

# World Journal of *Gastroenterology*

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## Contents

Weekly Volume 17 Number 13 April 7, 2011

### EDITORIAL

- 1655 Natural orifice transluminal endoscopic surgery: Progress in humans since white paper  
*Santos BF, Hungness ES*
- 1666 Isolated lymphoid follicles in colon: Switch points between inflammation and colorectal cancer?  
*Sipos F, Múzes G*

### TOPIC HIGHLIGHT

- 1674 Patterns of local recurrence in rectal cancer after a multidisciplinary approach  
*Enríquez-Navascués JM, Borda N, Lizerazu A, Placer C, Elosegui JL, Ciria JP, Lacasta A, Bujanda L*

### GUIDELINES FOR CLINICAL PRACTICE

- 1685 Therapeutic options for intermediate-advanced hepatocellular carcinoma  
*Zhang ZM, Guo JX, Zhang ZC, Jiang N, Zhang ZY, Pan LJ*

### REVIEW

- 1690 How to assess the severity of atrophic gastritis  
*Dai YC, Tang ZP, Zhang YL*

### ORIGINAL ARTICLE

- 1694 Legalon-SIL downregulates HCV core and NS5A in human hepatocytes expressing full-length HCV  
*Mehrab-Mohseni M, Sendi H, Steuerwald N, Ghosh S, Schrum LW, Bonkovsky HL*
- 1701 Endoscopic submucosal dissection for premalignant lesions and noninvasive early gastrointestinal cancers  
*Hulagu S, Senturk O, Aygun C, Kocaman O, Celebi A, Konduk T, Koc D, Sirin G, Korkmaz U, Duman AE, Bozkurt N, Dindar G, Attila T, Gurbuz Y, Tarcin O, Kalayci C*
- 1710 Discovery and validation of prognostic markers in gastric cancer by genome-wide expression profiling  
*Zhang YZ, Zhang LH, Gao Y, Li CH, Jia SQ, Liu N, Cheng F, Niu DY, Cho WCS, Ji JF, Zeng CQ*
- 1718 Differential expression of Bcl-2 and Bax during gastric ischemia-reperfusion of rats  
*Qiao WL, Wang GM, Shi Y, Wu JX, Qi YJ, Zhang JF, Sun H, Yan CD*

## BRIEF ARTICLE

- 1725 Intrahepatic natural killer T cell populations are increased in human hepatic steatosis  
*Adler M, Taylor S, Okebugwu K, Yee H, Fielding C, Fielding G, Poles M*
- 1732 Gastrotomy closure with a new tissue anchoring device: A porcine survival study  
*Guarner-Argente C, Córdova H, Martínez-Pallí G, Navarro-Ripoll R, Rodríguez-d'Jesús A, Rodríguez de Miguel C, Beltrán M, Fernández-Esparrach G*
- 1739 MR-arteriportography: A new technical approach for detection of liver lesions  
*Rennert J, Jung EM, Schreyer AG, Hoffstetter P, Heiss P, Feuerbach S, Zorger N*
- 1746 Carbachol promotes gastrointestinal function during oral resuscitation of burn shock  
*Hu S, Che JW, Tian YJ, Sheng ZY*
- 1753 Gastroesophageal reflux in cirrhotic patients without esophageal varices  
*Zhang J, Cui PL, Lv D, Yao SW, Xu YQ, Yang ZX*
- 1759 Association between polymorphism rs6983267 and gastric cancer risk in Chinese population  
*Guo Y, Fang J, Liu Y, Sheng HH, Zhang XY, Chai HN, Jin W, Zhang KH, Yang CQ, Gao HJ*
- 1766 EUS for choosing best endoscopic treatment of mesenchymal tumors of upper gastrointestinal tract  
*Zhou XX, Ji F, Xu L, Li L, Chen YP, Lu JJ, Wang CW, Huang W*
- 1772  $\beta$ -catenin accumulation in nuclei of hepatocellular carcinoma cells up-regulates glutathione-s-transferase M3 mRNA  
*Li YS, Liu M, Nakata Y, Tang HB*
- 1779 Nutrition support in surgical patients with colorectal cancer  
*Chen Y, Liu BL, Shang B, Chen AS, Liu SQ, Sun W, Yin HZ, Yin JQ, Su Q*

## CASE REPORT

- 1787 Application of a wire-guided side-viewing duodenoscope in total esophagectomy with colonic interposition  
*Yi CY, Chou JW, Peng YC, Chow WK*

## Contents

*World Journal of Gastroenterology*  
Volume 17 Number 13 April 7, 2011

**ACKNOWLEDGMENTS** I Acknowledgments to reviewers of *World Journal of Gastroenterology*

**APPENDIX** I Meetings  
I-VI Instructions to authors

**ABOUT COVER** Santos BF, Hungness ES. Natural orifice transluminal endoscopic surgery: Progress in humans since white paper.  
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## Natural orifice transluminal endoscopic surgery: Progress in humans since white paper

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### Abstract

Since the first description of the concept of natural orifice transluminal endoscopic surgery (NOTES), a substantial number of clinical NOTES reports have appeared in the literature. This editorial reviews the available human data addressing research questions originally proposed by the white paper, including determining the optimal method of access for NOTES, developing safe methods of luminal closure, suturing and anastomotic devices, advanced multitasking platforms, addressing the risk of infection, managing complications, addressing challenges with visualization, and training for NOTES procedures. An analysis of the literature reveals that so far transvaginal access and closure appear to be the most feasible techniques for NOTES, with a limited, but growing transgastric, transrectal, and transesophageal NOTES experience in humans. The theoretically increased risk of infection as a result of NOTES procedures has not been substantiated in transvaginal and transgastric procedures so far. Development of suturing and anastomotic devices and advanced platforms for NOTES has progressed slowly, with limited clinical data on their use so far. Data on

the optimal management and incidence of intraoperative complications remain sparse, although possible factors contributing to complications are discussed. Finally, this editorial discusses the likely direction of future NOTES development and its possible role in clinical practice.

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**Key words:** Natural orifice transluminal endoscopic surgery; Outcomes; Complications; Endoscopic; Surgery

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### INTRODUCTION

The concept of natural orifice transluminal endoscopic surgery (NOTES<sup>®</sup>) has generated intense interest in the surgical and gastroenterology communities. Accessing the peritoneal or thoracic spaces through internal, transvisceral incisions instead of transabdominal incisions has the potential benefits of decreasing postoperative pain, wound complications, improving cosmesis, decreasing the physiologic and immune response to surgery, decreasing



anesthesia requirements, accelerating patient recovery and return to normal function, and improving access to organs that are currently difficult to reach with conventional open or laparoscopic approaches (e.g. esophagus, rectum). Given the intense interest in NOTES and its potential to revolutionize current surgical therapy, several working groups throughout the world have been formed to help guide NOTES research and clinical development. These groups include EURO-NOTES, EATS (European Association for Transluminal Surgery™), D-NOTES, ASIA-NOTES, NOSLA (Natural Orifice Surgery Latin America), Japan-NOTES, India NOTES, NOTES Research Group Brazil, and NOSCART, which published a white paper in 2006 outlining the perceived barriers to the clinical adoption of NOTES<sup>[1]</sup>. These barriers included determining the optimal orifice to access the peritoneal cavity, developing a reliable means to close a viscotomy, minimizing the risk of infection as a result of access through a non-sterile orifice, developing an endoscopic suturing device, addressing difficulties with spatial orientation inherent to a NOTES technique, developing multi-tasking platforms to perform NOTES procedures, managing intraoperative complications, and developing NOTES training to allow safe, widespread adoption of the techniques. Although there have been numerous studies addressing some of these questions in animal and cadaver models, reports of clinical NOTES procedures in humans, and human data addressing these questions have only started to appear since 2007. This editorial will discuss the progress made on these questions by reviewing the currently available human outcomes data and clinical NOTES publications in the literature.

## ACCESS TO THE PERITONEAL CAVITY

A comprehensive review of the human NOTES literature was conducted using PubMed to search the MEDLINE database with the search terms of “human natural orifice surgery, human transvaginal, human transrectal, human transgastric, or human NOTES surgery,” for articles published between January 1, 2004 and September 1, 2010. Manuscripts describing clinical human NOTES procedures include the use of transgastric, transvaginal, transrectal, and transesophageal approaches. Currently, the most frequently used orifice for NOTES is the vagina, with cholecystectomy accounting for the highest number of cases in the published literature<sup>[2]</sup>. Transvaginal access has the longest history of use for intraperitoneal procedures, prior to the recent description of NOTES. In 1949, Bueno described a series of transvaginal appendectomies performed with open instruments (without an endoscope) at the time of hysterectomy<sup>[3]</sup>. Since then, transvaginal access for intraperitoneal procedures in the form of culdoscopy has developed as an accepted, safe procedure in the gynecology community<sup>[4-7]</sup>. Transvaginal access can be established using a posterior colpotomy created under direct vision with open instruments, or with the use of

direct trocar insertion under laparoscopic guidance. Establishment of transvaginal access does not require the use of a flexible endoscope or transanal endoscopic microsurgery (TEM) platform, unlike transgastric, transrectal, and transesophageal approaches that have been described to date. Likewise, closure of transvaginal access sites is performed with direct suturing using open instruments.

While transvaginal access is the most frequently used NOTES approach to date and can be safely performed, the potential for complications should not be overlooked. The close proximity of the rectum posteriorly, the ureters laterally, and the tendency for the small intestine to occupy the pelvis should be kept in mind while performing transvaginal NOTES. Reported complications of NOTES transvaginal access include rectal and colonic injuries, small bowel injuries, ureterovaginal fistula formation, vulvar lacerations, and bladder injuries<sup>[8-14]</sup>. Given the possibility of these complications, assistance from a gynecologist experienced in transvaginal access should be considered, at least initially, in the performance of transvaginal NOTES. In addition, simultaneous visualization of colpotomy creation with a transumbilical laparoscope, along with the use of a uterine manipulator to anteriorly retract the uterus may minimize the likelihood of rectal, bladder, or bowel injuries during the creation of transvaginal access. Most cases reported so far have utilized a “hybrid” NOTES approach, with at least one laparoscopic port used for initial visualization, retraction, and assistance with the dissection. Until instruments for NOTES improve, a “hybrid” NOTES approach may be preferable to a “pure” NOTES approach (without any percutaneous or laparoscopic assistance) in order to increase the safety of the procedures.

Transgastric access is the second-most frequently reported access route after transvaginal access in the literature. Experience with transgastric NOTES includes at least 70 transgastric peritoneoscopy procedures reported by Nau *et al.*<sup>[15,16]</sup> and Nikfarjam *et al.*<sup>[17]</sup>, as well as several series which have reported at least 42 cholecystectomies, 15 appendectomies, PEG rescue, and 6 cases of transgastric, stapled cystogastrostomy<sup>[11,15,16,18-24]</sup>. Transgastric access in all of these cases was obtained in the anterior stomach (antrum or body) using needle knife cautery and balloon dilation through a flexible endoscope, except in cases of PEG-rescue and cystogastrostomy. Most cases were performed with placement of a laparoscopic port prior to gastrotomy creation to allow laparoscopic guidance and insufflation, while some were performed without any previous laparoscopic ports or insufflation. It is interesting to note that although no bowel injuries were recorded in transgastric peritoneoscopy cases performed without prior laparoscopic port placement, the authors noted there were instances of cautery burns to the anterior peritoneum or the under surface of the liver that were discovered after subsequent abdominal inspection with a laparoscope<sup>[15]</sup>. As such, it is not surprising that the majority of transgastric cases have been performed in a hybrid fashion, with laparoscopic visualization of

the access point in order to prevent injuries to surrounding organs or the gastropiploic vessels, which may be difficult or impossible to see from inside the stomach<sup>[2]</sup>.

Transesophageal access has been used to perform esophageal myotomies in a series of 17 patients with achalasia, reported by Inoue *et al.*<sup>[25]</sup>. This procedure, termed Per-Oral Esophageal Myotomy (POEM), incises the inner circular muscle layer of the distal esophagus and lower esophageal sphincter, while completely avoiding the hiatal dissection and disruption of the phrenoesophageal ligament that occurs during laparoscopic Heller myotomy. Transesophageal access begins at the anterior, mid-esophagus with the creation of a submucosal bleb using a sclerotherapy needle. The submucosal bleb is then incised using electrocautery and the endoscope is advanced through the incision to create a submucosal tunnel distally past the gastro-esophageal (GE) junction onto the cardia of the stomach. The inner circular muscle distal to the mucosal incision is then incised. Currently transesophageal access has been used to perform only procedures on the esophageal wall. In one case, full separation of the outer longitudinal esophageal muscle layer occurred, exposing the mediastinum. Per the authors, however, this patient did not have any adverse consequences as a result; this suggests that as long as closure of the proximal mucosal incision is ensured, transesophageal mediastinal or thoracic access through a submucosal tunnel may be clinically feasible in the future. However, no clinical studies have been performed to date investigating the safety of transesophageal mediastinal or thoracic access.

In contrast to other forms of NOTES access, transrectal access has been the least reported in the literature. The only two published cases are of a proctosigmoidectomy for cancer<sup>[26]</sup>, and a transanal pull-through for Hirschsprung's disease<sup>[27]</sup>. The proctosigmoidectomy was performed using a TEM platform, with a circumferential rectal dissection proceeding cephalad from the distal rectum, assisted by laparoscopy. The transanal pull-through was performed in an infant, without the use of the TEM; instead the authors reported using trocars inserted directly through the rectal wall to allow passage of a rigid laparoscope and rigid instruments. Although no complications were reported to have occurred in either case, further data is needed in order to accurately determine the risks of this approach.

Although so far various access points for a variety of NOTES procedures have been attempted, the specific indications that are best suited for each orifice will need to be defined. For example, the ideal indications for transoral access may end up being limited to therapeutic esophageal or gastric procedures, or diagnostic procedures in the intraperitoneal cavity. Transoral access may be poorly suited to advanced therapeutic intraperitoneal procedures given the requirement for complex, flexible instrumentation, as well as the small native diameter of the esophagus which makes extraction of large, bulky specimens potentially hazardous. Similarly, transrectal access may be best suited

to colorectal applications, and transvaginal access may end up being ideally suited for gynecologic indications. However, if these two approaches prove to be the most forgiving in terms of ease of access, ability to reach the upper abdomen, complications, and the ability to introduce both flexible and rigid instruments through the orifice, it is possible that these approaches may become "workhorse" approaches for intraperitoneal NOTES procedures or specimen removal in female and male patients, respectively.

## VISCERAL CLOSURE

Transvaginal closure is currently the most feasible closure method for NOTES, as the incision is closed by direct suturing. Aside from potential injuries to surrounding structures as previously mentioned, there have been no reports of vaginal dehiscence or herniation through the vaginal incision. Also, the consequences of a vaginal wound dehiscence would likely not be as potentially dangerous as a gastric leak or a rectal leak, which would introduce highly caustic or infectious luminal contents into the abdomen.

In contrast to transvaginal closure, transgastric closure currently requires the use of flexible endoscopic clips or tissue anchors, with or without laparoscopic sutures to buttress the closure. Although several groups have reported successful performance of transgastric closures without leaks, data on the true safety of current transgastric closure techniques are sparse at best. In 2010, Zorron *et al.*<sup>[14]</sup> reported results from a prospective, multi-center NOTES registry, including data from 43 transgastric operations (29 cholecystectomies and 14 appendectomies), in which the stomach was closed using laparoscopic suturing. No gastric leaks were reported in this study. Similarly, reports of transgastric closure by other groups using endoscopic clips or anchors, with or without laparoscopic sutures, accounting for a total of approximately 30 patients, did not include any postoperative gastric leaks<sup>[18-21,23,24]</sup>. However, there has been at least one reported complication of gastric closure: a pneumothorax which occurred due to the aberrant placement of a tissue anchor through the diaphragm<sup>[24]</sup>. Innovative solutions for transgastric closure that have been reported in humans include the creation of a gastric valve mechanism made with tissue anchors, through which a gastrotomy is created<sup>[21]</sup>. The gastrotomy is then closed with additional tissue anchors once the procedure is finished. Although this technique has been successfully used in 5 patients so far, the majority of transgastric cases reported in the literature continue to rely on laparoscopic suturing alone or in combination with endoscopic instruments. Completely endoscopic means for closing gastrotomies will need to be developed and evaluated in human studies for transgastric NOTES to become feasible without laparoscopic assistance. Numerous prototype closure devices and techniques have been developed and tested in pre-clinical models. However, a detailed discussion of these devices and their results in animals are beyond the scope of this editorial.

Transesophageal NOTES closure has so far been reported using endoscopic clips to close the longitudinal mucosal incision at the entrance to the submucosal tunnel during POEM. No esophageal leaks or mediastinitis were reported in a series of 17 patients<sup>[25]</sup>. These clips slough off into the GI tract, with healing of the mucosal incision demonstrated on follow-up endoscopy.

Closure of transrectal NOTES access has so far been accomplished by incorporating the rectotomy into a hand-sewn coloanal anastomosis. This technique increases the safety of transrectal NOTES since it uses currently accepted anastomotic techniques, but it is limited to resections of the left colon and rectum. The safety of transrectal closures left in situ (not incorporated into the anastomosis) remains to be determined, although there is evidence from the TEM literature suggesting that intraperitoneal rectal closures can be performed as safely as those without peritoneal entry during full-thickness rectal tumor excision<sup>[28]</sup>. Research to test closure techniques for transrectal surgery will ultimately need to be performed on human tissue rather than porcine models for it to be useful. However, initial closure tests should be attempted on tissues that are already targeted for removal, such as in portions of the colon that will be removed following colectomy, to ensure patient safety.

## RISK OF INFECTION

Concerns about potentially higher rates of infection have repeatedly been raised in regards to NOTES. The notion of introducing surgical instruments through non-sterile orifices into the normally sterile peritoneal cavity runs counter to years of established surgical dogma. Many groups performing clinical NOTES have adopted the routine use of preoperative intravenous (IV) antibiotics combined with local application of antibiotic or antiseptic solutions such as povidone-iodine at the site of visceral entry as a precaution. Although the data are currently limited, concerns about increased infectious risk with a transvaginal approach compared to conventional laparoscopy have not been substantiated. The best data so far are from a large, prospective NOTES registry including 488 patients that underwent transvaginal cholecystectomy<sup>[13]</sup>. Complications reported in this registry included urinary tract infection, abscess in the pouch of Douglas, wound infection, vaginal mycosis, and bacterial vaginitis, with a combined incidence of 1%, which is comparable to the rate of infectious complications seen with conventional laparoscopic cholecystectomy<sup>[29]</sup>.

Bacterial contamination has been quantified during performance of laparoscopic roux-en-y gastric bypass (LRYGB) as a surrogate for NOTES, and during actual transgastric NOTES peritoneoscopy by investigators at Ohio State University<sup>[30,31]</sup>. These authors measured contamination in 50 patients undergoing LRYGB given preoperative IV Cefazolin alone without additional luminal decontamination, and showed that native levels of bacte-

ria in the stomach were higher (mean 22 303 CFU/mL) compared to that of the peritoneum after the operation (1102 CFU/mL), with significant correlation between these levels. These results indicate that some cross-contamination occurs during transgastric peritoneoscopy, but that the degree of contamination is not dependent on the pre-existing level of bacteria in the stomach. In addition, despite the documented levels of contamination, no clinically obvious infections were found with a minimum of 30 d of follow-up for all patients. A follow-up study in patients undergoing transgastric NOTES peritoneoscopy prior to a planned pancreaticoduodenectomy also showed minimal cross-contamination with insignificant levels of intra-operative peritoneal contamination (160 CFU/mL), and no infectious complications in a group of 10 patients with 30 d follow-up. An observation requiring further investigation, though, was that patients on proton-pump inhibitors (PPIs) had significantly higher levels of bacteria in the stomach (median 33 000 CFU/mL) compared to those not on PPIs (median 0 CFU/mL). Differences in post-operative peritoneal contamination between patients with or without PPI use preoperatively approached, but did not reach, statistical significance due to a limited number of patients in the study. Thus, while the risk of clinically significant infection as a result of transgastric NOTES appears to be low, the optimal perioperative management of patients on PPIs undergoing NOTES requires further study.

In contrast to transvaginal and transgastric NOTES access, transesophageal and transrectal access have a theoretically higher risk of infectious complications due to their proximity to the oropharyngeal and colonic flora, respectively. Unfortunately, to date no human studies have directly quantified the levels of bacterial contamination from either of these NOTES approaches, or the true incidence of clinically significant infections. Nevertheless, in the reported series of 17 POEM patients who received preoperative IV antibiotics and irrigation of the submucosal tunnel with dilute antibiotic solution prior to closure, no infectious complications were noted with a mean follow-up period of 5 mo (minimum 1 mo)<sup>[25]</sup>. In addition, the two transrectal NOTES procedures reported in the literature to date did not report any infectious complications. The rectosigmoid resection patient underwent preoperative mechanical bowel preparation with oral sodium phospho soda, received preoperative IV Cefoxitin, and had a dilute Betadine irrigation of the rectum<sup>[26]</sup>. The 5-d-old patient who underwent a NOTES transanal pull-through received perioperative systemic antibiotics for 24 h following the case with no reported complications. In short, more data are needed to accurately estimate the risk of infectious complications with transrectal and transesophageal NOTES approaches.

The fear of increased infectious risk from NOTES procedures has so far not been substantiated by examining available clinical outcomes and bacteriologic studies. It is likely that IV antibiotics alone for transgastric proce-



dures, along with some form of luminal disinfection for transvaginal, transrectal or transesophageal procedures will be the ultimate strategy adopted clinically.

## DEVELOPMENT OF ENDOSCOPIC SUTURING OR ANASTOMOTIC DEVICES

The development of endoscopic suturing and anastomotic devices was deemed by the white paper to be necessary in order for NOTES to ultimately be applied to the wide spectrum of current surgical therapy<sup>[1]</sup>. However, the development of these devices and their use in clinical trials has proceeded slowly since 2005. Currently, two types of endoscopic suturing devices have been approved: OverStitch™ (Apollo Endosurgery, Inc., Austin, TX, USA) and the Tissue Apposition System (TAS, Ethicon Endosurgery, Cincinnati, OH, USA). However, only use of the TAS has so far been reported clinically to approximate partial colonic wall defects at the time of laparoscopic-assisted polypectomy<sup>[32]</sup>. The TAS system works by sequentially deploying a threaded T-tag through the bowel wall on each side of a defect using an endoscopic hollow bore needle; once two threaded T-tags have been placed on either side of a defect, the two threads are cinched together and trimmed by a one-way locking mechanism in order to approximate both sides of the luminal defect. A similar endoscopic T-tag closure technique using instruments from Cook Medical (Bloomington, IN, USA) was employed by Park *et al.* to close gastrotomy defects during transgastric NOTES<sup>[24]</sup>. One of the difficulties with existing endoscopic T-tag systems, however, is the inability to directly visualize deployment of the T-tag from the extraluminal side of the defect without a laparoscope. This impaired visualization may contribute to the risk of inadvertent injury to surrounding organs, or deployment through a vessel. As mentioned previously, inadvertent deployment of a T-tag through the diaphragm during a gastric closure was reported to have resulted in a pneumothorax discovered post-operatively<sup>[24]</sup>. The OverStitch™, in contrast to T-tag based systems, employs a lateral needle-passing mechanism more similar to conventional suturing techniques. However, the OverStitch™ still requires assistance from an endoscopic grasper, and may be limited by the visual and mechanical constraints of conventional flexible endoscopes. Human use data will be needed to adequately evaluate the potential of the OverStitch™ for use in luminal closures.

The development of endoscopic anastomotic devices for NOTES has proceeded even more slowly than the development of suturing devices. The only reports of NOTES procedures with anastomoses have utilized hand-sewn coloanal anastomoses during colorectal resections, or a flexible, powered surgical stapler (SurgAssist™ SLC 55, Power Medical Interventions, Langhorne, PA, USA) during cystogastrostomy<sup>[33]</sup>. In the cystogastrostomy cases, the stapler was passed down the esophagus through an

overtube, alongside a flexible gastroscope. Although this stapler was used successfully to create a cystogastrostomy, the authors reported significant difficulty in passing the rigid part of the stapler through the esophagus, even through a previously placed overtube. In addition, the authors reported significant difficulty directing the stapler into the appropriate angle once inside the stomach. Unfortunately, since the publication of the study, the stapler has been removed from the market due to the acquisition of Power Medical, Inc. by Covidien (Mansfield, MA) and is not currently available for use. Development of flexible, articulating, low-profile staplers is needed to make creation of anastomoses or luminal closures during NOTES more feasible. Additional features which may make application of stapling technology to NOTES more feasible include the addition of visualization and steering capabilities. These features might allow staplers to be more precisely directed into difficult to reach areas and fired with more confidence.

## SPATIAL ORIENTATION

The difficulty with correct spatial orientation during NOTES and its consequences in hindering the performance of advanced procedures was foreseen in the white paper. These difficulties are inherent to the use of current flexible endoscopes to perform NOTES, and have the potential to create not only a difficult operation, but may also increase the risk of complications during NOTES. Perretta *et al.*<sup>[34]</sup> reported a case of misinterpretation of biliary anatomy during transgastric NOTES which was fortunately recognized, preventing the occurrence of a common bile duct injury. The authors in this case converted to a laparoscopic view temporarily to clarify the unclear biliary anatomy. As emphasized by the authors, current NOTES techniques may alter the usual surgical anatomy that is seen due to the difficulty in achieving adequate retraction without laparoscopic instruments, and the spatial confusion created by retroflexion when using a flexible endoscope. A solution to the problem of difficult spatial orientation during NOTES may be the use of rigid endoscopes whenever possible. However, the use of rigid endoscopes is potentially feasible only through transvaginal or transrectal approaches, or through the umbilicus in the case of transgastric surgery. Short of using a rigid endoscope routinely, surgeons performing NOTES with flexible endoscopes should have a low threshold to convert to a laparoscopic view, even temporarily, to resolve any confusion in regards to the surgical anatomy. Although image-guided systems have been described as having potential applications for NOTES, none of these systems have been applied in a clinical setting so far<sup>[35]</sup>. Future solutions to the problem of spatial orientation may also involve the use of small, wireless cameras that are able to provide a wider, overhead view of the surgical field, and can be moved to the appropriate location as needed. Use of this type of camera has been described for human single-incision laparoscopy (SIL) cases<sup>[36]</sup>. However,





**Figure 1** The Transanal Endoscopic Operations device from Karl-Storz allows the insertion of rigid or flexible instruments through the anus and is currently used for performing transanal endoscopic microsurgery excisions of rectal tumors. It also has the potential to serve as a stable transrectal natural orifice transluminal endoscopic surgery (NOTES®) platform. Image used with permission (©Karl Storz).



**Figure 2** The TransPort™ multi-channel access device from USGI has been used as a transgastric natural orifice transluminal endoscopic surgery platform. It has a steering mechanism similar to a flexible endoscope, along with multiple, large-diameter channels to accommodate a small-diameter flexible endoscope and other large caliber flexible endoscopic instruments (g-Prox® tissue anchor device is shown). Image used with permission (©USGI Medical).

it should be noted that these cameras are not currently FDA-approved.

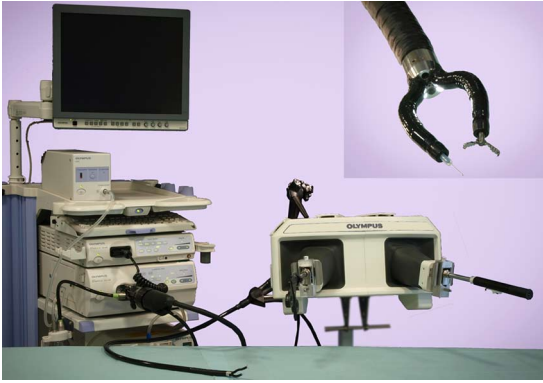
## DEVELOPMENT OF A MULTITASKING PLATFORM

The creation of a multitasking platform to allow the performance of multiple NOTES procedures with the same platform continues to be an issue of the highest priority in the development of NOTES as a viable technique. Although several types of advanced operations (nephrectomy<sup>[12,37]</sup>, partial gastrectomy<sup>[38,39]</sup>, sigmoidectomy<sup>[40,41]</sup>, and splenectomy<sup>[42]</sup>) have been reported using a NOTES technique, many of these procedures have relied heavily on laparoscopic instruments for visualization and dissection. In order for “pure” NOTES (without laparoscopic or percutaneous assistance) to become feasible, a multi-tasking platform that balances flexibility and maneuverability with



**Figure 3** The Anubis® platform from Karl-Storz is an advanced flexible natural orifice transluminal endoscopic surgery platform (in development), with a tip that opens to expose working instruments capable of multiple degrees of freedom controlled by the surgeon. Image used with permission (©Karl Storz).

the ability to provide powerful retraction and instrument mobility, as well as an intuitive interface for the surgeon will need to be developed. Examples of the novel application of multi-lumen operating platforms for NOTES include use of a TEM platform (Figure 1) to perform proctosigmoidectomy, and the use of the TransPort™ (USGI Medical, San Clemente, CA, USA) multi-channel access port for transgastric NOTES (Figure 2). Use of a TEM platform for transrectal surgery allows the simultaneous use of rigid instruments with a flexible or rigid endoscope to perform intra-abdominal surgery. This combination of operating instruments permits strong retraction, while allowing flexible visualization and dissection capabilities through the flexible endoscope. Current limitations of such a system, however, include the difficulty of reaching beyond the sacral promontory with rigid instruments, and the limitations of dissection performed with current flexible endoscopes. The TransPort™ device is a flexible, multi-channel device which allows passage of a flexible endoscope through one channel as well as additional flexible instruments through the other channels. This device is flexible enough to be passed transgastrically and has the ability to retroflex and assume a rigid configuration independent of the endoscope. While it has been used for transgastric cholecystectomy and appendectomy<sup>[19,21,23]</sup>, use of the flexible instruments through its channels is similar to the use of accessories through a conventional flexible endoscope in that the instruments have limited degrees of freedom and lack the ability to make lateral or vertical movements independent of the endoscope in an intuitive fashion. The limitations of both of these platforms make them less than ideal multi-tasking platforms for NOTES. However, the development of a system combining aspects of both platforms, along with robotic control may greatly facilitate the performance of NOTES procedures and may be the crucial enabling technology that would allow NOTES development to proceed exponentially, similar to the way the development of the charge-coupled device (CCD) camera revolutionized laparoscopy. Examples of experimental platforms with some of these



**Figure 4** EndoSamurai is a prototype, advanced platform in development by Olympus. To operate the system, a surgeon uses an intuitive, bi-manual interface to control instruments with multiple degrees of freedom (inset shows close-up of endoscope tip with working instruments). Image used with permission (©Olympus Medical Systems Corp.).

capabilities that may be seen in future clinical reports include Anubis (Karl-Storz, Tuttlingen, Germany, Figure 3), EndoSamurai (Olympus, Tokyo, Japan, Figure 4), and the Direct-Drive Endoscopic System (Boston Scientific, Natick, MA, USA, Figure 5). Economic concerns continue to be an issue with regard to the development of these platforms, however. The emergence of single-incision laparoscopy (SIL) has caused a tremendous amount of resources to be redirected away from NOTES, towards the development of SIL technology. Although some of this technology may end up being adapted for NOTES, the development of SIL will likely delay the development of NOTES-specific technology such as advanced multitasking platforms. Both industry and innovators in minimally-invasive surgery need to not lose sight of the potential promise of NOTES, while SIL occupies the spotlight.

## TRAINING

There are currently not enough data from human studies to make quantitative recommendations in regards to the ideal amount of previous endoscopic or laparoscopic clinical or laboratory NOTES training prior to the performance of clinical NOTES procedures. Nevertheless, a conservative approach described in the white paper recommends that NOTES procedures be performed by multi-disciplinary teams after a period of laboratory training in a properly equipped facility in order to maximize patient safety and ensure continuing regulatory acceptance of early NOTES development.

