DOACs are currently available: thrombin inhibitors (dabigatran) and coagulation factor Xa inhibitors (rivaroxaban, apixaban, and edoxaban). Current endoscopic and LGIB guidelines do not discuss the role of a heparin bridge sufficiently, nor management of DOACs in the acute GIB setting[13,14]. Evidence is mainly based on studies of UGIB or all types of GIB.

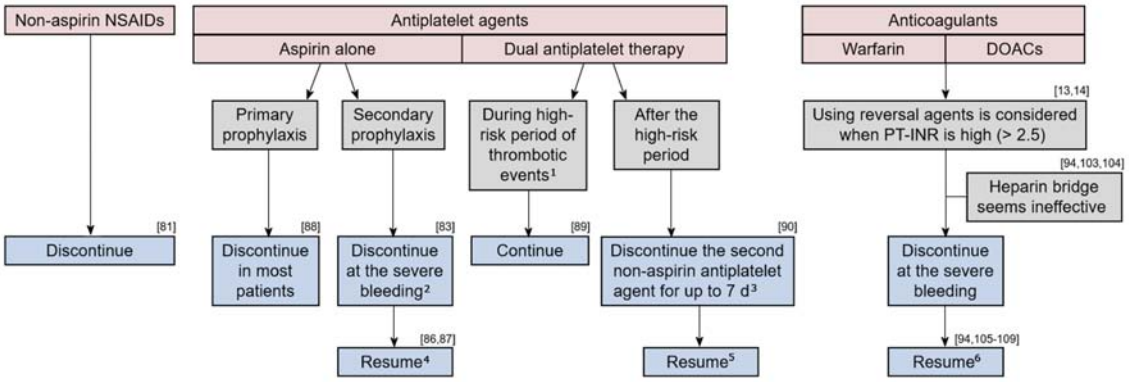

Figure 1 Recommendation for the management of medication based on current studies. [1]During the first 30 d following coronary stenting and during the first 90 d following acute coronary syndrome; [2]The influence of short-term discontinuation has not been determined; [3]Aspirin should be continued; [4]Resumption reduces cardiovascular events but may increase rebleeding; [5]The influence of long-term discontinuation has not been determined; [6]Changing to apixaban, or reducing the dose of dabigatran to 110 mg b.i.d may reduce rebleeding in GIB patients taking warfarin, dabigatran (150 mg b.i.d) or rivaroxaban. NSAIDs: Nonsteroidal anti-inflammatory drug; DOAC: Direct-acting oral anticoagulant; PT-INR: Prothrombin time-international normalized ratio.

Prothrombin time-international normalized ratio and the reverse method: Guidelines[13,14] recommend INR < 2.5 as being reasonable for endoscopy in the acute GIB setting, based on reports that a moderate elevation in INR does not increase the risk of rebleeding following endoscopic therapy for nonvariceal UGIB[91-93]. Guidelines also recommend using reversal agents before endoscopy for patients with an INR > 2.5, but the evidence for this is not well-established. Indeed, some retrospective studies found that a higher INR does not increase the rebleeding rate in LGIB[94] or all types of GIB[95]. Thus, an elevated INR appears not to carry a risk of rebleeding. However, an elevated INR at onset has been reported to be a predictor of thromboembolism within 90 d of endoscopy for all GIB (INR > 2.5, OR: 7.9)[94], and of mortality for nonvariceal UGIB (INR > 1.5, OR: 5.6)[96]. This is presumably because INR is an indicator of underlying comorbid diseases. In a study of all types of GIB, other factors related to anticoagulant management, such as the difference in onset and pre-endoscopic INR, reversal agent use, and anticoagulant interruption, were associated with thromboembolism[94]. Therefore, it might be unnecessary to actively reduce the INR. Rather, early endoscopy without using a reversal agent or interrupting anticoagulant therapy may be warranted for acute GIB.

Reversal of the anticoagulant effect should be considered for ongoing severe bleeding *via* intravenous vitamin K, fresh frozen plasma, or prothrombin complex concentrate (PCC) for warfarin users[97,98], and *via* oral charcoal, hemodialysis, idarucizumab, or PCC for DOAC users[99-102]. Oral charcoal is considered if a DOAC was taken within 2 h. Hemodialysis or idarucizumab is considered for dabigatran users. The effect of PCC on bleeding of DOAC users has not been established.

Heparin Bridge: Previous reports suggest that a heparin bridge might be ineffective in the acute GIB setting. A heparin bridge did not significantly alter the risk of rebleeding or thromboembolism in a recent retrospective study of patients with GIB[94]. In an RCT of warfarin users undergoing invasive procedures, the heparin bridge group suffered from more major bleeding than the non-bridged group, without a difference in the thromboembolism rate during the periprocedural period[103]. Furthermore, a similar result was found in a prospective observational study of DOAC users undergoing interventional procedures[104].

Resumption of anticoagulants: A meta-analysis concluded that resuming anticoagulants reduces the rate of thrombotic events in patients with disrupted use of anticoagulants due to GIB (HR: 0.68, 95%CI: 0.52-0.88), and mortality (HR: 0.76, 95%CI: 0.66-0.88), without significantly increasing the rebleeding rate (HR: 1.20, 95%CI: 0.97-1.48)[105]. This result was consistent with other reports[106-108]. Studies that compared warfarin and DOAC users reported that the rate of thrombotic events was similar between the two groups, during the 90 d after GIB[94] and during the anticoagulant-interrupted period[109]. A retrospective cohort study on DOAC users reported that the rate of thromboembolism within 90 d of GIB did not differ between those who resumed DOAC and those who did not[110]. In that study, a history of venous thromboembolism was associated with thromboembolism events (HR: 3.30, 95%CI: 1.29-7.38).

The optimal duration before restarting anticoagulants after an episode of GIB

remains uncertain. In a retrospective cohort study, the HRs of rebleeding, thromboembolism, and mortality in patients who resumed warfarin within 7 d were 3.27 (95%CI: 1.82-5.91), 0.76 (95%CI: 0.37-1.59), and 0.56 (95%CI: 0.33-0.93), respectively, compared with patients who resumed warfarin after 1 mo[107].

The bleeding risk of individual anticoagulants should be considered, when resuming anticoagulants in patients with high-risk GIB. Changing to apixaban, or reducing the dose of dabigatran to 110 mg b.i.d may reduce rebleeding in GIB patients taking warfarin, dabigatran (150 mg b.i.d) or rivaroxaban[111-115]. The HAS-BLED is a scoring system to evaluate bleeding risk among anticoagulants users[116]. However, the main outcome of the score is composite bleeding events, including intracerebral hemorrhage and GIB. One study focused specifically on the risk of acute GIB in anticoagulant users, and developed a new scoring model for acute GIB risk based on five factors (no PPI use, chronic kidney disease, chronic obstructive pulmonary disease, history of peptic ulcer disease, and liver cirrhosis). The c-statistic of the new score (0.65) was superior to that of the HAS-BLED score (0.57) for predicting acute GIB[117,118]. The utility of these scoring systems for predicting re-bleeding, and the strategy of changing anticoagulants would be important topics of study.

CONCLUSION

This literature review has summarized evidence for the initial management of acute LGIB. Assessing various clinical factors, including comorbidities, medication use, presenting symptoms, vital signs, and laboratory data is useful for risk stratification of severe LGIB. Early timing of colonoscopy could improve identification of the bleeding source and the rate of endoscopic intervention. CE-CT before colonoscopy may support identification, particularly for patients who can be examined immediately after the last hematochezia. How to deal with antithrombotic agents after hemostasis should be carefully considered. Further investigations are required to predict the need for early colonoscopy and hemostatic intervention in patients with LGIB.

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ORIGINAL ARTICLE

Basic Study

Endoscopic trans-esophageal submucosal tunneling surgery: A new therapeutic approach for diseases located around the aorta ventralis

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ARRIVE guidelines statement: The

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Abstract

AIM

To assess the efficiency of endoscopic trans-esophageal submucosal tunneling surgery (EESTS) technique for diseases located around the aorta ventralis.

METHODS

Nine pigs were assigned to EESTs. The procedures were as follows: First, a long esophageal submucosal tunnel was established. Second, full-thickness myotomy was created. Third, an endoscope was entered into the abdominal cavity through a muscle incision and the endoscope was around the aorta ventralis. Eventually, celiac trunk ganglion neurolysis, partial hepatectomy and splenectomy, partial tissue resection in the area of the posterior peritoneum, and endoscopic submucosal dissection (ESD) combined with lymph node dissection were performed. The animals were given antibiotics for 5 d and necropsied 7 d after surgery.

RESULTS

In all surgeries, one pig died from intraperitoneal hemorrhage after doing partial splenectomy, while the other pigs were alive after successfully operating other surgeries. For surgery of celiac trunk ganglion damage, at necropsy, there was no exudation in the abdominal cavity. Regarding surgery of partial hepatectomy, the wound with part healing was observed in the left hepatic lobe, and no bleeding or obvious exudation was seen. In surgery of partial splenectomy, massive hemorrhage was observed on the splenic wound surface, and the metal clips could not stop bleeding. After surgery of retroperitoneal tissue resection, mild

ARRIVE guidelines have been adopted.

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tissue adhesion was observed in the abdominal cavity of one animal, and another one suffered from severe infection. For surgery of ESD and lymph node dissection, a moderate tissue adhesion was observed.

CONCLUSION
EESTS is a feasible and safe technique for diseases located around the aorta ventralis.

Key words: Endoscopic trans-esophageal submucosal tunneling surgery; Diseases around the aorta ventralis; Endoscopic submucosal tunneling technique; Abdominal surgery; Animal model

Core tip: Endoscopic trans-esophageal submucosal tunneling surgery (EESTS) technique is a new branch of endoscopic tunneling technology for diagnosing and treating diseases located around the aorta ventralis. The objective of our study was to simulate surgeries in a porcine model and to assess the efficiency of this new strategy. The surgeries included celiac trunk ganglion neurolysis, partial hepatectomy and splenectomy, partial tissue resection in the area of the posterior peritoneum, and endoscopic submucosal dissection combined with lymph node dissection. And we confirmed that EESTS is a feasible and safe technique.

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INTRODUCTION

Nowadays, surgical procedures are shifting paradigms in minimally invasive surgery, including natural orifice transluminal endoscopic surgery (NOTES)[1]. NOTES is a technology of utilizing a flexible endoscope through a natural orifice (*e.g.*, the mouth, esophagus, stomach, rectum, vagina and urethra) to diagnose diseases and perform surgeries[2]. The emergence of endoscopic tunnel technique makes the diseases which used to need surgical or laparoscopic surgical treatments enter into the endoscopic therapy[3]. There is a significant question in scholars' mind why endoscopic tunnel technique has such an appropriate curative effect and has some advantages (*e.g.*, fewer complications). To answer this question, we can explain that the submucosal tunneling technique could well prevent the communication between the intra-luminal and the extra-luminal space by sealing the entry incision of the submucosal tunnel, and that gas or fluid within the lumen is prevented from entering the extra-luminal space, which could ensure the endoscopic therapy free of perforation[4,5]. In 2000, Kalloo *et al*[6] reported the first NOTES. They performed liver biopsy and examined the peritoneal cavity by using upper gastrointestinal endoscopy in a live swine model. In addition, they successfully confirmed the feasibility and safety of NOTES. In 2006, Rao *et al*[7] made the first attempt of transgastric NOTES in human. Subsequently, a number of scholars used NOTES to simulate a variety of intraperitoneal surgeries including cholecystectomy, colectomy, liver resection, and thyroidectomy in experimental animals[8-11]. A significant and hybrid model of NOTES was proposed for abdominal surgeries using transcutaneous rigid laparoscopes in combination with a flexible endoscope passing through a visceral incision[12,13]. Moreover, it was confirmed that NOTES had been less invasive, safer, and more cost-effective than either traditional or laparoscopic surgery[14].

However, there are several technical challenges in the development of NOTES[14-16]. First, it is difficult to close the entrance of natural orifices, which lacks an economic and precise suture technique. Second, the infection caused by liquid and air from natural orifices to the body cavity would unavoidably occur. Third, it would be lost in body cavity due to the insufficient localization and navigation. Forth, a flexible endoscope could not create a stable operative platform in order to perform a very

precise resection of tissues. Eventually, the loss of triangulation is a stubborn problem in either pure NOTES or laparoscopy. These challenges may puzzle endoscopists.

In this study, we attempted to put forward a new approach using endoscopic tunneling techniques to perform NOTES, which was named endoscopic trans-esophageal submucosal tunneling surgery (EESTS). Additionally, some preliminary studies were performed in a porcine model. The proposed technique can resolve the problem of closing entrance and infection. The aim of this study was to perform some endoscopic surgeries in animals' body cavity, including celiac trunk ganglion damage, partial hepatectomy, partial splenectomy, resection of the retroperitoneal organs, and endoscopic submucosal dissection (ESD) and lymph node dissection.

MATERIALS AND METHODS

Animals

Experiments were performed with the contribution of five males and four female porcine corpses at animal laboratory of Pinggu District Hospital (Beijing, China), and approved by the Ethics Committee of the Animal Facility of Chinese PLA General Hospital. All these animals were treated humanely and underwent a 3 d quarantine and acclimation period.

Preoperative preparation

All procedures were performed under general anesthesia, using the combination of chloramine hydrochloride (5 mg/kg) and Su Mian Xin II (0.1 mL/kg), as well as endotracheal intubation. All animals received antibiotic prophylaxis with a single dose of intravenous penicillin (3.2 million units) at 24 h before operating surgery. The pigs were in supine and raised right shoulder positions. Furthermore, the basal body temperature was measured before surgery. All endoscopic instruments were sterile whereas EESTS equipment underwent a high-level disinfection with 25% glutaraldehyde solution (Instrunet, Laboratorios Inibsa, Spain).

Performing EESTS

The procedures were performed by an experienced endoscopist. A gastroscope (GIF-Q260J, Olympus Medical Systems, Corp., Tokyo, Japan) was inserted through the pig's mouth which was secured with a self-made overtube. The procedure was maintained with CO_2 insufflation through the endoscope. The vital signs of pigs were closely monitored during the operation.

A submucosal tunnel was made as follows: (1) the esophagus and stomach cavity were rinsed repeatedly with saline, and this was followed by injecting 100 mL of metronidazole and retaining the fluid for 15 min before removing it; (2) the site of interest was the right posterior wall of the cardia and located by injecting 0.5 mL of methylene blue (1:2); (3) 10 mL of saline with methylene blue (1:10000) was injected to create a submucosal cushion in the right posterior wall at 5 cm above the esophagogastric junction; (4) inverted T-shaped incision was created in the mucosal layer of the esophagus with an electric knife (KD-V451M, Olympus Medical Systems, Corp., Tokyo, Japan) and an electrosurgical unit (PSD-30, Olympus Medical Systems, Corp., Tokyo, Japan); (5) the endoscope was introduced into the submucosal space and gently separated the submucosa from the muscularis propria, creating a submucosal tunnel. The dark blue localization spot in the cardia was then identified; (6) at the end of the tunnel, a progressive full-thickness incision was created through the muscularis propria with the needle-knife and the endoscope was entered into the abdominal cavity. Once the scope was moved into the lesser omental sac, blunt dissection with an electric knife was used to advance the scope until the aorta ventralis was identified; (7) an artificial pneumoperitoneum was created and a 20 mL needle was used to puncture exhaust to maintain relatively stable intra-abdominal pressure; and (8) after simulating the abdominal operation, the submucosal space was closed by applying clips at the mucosal entry site.

Abdominal operation procedure

Surgery of celiac trunk ganglion damage: At the end of the esophageal tunnel, the endoscope was inserted into the abdominal cavity from the posterior wall of the gastric fundus near the cardia. The aorta ventralis and its branches were observed in the endoscopic direct status. The abdominal ganglions were invisible in healthy pigs. Thus, the surgery was simulated by partially removing tissues in the angle formed between the aorta ventralis and the coeliac trunk using thermal biopsy forceps and an electric knife (Figure 1A and B)

Surgery of partial hepatectomy: The endoscope was inserted into the abdominal

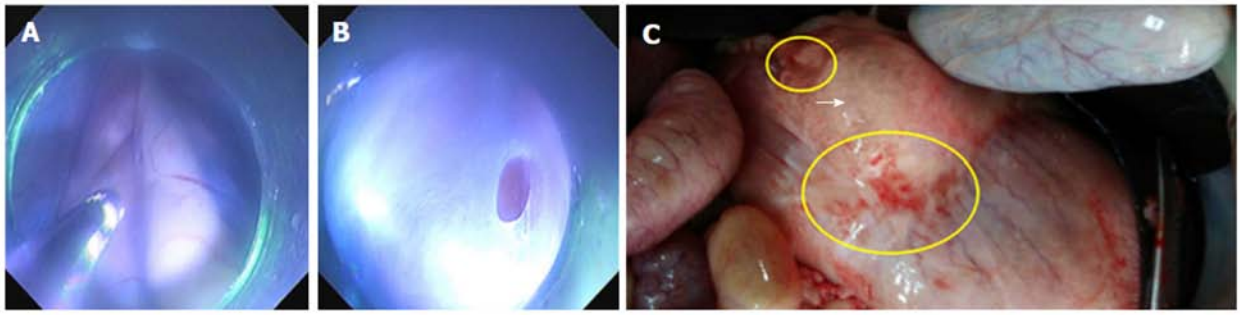

Figure 1 Surgery of celiac trunk ganglion damage. A: The aorta ventralis was observed in the endoscopic direct status and the aortic tissue was clamped by thermal biopsy forceps; B: The peritoneal tissue was partially removed by biopsy forceps for simulating ganglion destruction; C: Anatomic structure in the posterior gastric wall. The white arrow shows the lesser omental sac, and the two yellow loops show tissue adhesion.

cavity. The left hepatic lobe was observed by twisting the body of the endoscope to the right (clockwise) or left (counter-clockwise) direction. Wedge resection of the liver tissues was carried out with an electric knife using a hybrid cutting mode (electro-coagulation was higher than that for cutting). Specimens were collected and stored for diagnostic pathology investigation. The wound was treated with conventional maneuvers (coagulation forceps, Olympus Medical Systems, Corp., Tokyo, Japan) as displayed in Figure 2A and B)

Surgery of partial splenectomy: The endoscope was inserted into the area around the aorta ventralis. It was attempted to find the left hepatic lobe and then, explore the spleen by upward turning the endoscope. Pig's spleen is in banded shape and the spleen tail was relatively unfixed and in free status in the abdominal cavity. Wedge resection of the spleen tissues was done with an electric knife using a hybrid cutting mode. Specimens were collected and stored for diagnostic pathology investigation. The wound was treated with conventional maneuvers (coagulation forceps), as shown in Figure 3A and B.

Surgery of retroperitoneal tissue resection: The endoscope was inserted into the area around the aorta ventralis. The retroperitoneum was observed in the endoscopic direct status and the aorta ventralis was the predetermined structure for identification (Figure 4A and B). Tissues in the retroperitoneum were partially removed using an electric knife, avoiding thick blood vessels (Figure 4C and D). This aimed to simulate surgical removal of the retroperitoneal tumors. The omentum majus was observed when the endoscope was reversed continually. Afterwards, some tissues of the omentum majus were removed using endoloops (Olympus Medical Systems, Corp., Tokyo, Japan) (Figure 4E and F).

Surgery of ESD and lymph node dissection: The endoscope was inserted into the area around the aorta ventralis. In the endoscopic direct status, the gastric fundus and the posterior wall of the gastric body were observed. Blunt dissection of omental tissue outside the stomach wall was undertaken with an electric knife. The endoscope exited from the esophageal mucosal tunnel and entered into the gastric cavity. The simulation of ESD for early gastric carcinoma in posterior gastric body wall was undertaken. After completing submucosal dissection, 0.5 mL of methylene blue (1:2) and saline mixture was used as a marker and injected to the intrinsic muscle layer surrounding the wound. The endoscope was inserted into the posterior wall of the gastric fundus near the cardia in the abdominal cavity through the end of the esophageal tunnel. Methylene blue marker was identified and lymph nodes outside the gastric wall were then dissected. A full-thickness incision was created in the posterior part of the gastric body and the opening was enlarged to the extent that the metal clip could not close it. Another endoscope was inserted into the abdominal cavity through this esophageal tunnel. Lateral wall of the perforation was pulled by the tongs from outside the gastric wall for alignment and then, this perforation was closed by that endoscope inside the gastric cavity using metal clips (Olympus Medical Systems, Corp., Tokyo, Japan).

Postoperative care and necropsy: All animals were fasted within 24 h after doing surgery. They were orally administered with glucose (1000 g/d) for 2 d and then, were given regular meals. All animals received intravenous penicillin (3.2-million units/d) after surgery and were monitored for signs of abdominal infection and sepsis during the next 6 d. Necropsy was made for the evidence of ascites and signs of

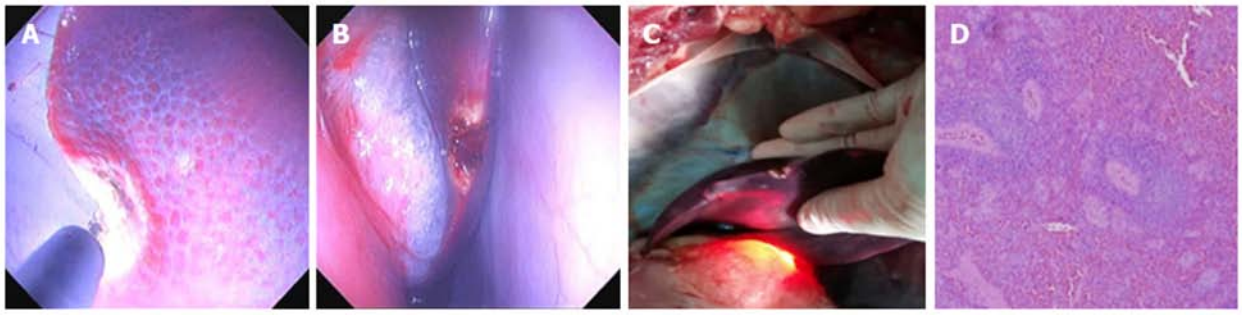

Figure 2 Surgery of partial hepatectomy. A and B: Partial hepatectomy using an electric knife; C: The wound in the left hepatic lobe; D: Hepatic tissue stained with hematoxylin and eosin (200 ×).

infection, injuries in other organs and tissue adhesion in surgery position. The surgical specimens were stained with hematoxylin and eosin for pathological diagnosis.

RESULTS

In this study, nine animals weighing 28.5 ± 5.2 kg were examined. In addition, eight animals, in which the surgery was completed, survived until they were euthanized on the 7th day.

Surgery of celiac trunk ganglion damage

Two pigs survived well after finishing the operation without complications such as delayed air leak, severe infection and injuries of other organs. But both had the complication of mild tissue adhesion. The procedure time was 32 min and 28 min, respectively, and the incision size was 5 mm for both. At necropsy, there was no exudation in the abdominal cavity. In addition, the lesser sac in the posterior gastric wall was adhered to adjacent tissue (Figure 1C). The postoperative pathology showed that one was in part healing and one was in minimal inflammatory reaction.

Surgery of partial hepatectomy

Two pigs survived well after doing the operation without the complications of delayed air leak and injuries of other organs. But one had the complications of severe infection and medium tissue adhesion. One did not have the complication of severe infection, but had the complication of tissue adhesion. The procedure time was 47 min and 39 min, and the incision size was 10 mm and 15 mm, respectively. At necropsy, the wound with part healing was observed in the left hepatic lobe, and no bleeding or obvious exudation was seen in the abdominal cavity (Figure 2C). Furthermore, the lesser sac in the posterior gastric wall was adhered to adjacent tissue. The liver tissue, which was excised by an electric knife, was further confirmed by a pathological examination as illustrated in Figure 2D. The postoperative pathology showed that both were in part healing.

Surgery of partial splenectomy

One pig was dead after completing the operation without the complications of delayed air leak and injuries of other organs. But this pig had the complications of severe infection and severe tissue adhesion. The procedure time was 92 min. The incision size was 15 mm. During the procedure, partial splenectomy was made in the spleen tail and massive hemorrhage was observed on the wound surface. The wound was repeatedly rinsed with norepinephrine saline (1:20000) and was treated with conventional maneuvers (coagulation forceps) without a proper effect. Then, metal clips were applied to clamp the splenic vessels, and bleeding significantly decreased. The splenic vessels could not be fully clamped, and limited by the small size of metal clips. Consequently, a small amount of bleeding was observed in the splenic wound (Figure 3A and B). After rapid recovery from the anesthesia in 3 h after doing the surgery, the animal's state was extremely poor and it immediately received antibiotics fluid transfusion. On the second day, the pig died. At necropsy, massive bleeding was seen in the abdominal cavity and diffuse blood oozing was observed in the splenic wound, as displayed in Figure 3C. The spleen tissue, which was excised by an electric knife, was further confirmed by a pathological examination (Figure 3D). The postoperative pathology showed that one pig was in intraperitoneal hemorrhage.