Future NOTES practitioners will likely need some form of fundamental surgical training, along with platform-specific and procedure-specific training once the field has undergone significant development. The current paradigm of performing NOTES primarily with flexible endoscopes is reaching the limits of practicality and safety, and will arguably become quickly obsolete with the availability of advanced multitasking platforms. Thus, rec-



**Figure 5** Direct-Drive Endoscopic System from Boston Scientific is a prototype, advanced multi-channel platform currently in development, featuring instruments with multiple degrees of freedom controlled through a bi-manual user interface. Inset figure shows close-up of device tip with a small diameter flexible endoscope in place. Image used with permission (©Boston Scientific).

ommendations made in regards to training surgeons for NOTES using currently available instruments and techniques may quickly become obsolete.

## COMPLICATIONS OF NOTES

Complications are an inevitable part of surgical practice, especially during the application of new techniques such as NOTES. Intraoperative complications inherent to all procedures, such as bleeding, will need to be managed appropriately to ensure patient safety. Along with the development of better endoscopic instruments to manage hemorrhage, surgical decision-making will need to evolve based on laboratory and clinical data. Although the method of hemorrhage control will always depend on the situation and surgeon judgment, it will be useful to determine what is realistically manageable using a pure versus hybrid NOTES technique, and when it would be most beneficial to convert to a full laparoscopic procedure. Unfortunately, data from human studies to answer this question are currently limited. The reported incidence of bleeding in a prospective NOTES registry of 488 transvaginal cholecystectomy patients was 0% for intraoperative bleeding, and 0.6% for postoperative bleeding, comparable with the incidence of bleeding during laparoscopic cholecystectomy<sup>[13]</sup>. However, it should be kept in mind that the dominant technique in these cases did not include the use of any flexible endoscopes or accessories, and relied heavily on dissection through a transumbilical laparoscopic port. These results may thus only apply to NOTES performed exclusively with rigid instruments, as opposed to NOTES performed using a combination of flexible and rigid instruments. A more accurate picture of NOTES outcomes from operations performed primarily with flexible endoscopes (with or without laparoscopic assistance) may be derived from the registry by Zorron *et al.*<sup>[14]</sup>, which reported the incidence of intraoperative bleeding to be approximately 2% for transvaginal cholecystectomy (all from

the cystic artery), 8% for transvaginal appendectomy (all from the appendiceal artery), and a combined incidence of 4.7% for all transgastric procedures (7% for the appendiceal artery and 3.4% from the gastroepiploic artery)<sup>[14]</sup>. Although these rates of bleeding may seem high, it should be kept in mind that all of these bleeding complications occurred intraoperatively and were managed laparoscopically or endoscopically with the exception of 1 instance of gastroepiploic bleeding during transgastric access which required conversion to an open procedure. In addition, no cases of delayed postoperative bleeding were reported. Future research on the optimal method to control hemorrhage during NOTES will likely need to be performed in animal models (for ethical reasons), and should involve both the development of new instruments and algorithms to help guide intraoperative decision-making.

In addition to bleeding, the authors of the white paper foresaw the possibility that physiologic complications and compression syndromes might be more frequently seen during NOTES procedures, compared to existing laparoscopic procedures. So far the incidence of these complications in the literature has been low (0.8% of 362 NOTES procedures), however, as reported by Zorron *et al*<sup>[14]</sup> in a large, multi-institutional registry. These complications consisted of two episodes of intraoperative abdominal hypertension which resolved with desufflation of gas and fluid therapy, as well as one episode of facial and cervical subcutaneous emphysema following transvaginal, retroperitoneal cyst excision from the kidney<sup>[14,43]</sup>. This complication was reported to have been managed with oxygen therapy and observation in the intensive care unit, without requiring re-intubation. Although it was reported that most groups in the registry used laparoscopic insufflators through a transabdominal port or Veress needle, the case with subcutaneous emphysema used a laparoscopic carbon dioxide (CO<sub>2</sub>) insufflator connected to one of the flexible endoscopic channels with pressure maintained between 12 to 16 mmHg. Although the overall incidence of physiologic and compression syndrome complications was low in this registry report, surgeons performing NOTES should be aware that these complications may still occur and the risk of their occurrence may depend on the insufflation gas or insufflators used, and the anatomic compartment where dissection is performed.

Although the use of pressure-controlled CO<sub>2</sub> insufflation is likely to continue being a key component of NOTES procedures, lower insufflation pressures compared to conventional laparoscopy may be feasible, further reducing the risk of compression syndromes and subcutaneous emphysema.

More serious complications during NOTES cases have been reported that would otherwise be rare in the corresponding laparoscopic operations. These are worth noting to caution those who might be tempted to prematurely or over-enthusiastically adopt this still nascent approach to intra-abdominal surgery, and also to prioritize areas for potential improvement through better patient selection or

technical modifications to NOTES procedures. Reported complications of this kind during transvaginal cholecystectomy include 4 bladder injuries (0.8%), 2 rectal injuries (0.4%), and 1 small bowel injury (0.2%). All bladder injuries were reported to have occurred in older, obese women. However, it was not clear from the report whether these injuries occurred during the establishment of transvaginal access using a transvaginal trocar inserted under laparoscopic guidance or whether they occurred during the latter parts of the procedures. The occurrence of these complications emphasizes the extreme care that should be taken when establishing transvaginal access, closing the defect, and with the use of rigid transvaginal laparoscopic instruments. Similarly, the registry report by Zorron *et al* noted 2 esophageal hematomas, 1 esophageal laceration, and 1 esophageal perforation during 29 transgastric cholecystectomies, accounting for a combined rate of 13.7% esophageal complications. This is an unacceptably high rate of complications compared to conventional laparoscopic cholecystectomy, which is normally performed with minimal morbidity and mortality. Investigators from the International Multicenter Trial on Clinical Natural Orifice Surgery (IMTN) investigators addressed this high rate of esophageal complications and recommended the use of esophageal overtubes to protect the esophagus during the procedures, especially during specimen extraction. In agreement with this recommendation, a study conducted by our group found that preoperative ultrasound measurements of gallbladder stones can be used to help predict which gallbladders are able to be extracted through an esophageal overtube<sup>[44]</sup>. Gallbladders found to be full of multiple small stones, in which the size of the largest stone cannot be determined, as well as those in which the largest gallstone is greater than or equal to 10 mm, are unlikely to pass through an overtube. Patients with these ultrasound findings may be better managed with conventional laparoscopic cholecystectomy. Criteria such as these may help improve patient selection for transgastric cholecystectomy, for example.

Ultimately, once more human data on the risks and benefits of NOTES procedures become available surgeons will have to decide whether the benefits of NOTES are worth the risks. It should be kept in mind that just because a procedure has an inherently higher rate of a specific complication doesn't mean it is not worthwhile. Laparoscopic cholecystectomy, for example, has been shown have increased rates of common bile duct injury compared to open cholecystectomy<sup>[45,46]</sup>. However, this risk is acceptable given that the other benefits of laparoscopic cholecystectomy (decreased postoperative pain, decreased wound complications, improved cosmesis, and a faster rate of recovery for patients) outweigh its potential for harm. The same type of analysis weighing the risks and benefits of NOTES will need to be applied to determine its ultimate role in surgical practice.

## NOTES MOVING FORWARD

In the five years since the publication of the NOTES



white paper, there has been a substantial proliferation of clinical NOTES publications. Progress has been made addressing the questions originally foreseen as the likely barriers to the introduction of NOTES into clinical practice. However, NOTES development in the next 10-15 years is likely to change surgical practice through a series of small incremental gains, rather than through an overnight revolution as in the case of laparoscopic surgery. This evolution may involve first a change in the practice of specimen extraction, with the use of natural orifices instead of the abdominal wall. Finally, once advanced platforms reach the clinical arena surgeons may shift to using the natural orifice not only for specimen extraction, but also for dissection. The evolution may also occur more quickly for some indications than others. For example, esophageal and colorectal NOTES applications may evolve more quickly compared to hernia, solid-organ, biliary, and general intra-abdominal applications given the already excellent outcomes and ease of laparoscopic techniques in performing these later procedures. NOTES may be able to more easily establish a niche in the thoracic esophagus, distal colon and rectum, or other anatomic locations where laparoscopic approaches are currently challenging or where there is still significant morbidity with traditional approaches. For example, limited resections performed through a natural orifice may replace the current practice of removing a large segment of colon or rectum for endoscopically unresectable polyps, assuming that oncologic outcomes can be maintained or equaled using alternative methods to assess or treat the possibility of disease in regional lymph nodes.

Given this likely evolutionary pattern for NOTES, the original goals of NOTES should be re-thought and re-prioritized. Transgastric NOTES in the near-term is unlikely to be useful to perform advanced therapeutic procedures or operations requiring removal of bulky specimens, even with the appearance of suturing devices for conventional flexible endoscopes. Rather transgastric NOTES may be better suited for diagnostic peritoneoscopy, using lower profile endoscopes that are able to traverse the gastric wall without the need for complicated closures of large, potentially dangerous gastrostomies. In addition to a decreased primary emphasis on transgastric NOTES as the access route of choice, the NOTES community should re-think its primary emphasis on flexible endoscopy as the preferred platform for NOTES, and instead be open to the use of rigid, pre-bent, or articulating instrumentation either in concert or instead of flexible instruments, until more advanced platforms become available.

## CONCLUSION

In summary, since the first description of the concept of NOTES, many clinical NOTES cases have been reported in the literature, adding to the body of human data with which to begin to answering questions raised by the white paper. So far, transvaginal access has been the most feasible access route for NOTES procedures, although there

is growing experience with transgastric, transesophageal, and transrectal approaches. Luminal closure appears to be most feasible with a transvaginal approach, with smaller but nevertheless good outcomes also reported for transgastric and transesophageal closures. Data on the feasibility of true, intraperitoneal transrectal closures remain limited by the fact that the only closures performed to date have been hand-sewn coloanal anastomoses. Infection appears to be a non-issue with regard to transvaginal and transgastric surgery with the use of preoperative IV antibiotics (and local disinfection in the case of transvaginal procedures), with additional data required to more accurately estimate the risk with transesophageal and transrectal procedures. Development of suturing and anastomotic devices for NOTES has progressed slowly, with limited clinical data on their use so far. Likewise, the development of true multitasking platforms for NOTES has been slow and has not yet reached the clinical arena. The optimal management of intraoperative complications has still not been determined, but the data suggest that intraoperative hemorrhage may not automatically require conversion to laparoscopy. The incidence of compression syndromes appears low, as long as procedures are performed primarily with controlled, laparoscopic insufflation using CO<sub>2</sub>. Additional major complications specific to NOTES procedures that would normally not occur during the corresponding laparoscopic operations have been noted in the literature. These types of complications absolutely need to be reported in order to constructively analyze the current status of NOTES and optimize patient selection and techniques to minimize their occurrence. As far as recommendations for NOTES training, there are no data to provide more specific recommendations outside of previous recommendations in the white paper and those from large NOTES registries. Finally, it may be useful to re-prioritize the development of NOTES to focus on high-yield colorectal and esophageal applications that are more likely to succeed in the near-term, instead of seeking the holy-grail of being able to perform entire, complicated procedures through transgastric access alone.

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## Isolated lymphoid follicles in colon: Switch points between inflammation and colorectal cancer?

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### Abstract

Gut-associated lymphoid tissue is supposed to play a central role in both the organization of colonic repair mechanisms and colorectal carcinogenesis. In inflammatory conditions, the number, diameter and density of isolated lymphoid follicles (ILFs) increases. They are not only involved in immune surveillance, but their presence is also indispensable in normal mucosal regeneration of the colon. In carcinogenesis, ILFs may play a dual role. On the one hand they may support tumor growth and the metastatic process by vascular endothelial growth factor receptor signaling and producing a specific cytokine and cellular milieu, but on the other hand their presence is sometimes associated with a better prognosis. The relation of ILFs to bone marrow derived stem cells, follicular dendritic cells, subepithelial myofibroblasts or crypt formation, which are all involved in mucosal repair and carcinogenesis, has not been directly studied. Data about the putative organizer role of ILFs is scattered in scientific literature.

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**Key words:** Isolated lymphoid follicle; Colon; Mucosal repair; Colorectal cancer; Epithelial stem cell; Myofibroblast; Follicular dendritic cell; Mesenchymal-epithelial transition; Epithelial-mesenchymal transition

### INTRODUCTION

The imbalance of colonic epithelial proliferation and apoptosis may lead to both ulcer- and carcinoma development of the mucosa. The final direction of this imbalance depends on complex pathogenetic pathways in which isolated lymphoid follicles (ILFs) seem to have a specific role.

Some steps of colonic epithelial regeneration are known, but the connection among them is not fully understood. The continuous reformation of the epithelial layer is important in avoiding the aggregation of pernicious mutations induced by intraluminal factors. In inflammation, the lack of regenerative factors and the disturbance of the regulation of regenerative mechanisms favour ulcer development. It has also been observed that in colonic inflammation there is a tight connection between the degree of epithelial damage and the number, diameter and cellular compounds of subepithelial lymphoid follicles<sup>[1,2]</sup>. The more severe the epithelial destruction that develops, the higher the number of ILFs that can be found in adjacent mucosa.

It has recently been reported that lymphoid follicles are also present in carcinomas of the lung<sup>[3]</sup>, endometrium<sup>[4]</sup>, liver<sup>[5]</sup>, and colon<sup>[1]</sup>. They are supposed to have immune-mediated anti-tumoral effects, as their elevated number is in positive correlation with a better prognosis and a longer survival<sup>[3]</sup>. However, the density of lymphoid

follicle-associated flat dysplastic aberrant crypt foci was significantly higher compared to the rest of the mucosa in azoxymethane-treated rats<sup>[6]</sup>. Several reports have investigated the association between lymphoid aggregates and colonic tumors in rodents<sup>[7,8]</sup>. The results indicate that colonic crypts overlying ILFs show a significantly higher proliferative activity, which may also influence genetically defected epithelial cells. Hence, the risk of carcinoma is increased in the colonic mucosa of ILFs compared to mucosa without ILFs. It has also been shown that the incidence of ILFs in early human colorectal cancers significantly differs by gender, location, macroscopic type and histology, but moreover, their localization significantly differs by their macroscopic type<sup>[9]</sup>.

However, the exact role of ILFs in colonic epithelial repair and colorectal carcinogenesis is not yet known. Some data show<sup>[10]</sup> that the lack of lymphoid follicles results in abnormal crypt formation in the case of epithelial destruction. On the other hand, Apc gene mutation causes impairment of developmental and apparent differentiation blockade in proliferative tissues, including those of the lymphoid follicles<sup>[11]</sup>. Whether ILFs act as a regenerative pool containing putative stem cells in case of mucosal damage, or they are responsible only for the optimal cytokine milieu for the differentiation of immigrating stem cells or invasive carcinoma cells<sup>[12]</sup> need to be further examined.

## THE ORGANIZATION OF THE GUT-ASSOCIATED LYMPHOID TISSUE

The gut-associated lymphoid tissue (GALT) is a component of the mucosa-associated lymphoid tissue, in which approximately 70 percent of the body's immune cells are found<sup>[13,14]</sup>. GALT differentiates between pathogens and commensal bacteria.

The majority of GALT is composed of isolated and aggregated lymphoid follicles dispersed throughout the small and large intestines<sup>[15]</sup>. These lymphoid follicles, including Peyer's patches (PPs) of the small intestine and ILFs of the large intestine, are composed of a specialised follicle associated epithelium (FAE), which overlies a sub-epithelial dome containing numerous macrophages, dendritic cells, T, B lymphocytes, and special antigen sampling microfold/M/cells<sup>[15-17]</sup>. The FAE has a crucial role in the initiation of the mucosal and systemic immune response<sup>[18]</sup>. ILFs have, in general, an average diameter of 0.1-0.7 mm and number of around 30 000 in humans<sup>[19]</sup>.

ILFs are innervated sites of GALT. Functionally, antigen-triggered mast cell and eosinophil activation affects both the secretory and motor functions of the intestines<sup>[20]</sup>, and these defensive reactions can be modulated by the enteric nervous system<sup>[21]</sup>. It has been recently recognised that there is a dense neuronal network at the level of the supra-follicular dome region, but not within the germinal centers in lymphoid follicles<sup>[22]</sup>. Neuronal alterations of PPs and ILFs, such as nerve-eosinophil associations or increasing neuronal cell adhesion molecule expression, may have consequences on the uptake of particular pathogens<sup>[16,23]</sup>.

## VASCULARIZATION OF ILFS

ILFs have rich blood and lymphatic vascularization<sup>[14,19]</sup>. Vasculogenesis may play a dual role in mucosal organization, in that it is not only necessary for nutritional and metabolic processes, but the homing of the repopulating bone marrow derived stem cells to the site of tissue damage may happen *via* blood vessels. In the case of cancer development, the vascular system is essentially involved in tumor growth, invasion and metastasis formation.

Revascularization is a key point of colonic mucosal repair. During inflammatory stages, due to cytokine action and intercellular adhesion, molecules signalling some of the vessels differentiate into high endothelial venules (HEVs)<sup>[24,25]</sup>. In the case of lymphocytes and neutrophils, it is supposed that they firstly reach the inflammatory sites *via* a transcellular pathway through the HEVs<sup>[26]</sup>, but an intercellular pathway is also known<sup>[27]</sup>. Upon epithelial injury the circulating bone marrow derived cells (BMDCs) migrate to the stromal layer of the damaged colonic wall, presumably *via* HEVs at an increased number regulated by overexpressed inflammatory chemokines<sup>[28]</sup>.

Based on the result of Witmer *et al.*<sup>[29]</sup>, it has been suggested that in lymphoid tissues, including GALT, the signaling system of the vascular endothelial growth factor (VEGF) and its receptor play a permanent role in the vasculogenesis of ILFs. Whereas the inhibition of VEGF has shown promising results in sporadic colon cancer, it has been recently published that VEGF receptor signaling acts as a direct growth factor for tumor cells in colitis-associated cancer, providing a molecular link between inflammation and the development of colon cancer<sup>[30]</sup>.

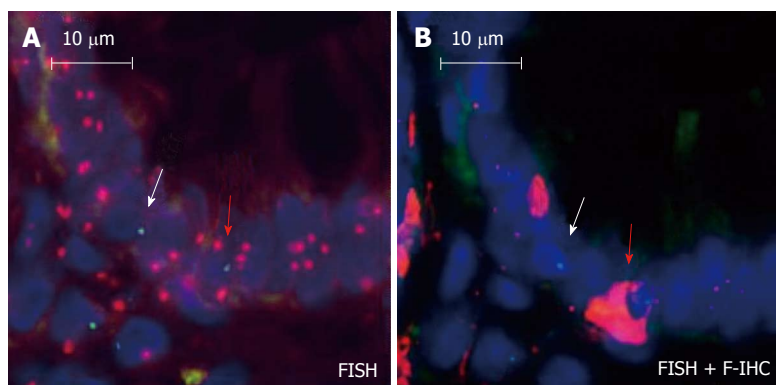
## BONE MARROW DERIVED STEM CELLS OF ILFS

Based on the former results<sup>[31-33]</sup>, emerging evidence suggests that bone marrow derived stem cells contribute to tissue regeneration partly by promoting neovascularization or arteriogenesis. After human hematopoietic cell transplantation epithelial tissue chimerism appears<sup>[34-36]</sup>.

The bone marrow origin of epithelial cells may be supposed by observations in which epithelial cell markers and leukocyte markers showed that double positive cells were found in inflamed mucosa adjacent to lymphoid aggregates<sup>[2,37-39]</sup>. The presence of cytokeratin, epithelial growth factor receptor, hepatocyte-derived growth factor receptor or CDX2 co-expression in CD45+ cells of ILFs may support the mesenchymal origin of epithelial stem cells. Based on these results, it seems that ILFs are involved in the homing and differentiation of BMDCs in the case of colonic mucosal damage (Figure 1).

The cause of metastasis remains elusive despite a vast amount of information on cancer cells. According to recent research, cancer cell fusion with macrophages or immigrating BMDCs provides an explanation<sup>[40,41]</sup>. BMDCs fused with tumor cells were present not just in animal tumor xenografts where they were associated with metastases, but in





**Figure 1** Intraepithelial male donor bone marrow origin CD45/Y-FISH+ cell (white arrow) and CD45/Y-FISH+ intraepithelial lymphocyte (red arrow) in the colonic biopsy specimen of a female acceptor. A: Chromosomal detection (green: Y-chromosome, red: X-chromosome; fluorescence *in situ* hybridization); B: CD45 and cytokeratin (green: cytokeratin, red: CD45; fluorescence immunohistochemistry; 130 × magnification).

human carcinomas, including colon cancer. BMDC-tumor cell fusion explains the epidermal-mesenchymal transition in cancer since BMDCs express mesodermal traits and epithelial-mesenchymal transition regulators (i.e.: Twist, SPARC). If BMDC-tumor cell fusion underlies invasion and metastasis in human cancer, new therapeutic strategies would be mandated.

## DENDRITIC CELLS IN ILFS

Follicular dendritic cells (FDCs) in lymphoid follicles retain native antigens in the form of immune complexes on their membrane for months, and present these antigens to B cells during the secondary response<sup>[42,43]</sup>. The origin and cell lineage of FDCs are controversial. Whereas their immune functions and expression of hemopoietic cell-associated antigens suggest that they belong to the hemopoietic lineage<sup>[44]</sup>, their spindle-shaped morphology “*in vitro*”, lack of CD45, and presence of antigens expressed by fibroblasts<sup>[45]</sup> indicate that FDCs may be mesenchymal cells. Based on studies with mouse radiation chimeras, Humphrey *et al.*<sup>[46]</sup> concluded that FDCs were not derived from the bone marrow, but came from a local mesenchymal precursor. However, Kapasi *et al.*<sup>[44]</sup>, using mice homozygous for the SCID mutation, which lack T, B lymphocytes, and FDCs, demonstrated that after reconstitution with bone marrow from donor mice, the FDCs of the reconstituted mice expressed the donor phenotype. These authors concluded that FDC precursors came from bone marrow.

According to the results of Muñoz-Fernández *et al.*<sup>[47]</sup>, FDCs seem to be a specialized form of myofibroblasts and derive from bone marrow stromal cell progenitors. The authors were able to isolate and culture 18 follicular dendritic cell lines from human tonsils. These cells were CD45-negative and expressed antigens associated with FDCs (CD21, CD23, CD35, CD40, CD73, BAFF, ICAM-1, and VCAM-1) and antigens specific for FDC (DRC-1, CNA.42, and HJ2). These cell lines were also able to bind B cells and secrete CXCL13, and they had functional activities characteristic of FDCs. Nevertheless, the additional expression of STRO-1, together with CD10, CD13, CD29, CD34, CD63, CD73, CD90, ICAM-1, VCAM-1, HLA-DR, al-

kaline phosphatase, and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA) indicated that FDCs are closely related to bone marrow stromal cell progenitors. The expression of  $\alpha$ -SMA also relates FDCs with myofibroblasts. Like myofibroblasts, FDC lines expressed stress fibers containing  $\alpha$ -SMA and were able to contract collagen gels under the effect of TGF $\beta$ 1 and platelet-derived growth factor.

In various inflammation models, tissue-derived dendritic cells have been shown to migrate from the inflammatory site *via* lymphatics to secondary lymphoid organs where they interact with lymphocytes<sup>[48]</sup>. Based on their dual phenotype, follicular dendritic cells may represent a transformation switch point among immigrating bone marrow derived stem cells in ILFs and the surrounding subepithelial myofibroblasts.

The origin of dendritic cells (DCs) in tumors remains obscure. Recent studies indicate that conventional DCs in lymphoid tissues arise from a distinct population of committed conventional DC precursors (pre-cDCs) that originate in bone marrow and migrate *via* blood. Diao *et al.*<sup>[49]</sup> showed that pre-cDCs are precursors for conventional DCs in tumors, and they migrate from blood into the tumor where they generate conventional DCs. The chemokine CCL3, which is markedly upregulated in tumors (including colon cancer) and in tumor-infiltrating stromal and immune cells, promotes pre-cDC recruitment. Both pre-cDCs and their conventional DC progeny actively proliferate within the tumor, and have the ability to mature and stimulate Ag-specific lymphocytes. This finding suggests that in several cases the migration of pre-cDCs to tumors may represent a normal response to inflammation. Further studies are needed to delineate the role of pre-cDCs in other inflammatory processes and to compare them with monocytes, which are currently considered the main source of inflammatory DCs in peripheral tissues<sup>[50,51]</sup>.

## MYOFIBROBLASTS SURROUNDING ILF ADJACENT EPITHELIUM

Subepithelial myofibroblasts (SEMFs) exist as a syncytium that extends throughout the colonic lamina propria, merg-

ing with the pericytes surrounding the blood vessels<sup>[52,53]</sup>. SEMFs are involved in two epithelial repair processes<sup>[54,55]</sup>. One process is called restitution<sup>[56]</sup>. This is an important response to minor to moderate injury. The other process is observed when the wound is deep, and the subepithelial tissues and the basement membrane need to be reconstituted<sup>[55]</sup>.

According to recent studies<sup>[54,57,58]</sup>, myofibroblasts are thought to derive from two major sources, bone marrow or locally activated fibroblasts, in response to transforming growth factor- $\beta$ 1. In the case of serious tissue injury (i.e. active ulcerative colitis) the regeneration capacity of local stem cells is not enough to complete tissue repair. In this case, bone marrow derived mesenchymal stem cells migrate into the gastrointestinal wall where they may contribute to the repair progress<sup>[59,60]</sup> as differentiated mesenchymal cells (e.g. myofibroblasts)<sup>[61]</sup>.

Despite the increasing number of publications illustrating the role of tumor-associated stromal cells in cancer progression, there still exists a significant ambiguity with respect to the identification of cancer-associated fibroblasts, myofibroblasts and peritumoral fibroblasts in the cancer tissue. SEMFs appear early in the cancer's development. The mutual interaction (through direct cell-cell contacts and paracrine signals) between cancer cells and SEMFs is essential for invasive growth and is translated into a poor clinical prognosis<sup>[62]</sup>.

## TOLL-LIKE RECEPTOR EXPRESSION IN ILFS

Beside immune functions, PPs and ILFs are supposed to be involved in mucosal repair *via* Toll-like receptors (TLRs). In ILFs, TLRs are expressed on the cells of the monocyte/macrophage system, on some kinds of T cells, as well as on intestinal epithelial, endothelial and stromal cells<sup>[63]</sup>. Using the dextran sodium sulfate (DSS) model of colitis, mice lacking TLR2, TLR4 or MyD88 all developed more severe colitis than wild type mice when exposed to orally administered DSS<sup>[64]</sup>. These findings suggest that signaling from commensal bacteria throughout TLRs resulted in protection from DSS colitis through enhanced epithelial cell proliferation, and worked as a compensatory factor against epithelial damage<sup>[64]</sup>.

TLRs can also bind endogenous ligands including necrotic cells, heat shock proteins, and extracellular matrix components<sup>[65-67]</sup>. Necrotic cells may activate NF- $\kappa$ B through TLR2, leading to the expression of tissue repair-associated genes<sup>[65]</sup>. It is supposed that necrosis induced inflammation in tissue damage may provide danger signals functioning as inducers of tissue repair responses through TLRs. The TLR ligands released from necrotic cells have not been identified, although heat shock proteins produced by damaged cells are known to be TLR ligands<sup>[66]</sup>. Components of the extracellular matrix, such as hyaluronan, can be an endogenous ligand for TLR4<sup>[67]</sup>. Increased hyaluronan production has been demonstrated in both DSS colitis in mice and in human Crohn's disease<sup>[68]</sup>. It

is possible that TLR activation may occur in the absence of microbial products<sup>[68]</sup>. In the case of inflammatory mucosal damage, ILFs may induce repair mechanisms *via* endogenous TLR activation.

TLR4 was also shown to be expressed on human colon carcinoma cells and functionally active. It may play important roles in promoting immune escape of human colon carcinoma cells by inducing immunosuppressive factors and apoptosis resistance, and it may also promote the proliferation and migration of cancer cells<sup>[69,70]</sup>.

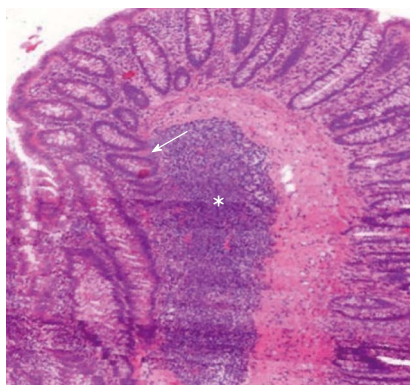
The analysis of isolated tumor cells from primary colon cancers showed co-expression of TLR7 and TLR8 with CD133 and gave evidence for a subpopulation of colon cancer-initiating cells<sup>[71]</sup>. Persistent TLR-specific activation of NF- $\kappa$ B in colorectal cancer, particularly in tumor-initiating cells, may sustain further tumor growth and progression through perpetuated signaling known from inflammatory and tissue repair mechanisms with consecutive self-renewal in pluripotent tumor cells. Activation through self-ligands or viral RNA fragments from tumor-associated lymphoid aggregates may putatively maintain this inflammatory process, suggesting a key role in cancer progression.

## THE EFFECT OF THE PRESENCE OF ILFS ON MUCOSAL REPAIR

The epithelia of intestinal crypts associated with ILFs and PPs have an increased proliferation rate<sup>[10,14]</sup>. Saxena *et al*<sup>[10]</sup> showed that PPs in rats have a facilitative effect on the healing of intestinal wounds by promoting both epithelial cell migration on the defect and epithelial cell proliferation in the crypts adjacent to the wound and by decreasing the rate of wound contraction.

In rats, a difference in epithelial apoptosis between the FAE of PPs and intestinal villi was described<sup>[72]</sup>. Onishi *et al*<sup>[72]</sup> showed that the progression of the apoptotic process in the epithelial cells of FAE occurs later than in the intestinal villi, so the possibility of epithelial differentiation might remain in FAE, unlike in intestinal villi. PPs are supposed to have a regulatory effect on the epithelial proliferation as well<sup>[73]</sup>.

The Wnt signaling pathway is critical for regulating a number of basic cell functions, such as cell proliferation, cell fate, polarity, differentiation, and migration, leading to morphogenesis and organogenesis<sup>[74,75]</sup>. There is strong genetic evidence that Wnt signaling play critical roles in the regulation of epithelial stem cells in the intestinal tract<sup>[76]</sup>. The Wnt target gene *Lgr5* has been recently identified as a novel stem cell marker of the intestinal epithelium and the hair follicle<sup>[77]</sup>. In the intestine, *Lgr5* is exclusively expressed in cycling crypt base columnar cells<sup>[77]</sup>. Many Wnt family proteins are expressed in hematopoietic tissues, and can also be secreted by lymphoid cells<sup>[78,79]</sup>. The Wnt-*Lgr5* pathway may be a potential switch between the ILFs and colonic epithelial renewal. Lymphoid cells of ILFs may produce Wnts which are essential components of a milieu in which bone marrow derived stem cells immigrated to ILFs to engage in epithelial differentiation (Figure 2).



**Figure 2** 3D reconstruction of a human colonic surgical sample (MIRAX Viewer, 3D, 3DHISTECH Ltd., Budapest). A large subepithelial isolated lymphoid follicle (white star) can be seen. Colonic crypts (white arrow) with no connection to the luminal surface “outgrow” from the isolated lymphoid follicle.

## THE EFFECT OF THE PRESENCE OF ILFS ON COLORECTAL CARCINOGENESIS

Results from experimental colon cancer studies indicated that ILFs might promote the development of adenocarcinomas<sup>[7,8]</sup>. However, studies in experimental animals have also shown that the intestinal lymphoid system plays an important role in immunologic defense mechanisms; that is, antigenic stimuli result in germinal center formation, antibody production, and finally enlargement of the follicles<sup>[80]</sup>. In humans, the presence of tumor-infiltrating lymphocytes is associated with an improved prognosis in colorectal cancers, as does the presence of high level DNA microsatellite instability<sup>[81]</sup>. These results suggest that ILFs in early colorectal neoplasms play an important role in defense rather than in promotion.