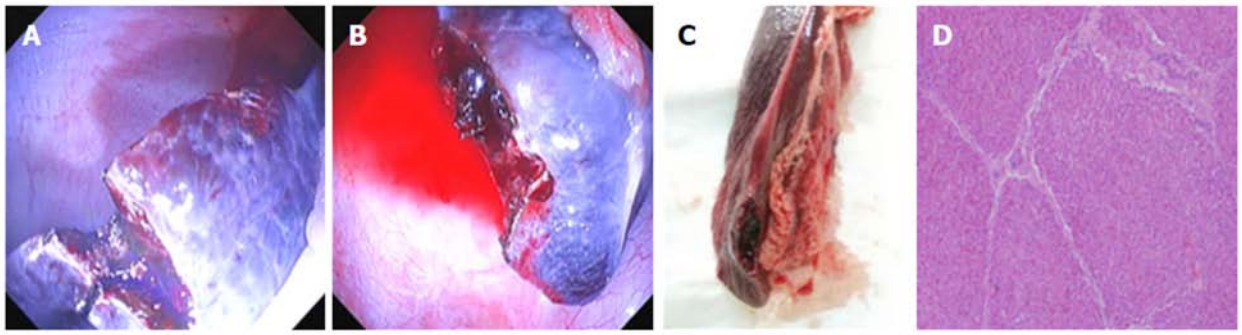

Figure 3 Surgery of partial splenectomy. A: Partial splenectomy using electric knife; B: Wedge resection of the spleen and massive bleeding during operation; C: The wound in the spleen tail; D: Spleen tissue stained with hematoxylin and eosin (200 ×).

Surgery of retroperitoneal tissue resection

Two pigs survived well after completing the operation without the complications of delayed air leak and injuries of other organs. But one had the complications of severe infection and moderate tissue adhesion. One did not have the complication of severe infection, but had the complication of mild tissue adhesion. The procedure time was 42 min and 51 min, and the incision sizes was 15 mm and 20 mm, respectively. At necropsy, mild tissue adhesion was observed between the posterior gastric wall and retroperitoneal space in one of the pigs. Besides, another one suffered from severe infection. Because of amounts of chyme in the stomach and longer operative time, chyme refluxed into the abdominal cavity through the entrance of the esophageal tunnel. During this procedure, plenty of saline was used to flush the abdominal cavity without an appropriate effect. The pig had a fever up to 43.2 °C. At necropsy, a severe tissue adhesion was observed between the posterior gastric wall and retroperitoneal space. The omentum majus tissue, which was excised with an electric knife, was further confirmed by a pathological examination (Figure 4D). The postoperative pathology showed that one was in minimal inflammatory reaction and one is in complete healing.

Surgery of ESD and lymph node dissection

Two pigs were employed to simulate the ESD of early gastric carcinoma, and dissection of lymph nodes outside the gastric wall survived well (Figure 5). All animals survived well after doing the operation without the complications of delayed air leak and injuries of other organs. But both had the complications of severe infection and medium tissue adhesion. The procedure time was 57 min and 66 min, and the incision size of ESD was 25 mm and 20 mm, respectively. The incision size of lymph node dissection were both 5 mm. At necropsy, a moderate tissue adhesion was observed in the abdominal cavity.

DISCUSSION

It is noteworthy that the area around the aorta ventralis is difficult to reach in laparoscopic surgery, which makes doing the operation to be difficult. However, the endoscope could easily approach to this area, which makes it simple and convenient.

In a previous study performed by authors, it confirmed some surgical techniques as follows: (1) Supine and raised right shoulder position was chosen as surgical positioning; (2) Methylene blue was injected to preoperative cardiac submucosa in the posterior wall as a marker of piercing site; and (3) An esophageal submucosal tunnel was established to the cardia, a progressive full-thickness incision through the muscularis propria was created, and an endoscope was inserted into the abdominal cavity. In this study, we inserted the endoscope into the area around the aorta ventralis to simulate intraperitoneal surgeries, including celiac trunk ganglion damage, partial hepatectomy, partial splenectomy, resection of regional tissues in the retroperitoneum, and ESD and lymph node dissection.

In the surgery of celiac trunk ganglion damage, it was revealed that the aorta ventralis could be identified as the predetermined structure in retroperitoneal space when the endoscope was in the direct status. The abdominal ganglions were scarcely found in healthy pigs. Thus, a part of tissue around the aorta ventralis was removed using thermal biopsy forceps and an electric knife. All animals received accurate postoperative care and survived after doing surgery as well; consequently, the

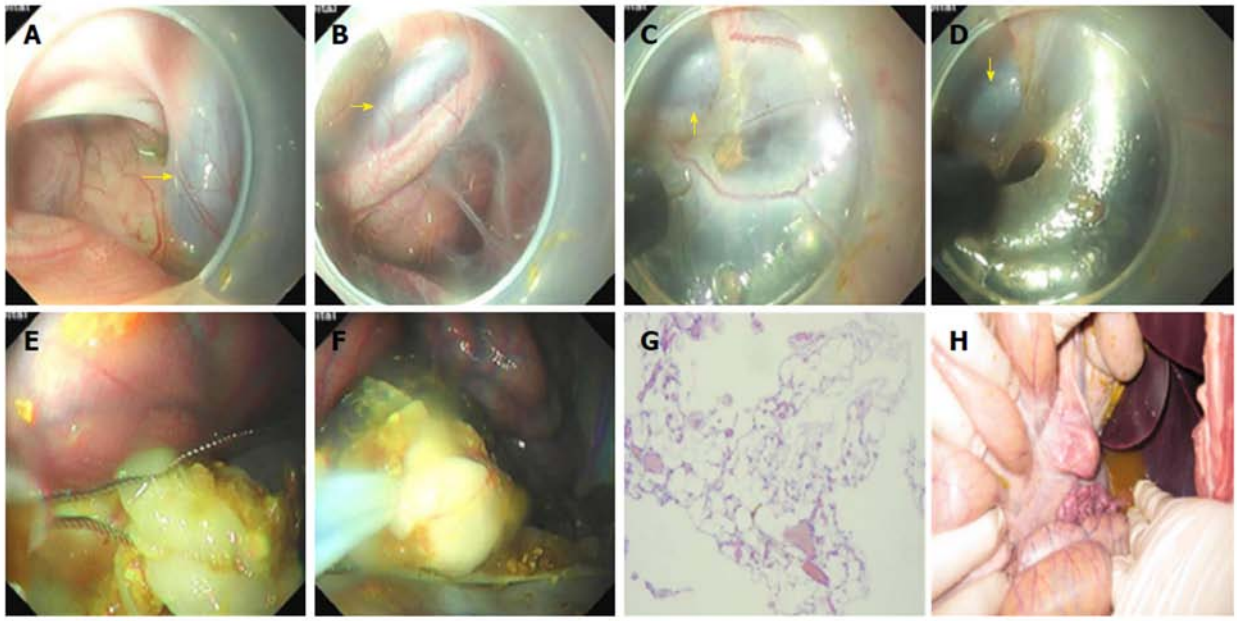

Figure 4 Surgery of retroperitoneal tissue resection. A: The aorta ventralis was observed in the endoscopic direct status; B: The retroperitoneal space was visible near the aorta ventralis; C and D: Partial resection of tissues in the retroperitoneal space using an electric knife; E and F: Partial resection of the omentum majus using an endoloop; G: Omentum majus tissue stained with hematoxylin and eosin (200 ×); H: A large amount of liquid and chyme in the abdominal cavity.

surgery was successful. However, finding and recognizing ganglions around the aorta ventralis was difficult. Thus, it necessitates that endoscopists should increase their knowledge about the anatomy of the abdomen and predetermined structure should be confirmed as well.

In the surgery of partial hepatectomy, the liver tissues were dissected with an electric knife using the hybrid cutting mode. No obvious bleeding occurred during the operation and all animals survived well after finishing surgery. The liver tissue was confirmed by a pathological examination. A massive liver resection could not be operated because of the restriction to the suitable endoscopic instruments and severe hemorrhage complications of large vessel injuries. Thus, further studies should concentrate on the development of endoscopic instruments and techniques.

In the surgery of partial splenectomy, the surgery was performed in the spleen tail and massive hemorrhage was found on the wound surface, while massive bleeding was seen in the abdominal cavity, and diffuse blood oozing was observed in the splenic wound. Several conventional maneuvers (coagulation forceps) were applied to stop bleeding, however, that failed. Then, metal clips were used to clamp the splenic vessels, while those metal clips could not fully block the splenic blood supply, which was limited to a specification for metal clips (5 mm of arm length and 135° of opening angle). A pig died on the second day after doing the operation. This can be justified as follows: (1) Because of the features of the spleen with fragile tissue and rich blood supply; and (2) A traumatic spleen rupture requires emergency total splenectomy by surgeons. Moreover, it is necessary to suture the splenic artery and vein in order to stop bleeding during the operation. Hence, this surgery could not be properly undertaken by endoscopic technique.

In the surgery of retroperitoneal tissue resection, some tissues were removed in the retroperitoneal space. No obvious complications occurred during the operation, and all animals survived after completing the surgery. However, one of the pigs suffered from severe infection, while that survived. Besides, omentum majus tissue was confirmed by a pathological examination.

Due to the development of endoscopic technology and the improvement of cancer screening systems, the diagnostic rate of early gastric cancer (EGC) has been remarkably increased. This has led to an increase of survival rate of gastric cancer. Recently, ESD has been preferred in order to treat EGC with strict indications within the superficial submucosa (SM1). In addition, the expanded indications within SM1 and the submucosa (SM2), particularly accompanied with lymph node metastasis (LNM), have been a subject of debate among clinicians[17]. EGC invasion into the deep submucosa (SM3) is an absolute indication of surgery[17]. Additionally, patients with EGC and LNM should receive lymph node dissection. However, subtotal gastrectomy is difficult to be accepted by the patients because of greater trauma and poor quality

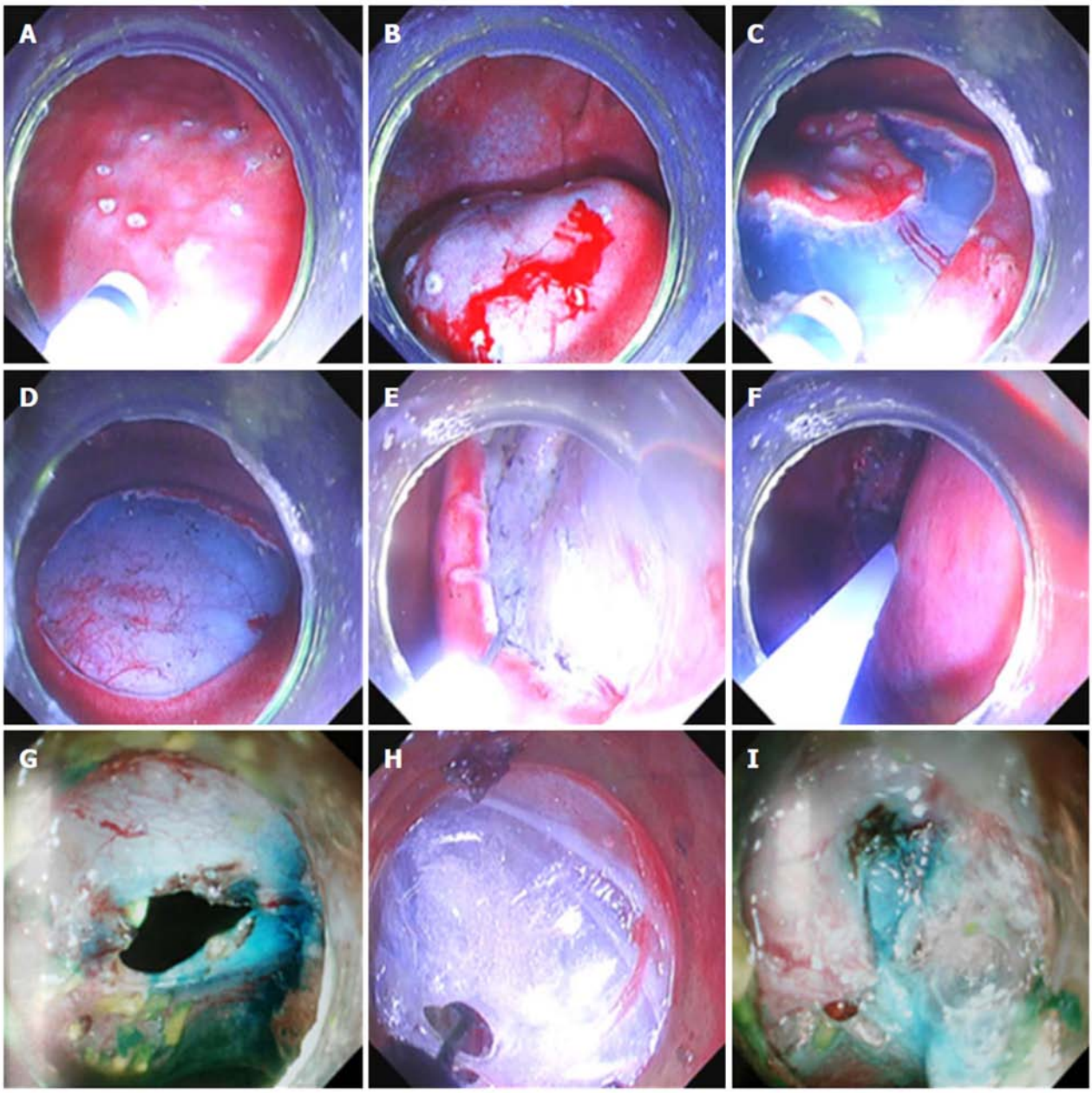

Figure 5 Surgery of endoscopic submucosal dissection and lymph node dissection. A-D: Simulation of ESD of early gastric carcinoma in pigs; E and F: Methylene blue marker injected to the intrinsic muscle layer surrounding the wound; G and H: Dissection of lymph nodes outside the gastric wall; G: Incision of the serosa and muscularis propria; H: Separation of tissues outside the gastric wall; I: A close large gastric perforation using two endoscopes. ESD: Endoscopic submucosal dissection.

of life after operation. Conversely, endoscopic physicians could not perform lymphadenectomy. Thus, a hybrid NOTES technique enables minimal surgical resection and would be a new alternative in EGC treatment. Hybrid NOTES utilizes the ESD technique for local excision of early carcinoma, which is simultaneously combined with a laparoscopic lymphadenectomy in case of EGC with a risk of LNM[13,17]. Satisfactory results were reported in some studies[13,18]. Although laparoscopic surgery is a minimally invasive treatment, a laparoscope is inserted into the abdominal cavity by cutting the abdominal wall.

In the surgery of ESD and lymph node dissection, two endoscopes were utilized instead of a laparoscope to clear the lymph nodes around the stomach. Moreover, it was confirmed that this surgery is feasible. A new and simple method of perforation closure with two endoscopes was also proposed in the experiment. One endoscope with tongs was used to pull the lateral wall of the perforation from outside the gastric wall for alignment, and another endoscope with metal clips was employed to close this perforation.

However, the indication of EESTS seems to be limited at the present time because it

contains several limitations. First, tunnel and penetration site could not be created in the same place because of scar healing. Second, various degrees of tissue adhesion and abdominal infection were inevitable regardless of the application of antibiotics before and after operation. Third, a flexible endoscope easily gets lost in the abdominal cavity. Sufficient localization and navigation of endoscope would be key points. Forth, instruments, which are specially designed for NOTES treatments in the abdominal cavity, have not been developed. Furthermore, in this study, an electric knife was used for ESD, thermal biopsy forceps and snare were utilized as well, which limited the operations. Therefore, only a small amount of tissues could be removed. Moreover, hemostasis was difficult, and the abdominal vessels could not be fully clipped.

In conclusion, EESTS by the submucosal tunneling technique to simulate surgeries around the aorta ventralis is feasible, efficient, and relatively safe in a porcine model. However, the safety of profile has to be improved before adopting in a clinical setting. And developing new endoscopic instruments suitable for the technique will be one of the future directions.

ARTICLE HIGHLIGHTS

Research background

Surgical procedures are shifting paradigms in minimally invasive surgery nowadays, including natural orifice transluminal endoscopic surgery (NOTES), which is a technology of utilizing a flexible endoscope through a natural orifice to diagnose diseases and perform surgeries. The emergence of endoscopic tunnel technique makes the diseases which used to need surgical or laparoscopic surgical treatments enter into the endoscopic therapy.

Research motivation

We attempted to put forward a new approach using endoscopic tunneling techniques to perform NOTES, which was named endoscopic trans-esophageal submucosal tunneling surgery (EESTS).

Research objectives

To assess the efficiency of endoscopic trans-esophageal submucosal tunneling surgery technique for diseases located around the aorta ventralis.

Research methods

We simulated surgeries in a porcine model and to assess the efficiency of this new strategy.

Research results

One pig died from intraperitoneal hemorrhage after doing partial splenectomy, while the other pigs were alive after successfully operating all surgeries.

Research conclusions

We confirmed that EESTS is feasible and safe.

Research perspectives

EESTS by the submucosal tunneling technique to simulate surgeries for diseases located around the aorta ventralis is feasible, efficient, and relatively safe in a porcine model at least. Developing new endoscopic instruments suitable for the technique will be one of the future directions. However, the safety of profile has to be improved before adopting in a clinical setting.

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ORIGINAL ARTICLE

Case Control Study

Autonomic functions and gastric motility in children with functional abdominal pain disorders

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Abstract

BACKGROUND

Abdominal pain-predominant functional gastrointestinal disorders (AP-FGIDs) are the most common cause of recurrent abdominal pain in children. Despite its high prevalence, the underlying pathophysiology of this condition is poorly understood.

AIM

To assess the role of gastric dysmotility and autonomic nervous system dysfunction in the pathophysiology of AP-FGIDs.

METHODS

One hundred children, fulfilling Rome III criteria for AP-FGIDs, and 50 healthy controls, aged 5 to 12 years, were recruited after obtaining parental consent. All patients were investigated for underlying organic disorders. Gastric motility and cardiovascular autonomic functions were assessed using validated non-invasive techniques.

RESULTS

The main gastric motility parameters assessed (gastric emptying rate [45.7 *vs* 59.6 in controls], amplitude [48.7 *vs* 58.2], frequency of antral contractions [8.3 *vs* 9.4], and antral motility index [4.1 *vs* 6.4]) were significantly lower in children with

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Data sharing statement: Technical appendix, statistical code and dataset available from the corresponding author at niranga@kln.ac.lk.

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AP-FGIDs ($P < 0.05$). The post-prandial antral dilatation at 1 min after the test meal significantly correlated with the severity of abdominal pain ($P < 0.05$). Assessment of autonomic functions in AP-FGID patients showed neither a significant difference compared to the control group, nor a correlation with gastric motility abnormalities ($P > 0.05$). The duration of pain episodes negatively correlated with the parasympathetic tone (maladaptive parasympathetic tone) ($P < 0.05$).

CONCLUSION
Children with AP-FGIDs have abnormal gastric motility but normal cardiovascular autonomic functions. There is no relationship between abnormal gastric motility and autonomic functions. The pathogenesis of AP-FGIDs is not related to cardiovascular autonomic dysfunction.

Key words: Abdominal pain; Functional gastrointestinal disorders; Autonomic function; Gastric motility

Core tip: In this study, we examined the relationship between cardiovascular autonomic functions and functional abdominal pain disorders in children. We failed to demonstrate a significant difference in autonomic functions and a significant relationship between gastric motor abnormalities and autonomic functions in affected children. In this paper, we propose functional extrinsic denervation and maladaptive parasympathetic division as possible contributing factors for the impaired gastric motility and symptoms in functional abdominal pain disorders, which is demonstrated in the 'Automatic Stomach' model.

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INTRODUCTION

Abdominal pain-predominant functional gastrointestinal disorders (AP-FGIDs) are one of the most recognized groups of gastrointestinal disorders in children across the world. It has an estimated global prevalence of 13.5% in community- and school-based surveys[1]. This group of disorders consists of irritable bowel syndrome (IBS), functional abdominal pain (FAP), functional dyspepsia (FD) and abdominal migraine[2]. Although not directly related to mortality, AP-FGIDs have a considerable effect on health-related quality of life and healthcare expenditure[3,4]. Even with the highly advanced modern technologies, pathophysiological mechanisms of FGIDs are not yet clearly understood. The recognized pathophysiological mechanisms include visceral hypersensitivity, altered gastrointestinal motility, immunological dysfunction, altered gastrointestinal microbiota, altered intestinal permeability, genetic factors and psychosocial disturbances[5].

Abnormalities in gastrointestinal motor function have been suggested as a potential pathophysiological mechanism in AP-FGIDs. They include, dilated gastric antrum at fasting period[6,7], delayed gastric emptying[6,8-11], impaired initial distribution of a meal[12], impaired gastric accommodation to a meal[13] and antral hypomotility[8-11,14].

The autonomic nervous system (ANS) is an integral part of the brain-gut axis that is involved in regulating gastrointestinal motility. Some studies have demonstrated dysfunctions in both sympathetic and parasympathetic divisions of the ANS in children and adults with functional gastrointestinal disorders (FGIDs)[15-17]. Elsenbruch and Orr have noted a significant correlation between vagal response and post-prandial abdominal symptoms in patients with diarrhoea-predominant IBS[18]. Abnormalities of gastric motility and underlying vagal defects have been demonstrated in adult patients with IBS[15,18]. In addition, the ANS is thought to play an important role in modulating visceral sensitivity in FGIDs[19]. However, the

relationship between autonomic function and gastric motility has not been studied in affected children.

The main objective of this study was to assess ANS functions in paediatric patients with AP-FGIDs and its relationship with gastric motor functions.

MATERIALS AND METHODS

Study design

This is a comparative, cross-sectional study to assess cardiovascular autonomic functions and gastric motility in children with AP-FGIDs.

Recruitment of the patients

All consecutive patients aged 5-12 years who were eligible according to the inclusion criteria were recruited from the paediatric out-patient clinics of North Colombo Teaching Hospital, Ragama, Sri Lanka and investigated in the Gastroenterology Research Laboratory, Faculty of Medicine, University of Kelaniya, Sri Lanka. A detailed history was taken from each subject and his or her parents after obtaining written informed consent. Details regarding pain characteristics and autonomic symptoms were obtained using an interviewer-administered pre-tested questionnaire. AP-FGIDS were diagnosed using Rome III criteria[2].

Inclusion criteria

(1) Fulfilment of Rome III criteria for at least one AP-FGID AND;

(2) Abdominal pain at least once per week for at least 2 mo prior to diagnosis AND;

(3) Pain severity more than 25 mm on a 100-mm visual analogue scale and severe enough to interrupt the activities of the child (*e.g*., sleep, play, schooling, *etc*).

All patients were screened for organic diseases using a detailed history, and complete physical examination, including growth parameters, stool microscopy, urine microscopy and culture, full blood count, C-reactive protein, liver function tests, renal function tests and ultrasound scan abdomen. Special investigations performed, based on clinical judgment of the consultant paediatrician who assessed the patients, included upper and lower gastrointestinal endoscopy, serum amylase and X-ray kidney-ureter-bladder. Patients were not screened for coeliac disease since it is extremely rare in Sri Lanka[20].

Exclusion criteria

(1) Clinical or laboratory evidence suggestive of an organic pathology.

(2) Chronic medical or surgical diseases other than AP-FGIDs.

(3) Long-term medication for any illness other than AP-FGIDs.

(4) Previous abdominal surgery.

(5) Subjects who had received prokinetic drugs or any other drugs that can alter gastrointestinal motility during the 30 d prior to the diagnosis being made.

Recruitment of controls

An age- and sex-compatible group of children were recruited from the community of the same geographical area as controls after obtaining written parental consent. None of the controls had acute or chronic disease or symptoms related to the gastrointestinal tract.

Subject preparation for testing

Autonomic functions and gastric motility were assessed on the same day (gastric motility from 8:30 am to 9:30 am and autonomic function test from 9:30 am to 10:30 am) under thermo-neutral conditions (26°C). All girls who had attained menarche underwent laboratory investigations during the proliferative phase of their menstrual cycles. All medications with adrenergic and cholinergic properties were discontinued for a period of at least five times the half-life of the specific medication. All subjects were advised to refrain from ingesting beverages containing caffeine, nicotine or alcohol for at least 8 h prior to testing. They were in a fasting state for at least six hours prior to the study. A standard breakfast was given with water after completion of gastric motility assessment. The autonomic function was assessed in all subjects 30 minutes after completion of breakfast.

Assessment of gastric motility

Gastric motility was measured in all children with AP-FGIDs and the controls by a previously reported and validated ultrasound method[21] using a high-resolution real-time scanner (Siemens ACUSON X300™) with 1.8 MHz to 6.4 MHz curve linear transducer and with facilities to record and playback. All gastric motility parameters

were assessed by the same investigator (NMD) who was blind to the diagnosis and results of the autonomic function tests. The main gastric motility parameters assessed were fasting antral area, gastric emptying rate, frequency and amplitude of antral contractions and antral motility index.

Assessment of cardiovascular autonomic functions

All subjects underwent autonomic cardiovascular tests according to the test battery described by Ewing *et al*[22], using the standard procedures described. All autonomic functions were assessed by the same investigator (AK) who was blind to the gastric motility status. The test battery consisted of four autonomic function tests conducted in the following order, and the results were recorded in a data sheet.

(1) Blood pressure (BP) response to standing from lying down position.
(2) Heart rate response to standing from lying down position.
(3) Heart rate variation with deep breathing.
(4) Valsalva test.

Before the test, the procedures were explained and mimicked for the benefit of each subject.

After instrumentation, children were subjected to ten minutes of mandatory rest. At the end of the rest period, the electrocardiogram (ECG) recording from lead II was started along with the BP recording. Thereafter, two readings of BP and heart rate were obtained at an interval of two minutes between two consecutive recordings. The average of the two readings was recorded as resting heart rate and BP.

Test 1 - BP response to standing from lying down position: BP readings were recorded one minute after the unaided standing up, maintaining the arm cuff at the level of the heart. The one-minute systolic BP was compared with the resting systolic BP, and the postural change in systolic BP was calculated. An automated BP machine (A&D Medical®) with a paediatric cuff, which was calibrated against a standard mercury sphygmomanometer, was used. The BP response to standing is dependent upon sympathetic adrenergic function[23].

Test 2 - Heart rate response to standing from lying down position: ECG was recorded for a further 60 seconds after standing. The heart rate ratio (30:15 ratio) was calculated as the ratio between the longest R-R interval at around the 30th beat (R-R 30) and the shortest R-R interval at or around the 15th beat after standing (R-R 15). The 30:15 ratio was calculated as R-R 30/R-R 15. An increment in the 30:15 ratio was considered an increased parasympathetic response[24].

Test 3 - Heart rate response to deep breathing: Subjects were instructed to sit quietly and to breathe deeply at six breaths per minute (five seconds in and five seconds out). The investigator guided them through the manoeuvre by counting. Continuous ECG recording (Lead II) was completed for three consecutive artefact-free cycles of deep inspiration and expiration. The difference between maximum and minimum heart rates during each cycle was calculated, and the mean difference of the three cycles was obtained. Impairment in heart rate variability is a sign of parasympathetic dysfunction[23]. Increased parasympathetic response is indicated by widening of the difference[25].

Test 4 - Valsalva test: Subjects were asked to exhale into a mouthpiece connected to a mercury manometer and to maintain the expiratory pressure at 20 mmHg for 15 s in the sitting position. ECG was recorded during this manoeuvre and for 45 seconds afterwards. Pre-testing showed that most of the children were not able to achieve 40 mmHg expiratory pressure proposed by Ewing. Therefore, we set the value at 20 mmHg, which was achieved by the children. The child was allowed to rest for one minute before repeating the Valsalva manoeuvre. The Valsalva ratio was calculated by dividing the maximum R-R interval following Valsalva manoeuvre with the minimum R-R interval during the Valsalva procedure. The mean ratio of the two attempts was calculated. A reduced ratio indicates parasympathetic dysfunction[23].

Tools used to assess symptoms

Autonomic symptoms were assessed by a modified composite autonomic symptom scale (commonly known as COMPASS) which was translated and validated for the local language[26].

Gastrointestinal symptoms were assessed using a translated and validated Rome III questionnaire[27,28]. The severity of abdominal symptoms was recorded on a 100-mm visual analogue scale.

Statistical methods

The sample size was calculated using the 30:15 ratio taken from a previous study done

on obese children aged 5-10 years in India[29]. The similarity with the race and age group was considered for selecting values from the Indian study. At a power of 90% and significance level of 95%, the minimum sample required was 26 per group.

All statistical analyses were completed using PSPP version 0.8.3-g5f9212 statistics software (Free Software Foundation, Inc. http://fsf.org/). Means and standard deviations were calculated for continuous variables, and frequencies and percentages were taken for categorical variables. For continuous data, nonparametric, a Mann Witney *U* test was used. For dichotomous data, a chi-square test was used to assess differences between the two groups. A two-tailed level of significance of 0.05 was used for the analysis.

Ethical approval

This study protocol was approved by the Ethics Review Committee, Faculty of Medicine, University of Kelaniya, Sri Lanka.

RESULTS

Sample characteristics

A total of 100 patients with AP-FGIDs [39 (39%) boys, mean age 7.9 years (SD 2.1 years)] and 50 healthy controls [20 (40%) boys, mean age 8.6 years (SD 1.9 years)] were recruited for this study. The AP-FGID group consisted of 54 (54%) children with FAP, 33 (33%) with IBS, and 13 (13%) with FD.

Autonomic symptoms in study subjects

The autonomic symptoms related to the gastrointestinal tract were significantly higher among the AP-FGID group (Table 1). The extra-intestinal symptoms, with the exception of the presence of cold feet, were higher among the AP-FGID group, though the difference was not statistically significant.

Autonomic parameters in study subjects

The autonomic parameters were not significantly different between AP-FGIDs and control groups (Table 2). All parasympathetic parameters were lower in the AP-FGIDs group, but these were not statistically significant. Resting heart rate, which is under parasympathetic inhibition, was higher among the AP-FGIDs group but with no statistical significance.

When pain characters of children with AP-FGIDs were correlated with autonomic parameters, the duration of pain episode negatively correlated with the 30:15 ratio (r = -0.21, P = 0.024, Pearson correlation coefficient). Other characteristics had no such correlation.

Gastric motility in study subjects

Gastric emptying rate, frequency of antral contractions, the amplitude of antral contractions and antral motility index were significantly lower in AP-FGIDs group (Table 3). Pain severity positively correlated with the antral area at 1 min (r = 0.2, P = 0.02).

Correlation between autonomic functions and gastric motility

In healthy controls, the gastric emptying rate and the frequency of antral contractions positively correlated with the 30:15 ratio. Furthermore, the Valsalva ratio positively correlated with the frequency of antral contractions in healthy controls (Table 4). There was no such correlation between autonomic functions and gastric motility in patients with AP-FGIDs.

DISCUSSION

The current study assessed the cardiovascular autonomic functions and gastric motility in children with AP-FGIDs. The assessment of autonomic functions in AP-FGID patients showed no significant difference when compared with the control group. The gastric motility parameters were significantly impaired in children with AP-FGIDs. None of the autonomic function tests showed significant correlation with any of the gastric motility parameters in the AP-FGIDs group.

The lack of differences in the autonomic parameters in the two groups indicates the possibility of normal autonomic function in children with AP-FGIDs. Since there are no tests that can directly assess the autonomic function of the gastrointestinal system and its interactions with the brain, the cardiovascular autonomic functions are used as

Table 1 Autonomic symptoms among children with abdominal pain-predominant functional gastrointestinal disorders and controls, *n* (%)

Symptoms	AP-FGIDs, *n* = 92	Controls, *n* = 50	*P* value
Dizziness/light-headedness	14 (15)	3 (6)	0.15[1]
Dry mouth or dry eye	3 (3)	1 (2)	1.00[2]
Cold feet	0	0	-
Reduced limb sweating	6 (7)	1 (2)	0.43[2]
Post prandial abdominal pain or discomfort	55 (60)	0	< 0.0001[1]
Constipation or diarrhoea	32 (35)	2 (4)	< 0.0001[1]

[1]Chi-square test;
[2]Fisher's exact test.

a proxy to assess autonomic function of the gastrointestinal system[30]. Chelimsky *et al*[16] noted orthostatic intolerance in six out of eight patients (reflected by excessive increase in heart rate or reduction in BP) and low Valsalva ratio in two patients. Heart rate response to deep breathing, which exclusively assesses parasympathetic function, was within the normal limits in all eight patients[16]. However, this was an observational study with no controls. In addition, the authors did not classify recurrent abdominal pain to definitive functional gastrointestinal disorders using the standard Rome criteria. Several studies have assessed heart rate variability (HRV) in adult patients with IBS using different methods[31]. However, no significant difference in vagal activity and sympatho-vagal balance between children with FAP/IBS and healthy controls have been shown in HRV assessments[32]. Furthermore, meta-analysis of studies assessing HRV found that there could be a significantly lower vagal influence in IBS patients compared to controls[33]. The studies included in these meta-analyses used a different method to assess autonomic function, and therefore, we cannot directly compare our findings with the meta-analyses. Similar to our findings, some other studies have failed to demonstrate a significant association between autonomic functions and functional gastrointestinal disorders[34,35].

In addition, the lack of significant difference of extra-intestinal autonomic symptoms between children with AP-FGIDs compared to controls potentially indicates that children with AP-FGIDs do not have generalized autonomic dysfunction. Similarly, Chelimsky *et al*[16] did not find extra-intestinal autonomic symptoms in children with AP-FGIDs.

Current autonomic tests are only a proxy measure of gastrointestinal autonomic function. Apart from that, contribution of the autonomic input of the local neuronal network within the stomach has not been taken into account during the testing. These factors may at least partially contributed to the lack of differences in autonomic function in children with AP-FGIDs. However, it is essential to understand that with the current knowledge and available tests, this is the closest that we could come to making a reasonable assessment of autonomic functions.

Gastric motility abnormalities have been reported in children and adults with FGIDs[6,8-11,14,36-38]. In the current study, we also noted a similar pattern of abnormalities in children with AP-FGIDs. In addition, we found a positive correlation between abdominal pain and abnormalities in gastric motility, similar to previous studies[6,10,11,39-41]. These findings suggest a potential pathophysiological relationship between gastric motility abnormalities and AP-FGIDs.

When we correlated gastric motility with autonomic parameters, we did not find a clear correlation between them in children with AP-FGIDs. However, the finding of a significant correlation in controls indicates parasympathetic control of gastrointestinal motor function. None of the other studies in children have assessed the association between autonomic function and gastric motility, and therefore, we could not make a clear comparison.

The ANS is a physiological stress system. It is involved in adapting to various stimuli. Available literature has shown that autonomic activity may present as being normal[38], hypo-functioning[42] or hyper-active[43] in functional abdominal pain. Dysfunction of the ANS can cause significant gastrointestinal problems[44]. At the central level, there is a strong connection between autonomic activation and nociception, which is supported by the anatomical and functional overlap of pain processing structures and autonomic regulating structures[45]. The interaction between pain and autonomic response becomes maladaptive in chronic pain[46]. In some chronic pain states, sympathetic hyperactivity contributes to increased sensitivity to

Table 2 Comparison of autonomic parameters between children with abdominal pain-predominant functional gastrointestinal disorders and controls

Autonomic test	Measurement	AP-FGIDs, *n* = 100	Controls, *n* = 50	*P* value[1]
		Median (range)	Median (range)	
Resting heart rate (beats/min)	Resting heart rate (beats/min)	89.0 (57-110)	87.0 (63-114)	0.18
Heart rate response to deep breathing	Maximum-minimum heart rate (beats/min)	30.8 (10-60)	32.0 (10-57)	0.90
Lying to standing heart rate response	30:15 ratio	1.2 (0.9-1.6)	1.2 (0.9-1.8)	0.67
Valsalva manoeuvre	Valsalva ratio	1.5 (1-2.1)	1.5 (1-2.5)	0.23

[1]Mann-Whitney *U* test.

pain[47]. In contrast, the pain can result in reduced parasympathetic activity[48]. Association of pain and low parasympathetic flow has been reported in women with IBS[49].

In the current study, pain duration negatively correlated with the 30:15 ratio (parasympathetic), which can be interpreted as increased pain duration when parasympathetic activity is reduced. The 30:15 ratio is a sensitive index to detect autonomic abnormalities in children[50]. Therefore, we suggest that parasympathetic division may adapt to the initial phase of the disease, as shown in Figure 1. However, a decrease in the parameters after 12 mo of disease may be a feature of mal-adapting autonomic flow. Furthermore, progressive autonomic dysfunction over time has been demonstrated in adults with IBS[51]. Therefore, the degree of parasympathetic functional impairment may present as a spectrum extending from normal to severe impairment. In this context, we may not see a similar response in every FGID patient.

The three cardinal findings in this study, such as lack of difference in extra-intestinal autonomic symptoms between AP-FGIDs and controls, lack of differences in autonomic functions between the two groups and lack of correlation between gastric motility and autonomic parameters in those with FGIDs, suggest that the ANS does not play a major role in the pathogenesis of AP-FGIDs in children. In this context, abnormalities in parasympathetic flow are unlikely to be the primary cause for impaired motility in AP-FGIDs. Therefore, the stomach's unresponsiveness to extrinsic autonomic signals (functional extrinsic denervation) would be a possible underlying primary pathophysiological mechanism for gastric motility abnormalities seen in AP-FGIDs.

Based on these observations, we have developed a hypothetical model to explain the possible mechanism of pathogenesis of AP-FGIDs, which we term ''automatic stomach in AP-FGIDs''. According to the proposed model, functional extrinsic denervation is able to impair motility by three mechanisms (Figure 1). Both impaired motility and maladapted parasympathetic flow have an impact on pain. Possible functional extrinsic denervation demonstrated in the current study would affect gastric motility by increasing dopaminergic inhibition on the stomach, possibly via DAR2 receptors, leading to an "automated stomach" that does not directly respond to the outflow of the autonomic nervous system (Figure 1). Additionally, we have incorporated the modulatory effects of peripheral dopamine receptors on the central dopaminergic system as another possible effect of functional extrinsic denervation[52].

There are several strengths of this study. We have employed well established, non-invasive techniques to assess cardiovascular autonomic functions and gastric motility. The two investigators who assessed gastric motility and autonomic functions were blinded to the diagnosis of the study subjects. In addition, the impact of diurnal variation on motility was minimized by conducting the study from 8:30 am to 10:30 am. The large sample size (100 AP-FGIDs and 50 controls) and detailed evaluation of patients (using history, examination and investigations) to exclude possible underlying organic disorders were the other strengths of the study. Therefore, we believe that our results can be applied to the whole population of children with AP-FGIDs. However, we did not separate children with AP-FGIDs into specific disease entities such as IBS, FD and FAP. In addition, we used cardiovascular autonomic functions to assess the autonomic functions of the gastrointestinal tract, which is only a proxy measure at best.

In conclusion, children with AP-FGIDs showed abnormal gastric motility parameters, while their cardiovascular autonomic functions were normal. There was no correlation between gastric motility parameters and autonomic functions, indicating that abnormalities in the autonomic nervous system do not play a major role in the pathogenesis of AP-FGIDs. However, we believe maladaptive

Table 3 Comparison of gastric motility parameters between children with abdominal pain-predominant functional gastrointestinal disorders and controls

Gastric motility parameter	AP-FGIDs, *n* = 100	Controls, *n* = 50	*P* value[1]
	Median (range)	Median (range)	
Fasting antral area, cm^2	1.6 (0.4-5.9)	1.3 (0.4-5.1)	0.19
Antral area at 1 min, cm^2	9.8 (3.8-14.6)	9.8 (3.4-19.1)	0.42
Antral area at 15 min, cm^2	4.8 (0.9-10.7)	3.6 (0.6-11.2)	< 0.0001
Gastric emptying rate, %	46.3 (16.0-77.9)	61.6 (10.0-88.5)	< 0.0001
Frequency of antral contraction, /3 min	8.0 (6-11)	9.0 (8-11)	< 0.0001
Amplitude of antral contraction, %	47.2 (22.8-83.0)	59.4 (32.6-85.4)	< 0.0001
Antral motility index	4.0 (1.7-7.5)	5.7 (2.9-8.1)	< 0.0001

[1]Mann-Whitney *U* test.

parasympathetic flow and the proposed automated stomach model can shed some light upon the pathophysiology of AP-FGIDs in children.

Table 4 Correlation between autonomic parameters and gastric motility parameters among children with abdominal pain-predominant functional gastrointestinal disorders and controls

	Gastric motility parameter	Valsalva ratio	Minimum -maximum heart rate	30:15 ratio
AP-FGIDs,	FAA	-0.01	0.04	-0.09
n = 100	AA1	-0.02	-0.02	-0.06
	AA15	-0.01	-0.03	-0.07
	GER	0.07	-0.03	0.06
	FAA	-0.02	-0.07	0.04
	AAC	0.07	-0.06	0.13
	MI	0.07	-0.07	0.14
Controls,	FAA	0.11	-0.05	-0.11
n = 50	AA1	0.03	0.01	-0.01
	AA15	0.07	0.05	0.17
	GER	0.2	0.1	0.39[a]
	FAC	0.7[a]	-0.09	0.14[a]
	AAC	-0.07	0.11	-0.2
	MI	0.05	0.17	0.07

Spearman correlation coefficient,
[a]P < 0.05. FAA: Fasting antral area; AA1: Antral area at 1 min; AA15: Antral area at 15 min; GER: Gastric emptying rate; FAC: Frequency of antral contraction; AAC: Amplitude of antral contraction; MI: Antral motility index.

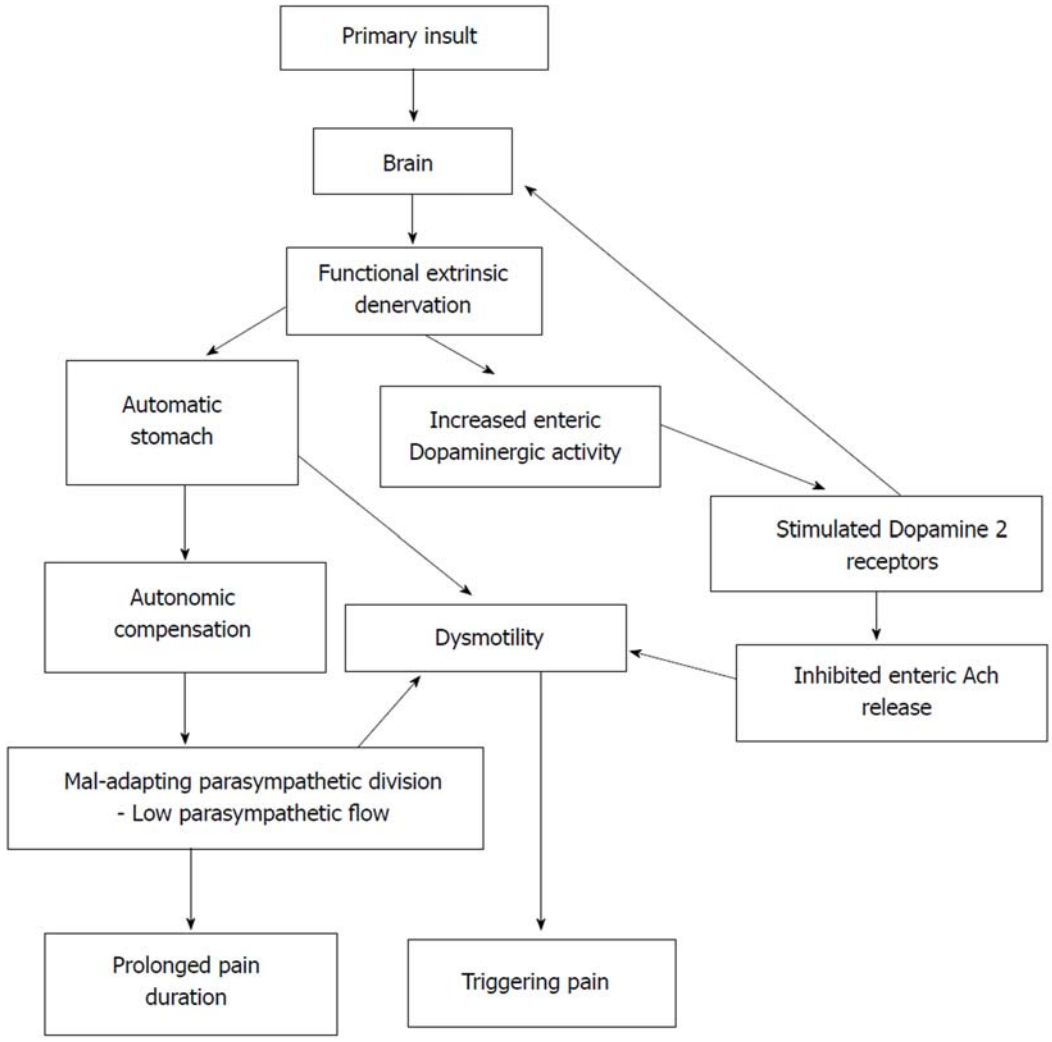

Figure 1 Development and consequences of automatic stomach in abdominal pain-predominant functional gastrointestinal disorders according to the proposed model. Ach: Acetylcholine.

ARTICLE HIGHLIGHTS

Research background

Abdominal pain-predominant functional gastrointestinal disorders (AP-FGIDs) are a common clinical problem in paediatric practice across the globe, with an estimated prevalence of 13.5%. Although thought to be benign in nature, as a group they are known to associate with poor health-related quality of life and high healthcare burden.

Research motivation

The pathophysiology of AP-FGIDs is not clearly understood. Previous studies have shown abnormalities in gastroduodenal motility, such as delayed gastric emptying, impaired antral motility, and impaired gastric accommodation as potential pathophysiological mechanisms in children. Studies among adults have found autonomic dysfunction in patients with IBS. However, the association between autonomic dysfunction and gastric motility in children with AP-FGIDs had not been previously evaluated.

Research objectives

The main objective of our study was to assess the autonomic functions in children with AP-FGIDs and their relationship to gastric motor functions.

Research methods

One hundred children fulfilling Rome III criteria for AP-FGIDs and 50 healthy controls aged 5 to 12 years were recruited for the study. All patients were thoroughly investigated to rule out underlying organic disorders. Gastric motility and cardiovascular autonomic functions were assessed using validated, non-invasive techniques.

Research results

Gastric emptying rate, amplitude of antral contractions, and antral motility index were significantly lower in children with AP-FGIDs. Autonomic functions, including blood pressure and heart rate responses to standing from lying down position, heart rate response to deep breathing, and Valsalva test, showed no difference between children with AP-FGIDs and controls. These parameters did not show any correlation with gastric motor functions. However, the duration of pain episodes negatively correlated with the parasympathetic tone.

Research conclusions

Although children with AP-FGIDs have abnormal gastric motility parameters, their cardiovascular autonomic functions are normal. In addition, there is no correlation between autonomic functions and gastric motility. Our findings indicate that the autonomic nervous system is not chronically abnormal in patients with AP-FGIDs. Based on currently available evidence, we propose maladaptive parasympathetic flow and an automated stomach model as a potential pathophysiological mechanism for AP-FGIDs.

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ORIGINAL ARTICLE

Retrospective Study

Usefulness of urinary trypsinogen-2 and trypsinogen activation peptide in acute pancreatitis: A multicenter study in Japan

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Abstract

BACKGROUND

Rapid urinary trypsinogen-2 dipstick test and levels of urinary trypsinogen-2 and trypsinogen activation peptide (TAP) concentration have been reported as prognostic markers for the diagnosis of acute pancreatitis.

AIM

To reconfirm the validity of all these markers in the diagnosis of acute pancreatitis by undertaking a multi-center study in Japan.

METHODS

Patients with acute abdominal pain were recruited from 17 medical institutions in Japan from April 2009 to December 2012. Urinary and serum samples were collected twice, at enrollment and on the following day for measuring target markers. The diagnosis and severity assessment of acute pancreatitis were assessed based on prognostic factors and computed tomography (CT) Grade of the Japanese Ministry of Health, Labour, and Welfare criteria.

RESULTS

A total of 94 patients were enrolled during the study period. The trypsinogen-2 dipstick test was positive in 57 of 78 patients with acute pancreatitis (sensitivity, 73.1%) and in 6 of 16 patients with abdominal pain but without any evidence of acute pancreatitis (specificity, 62.5%). The area under the curve (AUC) score of urinary trypsinogen-2 according to prognostic factors was 0.704, which was highest in all parameter. The AUC scores of urinary trypsinogen-2 and TAP according to CT Grade were 0.701 and 0.692, respectively, which shows higher

than other pancreatic enzymes. The levels of urinary trypsinogen-2 and TAP were significantly higher in patients with extended extra-pancreatic inflammation as evaluated by CT Grade.

CONCLUSION
We reconfirmed urinary trypsinogen-2 dipstick test is useful as a marker for the diagnosis of acute pancreatitis. Urinary trypsinogen-2 and TAP may be considered as useful markers to determine extra-pancreatic inflammation in acute pancreatitis.

Key words: Acute pancreatitis; Trypsinogen activation peptide; Urinary trypsinogen-2 dipstick test

Core tip: A total of 94 patients with acute abdominal pain were enrolled from 17 medical institutions in Japan from April 2009 to December 2012. The trypsinogen-2 dipstick test was positive in 57 of 78 patients with acute pancreatitis (sensitivity, 73.1%) and in 6 of 16 patients with abdominal pain but without any evidence of acute pancreatitis (specificity, 62.5%). The levels of urinary trypsinogen-2 and trypsinogen activation peptide (TAP) were significantly higher in patients with extended extra-pancreatic inflammation as evaluated by CT Grade. Urinary trypsinogen-2 and TAP may be considered as additional markers to determine extra-pancreatic inflammation in acute pancreatitis.

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INTRODUCTION

Acute pancreatitis is a common disease accompanied by acute abdominal pain. However, the early diagnosis of acute pancreatitis remains difficult, due to the difficulty of performing quick measurements of pancreatic enzymes in most clinics. Japanese guidelines for the management of acute pancreatitis in 2015 (JPN Guideline 2015)[1] recommended the measurement of serum lipase instead of serum amylase for the diagnosis of acute pancreatitis because of its higher specificity for pancreas. In fact, serum amylase is more often measured than serum lipase in Japan, because only amylase can be rapidly measured in most of the emergency centers. Trypsinogen-2 is a pancreatic enzyme known to remain elevated longer in patients with acute pancreatitis, with higher levels in the urine than in serum, compared to the commonly measured pancreatic enzyme amylase[2]. A rapid test strip for the detection of urinary trypsinogen-2 was developed in Finland and was reported to be useful for the diagnosis of acute pancreatitis[3-8], and its accuracy and usefulness was also verified in Japan[9].

Trypsinogen activation peptide (TAP) is the amino-terminus peptide released by the activation of trypsinogen. In experimental acute pancreatitis, the inappropriate activation of trypsinogen within the pancreas results in the release of TAP into the blood, urine, and peritoneum[10,11]. The concentration of urinary TAP is thought to correlate directly with the severity of acute pancreatitis, reflecting the degree of trypsinogen activation in the pancreas[12-14]. The concentration of urinary trypsinogen-2 has also been previously reported to be a candidate prognostic marker of severe acute pancreatitis[6].