In a recent study, Fu *et al.*<sup>[9]</sup> found that the incidence of ILFs in early human colorectal neoplasms significantly differs by gender, location, macroscopic type, and histology, but moreover, their localization significantly differs by macroscopic type.

In squamous cell carcinomas of the esophagus, cyclin A expression in the germinal center cells of ILFs beneath the superficial tumorous lesions was shown to be an immunological signal toward the proliferation and progression of the tumors<sup>[82]</sup>. Gutfeld *et al.*<sup>[83]</sup> found that the cells of colonic ILFs, inflammatory cells, ganglion cells, and endothelial cells express serum amyloid A, an acute phase reactant, whose level in the blood is elevated in response to trauma, infection, inflammation, and neoplasia, on both mRNA and protein levels. The serum amyloid A mRNA expression in epithelial cells was found to gradually increase as it progressed through different stages of dysplasia to overt carcinoma. While expression of the serum amyloid A1 and -4 genes in colon carcinomas was confirmed by RT-PCR analysis, this expression was barely detectable in normal colon tissues. Their findings indicate local and differential expression of serum amyloid A in human colon cancer and tumor-associated ILFs, and suggest its role in colorectal carcinogenesis.

## MESENCHYMAL-EPITHELIAL AND EPI-THELIAL-MESENCHYMAL TRANSITION IN ILFS

Epithelial-mesenchymal transition (EMT) is a physiological mechanism present during development, and is also encountered in several pathological situations such as renal interstitial fibrosis, endometrial adhesion, and cancer metastasis<sup>[84]</sup>. A reverse phenomenon, mesenchymal-epithelial transition (MET) also takes place during normal development in processes such as somitogenesis, kidney development and coelomic cavity formation<sup>[85]</sup>. In adult organisms, it has been proposed that restrictive mechanisms repress EMT and MET<sup>[86]</sup>. During tumor development, these mechanisms appear to fail, allowing EMT described in metastasis generation<sup>[87]</sup>.

In inflammation, MET can also be altered because mesenchymal stem cells are mobilized to these sites of injury and consequently subjected to the inflammatory response<sup>[88]</sup>. BMDCs could differentiate into mature-appearing epithelial cells in response to tissue damage<sup>[89]</sup>. It was recently published that versican, a large chondroitin sulfate proteoglycan, mediates MET<sup>[90]</sup>. The results of Hirose *et al.*<sup>[91]</sup> indicate that versican can bind specific chemokines through its chondroitin sulfate chains and that the binding tends to down-regulate the chemokine function. This raises the possibility that versican may act as a regenerative factor in colonic mucosa, and may be an important switch point between ILFs and MET. The presence of CDX2 and cytokeratin positive subepithelial cells in the marginal zone of ILFs also suggests that MET may take place in these immune formations<sup>[2]</sup>.

Stroma-tissue, including lymphoid aggregates and ILFs surrounding the cancer cells, plays an important role in the tumor behavior. Mesker *et al.*<sup>[92]</sup> analyzed the expression of markers involved in pathways related to stroma production and EMT ( $\beta$ -catenin, TGF- $\beta$ -R2, SMAD4) in high-risk colorectal cancer patients, and found that patients with stroma-high and SMAD4 loss are of high risk. The anti-EMT effect of SMAD4 was also proven in colon carcinoma cells<sup>[93]</sup>.

## CONCLUSION

Based on the summarized results of literature, it seems that ILFs act like a switch between colonic mucosal regeneration and colorectal carcinogenesis.

Subepithelial revascularization after mucosal damage takes place partly under the direction of ILFs with the prominent help of vascular endothelial growth factor and its receptors. Immigrating stem cells from bone marrow may leave circulation *via* high endothelial venules in ILFs and their surroundings. Their differentiation throughout mesenchymal-to-epithelial transition may also happen in ILFs, and follicular dendritic cells, as well as the subepithelial myofibroblasts, seem to be crucial parts of colonic crypt formation and epithelial renewal.

Vasculogenesis in ILFs supports not just tumor growth



and the metastatic process, but the VEGF receptor signaling acts like a direct growth factor for tumor cells. The fusion of BMDCs immigrating to ILFs with tumor cells may explain EMT in colorectal cancers. The presence of ILFs, dendritic cells and subepithelial myofibroblasts may also result in a specific milieu for tumor formation, growth and invasion.

Better understanding of the role of ILFs in mucosal repair may lead to the development of new therapeutic agents for inflammatory colon diseases that not only decrease the activity of inflammation, but also accelerate epithelial barrier recovery, hence dramatically decreasing clinical symptoms. Moreover, by revealing the exact connections between ILFs and colorectal carcinogenesis, the basis of individualized anti-cancer immunotherapies may be established.

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## Patterns of local recurrence in rectal cancer after a multidisciplinary approach

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chemoradiotherapy versus extended lymphadenectomy, though there is a trend towards posterior or presacral LR in patients in the Western world and lateral LR in Asia. Nevertheless, both may arise from the same mechanism. Moreover, as well as the mode of treatment, the type of LR is related to the height of the initial tumor. Nowadays most LRs are related to the advanced nature of the disease. Involvement of the circumferential radial margin and spillage of residual tumor cells from lymphatic leakage in the pelvic side wall are two plausible mechanisms for the genesis of LR. The patterns of pelvic recurrence itself (pelvic subsites) also have important implications for prognosis and are related to the potential success of salvage curative approach. The re-operability for cure and prognosis are generally better for anastomotic and anterior types than for presacral and lateral recurrences. Overall survival after LR diagnosis is lower with radio or chemoradiotherapy plus optimal surgery approaches, compared to optimal surgery alone.

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### Abstract

Improvements in surgery and the application of combined approaches to fight rectal cancer have succeeded in reducing the local recurrence (LR) rate and when there is LR it tends to appear later and less often in isolation. Moreover, a subtle change in the distribution of LRs with respect to the pelvis has been observed. In general terms, prior to total mesorectal excision the most common LRs were central types (perianastomotic and anterior) while lateral and posterior forms (presacral) have become more common since the growth in the use of combined treatments. No differences have been reported in the current pattern of LRs as a function of the type of approach used, that is, neo-adjuvant therapies (short-term or long-course radiotherapy, or

**Key words:** Rectal cancer; Local neoplasm recurrence pelvis; Pattern of recurrence multidisciplinary approach

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## INTRODUCTION

Over the past two decades, there have been improvements in the management of rectal cancer in terms of postoperative death (falling from 10% to 2%), locoregional failure (dropping from 30%-40% to less than 15%), conservative surgery rates (increasing from 20% to 60%) and survival, with advances made in the understanding of the biology of this type of tumor as well as staging and the use of combined therapies<sup>[1]</sup>. The anatomical and technical basis of tumor recurrence within the pelvis has been extensively investigated by surgeons and pathologists, and this has led to major improvements in surgical therapy. Nonetheless, although surgery remains the mainstay of treatment aimed at achieving locoregional control, nowadays the therapeutic approach to rectal cancer is eminently multidisciplinary.

The key to successful surgery is complete excision of the tumor proximally, distally and around its circumference with sufficient margin of normal tissue (R0 resection). In total mesorectal excision (TME) surgery the rectum and its perirectal lymphatic and fatty tissue (mesorectum) is completely mobilized by sharp dissection, as an intact package surrounded by an undamaged peri-mesorectal layer of proper rectal (visceral) fascia, avoiding spillage and growth of residual tumor cells into the pelvis and subsequent development of a local recurrence (LR). Educational programs aimed at training surgeons in this pelvic dissection technique have demonstrated reproducible results in achieving a reduction of the LR of rectal cancer rate by 40%<sup>[2]</sup> and even greater when associated with radiotherapy (RT)<sup>[3]</sup> or neoadjuvant chemoradiotherapy (CRT)<sup>[4]</sup>.

Nevertheless, LR of rectal cancer remains a significant clinical problem, associated with severe morbidity, low rates of success of salvage procedures, and eventual death in the majority of patients<sup>[5]</sup>.

It is important to review the patterns of treatment failure resulting after rectal cancer management. Improvements in surgical and adjuvant therapies may affect not only the likelihood of tumor recurrence in the pelvis but also the pattern of pelvic recurrence itself (i.e. pelvic subsites). Knowledge of the pattern and natural history of LR, the associated risk factors for their development and the mechanism by which they occur may serve as the foundation for efforts to improve the results of multidisciplinary care (i.e. RT field design, suitability of lymphadenectomies, strategy in the follow-up monitoring, *etc.*).

The aim of this review is to characterize and analyze the pattern of LR today following different curative approaches for rectal cancer, with special emphasis on the correlation between subsites of pelvic recurrences and treatment modalities.

## LIMITATIONS OF THIS REVIEW

Many of the studies that report patterns of pelvic recurrence have multiple limitations. Some are outdated or do not give exact anatomical information of the location of recurrent tumors in the pelvis. In particular, the diagnostic procedures, methods of documentation, acknowledge-

ment or confirmation of diagnosis, presence or absence of histology, the use of interval pain, anatomic definitions of the rectum, first site of recurrence and cumulative recurrence data, as well as the definition of the LR itself and other details all affect the analysis of incidence rates, timing, and patterns<sup>[6]</sup>.

Old literature concerning the pattern of local failure in rectal cancer was based on planned or symptomatic reoperations data or autopsy series. Planned "second look" procedures and symptomatic surgery were performed in the pre-TME surgery era and LR data obtained may be outdated. Furthermore, autopsies reveal only the end pattern of failure.

On the other hand, most recent reports are based on clinical or imaging data which can be also misleading as the methods of diagnosing and confirming LR and length of follow-up are not described consistently. The actual rate of pelvic recurrence may be somewhat higher than estimated by these reports, as some studies report only first sites of failure, and pelvic relapse later in the course of disease is not always assessed in patients under palliative chemotherapy for distant metastasis.

Trials of preoperative RT or CRT in resectable rectal cancer are characterized by multiple methodological problems because treatments are combined (RT and surgery) to address a heterogeneous condition (various populations and stages of rectal carcinoma) and to achieve a variety of goals (downstaging and improving resectability, as well as decreasing local and possibly distant recurrences and improving survival).

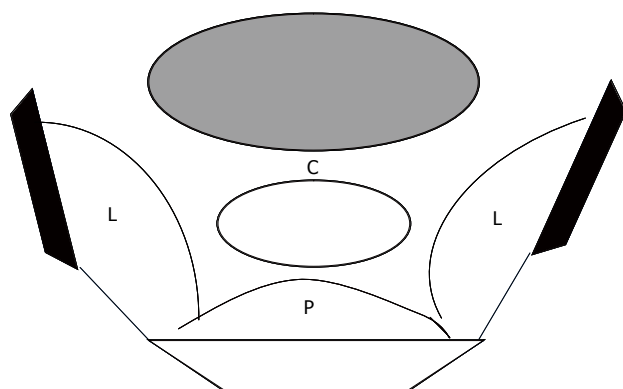
## DEFINITION AND CLASSIFICATION OF LOCAL RECURRENCE

Although by definition the term LR is only applicable when the initial or primary surgery is expected to be curative (no remaining macroscopic evidence of disease locally, that is R0 and R1 according to the UICC (International Union Against Cancer), it must be seen as the further development of tumor cell remnants: there is a close biological similarity between a primary tumor and an LR, in contrast to the situation with corresponding organ metastases<sup>[7]</sup>.

Recurrent rectal cancer may be isolated (local or metastatic) or combined (local and metastasis). Indeed LR can be defined as any tumor located within the pelvis, either alone or in conjunction with metastases<sup>[6]</sup>. Several authors have classified locoregional pelvic recurrence in order to facilitate treatment and compare outcomes. Specifically, the distinction between localized and diffuse pelvic recurrence is pivotal in defining subsequent management and prognosis.

The Mayo Clinic<sup>[8]</sup> described recurrence in terms of degree of fixation both in term of site (anterior, sacral, right or left) and number of points of fixation (F0-F3). Wanebo *et al*<sup>[9]</sup> proposed a classification based on the UICC TNM system: TR1 and TR2 corresponding to intraluminal LR, either following local excision or at the anastomosis; TR3 corresponding to LR at or around the level of the anastomosis with limited extramural spread





**Figure 1** Types of pelvic recurrence. C: Central; L: Lateral; P: Posterior.

and without pelvic fixation; TR4 corresponding to invasion into either adjacent urogenital organs or presacral tissues with tethering but no fixation; and TR5 corresponding to invasion into the sacrum or pelvic side walls. On the other hand, others divided pelvic local failures into just three basic types of recurrence: localized (central), sacral and pelvic sidewall.

The Memorial Sloan Kettering<sup>[10]</sup> group describe a nomenclature based on the anatomical region of the pelvis that is involved (Figure 1). Accordingly, LR is defined as either: axial, subdivided into anastomotic, mesorectal (residual mesorectum) or perirectal soft tissue within the center of the pelvis or perineum following an abdominoperineal resection (APR); anterior, involving the genitourinary tract; posterior, involving the sacrum and presacral fascia and sacral root sheaths; or lateral, involving the muscles (piriformis, elevator), soft tissue of the pelvic sidewall, lymph nodes, major iliac vessels, sacral nerve plexus and lateral bony pelvis. Lastly, the Dutch TME trial<sup>[11]</sup> classifies LR based on the same pelvic subsites, although the perineum and anastomotic recurrences are grouped separately. We have found the latter to be the most useful classification, although it does not distinguish between two different origins of the pelvic sidewall involvement itself: true lateral involvement by growth of tumor deposit in lymph nodes along the iliac vessels and continuous extension of tumors of central origin.

## DIAGNOSIS OF RECURRENT RECTAL CANCER

Patients with recurrent cancer are a heterogeneous group. To establish LR or pelvic disease after definitive resection of rectal cancer<sup>[12]</sup>, most authors accept at least one of the following major criteria: (1) Histological confirmation; (2) Palpable or evident disease with subsequent clinical progress; (3) Clear evidence of bone destruction; and (4) Positive positron emission tomography examination, and at least one of the minor criteria: (1) Progressive enlargement of soft tissue mass on repeated computed tomography (CT) or magnetic resonance (MRI) examination; (2) invasion of adjacent organs; (3) subsequent rise in tumor

markers; and (4) typical appearance in endoscopic ultrasound, CT or MRI imaging.

Note that according to these major criteria no patient should be accepted as having pelvic recurrence by diffuse pelvic pain.

## RISK FACTORS FOR LR

Many factors affect the risk of local recurrence.

### Pathological factors

The pelvic recurrence rate is tumor stage dependent: the more advanced the stage the more likely it is that rectal cancer will recur<sup>[13]</sup>. Many authors have confirmed the association between advanced UICC or Dukes' stage and the likelihood of recurrence. Not surprisingly, the extent of invasion beyond the rectum affects recurrence, with an incidence of less than 1% in patients in whom no local extension is noted, compared to between 5% and 10% in patients with moderate spread and 15%-25% in those with more extensive spread<sup>[14,15]</sup>. The number of positive lymph nodes<sup>[16]</sup> as well as a positive circumferential resection margin (CRM) also influence both LR and survival<sup>[11]</sup>. Even with combined treatments, the incidence of LR in patients with one of these risk factors (that is, TMN stage IV, T4 tumor, N2 disease or positive CRM in T3 disease) reaches 20%, compared to less than 5% in patients who do not have these characteristics<sup>[11]</sup>. It is also clear that the combination of risk factors is also important: in patients at T1-T2 the incidence of LR is 1% with a negative CRM but this rises to 12% for a positive CRM, while for those at T3-T4 it is 15% for a negative CRM but 25% for a positive CRM<sup>[11]</sup>. In patients undergoing surgery with or without preoperative RT, the combination of CRM and lymph node status has been shown to be a more effective discriminator of prognosis than TNM staging<sup>[11]</sup>.

Height of the tumor is also a critical factor as LR is also more likely with tumors in the lower third of the rectum (10%-15%) than in patients with tumors in either the middle third (5%-10%) or upper third (2%-5%)<sup>[3,5,11,15]</sup>. The risk of LR is also related to the position of the tumor within the circumference of the rectum. In the series of Chan *et al.*<sup>[17]</sup> the rate of LR was 15% (95% CI, 11-22) for tumors affecting the anterior side of the rectum but was 5.8% (95% CI, 3-11) for other locations. Anterior tumors tend to be more advanced, at least in male patients, and the anterior aspect of the TME dissection more difficult to perform in the narrow male pelvis, presenting a higher risk of LR and death than tumors in other sites<sup>[18]</sup>.

The shape (exophytic versus non-exophytic) of the tumor, the presence or absence of budding, lymphatic, venous or perineural invasion, the presence of obstruction or perforation of the tumor together with the degree of tumor differentiation, and fixity of the tumor, all influence the risk of local recurrence adversely<sup>[13,19]</sup>.

### Therapeutic factors

Inadequate removal of the primary tumor is the most

Table 1 Patterns and rates of recurrences after different approaches

Authors - year	Treatment	5-year local recurrence (%)			Metastases as a first site of recurrence (%)	Median time to LR
		Total	Isolated	Combined		
Pilipshen <i>et al</i> <sup>[32]</sup> (1968-1976)	Pre-TME surgery	25.5	13.3	12.3	NA	16 mo
Heald <i>et al</i> <sup>[34]</sup> (1978-1986)	TME surgery	3.7	2.6	1.1	NA	14 mo
Swedish Trial <sup>[39]</sup>	Pre-TME surgery/	23.0	13.0	10.0	11	NA
(1987-1990)	sRT+ pre-TME	9.0	5.0	4.0	19	
Mohiuddin <i>et al</i> <sup>[67]</sup> (1976-1989)	LRT+ Pre-TME	13.0	6.0	7.0	17	25 mo
German Trial <sup>[25]</sup> (1995-2002)	CRT+ TME/	6.0	3.0	3.0	20	NA
	TME + CRT	13.0	7.0	6.0	20	
British Trial <sup>[44]</sup> (1998-2005)	sRT +TME/	4.7	2.0	2.7	19	NA
	TME+ selective CRT	11.5	6.1	5.4	21	
Dutch Trial <sup>[41]</sup> (1996-1999)	TME surgery/	11.3	6.6	4.7	17	18 mo
	sRT+ TME	5.8	2.3	3.5	19.3	30 mo
Guillem <i>et al</i> <sup>[4]</sup> (1988-2002)	CRT+TME	4.3	2.3	2.0	22	23 mo
Yu <i>et al</i> <sup>[54]</sup> (1989-2001)	CRT+TME	8.3	6.3	2.0	NA	NA
Kim <i>et al</i> <sup>[56]</sup> (2001-2005)	CRT+TME	8.0	3.7	4.3	19	24 mo central 18 mo lateral
Kusters <i>et al</i> <sup>[58]</sup> (1993-2002)	Unilateral EL/	15.4 (only T3,T4 tumors)			NA	NA
	Bilateral EL	8.3 (only T3-T4 tumors)				
Moriya <i>et al</i> <sup>[57]</sup> (1982-1991)	Nerve-sparing EL	6.2 (14 for N+ tumors)			12	17 mo

TME: Total mesorectal excision; sRT: Short-term preoperative radiotherapy; LRT: Preoperative long-term radiotherapy; CRT+TME: Pre-operative chemoradiotherapy; TME+CRT: Post-operative chemoradiotherapy; EL: Extended lymphadenectomy; NA: Not available; LR: Local recurrence.

important factor determining whether the tumor recurs<sup>[20]</sup>. In addition to the involvement of the CRM, the plane of surgery achieved, as a measure of the quality of mesorectal excision, has been shown to be an important prognostic factor for LR<sup>[21]</sup>. Surgeon variability is also a widely studied phenomenon. In multivariate analysis “the surgeon factor” has emerged as a critical treatment-independent variable, and is not only related to the volume of operations performed<sup>[22]</sup>.

The incidence of lateral lymph node involvement has been extensively investigated by Japanese authors. The term “extended lymphadenectomy” (EL) refers to the removal of the lymph nodes in the extra-mesenteric area. Generally rates of node involvement have been reported to be 5%-10%, but are markedly higher in stage III tumors in the lower third of the rectum, with rates of up to 15%-25%<sup>[23]</sup>. However, it is doubtful that remaining lateral lymph nodes after an R0 resection is the major or only source of tumor regrowth<sup>[24]</sup>.

There is abundant clinical data supporting the importance of the pathologic response and downstaging to preoperative CRT. All patients who developed cancer recurrence in the German trial had positive lymph node involvement post-treatment<sup>[25]</sup>. A pathological complete response (pCR) or greater than 95% CR in the post-CRT is a predictive factor of low LR rate and good prognosis, and several studies have shown that this is the most important independent prognostic factor in multivariate analysis for disease-free survival<sup>[4,26]</sup>.

While attention was traditionally focused solely on the optimal distal mucosal margins required to achieve an oncologically safe resection, not only have these margins been reduced significantly, but also greater importance has come to be placed on the lateral margins for achieving an R0 resection. Short (but negative) distal margins have

repeatedly been found not to be associated with pelvic recurrence<sup>[27,28]</sup>. The risk is not so much of intramural spread as of intramesorectal spread, and probably the risk for mesorectal tumor deposit is higher in node-positive than in node-negative patients<sup>[27]</sup>.

Other factors that may increase a patient's risk of LR are related to increasing body mass index<sup>[29]</sup>. It has been shown that obese men are more likely than normal weight males to develop an LR, and that adiposity is a strong predictor of requiring an APR<sup>[30]</sup>.

## SITES OF LOCAL RECURRENCE AFTER SURGERY ALONE

In the late 1970s, areas of failure found on planned reoperation (“second look” procedures) of patients at risk of recurrence, in spite of an initial curative resection, were investigated. The seminal article by Gunderson and Sosin<sup>[31]</sup> showed that as distant metastases alone were uncommon (7%), LR as the only type of failure occurred in nearly 50% of cases of recurrence and as some component in 92%. They also depicted the pattern of pelvic subsites LR (Table 1), showed that disease relapse rates were indeed related to the degree of bowel wall penetration and the extent of nodal disease, and paved the way for the exploration of radiation therapy.

In the era before the adoption of TME, surgery alone was associated with local failures of up to 30%-50%. The classic article of Pilipshen *et al*<sup>[32]</sup> describes the pattern of LR at a prestigious institution (Sloan-Kettering) in the pre-TME era, with LR rates of 31% in cases at T3-T4 and 49% in cases at N2. Hruby *et al*<sup>[33]</sup> provide a detailed analysis of the sites of LR after undergoing surgery for rectal cancer in a series of 269 patients mainly during the 1980s. As can be seen in Table 1, at that time most LR was axial

or central, in the block of fatty tissue surrounding the rectal wall, within or contiguous to the operative site, and appeared 6-16 mo before the appearance of metastasis.

In the 1980s details emerged of the first series of patients treated with TME surgery. Heald *et al*<sup>[34]</sup> published an accumulated local recurrence rate of 3.7% in a personal series of 115 cases of “curative” low anterior resections<sup>[5]</sup>. Ten years later, the same author reported actuarial 5- and 10-year recurrence rates of 6% (95% CI, 2%-10%) and 8% (95% CI, 2%-14%) respectively with TME surgery alone. Another TME pioneer, Enker, published a rate of 4.1% for Dukes' B and 8.2% for Dukes' C patients<sup>[35]</sup>.

In the 1990s large reductions in local and distant recurrences were reported with TME and so, even without a proper randomized control trial, the TME resection became the new gold standard of surgery for rectal cancer.

Although regarded as equivalent in the pre-TME surgical era, the superiority in terms of LR of the TME low anterior resection (LAR) over the “traditionally” executed APR soon became clear. “Standard” or “traditional” APR for low rectal cancer is associated with a higher rate of positive CRM (30%-60%) and operative perforation (20%-33%), leading to higher LR and poorer survival rates than with LAR<sup>[36]</sup>. Recently a more radical excision of the levators and puborectalis muscles, carried out in the prone jack-knife position, has been proposed (“extralevator APR”)<sup>[37]</sup>.

As the surgical techniques worldwide evolved to TME resections, European researchers focused on the delivery of a short course of high-dose preoperative RT, without chemotherapy, after ascertaining that adjuvant RT, both before and after surgery, substantially reduced the risk of LR when biologically effective doses of 30 Gy or more were used<sup>[38]</sup>.

## LOCAL FAILURES AFTER SHORT-TERM PREOPERATIVE RT

The Swedish Rectal Cancer Trial<sup>[39]</sup> randomized 1168 patients to surgery alone or to surgery following a 1-wk of pelvic RT (25 Gy in 5 daily fractions), and showed that not only was the 5-year LR rate significantly improved with preoperative RT (23% *vs* 9%, among the curatively treated patients) but also the 5-year survival rate significantly improved (58% *vs* 48%). However, this trial was conducted in the surgical era prior to the adoption of TME.

Syk *et al*<sup>[40]</sup> reviewed the incidence and location of LR in a group of 880 patients from Stockholm after the introduction of TME surgery, and half of the group also received short-term preoperative RT. In this study, 42% of LR originated from tumors in the upper rectum, and a majority of these patients had not received RT. In all these cases, the recurrence was at the anastomosis and virtually all had visible signs of residual mesorectal fat. Eighteen percent of the patients had LR involving the lateral wall of the pelvis, but only 6% of the tumors involved sites consistent with recurrence in iliac lymph nodes. The authors concluded that lateral pelvic lymph node metastases are not a major cause of local recurrence after TME, and

that partial mesorectal excision may be associated with an increased risk of local recurrence due to presacral and/or pelvic sidewall involvement in the upper rectum.

As surgery improved, Dutch researchers then asked whether preoperative short-term RT would still be beneficial in the setting of TME resection properly executed. In the Dutch TME trial a significant benefit was seen with preoperative RT in patients with TNM stage II and III disease, with the two-year local relapse rates decreasing from 5.6% to 1% and from 15% to 4.3%, respectively<sup>[41]</sup>. The update of the trial reported in 2007 noted a drop in local relapse from 22% to 11% for stage III patients but no significant reduction for stage II patients, and no difference in distant metastasis rate or 5-year overall survival<sup>[42]</sup>.

Subgroup analysis showed a significant fall in patients with cancer in the lower rectum, with nodal involvement but uninvolved CRM. Although those CRM positive patients who received preoperative RT had a lower LR rate than the group with TME alone, this difference was not statistically significant (9.3% *vs* 16.4%, *P* = 0.08). The authors arguably concluded that short-term preoperative RT “hardly compensate” for involved CRM<sup>[43]</sup>.

The Dutch group has also recently published a complete and updated analysis of the pattern of LR and the most likely mechanism of recurrence in the trial<sup>[11]</sup>. They showed that preoperative RT reduces LR in all subsites. However the appearance of LR was slower in the group who underwent RT (2.6 years *vs* 1.5 years), and if distant metastases diagnosed within 1 mo of LR diagnosis were also considered to have occurred simultaneously, the rate of combined recurrences was higher in the RT + TME group (74% *vs* 40% in the TME group). In the TME group the recurrences were predominantly anastomotic and posterior, while in the RT + TME group most were posterior and lateral. The anastomotic recurrences were significantly more common in the TME only group, suggesting that RT is especially effective in preventing anastomotic recurrence. Lateral recurrences represented more than 25% of the total in the RT + TME group and most appeared together with metastasis. After LAR, the recurrences were mostly perianastomotic while after APR they were mostly presacral. Perineal LR were found after APR in the TME only group, but not in the RT + TME group. TME alone in node positive disease resulted in considerable local recurrence when the distal margin was 2 cm or less, while RT resulted in a small number of LRs, except when distal margins were less than 5 mm. In total 17% of the patients had a positive CRM, and of those 17% developed LRs (12% in those with T1-T2 tumors, and 24% in T3-T4 tumors with positive CRM).

Given the higher LR rates with narrow CRM, the question of whether selective postoperative RT could improve outcomes in this setting was addressed by researchers in the United Kingdom. The MRC CR07 trial<sup>[44]</sup> randomized 1350 patients to TME preceded by short-term RT (25 Gy in 1 wk) *vs* TME followed by CRT (45 Gy plus 5-fluorouracil) if the CRM was < 1 mm. The results showed that the 5-year local recurrence rate was significantly better in



the preoperative RT group (4.7%) than the postoperative CRT group (11.5%). However, in those patients with a positive CRM, the LR rates were not statistically different (16% preoperative *vs* 23% postoperative).

## PATTERN OF PELVIC FAILURE AFTER LONG COURSE RT PLUS CHEMOTHERAPY

Postoperative adjuvant strategies to improve outcomes following rectal resection have mainly been explored in the United States. Indeed, in 1990, after positive trials conducted by the Gastrointestinal Tumor Study Group<sup>[45]</sup> and the Mayo Clinic/North Central Cancer Treatment Group<sup>[46]</sup>, the NCI issued a statement declaring combined postoperative therapy the new standard of care in this setting<sup>[47]</sup>.

Researchers, however, were questioning whether preoperative combined therapy would be even more beneficial. In the 1990s, data from the Memorial Sloan-Kettering Cancer Center and the MD Anderson Cancer Center accumulated<sup>[48]</sup> in support of that benefit. Moreover, results from three randomized trials (the Uppsala trial<sup>[49]</sup>, NSABP R03<sup>[50]</sup> and above all the German CAO/ARO/AIO trial<sup>[25]</sup>), demonstrated the clear superiority of preoperative RT regimens over postoperative therapy in terms of local control with better compliance to treatment and lower toxicity.

The next step was to test the hypothesis that chemotherapy plus preoperative RT significantly improved local control, tumor downsizing and downstaging compared with RT alone. Two randomized trials compared preoperative RT *vs* preoperative CRT, the study by the Fédération Francophone de Cancérologie Digestive (FFCD 9203)<sup>[51]</sup> and the EORTC 22921 trial<sup>[52]</sup>, and similar results were reported. In the latter, the five-year results showed that chemotherapy increased the rate of pCR (14% *vs* 5.3%), translated into a 3% benefit in terms of sphincter preservation and significantly reduced LR rate from 17% without chemotherapy down to 8% with CRT. Thus chemotherapy, regardless of whether it is administered before or after surgery, confers a significant benefit with respect to local control. The main criticism that can be made of those trials is that TME resections were not uniformly implemented.

On the other hand, the favorable effect of delaying surgery after CRT on downstaging (and possibly also sphincter preservation) was shown in the Lyon R90-01 trial<sup>[53]</sup>.

There is, however, limited data on patterns of relapse in rectal cancer patients treated with TME surgery and CRT. Such information might help determine whether modifications in RT dose or field design are warranted (i.e. a local recurrence after RT may be inside or outside the RT field; recurrence outside the field requires an increase in the size of the field, and recurrence inside the field implies the need for an increase in the total dose). We have identified reports on only four series of patients that contain detailed analysis of the pattern of pelvic recurrence after CRT and TME surgery.

From the MD Anderson Cancer center, Yu *et al*<sup>[54]</sup> presented a thorough study attempting to identify subsites of pelvic LR in an effort to correlate sites of relapse on

CT images with RT simulation films in 46 rectal cancer patients. Of all the LR, approximately two-thirds were in-field (within the radiation field) recurrences and only one-third were marginal (inside but within 1 cm of the border of the field) or out-of-field (more than 1 cm from the border) recurrences. Of the in-field recurrences, nearly 80% occurred in the low pelvic and presacral regions. Multivariate analysis showed that the risk of in-field LR was significantly associated with pathological N stage, while it was notably not with positive CRM or downstaging. The authors suggested various strategies to improve locoregional control in low pelvic and presacral regions.

Höthch *et al*<sup>[12]</sup> published a large-scale multicenter study based in Germany to evaluate pelvic sites of recurrence with special attention to radiation ports. Nearly 80% of LR occurred within the treated volume, in the central pelvis, and the pelvic sidewall structures were involved in fewer than 5% of tumor relapses. They found no significant differences in the incidence of pelvic sidewall involvement between APR and LAR cases, however there was a significant difference in the spread of recurrent tumors in the inferior part of the pelvis.