The criteria for severity assessment of acute pancreatitis was fully revised in Japan in 2008[15], in which the diagnosis of severe acute pancreatitis can be made according to 9 prognostic factors and/or on computed tomography (CT) grading based on

contrast-enhanced CT. These criteria emphasize that the assessment of severity at the initial medical examination plays an important role in introducing adequate early treatment and the transfer of patients to a medical facility that is able to provide intensive treatment. However, CT has a problem for the radiation exposure and contrast-enhanced CT may cause worsening of renal dysfunction, often accompanied by severe acute pancreatitis. Therefore, the establishment of a simple marker and method in clinical practice is strongly warranted to diagnose severe acute pancreatitis.

In Japan, any examination or investigation without health insurance coverage is hardly performed, because we have national public health care system to cover them. We need clinical evidence of a useful marker to predict severity level of acute pancreatitis in order to be approved by and consider future inclusion under national health insurance system in Japan.

In the present study, in a multi-center study we performed rapid urinary trypsinogen-2 dipstick test to reconfirm its validity in the diagnosis of acute pancreatitis. In addition, we measured trypsinogen-2 and TAP levels in urine samples to evaluate their usefulness as possible prognostic markers of severe acute pancreatitis as assessed by Japanese criteria.

MATERIALS AND METHODS

Patients

Patients with acute abdominal pain were enrolled prospectively in this study. All patients who were seen in the emergency centers and hospitalized at 17 medical institutions in Japan from April 2009 to December 2012 were considered eligible for this study. The Institutional Review Board committee of each institution approved this study and an informed written consent was obtained from all patients before inclusion. This study was registered with the UMIN Clinical Trials Registry (reference no. UMIN000001622).

Study design

Urinary and serum samples were collected from all study participants twice, at enrollment and on the following day within 48 h after admission. These samples were frozen immediately and stored at -20 °C until analysis. The qualitative analyses of urinary trypsinogen-2 were performed using a dipstick test (Actim Pancreatitis, Medix Biochmica, Kauniainen, Finland). A quantitative immunoenzymometric assay (Trypsinogen-2 Iema Test, Medix Biochmica) of urinary trypsinogen-2 was performed using commercially available kit of Unitika Ltd. (Osaka, Japan). The concentration of TAP in urine was measured by ELISA (Oriental Yeast Co. Ltd., Tokyo Japan). The urine and serum levels of amylase, lipase, and creatinine was measured by each institution or BML Inc. (Tokyo, Japan).

Acute pancreatitis was diagnosed according to the diagnostic criteria established by the Japan Ministry of Health, Labour, and Welfare (JMHLW) (2008)[16]. These criteria are composed of 3 items: (1) acute abdominal pain and tenderness in the upper abdomen; (2) elevated levels of pancreatic enzymes in the blood or urine; and (3) findings of acute pancreatitis detected by ultrasonography (US), CT, or magnetic resonance imaging (MRI). Patients who presented with at least 2 of the above 3 manifestations and in whom other pancreatic and acute abdominal diseases had been ruled out were diagnosed as having acute pancreatitis.

The severity of acute pancreatitis within 48 h of entry was evaluated using the criteria established by the JMHLW (2008) for severity assessment of acute pancreatitis, in which patients were diagnosed as severe acute pancreatitis based on ≥ 3 of 9 prognostic factors and/or CT grading ≥ 2 based on contrast-enhanced CT scan (Table 1)[15].

The primary outcome of this study was to find the association between the values of urinary trypsinogen-2 and TAP and the severity levels of acute pancreatitis. We investigated the relative accuracy of the urine trypsinogen-2 dipstick test for the diagnosis of acute pancreatitis and attempted to reconfirm its validity with published results.

Statistical analysis

In the qualitative evaluation of urinary trypsinogen-2 dipstick test for the diagnosis of acute pancreatitis, the sensitivity and of specificity with 95% confidence interval (CI), lower bound to upper bound were calculated. In the quantitative measurement of urinary trypsinogen-2 and TAP, data were expressed as median and lower and upper quartile. Their area under the curve (AUC) scores between severe and mild pancreatitis groups were calculated by logistic regression analysis. The relationship

Table 1 The severity scoring system of acute pancreatitis of the Japanese Ministry of Health, Labour and Welfare (2008)[15]

Prognostic factors (1 point for each factor)	
Base excess ≤ 3 mEq/L or shock (systolic blood pressure < 80 mmHg)	
PaO_2 ≤ 60 mmHg (room air) or respiratory failure (respirator management is needed)	
BUN ≥ 40 mg/dL (or Cr ≥ 2.0 mg/dL) or oliguria (daily urine output <400 mL even after fluid replacement)	
LDH ≥ 2 times of upper limit of normal	
Platelet count ≤ 100000/mm^3	
Serum Ca ≤ 7.5 mg/dL	
CRP ≥ 15 mg/dL	
Number of positive measures in SIRS criteria ≥3	
Age ≥ 70 yr	
CT Grade by CECT	
Extra-pancreatic progression of inflammation	
Anterior pararenal space	0 point
Root of mesocolon	1 point
Beyond lower pole of kidney	2 points
Hypo-enhanced lesion of the pancreas	
The pancreas is conveniently divided into three segments (head, body, and tail).	
Localized in each segment or only surrounding the pancreas	0 point
Covers 2 segments	1 point
Occupies entire 2 segments or more	2 points
1 + 2 = total scores	
Total score = 0 or 1	Grade 1
Total score = 2	Grade 2
Total score = 3 or more	Grade 3
Assessment of severity	
(1) If prognostic factors are scored as 3 points or more, or (2) If CT Grade grade is judged as Grade grade 2 or more, the severity grading is evaluated to be as ''severe''.	
Measures in SIRS diagnostic criteria: (1) Temperature > 38 °C or < 36 °C; (2) Heart rate > 90 beats/min; (3) Respiratory rate > 20 breaths/min or $PaCO_2$ < 32 torr; and (4) WBC > 12000 cells/mm^3, < 4000 cells/mm^3, or > 10% immature (band) forms.	

WBC: White blood cell; CT: Computed tomography; LDH: lactate dehydrogenase; CRP: C-reaction protein.

among 3 groups evaluated by scores of CT Grade was analyzed using ordinal logistic regression. Significance was defined by a P value of < 0.05. All statistical analyses were performed using JMP® statistical software, version 8 (SAS Institute Inc., Cary, NC, United States).

RESULTS

A total of 94 patients with acute abdominal pain who were seen in the emergency centers and hospitalized at 17 medical institutions in Japan were included in this study. The mean age was 58.0 years (range: 25 to 92 years). Of these patients, 78 (82.9%) were diagnosed with acute pancreatitis and 16 (17.1%) with different diseases such as acute gastritis, biliary stones, and peptic ulcer. The characteristics of patients at enrollment are summarized in Table 2.

The results of the urinary trypsinogen-2 dipstick test were positive in 57 of the 78 patients with acute pancreatitis (sensitivity: 73.1%, 95%CI: 0.62-0.82). Dipstick results were also positive in 6 of 16 patients with abdominal pain but no evidence of acute pancreatitis (specificity: 62.5%, 95%CI: 0.39-0.82). The positive and negative predictive values of the trypsinogen-2 dipstick test for the diagnosis of acute pancreatitis were 90.5% and 32.3%, respectively.

When we distributed our data at enrollment based on prognostic factors of the JMHLW criteria, the median levels of urinary trypsinogen-2 were 2.87 and 6.49 mg/dL in patients with mild and severe pancreatitis and the AUC score was 0.704, which was highest among all parameters (Table 3). It was obvious that AUC score of

Table 2 The characteristics of patients at enrollment

	Acute pancreatitis (*n* = 78)	Other disease (*n* = 16)
Age, median (IQR), yr	57 (28)	61 (26)
Sex, *n*: male/female	50/26	9/7
Etiology of acute pancreatitis, *n* (%)		
Alcohol	26 (33.3)	
Gallstones	13 (16.7)	
Idiopathic	12 (15.4)	
Post-ERCP	5 (6.4)	
Others	22 (28.2)	
Prognostic factor score by JMHLW (2008) criteria (*n* = 78)		
Mean (SD)	0.9 (1.2)	
Severe acute pancreatitis (≥ 3), *n* (%)	9 (11.5)	
Score of CT Grade by JMHLW (2008) criteria (*n* = 70)		
Mean (SD)	1.0 (1.2)	
Severe acute pancreatitis (Score ≥ 2), *n* (%)	28 (40)	

serum creatinine was high, because it was one of prognostic factors.

The median levels of urinary trypsinogen-2 and TAP were 2.69 mg/dL and 2.07 ng/mL, respectively in patients with mild pancreatitis, and 14.68 mg/dL and 3.98 ng/mL, respectively in those with severe pancreatitis, according to CT Grade of the JMHLW criteria (Table 4). Their AUC scores were 0.701 and 0.692, respectively, which are higher than other pancreatic enzymes. The ratio of urinary trypsinogen-2 or TAP to urinary creatinine was calculated to correct the influence of dehydration. Their AUC scores of the urinary trypsinogen-2 and TAP to creatinine ratio were also high (Table 4). Compared with the levels of urinary trypsinogen-2 and TAP at enrollment, both of the levels on the following day and the values that subtracted the level at enrollment from that on the following day were not related to disease severity (data not shown).

The levels of urinary trypsinogen-2 and TAP showed significant differences between different scores of extra-pancreatic progression of inflammation, but no significant differences were observed between the different scores of hypo-enhanced pancreas lesions (Table 5). Furthermore, the levels of urinary trypsinogen-2 to creatinine ratio (trypsinogen-2/cre) and TAP to creatinine ratio (TAP/cre) also showed significant differences between different scores of extra-pancreatic progression of inflammation.

DISCUSSION

In this multi-center study, we reconfirmed the validity of performing urinary trypsinogen-2 dipstick test to isolate patients with acute pancreatitis who attends emergency clinics with acute abdominal pain. We also found that sensitivity and positive predictive value of this test in patients with acute pancreatitis is quite high and as such further strengthens its applicability in clinical practice. The frequency of acute pancreatitis diagnosed in patients with acute abdominal pain was reported to be approximately 5% in a previous Japanese multicenter study[17]. This simple and easy laboratory procedure (urinary trypsinogen-2 dipstick test) may be able to make a quick decision for diagnosing patients with acute pancreatitis without diverging to measure other conventional pancreatic enzymes such as amylase, lipase, and trypsin, which needs laboratory technicians and/or expensive instruments.

In Japan, the present diagnostic criteria of acute pancreatitis as established by the JMHLW were revised in part in 2008[16] and includes at least 2 of the 3 manifestations and exclusion of other diseases compatible with acute abdominal pain. In contrast, Revised Atlanta Criteria[18] are composed of similar 3 items, strict in elevated levels of pancreatic enzymes and do not need to exclude other diseases. If the diagnosis of acute pancreatitis is established by abdominal pain and by increases in the serum pancreatic enzyme activities, a contrast-enhanced CT is not usually required for diagnosis. Therefore, Japanese Criteria seems to be more sensitive and specific than Revised Atlanta Criteria. The sensitivity and specificity of urinary trypsinogen-2 dipstick test in this study coincided with the findings of previous reports[3-9,19].

Table 3 Urinary marker levels at enrollment in patients with severe and mild pancreatitis by prognostic factors according to the Japanese Ministry of Health, Labour and Welfare criteria (2008)[15]

	Severity by prognostic factors				
	Severe		Mild		
No. cases	9		69		
Prognostic factors: mean (SD)	2.89	(1.83)	0.57	(0.69)	
Age: median (LQ, UQ), yr	48.5	(45, 69.75)	58	(44, 72)	
Sex: male/female	5/2		45/24		
	Median (LQ, UQ)				AUC
Urinary trypsinogen-2, mg/dL	6.49	(2.41, 208.76)	2.87	(0.22, 19.98)	0.704
Urinary trypsinogen-2/cre	11.2	(2.43, 214.05)	6.36	(0.31, 33.02)	0.592
Urinary TAP, ng/mL	2.68	(2.07, 5.22)	2.79	(1.25, 5.53)	0.458
Urinary TAP/cre, × 0.0001	6.7	(2.40, 11.50)	4.1	(2.25, 7.20)	0.631
Urinary amylase, × 1000 U/L	1.42	(0.50, 3.16)	1.01	(0.40, 2.68)	0.563
Urinary amylase/cre, U/mg	3.65	(0.38, 8.47)	2.22	(0.81, 4.00)	0.58
Urinary creatinine, × 10 mg/dL	3.63	(3.16, 8.65)	6.09	(3.89, 10.24)	0.599
Serum amylase, × 100 U/L	11.37	(2.44, 23.37)	6.38	(3.46, 11.72)	0.581
Serum lipase, × 100 U/L	5.85	(4.41, 13.57)	6.95	(2.46, 16.71)	0.508
Serum creatinine, × 0.1mg/dL	9.7	(5.95, 19.65)	6.75	(5.83, 8.55)	0.676

Their area under the curve scores were calculated between severe and mild pancreatitis groups by logistic regression analysis. The cases with no available data were excluded from analysis. All data were showed by median, lower quartile, and upper quartile. AUC: Area under the curve; LQ: Lower quartile; UQ: Upper quartile; TAP: Trypsinogen activation peptide.

Mayumi *et al*[9] reported in Japan for the first time that the urinary trypsinogen-2 dipstick test was able to diagnose or rule out most cases of acute pancreatitis, and the present results are in agreement with the clinical usefulness of the dipstick test despite the small number of patients. The urinary trypsinogen-2 dipstick test is based on the same rapid qualitative analysis method as was the rapid influenza diagnostic tests as well as the rapid panel tests for heart-type fatty acid-binding protein. However, the urinary trypsinogen-2 dipstick test is not widely utilized due to its relative unavailability compared to the rapid influenza and heart-type fatty acid-binding protein tests, which are already made available and approved under the national health insurance of Japan. We emphasize that our current findings will be helpful to achieve the national health insurance approval of the urinary trypsinogen-2 dipstick test as a diagnostic tool of acute pancreatitis.

In Revised Atlanta Criteria[18], disease severity is classified as mild, moderate or severe. The prediction of severity levels of acute pancreatitis can be globally made by using scoring systems, the Ranson score, the Glasgow score, and the Acute Physiology and Chronic Health Evaluation (APACHE) II score. In Japan, the criteria established by the Research Committee of Intractable Pancreatic Disease supported by the JMHLW in 2008[15] was used for severity assessment of acute pancreatitis. As mentioned above, in the new Japanese criteria, the diagnosis of severe acute pancreatitis can be made according to 9 prognostic factors and/or CT grading based on contrast-enhanced CT[15]. The new Japanese criteria predicted the mortality rate and were largely as useful as the old criteria, the Ranson Score and the APACHE II score for severity assessment[15,20,21]. The present study examined whether the level of urinary trypsinogen-2, TAP, and amylase as well as serum amylase and lipase could be used as predicting markers of severe acute pancreatitis. The level of urinary trypsinogen-2 and TAP were higher in patients with severe pancreatitis by CT Grade, furthermore their AUC scores were higher than those of urinary amylase and serum pancreatic enzymes. In severe acute pancreatitis, the level of urinary creatinine is known to be elevated due to dehydration and renal dysfunction, and is one of prognostic factors according to the JMHLW criteria (2008). Subsequently, the urinary trypsinogen-2/cre ratio and TAP/cre ratio were evaluated to exclude the possibility of the elevation of urinary trypsinogen-2 and TAP due to dehydration, demonstrating that both the trypsinogen-2/cre ratio and the TAP/cre ratio were higher in patients with severe pancreatitis by CT Grade. Furthermore, the levels of urinary trypsinogen-2 and TAP were related to CT Grade, especially extra-pancreatic progression of

Table 4 Urinary marker levels at enrollment between patients with severe and mild pancreatitis by computed tomography Grade according to the Japanese Ministry of Health, Labour and Welfare criteria (2008)[15]

	Severity by CT Grade				
	Severe		Mild		
No. cases	28		42		
Score of CT Grade: mean (SD)	2.37 (0.69)		0.17 (0.38)		
Extra-pancreatic progression of inflammation (Score 0/1/2)	0/1/27		35/7/0		
Hypo-enhanced lesion of the pancreas (Score 0/1/2)	19/3/4		42/0/0		
Age: median (LQ, UQ), yr	53 (44, 67)		61 (47.5, 73.25)		
Sex: male/female, *n*	20/6		24/18		
	Median (LQ, UQ)				AUC
Urinary trypsinogen-2, mg/dL	14.68	(2.10, 66.90)	2.69	(0.20, 17.11)	0.701
Urinary trypsinogen-2/cre	14.40	(4.29, 104.03)	6.36	(0.32, 17.94)	0.678
Urinary TAP, ng/mL	3.98	(2.20, 7.81)	2.07	(0.96, 3.87)	0.692
Urinary TAP/cre, × 0.0001	6.70	(4.15, 10.90)	3.10	(2.20, 6.00)	0.727
Urinary amylase, × 1000 U/L	1.94	(0.64, 3.62)	0.97	(0.47, 2.29)	0.615
Urinary amylase/cre, U/mg	3.54	(0.63, 5.88)	2.23	(0.88, 3.65)	0.588
Urinary creatinine, × 10 mg/dL	6.55	(3.54, 9.24)	5.54	(3.49, 10.47)	0.472
Serum amylase, × 100 U/L	9.28	(2.88, 15.22)	6.28	(3.91, 11.74)	0.588
Serum lipase, × 100 U/L	8.10	(1.82, 18.49)	6.95	(4.32, 15.73)	0.521
Serum creatinine, × 0.1 mg/dL	7.25	(5.40, 8.90)	6.90	(5.75, 7.95)	0.574

Their area under the curve scores were calculated between severe and mild pancreatitis groups by logistic regression analysis. The cases with no available data were excluded from analysis. All data were showed by median, lower quartile, and upper quartile. AUC: Area under the curve; LQ: Lower quartile; UQ: Upper quartile; CT: Computed tomography; TAP: Trypsinogen activation peptide.

inflammation, but not hypo-enhanced lesion of the pancreas, which may indicate that trypsinogen-2 and TAP generated in the pancreas did not release into blood, urine, and peritoneum in patients with hypo-enhanced lesion of the pancreas due to decreased pancreatic perfusion as reported by Takaoka *et al*. in the experimental acute pancreatitis[11]. Furthermore, unlike amylase, only trypsin such as trypsinogen-2 and TAP was related to extra-pancreatic progression of inflammation, which may indicate that extra-pancreatic inflammation may be caused by the extra-pancreatic release of trypsin and not amylase. These results indicated that the level of urinary trypsinogen-2 and TAP may be useful for the determination of extra-pancreatic inflammation, particularly in cases who are not able to undergo CT examination and may be expected as predicting markers for severe acute pancreatitis. We expected urinary trypsinogen-2 and TAP as a marker, which can select the patients who should have CT examination, but they were not useful enough for the determination of hypo-enhanced lesion of the pancreas.

The main limitations of the present study are as follows: (1) small sample size and the indirect analysis; and (2) we measured urinary trpsionogen-2 and TAP levels as prognostic markers of severe acute pancreatitis but we did not compare them with morbidity/mortality or complications. Future large study may answer these unresolved issues.

In conclusion, we reconfirmed that urinary trypsinogen-2 dipstick test may be useful as a predictive marker to diagnose acute pancreatitis in emergency clinical setting. In addition, the levels of urinary trypsinogen-2 and TAP may be considered as suitable markers to determine extra-pancreatic inflammation in acute pancreatitis. Further studies with large number of samples may strengthen our current findings.

Table 5 Urinary marker levels at enrollment according to the score of computed tomography Grade by the Japanese Ministry of Health, Labour and Welfare criteria (2008)[15]

Extra-pancreatic progression of inflammation					
Score of CT Grade		0	1	2	*P* value
n		32	8	24	
Urinary trypsinogen-2 (mg/dL)	Median	1.26	27.65	16.98	0.001[a]
	(LQ, UQ)	(0.15, 11.20)	(2.67, 91.61)	(3.04, 71.25)	
Urinary trypsinogen-2/cre	Median	3.11	28.5	15.77	0.046[a]
	(LQ, UQ)	(0.31, 13.17)	(6.70, 130.83)	(3.76, 106.64)	
Urinary TAP (ng/mL)	Median	1.97	2.7	4.19	0.001[a]
	(LQ, UQ)	(0.95, 3.79)	(1.15, 4.73)	(2.55, 8.06)	
Urinary TAP/cre (× 0.0001)	Median	2.75	3.65	6.95	0.003[a]
	(LQ, UQ)	(2.03, 6.53)	(3.10, 4.73)	(4.63, 11.20)	
Urinary amylase (× 1000 U/L)	Median	0.94	1.44	2.11	0.06
	(LQ, UQ)	(4.63, 17.14)	(3.13, 41.84)	(8.25, 36.65)	
Urinary amylase/cre (U/mg)	Median	2.22	2.02	3.72	0.22
	(LQ, UQ)	(0.85, 3.54)	(0.56, 4.36)	(0.91, 6.15)	
Urinary creatinine (× 10 mg/dL)	Median	4.68	6.5	6.7	0.94
	(LQ, UQ)	(3.48, 10.20)	(3.24, 10.14)	(3.95, 9.57)	
Serum creatinine (× 0.1 mg/dL)	Median	6.75	6.6	7.5	0.042[a]
	(LQ, UQ)	(5.88, 8.33)	(4.95, 7.20)	(5.20, 9.00)	
Hypo-enhanced lesion of the pancreas					
Score of CT Grade		0	1	2	*P* value
n		55	3	4	
Urinary trypsinogen-2 (mg/dL)	Median	4.51	44.33	6.77	0.63
	(LQ, UQ)	(0.47, 30.35)	(1.78, 100.99)	(2.45, 14.81)	
Urinary trypsinogen-2/cre	Median	8.37	148.96	9.07	0.84
	(LQ, UQ)	(0.53, 40.84)	(2.27, 179.09)	(6.54, 15.45)	
Urinary TAP (ng/mL)	Median	2.61	3.07	5.88	0.45
	(LQ, UQ)	(1.33, 5.63)	(2.79, 5.22)	(1.04, 12.48)	
Urinary TAP/cre (× 0.0001)	Median	4.1	6.7	6.85	0.65
	(LQ, UQ)	(2.30, 7.20)	(4.90, 10.30)	(3.25, 11.80)	
Urinary amylase (× 1000 U/L)	Median	1.24	1.01	2.22	0.83
	(LQ, UQ)	(4.95, 28.55)	(7.87, 22.70)	(2.94, 35.16)	
Urinary amylase/cre (U/mg)	Median	2.59	3.39	2.21	0.72
	(LQ, UQ)	(0.88, 4.56)	(1.00, 4.03)	(0.46, 6.13)	
Urinary creatinine (× 10 mg/dL)	Median	6.37	5.64	7.47	0.56
	(LQ, UQ)	(3.70, 10.45)	(2.98, 7.85)	(3.11, 10.53)	
Serum creatinine (× 0.1 mg/dL)	Median	6.95	7.8	6.25	0.3
	(LQ, UQ)	(5.83, 8.60)	(4.90, 8.60)	(4.70, 7.88)	

Extra-pancreatic progression of inflammation: 0 = anterior pararenal space, 1 = root of mesocolon, 2 = beyond the lower pole of the kidney. Hypoenhanced lesion of the pancreas: The pancreas was divided into three segments. 0 = signal was localized in each segment or only the surrounding pancreas, 1 = covers two segments, 2 = entirely covered two or more segments. Data are expressed as the median (lower quartile, and upper quartile). Statistical significance is expressed as

[a]$P < 0.05$ among the three groups by ordinal logistic regression analysis. LQ: Lower quartile; UQ: Upper quartile; CT: Computed tomography; TAP: Trypsinogen activation peptide.

ARTICLE HIGHLIGHTS

Research background

Rapid urinary trypsinogen-2 dipstick test and measurements of urinary trypsinogen-2 and trypsinogen activation peptide (TAP) has not covered by the national health insurance program in Japan. On the other hands, rapid urinary trypsinogen-2 dipstick spreads to Europe.

Research motivation

We would like to know how to diagnose acute pancreatitis earlier and estimate exacerbation risk of acute pancreatitis.

Research objectives
We would like to reconfirm the accuracy and accessibility of rapid urinary trypsinogen-2 dipstick test in a multicenter study in Japan for acceptance in the national health insurance program. Furthermore, we would like to verify usefulness of urinary trypsinogen-2 and TAP as prognostic factor of acute pancreatitis.

Research methods
This is a retrospective study by 17 medical institutions in Japan. Patients with acute abdominal pain were enrolled prospectively. Urinary and serum samples were collected twice, at enrollment and on the following day for measuring pancreatic enzymes. We investigated the association between the values of urinary and serum pancreatic enzymes and the severity levels of acute pancreatitis based on the JMHLW criteria (2008).

Research results
The sensitivity and specificity of the urinary trypsinogen-2 dipstick test were 73.1% and 62.5%, respectively. The area under the curve (AUC) score of urinary trypsinogen-2 according to prognostic factors of the JMHLW criteria was highest in all parameter. The AUC scores of urinary trypsinogen-2 and TAP according to computed tomography (CT) Grade of the JMHLW criteria were higher than other pancreatic enzymes. The levels of urinary trypsinogen-2 and TAP were significantly higher in patients with extended extra-pancreatic inflammation as evaluated by CT Grade of the JMHLW criteria.

Research conclusions
The levels of urinary trypsinogen-2 and TAP were significantly higher in patients with extended extra-pancreatic inflammation as evaluated by CT Grade, but not significantly higher in patients with hypo-enhanced pancreas lesions. Therefore, the measurement of urinary trypsinogen-2 and TAP could not select the patients who should have CT examination.

Research perspectives
We need a serum or urinary marker, which can select the patients who should have CT examination.

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ORIGINAL ARTICLE

Retrospective Study

Nomograms for predicting pathological response to neoadjuvant treatments in patients with rectal cancer

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Abstract

BACKGROUND

In recent decades, neoadjuvant therapy (NT) has been the standardized treatment for locally advanced rectal cancer (LARC). Approximately 8%-35% of patients with LARC who received NT were reported to have achieved a complete pathological response (pCR). If the pathological response (PR) can be accurately predicted, these patients may not need surgery. In addition, no response after NT implies that the tumor is destructive, resistant to both chemotherapy and radiotherapy, and prone to having a high metastatic potential. Therefore, developing accurate models to predict PR has great clinical significance and can help achieve individualized treatment in LARC patients.

AIM

To establish nomograms for predicting PR to different NT regimens based on pretreatment parameters for patients with LARC.

METHODS

No. 16ykpy35; Program of Introducing Talents of Discipline to Universities; and National Key Clinical Discipline.

Institutional review board statement: This study was reviewed and approved by the Ethics Committee of The Sixth Affiliated Hospital, Sun Yat-sen University.

Informed consent statement: Patients were not required to give informed consent for the study because the analysis used anonymous clinical data obtained via written consent after each patient agreed to treatment.

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Rectal cancer patients were identified from the database of The Sixth Affiliated Hospital, Sun Yat-sen University from January 2012 to December 2016. Logistic regression and nomograms were developed to predict the probability of pCR and good downstaging to ypT0-2N0M0 (ypTNM 0-I), respectively, based on pretreatment parameters for all LARC patients. Nomograms were also developed for three NT regimens (capecitabine/deGramont-RT, mFOLFOX6, and mFOLFOX6-RT) to predict pCR probability.

RESULTS

Four hundred and three patients were included in this study; 72 (17.9%) had pCR at the final pathology report, and 177 (43.9%) achieved good downstaging to ypT0-2N0M0 (ypTNM 0-I). The nomogram for predicting pCR probability showed that NT regimens, tumor differentiation, mesorectal fascia (MRF) status, and tumor length significantly influenced pCR probability. When predicting the probability of good downstaging, tumor differentiation, MRF status, and clinical T stage were the significant factors. Nomograms were developed based on NT regimens. For the capecitabine/de Gramont-RT group, the multivariate analysis showed that the neutrophil-lymphocyte ratio (NLR) was the only significant factor, thus we could not develop a nomogram for this regimen. For the mFOLFOX6-RT group, the analysis showed that the significant factors were tumor length and MRF status; and for the mFOLFOX6 group, the significant factors were tumor length and tumor differentiation.

CONCLUSION

We established accurate nomograms for predicting the PR to preoperative NT regimens based on pretreatment parameters for LARC patients.

Key words: Neoadjuvant therapy; Locally advanced rectal cancer; Nomogram; Prediction of pathological response; Complete pathological response; Good downstaging

Core tip: In this study, we established accurate nomograms for predicting the pathological response (PR) to preoperative neoadjuvant therapy (NT) regimens based on pretreatment parameters for locally advanced rectal cancer (LARC) patients. Logistic regression and nomograms were developed to predict the probability of complete pathological response and good downstaging, respectively, for all patients and for subgroups based on NT regimens. In conclusion, nomograms have been established for predicting the PR to different NT regimens for LARC patients; and these nomograms can be used to facilitate developing individualized treatments.

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INTRODUCTION

In recent decades, neoadjuvant therapy (NT) has been the standardized treatment for locally advanced rectal cancer (LARC)[1]. NT was reported to decrease the risk of local recurrence and have reduced toxicity[2,3]. Pathological complete response (pCR) is characterized as complete elimination of malignant cells in a resected specimen[4,5]. Approximately 8%-35% of patients with LARC who received NT was reported to have achieved pCR[6-9]. Researchers have also found that good pathological response are associated with a longer disease-free survival (DFS) and lower local and distant recurrence rates[10-15].

Individualized treatment for LARC patients can be achieved by developing an accurate model to predict the probability of pCR or good downstaging. Some authors suggest that if pCR can be accurately predicted, these patients can be strictly followed

without requiring surgery[16-18]. Radical surgery can drastically reduce the quality of life by impairing normal intestinal and genitourinary functions[19]. However, other authors argue that follow-up alone is unsafe and that the pathology cannot be accurately assessed without surgery after NT[20]. In addition, no tumor response or progression after NT implies that the tumor is destructive, resistant to both chemotherapy and radiotherapy, and prone to having a high metastatic potential. Thus, identifying potential responders and non-responders may aid in predicting treatment outcomes and choices.

Previous studies have reported that low carcinoembryonic antigen (CEA) levels[21,22], high pretreatment hemoglobin (HB) levels, early clinical T stage (cT)[23], early clinical N stage (cN), small tumor size, and long radiation surgery interval[6,23-25] are related to pCR probability. However, few models or nomograms have been established and even fewer are used clinically to predict a good pathological response after NT for LARC. Additionally, few models are available to predict neoadjuvant treatments. Therefore, developing accurate models to predict pathological responses has great clinical significance and remains a great challenge.

In this study, by analyzing pretreatment parameters in LARC patients before NT at our institution, we established accurate models and nomograms to predict the probability of pCR and good downstaging, respectively, with currently available pretreatment parameters that can be easily used in clinical decision-making.

MATERIALS AND METHODS

Patients

Rectal cancer patients were identified from the database of The Sixth Affiliated Hospital, Sun Yat-sen University from January 2012 to December 2016. Four hundred and three patients who met the following criteria were included: histopathologically confirmed rectal adenocarcinoma, >18 years old, tumor located no more than 12 cm above the anal verge, clinical stage of cT3/4 or lymph node (+), and non-metastatic. All patients were assessed *via* abdominal-pelvic computed tomography (CT) and pelvic magnetic resonance imaging (MRI), and 44 (11%) patients received transrectal ultrasound testing. All received NT followed by total mesorectal excision (TME) radical surgery.

We collected all available clinical information before treatment: gender, age, body mass index (BMI), cT, cN, mesorectal fascia (MRF) status, tumor differentiation, tumor length (TL), distance of tumor from the anal verge (DTAV), tumor circumferential extent (TCE), serum tumor marker CEA, HB, neutrophil-lymphocyte ratio (NLR), platelets (PLT), apolipoprotein A-1 (ApoA1), apolipoprotein B (ApoB), and NT regimen type. All tumor-related parameters such as cT, cN, MRF status, DTAV, and TCE were assessed by MRI. Tumor length was also assessed by using MRI to measure the maximum diameter of tumor. CT, transrectal ultrasound, and endoscopy provided additional verification. Tumor differentiation was identified by enteroscopic pathology.

This retrospective study was approved by the Institutional Review Board of The Sixth Affiliated Hospital, Sun Yat-sen University.

Therapy

During the period we identified patients for the current study, a clinical trial (FOWARC) was conducted at our institution comparing the effectiveness and safety of administering only chemotherapy with mFOLFOX6 or mFOLFOX6 plus radiotherapy to LARC patients with the effectiveness and safety in patients undergoing a standard NT regimen with fluorouracil plus radiotherapy. Consequently, 273 (67.7%) patients in our study were included in the FOWARC trial. The NT regimens included in our study were capecitabine /fluorouracil plus radiotherapy (standard group, capecitabine/deGramont-RT), mFOLFOX6 without radiotherapy (mFOLFOX6), and mFOLFOX6 plus radiotherapy (mFOLFOX6-RT). Details of all these treatments have been reported in previous studies[26,27]. The radiation dose for the radiotherapy was 46.0-50.4 Gy, delivered as 1.8-2.0 Gy/d, and the dose was the same in the capecitabine/deGramont-RT and mFOLFOX6-RT groups. Patients in the capecitabine/deGramont-RT and mFOLFOX6-RT groups underwent standard TME radical surgery after NT. The interval between radiation and surgery was 6-12 wk in mFOLFOX6-RT and de Gramont–RT groups. The interval between chemotherapy and TME radical surgery was about 2-4 wk in the mFOLFOX6 group.

Pathological assessment

All resected specimens were examined to determine the post-TN staging according to the American Joint Committee on Cancer-International Union Against Cancer (seventh edition), which is currently considered the most accurate and standard staging system in this period[28]. pCR was defined as no malignant cells found in the resected specimens, including the primary tumor and lymph nodes, and ypT0-2N0M0 (ypTNM 0-I) was classified as good downstaging.

Statistical analysis

Chi-square analysis was selected for the univariate logistic regression analysis of the countable data. Normal distribution tests were performed for the metrological data, nonparametric test was used for the indicators that were not normally distributed, and the expression form of the median (upper quartile to lower quartile) is used.

Parameters such as age (≤ 60 years *vs* > 60 years), BMI (< 25 kg/cm^2*vs* ≥ 25 kg/cm^2), CEA (> 5 ng/mL *vs* ≤ 5 ng/mL), HB (≤ 125 g/L *vs* > 125 g/L), NLR (> 3 *vs* ≤ 3), DTAV (< 5 cm *vs* ≥ 5 cm) and TL (> 3 cm *vs* ≤ 3 cm) were dichotomized according to previous studies[24,29,30]. PLT, ApoA1, ApoB, and the interval were used as continuous variables, however, all these variables were not normally distributed, so a nonparametric test was used.

Univariate logistic regression analysis was used to analyze variables related to the probability of pCR or good downstaging. Variables that achieved significance at $P \leq 0.05$ in the univariate logistic regression analysis were further analyzed into the forward stepwise multivariable logistic regression. Multivariate logistic regression analysis was used to construct nomograms. Because the NT regimen was a statistically significant factor for predicting pCR probability, all patients were divided into three subgroups (the capecitabine/deGramont-RT, mFOLFOX6-RT, or mFOLFOX6 groups) based on the NT regimens. We then attempted to develop three nomograms based on the different NT regimens to predict pCR probability. The C-index was acquired for the nomogram, and internal validation using the bootstrap method was performed to determine the adjusted C-index. Calibration curves of the nomograms were generated to show the relationship between the predicted and observed outcomes. All statistical analyses were performed using SPSS 24.0 and R 3.5.1.

RESULTS

Of the 403 patients in our study, 281 (69%) were men. As assessed pathologically, 72 (17.86%) individuals achieved pCR; 177 (43.9%) patients achieved ypTNM 0-I and were classified as having good downstaging.

The interval between radiation and surgery was 52 (47-59) d in the mFOLFOX6-RT group and 54 (49-58.25) d in the deGramont–RT group, and there was no significance difference between the two groups. The interval between chemotherapy and surgery was 22 (18-25.75) d in the mFOLFOX6 group, which was much shorter than those in the other two groups.

All patients received TME surgery (28 underwent APR and 375 underwent sphincter-saving surgery).

pCR patients and non-pCR patients did not differ significantly in terms of gender, BMI, CEA, NLR, HB, PLT, ApoA1, ApoB, cT, cN, or TCE in the univariate analysis ($P > 0.05$); however, significant differences were found for age, tumor differentiation, TL, DTAV, MRF status, interval, and NT regimen (Table 1). Statistically significant factors in the univariate logistic regression analysis ($P \leq 0.05$) to predict pCR were entered into a multivariate analysis, in which NT regimen types ($^aP < 0.05$), tumor differentiation ($^bP < 0.05$), TL ($^cP < 0.05$), and MRF status ($^dP < 0.05$) were significantly associated with pCR probability (Table 2). For the NT regimens, the odds ratio (OR) was 5.339 [95% confidence interval (CI): 2.394-11.903] for the mFOLFOX6-RT regimen compared with the capecitabine/deGramont-RT regimen. The mFOLFOX6 regimen and capecitabine/deGramont-RT regimen did not differ significantly. For tumor differentiation, the OR was 2.966 (95%CI: 1.449-6.069) for well tumor differentiation compared with moderate-poor differentiation. For TL (> 3 cm) compared with TL (≤ 3 cm), the OR was 2.608 (95%CI: 1.347-5.052), and for MRF(-) compared with MRF(+), the OR was 2.729 (95%CI: 1.199-6.211).

Patients with good downstaging and bad downstaging did not significantly differ in terms of age, gender, BMI, NLR, HB, PLT, ApoA1, ApoB, cN, TCE, the interval, or NT regimen in the univariate analysis ($P > 0.05$); however, significant differences were found for CEA, tumor differentiation, DTAV, TL, cT, and MRF status in the univariate logistic regression analysis for good downstaging (Table 3). In the multivariate analysis, tumor differentiation ($^eP < 0.05$), MRF statuses ($^fP < 0.05$), and cT ($^gP < 0.05$)

Table 1 Predictive factors for complete pathological response in univariate logistic regression analysis for all patients

Variable		Non-pCR (n = 331) n P_{50} (P_{25}-P_{75})	pCR (n = 72) n P_{50} (P_{25}-P_{75})	pCR rate	*P*-value
Gender	Male	232	49	17.44%	0.653
	Female	96	23	19.33%	
Age (yr)	≤ 60	210	54	20.45%	0.07
	> 60	119	18	13.14%	
BMI (kg/cm^2)	< 25	231	53	18.66%	0.581
	≥ 25	78	15	16.13%	
Hemoglobin (g/L)	≤ 125	109	16	12.80%	0.126
	> 125	172	41	19.25%	
NLR	> 3	37	11	22.92%	0.227
	≤ 3	244	46	15.86%	
Platelet (× 10^9/L)		237.5 (200.25-286.75)	246 (200.5-268.5)		0.981
ApoA1 (g/L)		1.29 (1.13-1.44)	1.3 (1.15-1.52)		0.454
ApoB (g/L)		0.98 (0.79-1.14)	0.97 (0.82-1.19)		0.382
The interval (d)		39 (22.25-54)	50 (42-56.5)		0
CEA (ng/mL)	> 5	163	28	14.66%	0.217
	≤ 5	118	29	19.73%	
Differentiation	Moderate-poor	274	44	13.84%	0.001
	Well	44	20	31.25%	
DTAV (cm)	< 5	140	41	22.65%	0.024
	≥ 5	191	31	13.96%	
TL (cm)	> 3	237	41	14.75%	0.001
	≤ 3	73	30	29.13%	
TCE	< 50%	37	7	15.91%	0.772
	≥ 50%	256	55	17.68%	
cT	2	16	5	23.81%	0.405
	3	234	56	19.31%	
	4	54	8	12.90%	
cN	+	235	57	19.52%	0.465
	-	78	15	16.13%	
MRF	-	231	61	20.89%	0.013
	+	97	11	10.19%	
NT regimen	Capecitabine/de Gramont-RT	102	13	11.30%	0
	mFOLFOX6	148	14	8.64%	
	mFOLFOX6-RT	81	45	35.71%	

Platelet, apolipoprotein A-1, apolipoprotein B, and the interval were calculated as metrological data, and others are counting data. pCR: Complete pathological response; NLR: Neutrophil-lymphocyte ratio; DTAV: Distance of tumor from the anal verge; TL: Tumor length; TCE: Tumor circumferential extent; NT: Neoadjuvant therapy; PLT: Platelet; ApoA1: Apolipoprotein A-1; ApoB: Apolipoprotein B; MRF: Mesorectal fascia; CEA: Carcinoembryonic antigen.

were significantly associated with the probability of good downstaging (Table 4). The OR was 4.814 (95%CI: 2.343-9.892) for well differentiation compared with moderate-poor differentiation, 4.226 (95%CI: 1.894-9.426) for MRF(-) compared with MRF(+), and 0.248 (95%CI: 0.063-0.974) for cT3 compared with cT2.

Because the type of NT regimen was a statistically significant factor for predicting pCR probability, patients were divided into three subgroups (the capecitabine/deGramont-RT, mFOLFOX6, and mFOLFOX6-RT groups) based on the NT regimen. Table 5 shows the distribution of pretreatment clinical parameters in the NT regimen groups. No differences were found in any factors between the three groups except age ($^hP < 0.05$) and DTAV ($^iP < 0.05$). In the univariate analysis of the capecitabine/deGramont-RT group, NLR was the only significant factor for predicting pCR probability (Table 6). NLR (> 3) ($^iP < 0.05$) was the only significant factor, with an OR of 4.278 (95%CI: 1.051-17.413) compared with NLR ≤ 3 in the further multivariate analysis (Table 7). We could not develop a nomogram to predict pCR probability in this case.

Table 8 shows that TL and MRF status were significant factors predicting pCR

Table 2 Predictive factors for complete pathological response in multivariate logistic regression analysis for all patients

Variable		*P*-value	OR	95%CI
Age (yr)	≤ 60	0.703	0.873	0.434-1.756
	> 60		1	
Differentiation	Well	0.003	2.966	1.449-6.069
	Moderate-poor		1	
TL (cm)	≤ 3	0.004	2.608	1.347-5.052
	> 3		1	
DTAV	≥ 5	0.07	0.56	0.299-1.049
	< 5		1	
MRF	-	0.017	2.729	1.199-6.211
	+		1	
NT regimen	mFOLFOX6-RT	0	5.339	2.394-11.903
	mFOLFOX6	0.402	1.821	0.449-7.387
	Capecitabine/de Gramont-RT		1	
The interval		0.093	1.029	0.995-1.064

pCR: Complete pathological response; TL: Tumor length; DTAV: Distance of tumor from the anal verge; NT: Neoadjuvant therapy; MRF: Mesorectal fascia.

probability in the univariate analysis of the mFOLFOX6-RT regimen. TL ($^{k}P < 0.05$) and MRF(+) ($^{l}P < 0.05$) were significant factors, with an OR of 2.452 (95%CI: 1.015-5.926) for TL(≤ 3 cm) compared with TL(> 3 cm) and an OR of 3.829 (95%CI: 1.42-10.325) for MRF(-) compared with MRF(+) in the further multivariate analysis (Table 9).

In the univariate analysis of the mFOLFOX6 regimen, tumor differentiation and TL were significant factors for predicting pCR probability (Table 10). Further multivariate analysis showed that differentiation ($^{m}P < 0.05$) and TL ($^{n}P < 0.05$) were significant factors, with an OR of 8.881 (95%CI: 2.263-34.85) for well tumor differentiation compared with moderate-poor differentiation and an OR of 4.805 (95%CI: 1.25-18.466) for TL (≤ 3 cm) compared with TL (> 3 cm) (Table 11).

Predictive nomograms established for pCR and good downstaging

Nomograms were developed based on the significant factors in the multivariate logistic regression analysis. The nomogram for predicting pCR probability showed that NT regimen and tumor differentiation influenced the probability of pCR, followed by TL and MRF status (Figure 1). When developing the nomogram to predict the probability of good downstaging, tumor differentiation and MRF status were the most important, followed by cT (Figure 2). We attempted to develop three nomograms to predict pCR probability based on NT regimens, because only one significant factor was found for the capecitabine/deGramont-RT regimen, we could not develop a nomogram. MRF status and TL were the significant factors for the mFOLFOX6-RT group (Figure 3). For the mFOLFOX6 group, tumor differentiation and TL were the significant factors in the nomogram for predicting pCR probability (Figure 4). Using the nomograms, we could easily calculate the probability of pCR and ypTNM (0-I), and we calculated pCR probabilities based on the NT regimens.

We used 1000 bootstrap resamples to compute an adjusted C-index, which was 79.34% for predicting pCR (95%CI: 73.48%-85.21%) for all patients, with a C-index of 69.85% (95%CI: 60.94%-78.76%) for the mFOLFOX6-RT group and 83.39% (95%CI: 67.26%-93.52%) for the mFOLFOX6 group. For predicting good downstaging, the adjusted C-index was 68.08% (95%CI: 63.08%-73.07%) for all patients. Calibration curves between predicted and actual observations by internal validation demonstrated that these nomograms showed good statistical performance for predicting the probability of pCR and good downstaging. Figures 5-8 show the calibration curve between the predicted and actual observations by internal validation and demonstrates that these nomograms showed good statistical performance for predicting the probability of pCR and good downstaging.

Table 3 Predictive factors for good downstaging in univariate logistic regression analysis for all patients

Variable		Bad downstaging (n = 226) n P_{50} (P_{25}-P_{75})	Good downstaging (n = 177) n P_{50} (P_{25}-P_{75})	Good downstaging rate	*P*-value
Gender	Male	164	117	41.64%	0.106
	Female	59	60	50.42%	
Age (yr)	≤ 60	144	120	45.45%	0.462
	> 60	80	57	41.61%	
BMI (kg/cm^2)	< 25	159	125	44.01%	0.475
	≥ 25	56	37	39.78%	
Hemoglobin (g/L)	≤ 125	69	56	44.80%	0.426
	> 125	127	86	40.38%	
NLR	> 3	27	21	43.75%	0.792
	≤ 3	169	121	41.72%	
Platelet (×10^9/L)		241 (207-294)	236 (193-272.25)		0.125
ApoA1 (g/L)		1.27 (1.13-1.44)	1.31 (1.15-1.48)		0.228
ApoB (g/L)		0.98 (0.79-1.13)	0.97 (0.79-1.18)		0.88
The interval		39 (23-54)	48 (25.75-55)		0.062
CEA (ng/mL)	> 5	125	66	34.55%	0.002
	≤ 5	71	76	51.70%	
Differentiation	Moderate-poor	194	124	38.99%	0
	Well	20	44	68.75%	
DTAV (cm)	< 5	85	96	53.04%	0.001
	≥ 5	141	81	36.49%	
TL (cm)	> 3	167	111	39.93%	0.002
	≤ 3	44	59	57.28%	
TCE	< 50%	23	21	47.73%	0.508
	≥ 50%	179	132	42.44%	
cT	2	4	17	80.95%	0
	3	160	130	44.83%	
	4	46	16	25.81%	
cN	+	166	126	43.15%	0.074
	-	43	50	53.76%	
MRF	-	142	150	51.37%	0
	+	81	27	25.00%	
NT regimen	Capecitabine/de Gramont-RT	64	51	44.35%	0.061
	mFOLFOX6-RT	61	65	51.59%	
	mFOLFOX6	101	61	37.65%	

Platelet, apolipoprotein A-1, apolipoprotein B, and the interval were calculated as metrological data, and others are counting data. NLR: Neutrophil-lymphocyte ratio; DTAV: Distance of tumor from the anal verge; TL: Tumor length; TCE: Tumor circumferential extent; NT: Neoadjuvant therapy; PLT: Platelet; ApoA1: Apolipoprotein A-1; ApoB: Apolipoprotein B; MRF: Mesorectal fascia; CEA: Carcinoembryonic antigen.

DISCUSSION

At present, preoperative NT is the standard treatment for patients with LARC. Patients who respond well to preoperative treatment have shown to have an excellent long-term prognosis. Knowledge of these factors ultimately leads to individualized treatment strategies; for example, patients who do not respond to the usual management can choose an aggressive preoperative regimen before NT. Conversely, to accurately determine an excellent pathological response after NT, surgeons may choose to perform local excision or a "watch and wait" strategy. In some cases, radical surgical resection may not benefit for some patients who achieve a good response because radical surgical resection may be associated with high rates of temporary or permanent stomas, defecatory disorders, urinary and sexual dysfunction, and unnecessary mortality[31,32]. pCR after NT is reported to have an excellent long-term prognosis irrespective of the treatment strategy, so noninvasive treatment strategies, such as a "watch and wait" strategy, have become more popular for patients who

Table 4 Predictive factors for good downstaging in multivariate logistic regression analysis for all patients

Variable		*P*-value	OR	95%CI
CEA (ng/mL)	≤ 5	0.095	1.565	0.925-2.647
	> 5		1	
Differentiation	Well	0	4.814	2.343-9.892
	Moderate-poor		1	
DTAV (cm)	≥ 5	0.052	0.588	0.345-1.004
	< 5		1	
TL (cm)	≤ 3	0.9	1.04	0.566-1.909
	> 3		1	
cT	3	0.046	0.248	0.063-0.974
	4	0.127	0.282	0.056-1.434
	2		1	
MRF	-	0	4.226	1.894-9.426
	+		1	

DTAV: Distance of tumor from the anal verge; TL: Tumor length; MRF: Mesorectal fascia; CEA: Carcinoembryonic antigen.

achieve a good response[33,34]. Thus, understanding the factors that predict the pathological response to NT is becoming crucial.

Our study identified clinical variables related to the pCR and good downstaging of LARC patients after NT. In the nomogram, we demonstrated that type of NT regimen, tumor differentiation, MRF status, and TL predicted pCR, whereas tumor differentiation, MRF status, and cT predicted good downstaging.

In our model, the mFOLFOX6-RT group had a higher probability of pCR compared with the capecitabine/de Gramont-RT group. We acknowledge that a potential selection bias may contribute to this high pCR rate. The data missing were more frequently in patients not reaching pCR than those with pCR, possibly resulting from more attentions pCR-patients got in clinical practice, follow-up, or research work. The pCR rate was 35.7% for mFOLFOX6-RT, which is higher than that of FOWARC[26,27]. It is expected since this is a single-center statistic result, while the FORWARC trial is a muli-center study. Although the benefits of oxaliplatin have not been demonstrated and it is not part of standard NT regimens, oxaliplatin is a standard component of chemotherapy for treating colon cancer[35]. Importantly, it has been reported in more and more studies[36,37] that the regimen combining mFOLFOX 6 with RT is getting a higher pCR rate of 38% in a clinical trial published in Lancet Oncology[38]. However, the role of oxaliplatin adding to fluorouracil-based neoadjuvant chemoradiotherapy (CRT) is unclear for LARC patients, and more studies are needed in the future.

pCR probabilities did not significantly differ between the capecitabine/deGramont-RT and themFOLFOX6 groups. Additionally, NT regimen was not a significant factor for predicting the probability of good downstaging. To avoid radiotherapeutic harm to LARC patients, the use of neoadjuvant chemotherapy alone has been proposed. Our model showed that patients treated with the mFOLFOX6 regimen alone had an acceptable probability of pCR and good downstaging. Thus, some chemosensitive patients can avoid radiation therapy.