However, a quite different picture has been reported from Korea, where Kim *et al*<sup>[56]</sup> examined the patterns of locoregional recurrences in 366 patients with locally advanced rectal cancer who underwent preoperative CRT and curative TME surgery, and assessed the effect of clinical parameters on lateral pelvic recurrence. Eight percent of the patients had LR, of which around 20% and 80% occurred in central and lateral pelvic areas respectively. Multivariable analysis showed that lateral pelvic recurrence was significantly associated with ypN classification (lymph node status after preoperative CRT) and lateral lymph node size. The authors suggested that lateral lymph node metastasis is a risk factor for LR and could be a potentially curable regional disease rather than a sign of systemic disease. Accordingly, they suggested that patients with lateral lymph node size of > 10 mm and ypN0 or lateral lymph node size of 5 mm and ypN+ are a potential subgroup of patients who might benefit from lateral lymph node dissection.

In tumors of the middle and lower rectum, lateral lymph nodes remain a potential cause of locoregional recurrence after conventional TME because they are not removed. EL has been championed mainly by Asian surgeons, who are internationally renowned for their skills in radical surgery.

## PATTERN OF RECURRENCES AFTER EXTENDED LYMPHADENECTOMY AND TME, WITH OR WITHOUT ADJUVANT TREATMENT

Around 40% of patients treated for rectal cancer present with lymph node metastases, which occur along the mesorectal nodal chain, along the inferior mesenteric artery lymph nodes or in the lateral pelvic lymph nodes (along the obturator, internal iliac or medial aspect of the external



iliac artery). Whether pelvic sidewall lymph nodes should be considered metastatic disease as suggested by the TNM classification (M1) or part of the regional lymphatics (N3) as outlined in Japanese guidelines that are amenable to curative resection, is a contentious issue. Japanese surgeons have adopted the technique of EL to supplement TME, with the aim of minimizing LR and improving survival. Western surgeons do not use EL regularly, and this might pose a risk of local recurrence in the pelvic sidewall in patients operated on without preoperative RT.

In a recent detailed topological analysis of the pattern of lymphatic spread in 605 cases of rectal cancer, 285 cases (47%) were identified as having lymph node metastases. Of this total, 71.5% were mesenteric, 21.5% were lateral and mesenteric, and only 4.7% were exclusively lateral (so-called skip metastases), while among the cases of lateral metastases slightly more than a third were bilateral. The authors<sup>[23]</sup> concluded that lateral lymph node status is reflective of overall mesenteric lymph node status and that evidence of lateral lymph node involvement may be an ominous sign of advanced disease with an inherent dismal prognosis.

EL is associated with high degrees of urinary and sexual dysfunction and while it is possible to undertake lateral node dissection with autonomic nerve-sparing surgery (NSEL), there are problems with this surgical technique in terms of worldwide uniformity. Moriya *et al.*<sup>[57]</sup> have studied the pattern of recurrence after NSEL surgery in 306 patients, of which 14% were in Dukes' stage C, and found an overall LR rate of 6.2%. Dukes' A and B patients with relapse had suture-line recurrence. In contrast, in the Dukes' C group, 70% of the recurrences were in patients who had had involvement of more than 5 lymph nodes and 40% in those in whom there had been lateral spread, the number of mesenteric lymph node metastases being the factor which had the strongest impact on the LR. The authors judged that the LR rate with NSEL is similar to that obtained with conventional EL.

Kusters and Van de Velde<sup>[58]</sup> reviewed 351 patients operated for rectal carcinoma at or below the peritoneal reflection at the National Cancer Center in Tokyo. Standard TME surgery was performed for T1 and T2 ( $n = 145$ ), and NSEL was added to TME for T3 and T4 (unilateral = 73; bilateral, when the tumor was located centrally,  $n = 133$ ). They noted that overall there was lymph node involvement in 42% of cases, and lateral involvement in 10%, with "skip" metastases in 3% (mesorectal nodes negative and lateral nodes positive). Overall the 5-year LR rate was 6.6%, while for node-positive (N+) patients the difference between the uni- and bilateral NSEL (32% *vs* 14%, respectively) was significant.

On the other hand, studies of EL are observational and the reported outcomes are far from uniform even within series reported from Japan. Moreira *et al.*<sup>[24]</sup> indicated that EL has no advantages for patients in Dukes' stages A and B and that for cases in stage C it does not significantly reduce LR rates compared to TME. Other series, from both Western and Asian countries, suggested that despite undergoing EL few patients survive for 5 years or more if carcinoma has spread to the pelvic lymph nodes.

Yano *et al.*<sup>[59]</sup> proposed selective use of extra-mesenteric nerve-sparing lymphadenectomy for those cases in which lateral node metastasis is detected in the CT scan. These authors reported a high level of sensitivity and accuracy (88%) of CT scans for detecting lateral node metastasis, in marked contrast to their diagnostic accuracy for mesorectal lymph node involvement. This same group has published a recent review of lateral lymph node spread in a Japanese journal, emphasizing in particular the rates (20%) of lateral node involvement in T3-T4 cases of low rectal cancer with positive mesorectal nodes. Similarly, Min *et al.*<sup>[60]</sup> reserved EL for cases in which high-resolution MRI detected extra-mesenteric lymph node metastasis. These authors found a positive predictive value of MRI of 86.4% and 40% for the lateral and paraaortic nodes respectively, and that the location of the lymph node metastasis was the only prognostic factor for cancer-specific survival, with disease in the paraaortic area indicating a worse prognosis than lateral or mesenteric involvement. This suggestion accords with the recommendation in the Guidelines 2000 for Colon and Rectal Cancer Surgery that dissection should be attempted to remove clinically suspected lateral lymph node disease, as far as is technically feasible<sup>[61]</sup>.

A different approach would be to consider EL in various combinations with RT. In the only randomized control trial of EL ( $n = 23$ ) versus non-EL ( $n = 22$ ) after preoperative RT (50 Gy) in both groups, Nagawa and colleagues<sup>[62]</sup> reported no difference in disease-free survival or local recurrence. However, given the small sample size and the fact that the study did not include patients with lateral pelvic lymph node involvement, no safe conclusion can be drawn on the role of either RT or EL in this particular group of patients. Wanatabe *et al.*<sup>[63]</sup> in a retrospective non-randomized study of four patient groups comparing EL with non-EL using either 50 Gy pre-operative RT or no RT found a 5-year survival advantage in the RT group. In addition, the authors reported no significant survival difference between the patients who had preoperative RT with conventional TME surgery compared with those who had EL without preoperative RT, and concluded that preoperative RT could be an alternative to EL.

A comparative non-randomized study by Kim *et al.*<sup>[64]</sup> recently reported a higher local recurrence rate in patients with TME plus EL than in those with TME plus postoperative CRT. Among those with stage III lower rectal cancer, they successfully demonstrated that the EL group showed a 2.2-fold increase in local recurrence rate compared to the CRT group. However, the 5-year LR rate in stage III in the EL group was much higher than the rate previously reported by the same Japanese group (16.7% *vs* 7.4%), therefore either a patient selection bias or a different definition of LR cannot be ruled out in this study.

A recent meta-analysis<sup>[65]</sup> comparing EL and non-EL TME surgery showed no overall difference in cancer-specific outcomes (5-year survival, 5-year disease-free survival and local or distant recurrence). However, as the authors state, the question of whether EL provides benefits in terms of survival or just local control in a subset of

Table 2 Relative incidence of sub-site locations of pelvic recurrences

Authors (yr)	Treatment	Axial or central (%)				Lateral (%)	Other (%)
		Anastomotic (perianastomotic)	Anterior (genitourinary)	Posterior (presacral)	Perineal		
Gunderson <i>et al</i> <sup>[31]</sup>	Pre-TME surgery	- <sup>1</sup>	40	19	31	10	
Pilipshen <i>et al</i> <sup>[32]</sup> (1968-1976)	Pre-TME surgery	40	10		40 <sup>2</sup>	10	
Hruby <i>et al</i> <sup>[33]</sup> (1979-1996)	Pre-TME surgery	21	10.7	47	11	11	
Dutch Trial <sup>[41]</sup> (1996-1999)	TME surgery	24	18	32	5	18	2.5
Dutch Trial <sup>[41]</sup> (1996-1999)	sRT+ TME	13	16	41	-	25	2.7
Syk <i>et al</i> <sup>[40]</sup> (1995-1999)	sRT +TME	37 <sup>3</sup>	30	10		18	
Yu <i>et al</i> <sup>[54]</sup> (1989-2001)	CRT+ CRT	-	44	28	-	10	18.0
Höthch <i>et al</i> <sup>[12]</sup> (1998-2001)	CRT+TME		60 <sup>4</sup>	29		10	
Kim <i>et al</i> <sup>[56]</sup> (2001-2005)	CRT+TME		20 <sup>5</sup>			80	
Kusters <i>et al</i> <sup>[58]</sup> (1993-2002)	Unilateral EL	25	-	16	16	40 <sup>6</sup>	
Kusters <i>et al</i> <sup>[58]</sup> (1993-2002)	Bilateral EL	20	10	16	16	40	

APR: Abdominoperineal resection; Anast: Anastomotic; perin: Perineal; Ant: Anterior. <sup>1</sup>0% were APR; <sup>2</sup>Post + perineum; <sup>3</sup>Anast + ant; <sup>4</sup>Perin + ant + anast; <sup>5</sup>Axial o central; <sup>6</sup>20% ipsilateral and 20% contralateral. TME: Total mesorectal excision; sRT: Short-term preoperative radiotherapy; EL: Extended lymphadenectomy; CRT: Chemoradiotherapy.

patients with advanced rectal cancer could not be safely answered by this meta-analysis.

## PATTERNS OF RECURRENCE AFTER INTRA-OPERATIVE RADIOTHERAPY PLUS EXTENDED SURGERY

Several authors reported the impact of intra-operative radiotherapy (IORT) with or without preoperative external beam irradiation and surgical resection in patients with locally advanced or recurrent cancer. The overall available data on IORT showed a favorable impact on local control and in overall survival for patients resected for cure (R0, R1); However, there is a need for randomized studies of the effect of IORT. From a Dutch national referral center, the pattern of LR in 247 patients with locally advanced rectal carcinoma after IORT including multimodal treatment (preoperative CRT and extended surgery) has been analyzed in detail<sup>[55]</sup>. The 5-year LR rate was 13.2% (7.5% after R0 resections). The most prominent sites of LR were the presacral (44%) followed by the anterior (21%) subsites and lateral spread accounted for less than 10% of recurrences. Around 50% of the LRs appeared in the IORT field, particularly high rates of infield recurrences being observed after dorsal IORT (75%). The authors hypothesize that migration of remaining tumor cells to the presacral space would explain the occurrence of this LR.

## PATTERNS OF RECURRENCE FOLLOWING COMPLETE CLINICAL RESPONSE AFTER CRT

The definitive role of an initially non-surgical approach to treatment following complete clinical response (cCR) after CRT has not yet been determined and no definitive conclusions can be drawn before long-term results concerning LR and distant failure are available.

However, Habr-Gama *et al*<sup>[66]</sup> have reported the pattern of recurrence and survival of 99 patients with distal rectal cancer (0-7 cm) and cCR following adjuvant CRT, sustained for at least 12 mo, managed by initial non-operative treatment. They observed 13 recurrences: five endorectal (limited to the rectal wall), seven systemic, one combined (endorectal and distant), and no pelvic recurrence outside the rectal wall was detected. There were no significant clinical differences either between patients with and without recurrence, or these same patients according to the location of the recurrence. Surprisingly, systemic recurrence occurred sooner than LR. The authors suggest that a change in the approach to follow-up monitoring may be necessary.

## CLOSING REMARKS

The reported patterns and rates of local recurrence, after the aforementioned range of treatment approaches to rectal cancer, used in isolation and as combined therapies are summarized in Table 1. Overall, combining therapies reduces LR rates, delays the appearance of LR and means that when there is recurrence it is less often isolated than after surgery alone.

Table 2 lists the relative frequency of LRs in various pelvic subsites. In recent years, a subtle change has been observed in the distribution of LRs in terms of location within the pelvis, implying the involvement of a different mechanism in their development. In general terms, in the pre-TME years most recurrences were central, perianastomotic and anterior and since the adoption of combined therapies lateral and posterior (presacral) forms dominate. However, the LR distribution is not only related to therapy modality but also to the height of the tumor. LRs in the upper rectum are relatively rare, but when they do occur are usually perianastomotic, originating in the residual mesorectal fatty tissue (as they are treated with partial mesorectal excision, transecting the rectum at about 5 cm below the tumor), and are comparatively more common when only surgical treatment is used. This suggests that while

preoperative RT helps to prevent LR at all sites, it is especially effective in preventing anastomotic recurrences<sup>[67]</sup>. Isolated anastomotic recurrences are also seen in select cases of very low rectal cancer treated using intersphincteric resection (ISR)<sup>[68]</sup>. Surgical technique and attention to distal margin can also play a role in preventing this type of LR. The LR rate after ISR is higher in poorly selected cases of pT3 with no previous RT, due to accidental tumor spillage into the intersphincteric space or positive CRM<sup>[69]</sup>. A lower LR rate has been reported with stapled coloanal anastomosis than for ISR even in T1-T2 patients<sup>[70]</sup>. As expected, after transanal endoscopic microsurgery, intramural recurrence is the most common type of LR<sup>[71]</sup>.

Most local recurrences of mid-rectal cancers treated with RT or CRT + TME are related to the advanced nature of the disease. Tumor height of 5 cm or more is associated with a higher incidence of presacral and lateral LR<sup>[72]</sup>. If the CRM is found to be positive after CRT, the hazard ratio for LR after surgery is significantly higher than if the CMR is involved when no preoperative CRT has been administered (6.3 *vs* 2.0), possibly because of selection of a population of tumor cells that are resistant to therapy<sup>[73]</sup>. LR may also sometimes occur even in the absence of an involved CRM possibly owing to lymphatic spread from the distal rectum to lymph nodes in the pelvic side wall<sup>[57]</sup>. Unilateral EL (lateral lymph nodes on one side of the pelvis are left intact) result in more LR than bilateral ELs, and it has been suggested that the mechanism for the formation of posterior-lateral recurrences may be the migration of tumor cells through the lateral lymphatic vessels to the presacral space under gravity<sup>[55]</sup>. This would explain why presacral local recurrence is more common in advanced disease than in limited disease.

The highest rates of positive CRM are found with lower rectum tumors. Indeed, TME surgery is not a universal solution for all rectal carcinomas: in low rectal cancer TME may be insufficient to obtain the desired circumferential clearance because of this lack of mesorectum at the level of the pelvic floor. On the other hand, APR surgery mainly results in perineal and presacral LR, which may be prevented by a wider resection<sup>[57]</sup>.

The pelvic pattern of recurrence itself (i.e. pelvic sub-sites) also has important prognostic value and is related to the potential success of repeat curative intent surgery<sup>[74,75]</sup>. The operability for cure and prognosis for anastomotic and anterior recurrence are generally better than for presacral and lateral recurrences<sup>[75]</sup>. Moreover, the upper sacral/lateral invasive type of LR is often associated with synchronous metastatic disease<sup>[74]</sup>. The type of pelvic invasion is also closely associated with survival after re-resection<sup>[74-76]</sup>. Finally, it is worth noting that overall survival after LR diagnosis is lower with RT and CRT+TME approaches, than after TME surgery alone<sup>[74]</sup>.

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## Therapeutic options for intermediate-advanced hepatocellular carcinoma

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on the optional therapeutic modalities for intermediate-advanced HCC.

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### Abstract

Hepatocellular carcinoma (HCC) is one of the most common malignancies, ranking the sixth in the world, with 55% of cases occurring in China. Usually, patients with HCC did not present until the late stage of the disease, thus limiting their therapeutic options. Although surgical resection is a potentially curative modality for HCC, most patients with intermediate-advanced HCC are not suitable candidates. The current therapeutic modalities for intermediate-advanced HCC include: (1) surgical procedures, such as radical resection, palliative resection, intraoperative radiofrequency ablation or cryosurgical ablation, intraoperative hepatic artery and portal vein chemotherapeutic pump placement, two-stage hepatectomy and liver transplantation; (2) interventional treatment, such as transcatheter arterial chemoembolization, portal vein embolization and image-guided locoregional therapies; and (3) molecularly targeted therapies. So far, how to choose the therapeutic modalities remains controversial. Surgeons are faced with the challenge of providing the most appropriate treatment for patients with intermediate-advanced HCC. This review focuses

### INTRODUCTION

Hepatocellular carcinoma (HCC) is the sixth most common cancer in the world and the third most common cause of cancer-related death<sup>[1]</sup>. Patients at the early stage are those who present with an asymptomatic single HCC with the nodule < 5 cm in diameter or ≤ 3 in number. Patients exceeding these limits, but free of cancer-related symptoms and vascular invasion or extrahepatic spread, are considered at the intermediate stage. The patients with the cancer-related symptoms and vascular invasion or extrahepatic spread are deemed at the advanced stage. HCC is frequently diagnosed at the late stage and has a high mortality rate. Surgical resection is a potentially curative therapy for HCC, however, only 10%-30% of patients with HCC are eligible for curative hepatectomy. Comprehensive therapy for HCC has become the focus of interest in recent years<sup>[2-6]</sup>. The current therapeutic modalities

for intermediate-advanced HCC are collected and evaluated as follows.

## SURGICAL PROCEDURES

Radical resection is still the first choice for treatment of HCC<sup>[7,8]</sup>, even at the intermediate or advanced stage<sup>[9,10]</sup>. If radical resection is impractical, palliative resection combined with comprehensive therapy can significantly prolong patients' survival time<sup>[11,12]</sup>. Intraoperative comprehensive therapy includes radiofrequency ablation, cryosurgical ablation, and hepatic artery and portal vein chemotherapeutic pump placement. Two-stage hepatectomy can improve the survival rate in selected patients with advanced HCC<sup>[13,14]</sup>. Liver transplantation has been shown to achieve excellent survival rate in appropriate HCC patients<sup>[15,16]</sup>.

### Radical resection

Radical resection for intermediate-advanced HCC is indicated as follows: (1) single HCC with large or huge tumor nodule, swelling outward, clear border or pseudocapsule, and less than 30% hepatic tissue destroyed measured by computed tomography (CT) or magnetic resonance imaging (MRI) scan, or more than 50% compensatory hepatic hypertrophy; (2) multiple HCC with 3 or fewer nodules localized in one lobe or segment of the liver<sup>[17,18]</sup>. It should be pointed out that tumor nodules limited to the liver are not the absolute operative indication. The outcome of radical resection could be affected by multicentric occurrence of HCC, tumor nodule adjacent to major blood vessel or bile duct, and the hepatic insufficiency induced by coexisting cirrhosis<sup>[19]</sup>.

With the deeper recognition of the pathology of HCC, the rational criteria of negative surgical margin are initially determined as follows: (1) > 2 cm margin free from tumors < 5 cm in diameter; (2) > 1 cm margin free from tumors 5-10 cm; and (3) > 0.5 cm margin free from tumors > 10 cm. More than 90% hepatectomies fulfilling the above-mentioned criteria can achieve negative surgical margin<sup>[20]</sup>. Thereby, healthy hepatic tissue should be reserved as much as possible during radical resection so as to enhance the operative security, to facilitate the postoperative recovery and to help with further treatment.

### Palliative resection

The indications of palliative resection for intermediate-advanced HCC are: (1) multiple HCC with 3-5 tumor nodules, exceeding half of the liver; (2) multiple HCC with nodules localized in 2-3 adjacent segments or half of the liver, more than 50% compensatory hypertrophy in the tumor-free liver demonstrated by image examinations; (3) central HCC with more than 50% compensatory hypertrophy in the tumor-free liver; (4) hilar lymph node metastasis should be cleared up during hepatectomy; and (5) invaded organs around the liver, such as colon, stomach, diaphragm, right adrenal gland, *etc.*, and single metastatic neoplasm far from the liver (e.g. lung metastasis) should be resected<sup>[17]</sup>.

### Intraoperative radiofrequency ablation

Radiofrequency ablation (RFA) is a technique in which an electromagnetic energy deposition is used to thermally ablate the hepatic tumor tissue<sup>[21]</sup>. During RFA treatment, heat energy generated by high-frequency alternating currents targeted at the living tissues causes protein denaturation at a temperature of 60-110°C through ionic vibration, resulting in coagulative necrosis of the target lesion. In addition, RFA treatment stimulates the immune system and provides an easy way to achieve *in vivo* vaccination against tumoral antigens<sup>[22]</sup>.

RFA is generally indicated for HCC patients who are not candidates for either liver resection or transplantation<sup>[23]</sup>. HCC patients are required to have  $\leq 5$  nodules, each < 3 cm in diameter, no evidence of vascular invasion or extrahepatic spread, 0 score performance status of the Eastern Cooperative Oncology Group (ECOG), and liver cirrhosis in Child-Pugh class A or B. The more versatile radiofrequency probes allow ablation of nodule > 5 cm. When complete resection by major hepatectomy is dangerous because of difficult nodule location, selective use of intraoperative RFA will be helpful<sup>[24]</sup>. The integration of intraoperative RFA into resection surgery contributes to complete removal of nodules with adequate margin, diminishes the extent of parenchymal resection, and improves the resectability rate for patients with advanced HCC<sup>[24]</sup>.

Pretreatment imaging must carefully define the location of tumor nodule with respect to the surrounding structures for RFA in HCC: nodules located on the surface of the liver can be considered; nodules adjacent to the hepatic vessels may be considered because flowing blood usually protects the vascular wall from thermal injury; nodules adjacent to the hepatic hilum represents a relative contraindication due to the risk of thermal injury of the biliary tract; and nodules adjacent to any part of the gastrointestinal tract must be avoided<sup>[25]</sup>.

### Intraoperative cryosurgical ablation

Although RFA has been the most widely utilized ablation modality for HCC, cryosurgical ablation has several advantages (most significantly, the ability to produce larger and more precise zones of ablation) over RFA<sup>[26]</sup>.

Cryosurgical ablation for HCC patient relies on nonspecific tissue necrosis due to freezing as well as microvascular thrombosis. Argon-helium cryosurgical ablation is able to induce the necrosis of tumor cells through the formation of extracellular and intracellular ice crystals and then cell dehydration due to rapidly freezing (< -140°C) as well as rapidly thawing (20-40°C) the tumor tissues with argon/helium gas. Therefore, argon-helium cryosurgical ablation has become one of the major therapeutic approaches for unresectable intermediate-advanced HCC.

The indications of cryosurgical ablation for HCC patient are: (1) nodules < 5 cm in diameter,  $\leq 3$  in number; (2) nodule > 5 cm with irregular margin, may be given intraoperative cryosurgical ablation with or without excision of nodule. Intraoperative cryosurgical ablation offers

an effective and safe option for management of advanced HCC<sup>[27]</sup>. HCC patients with diffuse infiltrative disease or large bilobar nodules (> 50% of liver volume) are not candidates for cryosurgical ablation because complete ablation of the nodules might induce hepatic failure.

### **Intraoperative hepatic artery and portal vein chemotherapeutic pump placement**

The liver has a dual blood supply from the hepatic artery and the portal venous system. For HCC patients who are not suitable for hepatectomy confirmed by intraoperative exploration, two chemotherapeutic pumps could be implanted subcutaneously into the upper abdominal wall near the incision, with the tip of pump catheter separately inserted into the hepatic artery and portal vein during the operation, followed by postoperative chemotherapy. The advantage of intraoperatively implanted chemotherapeutic pump is the ability to accurately and selectively place into the main trunk or branch of hepatic artery and portal vein. For resectable intermediate-advanced HCC, the postoperative hepatic artery and portal vein dual perfusion chemotherapy *via* chemotherapeutic pumps could prevent tumor recurrence<sup>[28]</sup>.

### **Two-stage hepatectomy**

Two-stage hepatectomy has been developed as a surgical strategy for extremely difficult patients with intermediate-advanced HCC<sup>[7]</sup>. This strategy is applied when it is impossible to resect the tumor in a single procedure. The main principles of this strategy are: huge HCC with the remnant liver volume cannot maintain hepatic function after hepatectomy; central or hilar HCC adjacent to or invaded major blood vessel; and serious cirrhosis with possible hepatic decompensation after hepatectomy.

For unresectable HCC, preoperative intervention with transcatheter arterial chemoembolization (TACE)<sup>[29]</sup>, portal vein embolization (PVE)<sup>[30,31]</sup>, or percutaneous RFA could control tumor progression and invasion, downstage tumor status, increase remnant liver volume, and decrease tumor recurrence rate, thus making the two-stage hepatectomy possible. The indication of two-stage hepatectomy is that tumor diameter reduced to 50% of the initial size, and nontumorous liver tissue had significant compensatory hyperplasia. Sequential TACE and PVE could broaden the surgical indication and the safety of major hepatic resection for advanced HCC patient with damaged liver<sup>[32,33]</sup>.

Non-anatomic local excision of liver cancer or hepatic segmentectomy should be used in the two-stage hepatectomy so as to maximally preserve the normal liver tissue. For the patients with HCC invading the hepatic hilum and inferior vena cava, total hepatic vascular exclusion (HVE) should be prepared to avoid massive hemorrhage during hepatectomy.

### **Liver transplantation**

Liver transplantation is an ideal treatment option, as it simultaneously cures HCC. However, up to date, there are no uniform criteria of liver transplantation for HCC

patients in China. The United Network for Organ Sharing (UNOS) criteria for liver transplantation are usually adopted in the world: single tumor  $\leq 5$  cm; 2-3 tumors, each  $\leq 3$  cm; no macrovascular invasion; and no extrahepatic spread to surrounding lymph nodes, lungs, abdominal organs, or bones<sup>[34]</sup>. However, if the UNOS criteria are strictly adopted in China, it means that most HCC patients will lose the opportunity of liver transplantation, because more than 100 000 patients die of advanced HCC each year. For this reason, the indication of liver transplantation for advanced HCC should be relatively loose in China. For the patients with unresectable huge or multiple HCC, if no vascular invasion and no extrahepatic spread, liver transplantation is the treatment of choice. Considering the limited organ supply, high cost, and considerable risk, we suggest that only those HCC patients with a high probability of survival benefit should be selected to receive liver transplantation. The shortage of donor livers is the major constraint of liver transplantation.

## **INTERVENTIONAL TREATMENT**

Although surgical resection has been the first choice for treatment of HCC, a simple surgical exploration could accelerate the process of disease and even cause death due to the postoperative complication of patients with unresectable HCC. With advances of medical imaging and improvement of interventional technology, interventional treatment has become an effective approach to inoperable HCC<sup>[35-37]</sup>. The common approaches of interventional treatments for inoperable HCC include transcatheter arterial chemoembolization, portal vein embolization, and image-guided locoregional therapies.

### **Transcatheter arterial chemoembolization**

For the treatment of inoperable HCC demonstrated by preoperative image examination, the priority is transcatheter arterial chemoembolization (TACE). The theoretical basis of TACE is the special vascular supply of liver and HCC. Liver derives dual blood supply from portal vein and hepatic artery, the former accounts for 2/3 to 3/4 while the latter for only 1/4 to 1/3. HCC derives 90% blood supply from hepatic artery and only 10% from portal vein. Thus, TACE provides a higher local concentration of chemotherapeutic drugs into tumor compared with intravenous perfusion chemotherapy, and meanwhile, it blocks blood supply of HCC, but only exerts little influence on blood supply of the liver. The consequence is that the major portion of cancer nodule becomes necrotic, while hepatic function remains unchanged or little impaired.

Better patient selection and selective segmental chemoembolization may improve the benefit-risk ratio of TACE<sup>[38]</sup>. TACE is indicated in intermediate-advanced HCC even in the setting of portal vein involvement (excluding main portal vein)<sup>[39]</sup>. The presence of main portal vein thrombosis, extrahepatic metastasis, Child-Pugh class C liver function, and severe hepatic arterio-portal shunts is considered as contraindications for TACE.



### Portal vein embolization

Percutaneous transhepatic portal vein embolization (PVE) is a useful procedure for the preoperative intervention of advanced HCC patients selected for hepatectomy. PVE could increase the volume and function of the future remnant liver through the acceleration of hepatocyte proliferation, and embolize possible hepatic arterio-portal shunts, so as to prevent postoperative liver insufficiency.

For the treatment of intermediate-advanced HCC, the combination of TACE and PVE not only blocks most blood supply of main tumor and satellite lesions, but also increases the local concentration of chemotherapeutic drugs into tumor, so as to more effectively control the tumor growth and decrease tumor recurrence. Contraindications to PVE include distant metastases, uncontrolled coagulopathy, active cholangitis, portal hypertension, and renal failure<sup>[40]</sup>.

### Image-guided locoregional therapies

Ultrasound or CT guided locoregional therapies have a therapeutic effect in advanced HCC patients by means of thermoablative therapy (radiofrequency ablation, microwave coagulation, laser ablation), cryotherapy (argon-helium knife, liquid nitrogen), or chemical therapy (ethanol injection, acetic acid injection) to destroy tumor tissues. To date, the commonly used therapies include percutaneous RFA, microwave coagulation, cryoablation therapy, and ethanol injection, especially with percutaneous RFA as the first choice to inoperable HCC. The roles of different locoregional therapies may change with further development of technology and availability of data from future prospective randomized trials<sup>[38,41-43]</sup>.

## MOLECULARLY TARGETED THERAPIES

Recently, molecularly targeted therapies, including sorafenib, sunitinib, brivanib, cetuximab, erlotinib plus bevacizumab, and lapatinib, have emerged as promising therapeutic approaches for advanced HCC<sup>[44,45]</sup>. Sorafenib, as an orally-active multikinase inhibitor targeting both tumor cells and the tumor vasculature, and the first agent to improve the overall survival status for patients with advanced HCC, has been approved for systemic therapy in patients with advanced HCC in Eastern and Western countries<sup>[3,46-48]</sup>. Many other molecularly targeted agents of blocking epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptor (VEGFR), platelet-derived growth factor receptor (PDGFR), and mammalian target of rapamycin (mTOR) are at different stages of clinical development for the treatment of advanced HCC<sup>[49-51]</sup>.

## CONCLUSION

For the treatment of intermediate-advanced HCC, various surgical procedures may produce the definite therapeutic effects. The interventional treatment can also improve the prognosis to a great extent, but so far there is still lack of a special effective approach. In recent years, the model of comprehensive therapies mainly based on surgical

resection has been adopted to further enhance the curative effect, prolong the survival time, and improve the life quality of the patients. According to the indications and advantages of each therapeutic method, combined with the patient's clinical stage, the selection of therapeutic approaches to maximize the efficacy and minimize the adverse effect is very important for designing a more rational therapeutic plan for intermediate-advanced HCC.

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## How to assess the severity of atrophic gastritis

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### Abstract

Atrophic gastritis, is the main consequence of long-standing *Helicobacter pylori* infection, and is linked to the development of gastric cancer. The severity of atrophic gastritis is related to the lifetime risk of gastric cancer development, especially in terms of its degree and extent of mucosal damage. Therefore, it is important for clinicians to assess the severity of atrophic gastritis, interfere with the disease progress, and reverse gastric mucosal atrophy. In the article, we demonstrated some methods (conventional endoscopy, modern endoscopic technology and noninvasive methods) that may help assess the severity of atrophic gastritis and select the reasonable treatment protocols.

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**Key words:** Atrophic gastritis; Endoscopy; Pepsinogen

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### INTRODUCTION

Atrophic gastritis (AG) is a histopathological entity that is characterized by chronic inflammation of the gastric mucosa with loss of gastric glandular cells and replacement by intestinal-type epithelium, pyloric-type glands, and fibrous tissue. Atrophy of the gastric mucosa is the endpoint of chronic processes, such as chronic gastritis associated with *Helicobacter pylori* (*H. pylori*) infection, other unidentified environmental factors, and autoimmunity directed against gastric glandular cells<sup>[1]</sup>. It has been established that people with AG have a high risk for gastric cancer<sup>[2,3]</sup>, and it has been reported that about 10% of the patients with moderate-severe AG will develop gastric malignancies during a mean follow-up of 7.8 years<sup>[4]</sup>. Thus, the assessment of the severity of AG may be an important challenge for the management of these patients because its features (i.e. extension of atrophy and intestinal metaplasia, and hypochlorhydria) may be considered as potential surrogate markers for the increased risk for gastric cancer. Here, we demonstrate some methods used to assess the severity of AG.

### DEFINITION AND CLASSIFICATION OF AG

Gastric mucosal atrophy is defined as the loss of appropriate glands, which occurs when glands damaged by inflammation are replaced either by connective tissue (scarring) or by glandular structures inappropriate for location (metaplasia). Most often, as in the antral mucosa, the metaplastic transformation assumes the phenotype of the glands lined by intestinal-type epithelium (IM), but in the oxyntic mucosa, it may also take the form of mucin-secreting antral glands (pseudopyloric metaplasia)<sup>[5]</sup>. Traditionally, AG can be divided into gastric body atrophy



and sinuses ventriculi atrophy: the former is mostly associated with autoimmune diseases, and the latter is often associated with *H. pylori* infection<sup>[6,7]</sup>. However, in general practice, the diagnosis of atrophy and IM is troublesome due to an unsatisfactory interobserver agreement among pathologists, therefore in 2000, an international group of pathologists from Atrophy Club reviewed once again the spectrum of gastric atrophy and IM, and proposed a simplified definition of atrophy, which includes a metaplastic and a non-metaplastic category, thus making metaplasia an absolute concept to demonstrate the severity of the disease<sup>[5]</sup>.

## CONVENTIONAL ENDOSCOPY AND AG

In 2003, the Chinese Society of Digestive Endoscopy established endoscopic criteria for chronic gastritis in Dalian meeting. The scar lesions were characterized by the following attributes: mucosal atrophy, granular mucosa, flattened folds, gray intestinal-type epithelium and blood vessel permeability. AG was classified into three patterns of ridges: (1) fine granular mucosa, permeability of some blood vessels and a single nodule of gray intestinal-type epithelium; (2) medium granular mucosa, permeability of blood vessels, multiple nodules of gray intestinal-type epithelium; and (3) coarse granular mucosa, blood vessels can be seen up to the surface, diffuse nodules of gray intestinal-type epithelium<sup>[8]</sup>.

## MAGNIFYING ENDOSCOPY AND AG

Magnifying endoscopy has been developed to visualize the microstructure of gastrointestinal surface mucosa and mucosal vascularity, which provides a magnified image of up to 200 times<sup>[9]</sup>. The pit patterns observed on the mucosal surface are considered to reflect the arrangement and structure of surface epithelia, morphology, number, distribution and function of glands, mucosal edema and inflammation, and vascular morphology, arrangement, number and distribution. The basic units of the microstructures on the surface of gastric mucosa are countless gastric pits that form gastric areas separated by minor gastric grooves (also called interval grooves). As the openings of glands, gastric pits are the first to undergo structural change due to gastric mucosal lesions. Yagi *et al*<sup>[10]</sup> thought that the presentation of gastric mucosal atrophy was that gastric pit became white, expanded in size, and was surrounded by areas of erythema. In the study of Sakaki *et al*, magnifying endoscopy patterns of gastric erosion pits were classified into six types: A (round spot pits), B (short rod pits), C (sparsely and thickly linear), D (patchy), E (villous) and F (unclear or disappearance of pits or abnormal hyperplasia blood capillary)<sup>[11]</sup>. Yuan *et al*<sup>[12]</sup> used magnifying endoscopy in combination with methylene blue staining to examine the microstructures of gastric mucosa in 180 patients with gastric erosion. Their results showed that types A and B were found in normal gastric mucosa, while types C-F were found in gastric mucosa with active inflammation, atrophic

inflammation, intestinal metaplasia and dysplasia of varying degrees. Type E mucosa (81.8%) suggested intestinal metaplasia, type F indicated existence of dysplasia (86.3%), and type F with abnormal hyperplasia blood capillary suggested dysplasia (89.9%).

## MAGNIFYING NARROW-BAND IMAGING AND AG

Narrow-band imaging (NBI) is an endoscopic imaging technique for the enhanced visualization of mucosal microscopic structure and capillaries of the superficial mucosal layer. Images are obtained using narrower bands of red, blue and green filters, which are different from conventional red-green-blue filters<sup>[13]</sup>. Combining the NBI system and magnifying endoscopy allows for simple and clear visualization of microscopic structures of the superficial mucosa and its capillary patterns<sup>[14]</sup>. In the study of Tahara *et al*<sup>[15]</sup>, gastric mucosal patterns seen with magnifying NBI in uninvolved gastric corpus were divided into the following categories: normal small, round pits with regular subepithelial capillary networks; type 1, slightly enlarged, round pits with unclear or irregular subepithelial capillary networks; type 2, obviously enlarged, oval or prolonged pits with increased density of irregular vessels; and type 3, well-demarcated oval or tubulovillous pits with clearly visible coiled or wavy vessels. They found that the mucosal patterns were associated with the degree of endoscopic gastric atrophy. As mucosal patterns advanced from normal to types 1, 2 and 3, the degree of endoscopic gastric mucosal atrophy increased simultaneously. The sensitivity and specificity for types 1, 2 and 3 for detection of *H. pylori* infection and type 3 for detection of intestinal metaplasia were 95.2%, 82.2%, 73.3%, and 95.6%, respectively. Uedo *et al*<sup>[16]</sup> found in their study that the appearance of a light blue crest on the epithelial surface was correlated with histological evidence of intestinal metaplasia with a sensitivity of 89% (95% CI: 83-96), specificity of 93% (95% CI: 88-97), positive predictive value of 91% (95% CI: 85-96), negative predictive value of 92% (95% CI: 87-97), and accuracy of 91% (95% CI: 88-95).

## AUTO-FLUORESCENCE IMAGING VIDEOENDOSCOPY AND AG

Auto-fluorescence imaging (AFI) produces real-time pseudocolor images based on natural tissue auto-fluorescence emitted by light excitation from endogenous fluorophores such as collagen, nicotinamide, adenine dinucleotide, flavin and porphyrins. AFI enables the detection of mucosal features not visible with conventional endoscopy, therefore, it might improve the identification and characterization of the premalignant status in gastric mucosa<sup>[17,18]</sup>.

The fluorescence is almost purple, weaker in the normal gastric gland mucosa than that in the pyloric gland mucosa.



When gastric mucosa is atrophic, the color is green, which is the same as that in the pyloric gland mucosa. Gastric biopsy is taken separately from purple and green region for pathological studies, and the green region is significantly increased in AG and intestinal metaplasia<sup>[19]</sup>. The extent of chronic atrophic fundal gastritis (CAFG) was considered to be the green areas in the gastric body and was classified into six categories by Inoue *et al.*<sup>[20]</sup>: AF-C-I, the entire gastric body appears purple to dark green; AF-C-II, a color border on the lesser curvature was observed at a lower part of the gastric body; AF-C-III, a color border on the lesser curvature at an upper part of the gastric body; AF-O-I, a color border between the lesser curvature and the anterior wall; AF-O-II, a color border between the anterior wall and the greater curvature; and AF-O-III, a color border on the greater curvature proximal to the lower gastric body. They found that the diagnostic accuracy of green areas in the gastric body of the patients in the activity, inflammation, atrophy and intestinal metaplasia was 64%, 93%, 88% and 81%, respectively. However, the diagnostic accuracy of AFI was not compared with that of white-light images in relation to the histology. Therefore, whether the accuracy of AFI is superior to that of white-light images is not known.

## SERUM BIOMARKERS AND AG

### Pepsinogen I and II

Pepsinogens (PGs) are aspartic proteinases that are mainly secreted by gastric cells. They can be immunologically classified into two major types: pepsinogen I (PG I) and pepsinogen II (PG II). PGI is secreted only from the gastric fundic mucosa, whereas PG II is secreted from the cardiac, fundic and antral mucosa of the stomach, and also from the duodenal mucosa<sup>[21]</sup>. Patients with gastric fundic atrophy have a lower mean serum PG I concentration than those without atrophy. Both mucosal types secrete PG II, however, serum PG II levels remain stable or are increased during progression from a normal stomach to one with severe atrophy<sup>[22]</sup>. The net effects of severe atrophy on serum PG concentrations are lower PGI and a stable or increased PG II, and this leads to a lower PG I / II ratio<sup>[22]</sup>. Ren *et al.*<sup>[23]</sup> have confirmed a strong association between gastric fundic atrophy and PGs, as estimated by a low serum PGI and PG I / II ratio in a prospective study. They have found that compared to the subjects with a PG I / II ratio of > 4, those with a ratio ≤ 4 had hazard ratios (HRs) of 2.72 (95% CI: 1.77-4.20) and 2.12 (95% CI: 1.42-3.16) for non-cardiac and cardiac gastric adenocarcinoma, respectively. Storskrubb *et al.*<sup>[24]</sup> found that the phenotype of gastritis is characterized by normal levels of serum PGs (PG I ≥ 25 ng/mL and PG I / PG II ratio ≥ 3 indicate that the corpus mucosa is normal). For the diagnosis of atrophic corpus gastritis, three different criteria have been used as follows<sup>[25-27]</sup>: Mild: PG I ≤ 70 ng/mL and PG I / II ratio ≤ 3.0; Moderate: PG I ≤ 50 ng/mL and PG I / II ≤ 3.0; Strict: PG I ≤ 30 ng/mL and PG I / II ≤ 2.0. Both cut-offs for PG I and PG I / II should be fulfilled at the same time for each criterion.

### Gastrin-17

Gastrin-17 (G-17) is secreted exclusively by the G-cells of

the gastric antrum. The levels of G-17 are depressed in cases of atrophy in this area<sup>[28]</sup>. Leja *et al.*<sup>[29]</sup> found that G-17 < 5 pmol/L is related to atrophy in the antral region ( $P = 0.007$ ) with a 36.8% sensitivity and a 86.5% specificity. They indicated that G-17 used for the detection of atrophy in the antral part of the stomach requires further evaluations due to its low sensitivity.

### *H. pylori* testing

*H. pylori* is now recognized as a major cause of gastric cancer and is classified as a group I carcinogen by the WHO<sup>[30,31]</sup>. *H. pylori* infection causes persistent chronic gastritis, which in susceptible individuals can progress to atrophy, intestinal metaplasia and dysplasia, and finally, intestinal-type gastric cancer<sup>[31,32]</sup>. Nearly all infected individuals (> 90%) exhibit *H. pylori*-specific IgG antibodies. Most (70%) of these individuals also exhibit IgA antibodies and *H. Pylori* proteins, including cytotoxin-associated gene A (CagA) protein and vacuolating cytotoxin A (VacA) protein. These proteins are used for *H. pylori* testing. A combined use of the serological biomarkers (PGI, PGII, G-17 and *H. pylori* antibodies) shows a high accuracy as a noninvasive method to diagnose gastric atrophy, which is common in the general population<sup>[23,24,33]</sup>.

## CONCLUSION

In the cases of AG, its severity is mainly related to the lifetime risk to develop gastric cancer, especially in terms of the degree and extension of mucosal damage. The application of conventional endoscopy, modern endoscopic technology and noninvasive methods is useful for the identification of those patients with atrophic gastritis at higher risk for gastric malignancies. Using these technologies to assess the severity of atrophic gastritis, interfering with the disease progress, and reversing gastric mucosal atrophy are the important issues for clinicians.

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## Legalon-SIL downregulates HCV core and NS5A in human hepatocytes expressing full-length HCV

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### Abstract

**AIM:** To determine the effect of Legalon-SIL (LS) on hepatitis C virus (HCV) core and NS5A expression and on heme oxygenase-1 (HMOX-1) and its transcriptional regulators in human hepatoma cells expressing full length HCV genotype 1b.

**METHODS:** CON1 cells were treated with 50  $\mu\text{mol/L}$  or 200  $\mu\text{mol/L}$  LS. Cells were harvested after 2, 6 and 24 h. HCV RNA and protein levels were determined by quantitative real-time polymerase chain reaction and Western blotting, respectively.

**RESULTS:** HCV RNA (core and NS5A regions) was

decreased after 6 h with LS 200  $\mu\text{mol/L}$  ( $P < 0.05$ ). Both 50 and 200  $\mu\text{mol/L}$  LS decreased HCV RNA levels [core region (by 55% and 88%, respectively) and NS5A region (by 62% and 87%, respectively) after 24 h compared with vehicle (dimethyl sulphoxide) control ( $P < 0.01$ ). Similarly HCV core and NS5A protein were decreased (by 85%,  $P < 0.01$  and by 65%,  $P < 0.05$ , respectively) by LS 200  $\mu\text{mol/L}$ . Bach1 and HMOX-1 RNA were also downregulated by LS treatment ( $P < 0.01$ ), while Nrf2 protein was increased ( $P < 0.05$ ).

**CONCLUSION:** Our results demonstrate that treatment with LS downregulates HCV core and NS5A expression in CON1 cells which express full length HCV genotype 1b, and suggests that LS may prove to be a valuable alternative or adjunctive therapy for the treatment of HCV infection.

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**Key words:** Hepatitis; Hepatitis C virus; Silymarin; Silybin; Genotype; Huh7.5; CON1

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### INTRODUCTION

Over 170 million people are infected by hepatitis C virus (HCV) worldwide, and of these, approximately 85% will develop chronic hepatitis C (CHC). This could potentially



lead to fibrosis, cirrhosis, end-stage liver disease and hepatocellular carcinoma<sup>[1]</sup>. The ultimate goal of antiviral treatment for hepatitis C is the sustained elimination of HCV. Currently, the standard of care for individuals with CHC is pegylated interferon (IFN)  $\alpha$ -2a or IFN  $\alpha$ -2b plus ribavirin. However, this protocol is far from ideal. Even under the best conditions of sponsored clinical trials, sustained virologic responses have been achieved in only 40%-50% of those with HCV genotype 1 infection<sup>[2,3]</sup>. Furthermore, serious side effects are associated with this therapy. The paucity of effective and affordable treatments for HCV-infected patients has led scientists to seek alternative therapies. At present, novel therapeutic agents with various mechanisms of action are under development or in clinical trials.

Although the precise mechanisms underlying hepatocellular injury associated with HCV has yet to be determined, there is compelling evidence that HCV produces increased oxidative stress in human liver cells that is linked to the production of reactive oxygen species (ROS), and consequent increases in cellular lipid peroxidation and other oxidative damage. Oxidative stress appears to be an important aspect of HCV-induced hepatocellular injury<sup>[4]</sup>. Microsomal heme oxygenase-1 (HMOX-1) is an inducible cytoprotective enzyme that catalyzes the initial and rate-limiting reaction in heme catabolism to release free iron and equimolar amounts of carbon monoxide and biliverdin<sup>[5,6]</sup>. A variety of DNA-binding proteins interact with regions that contain multiple antioxidant response elements (ARE). Among these are nuclear factor erythroid 2-related factor 2 (Nrf2) and Bach1, a leucine b-zipper transcription protein, which form heterodimers with the small Maf proteins<sup>[7,8]</sup>. Nrf2 is known to be associated with activation of HMOX-1 and numerous other antioxidant genes in response to multiple agents<sup>[9]</sup>, while Bach1 is a negative regulator of HMOX-1<sup>[10]</sup>.

Milk thistle (*Silybum marianum*) has been used since ancient times as a liver tonic. Silymarin (SI), a purified extract of polyphenolic flavonoids isolated from milk thistle, is composed mainly of silychristin, silydianin, silybin A, silybin B, isosilybin A and isosilybin B. After oral administration, the SI flavonolignans are rapidly metabolized<sup>[11]</sup>. Silybin (SBN) constitutes approximately 50% of SI and is the most biologically active component<sup>[12]</sup>. A number of studies have shown that SI has potent antioxidant and immunomodulatory effects in addition to numerous metabolic actions that may contribute to its purported hepatoprotective actions<sup>[13-15]</sup>.

We recently showed that SI downregulates HCV RNA (core region) and protein in CNS3 cells that stably express HCV RNA core to the amino terminal of NS3 proteins<sup>[16]</sup>. Another recent study *in vitro* showed that SI exerts antiviral and antiinflammatory effects in hepatoma cell lines expressing the HCV full length genome of genotype 2a<sup>[17]</sup>. On the other hand, a randomized, double-blind, placebo-controlled study administering oral SI to CHC patients failed to show a significant effect on either serum aminotransferase levels or quality-of-life measures<sup>[18]</sup>.

Legalon-SIL (LS) is a form of SBN which is a water-soluble formulation of the dihydro-succinate sodium salt of SBN A and SBN B in equal proportion. Recent results

from a pilot study in patients with chronic HCV using LS indicate that some SI flavonolignans may have antiviral activity<sup>[19]</sup>. In this study we assessed the effects of LS on HCV RNA and protein levels in cell lines expressing the full length genome of HCV genotype 1b. We also determined the effects of LS on HMOX-1, Bach1, and Nrf2 expression in these cells.

## MATERIALS AND METHODS

### Chemicals and antibodies

LS was obtained from Rottapharm-Madaus (Italy). Dimethyl sulphoxide (DMSO) was purchased from Thermo Fisher Scientific Inc (Rockford, IL, USA). A 100 mmol/L LS stock solution (molecular weight = 726) was prepared in DMSO and filtered through a 0.2  $\mu$ mol/L nylon filter. LS was prepared fresh just prior to use in each experiment. Mouse monoclonal antibody against HCV core protein was purchased from Abcam (Cambridge, MA, USA). Mouse monoclonal antibody against HCV NS5A was purchased from Virogen Corporation (Watertown, MA, USA). Rabbit polyclonal antibody against HMOX-1 was purchased from Stress Gene (Ann Arbor, MI, USA). Goat monoclonal antibody against Bach1, rabbit polyclonal antibody against Nrf2, and mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) monoclonal antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Enhanced chemiluminescence (ECL)-Plus Western blotting detection reagent was obtained from Amersham Biosciences (Piscataway, NJ, USA).

### Cell cultures and treatments

Huh-7.5 and CON1 subgenomic genotype 1b HCV cell lines were from Apath LLC (St. Louis, MO, USA). Huh-7.5 is a highly permissive, IFN-cured Huh-7 human hepatocellular carcinoma cell line derivative. The CON1 cell line is a Huh-7.5 cell population containing the full-length HCV genotype 1b replicon.

Huh-7.5 and CON1 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100  $\mu$ g/mL streptomycin, and selection antibiotic for CON1 cells (750  $\mu$ g/mL G418).

### Colorimetric MTT assay

Cellular proliferation of treated CON1 cells was assessed by measuring the conversion of MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] to MTT formazan (Sigma-Aldrich). The absorbance was measured on a Synergy HT microtiter plate reader (Biotek Instruments, Winooski, VT, USA), at a wavelength of 570 nm with background subtraction at 690 nm. Decreases in absorption were taken as an index of decreased cellular proliferation.

### Propidium iodide assay

The viability of CON1 cells treated with LS was also confirmed by the standard propidium iodide [(PI); Invitrogen,



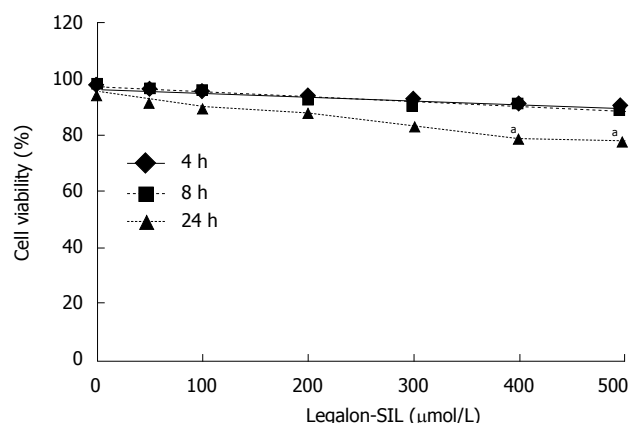
USA)] assay. The experiment was performed according to the manufacturer's recommended protocol. CON1 cells were plated in 12-well plates 24 h before treatment and incubated at 37°C. LS was dissolved in DMSO and added to the cell culture medium. The effects of LS on cell viability were studied at various concentrations (0, 50, 100, 200, 300, 400 and 500)  $\mu\text{mol/L}$  and at different time points (4, 8, and 24 h). Percent cell viability was determined by counting cell density in drug-treated cells and in DMSO-treated cells as control in the same incubation period [percentage of cellular viability = (total cell count-PI positive cell count)/total cell count\*100]. All experiments were repeated 3 times.

### RNA isolation and quantitative reverse transcriptase polymerase chain reaction

RNA from treated cells was isolated by TRIZOL reagent (Invitrogen). The RNA concentration and purity were determined by measuring absorbance at 260/280 nm. Reverse transcription was performed on 1  $\mu\text{g}$  of total RNA to generate cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Quantitative real time reverse transcriptase (RT)-polymerase chain reaction (PCR) was performed using a CFX-96 Real-Time PCR Detection System (Bio-Rad Laboratories) and iQTM SYBR Green Supermix (Bio-Rad Laboratories). Sequence specific primers for HMOX-1, Bach1, and GAPDH were designed as described<sup>[20]</sup>. Nucleotide sequences of other primers are as follows: NS5A: Forward primer, 5'-CG-GACGTAGCAGTGCTCACTTC-3' and reverse primer, 5'-TGATGAGCTGGCCAAGGAGG-3'; Nrf2: Forward primer, 5'-CCTTCTCGCCTAGGCATCA-3', reverse primer, 5'-CCCTTCAGCTCTCCCTACCG-3'. Fold change values were calculated by comparative cycle threshold (Ct) value analysis after normalizing for the quantity of GAPDH mRNA in samples.

### Protein preparation and Western blotting

Cells were grown to near confluence and washed with phosphate-buffered saline (PBS), lysed in a buffer containing 1% Triton X-100 with PBS and Halt Protease Inhibitor Cocktail (Pierce Chemicals, Rockford, IL, USA). Protein concentrations were measured using the bicinchoninic acid method. Total proteins (10  $\mu\text{g}$ ) were separated on 4%-12% gradient sodium dodecyl sulphate-polyacrylamide gel (Invitrogen Laboratories) and electrophoretically transferred onto an Immun-Blot PVDF (Invitrogen Laboratories). The membranes were blocked for 1 h in PBS containing 5% nonfat dry milk, and then incubated for 1 h with the primary antibody at room temperature. The dilutions of the primary antibodies were as follows: 1:1000 for anti-HCV core antibody; 1:1000 for anti-HCV NS5A antibody; 1:500 for anti-HMOX-1, and 1:1000 for anti-Bach1, anti-Nrf2, and anti-GAPDH antibody. After 4 washes with 0.1% Tween 20 in PBS (PBS-T), the membranes were incubated for 1 h with a secondary antibody (anti-rabbit, anti-goat or anti-mouse immunoglobulin G; dilution 1:10 000). Finally, the membranes were washed 4 times with PBS-T, and the



**Figure 1** Effects of legalon-SIL on CON1 cell viability. Cellular viability of CON1 cells was measured using the propidium iodide assay after 4, 8, and 24 h of exposure to legalon-SIL (LS) at varying concentrations ranging from 0-500  $\mu\text{mol/L}$ . The mean  $\pm$  SE from 3 independent experiments. <sup>a</sup> $P < 0.05$  vs DMSO control.

bound antibodies were visualized with the ECL-Plus chemiluminescence system. A computer based imaging system, LAS 3000 (Fuji Film, USA) was used to measure the relative optical density of each specific band obtained after Western blotting.

### Statistical analysis

Data are expressed as mean  $\pm$  SE of the mean. Statistical differences between groups were analyzed by analysis of variance followed by Dunnett's test.  $P < 0.05$  was considered significant.

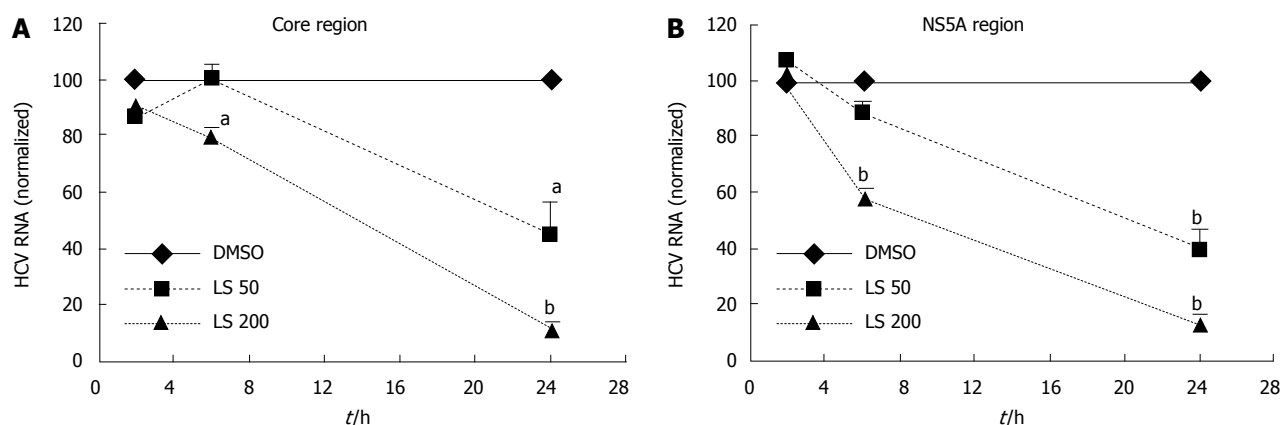
## RESULTS

### Cytotoxicity of Legalon-SIL in CON1

Appropriate doses of LS in the cell lines were determined. Effects of different doses of LS on cell viability in CON1 cells were assessed by PI staining (Figure 1). LS concentrations of 50-300  $\mu\text{mol/L}$  had no significant effect on cell viability, whereas concentrations equal to or more than 400  $\mu\text{mol/L}$  caused significant cytotoxicity in the cells ( $P < 0.05$ ). Similar results were demonstrated with the MTT proliferation assay (data not shown).

### Legalon-SIL downregulates HCV RNA as well as HCV core and NS5A proteins in CON1 cells

LS downregulated HCV RNA (core region) in a dose-dependent and also a time-dependent manner in CON1 cells. The HCV RNA (core region) level was decreased 21% following 6 h treatment with LS 200  $\mu\text{mol/L}$  compared with the DMSO control ( $P < 0.05$ , Figure 2A). HCV RNA (core region) levels were further decreased after 24 h treatment by both LS 50  $\mu\text{mol/L}$  (55% decrease,  $P < 0.05$ ) and 200  $\mu\text{mol/L}$  (88% decrease,  $P < 0.01$ ) when compared with vehicle (DMSO) control (Figure 2A). The HCV RNA (NS5A region) level was also decreased 43% following 6 h treatment with LS 200  $\mu\text{mol/L}$  compared with DMSO control ( $P < 0.01$  Figure 2B), and was also further decreased after



**Figure 2** Time course of effects of legalon-SIL on hepatitis C virus RNA in CON1 cells. CON1 cells were grown to near confluence and the medium was changed to 5% fetal bovine serum (FBS) plus Dulbecco's modified Eagle's medium, then treated with vehicle only (DMSO) or 50 or 200  $\mu\text{mol/L}$  legalon-SIL (LS). The cells were harvested after 2, 6, and 24 h after treatment. The levels of hepatitis C virus (HCV) RNA [core region (A) and NS5A region (B)] were quantified using qRT-PCR as described in "Materials and Methods". The amounts of HCV RNA were normalized to glyceraldehyde-3-phosphate dehydrogenase. A: LS 50  $\mu\text{mol/L}$  downregulated HCV RNA (core region) after 24 h. LS 200  $\mu\text{mol/L}$  downregulated HCV RNA (core region) after 6 and 24 h; B: LS 50  $\mu\text{mol/L}$  downregulated HCV RNA (NS5A region) after 24 h. LS 200  $\mu\text{M}$  downregulated HCV RNA (core region) after 6 and 24 h. Data for RNA levels are mean  $\pm$  SE ( $n = 3$  independent experiments). <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs DMSO control.

24 h treatment by both LS 50  $\mu\text{mol/L}$  (62% decrease,  $P < 0.01$ ) and 200  $\mu\text{mol/L}$  (87% decrease,  $P < 0.01$ ) (Figure 2B). LS 200  $\mu\text{mol/L}$  also downregulated HCV core (by 57%) and NS5A protein (by 49%) after 24 h of treatment although this was statistically significant only for HCV core protein,  $P < 0.05$ ). This effect was more pronounced following 48 h of treatment: LS 200  $\mu\text{mol/L}$  decreased HCV NS5A protein expression by 65% ( $P < 0.05$ ), while LS significantly decreased HCV core protein expression in a dose-dependent manner (52% reduction at 50  $\mu\text{mol/L}$  and 85% reduction at 200  $\mu\text{mol/L}$   $P < 0.01$ ) (Figure 3).

#### Legalon-SIL downregulates HMOX-1 and Bach1 mRNA levels in CON1 cells while it upregulates Nrf2 protein expression

HMOX-1 and Bach1 mRNA levels were significantly decreased following 24 h treatment by both LS 50  $\mu\text{mol/L}$  and 200  $\mu\text{mol/L}$  when compared with the DMSO control (HMOX-1 decreased by 40%,  $P < 0.01$ ; Bach1 decreased by 35%,  $P < 0.01$ ; Figure 4). LS treatment decreased Bach1 protein level, although not significantly, while it significantly increased Nrf2 protein expression ( $P < 0.05$ ) (Figure 5).

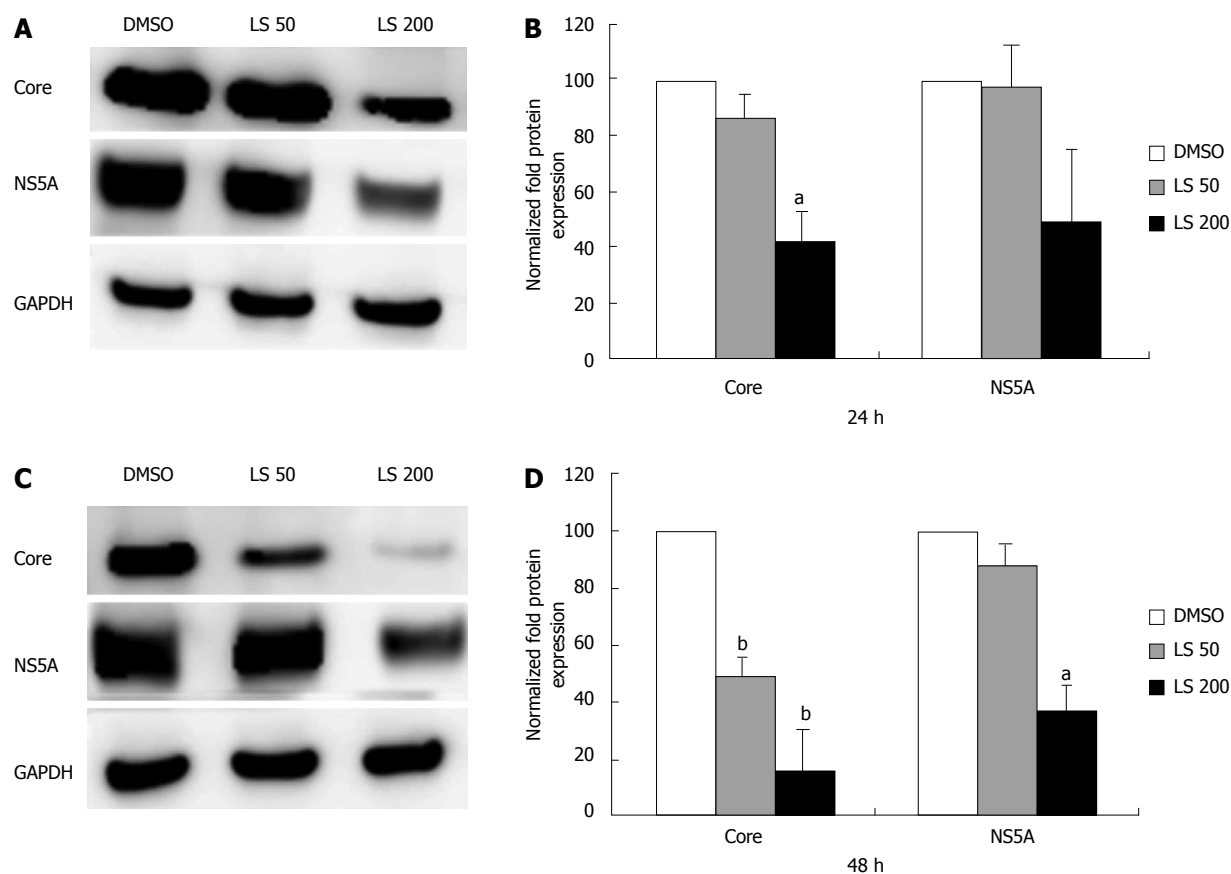
#### HMOX-1 expression is increased in CON1 cells in comparison with its expression in the parental Huh7.5 cell line

For investigation of possible effects of stable transfection of the HCV genome in CON1 replicon system, we compared HMOX-1, Bach1, and Nrf2 mRNA levels between CON1 cells and Huh7.5 cells, the 'parental' cell line. In untreated CON1 cells, the HMOX-1 mRNA level was 3-fold higher than in untreated Huh7.5 cells ( $P < 0.01$ ), while there was no significant difference in the level of Bach1 or Nrf2 mRNA between CON1 and Huh7.5 cells (Figure 6).