Tumor differentiation was associated with both pCR and good downstaging. Well differentiation was associated with a higher pCR probability, which is consistent with previous studies[23,39], and was related to good downstaging compared with moderate-poor differentiation. Patients with a well differentiated tumor have a higher pCR probability indicating that a mild NT regimen, local resection, or "watch and wait" strategy can be considered after NT. Patients with moderate-poor differentiation may have a poor likelihood of pCR and good downstaging indicating that a "watch and wait" strategy requires careful selection.

Factors associated with pCR and good downstaging both included MRF status. MRF(+) implies that the tumor is aggressive, and even after NT, patients with MRF(+) may have a poor likelihood of pCR and good downstaging, indicating that an enhanced NT regimen and radical surgery are needed and a "watch and wait" strategy requires careful selection. While patients with MRF(-) may have a higher pCR probability indicating that a mild NT regimen, local resection, or "watch and wait" strategy can be considered after NT.

Table 5 Distribution of pretreatment clinical parameters in different neoadjuvant therapy regimen groups

Variable		Capecitabine/deGramont-RT *n* (%) P_{50} (P_{25}-P_{75})	mFOLFOX6 *n* (%) P_{50} (P_{25}-P_{75})	mFOLFOX6-RT *n* (%) P_{50} (P_{25}-P_{75})	*P*-value
Gender	Male	76 (66.67)	107 (66.88)	98 (77.78)	0.083
	Female	38 (33.33)	53 (33.13)	28 (22.22)	
Age (yr)	≤ 60	66 (57.39)	102 (63.75)	96 (76.19)	0.007
	> 60	49 (42.61)	58 (36.25)	30 (23.81)	
BMI (kg/cm^2)	< 25	83 (76.85)	118 (76.62)	83 (72.17)	0.641
	≥ 25	25 (23.15)	36 (23.38)	32 (27.83)	
Hemoglobin (g/L)	≤ 125	34 (34.34)	55 (39.29)	36 (36.36)	0.729
	> 125	65 (65.66)	85 (60.71)	63 (63.64)	
NLR	> 3	16 (16.16)	17 (12.14)	15 (15.15)	0.646
	≤ 3	83 (83.84)	123 (87.86)	84 (84.85)	
CEA (ng/mL)	> 5	56 (56.57)	79 (56.43)	56 (56.57)	1
	≤ 5	43 (43.43)	61 (43.57)	43 (43.43)	
Differentiation	Moderate-poor	90 (81.08)	135 (87.1)	93 (80.17)	0.246
	Well	21 (18.92)	20 (12.9)	23 (19.83)	
DTAV (cm)	< 5	61 (53.04)	62 (38.27)	58 (46.03)	0.049
	≥ 5	54 (46.96)	100 (61.73)	68 (53.97)	
TL (cm)	> 3	82 (74.55)	105 (70)	91 (75.21)	0.572
	≤ 3	28 (25.45)	45 (30)	30 (24.79)	
TCE	< 50%	10 (10)	22 (15.28)	12 (10.81)	0.389
	≥ 50%	90 (90)	122 (84.72)	99 (89.19)	
cN	+	84 (75)	110 (73.33)	98 (79.67)	0.462
	-	28 (25)	40 (26.67)	25 (20.33)	
MRF	-	85 (74.56)	119 (74.38)	88 (69.84)	0.627
	+	29 (25.44)	41 (25.63)	38 (30.16)	
pCR	Non-pCR	102 (88.7)	148 (91.36)	81 (64.29)	0
	pCR	13 (11.3)	14 (8.64)	45 (35.71)	
Downstaging	Bad	64 (55.65)	101 (62.35)	61 (48.41)	0.061
	Good	51 (44.35)	61 (37.65)	65 (51.59)	
cT	2	9 (8.26)	10 (6.85)	2 (1.69)	0.19
	3	85 (77.98)	112 (76.71)	93 (78.81)	
	4	15 (13.76)	24 (16.44)	23 (19.49)	
PLT (×10^9/L)		230 (188.75-267.25)	236.5 (200.25-290.75)	244 (212-281)	0.168
ApoA1 (g/L)		1.31 (1.14-1.47)	1.29 (1.12-1.44)	1.28 (1.15-1.5)	0.73
ApoB (g/L)		0.97 (0.8-1.09)	0.98 (0.78-1.14)	0.98 (0.81-1.21)	0.425
The interval		54 (49-58.25)	22 (18-25.75)	52 (47-59)	0

Platelet, apolipoprotein A-1, apolipoprotein B, and the interval were calculated as metrological data, and others are counting data. pCR: Complete pathological response; NLR: Neutrophil-lymphocyte ratio; DTAV: Distance of tumor from the anal verge; TL: Tumor length; TCE: Tumor circumferential extent; NT: Neoadjuvant therapy; PLT: Platelet; ApoA1: Apolipoprotein A-1; ApoB: Apolipoprotein B; MRF: Mesorectal fascia; CEA: Carcinoembryonic antigen.

TL was also a significant factor in the multivariate logistic regression analysis for predicting pCR probability in all patients. Van Stiphout *et al*[40] reported that TL was related to the probability of pCR after NT, although this study was based on data from positron emission tomography (PET)-CT results. TL (> 3 cm) implies an aggressive tumor, and even after NT, patients with TL (> 3 cm) may have a lower pCR probability indicating that an enhanced NT regimen and radical surgery are needed. While for patients with TL (≤ 3 cm) may have a higher pCR probability indicating that a mild NT regimen, local resection, or "watch and wait" strategy can be considered after NT.

For predicting the probability of good downstaging, cT was also a significant factor in the multivariate logistic regression analysis. In a study performed by Joye *et al*[41], a low cT stage was linked with ypT0-1N0, and it, together with other factors, could be

Table 6 Predictive factors for complete pathological response in univariate logistic regression analysis for the capecitabine/deGramont-RT regimen

Variable		Non-pCR (n = 102) n P_{50} (P_{25}-P_{75})	pCR (n = 13) n P_{50} (P_{25}-P_{75})	pCR rate	P-value
Gender	Male	70	6	7.89%	0.096
	Female	31	7	18.42%	
Age (yr)	≤ 60	56	10	15.15%	0.131
	> 60	46	3	6.12%	
BMI (kg/cm^2)	< 25	75	8	9.64%	0.375
	≥ 25	21	4	16.00%	
Hemoglobin (g/L)	≤ 125	33	1	2.94%	0.087
	> 125	56	9	13.85%	
NLR	> 3	12	4	25.00%	0.031
	≤ 3	77	6	7.23%	
PLT (×10^9/L)		228 (188.25-266.75)	252 (188.75-319)		0.338
ApoA1 (g/L)		1.3 (1.14-1.48)	1.34 (1.11-1.46)		0.912
ApoB (g/L)		0.97 (0.8-1.09)	0.92 (0.78-1.02)		0.667
The interval		53.97 ± 8.94	52.38 ± 10.79		0.588
CEA (ng/mL)	> 5	51	5	8.93%	0.659
	≤ 5	38	5	11.63%	
Differentiation	Moderate-poor	82	8	8.89%	0.177
	Well	17	4	19.05%	
DTAV (cm)	< 5	52	9	14.75%	0.214
	≥ 5	50	4	7.41%	
TL (cm)	> 3	74	8	9.76%	0.252
	≤ 3	23	5	17.86%	
TCE	≤ 50%	10	0	0.00%	0.241
	> 50%	79	11	12.22%	
cT	2	7	2	22.22%	0.521
	3	75	10	11.76%	
	4	14	1	6.67%	
cN	+	73	11	13.10%	0.394
	-	26	2	7.14%	
MRF	-	74	11	12.94%	0.377
	+	27	2	6.90%	

Platelet, apolipoprotein A-1, apolipoprotein B, and the interval were calculated as metrological data, and others are counting data. pCR: Complete pathological response; NLR: Neutrophil-lymphocyte ratio; DTAV: Distance of tumor from the anal verge; TL: Tumor length; TCE: Tumor circumferential extent; PLT: Platelet; ApoA1: Apolipoprotein A-1; ApoB: Apolipoprotein B; MRF: Mesorectal fascia; CEA: Carcinoembryonic antigen.

used as a selection tool for organ-preserving strategies. Our study also showed that low cT stage was more likely to achieve good downstaging with NT, and indicated that less invasive surgery can be selected.

For the capecitabine/de Gramont-RT regimen, the only significant factor was the NLR. Kim *et al*[42] showed that an elevated NLR before CRT can be used to predict poor tumor response and adverse prognostic factors. As lymphocytes decrease and neutrophils increase, NLR affects the adverse tumor reaction and adverse prognosis. Our study showed that the NLR before NT was related to better pathological responses to the capecitabine/deGramont-RT regimen; thus, further studies are needed to validate the relationship between NLR and pathological response to NT.

For the mFOLFOX6-RT regimen, the significant factors for predicting pCR probability were MRF status and TL. MRF(+) and long TL indicated that the tumor was aggressive and patients have heavy tumor load, and were related to poor neoadjuvant pathological responses. Patients with moderate-poor differentiation and TL (> 3 cm) have a lower pCR probability indicating that the efficacy of CRT is poor for these patients, and radical surgery can be directly selected without NT to avoid complications caused by CRT.

For the mFOLFOX6 regimen, the nomogram for predicting pCR probability showed that differentiation and TL were significant factors. Poor differentiation and

Table 7 Predictive factors for complete pathological response in multivariate logistic regression analysis for the capecitabine/deGramont-RT regimen

Variable		*P* value	OR	95%CI
NLR	> 3	0.042	4.278	1.051-17.413
	≤ 3		1	

NLR: Neutrophil-lymphocyte ratio.

long TL indicated an aggressive tumor, and they were related to a poor neoadjuvant pathological response. Patients with moderate-poor differentiation and TL (> 3 cm) will have a lower probability of pCR indicating that radical surgery after NT is needed, or mFOLFOX6-RT regimen is chosen to increase pCR probability. However, good differentiation and short TL were related to a good neoadjuvant pathological response and high probability of pCR indicating that local resection or a "watch and wait" strategy can be chosen.

To the best of our knowledge, our study is the first to use different NT regimen types to predict a pathological response. We established an accurate model with easily obtained variables to predict the probability of pCR and good downstaging. Our analysis was also strengthened through cross-validation. These models can be used to assist with individualized therapy as follows. For LARC patients expected to have a poor pathological response, NT and NT-related harm can be avoided. For patients expected to have good pathological responses to chemotherapy alone, radiotherapy can be avoided. For patients who are not expected to have good pathological response from a standard NT regimen, an enhanced mFOFOLX6-RT regimen can be considered. For patients who are not expected to have good pathological response from an enhanced regimen, radical surgery can be directly chosen without NT to avoid complications caused by CRT. For patients with a high probability of pCR after NT, local resection or a "watch and wait" strategy can be used to avoid complications.

Our analysis had several limitations. First, this was a retrospective study, in which some factors associated with pCR were unavailable, such as smoking status, molecular subtype and so on. Second, mFOLFOX6 and mFOLFOX6-RT are not the standard regimens for LARC, and both regimens remain in the clinical trial phase. Finally, our nomograms are based on the experience of our single institution. These results must be validated in a group of independent external institutions.

The nomograms established in our study can be used to evaluate the probability of a pathological responses before NT and after NT. However, additional studies are required to answer clinical questions, regarding which patients can be treated only with neoadjuvant chemotherapy, which patients need oxaliplatin added to the neoadjuvant CRT, which patients need radical surgery, which patients can undergo local excision, and which patients can be managed with a "watch and wait" strategy after achieving a good response.

We established accurate nomograms to predicting the pathological responses to different preoperative NT regimens based on pretreatment parameters for LARC patients. These nomograms can be used to distinguish patient types and facilitate developing individualized treatments.

Table 8 Predictive factors for complete pathological response in univariate logistic regression analysis for the mFOLFOX6-RT regimen

Variable		Non-pCR (n = 81) n P_{50} (P_{25}-P_{75})	pCR (n = 45) n P_{50} (P_{25}-P_{75})	pCR rate	*P*-value
Gender	Male	63	35	35.71%	1
	Female	18	10	35.71%	
Age (yr)	≤ 60	62	34	35.42%	0.901
	> 60	19	11	36.67%	
BMI (kg/cm^2)	< 25	49	34	40.96%	0.202
	≥ 25	23	9	28.13%	
Hemoglobin (g/L)	≤ 125	23	13	36.11%	0.905
	> 125	41	22	34.92%	
NLR	> 3	9	6	40.00%	0.683
	≤ 3	55	29	34.52%	
PLT (×10^9/L)		246.5 (214.25-289.75)	239 (197-269)		0.22
ApoA1 (g/L)		1.26 (1.15-1.44)	1.3 (1.15-1.55)		0.453
ApoB (g/L)		1 (0.81-1.24)	0.97 (0.81-1.17)		0.725
The interval		51.5 (43-58.75)	54 (50-62)		0.116
CEA (ng/mL)	> 5	40	16	28.57%	0.107
	≤ 5	24	19	44.19%	
Differentiation	Moderate-poor	63	30	32.26%	0.311
	Well	13	10	43.48%	
DTAV (cm)	< 5	33	25	43.10%	0.11
	≥ 5	48	20	29.41%	
TL (cm)	> 3	64	27	29.67%	0.008
	≤ 3	13	17	56.67%	
TCE	< 50%	8	4	33.33%	0.944
	≥ 50%	65	34	34.34%	
cT	2	0	2	100.00%	0.061
	3	58	35	37.63%	
	4	18	5	21.74%	
cN	+	62	36	36.73%	0.946
	-	16	9	36.00%	
MRF	-	49	39	44.32%	0.002
	+	32	6	15.79%	

Platelet, apolipoprotein A-1, apolipoprotein B, and the interval were calculated as metrological data, and others are counting data. pCR: Complete pathological response; NLR: Neutrophil-lymphocyte ratio; DTAV: Distance of tumor from the anal verge; TL: Tumor length; TCE: Tumor circumferential extent; PLT: Platelet; ApoA1: Apolipoprotein A-1; ApoB: Apolipoprotein B; MRF: Mesorectal fascia; CEA: Carcinoembryonic antigen.

Table 9 Predictive factors for complete pathological response in multivariate logistic regression analysis for the mFOLFOX6-RT regimen

Variable		*P* value	OR	95%CI
TL (cm)	≤ 3	0.046	2.452	1.015-5.926
	> 3		1	
MRF	-	0.008	3.829	1.42-10.325
	+		1	

TL: Tumor length; MRF: Mesorectal fascia.

Table 10 Predictive factors for complete pathological response in univariate logistic regression analysis for the mFOLFOX6 regimen

Variable		Non-pCR (n = 148) n P_{50} (P_{25}-P_{75})	pCR (n = 14) n P_{50} (P_{25}-P_{75})	pCR rate	*P*-value
Gender	Male	99	8	7.48%	0.418
	Female	47	6	11.32%	

Age (yr)	≤ 60	92	10	9.80%	0.532
	> 60	54	4	6.90%	
BMI (kg/cm^2)	< 25	107	11	9.32%	0.477
	≥ 25	34	2	5.56%	
Hemoglobin (g/L)	≤ 125	53	2	3.64%	0.093
	> 125	75	10	11.76%	
NLR	> 3	16	1	5.88%	0.673
	≤ 3	112	11	8.94%	
PLT (×10^9/L)		127.5 (117.5-139)	137.5 (127-142.25)		0.82
ApoA1 (g/L)		1.28 (1.11-1.42)	1.3 (1.15-1.46)		0.542
ApoB (g/L)		0.96 (0.77-1.13)	1.13 (0.86-1.3)		0.051
The interval		21.5 (18-25)	25 (19.25-26.75)		0.09
CEA (ng/mL)	> 5	72	7	8.86%	0.889
	≤ 5	56	5	8.20%	
Differentiation	Moderate-poor	129	6	4.44%	0
	Well	14	6	30.00%	
DTAV (cm)	< 5	55	7	11.29%	0.345
	≥ 5	93	7	7.00%	
TL (cm)	> 3	99	6	5.71%	0.02
	≤ 3	37	8	17.78%	
TCE	< 50%	19	3	13.64%	0.413
	≥ 50%	112	10	8.20%	
cT	2	9	1	10.00%	0.974
	3	101	11	9.82%	
	4	22	2	8.33%	
cN	+	100	10	9.09%	0.866
	-	36	4	10.00%	
MRF	-	108	11	9.24%	0.707
	+	38	3	7.32%	

Platelet, apolipoprotein A-1, apolipoprotein B, and the interval were calculated as metrological data, and others are counting data. pCR: Complete pathological response; NLR: Neutrophil-lymphocyte ratio; DTAV: Distance of tumor from the anal verge; TL: Tumor length; TCE: Tumor circumferential extent; PLT: Platelet; ApoA1: Apolipoprotein A-1; ApoB: Apolipoprotein B; MRF: Mesorectal fascia; CEA: Carcinoembryonic antigen.

Table 11 Predictive factors for complete pathological response in multivariate logistic regression analysis for the mFOLFOX6 regimen

Variable		*P*-value	OR	95%CI
Differentiation	Well	0.002	8.881	2.263-34.85
	Moderate-poor		1	
TL	≤ 3	0.022	4.805	1.25-18.466
	> 3		1	

TL: Tumor length.

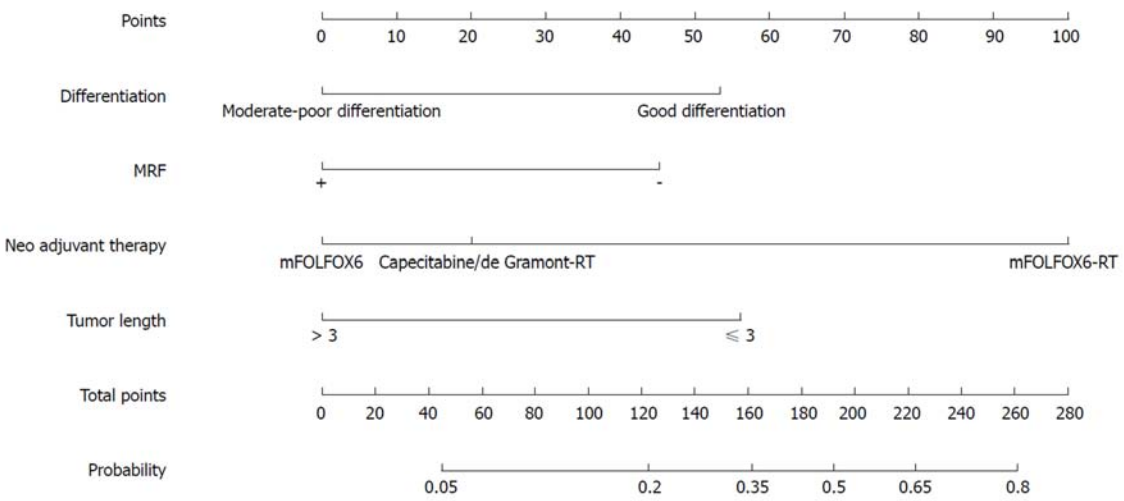

Figure 1 Nomogram for predicting the probability of pathological complete response for all patients. MRF: Mesorectal fascia.

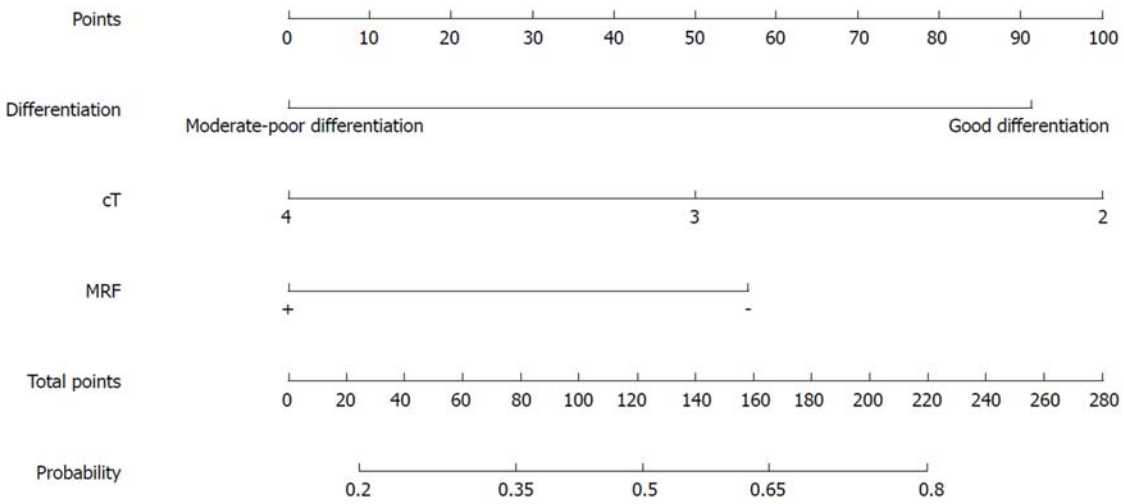

Figure 2 Nomogram for predicting the probability of good downstaging (ypTNM stage 0-I) for all patients. MRF: Mesorectal fascia.

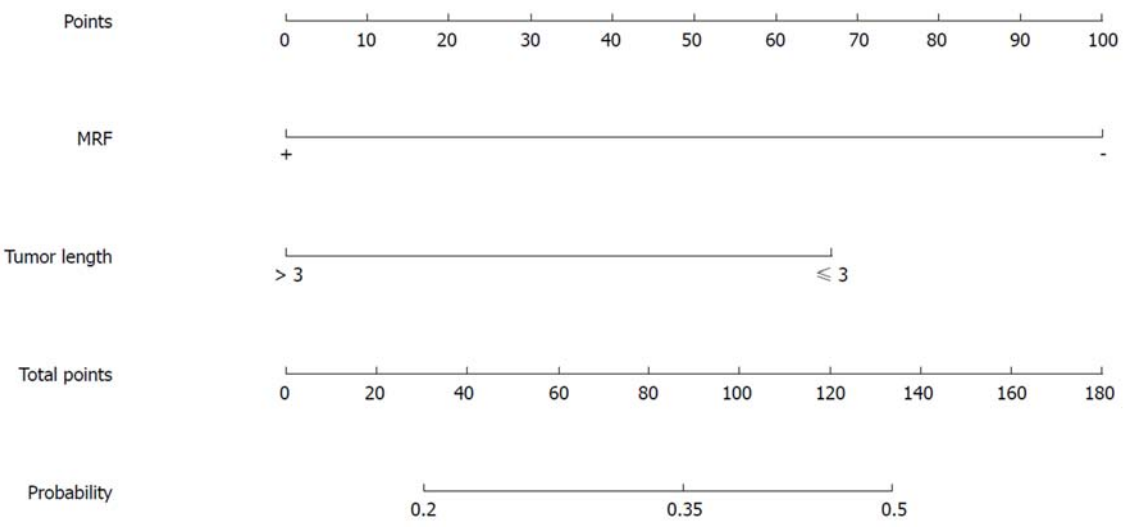

Figure 3 Nomogram for predicting the probability of pathological complete response for the mFOLFOX6-RT regimen. MRF: Mesorectal fascia.

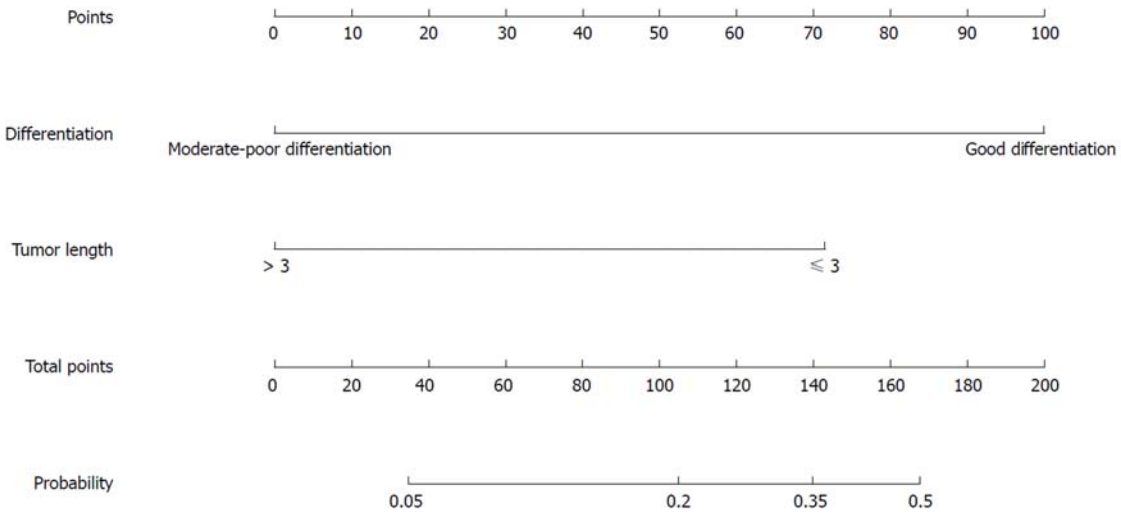

Figure 4 Nomogram for predicting the probability of pathological complete response for the mFOLFOX6 regimen.

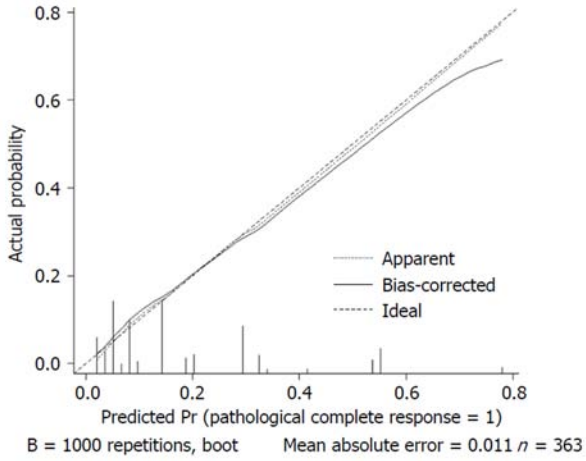

Figure 5 Calibration curve of the predicted and observed probabilities of pathological complete response for all patients.