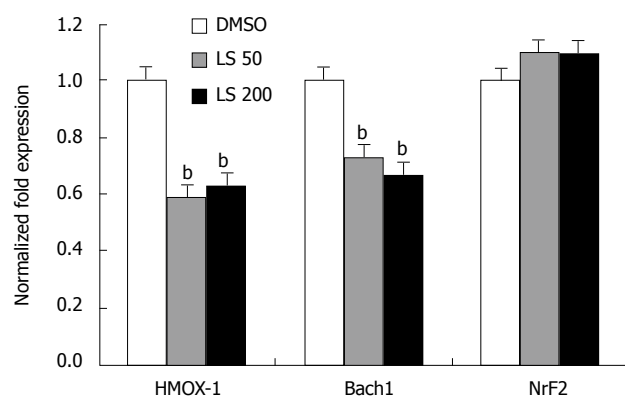
## DISCUSSION

The current treatment for CHC is a combination of pe-

gylated IFN- $\alpha$  and ribavirin which is effective only in 40%-50% of treated patients infected with genotype-1, by far the most frequent HCV genotype worldwide. This therapeutic regimen is expensive, prolonged (usually at least 48 wk) and causes serious side effects. Therefore investigations continue to search for alternative treatments for hepatitis C. SI is an herbal remedy that has been used to treat acute and chronic liver diseases for millennia<sup>[12-22]</sup>. Despite this broad use, the exact molecular mechanism by which SI confers hepatoprotection is yet to be elucidated. Ferenci *et al.*<sup>[19]</sup> recently showed that LS, as used in our studies, significantly reduced serum HCV RNA level in patients who had not responded to combination therapy with full dose pegylated IFN and ribavirin. They showed that SBN was effective only when administered intravenously, as LS, and that the antiviral effect was dose-dependent. They reported that HCV RNA was undetectable in 7 (out of 14) patients receiving 15 and 20 mg/kg SBN as LS. In a recent study *in vitro*<sup>[23]</sup>, SBN A, SBN B, a mixture of SBN A and SBN B, and LS were shown to inhibit HCV RNA-dependent RNA polymerase (RdRP) function and inhibited HCV genotype 1b sub-genomic replicon replication and HCV genotype 2a strain JFH1 replication in cell culture. Our results extend these findings showing that LS inhibits HCV replication in human hepatoma cells expressing full-length HCV genotype 1b. In this study, we also showed that LS doses equal to 400  $\mu\text{mol/L}$  and higher are cytotoxic for human hepatoma cells similar to other recent results<sup>[23]</sup>. The exact pharmacokinetics of SI and LS remain to be determined. However, it is suggested that oral doses of SI up to 2.1 g/d are safe and well-tolerated<sup>[24]</sup>. In the present study, we showed that LS started to downregulate HCV RNA in a dose-dependent manner after 2 h, but the significant effect was observed after 6 h of treatment. Although LS 200  $\mu\text{mol/L}$  was found to downregulate HCV RNA and proteins significantly in CON1 cells, LS 50  $\mu\text{mol/L}$  was also found to be effective in downregulation of HCV RNA.



**Figure 3** Effects of legalon-SIL on hepatitis C virus core and NS5A protein levels in CON1 cells after 24 and 48 h. CON1 cells were grown to near confluence and the medium was changed to 5% FBS plus Dulbecco's modified Eagle's medium, then treated with vehicle only (DMSO) or 50 or 200  $\mu\text{mol/L}$  legalon-SIL (LS). The cells were harvested 24–48 h after treatment. Total protein (10  $\mu\text{g}$ ) was separated on 4%–12% gradient sodium dodecyl sulphate-polyacrylamide gel electrophoresis and electrophoretically transferred onto an Immun-Blot PVDF. Hepatitis C virus core and NS5A protein levels were assessed through Western blotting. Representative Western blots, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-normalized quantifications are shown after 24 h (A, B), and 48 h (C, D). Data for protein levels are mean  $\pm$  SE ( $n = 3$  independent experiments). <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs DMSO control.

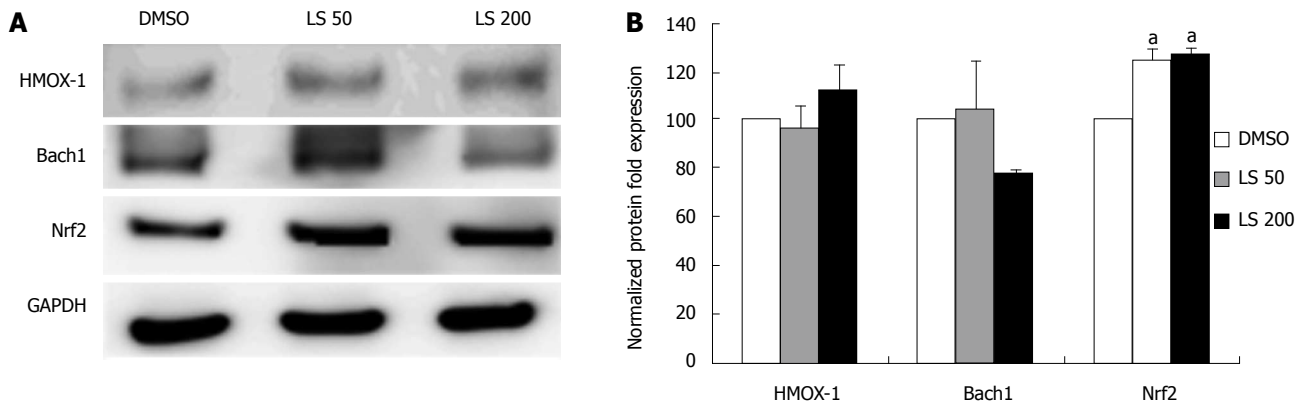


**Figure 4** Effects of legalon-SIL on heme oxygenase-1, Bach1, and nuclear factor erythroid 2-related factor 2 mRNA levels after 24 h. CON1 cells were grown to near confluence and medium was changed to 5% FBS plus Dulbecco's modified Eagle's medium, then treated with vehicle only (DMSO) or 50 or 200  $\mu\text{mol/L}$  legalon-SIL (LS). The cells were harvested 24 h after treatment. The levels of HMOX-1, Bach1, and nuclear factor erythroid 2-related factor 2 (Nrf2) mRNA were quantified using quantitative reverse transcriptase polymerase chain reaction as described in "Materials and Methods". The amounts of HMOX-1, Bach1, and Nrf2 mRNA were normalized to glyceraldehyde-3-phosphate dehydrogenase. Data are presented as the mean  $\pm$  SE experiments were performed 3 times. <sup>b</sup> $P < 0.01$  vs DMSO control.

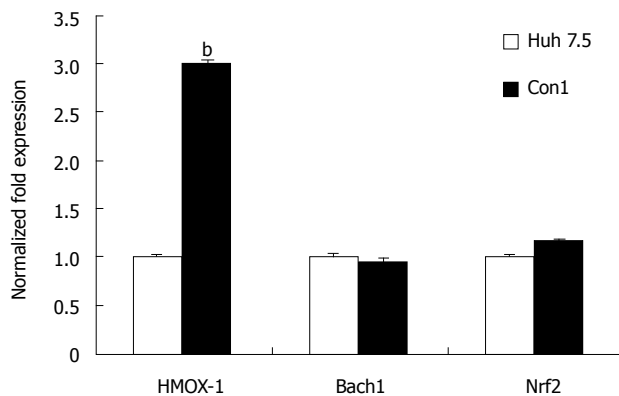
In addition to a direct effect of LS on HCV replication,

for instance inhibition of RdRP, LS might downregulate HCV through interaction with signaling molecules or other as yet unknown host factors. For example, Polyak *et al*<sup>[25]</sup> reported that SI suppresses TNF- $\alpha$  activation of nuclear factor- $\kappa$ B-dependent transcription, without affecting binding of p50 and p65 to DNA. They also reported that all SI-related compounds which they studied blocked JFH-1 virus-induced oxidative stress, including compounds that lacked antiviral activity.

In the present study, we found that LS decreased HMOX-1 mRNA in CON1 cells as well as the mRNA level of Bach1, which is its transcriptional repressor. However, no effect was observed on HMOX-1 and Bach1 protein levels, despite modest upregulation of Nrf2 protein. Bach1 and Nrf2 compete for binding to the ARE and exert their Bach1-induced repressive, or Nrf2-induced activating effects on HMOX-1 transcription. It is noteworthy to mention that for Nrf2 to bind to ARE, Bach1 needs to be dislocated from the binding sites<sup>[26]</sup>. Therefore, a lower level of Bach1 is necessary for the maximal effect of Nrf2 on HMOX-1 transcription. As our study did not show downregulation of Bach1 protein, it is possible to hypothesize that Bach1 does not dissociate from ARE to allow Nrf2 to bind. Therefore HMOX-1 is not upregulated despite mod-



**Figure 5** Effects of legalon-SIL on HMOX-1, Bach1, and Nrf2 protein levels in CON1 cells after 48 h. CON1 cells were grown to near confluence and the medium was changed to 5% FBS plus Dulbecco's modified Eagle's medium, and then treated with vehicle only (DMSO) or 50 or 200  $\mu\text{mol/L}$  legalon-SIL (LS). The cells were harvested 48 h after treatment. Total protein (10  $\mu\text{g}$ ) was separated on 4%-12% gradient sodium dodecyl sulphate-polyacrylamide gel electrophoresis and electrophoretically transferred onto an Immun-Blot PVDF. HMOX-1, Bach1, and Nrf2 protein levels were assessed through Western blotting, and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). A: Representative Western blotting. B: Results of these independent experiments quantified and normalized to GAPDH. Data for protein levels are mean  $\pm$  SE ( $n = 3$  independent experiments). <sup>a</sup> $P < 0.05$  vs DMSO control.



**Figure 6** HMOX-1, Bach1, and Nrf2 mRNA levels in CON1 vs Huh 7.5 cells. The levels of HMOX-1, Bach1, and Nrf2 mRNA in untreated Huh 7.5 and CON1 cells were quantified using quantitative reverse transcriptase polymerase chain reaction as described in "Materials and Methods". The amounts of HMOX-1, Bach1 and Nrf2 mRNA were normalized to GAPDH. Data for mRNA levels are mean  $\pm$  SE. <sup>b</sup> $P < 0.01$  vs Huh7.5.

est elevation of Nrf2 protein. Additionally, other HMOX-1 transcriptional factors may be affected by LS (e.g. changes in Keap1 or Kelch, glutathione, other thiol-containing compounds, levels of heme, other metalloporphyrins, *etc.*) which were not all examined in this study<sup>[27]</sup>.

Oxidative stress plays an important role in various diseases, including viral infection and chronic inflammation. HCV gene expression, in particular HCV RNA (core region) expression, can increase the levels of ROS<sup>[28]</sup>, and therefore upregulate HMOX-1<sup>[20,29]</sup>. We also compared HMOX-1 mRNA levels between CON1 cells and its parental cell line (Huh7.5), and we identified a 3-fold upregulation of HMOX-1 in the HCV replicon system compared with the parental cell line. Although SBN is a well-known antioxidant and cytoprotective enzyme, and upregulation of Nrf2 protein could be attributed to this antioxidant effect of LS, lack of HMOX-1 upregulation could be explained by anti-HCV activity of LS. Since HMOX-1 is already

increased by HCV infection in CON1 cells, its level is decreased toward normal levels as LS attenuates HCV infection. This downregulatory effect of LS on HMOX-1 seems to be more pronounced at the RNA level, and is compensated by an Nrf2 increase at the protein level.

In conclusion, LS downregulates HCV in hepatoma cell lines expressing full length HCV genotype 1b. LS treatment is also associated with downregulation of HMOX-1 mRNA and upregulation of Nrf2 protein. Further research is needed to further delineate mechanisms of the LS effect on HCV replication and on CHC.

## COMMENTS

### Background

Chronic hepatitis C virus (HCV) infection is a global health problem, and one causal factor for development of liver cirrhosis and hepatocellular carcinoma. The current therapy for HCV is interferon (IFN)  $\alpha$ -2a or IFN $\alpha$ -2b plus ribavirin which is effective in only 40%-50% of those with HCV genotype 1 infection. Furthermore, serious side effects are associated with this therapy. The paucity of effective and affordable treatments for HCV-infected patients has led scientists to seek alternative therapies.

### Research frontiers

Silymarin, also known as milk thistle extract, inhibits HCV infection and also displays antioxidant, antiinflammatory, and immunomodulatory actions that contribute to its hepatoprotective effects. In this study the authors demonstrated that Legalon-SIL (LS), a water-soluble formulation of silybin A and silybin B downregulates expression of HCV mRNA and protein in replicon CON1 cells which are human hepatoma cell lines stably transfected with full-length HCV genotype 1b.

### Innovations and breakthroughs

This report highlighted that LS decreases HCV core and NS5A expression. Additionally, the authors demonstrated that LS modulates expression of heme oxygenase-1 and its transcriptional regulators, Bach1 and Nrf2. This is the first study examining the effects of LS using a human hepatoma cell line expressing full-length HCV genotype 1b.

### Applications

Our results suggest that LS may prove to be a valuable alternative or adjunctive therapy for the treatment of HCV infection.

### Terminology

LS is a form of SBN which is a water-soluble formulation of the dihydro-succinate sodium salt of silybin A and silybin B in equal proportions.



# Peer review

The paper will appeal to clinicians as well as researchers involved in the field of elucidating the mechanisms of silymarin in HCV positive patients.

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## Endoscopic submucosal dissection for premalignant lesions and noninvasive early gastrointestinal cancers

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**Author contributions:** Hulagu S performed all ESD procedures; Senturk O assisted during the ESD procedure and literature review; Aygun C assisted during the ESD procedure, data collection and statistical analysis; Kocaman O, Celebi A, Konduk T, Koc D, Sirin G, Korkmaz U, Duman AE, Bozkurt N and Dindar G assisted during the ESD procedure and data collection; Attila T, Tarcin O and Kalayci C performed the endosonographic evaluation of patients; Gurbuz Y performed the histologic examination of ESD specimens.

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had premalignant lesions or non-invasive early cancers of the gastrointestinal tract and had endoscopic and histological diagnoses. Early cancers were considered to be confined to the submucosa, with no lymph node involvement by means of computed tomography and endosonography.

**RESULTS:** Sixty ESD procedures were performed. The indications were epithelial lesions ( $n = 39$ ) (33/39 adenoma with high grade dysplasia, 6/39 adenoma with low grade dysplasia), neuroendocrine tumor ( $n = 7$ ), cancer ( $n = 7$ ) (5/7 early colorectal cancer, 2/7 early gastric cancer), granular cell tumor ( $n = 3$ ), gastrointestinal stromal tumor ( $n = 2$ ), and leiomyoma ( $n = 2$ ). En bloc and piecemeal resection rates were 91.6% (55/60) and 8.3% (5/60), respectively. Complete and incomplete resection rates were 96.6% (58/60) and 3.3% (2/60), respectively. Complications were major bleeding [ $n = 3$  (5%)] and perforations [ $n = 5$  (8.3%)] (4 colon, 1 stomach). Two patients with colonic perforations and two patients with submucosal lymphatic and microvasculature invasion (1 gastric carcinoid tumor, 1 colonic adenocarcinoma) were referred to surgery. During a mean follow-up of 12 mo, 1 patient with adenoma with high grade dysplasia underwent a second ESD procedure to resect a local recurrence.

**CONCLUSION:** ESD is a feasible and safe method for treatment of premalignant lesions and early malignant gastrointestinal epithelial and subepithelial lesions. Successful en bloc and complete resection of lesions yield high cure rates with low recurrence.

**Key words:** Endoscopic submucosal dissection; Premalignant gastrointestinal lesion; Noninvasive early gastrointestinal cancer; Neuroendocrine tumor; Gastrointestinal stromal tumor

**Peer reviewers:** Marcela Kopacova, Associate Professor, MD, PhD, 2nd Department of Internal Medicine, Charles University

### Abstract

**AIM:** To investigate the indication, feasibility, safety, and clinical utility of endoscopic submucosal dissection (ESD) in the management of various gastrointestinal pathologies.

**METHODS:** The medical records of 60 consecutive patients (34 female, 26 male) who underwent ESD at the gastroenterology department of Kocaeli University from 2006-2010 were examined. Patients selected for ESD

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## INTRODUCTION

New developments in the optical technology of endoscopes allow early detection of mucosal abnormalities that are amenable to endoscopic therapies. Endoscopic therapies are used for premalignant lesions and noninvasive early cancers with low risk of lymph node metastasis. Endoscopic therapies include ablation and resection-based modalities. Resection-based modalities consist of endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD). The major advantage of resection-based modalities is the recovery of the specimen for histopathological analysis. A recent study has shown the long-term prognosis of complete en bloc EMR to be comparable to surgery for differentiated early gastric cancer with 10-year survival rates of 99%<sup>[1]</sup>. Although lesions with a diameter of less than 20 mm can be resected in an en bloc fashion with EMR, larger lesions can only be removed in a piecemeal fashion. It is difficult to have an accurate histopathological evaluation of a lesion that is removed in a piecemeal fashion. Furthermore, the risk of local recurrence after piecemeal resection is higher than that of en bloc resection<sup>[1-3]</sup>.

ESD is a newly developed technique that allows en bloc resection of larger (usually more than 20 mm) mucosal as well as subepithelial gastrointestinal lesions above the muscularis propria with the use of cutting devices. En bloc resection rate of ESD ranges between 83%-98%, which is significantly higher than EMR. Local recurrence rates range between 0%-3%<sup>[4]</sup>. However, compared to EMR, ESD is technically more challenging, requires higher endoscopic skills, is time consuming and has a prolonged learning curve<sup>[5-7]</sup>. Although ESD has been accepted in the armamentarium of endoscopic management of premalignant and noninvasive early gastrointestinal cancers in Japan and Asia, the Western experience with this new modality has been quite limited. This may be related to differences in the epidemiology of certain gastrointestinal diseases (e.g. gastric cancer), differences in technical expertise, due to its prolonged procedure time, due to its long learning curve, or possibly due to legal concerns, as well as procedural reimbursement. Our study is the first series of ESD cases from Turkey and among the few studies performed in Europe. The aim of this study is to describe indications, feasibility, safety, complications, and recurrence rate of the

mucosal and subepithelial ESD cases in the upper and lower gastrointestinal tract.

## MATERIALS AND METHODS

### Patients

From September 2006 to June 2010, a consecutive series of patients who underwent an ESD at a tertiary referral center (Kocaeli University Hospital) were reviewed. Premalignant lesions larger than 15 mm in size and noninvasive early cancers with low risk for lymph node metastasis larger than 10 mm were included in the study. The inclusion criteria for carcinoma were histological well differentiation, diameter of ulceration  $\leq$  30 mm, lack of submucosal invasion and lymph node involvement detected with computed tomography (CT) or endoscopic ultrasound (EUS). Prior to an ESD attempt, each case was reviewed by a team consisting of an oncologist, a general surgeon, and an anesthesiologist. ESD indications, procedural information (instruments, sedation, procedure duration, findings, interventions, outcome, and complications) were retrospectively collected and analyzed. The research protocol was approved by the local ethics committee. Both oral and written informed consents were obtained from the patients. This study was conducted in accordance with the ethical principles of the Declaration of Helsinki and in compliance with good clinical practice.

### Equipment and procedure

All ESD procedures were performed by a single operator (S.H.) who had studied ESD in 2006 for three months (under the supervision of Hironori Yamamoto, MD; Jichi Medical School, Tochigi, Japan). During his study as a visiting professor he studied the indication, techniques and other basic knowledge regarding ESD for the esophagus, stomach and colon in the endoscopy unit of Jichi Medical University. The author also joined ESD courses in porcine stomach models organized by the gastrointestinal endoscopy society during national gastroenterology weeks. In these courses he achieved more experience, but also contributed to the education of physicians interested in this area.

Prior to an ESD attempt, all lesions were examined with an optical magnifying endoscope (EG-450 ZW5; Fujinon) and colonoscope (-EC-590 Z/WL; Fujinon), with 1% Indigo Carmine used as an adjunct to magnification. The lesion size was determined upon the comparison of standard open biopsy forceps with the lesion. The invasion depth of the lesions was examined either with high frequency ultrasound mini probes (P1912-MB, P1915-MB, P2012-M, 12-20 MHz; Fujinon) or echoendoscopes (GF-UE 160-AL5; Olympus) depending on the size and location of the lesion. Superficial lesions were classified according to the Paris classification system as type I (protruding), type II (flat) a, b, c and type III (excavated)<sup>[8]</sup>. Kudo classification was used for characterization of colonic pit patterns<sup>[9]</sup>.

ESD procedures were performed in a standardized way. The margins of the lesion were marked with an



electrocautery (30 W soft coagulation) to determine the resection border (except in the colon). Then submucosal injection was performed to lift the lesion. For the injection, a special mixture (1 unit of 2% sodium hyaluronic acid, 3 units of saline, 0.5 mL of epinephrine (1/10000) and 0.5% of indigo carmine) was used. After sufficient lifting, a flush knife (DK2618JN 20; Fujinon), insulated-tip knife (KD-610L; Olympus) or needle knife (KD-11Q-1) was used to create a circumferential incision around the lesion extending into the submucosa. After circumferential incision, a submucosal dissection was performed to remove the lesion in an en bloc fashion.

A high frequency generator with an automatically controlled system (Endo-cut mode; ERBE ICC 200, Elektromedizin GmbH, Germany) was used for dissection and coagulation. A specialized cap (EMR ST Hood DH 15CR, Fujinon) was placed on the tip of the endoscope to make the dissection easier by increasing stability. Initially marked lesions were dissected with a diathermic knife (Olympus or Fujinon) circumferentially using endo-cut mode (2-3/80 W). An insulated-tip (IT) knife (KD-610L; Olympus) was used to dissect the borders of the lesions with a high perforation risk (wide based lesions and colonic lesions between haustral folds) using endo-cut mode (3/120 W). Submucosal dissection was performed with spray coagulation (45 W). Small vessels were coagulated with spray coagulation. Larger vessels or arteries with high bleeding risk were coagulated with hemostatic forceps (Fujinon).

Circumferential incision was completed in all cases. In colonic lesions, semi-circumferential incision was performed initially. After submucosal dissection, circumferential incision was completed. A few cases were finished with snare resection, but only after 80% of ESDs were completed. Standard or therapeutic gastroscopes (Fujinon EG-530 D) were used for lesions located in the rectum and sigmoid colon. Lesions close to the anus were treated in the retroflexion position. Colonoscopes were used for lesions proximal to the splenic flexure.

The first gastric lesions treated by ESD were located at the antrum in our series, as most gastric lesions were. Later on, cardiac lesions were treated by ESD in the retroflexion position.

All of the ESD procedures were performed under deep sedation. A combination of propofol and fentanyl was provided by an anesthesiologist. Patients were continuously monitored with an electrocardiogram, and blood pressure and oxygen saturation were monitored. The position of the patient could be easily changed whenever required with the help of medical attendants under the control of the anesthesiologist.

### Definitions and follow-up strategy

All the specimens were examined by a single pathologist who is specialized in gastrointestinal pathology. En bloc resection was defined as the removal of a lesion in a single piece. Piecemeal resection was defined as the removal of a lesion in more than one piece.

A recurrent disease was defined as the reappearance of neoplastic tissue at the site of initial ESD at the 6th mo

follow-up endoscopy. In the case of a perforation, hemoclips were used. Bleeding that could be managed with endoscopic intervention was considered as minor bleeding. Bleeding with hemodynamic instability and blood transfusion requirement with or without the need for surgical intervention was considered as major bleeding.

A lesion was considered to be completely removed (R0 resection), when the vertical and lateral surgical margins were 2 mm away from the lesion. When neoplastic cells were present at surgical margins, this was considered as an incomplete resection (R1). Patients found to have undifferentiated or signet cell adenocarcinoma and submucosal/lymphovascular invasion on histopathological evaluation were referred to surgery. Patients were hospitalized for observation after the procedure and underwent a control endoscopy within 2 d of the ESD procedure. Patients underwent follow-up endoscopies at 3 and 6 mo. After a normal endoscopy at the 6th mo, annual follow-up was offered.

### Statistical analysis

A median of continuous variables was used to present data. The Kruskal-Wallis test was used to compare median procedure time of ESD groups. When Kruskal-Wallis test results were statistically significant ( $P < 0.05$ ), a Mann-Whitney test using Bonferroni correction was used to compare median procedure time between ESD groups.  $P < 0.01$  was accepted as statistically significant. Statistical Packages for Social Sciences version 16.0 for Windows (SPSS, Chicago, IL, USA) was used for statistical analysis.

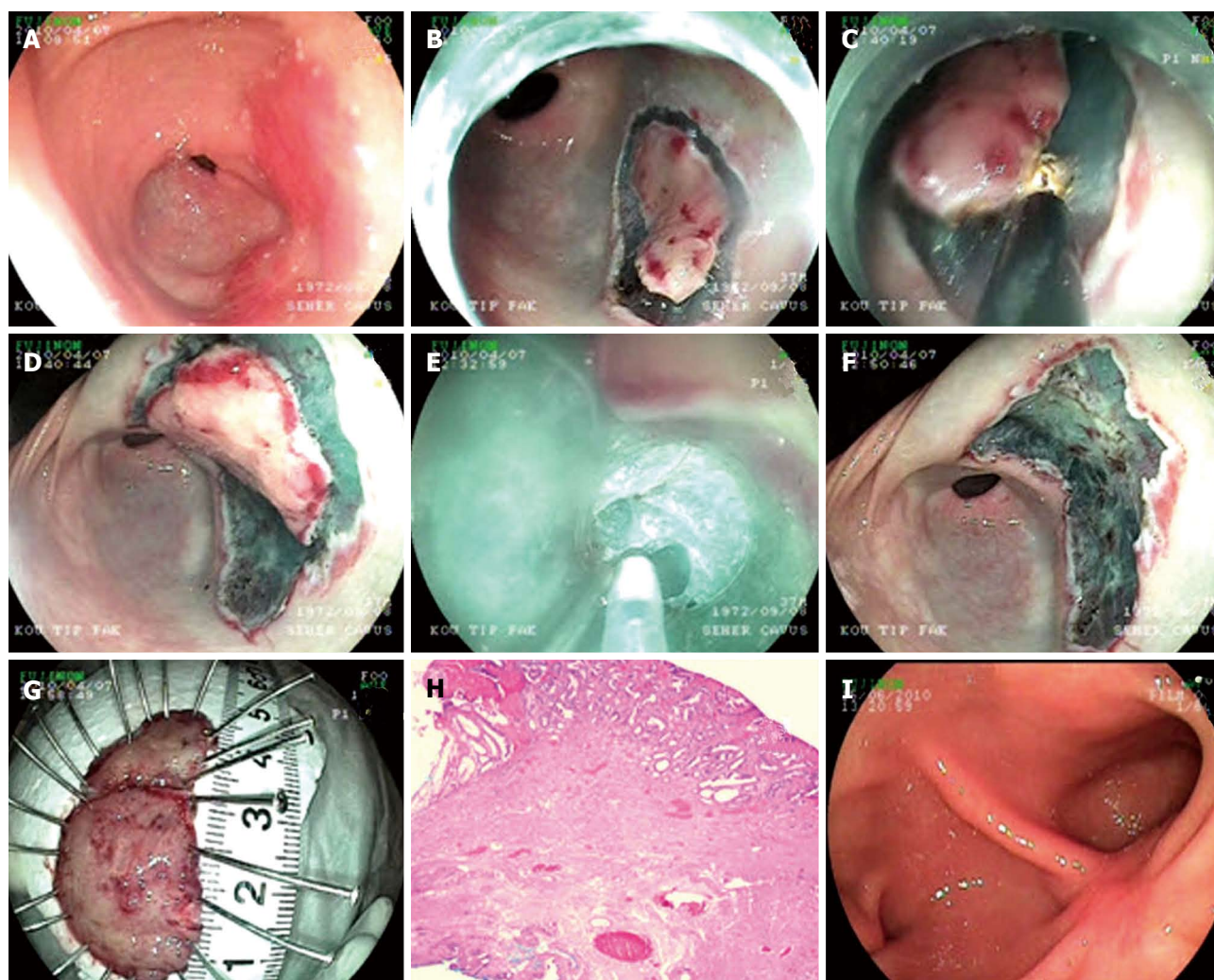
## RESULTS

Over the 46-mo period, 60 ESD procedures were performed by a single operator (S.H.). There were 34 female (56.6%) and 26 male (43.3%) patients. The mean age (*via* standard deviation) of patients was 54.6 ( $\pm 14.1$ ) years.

The majority of ESD procedures (65%) were performed for intraepithelial lesions ( $n = 39$ ) (33/39 adenoma with high grade dysplasia, 6/39 adenoma with low grade dysplasia). The indication of the remaining ESD procedures were as following: neuroendocrine tumor (NET) ( $n = 7$ ), cancer ( $n = 7$ ) [5/7 early colorectal cancer (ECC), 2/7 early gastric cancer (EGC)], granular cell tumor ( $n = 3$ ), gastrointestinal stromal tumor (GIST) ( $n = 2$ ), and leiomyoma ( $n = 2$ ). Microscopic types of the lesions in the different locations according to Paris classification is given in Table 1. En bloc and piecemeal resection rates were 91.6% (55/60) and 8.3% (5/60), complete and incomplete resection rates were 96.6% (58/60) and 3.3% (2/60), respectively.

An adenoma (piecemeal resection is done on purpose, Figure 1), early gastric cancer located in the antrum, and three flat adenomas with lateral invasion in the colon were resected in piecemeal fashion. A patient with a NET that was incompletely resected was referred to surgery due to vascular invasion noted on histopathologic evaluation. A patient with an incompletely resected early colon cancer (ECC) due to lateral spreading was referred to surgery. Invasion into the muscularis propria was seen





**Figure 1** Endoscopic submucosal dissection procedure for adenoma with high grade dysplasia at antrum. A: Endoscopic view flat adenoma at antrum; B: Cutting of the first piece of the lesion which was decided to be extracted in two pieces; C: Submucosal dissection of the first piece; D: Cutting of the second piece of the lesion; E: Submucosal dissection of the second piece; F: Endoscopic view after the lesion is being extracted; G: Microscopic view of the lesion; H: Histology; Adenoma including fields of marked glandular atypia and distortion (HE  $\times 20$ ); I: Endoscopic view ten weeks after the procedure.

**Table 1** Paris classification according to the endoscopic imaging of lesions

	I a	II a	II b	II a + II c
Stomach	-	14 <sup>1</sup>	-	10
Small bowel	-	3	-	1
Colon	-	12 <sup>2</sup>	-	13 <sup>3</sup>
Esophagus	1	5 <sup>1</sup>	-	1

<sup>1</sup>Two cases are submucosal; <sup>2</sup>Nine granular type lateral spreading tumor (LST); <sup>3</sup>Seven pseudo-depressed type LST.

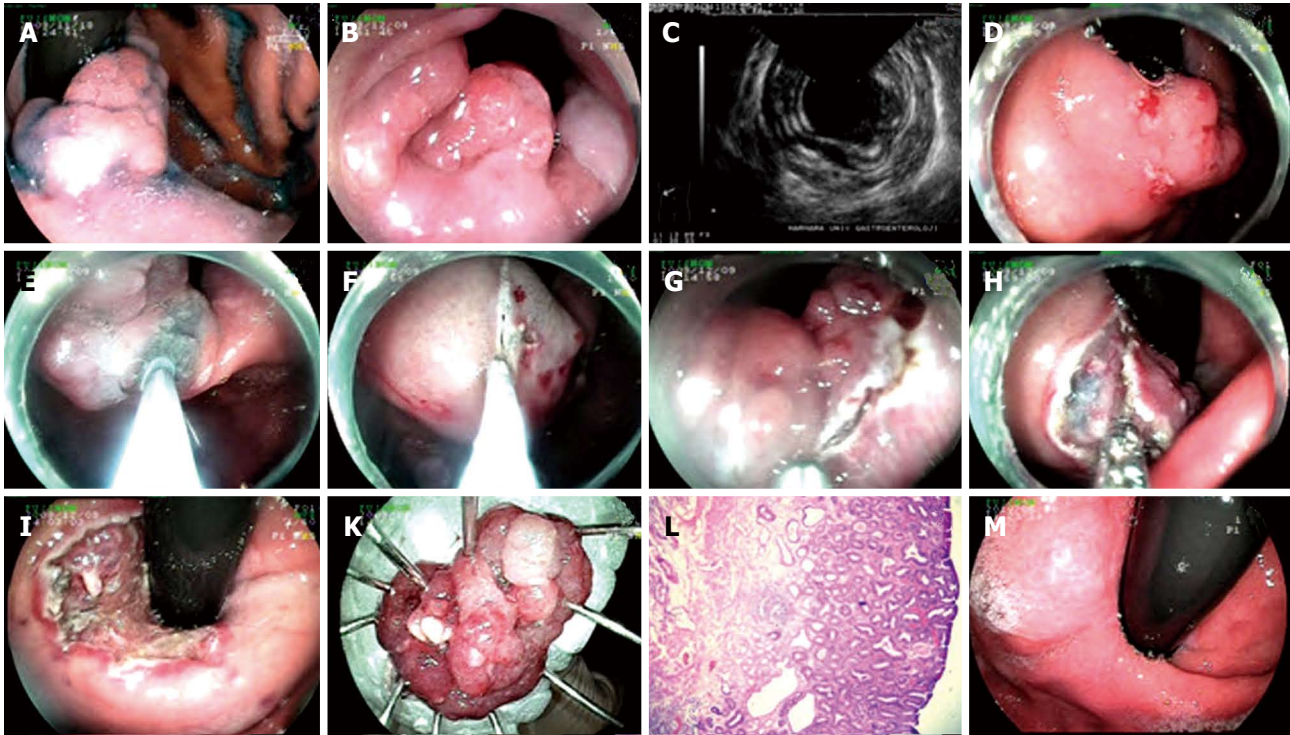
in the surgical specimen of the patient with gastric NET referred for surgery. But there was no invasion in the surgical specimen of the patient with ECC (this patient was referred for surgery because histology revealed neoplastic cells at the surgical margins of the specimen resected in en bloc fashion). En bloc, piecemeal, complete, and incomplete resection rates are shown in Table 2.

The duration of ESD procedures per cm<sup>2</sup> were as follows; 27.8, 21.8, and 18.3 min for stomach, esophagus, and

colon, respectively. However, the mean procedure durations were as follows; 158, 90.4 and 50.5 min for colon, stomach, and esophagus, respectively. The mean procedure time of colonic and gastric lesions was significantly longer than esophageal lesions [Kruskal-Wallis test ( $P < 0.01$ )]. This is due to the differences in size of the lesions removed in different anatomic locations; colon (8.61 cm<sup>2</sup>) > stomach (3.25 cm<sup>2</sup>) > esophagus (2.34 cm<sup>2</sup>). Technical challenges related to the anatomic location of lesions as well as nature of lesions contribute to the procedure time (endoscopic resection should be done in the retroflexed position for lesions located in the cardia and proximal corpus, neuroendocrine tumors (NET) with rich vascularization have more bleeding).

### Esophageal lesions

Seven esophageal lesions consisting of 3 granular cell tumors, 2 adenomas with high grade dysplasia, 1 gastrointestinal stromal tumor (submucosal), and 1 leiomyoma (submucosal) were treated with ESD (Table 3). No complications occurred in patients with esophageal lesions that were removed with ESD. No recurrences were not-



**Figure 2** Endoscopic submucosal dissection procedure for adenoma with high grade dysplasia at cardia. A: Adenoma at cardia; B: View of the lesion from esophageal aspect; C: Endosonographic image of the lesion; D, E: Marking the borders of the lesion with needle knife and lifting it; F, G: Cutting the lesion circumferentially with endo-cut above Z line, in retroflexion; H: Dissection of the submucosal area; I: Appearance of the mucosa after the lesion being extracted; K: Microscopic view of the lesion; L: Histology: mucosa, muscularis mucosa and superficial submucosa of stomach (HE  $\times 20$ ). Adenoma structure including adenomatous epithelium formed by irregular glands at mucosa; M: Endoscopic view six months after the procedure.

**Table 2** En bloc, piecemeal, complete, incomplete resection rates, median follow up and recurrence rates

	En bloc res. rate	Piecemeal res. rate	Complete res. rate	Incomplete res. rate	Median follow-up (mo)	Local recurrence
Esophagus	7/7 (100%)	0	7/7 (100%)	0	15	0
Stomach	22/24 (91.7%)	2/24 (8.3%)	23/24 (95.8%)	1/24 (4.1%)	11.8	1
Small intestine and colon	26/29 (89.7%)	3/29 (10.3%)	28/29 (96.5%)	1/29 (3.4%)	12.5	0
Total	55/60 (91.6%)	5/60 (8.3%)	58/60 (96.6%)	2/60 (3.3%)	12.51	1/58 (1.7%) <sup>1</sup>

<sup>1</sup>Two patients who underwent surgery were excluded. Res: Resection.

**Table 3** Esophageal endoscopic submucosal dissection cases

Location	Number	Histology (n)
Proximal esophagus	1	Granular cell tumor (1)
Middle esophagus	2	Granular cell tumor (2)
Distal esophagus	4	GIST (1) HGD-A (2) Leiomyoma (1)
Specimen size (median)		2.34 cm <sup>2</sup> (1.5-3 cm <sup>2</sup> )
Procedure time (median)		50.5 min (21.8 min/cm <sup>2</sup> )

GIST: Gastrointestinal stromal tumor; HGD-A: Adenoma with high grade dysplasia.

ed in the 7 patients that had follow-up data. The mean follow-up period for these 7 patients was 15 mo.

### Gastric lesions

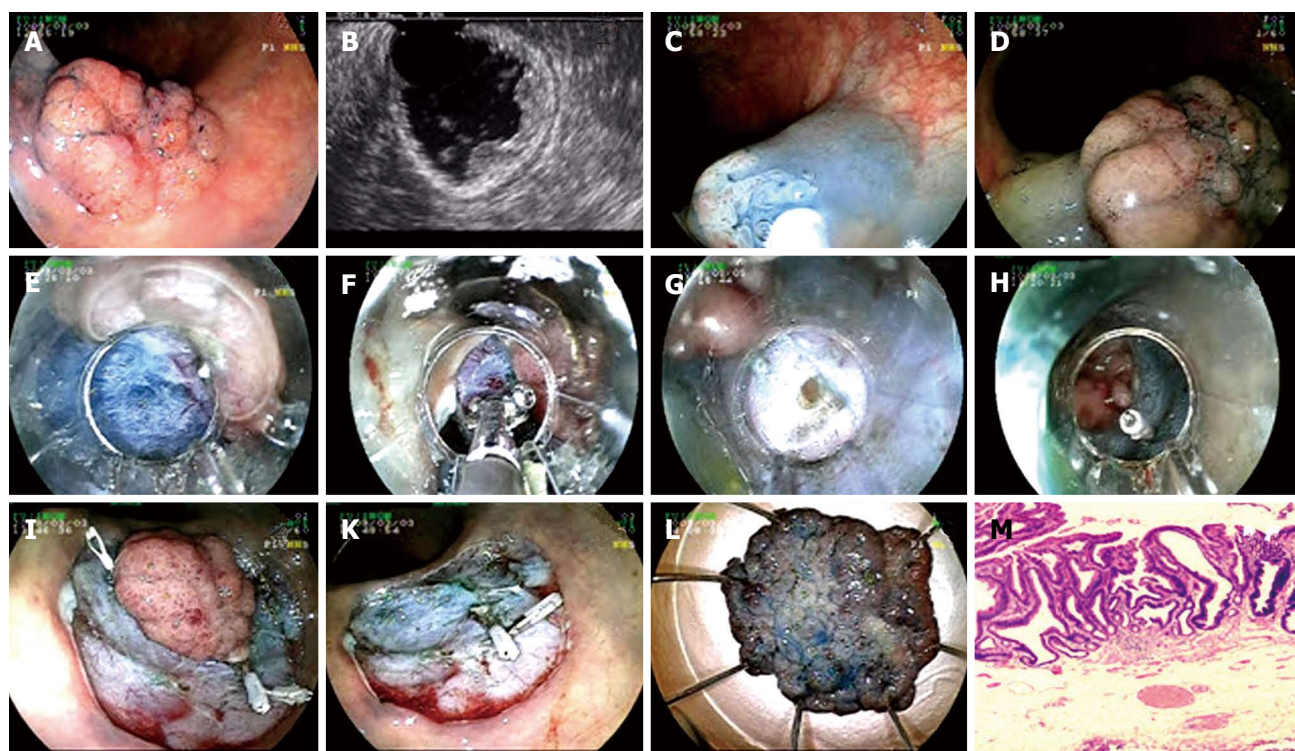
Twenty four gastric lesions, consisting of 16 adenomas (12 adenomas with HGD, 4 adenomas with LGD), 4 carci-

noid tumors, 2 early gastric cancers, 1 GIST (submucosal), and 1 leiomyoma (submucosal) were treated with ESD (Table 4) (Figures 1 and 2). One lesion was located in the cardia (adenoma with HGD), 3 lesions were located in the gastric corpus (3 carcinoid tumors), and 20 lesions were located in the antrum (11 adenomas with HGD, 4 adenomas with LGD, 2 EGC, 1 GIST, 1 carcinoid tumor, 1 leiomyoma). A patient with a gastric adenoma with HGD had recurrence at the site of prior resection. This patient had a second ESD, 12 mo after the first ESD.

### Colonic and small intestinal lesions

Twenty-nine lesions including 21 adenomas (2 tubulovillous adenoma with HGD in duodenal bulb, 1 tubulovillous adenoma with HGD in cecum, 2 tubulovillous adenoma with HGD in transverse colon, 3 tubulovillous adenoma with HGD in sigmoid colon, 1 tubular adenoma in sigmoid colon, 9 tubulovillous adenoma with HGD in rectum, and 3 tubular adenoma in rectum), 3 carcinoid





**Figure 3** Endoscopic submucosal dissection procedure for tubulovillous adenoma with high grade dysplasia at sigmoid colon. A: Adenoma at sigmoid colon; B: Endosonographic image of tubulovillous adenoma; C-D: Lifting the lesion; E: Cutting with endo-cut; F: Coagulation of submucosal vein with hemostatic forceps; G: Mini perforation during the procedure; H: Fixing perforation with hemoclip; I: Hemoclip application to control bleeding that occurred after cutting the lesion circumferentially with endo-cut; K: Appearance of the mucosa after the lesion being extracted; L: Microscopic view of the lesion; M: Histology; tubulovillous adenoma with high grade dysplasia (HE × 40).

Table 4 Gastric endoscopic submucosal dissection cases		
Location	Number	Histology (n)
Cardia	1	HGD (TVA) (1)
Corpus	3	NET (3)
Antrum	20	HGD-A (11)
		LGD-A (4)
		NET (1)
		EGC (2)
		GIST (1)
		Leiomyoma (1)
Specimen size (median)		3.25 cm <sup>2</sup> (1.5-12 cm <sup>2</sup> )
Procedure time (median)		90.4 min (27.8 min/cm <sup>2</sup> )

HGD: High grade dysplasia; NET: Neuroendocrine tumor; HGD-A: Adenoma with high grade dysplasia; LGD-A: Adenoma with low grade dysplasia; EGC: Early gastric cancer; LGD: Low grade dysplasia; GIST: Gastrointestinal stromal tumor; TVA: Tubulovillous adenoma.

tumors (1 in duodenal bulb, 1 in terminal ileum and 1 in rectum), and 5 early colon cancers (4 in rectum and 1 transverse colon) were treated with ESD (Table 5) (Figures 3 and 4). All early colon cancers were classified as II a + II c according to the Paris classification.

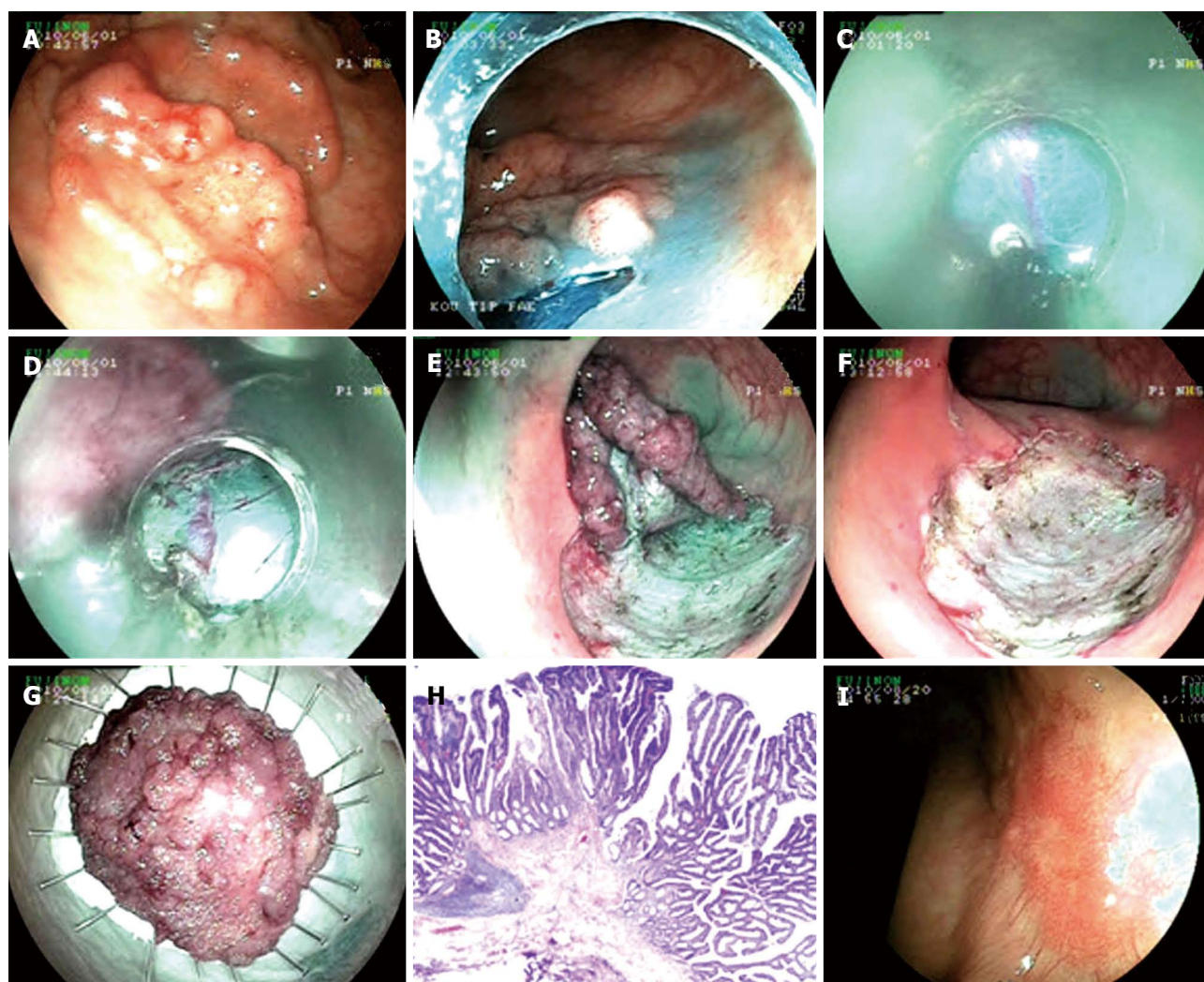
**Safety**

Among 5 perforations (8.3%), four were located in the colon (1 early colon cancer, 2 laterally spreading lesions and 1 large tubulovillous adenoma located on a colonic haustra) and one (carcinoid tumor) was located in the stomach.

Table 5 Colonic and intestinal endoscopic submucosal dissection cases		
Location	Number	Histology (n)
Duodenal bulb	3	HGD (TVA) (2)
		NET (1)
Terminal ileum	1	NET (1)
Cecum	1	HGD (TVA) (1)
Transverse colon	3	HGD (TVA) (2)
		ECC (1)
Sigmoid colon	4	HGD (TVA) (3)
		LGD-TA (1)
Rectum	17	ECC (4)
		HGD (9 TVA, 2 TA)
		LGD-TA (1)
		NET (1)
Specimen size (median)		8.61 cm <sup>2</sup> (1.5-25 cm <sup>2</sup> )
Procedure time (median)		158 min (18.3 min/cm <sup>2</sup> )

HGD: High grade dysplasia; NET: Neuroendocrine tumor; TA: Tubular adenoma; ECC: Early colonic cancer; LGD: Low grade dysplasia; TVA: Tubulovillous adenoma.

Two colonic perforations were managed with surgical intervention and the other three were managed with hemoclip application. Those managed with hemoclip application were smaller than 5 mm in size; hence it was easy to treat them. The patients with perforation were hospitalized for an average of ten days. There was no association of perforation with scarring and fibrosis from previous procedures. No post-ESD stenosis was noted. Major bleeding occurred



**Figure 4** Endoscopic submucosal dissection procedure for pseudo-depressed type lateral spreading tumor with high grade dysplasia at rectum. A: Pseudo-depressed type lateral spreading tumor at rectum; B: Cutting the lesion circumferentially with endo-cut; C, D: Submucosal dissection with semipermeable cap; E: Endoscopic view just before completing submucosal dissection; F: Appearance of the mucosa after the lesion being extracted; G: Microscopic view of the lesion; H: Histology; tubulovillous adenoma including fields of focal pattern loss and dysplasia (HE  $\times 20$ ); I: Endoscopic view ten weeks after the procedure.

in three patients (5%). One of these patients had a lesion located in the colon and two of them had lesions located in the stomach. Contributing factors to major bleeding may possibly be the nature as well as the location of the lesion, possibly due to the rich vascularization of the lesion. Both of the bleeding lesions in the stomach were NETs and located in the lesser curvature. Bleeding from gastric lesions was delayed and required blood transfusion. The colonic case that had a major bleed was an early colonic cancer. Minor bleeding occurred in 13 cases (7 lesions located in the colon and 6 lesions located in the stomach) (21.7%). Table 6 shows complications in detail.

## DISCUSSION

Although endoscopic resection-based therapeutic modalities (EMR and ESD) are considered to be the treatment of choice for premalignant and early gastrointestinal neoplasias in Japan, they are not widely practiced by Western endoscopists<sup>[10]</sup>. This is the first study from Turkey and among the few studies from outside of Japan and Asia on

the application of ESD in the management of premalignant and noninvasive early gastrointestinal cancers from various anatomic locations in the gastrointestinal tract.

It is difficult or impossible to remove large lesions with EMR technique in one fragment. Removal of a lesion in one piece is very important to accurately diagnose the tumor depth as well as decreasing the risk of local recurrence. A recent study has illustrated that this problem can be solved with the use of ESD for larger lesions<sup>[11]</sup>. In early esophageal cancers with a diameter of less than 20 mm, en bloc and curative resection rate of ESD (97%) was found to be significantly higher than that of EMR using a transparent cap (71%) and 2-channel EMR (46%)<sup>[11]</sup>. However, no significant difference was found between ESD and EMR using a transparent cap in en bloc and curative resection rate of lesions less than 15 mm in diameter. Therefore, ESD would be a better therapeutic modality than EMR for esophageal lesions with a diameter of greater than 15 mm. Given the size (median size of esophageal/gastric/colonic lesions 2.34 cm<sup>2</sup>/3.25 cm<sup>2</sup>/8.61 cm<sup>2</sup> respectively), various anatomic location of



**Table 6** Complications with regard to location and diagnosis

	<i>n</i>	Diagnosis	Minor bleeding	Major bleeding	Perforation
Esophagus	7	3 granular cell tumor 1 GIST 2 Premalignant lesions 1 Leiomyoma			
Stomach	24	4 NETs 2 EGC 18 Premalignant lesions	4 2	2	1
Small intestine	4	2 Premalignant lesions 2 NETs			
Colon	25	5 ECCs 19 Premalignant lesions 1 NETs	3 4	1	1 3
Total	60		13 (21.7%)	3 (5%)	5 (8.3%)

GIST: Gastrointestinal stromal tumor; NET: Neuroendocrine tumor; EGC: Early gastric cancer; ECC: Early colorectal cancer.

lesions and subepithelial nature of some lesions, ESD would be the treatment of choice for our cases.

There are few studies from Europe that were published in full manuscript format. Dinis-Ribeiro *et al*<sup>[12]</sup> evaluated feasibility and effectiveness of ESD in 19 gastric superficial lesions with HGD, LGD and noninvasive epithelial neoplasias. Probst *et al*<sup>[13]</sup> evaluated ESD in 71 flat adenomas, early cancers and submucosal tumors located in various locations of the gastrointestinal tract (51 gastric, 17 rectal, 2 esophageal and 1 duodenal). In the study of Dinis-Ribeiro *et al*, complete and en bloc resection rates were 89% and 79%, respectively. Major bleeding occurred in 1 case (5%). There were no perforations. Recurrence of a lesion (5%) was noted within a mean follow-up of 10 mo. In order to evaluate ESD learning curve, Probst *et al*<sup>[13]</sup> compared various aspects of ESD procedures performed in the first and second halves of the study.

A statistically significant increase in specimen size and decrease in procedural duration were noted between the two groups. En bloc resection rates and R0 en bloc resection rates in the first half of the study (77.1% and 65.7%, respectively) increased when compared with the second half of the study (86.1% and 72.2%, respectively), however this difference did not reach statistical significance. No recurrence occurred after R0 en bloc resection; however 38% recurrence occurred after piecemeal resection. Complications in the study of Probst included 2 perforations (gastric submucosal tumors) that required surgery (2.7%), 2 other perforations (large flat rectal lesions) (2.7%), 8 minor bleedings (10.9%) and 3 pyloric stenosis (4.1%) that were endoscopically managed.

ESD complication rates among the published studies have been variable depending on the size of the study as well as the experience of the operator. In our study, a patient (1.7%) was found to have a recurrence of an adenoma with HGD at the site of prior ESD on 12-mo follow-up endoscopy. The recurrent lesion was treated with a repeat ESD. The patient was free of disease at the 6-mo follow-up. No recurrences were noted with esophageal and colonic lesions. Compared to other studies, lower

recurrence rate in our study may be related to the relatively shorter follow up (median = 12.5 mo) of the patients after ESD. Our bleeding rate is consistent with other studies. Our perforation rate is higher than the study of Probst *et al*<sup>[13]</sup>. However, all of our perforations occurred at the first half of the study, which is a reflection of the impact of the operator's experience with the success of ESD procedures. Eighty percent of perforations occurred with colonic cases, which may be related to the relatively thinner colonic wall thickness and larger size of colonic lesions. Most perforations took place in initial cases. In those cases needle knives were used. We believe that the use of these knives also contributed to this relatively high number of perforations. After providing IT-knives we did not encounter any perforations. As stated above we could not refuse the patients and it is true that we had to perform colorectal cases with insufficient experience in gastric ESD, resulting in relatively high perforation rates.

In a review article from Japan, the en bloc resection rate of early gastric cancers was reported to be 79%-100%, with local recurrence, bleeding and perforation rates of 0%-1%, 1.7%-38%, 0%-5%, respectively<sup>[14]</sup>. Another study from Japan evaluating ESD in colorectal epithelial neoplasms revealed the rate of en bloc resection and en bloc resection with tumor free margins to be 91.5% and 70.5%<sup>[15]</sup>. In this study, perforation and local recurrence rates were found to be 5% and 1.7%, respectively. The sample size of studies coming from outside of Japan and Asia is quite modest; therefore it is premature to compare Western experience with the Asian one.

Ideally one should begin with gastric cases located in the antrum. After getting sufficient experience in gastric cases they can proceed with esophageal and colorectal cases, which are more risky. Our practice seems incompatible with this idea. But ESD is only performed in our institute in Turkey and patients are referred to our hospital from the entire country. So we did not have the chance to refuse the patients and performed esophageal and colorectal cases before having sufficient experience in gastric cases.

We believe that, besides EMR and endoscopic piecemeal mucosal resection, ESD will be a good alternative in the treatment of non-epithelial esophageal lesions. We observed that neuroendocrine tumors which have rich vascularization are more likely to bleed, so more attention should be paid when operating on them. During the ESD procedures in the colon and esophagus, a needle knife should be avoided in endo-cutting because of the perforation risk. Perforation risk is even higher in laterally spreading colonic lesions so IT-knife is a better choice for those lesions.

In summary, ESD, which originates in Japan, has been gaining popularity in other parts of the world as well. Comparable outcomes of ESD to surgery play an important role in the rapid propagation of this therapeutic endoscopic modality. Although no procedure related mortality has been reported, there is considerable morbidity with this technique. There is a significant learning curve to achieve proficiency in order to acquire skills to perform ESD safely and effectively. Therefore, importance of training can not be overemphasized. Further studies from outside of Japan and Asia are needed to better determine the global role of

ESD in the management of premalignant and early malignant epithelial, as well as subepithelial lesions.

## COMMENTS

### Background

Advances in endoscopic diagnosis techniques allowed premalignant lesions and non-invasive cancers of the gastrointestinal system to be detected early and be treated effectively and safely by endoscopic methods. Among these methods, endoscopic submucosal dissection (ESD) is being used more and more commonly and has pleasing results.

### Research frontiers

ESD is a safe and effective modality for the treatment of premalignant lesions and early non-invasive cancers of the gastrointestinal system and when compared to surgery it has the advantage of preserving the gastrointestinal system and its functions. This is the first study on ESD reported from Turkey.

### Innovations and breakthroughs

Complications related to ESD are similar to other studies in the literature except for the complication rate. Recurrence rates are lower compared to other studies. In this study we observed that neuroendocrine tumors had higher bleeding rates due to hypervascularization and the use of a needle knife in colonic and esophageal lesions increased the risk of perforation.

### Applications

When performed by experienced endoscopists ESD has pleasing results and can be safely performed for the treatment of premalignant lesions and early cancers of gastrointestinal system.

### Terminology

ESD is being used for the treatment of premalignant and lesions (with no lymph node involvement) of the gastrointestinal tractus confined to mucosa and submucosa. After lifting the lesion by injecting the specially prepared solution (Na - Hyaluronate + Adrenalin + Saline + Indigo carmine), the basement of the lesion, along with the surrounding area, are cut with special knives and the lesion is extracted.

### Peer review

This retrospective study sets out to evaluate the feasibility, safety and clinical outcomes of ESD for premalignant lesions and early gastrointestinal cancers. It establishes that the rates of en bloc and complete resection of these lesions were good, and comparable those of previous studies.

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## Discovery and validation of prognostic markers in gastric cancer by genome-wide expression profiling

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### Abstract

**AIM:** To develop a prognostic gene set that can predict patient overall survival status based on the whole genome expression analysis.

**METHODS:** Using Illumina HumanWG-6 BeadChip followed by semi-supervised analysis, we analyzed the expression of 47 296 transcripts in two batches of gastric cancer patients who underwent surgical resection. Thirty-nine samples in the first batch were used as the training set to discover candidate markers correlated to overall survival, and thirty-three samples in the second batch were used for validation.

**RESULTS:** A panel of ten genes were identified as prognostic marker in the first batch samples and classified patients into a low- and a high-risk group with significantly different survival times ( $P = 0.000047$ ). This prognostic marker was then verified in an independent validation sample batch ( $P = 0.0009$ ). By comparing with the traditional Tumor-node-metastasis (TNM) staging system, this ten-gene prognostic marker showed consistent prognosis results. It was the only independent prognostic value by multivariate Cox regression analysis ( $P = 0.007$ ). Interestingly, six of these ten genes are ribosomal proteins, suggesting a possible association between the deregulation of ribosome related gene expression and the poor prognosis.

**CONCLUSION:** A ten-gene marker correlated with overall prognosis, including 6 ribosomal proteins, was identified and verified, which may complement the predictive value of TNM staging system.

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**Key words:** Gastric cancer; Gene expression profiling; Survival markers; Prognosis; Ribosomal proteins



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## INTRODUCTION

Gastric cancer is the second leading cause of cancer related death worldwide<sup>[1]</sup>. As a complex and heterogeneous disease, it comprises multiple tumor entities associated with distinctive histological patterns and biological features, as well as clinical behaviours<sup>[2]</sup>. The 5-year survival rate of patients with advanced disease is only 20%-30%<sup>[3]</sup>. The current treatment plan and prognosis prediction for gastric cancer mainly depend on the clinicopathologic staging of the disease, and TNM staging system is still the golden standard for survival prediction among gastric cancer patients. However, prognosis varies among patients with a similar tumor stage, therefore disease staging alone can not accurately predict the outcome for individual patients.

Although great efforts have been made in the identification of prognostic markers from gene expression profiling to improve prognosis prediction for many cancers especially breast cancer<sup>[4]</sup>, limited research has been conducted in the field of gastric cancer. To date, most studies on the selection of prognosis markers were conducted by cDNA array or quantitative RT-PCR, in which only a few thousand genes were analyzed<sup>[5-9]</sup>. In an attempt to predict peritoneal relapse after gastrectomy for gastric cancer, the whole genome microarray consisting of 30K transcripts was employed in a very recent gene expression analysis<sup>[10]</sup>. Such a robust approach may provide not only more signals in marker selection, but also more comprehensive information in understanding molecular mechanisms of tumor-related processes. In this study, we explored the gene expression by microarray containing over 47K probes in two batches of surgical samples from 79 Chinese gastric cancer patients. A ten-gene marker for overall survival was identified and verified in an independent batch of samples.

## MATERIALS AND METHODS

### Patients and samples

Seventy-nine tissue samples from patients who were surgically treated for primary gastric carcinoma were procured at the Beijing Cancer Hospital (Peking University, School of Oncology) from 1999 to 2003. No patient received chemotherapy or radiotherapy before surgery. All patients were treated with curative surgical resection, which was, in some cases, followed by second-line treatment at the time

of recurrence. Macroscopic and microscopic evaluations were conducted by pathologist according to the general rules for gastric cancer. Follow-up was performed every three month for the first two years, and every three to six months thereafter. Stage of gastric cancer was classified according to 2002 tumor-node-metastasis (TNM) classification system recommended by the American Joint Committee on Cancer.