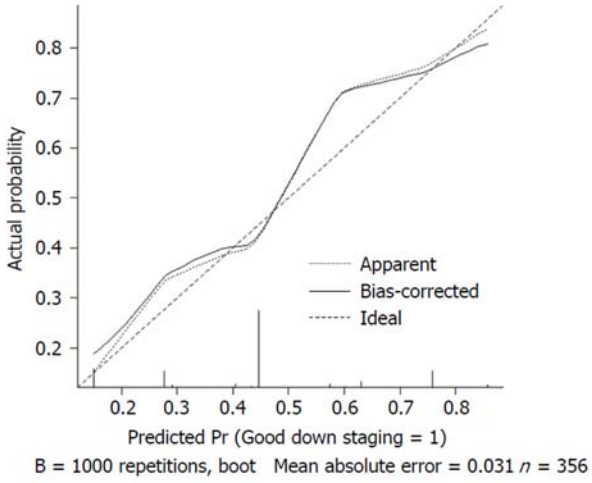

Figure 6 Calibration curve of the predicted and observed probabilities of good downstaging for all patients.

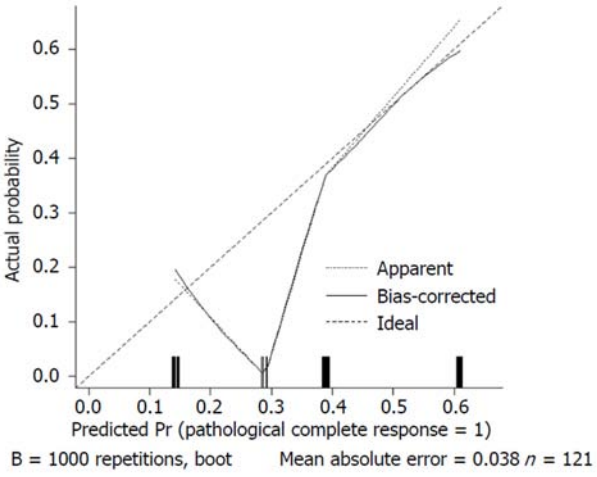

Figure 7 Calibration curve of the predicted and observed probabilities of pathological complete response for the mFOLFOX6-RT regimen.

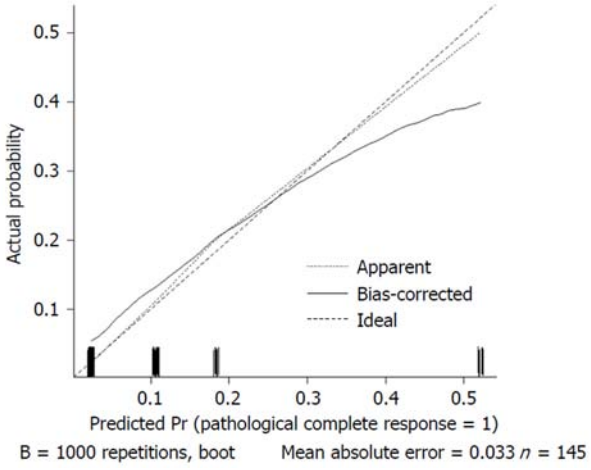

Figure 8 Calibration curve of the predicted and observed probabilities of pathological complete response for the mFOLFOX6 regimen.

ARTICLE HIGHLIGHTS

Research background

In recent decades, neoadjuvant therapy (NT) has been the standardized treatment for locally advanced rectal cancer (LARC). Approximately 8-35% of patients with LARC who received NT were reported to have achieved a complete pathological response (pCR). If the pathological response can be accurately predicted, these patients may not need surgery. In addition, no response after NT implies that the tumor is destructive, resistant to both chemotherapy and radiotherapy, and prone to having a high metastatic potential.

Few models or nomograms have been established and even fewer are used clinically to predict a good pathological response after NT for LARC. Therefore, developing accurate models to predict pathological response (PR) has great clinical significance and can help achieve individualized treatment in LARC patients.

Research motivation

Our goal was to establish nomograms that can be used to assist with individualized therapy as follows: for which patients NT and NT-related harm can be avoided; which patients will have good pathological responses to chemotherapy alone and radiotherapy can be avoided; which patients will have a good pathological response from a standard NT regimen, which patients need an enhanced mFOFOLX6-RT regimen; and which patients can use local resection or a "watch and wait" strategy to avoid complications. Solving these problems may aid in clinical treatment choices.

Research objectives

Our main objective was to establish nomograms for predicting a pathological response to different NT regimens based on pretreatment parameters for patients with LARC. We established accurate nomograms for predicting the pathological response to preoperative NT

regimens based on pretreatment parameters for LARC patients. These nomograms can be used to distinguish patient types and facilitate developing individualized treatments.

Research methods

Rectal cancer patients were identified from the database of The Sixth Affiliated Hospital, Sun Yat-sen University from January 2012 to December 2016. Four hundred and three patients who met the criteria were included. We collected all available clinical information before treatment.

The NT regimens included in our study were capecitabine/fluorouracil plus radiotherapy (standard group, capecitabine/deGramont-RT), mFOLFOX6 without radiotherapy (mFOLFOX6), and mFOLFOX6 plus radiotherapy (mFOLFOX6-RT). The radiation dose for the radiotherapy was 46.0-50.4 Gy, delivered as 1.8-2.0 Gy/d.

pCR was defined as no malignant cells found in the resected specimens, including the primary tumor and lymph nodes, and ypT0-2N0M0 (ypTNM 0-I) was classified as good downstaging.

Univariate logistic regression analysis was used to analyze variables related to the probability of pCR or good downstaging. Variables that achieved significance at $P \leq 0.05$ in the univariate logistic regression analysis were further analyzed into the forward stepwise multivariable logistic regression. Multivariate logistic regression analysis was used to construct the nomograms. Because the NT regimen was a statistically significant factor for predicting pCR probability, we then attempted to develop three nomograms based on the different NT regimens to predict pCR probability. The C-index was acquired for the nomogram, and internally validated using the bootstrap method to determine the adjusted C-index. Calibration curves of the nomograms were generated to show the relationship between the predicted and observed outcomes.

All statistical analyses were performed using SPSS 24.0 and R 3.5.1.

Research results

Of the 403 patients in our study, 281 (69%) were men. As assessed pathologically, 72 (17.86%) individuals achieved a pCR; 177 (43.9%) patients achieved ypTNM 0-I and were classified as having good downstaging.

Significant differences were found for age, tumor differentiation, TL, DTAV, mesorectal fascia (MRF) status, interval, and NT regimen in the univariate analysis. In the multivariate analysis, NT regimen types, tumor differentiation, TL, and MRF status were significantly associated with pCR probability.

Significant differences were found for carcinoembryonic antigen (CEA), tumor differentiation, distance of tumor from the anal verge (DTAV), tumor length (TL), cT, and MRF status in the univariate logistic regression analysis for good downstaging. In the multivariate analysis, tumor differentiation, MRF statuses, and cT were significantly associated with the probability of good downstaging.

Table 5 shows the distribution of pretreatment clinical parameters in the NT regimen groups. No differences were found in any factors between the three groups except age and DTAV.

In the univariate analysis of the capecitabine/deGramont-RT group, NLR was the only significant factor for predicting pCR probability. NLR (> 3) was the only significant factor compared with NLR ≤ 3 in the further multivariate analysis. We could not develop a nomogram to predict pCR probability in this case.

In the univariate analysis of the mFOLFOX6-RT regimen, TL and MRF status were significant factors predicting pCR probability. TL and MRF(+) were significant factors in multivariate analysis.

In the univariate analysis of the mFOLFOX6 regimen, tumor differentiation and TL were significant factors for predicting pCR probability. Further multivariate analysis showed that differentiation and TL were significant factors.

Nomograms were developed based on the significant factors in the multivariate logistic regression analysis. We used 1000 bootstrap resamples to compute an adjusted C-index. Calibration curves between predicted and actual observations by internal validation demonstrated that these nomograms showed good statistical performance for predicting the probability of pCR and good downstaging.

Research conclusions

We established accurate nomograms to predicting the pathological responses to different preoperative NT regimens based on pretreatment parameters for LARC patients. These nomograms can be used to distinguish patient types and facilitate developing individualized treatments.

To the best of our knowledge, our study is the first to use different NT regimen types to predict a pathological response. We established an accurate model with easily obtained variables to predict the probability of pCR and good downstaging. Our analysis was also strengthened through cross-validation. These models can be used to assist with individualized therapy as follows. For LARC patients expected to have a poor pathological response, NT and NT-related harm can be avoided. For patients expected to have good pathological responses to chemotherapy alone, radiotherapy can be avoided. For patients who are not expected to have good pathological response from a standard NT regimen, an enhanced mFOFOLX6-RT regimen can be considered. For patients with a high probability of pCR after NT, local resection or a "watch and wait" strategy can be used to avoid complications.

Our analysis had several limitations. First, this was a retrospective study, in which some factors associated with pCR were unavailable, such as smoking status, molecular subtypes and so on. Second, mFOLFOX6 and mFOLFOX6-RT are not the standard regimens for LARC, and both regimens remain in the clinical trial phase. Finally, our nomograms are based on the

experience of our single institution. These results must be validated in a group of independent external institutions.

The nomograms established in our study can be used to evaluate the probability of a pathological responses before NT and after NT. However, additional studies are required to answer clinical questions regarding which patients can be treated only with neoadjuvant chemotherapy, which patients need oxaliplatin added to the neoadjuvant chemoradiotherapy, which patients need radical surgery, which patients can undergo local excision, and which patients can be managed with a "watch and wait" strategy after achieving a good response.

Research perspectives

In the future, we plan to include a larger number of patients to enhance the accuracy of the prediction. On the other hand, we plan to add a second external cohort for validation to strengthen the reliability of the nomogram.

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ORIGINAL ARTICLE

Clinical Trials Study

Molecular detection of epithelial-mesenchymal transition markers in circulating tumor cells from pancreatic cancer patients: Potential role in clinical practice

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Abstract

AIM

To evaluate the clinical properties of three subpopulations of circulating tumor cells (CTCs) undergoing epithelial-mesenchymal transition (EMT) in pancreatic ductal adenocarcinoma (PDAC) patients.

METHODS

We identified CTCs for expression of the epithelial cell marker cytokeratin or epithelial cell adhesion molecule (EpCAM) (E-CTC), the mesenchymal cell markers vimentin and twist (M-CTC), or both (E/M-CTC) using the CanPatrol system. Between July 2014 and July 2016, 107 patients with PDAC were enrolled for CTC evaluation. CTC enumeration and classification were correlated with patient clinicopathological features and outcomes.

RESULTS

CTCs were detected in 78.5% of PDAC patients. The number of total CTCs ranged from 0 to 26 across all 107 patients, with a median value of six. CTC status correlated with lymph node metastasis, TNM stage, distant metastasis, blood lymphocyte counts, and neutrophil-to-lymphocyte ratio (NLR). Kaplan-Meier survival analysis showed that patients with ≥ 6 total CTCs had significantly decreased overall survival and progression-free survival compared with patients

additional data are available.

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with < 6 total CTCs. The presence of M-CTCs was positively correlated with TNM stage ($P < 0.01$) and distant metastasis ($P < 0.01$). Additionally, lymphocyte counts and NLR in patients without CTCs were significantly different from those in patients testing positive for each CTC subpopulation ($P < 0.01$).

CONCLUSION
Classifying CTCs by EMT markers helps to identify the more aggressive CTC subpopulations and provides useful evidence for determining a suitable clinical approach.

Key words: Pancreatic ductal adenocarcinoma; Circulating tumor cells; Epithelial-mesenchymal transition; Metastasis; Neutrophil-to-lymphocyte ratio

Core tip: In the present study, circulating tumor cell (CTC) enumeration and classification in pancreatic ductal adenocarcinoma (PDAC) patients were examined using the CanPatrol system. We explored the relationship between CTC status and survival and prognosis in 107 PDAC patients in China. CTC status was correlated with lymph node metastasis, TNM stage, distant metastasis, blood lymphocyte counts, neutrophil-to-lymphocyte ratio, and patient prognosis. Our findings demonstrate that CTCs show promise as a prognostic biomarker and provide useful evidence for determining an appropriate clinical approach for pancreatic adenocarcinoma patients.

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INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC), which is derived from the ductal epithelium, is the most common histological subtype of pancreatic malignancy, accounting for 90% of all cases[1,2] . It ranks fourth in cancer-related mortality. Because of its late presentation and propensity to invade adjacent organs and metastasize, PDAC remains one of the most lethal solid malignancies[3,4]. Endoscopic ultrasound (EUS) is the most sensitive nonoperative imaging test for the detection of benign or malignant pancreatic lesions, while fine needle aspiration (FNA) is a minimally invasive sampling technique that has proved to be a safe and accurate method of tissue acquisition. It has been demonstrated that the operating characteristics of EUS-FNA of solid pancreatic masses are: sensitivity 95%, specificity 92%, positive predictive value 98%, and negative predictive value 80%. The overall accuracy of EUS-FNA is 94.1%. Thus, EUS-FNA is the current gold standard technique for tissue acquisition in patients with unresectable pancreatic cancer[3,4]. However, EUS-FNA is costly and inconvenient, and is correlated with risk of complications, including pancreatitis and bowel perforations[5]. Computed tomography (CT) and magnetic resonance imaging (MRI) are commonly used in tumor staging, but they show limited ability in detecting small-volume metastatic disease, leading to routine under-staging[6]. Thus, identification of a biomarker for early diagnosis and accurate staging at the time of disease presentation can better inform first-line therapy.

Circulating tumor cells (CTCs) are disseminated cancer cells that escape from the primary lesion or from metastatic foci and enter the bloodstream, exemplifying the switch from localized to systemic disease[7,8]. CTCs are thought to represent the intravasated tumor stage between the formation of an invasive cancer and its eventual distant metastasis[9,10]. CTCs have been examined in numerous cancer types, such as prostate, colorectal, breast, gastric, and lung cancers for guiding clinical management, evaluating curative efficacy, predicting prognosis, and monitoring tumor recurrence[11-14] . In PDAC, CTCs have also been explored. Initial studies have confirmed the presence of CTCs in patients with PDAC[15,16]. A study reported by Liu

et al[17] classified cells as triploid, tetraploid, and multiploid CTCs based on chromosome 8 copy number, and found that both total CTC number and CTC subtype number are useful in PDAC diagnosis. Additionally, CTC positivity was also associated with poor tumor differentiation, shorter overall survival (OS), and increased metastasis in PDAC[18,19]. These data provide support for further exploration of CTC counts as indicators of PDAC progression. However, to date, CTCs are not as well established as a biomarker as compared with other solid cancers[17,20]. One reason may be the low sensitivity of previous technology in detecting CTCs from peripheral blood in PDAC.

Early and widespread metastasis remains a major challenge in the effective treatment of PDAC. Epithelial-mesenchymal transition (EMT), which is indispensable for PDAC metastasis, is a multi-step process involving many molecular and cellular changes, including the downregulation of epithelial proteins and the upregulation of mesenchymal proteins, endowing the cells with increased motility and invasiveness[21-23]. Recent studies have revealed that the EMT phenotype in CTCs may facilitate tumor metastasis. Characterizing the epithelial *vs* mesenchymal phenotypes of CTCs may be helpful in identifying the most aggressive CTC subpopulations and determining an appropriate therapy[24-26]. Recent work described a new technique called CanPatrol CTC enrichment, which evaluates CTC classification based on the EMT phenotype. Using this technique, CTCs are classified into three subpopulations based on the expression of epithelial (E-CTCs), biphenotypic epithelial/mesenchymal (E/M-CTCs), and mesenchymal markers (M-CTCs).

In the present study, CTCs from 107 PDAC patients were isolated and their EMT phenotype was characterized using the CanPatrol CTC enrichment technique. In addition, the relationships between clinicopathological parameters and the relative abundance of the three circulating EMT-CTC subpopulations were evaluated.

MATERIALS AND METHODS

Patients and clinical samples

From July 2014 and July 2016, 107 patients with newly diagnosed PDAC at Sun Yat-sen Memorial Hospital of Sun Yat-sen University were enrolled in the study. Informed consent was obtained from all patients before sample collection. All patients selected met the following criteria: pathological diagnoses were clear and definite; and no preoperative chemotherapy or radiotherapy had been administered. Follow-up was carried out completely and OS was defined as the time interval between the date of surgery and the date of death or the end of follow-up. This retrospective study was conducted in compliance with the institutional policy to protect private patient information and was approved by the institutional review board of Sun Yat-sen Memorial Hospital.

Isolation and enumeration of CTCs using CanPatrol system

CTC isolation was conducted using the CanPatrol CTC filtration system. For PDAC patients, 5-mL peripheral blood samples were collected in EDTA tubes by venipuncture and filtered with a calibrated membrane with 8-μm diameter pores. The filtration system included the membrane (Sur Exam, Guangzhou, China), a manifold vacuum plate with valve settings (SurExam, Guangzhou, China), an E-Z96 vacuum manifold (Omega, Norcrss, GA, United States), and a vacuum pump (Auto Science, Tianjin, China). Prior to filtration, red blood cell lysis was applied to remove erythrocytes, then PBS with 4% formaldehyde was used to resuspend the cells for 5 min. The cell suspension was transferred to a filtration tube and pumped with at least 0.08 MPa.

CTC subpopulations were identified using a multiplex RNA-ISH assay. Four epithelial biomarkers [epithelial cell adhesion molecule (EpCAM) and cytokeratin (CK) 8/18/19], two mesenchymal biomarkers (vimentin and twist), and a leukocyte biomarker, leukocyte common antigen (CD45), were applied to capture and characterize the CTCs. The hybridization assay was performed as previously described[22]. The assay was performed in a 24-well plate (Corning, NY, United States), and the cells on the membrane were treated with protease (Qiagen, Hilden, Germany) and subsequently subjected to serial hybridization reactions with capture probes that were specific for the intended examined genes (Supplementary Table 1). We applied 4′,6-diamindino-2-phenylindole (DAPI) to stain the nuclei, and the cells were analyzed with a fluorescence microscope.

Statistical analysis

All statistical analyses were performed using SPSS Statistics 22.0 and GraphPad Prism

Table 1 Baseline circulating tumor cell characteristics of treatment-naïve patients with advanced pancreatic ductal adenocarcinoma according to circulating tumor cell status

Group	Patients *n* (%)
Number of patients with no CTC	23/107 (21.5)
Number of patients with ≥ 1 CTC	84/107 (78.5)
Number of patients with ≥ 6 CTC	55/107 (51.4)
Number of patients with E-CTCs	65/107(60.7)
Number of patients with E/M-CTCs	39/107 (36.4)
Number of patients with M-CTCs	49/107 (45.8)
CTC dynamic range	
Total CTC	0-26
Median	6
E-CTC	1-11
E/M-CTC	0-26
M-CTC	0-9

CTC: Circulating tumor cells; E-CTC: Epithelial circulating tumor cells; M-CTC: Mesenchymal circulating tumor cells.

7.0. The results are presented as percentages for categorical variables. The Mann-Whitney *U* test was used to assess the differences between two groups because the data were not normally distributed, and the Kruskal-Wallis *H* test was used for multi-group analysis. Differences in patient survival were assessed using the Kaplan-Meier method and analyzed using the log-rank test in a univariate analysis. *P*-values less than 0.05 were considered statistically significant. Cox regression analyses were performed to assess the relative risk for each factor. A receiver operating characteristic (ROC) curve was established to evaluate the diagnostic value of CTCs. The area under the curve (AUC) was used to assess the predictive power. A two-tailed *P*-value of < 0.05 was considered statistically significant ($^{a}P < 0.05$, $^{b}P < 0.01$).

RESULTS

Detection of CTCs in the peripheral blood of patients with PDAC

CTCs from 107 PDAC patients were identified using the CanPatrol system. As shown in Figure 1, epithelial CTCs showed red fluorescence (Figure 1A), while mesenchymal CTCs exhibited green fluorescence (Figure 1B), corresponding to their specific genes (*i.e.*, EpCAM or CK, and vimentin and twist), respectively. Additionally, a third hybrid population of CTCs expressing both epithelial- and mesenchymal-specific genes was also observed (Figure 1C). Of the total population, 84 (78.5%) patients had positive CTC counts at baseline. The number of total CTCs ranged from 0 to 26 across all 107 patients, with a median value of six. Overall, ≥6 total CTC(s) were present in 51.4% of samples. The positive rates of epithelial, hybrid, and mesenchymal CTCs were 60.7%, 36.4%, and 45.8%, respectively (Table 1).

Correlation of CTCs with clinicopathological features

As shown in Table 2, 107 PDAC patients were included in the analysis. The presence of CTCs was positively correlated with TNM stage ($P < 0.001$), lymph node metastasis ($P = 0.016$), and distant metastasis ($P < 0.001$) (Figure 2A-C). The other clinicopathological features showed no statistical relationship with CTC status. Univariate analysis of OS revealed that lymph node metastasis ($P = 0.007$), TNM stage ($P < 0.001$), distant metastasis ($P < 0.001$), and ≥ 6 total CTCs ($P < 0.001$) were prognostic indicators. Multivariate analysis revealed that lymph node metastasis ($P = 0.011$), TNM stage ($P < 0.001$), distant metastasis ($P < 0.001$), and ≥ 6 total CTCs ($P < 0.001$) were all independent prognostic indicators for OS of patients with PDAC (Table 3). We constructed a ROC curve analysis (Figure 2D) to distinguish metastatic PDAC patients from those without metastasis; the area under the ROC curve (AUROC) was 0.8 ($P < 0.0001$) with an optimal cut-off point of 7 (sensitivity = 0.893, specificity = 0.633). For comparison, CA-199 at the optimum cut-off of 884 U/mL distinguished metastatic PDAC patients from those without metastasis, with a sensitivity of 0.536, specificity of 0.785, and AUROC of 0.637. Kaplan-Meier survival analysis showed that patients with ≥ 6 total CTCs had significantly decreased OS and

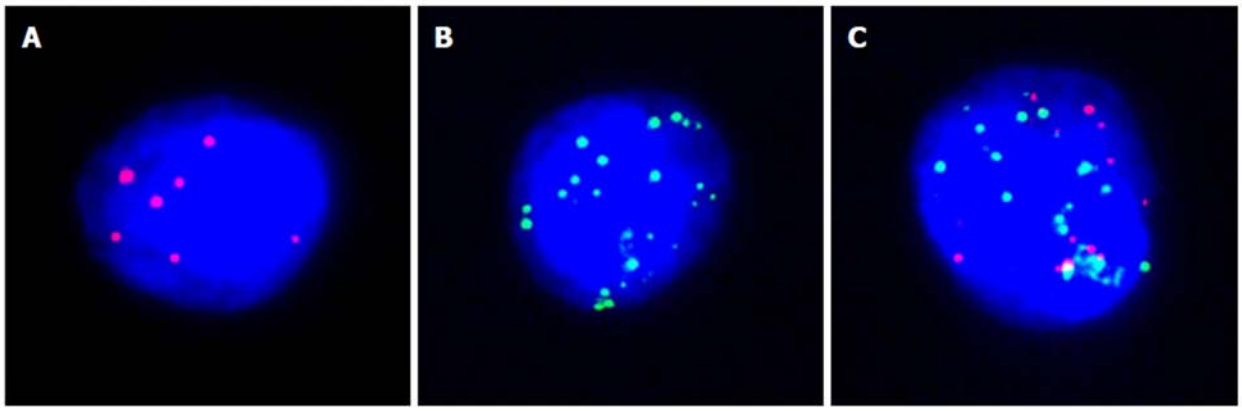

Figure 1 Circulating tumor cell subpopulations classified by categorical markers. (Red dots: epithelial biomarker expression; green dots: mesenchymal biomarker expression). A: Epithelial circulating tumor cells (E-CTCs); B: Mesenchymal CTCs (M-CTC); C: Biophenotypic epithelial/mesenchymal CTCs (E/M-CTCs).

progression-free survival (PFS) as compared with patients with < 6 total CTCs (OS: median 11 mo *vs* 18 mo; HR = 0.504; 95%CI: 0.330-0.768; $P < 0.001$. PFS: median 8 mo *vs* 13 mo; HR = 0.520; 95%CI: 0.342-0.791) (Figure 2E and F).

We quantified the immune cells in the peripheral blood of patients with PDAC and analyzed the associations between immune cell parameters and the presence of CTCs (Figure 2G and H). There were no significant differences between the CTC-negative and CTC-positive patient groups with respect to total white blood cell (WBC), neutrophil, or monocyte counts. However, there was a significant difference in lymphocyte count and the neutrophil-to-lymphocyte ratio (NLR) between the CTC-positive and CTC-negative patient groups ($P < 0.001$).

Association between CTC-EMT subpopulations and clinicopathological features

The associations between the presence of CTC subpopulations of each EMT phenotype and clinicopathological features were analyzed. Table 4 shows that E-CTCs were present in 65 (60.7%) patients, while E/M-CTCs and M-CTCs were present in 39 (36.4%) and 49 (45.8%) patients, respectively. The presence of M-CTCs was positively correlated with TNM stage ($P < 0.01$) and distant metastasis ($P < 0.01$). As shown in Figure 3A and B, M-CTCs were present in significantly more patients with distant metastasis than in those without. Regarding TNM stage, all patients with stage IV PDAC were CTC-positive and M-CTCs were more common in patients with stages III and IV disease.

We analyzed the correlation of different CTC subpopulations with the EMT phenotype and WBC count. There were no significant differences between different subpopulations with respect to total WBC, neutrophil, or monocyte counts (Figure 3C, D, and F). Figure 3E and G demonstrates that patients with any CTC subpopulations had significantly lower lymphocyte counts and higher NLRs than patients without CTCs ($P < 0.001$).

DISCUSSION

PDAC remains one of the most devastating diseases because of its late presentation and resistance to systemic therapy. Cross-sectional imaging alone often results in under-staging of PDAC patients[23,24,27]. Many studies have indicated that a "surgery first" paradigm for borderline resectable, and even early-stage, patients does not lead to a survival benefit and may in fact impair survival because of high recurrence and metastasis rates[27]. Therefore, there is an urgent need for novel biomarkers to complement cross-sectional imaging, especially for the identification of patients likely to benefit from non-surgical treatments first.

In recent years, the detection and characterization of CTCs have received tremendous attention because of the minimally invasive approaches applied to obtain sequential blood specimens from cancer patients and their potential clinical implications. It is anticipated that quantification and molecular subtyping of CTCs could be adopted for monitoring tumor burden and metastasis of PDAC. To date, published CTC data for PDAC mostly rely on small patient cohorts at different disease stages and use various CTC techniques, showing contradictory results. A large cohort of 154 patients had discovered CTCs using RT-PCR for CK20, obtaining a CTC rate of 34%. This study demonstrated that CTCs had a significantly negative

Table 2 Correlation between total circulating tumor cell status and clinicopathological factors of patients with pancreatic cancer

	Total CTC-negative patients (%)	Total CTC-positive patients (%)	*P*-value
Age, yr (median: 63, IQR: 52-76)			
< 60	8/23 (34.8)	32/84 (38.1)	0.769
≥ 60	15/23 (65.2)	52/84 (61.9)	
Gender			
Male	16/23 (69.6)	47/84 (56.0)	0.313
Female	7/23 (30.4)	37/84 (44.0)	
Ethnic group			
Han ethnic group	14/23 (60.9)	59/84 (70.2)	0.346
Zhuang ethnic group	5/23 (21.7)	16/84 (19.1)	
Li ethnic group	4/23 (17.4)	9/84 (10.7)	
Differentiation			
Well	3/23 (13.0)	7/84 (8.3)	0.435
Moderate	13/23 (56.5)	54/84 (64.3)	
Poor	7/23 (30.5)	23/84 (27.4)	
TNM stage			
I	6/23 (26.1)	4/84 (4.8)	< 0.001
II	7/23 (30.4)	13/84 (15.5)	
III	10/23 (43.5)	39/84 (46.4)	
IV	0/23 (0)	28/84 (33.3)	
Lymph node metastasis			
Negative	13/23 (56.5)	34/84 (40.5)	0.023
Positive	10/23 (43.5)	50/84 (59.5)	
Neural invasion			
Negative	10/23 (43.5)	29/84 (34.5)	0.247
Positive	13/23 (56.5)	55/84 (65.5)	
Distant metastasis			
Negative	23/23 (100)	56/84 (66.7)	< 0.001
Positive	0/23 (0)	28/84 (33.3)	

CTC: Circulating tumor cell; IQR: Interquartile range.

prognostic impact on patient survival[28]. In 2013, CTC detection rates and prognostic value were studied in a prospective cohort of locally advanced pancreatic carcinoma patients. This study used the Cellsearch technique to detect CTCs and demonstrated that CTC positivity was associated with poor tumor differentiation and shorter OS[19]. Another study used the microfluidic Nano Velcro assay and identified CTCs in 78% of 126 PDAC patients. The most significant finding of their study was the predictive ability of CTCs for occult metastatic disease in the preoperative setting[29]. Additionally, a recent study also detected CTCs through immunofluorescence staining for CK 19 or EpCAM in portal vein blood from PDAC patients. Their results demonstrated that CTC counts in portal vein blood were highly associated with intrahepatic metastases and poorer prognosis[30]. In our study, a novel technology applying a combination of epithelial and mesenchymal markers was used to isolate CTCs in peripheral blood from 107 patients with PDAC. We detected CTCs in 84 out of 107 (78.5%) patients. Our results demonstrated that positive CTC status was significantly correlated with lymph node metastasis, distant metastasis, and late TNM stage. Meanwhile, CTCs were associated with a significantly higher risk of death independent of other clinical risk factors.

Although millions of disseminated tumor cells are shed from primary lesions into the peripheral blood during metastasis, they very rarely survive to form new lesions[31]. A recent study demonstrated that CTC status is influenced both by the type of primary tumor and by the number of immune cells in the bloodstream[32]. To study this further, we evaluated the correlation between CTCs and immune cell counts. The inflammatory response is correlated with the progression of various tumors, including lung, colorectal, and pancreatic cancers[33-36]. Chronic pancreatitis is one of the risk factors for pancreatic cancer[33-36]. Some studies demonstrated that a high NLR, which is a circulating systemic inflammation marker, is a poor prognostic factor in

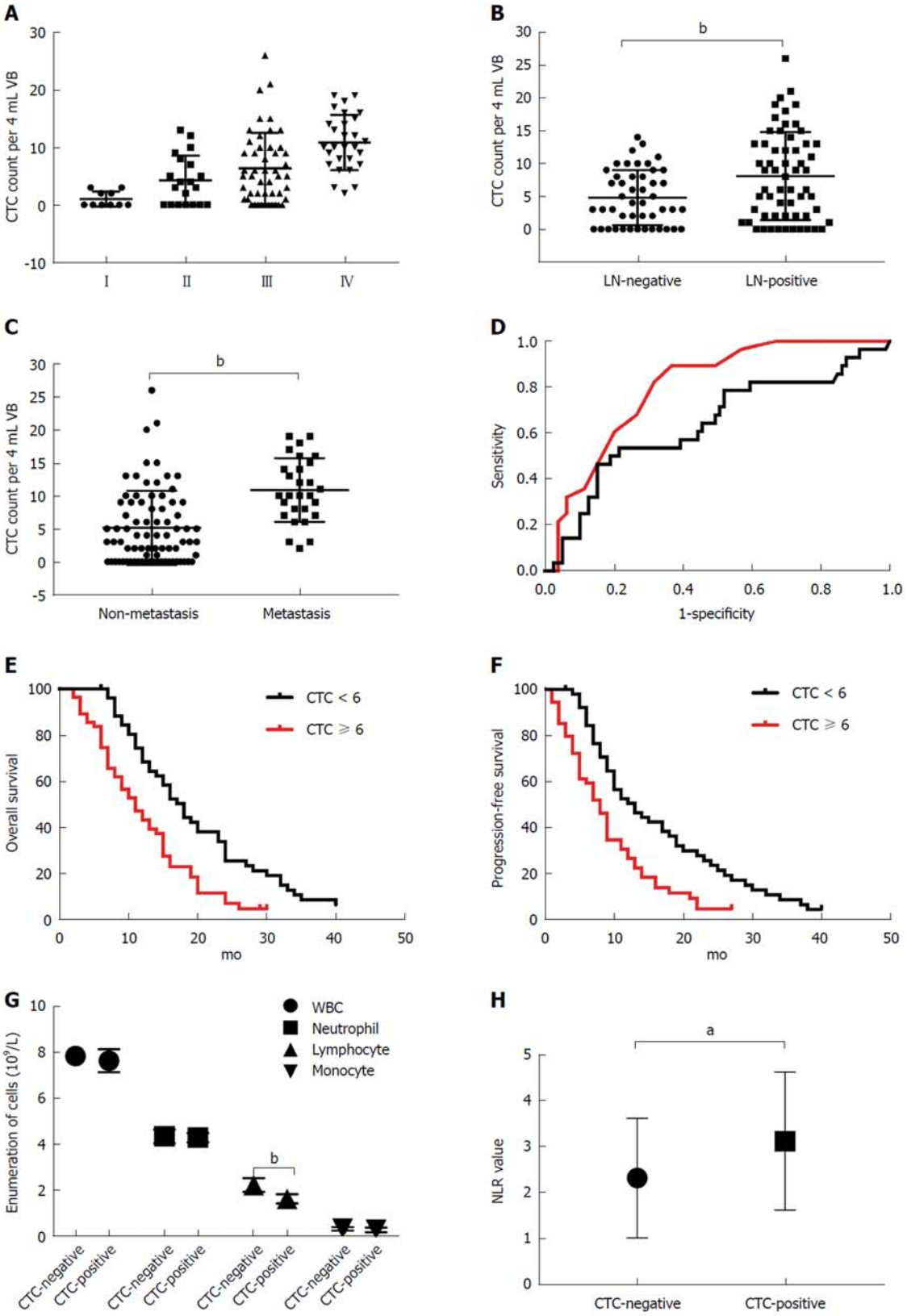

Figure 2 The presence of circulating tumor cells was correlated with a poor patient prognosis. A: Circulating tumor cell (CTC) count by AJCC stage of disease. B: CTC status in the lymph node invasion-negative group and invasion-positive group. C: CTC status in the metastasis-negative group and metastasis-positive group. D: Receiver operating characteristic curve analysis showing the performance of CTCs in predicting metastatic disease. E: Kaplan-Meier survival curves showing different overall survival in groups of pancreatic ductal adenocarcinoma (PDAC) patients with ≥ 6 or < 6 total CTCs. F: Kaplan-Meier survival curves showing different progression-free survival in groups of PDAC patients with ≥ 6 or < 6 total CTCs. G: White blood cell, neutrophil, lymphocyte, and monocyte counts in CTC-positive and CTC-negative patients. H: Neutrophil-to-lymphocyte ratio in CTC-positive and CTC-negative patients. $^{a}P < 0.05$; $^{b}P < 0.01$. CTC: Circulating tumor cells.

PDAC[37]. Our results demonstrated that lymphocyte count was reduced while NLR was significantly increased in the CTC-positive group compared with the negative

Table 3 Univariate and multivariate Cox regression analyses of prognostic factors for overall survival in pancreatic cancer

Predictor	Univariate analysis			Multivariate analysis		
	HR	95%CI	*P*-value	HR	95%CI	*P*-value
Age, < 60 *vs* ≥ 60	0.818	0.798-1.017	0.792			
Gender (female *vs* male)	0.928	0.781-1.130	0.952			
Differentiation (well *vs* moderate *vs* poor)	1.045	0.873-1.186	0.134			
Ethnic group (Han/Zhuang/Li)	0.917	0.834-1.048	0.866			
TNM stage (I/II *vs* III/IV)	1.452	1.215-1.475	< 0.001	1.148	1.031-1.268	0.011
Lymph node metastasis (negative *vs* positive)	1.287	1.048-1.364	0.007	1.198	1.036-1.380	0.017
Neural invasion (negative *vs* positive)	1.008	0.781-1.155	0.949			
Distant metastasis (negative *vs* positive)	1.426	1.236-1.710	< 0.001	1.521	1.256-1.887	< 0.001
CTC, < 6 *vs* ≥ 6	1.849	1.717-2.238	< 0.001	1.851	1.637-2.173	< 0.001

CTC: Circulating tumor cells.

group. Furthermore, there was a negative correlation between CTCs and lymphocytes and a positive correlation between CTCs and NLR. These results indicated that lymphocytes may have an important role in the clearance of CTCs.

In previous studies, CTCs were detected by examining the expression of epithelial-specific markers, such as EpCAM and CK. Thus far, the CellSearch system is the only Food and Drug Administration-approved CTC enumeration assay, which defines a CTC according to its size, positivity for EpCAM and CK, and negative CD45 expression[38]. However, multiple studies have reported that phenotypic alterations, such as the upregulation of mesenchymal markers and the loss of epithelial markers, are common in CTCs in general, suggesting that methods that rely on the expression of epithelial markers most likely overlook CTCs undergoing EMT[39,40]. Several studies exploring the EMT phenomenon of CTCs have showed that mesenchymal CTCs were associated with tumor progression and therapy resistance in some cancer types[41-43] . However, to date, there is no research describing the EMT phenomenon in CTCs in PDAC. In the present study, we detected and characterized CTCs from patients with PDAC using the cell size- and phenotype-based CanPatrol technique, which has been reported to detect CTCs with high efficiency. We identified CTCs with different EMT phenotypes in 84 patients. Furthermore, it was discovered that M-CTCs were most common in patients with advanced cancer. However, circulating tumor microemboli were not detected in the present study. Our results support the notion of a role for EMT in tumor metastasis and indicate that the role of M-CTCs might be more essential than the other subpopulations in terms of the risk of disease progression. Moreover, we also observed that M-CTCs were negatively correlated with lymphocyte counts and that patients who were positive for M-CTC had significantly lower circulating lymphocyte counts than the CTC-negative group or the group lacking M-CTCs.

There is no doubt that liquid biopsy is superior to conventional methods for dynamically monitoring cancer status, and the detection of CTCs is likely to gain popularity in the clinic. To our knowledge, here we report for the first time the prognostic utility of CTCs, as detected by the CanPatrol CTC enrichment technique, in patients with PDAC. Our data support the potential clinical value of PDAC CTCs. Both total CTC number and CTC EMT phenotype may act as potential biomarkers for PDAC prognosis. However, our study suffered from a small sample size, and the results should be interpreted with caution. Large well-designed clinical trials are required to elucidate the potential value of EMT markers in CTCs.

Table 4 Correlation between total circulating tumor cell status and clinicopathological factors of patients with pancreatic cancer

		Epithelia CTC (%)			Hybrid CTC (%)			Mesenchymal CTC (%)		
		Positive	Negative	*P*-value	Positive	Negative	*P*-value	Positive	Negative	*P*-value
Age (yr)	< 60	24/65	16/42	> 0.9	12/39	28/68	0.18	21/49	19/58	0.19
		(36.9)	(38.1)		(30.8)	(41.2)		(42.9)	(32.8)	
	≥ 60	41/65	26/42		27/39	40/68		28/49	39/58	
		(63.1)	(61.9)		(69.2)	(58.8)		(57.1)	(67.2)	
Gender	Male	37/65	26/42	0.56	24/39	39/68	0.56	28/49	35/58	0.77
		(56.9)	(61.9)		(61.5)	(57.4)		(57.1)	(60.3)	
	Female	28/65	16/42		15/39	29/68		21/49	23/58	
		(43.1)	(38.1)		(38.5)	(42.6)		(42.9)	(39.7)	
Differentiation	Well	6/65	4/42	0.21	4/39	6/68	0.97	5/49	5/58	0.84
		(9.2)	(9.5)		(10.3)	(8.8)		(8.1)	(9.3)	
	Moderate	38/65	29/42		24/39	43/68		31/49	36/58	
		(58.5)	(69.1)		(61.5)	(63.2)		(64.9)	(60.5)	
	Poor	21/65	9/42		11/39	19/68		13/49	17/58	
		(32.3)	(21.4)		(28.2)	(28)		(27)	(30.2)	
TNM stage	I	4/65	6/42	0.29	3/39	7/68	0.59	1/49	9/58	< 0.01
		(6.1)	(14.3)		(7.7)	(10.3)		(2)	(15.5)	
	II	13/65	7/42		9/39	11/68		5/49	15/58	
		(20)	(16.7)		(23.1)	(16.2)		(10.2)	(25.9)	
	III	30/65	19/42		17/39	32/68		22/49	27/58	
		(46.2)	(45.2)		(43.6)	(47.1)		(44.9)	(46.6)	
	IV	18/65	10/42		10/39	18/68		21/49	7/58	
		(27.7)	(23.8)		(25.6)	(26.4)		(42.9)	(12)	
Lymph node metastasis	Negative	25/65	22/42	0.08	18/39	29/68	0.78	24/49(49.0)	23/58	0.26
		(38.5)	(52.4)		(46.2)	(42.6)			(39.7)	
	Positive	40/65	20/42		21/39	39/68		25/49	35/58	
		(61.5)	(47.6)		(53.8)	(57.4)		(51)	(60.3)	
Neural invasion	Negative	22/65	17/42	0.38	18/39	21/68	0.78	17/49	22/58	0.77
		(33.8)	(40.5)		(46.2)	(42.6)		(34.7)	(37.9)	
	Positive	43/65	25/42		21/39	39/68		32/49	36/58	
		(66.2)	(59.5)		(53.8)	(57.4)		(65.3)	(62.1)	
Distant metastasis	Negative	46/65	33/42	0.25	27/39	52/68	0.26	25/49	54/58	< 0.01
		(70.8)	(78.6)		(69.2)	(76.5)		(51)	(93.1)	
	Positive	19/65	9/42		12/39	16/68		24/49	4/58	
		(29.2)	(21.4)		(30.8)	(23.5)		(49)	(6.9)	
Ethnic group	Li ethnic group	8/65	5/42	0.98	5/39	8/68	0.52	5/49	8/58	0.24
		(12.3)	(11.9)		(12.8)	(11.8)		(10.2)	(13.8)	
	Han ethnic group	44/65	29/42		25/39	48/68		32/49	41/58	
		(67.7)	(69.1)		(64.1)	(70.6)		(65.3)	(70.7)	
	Zhuang ethnic group	13/65	8/42		9/39	12/68		12/49	9/58	
		(20)	(19)		(23.1)	(17.6)		(24.5)	(15.5)	

CTC: Circulating tumor cells.

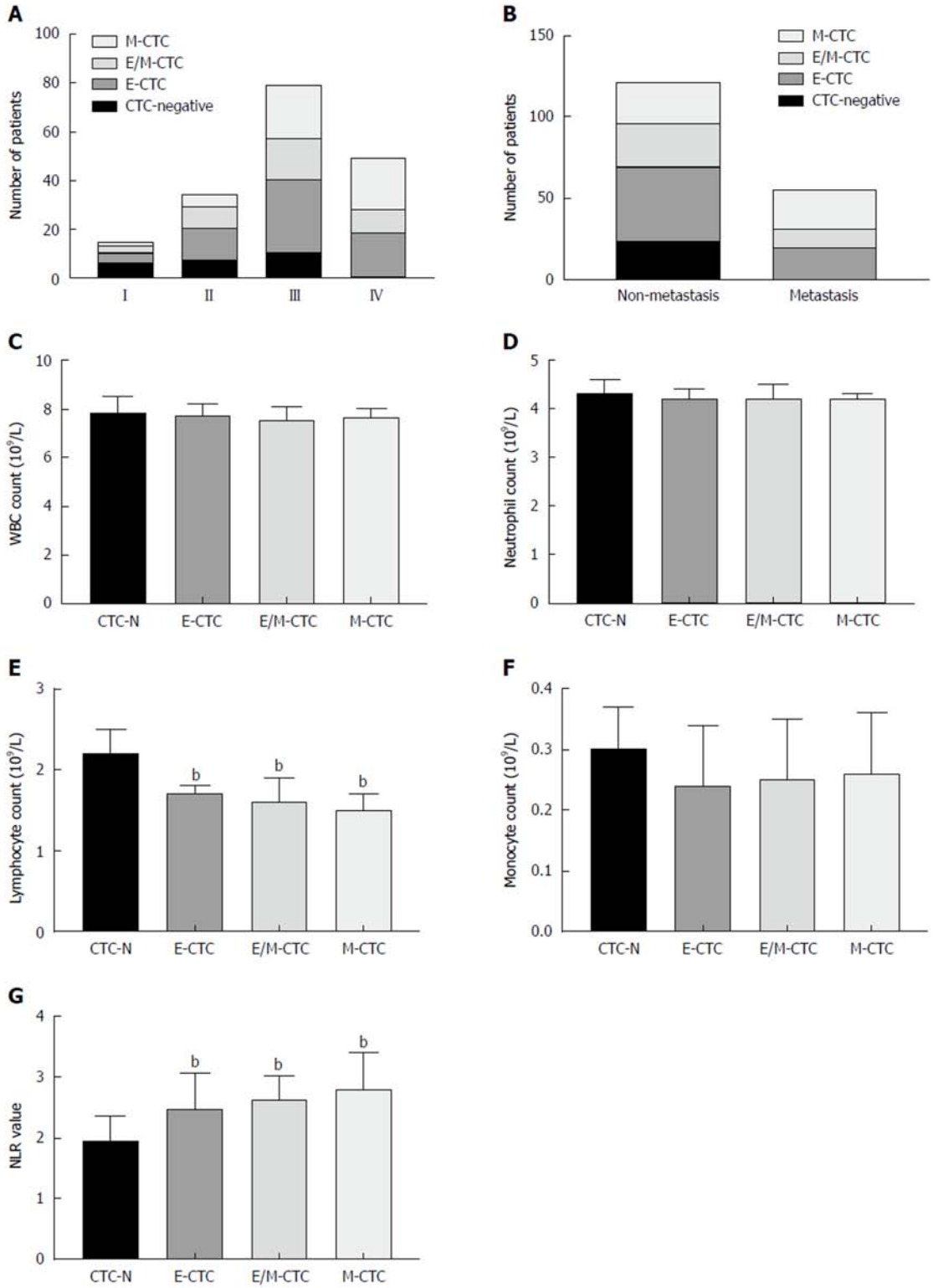

Figure 3 Correlation between circulating tumor cell-epithelial-to-mesenchymal transition subpopulations and clinicopathological characteristics. A: Distribution of circulating tumor cell (CTC) subpopulations in patients at different AJCC stages of pancreatic ductal adenocarcinoma (PDAC). B: Distribution of CTC subpopulations in patients with or without metastatic disease. C: White blood cell counts in patients without CTCs and positive for epithelial (E)-CTCs, mesenchymal (M)-CTCs, or E/M-CTCs. D: Neutrophil counts in patients without CTCs and positive for E-CTCs, M-CTCs, or E/M-CTCs. E: Lymphocyte counts in patients without CTCs and positive for E-CTCs, M-CTCs, or E/M-CTCs. F: Monocyte counts in patients without CTCs and positive for E-CTCs, M-CTCs, or E/M-CTCs. G: Neutrophil-to-lymphocyte ratio in patients without CTCs and positive for E-CTCs, M-CTCs, or E/M-CTCs. $^{b}P < 0.01$. CTC: Circulating tumor cells; E-CTC: Epithelial circulating tumor cells; M-CTC: Mesenchymal circulating tumor cells.

ARTICLE HIGHLIGHTS

Research background

Circulating tumor cells (CTCs) have been demonstrated to be a prognostic indicator in numerous cancers. However, in pancreatic ductal adenocarcinoma (PDAC), CTCs remain to be studied. Here, we report for the first time the prognostic utility of CTCs, as detected by CanPatrol CTC enrichment technique, in patients with PDAC. Our data support the potential clinical value of PDAC CTCs. Both total CTC number and CTC epithelial-to-mesenchymal transition (EMT) phenotype may act as potential biomarkers for PDAC prognosis.

Research motivation

In the present study, we explored the relationships between clinicopathological parameters and the relative abundance of three circulating EMT-CTC subpopulations. We found that CTC status correlated with lymph node metastasis, TNM stage, distant metastasis, blood lymphocyte counts, and the neutrophil-to-lymphocyte ratio (NLR). Kaplan-Meier survival analysis showed that patients with ≥ 6 total CTCs had significantly decreased OS and PFS compared to patients with < 6 total CTCs. The presence of M-CTCs was positively correlated with TNM stage and distant metastasis. Additionally, lymphocyte counts and NLR in patients without CTCs were significantly different from those in patients testing positive for each CTC subpopulation. Our data support the potential clinical value of PDAC CTCs. Furthermore, our data also provide support for further large well-designed clinical trials to explore CTC counts as indicators of PDAC progression.

Research objectives

The objective of this research was to explore the relationships between clinicopathological parameters and the relative abundance of the three circulating EMT-CTC subpopulations in PDAC. This research demonstrated that positive CTC status was significantly correlated with lymph node metastasis, distant metastasis, late TNM stage, and poor patient prognosis. Meanwhile, M-CTCs were most common in patients with advanced cancer. These results demonstrated that classifying CTCs by EMT markers helps to identify the more aggressive CTC subpopulations and provides useful evidence for determining a suitable clinical approach.

Research Methods

This research utilized the cell size- and phenotype-based CanPatrol CTC filtration system to isolate CTCs. CTC subpopulations were identified using a multiplex RNA-ISH assay. Four epithelial biomarkers (epithelial cell adhesion molecule and cytokeratin 8/18/19), two mesenchymal biomarkers (vimentin and twist), and a leukocyte biomarker, CD45, were applied to capture and characterize the CTCs.

Research results

This research indicated that the presence of CTCs was significantly associated with PDAC poor prognosis. Moreover, M-CTCs were most common in patients with advanced cancer. These results demonstrate that CTCs are promising biomarker for PDAC prognosis and identification of EMT markers in CTCs provide more information on tumor progression.

Research conclusions

In the present study, a novel technology called CanPatrol CTC filtration system applying a combination of epithelial and mesenchymal markers was used to detect CTCs in peripheral blood from 107 patients with PDAC. We found that CTC positivity was correlated with clinicopathologic variables and outcomes. Meanwhile, the presence M-CTCs was associated with advanced stage and distant metastasis. These results demonstrate that CTC enumeration and classification show promise as a prognostic biomarker and may provide useful evidence for determining a suitable clinical approach.

Research perspectives

This research supports the potential clinical value of PDAC CTCs. Both total CTC number and CTC EMT phenotype may act as potential biomarkers for PDAC prognosis. However, our study suffered from a small sample size, and the results should be interpreted with caution. Large well-designed clinical trials are required to elucidate the potential value of EMT markers in CTCs.

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