Overall survival was calculated from the date of primary surgery to the date of last follow-up or to the date of death due to cancer relapse or metastasis. All tumor samples were obtained at the surgery, followed by fresh freezing in liquid nitrogen and stored at -80°C. Informed consent was obtained from each patient for the collection and storage of tissue samples in a tissue bank for future research. This investigation was performed after approval by Ethics Committee of Peking University.

### RNA preparation and microarray analysis

Total RNA was purified from clinical samples using TRIzol reagent (GibcoBRL, Grand Island, New York, USA). And mRNA was linearly amplified by in vitro transcription using T7 RNA polymerase (MEGAscript T7 kit, Ambion, Inc, USA). The quality and integrity of total and amplified mRNA (cRNA) was monitored by both spectrophotometry (OD UV 260/280 ratio > 1.8) and agarose gel electrophoresis.

Gene-expression profiling was performed using Illumina HumanWG-6 BeadChip, which contains 47 296 transcripts. BeadChips were scanned with a BeadStation 500 GX and data are available at Gene Expression Omnibus (GSE21983). <http://www.ncbi.nlm.nih.gov/geo>.

### Statistical analysis

Average normalization in BeadStudio software was conducted for probe level average normalization and background correction. A detection *P* value was used in BeadChip to calculate probability to see a certain signal level without specific probe-target hybridization. All genes and probes with *P* value > 0.01 were filtered and removed from the analysis. Among 47 296 transcripts, 18 819 were expressed with *P* values < 0.01.

The supervised principal components method was used for survival profiling<sup>[11]</sup>. In the training set, we calculated the modified univariate Cox proportional-hazard scores for all genes (*n* = 18 819), which were measured to identify genes with their expression correlated to the duration of survival. We selected a set of genes whose absolute Cox score exceeded a threshold using cross-validation. For each iteration of the complete cross-validation, 10% of the cases were omitted, and principal components derived from the remaining 90% of the cases were included in a Cox model to predict the survival in 10% of the cases. By repeating the iteration process for 10 times, we found that a threshold of 2.6 yielded the highest average partial log-likelihood ratio. Principal component analysis (PCA) was then performed using 10 transcripts whose absolute Cox score equalled or exceeded the threshold for all cases in the training data set.

Table 1 Clinicopathological characteristics of all patients

Variables	Cases	Training dataset ( <i>n</i> = 39)	Validation dataset ( <i>n</i> = 33)	<i>P</i>
Sex				
Male	53	28	25	0.79
Female	19	11	8	
Age (yr)				
mean ± SE	72	60.9 ± 1.5	61.6 ± 1.3	0.74
Depth of wall invasion				
T2	4	3	1	0.12 <sup>1</sup>
T3	56	32	24	
T4	12	4	8	
Differentiation				
Well	7	5	2	0.18 <sup>1</sup>
Moderate	31	14	17	
Poor	27	18	9	
Undifferentiated <sup>2</sup>	7			
Lymph node metastasis				
Negative	16	10	6	0.57
Positive	56	29	27	
Distance metastasis				
M0	66	38	28	0.09
M1	6	1	5	
TNM stages				
I + II	17	12	5	0.30 <sup>1</sup>
III	33	18	15	
IV	22	9	13	

<sup>1</sup>The multiple comparisons of different subclasses; <sup>2</sup>Data was incomplete.

Kaplan-Meier survival curves were then plotted to predict overall survival. All analysis and plotting were conducted using R package superpc (<http://www-stat.stanford.edu/~tibs/superpc>).

Based on the transcript level in the 10 transcripts and the weight assigned to each transcript from the training set, a discrete risk score (the supervised principal components risk score) was then calculated for each patient in the validation dataset.

Multivariate analysis was conducted to evaluate the prediction accuracy of our survival profile in comparison with the standard clinicopathological covariates by Cox proportional hazards regression using SPSS software.

Functional gene set enrichment analysis was performed to find the pathways associated with prolonged and poor survivals. A total of 249 sets of canonical pathways (Gene Set Enrichment Analysis-Molecular Signatures Database) were analysed to indicate their correlations with overall survival to a greater degree than expected by chance<sup>[12]</sup>.

## RESULTS

### Patient characteristics

Totally, 79 gastric cancer patients treated with surgical resection were recruited in this study. Samples were randomly separated into two batches with no significant differences between the two sets with respect to age, sex and other clinicopathological features. Microarray was conducted in all samples, and the data of batch one served as the training dataset for marker discovery and data of batch two as the validation dataset. In batch one, microarray Quality Control

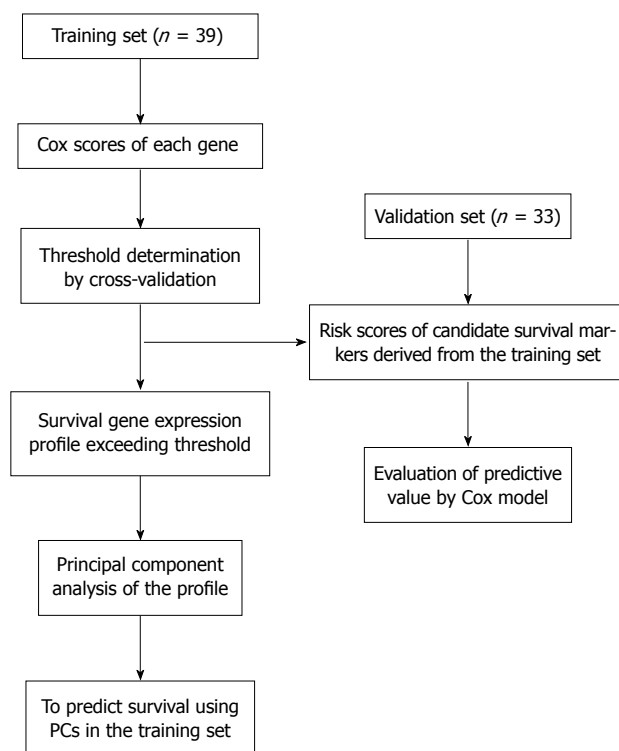
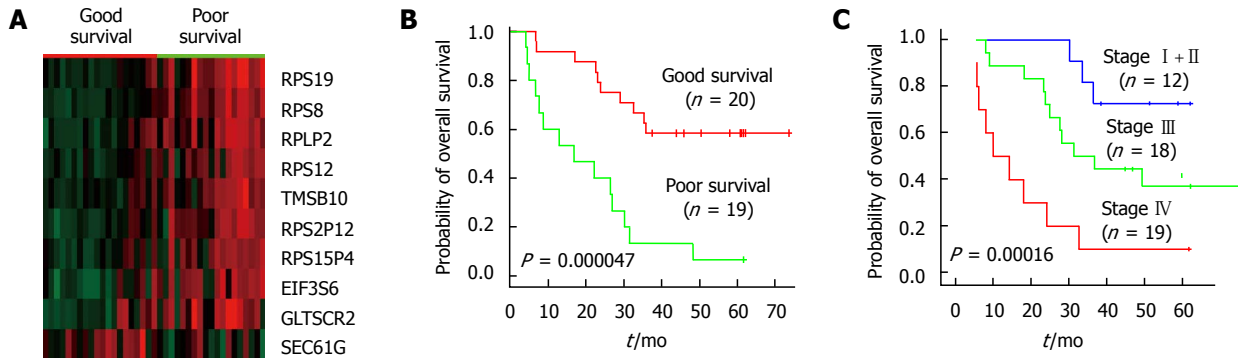


Figure 1 Overview of the strategy used for the development and validation of prognostic markers.

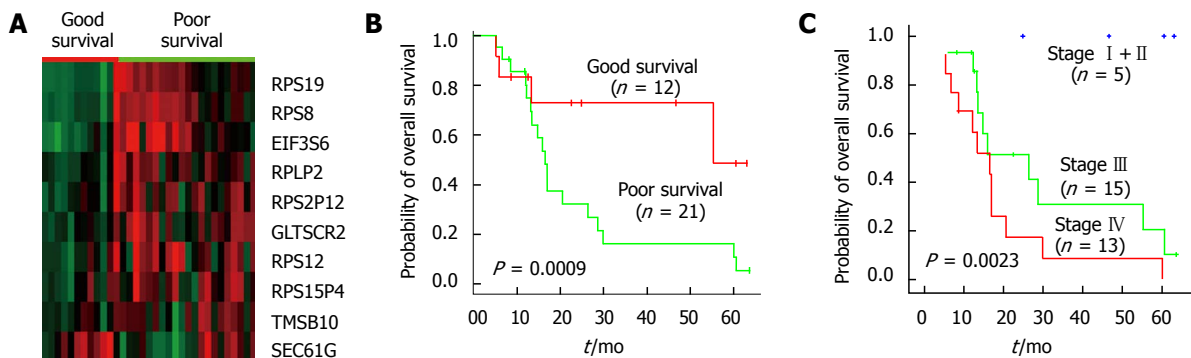
(QC) removed 7 samples due to failure in hybridization or failure to meet the analysis criteria, resulting in a total of 39 samples included in the training set. All of 33 samples in the second batch passed QC and were used in validation phase. The characteristics of the 72 patients are summarized in Table 1. The median overall survival time of all samples was 31 mo, ranging from 4.2 to 73.6 mo, and the 5-year overall survival was 33%.

### Gene expression profile associated with the overall survival

The “semi-supervised” learning approach was used to identify the gene expression profile related to the overall survival in the training dataset<sup>[12]</sup> (Figure 1). A total of 18 819 expression signals passed QC. First, we calculated the Cox scores of all 18 819 genes based on the survival times versus the expression levels obtained in 39 training observations. To choose the genes with the best prediction power, the threshold of Cox scores was calculated by 10-fold cross-validation. The expression profile of 10 transcripts whose Cox score equalled or exceeded the threshold was obtained (Table 2). Next, we performed PCA on the entire training set. For each case, a risk score that represents the sum of the weighted expression levels of the 10 prognostic transcripts was computed by supervised component analysis in a regression model. As shown in the Kaplan-Meier survival curves in Figure 2A and B, the patients were categorized into two groups based on their scores above or below the median risk of death. The low-risk group (*n* = 20) had a median survival of 42.1 mo, whereas the high-risk group (*n* = 19) had a median survival of only 26.5 mo.



**Figure 2 Overall survival curves and the expression profile of the ten-gene prognostic marker in the training dataset.** A: The gene expression pattern of the ten-gene prognostic marker. Nine genes were associated with the prolonged survival and one gene with poor survival. Red, high expression; green, low expression; B: Kaplan-Meier survival curves based on the expression profile of the ten-gene prognostic marker; C: Overall survival curves according to the tumor-node-metastasis stages.



**Figure 3 Overall survival curves and the expression profile of the ten-gene prognostic marker in the validation dataset.** A: The gene expression pattern of the ten-gene prognostic marker. Red, high expression; green, low expression; B: Kaplan-Meier survival curves based on the expression profile of the ten-gene prognostic marker; C: Overall survival curves according to the tumor-node-metastasis stages.

**Table 2 The ten-gene prognostic marker correlated to patients' survival**

Symbol	Cox score	Description
RPS19	2.93	Ribosomal protein S19
RPS8	2.90	Ribosomal protein S8
RPS2P12	2.62	Predicted ribosomal protein S2
RPS12	2.59	Ribosomal protein S12
RPS15P4	2.57	Predicted 40S ribosomal protein S15
RPLP2	2.51	Ribosomal protein, large, P2
EIF3S6	2.61	Eukaryotic translation initiation factor 3
GLTSCR2	2.67	Tumor suppressor candidate region gene 2
TMSB10	2.47	Thymosin, beta 10
SEC61G	-2.98	Sec61 gamma subunit

The correlation of the risk score and the survival status ( $P = 0.000047$ , log-rank) indicates that this transcriptional pattern was associated with the patient outcomes. A similar classification was also seen by TNM staging ( $P = 0.00016$ , log-rank; Figure 3A).

Among the ten-gene prognostic markers, high expression levels of 9 genes were associated with poor survival (Figure 2B). SEC61G, a subunit of the heteromeric SEC61 complex, was the only gene with its high expression associated with prolonged survival. Interestingly, six out of the

10 genes in this profile are either identified or predicted ribosomal proteins, including RPLP2, RPS12, RPS8, RPS19, RPS2P12, and RPS15P4. The involvement of numerous ribosomal genes with survival times suggested either their regulation by tumor suppressor or oncogenes, or their direct participation in certain pathways other than protein synthesis. Furthermore, RPS8 and RPS12 were previously reported as cancer related markers in colorectal tumor and cervical squamous cell carcinoma<sup>[13,14]</sup>. EIF3S6, a member of eukaryotic translation initiation factor 3, was identified as a prognostic factor in Stage I non-small cell lung cancers<sup>[15]</sup>. To test if the survival categories relate to known pathways, we applied gene set enrichment analysis (GSEA) to microarray data of 18 819 transcripts using 249 canonical gene sets collected by MsigDB. Glucocorticoid receptor (GCR) pathway gene set, referring to glucocorticoid receptor-related inhibition of inflammatory response, was significantly associated with the overall survival (FDR = 0.15,  $P = 0.004$ ).

#### Independent validation of prognostic markers

Next we evaluated the ten-gene prognostic marker in an independent dataset containing 33 cancer samples. As shown in Figure 3A and B, based on the expression of these 10 genes, the patients were classified into either a



**Table 3** Association between different prognosis groups identified by the ten-gene marker and the clinicopathological characteristics

Variables	Cases	Prolonged survival ( <i>n</i> = 12)	Poor survival ( <i>n</i> = 21)	<i>P</i>
Sex				
Male	14	9	5	0.070
Female	18	3	15	
Age (yr)				
mean ± SE	33	58.1 ± 2.2	63.5 ± 1.5	0.040
Depth of wall invasion				
T2	1	1	0	0.120
T3	24	11	13	
T4	8	0	8	
Differentiation				
Well	2	1	1	0.490
Moderate	17	8	9	
Poor	9	2	7	
Undifferentiated <sup>1</sup>	5			
Lymph Node Metastasis				
Negative	6	4	2	0.160
Positive	27	8	19	
Distance metastasis				
M0	28	12	16	0.080
M1	5	0	5	
TNM stages				
II	5	5	0	0.002
III	15	5	10	
IV	13	2	11	

<sup>1</sup>Data was incomplete. *P* values for stage, grade and location of tumors were derived from the Pearson  $\chi^2$  test. *P* value for age was derived from the *t* test.

“low-risk group” or “high-risk group” with significantly different survival times ( $P = 0.0009$ , log-rank). The low-risk group patients had a median survival of 31.7 mo whereas the high-risk group one had a median survival of 21.4 mo. The expression patterns of these 10 genes in validation set were also similar to the observation in the data training set (Figure 3B). The results in validation dataset showed the consistency of our ten-gene prognostic marker in survival prediction. For pathway analysis, no significant association was observed by GSEA.

#### Analysis of candidate survival markers with clinicopathological parameters

As certain clinicopathological parameters, especially TNM staging, have been used as prognosis indicators, we also compared our ten-gene prognostic marker with the clinicopathological characteristics in the validation dataset in order to assess the impact of clinicopathological factors on overall survival. First we examined the distribution of prognostic factors as a function of risk assignment based on our ten-gene prognostic marker (Table 3). Certain variation such as age was seen between high- and low-risk groups, while gender, tumor location and differentiation grade, the depth of wall invasion, and metastasis showed no significant difference between the two groups except for the TNM staging ( $P = 0.0023$ ).

Since the TNM staging is used most widely in clinical

**Table 4** Multivariate Cox regression for overall survival in validation dataset

Variables	<i>P</i>	HR	CI (95%)
Depth of wall invasion	0.370	1.66	0.55-4.90
Differentiation	0.240	4.93	0.15-1.58
Lymph node metastasis	0.780	0.77	0.13-4.53
Distance metastasis	0.120	0.32	0.08-1.35
Ten-gene prognostic marker	0.007	0.13	0.29-0.56

CI: Confidence interval; HR: Hazard ratio.

prognosis prediction, we compared the predictive power of our survival markers with the TNM staging. All the TNM stage II patients were categorized into the low-risk group and the entire high-risk group patients were in stage III or IV. A consistency between TNM staging and the staging was found by the ten-gene marker. However, two IIb patients of the low-risk group had a survival of 63.7 and 22.5 mo at last follow-up, respectively. By Kaplan-Meier survival plots and log-rank tests, we assessed the patient survival status predicted by our prognosis candidates and TNM staging. Relatively more accurate predictions were shown by the ten-gene prognostic marker in both sample groups (TNM,  $P = 0.00016$  and  $P = 0.0023$ ; survival markers  $P = 0.000047$  and  $P = 0.0009$ , Figure 2B and C, Figure 3B and C). As a result, in multivariate analysis, our ten-gene marker was the only independent indicator in prognosis prediction with statistical significance ( $P = 0.007$ ; Hazard ratio 0.13; 95% CI: 0.29-0.56, Table 4).

## DISCUSSION

It has been known that the environment and genetic background among ethnic groups correlate to the genesis and development of gastric cancer<sup>[16]</sup>. Up until now, very limited studies have been conducted in finding prognosis markers for gastric cancer, especially in the Chinese population. Moreover, only one set of prognosis markers was reported recently using the whole genome microarray ( $> 30K$ ), which, however, could predict peritoneal relapse but not overall survival<sup>[10]</sup>. In addition, in previous reports, by the classical supervised method to select survival markers, “low-risk” and “high-risk” subgroups are contrived based on survival times before analysis. Such a subjective step may result in bias for next process or lead to the classification which is not biologically meaningful. Therefore, in this study, we adopted a supervised PCA strategy to build prognosis profiles with the consideration of survival time as continuous parameters<sup>[11]</sup>. And based on the whole genome expression profiling, we found and verified a set of ten genes as candidate survival markers from the discovery panel of 39 samples and the validation panel of 33 samples.

In these 10 survival genes markers, 3 genes (RPS12, EIF3S6, RPS19) were previously reported as candidate

markers of diagnosis or prognosis in various types of cancers<sup>[13,17,18]</sup>. TMSB10, a migration-inducing gene, was shown to relate to cancer metastasis<sup>[19]</sup>. Additionally, a few genes (RPS19, RPLP2, GLTSCR2) are known factors involved in cell cycle control and apoptosis<sup>[20-22]</sup>. None of our 10 markers was reported in other sets of candidate genes for gastric cancer prognosis<sup>[5-8]</sup>. This is not a surprise since these 10 genes were selected based on the whole genome expression profiling followed by supervised PCA, whereas much less genes were included in earlier studies with the analysis strategy of supervised classification. Patients' genetic background may also contribute to such diversity.

Unexpectedly but also interestingly, 6 out of 10 candidate markers identified are ribosomal proteins (RPs). There may be a few explanations for this phenomenon. First, RPs have been shown to be the targets of several tumor suppressors and proto-oncogenes which affect the formation of the mature ribosomes or regulate the activity of proteins<sup>[23]</sup>. Moreover, the deregulated expression of RPs was reported to associate with the carcinogenesis and metastasis of various cancers<sup>[14]</sup>. Therefore, besides their unknown mechanisms possibly related to p53 and MYC<sup>[24,25]</sup>, RPs appear to have various cellular roles independent of protein biosynthesis, including their functions in DNA replication and DNA repair, transcription, RNA splicing and modification, cell proliferation, apoptosis, and cellular transformation.<sup>[26]</sup> Among 6 RPs of our candidate prognosis markers, RPLP2, RPL19, RPS8 and RPS12 were all found to be involved in the carcinogenesis and progression of various cancers<sup>[13,17,27]</sup>. RPS12 was also seen to have significant higher expression in gastric tumors in comparison with normal tissues in Chinese<sup>[28]</sup>.

In a number of diagnosis and prognosis sets identified in expression profiling from various cancer researches, the gene profile in most panels came from various pathways with different cellular functions. The result of our ten-gene prognostic marker containing 6 RPs raised another interesting issue on the molecular composition of biomarkers, i.e. which type is more powerful and more accurate in prediction, a set consisting of single gene tags from multiple individual pathways, or a group of genes from a few and related pathways. This issue needs more tests and evaluations for convincing answers. At this point, however, a few facts shall be brought into attention. First, our prognostic marker resulted from systematic analysis of whole genome expression profiling, and our strategy of supervised PCA largely reduced subjective attribution in analysis. Thus, a group of pinpointed signals will be more representative in biological meaning, thus providing more accurate prediction. Second, obviously in comparison with individual single signatures from multi-pathways, a group of signals would significantly overcome the individual bias, in which the pathway components in tumors vary widely<sup>[29]</sup>. Finally, it has been shown that even for genetic alterations of a large number of genes in cancer, these variations may function through a relatively small number of pathways and processes<sup>[29]</sup>. In our prognosis marker, although the details of the interrelationship

among those 6 RPs are still unknown, they have the same elevation in high-risk group, indicating the concordance of their functions in gastric cancer.

To reduce the heterogeneity among patients and samples which may bring bias to the analysis in this study, samples were randomly separated into training and validation batches. And no significant difference with respect to age, sex and other clinicopathological factors was found between the two batches (Table 1). And, by comparing clinicopathological factors between the high-risk group and low-risk group predicted by ten-gene markers, there was no significant difference between the two groups except for TNM staging (Table 3). Then we compared this ten-gene prognostic marker with TNM staging system. Both ten-gene prognostic marker and TNM classification can predict survival with statistical significances in discovery and validation sample batches (Figure 2C and 3C), indicating that our prognosis set can effectively complement traditional clinicopathological staging (Figure 2B and C, Figure 3B and C). The applicability of a marker with only 10 genes also suggests its potential to be developed as the prognosis marker panel for pre-operative molecular staging from endoscopic biopsy. Further validation with large scale samples are warranted for clinical application.

In conclusion, based on the whole genome expression profiling, we found and validated a ten-gene prognostic marker for overall survival prognosis of gastric cancer patients, which may be used with the TNM staging system as a parallel and complementary approach. However, the predominance of ribosome protein genes in our molecular prognostic marker warrants further research on their roles in cancer progression.

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## COMMENTS

### Background

Gastric cancer is the second leading cause of cancer related death in China and worldwide. The 5-year survival rate of patients with advanced disease is very poor. Currently, treatment plan and prognosis prediction for gastric cancer mainly depend on the clinicopathological staging. However, prognosis varies among patients with the same clinical pathological stage. An individualized expression test for selected markers in biopsy and surgical samples will complement the current staging system, especially for prognosis prediction.

### Research frontiers

The gene expression profiling has enabled researchers to quantify the biological states and consequently to uncover the subtle phenotypes in cancer. Such analyses have provided unique opportunities to develop various profiles that can distinguish, identify, and classify discrete subsets of disease, predict the disease outcome, and even predict the response to therapy.

### Innovations and breakthroughs

In this study, based on the whole genome expression profiling, the authors identified and validated a ten-gene set that can be further developed as clinical prognosis markers to predict overall survival of gastric cancer patients. This marker set showed consistent prognosis results with the traditional Tumor-

node-metastasis (TNM) staging system. The findings in this study also provided new clues about the possible association between the deregulation of ribosome related gene expression and survival status of the patients after surgery.

### Applications

Based on the whole genome expression profiling, a ten-gene prognostic marker set for overall survival prognosis of gastric cancer patients may be applied in combination with the TNM staging system as a parallel and complementary approach. However, the predominance of ribosome protein genes in these molecular prognostic markers awaits for further research on their roles in cancer progression.

### Terminology

**TNM:** The TNM system is one of the most widely used staging systems in tumor classification. The system is based on the extent of the tumor (T), the extent of spread to the lymph nodes (N), and the presence of distant metastasis (M). A number is added to each letter to indicate the size or extent of the primary tumor and the extent of cancer spread. **Principal component analysis (PCA):** A mathematical tool used to reduce the number of variables while retaining the original variability of the data. The first principal component accounts for as much of the variability in the data as possible, and each succeeding component accounts for as much of the remaining variability as possible. **Gene set enrichment analysis (GSEA):** A computational method that determines whether a prior identified set of genes shows statistically significant, concordant differences between two biological states. It is a method which focuses on the analysis at the level of functional related gene sets instead of a single gene. It helps biologists to interpret the DNA microarray data by their previous biological knowledge of the genes in a gene set. GSEA has been shown to efficiently identify gene sets containing known disease-related genes in the real experiments.

### Peer review

A ten-gene prognostic marker, including 6 ribosomal proteins, for overall survival prognosis of gastric cancer were identified and validated based on whole genome expression profiling. By comparing with the traditional TNM staging system, this ten-gene prognostic marker showed consistent prognosis results, which may complement the predictive value of current TNM staging system.

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## Differential expression of Bcl-2 and Bax during gastric ischemia-reperfusion of rats

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the early phases of reperfusion, reached its minimum at 1 h ( $P < 0.05$ ); it then increased, reaching its peak at 24 h of reperfusion ( $P < 0.05$ ). The pattern of Bax expression was opposite to that of Bcl-2. Bax expression increased after reperfusion, with its peak at 1 h of reperfusion ( $P < 0.05$ ), and then it decreased gradually to a minimum at 24 h after reperfusion ( $P < 0.05$ ). On the other hand, inhibition of activation of ERK1/2 induced by PD98059, a specific upstream MEK inhibitor, had significant effects on Bcl-2 and Bax in GI-R. Compared with GI-R treatment only at 3 h of reperfusion, PD98059 reduced the number of Bcl-2 positive cells (0.58% of R3h group,  $P < 0.05$ ) and Bcl-2 protein level (74% of R3h group,  $P < 0.05$ ) but increased the number of Bax-positive cells (1.33-fold vs R3h group,  $P < 0.05$ ) and Bax protein level (1.35-fold of R3h group,  $P < 0.05$ ).

**CONCLUSION:** These results indicated that the Bcl-2 and Bax played a pivotal role in the gastric mucosal I-R injury and repair by activation of ERK1/2.

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**Key words:** Stomach; Ischemia-reperfusion; Bcl-2; Bax; Extracellular signal-regulated kinase 1/2

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### Abstract

**AIM:** To investigate expression of Bcl-2 and Bax in gastric ischemia-reperfusion (GI-R) and involvement of extracellular signal-regulated kinase (ERK) 1/2 activation.

**METHODS:** The GI-R model was established by ligation of the celiac artery for 30 min and reperfusion in Sprague-Dawley rats. Rats were assigned to groups in accordance with their evaluation period: control, 0, 0.5, 1, 3, 6, 24, 48, and 72 h. Expression and distribution of Bcl-2 and Bax proteins were analyzed by immunohistochemistry and western blotting in gastric tissue samples after sacrifice.

**RESULTS:** Compared with controls, the percentage of positive cells and protein levels of Bcl-2 decreased in

### INTRODUCTION

Current research into gastric ischemia-reperfusion (GI-R)

has focused on its pathogenic and underlying molecular mechanism<sup>[1-8]</sup>. GI-R injury and repair is related to the changes in gastric mucosal cellular apoptosis and proliferation induced by GI-R in rats. In our previous experiments, we have explored the time course of gastric mucosal apoptosis and proliferation induced by GI-R, and the role of the extracellular signal-regulated kinase 1 and 2 (ERK1/2) signaling pathway in GI-R-induced gastric mucosal injury and repair<sup>[7]</sup>. We have found that serious gastric mucosal damage occurs rapidly at the early stage of reperfusion and is closely related to the suppression of ERK1/2 activation. The activity of ERK1/2 increases as the time of reperfusion is extended, and the activated ERK1/2 might inhibit apoptosis and promote proliferation in gastric mucosal cells. However, the precise mechanisms by which activated ERK1/2 accomplishes gastric mucosal apoptosis and proliferation are unknown.

The balance between apoptosis and cellular proliferation is a key in gastric injury and repair, and is regulated by several genes, including p53 and members of the Bcl-2 family such as Bax and Bcl-2<sup>[9-11]</sup>. The Bax gene is a proliferative suppressor gene that encodes Bax protein that promotes apoptosis. On the other hand, bcl gene encodes Bcl-2 protein that blocks wild type p53-mediated apoptosis, and heterodimers with Bax, antagonizing the function of Bax<sup>[12]</sup>.

Therefore, it is conceivable that increased cellular apoptosis and proliferation, because of altered expression of the regulating proteins such as Bax and Bcl-2, may be associated with gastric injury and repair induced by GI-R. Although Bcl-2 and Bax are expressed in gastric mucosa, their presence in normal gastric mucosa is controversial. Liu *et al.*<sup>[13]</sup> have reported that Bcl-1 mRNA and protein are expressed in the gastric gland zone at a middle level and Bax protein is expressed in the epithelial cells of normal gastric mucosa. Xia *et al.*<sup>[14]</sup> have found that, in intact gastric tissue, Bcl-2 and Bax are localized predominantly in the glandular base region in chief cells in normal rat gastric mucosa. However, a conflicting study has found that no expression of Bcl-2 protein is detected in the glandular epithelium of normal gastric mucosa<sup>[15]</sup>. On the other hand, Bcl-2 and Bax show significant changes in many conditions including gastric cancer, gastritis, and GI-R<sup>[15-18]</sup>. El Eter *et al.*<sup>[15]</sup> have reported that cytoplasmic expression of Bcl-2 protein is observed in the superficial portion of gastric mucosa sections obtained from rats subjected to GI-R injury.

The available data on expression of Bcl-2 and Bax proteins in the stomach, and their relation to apoptosis of gastric mucosal cells, seem equivocal, thus, a further study of Bcl-2 and Bax expression in the stomach is clearly important. In the present study, we used an immunohistochemical assay and western blotting to determine the changed courses of Bcl-2 and Bax at different reperfusion durations after GI-R, and whether ERK1/2 activation was involved in this process.

## MATERIALS AND METHODS

### Animals

Groups of six adult Sprague-Dawley rats, regardless of

sex, weighing 220-270 g, were provided by the Experimental Animal Centre of Xuzhou Medical College. All experiments were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Rats were housed under controlled temperature (22-24°C) and photoperiod (12 h light/12 h dark), and allowed food and water *ad libitum*. Rats were fasted for 24 h before the experiment, but were allowed free access to tap water. Animals were randomly assigned to groups: GI-R (different reperfusion time points after 30 min of ischemia); PD98059 + R3h (PD98059 + reperfusion for 3 h after 30 min of ischemia); and vehicle control (PD98059 replaced with vehicle but otherwise the same as PD98059 + R3h). PD98059 was given 20 min before operation [150 µg/kg, administered intraperitoneally (i.p.), dissolved in dimethyl sulfoxide]. A sham group in which only the same surgical procedure without clamping the celiac artery was performed served as a control.

### Reagents

PowerVision™ two-step immunohistochemistry detection kit were purchased from Zhongshan Biotech Co. (Beijing, China), anti-Bcl-2 and anti-Bax polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), alkaline-phosphorylase-tagged goat anti-rat IgG antibody, PD98059 was from Promega (Madison, WI, USA), and sodium pentobarbital was purchased from Sigma (St. Louis, MO, USA).

### Preparation of GI-R model

GI-R models were induced according to the method of Qiao *et al.*<sup>[7]</sup>. The randomly grouped rats were all anesthetized with sodium pentobarbital (40 mg/kg, i.p.). Their abdomens were incised along the midline, and the celiac artery and its adjacent tissues were carefully isolated. The celiac artery was clamped with a small non-traumatic vascular clamp for 30 min to induce gastric ischemia and then released for 0, 0.5, 1, 3, 6, 24, 48 and 72 h to allow reperfusion. Following reperfusion, the rats were sacrificed and the stomachs were removed immediately. The stomachs were incised along the greater curvature and flushed with ice-cold PBS (0.1 mol/L). One half of the gastric mucosa was frozen at -80°C for western blotting, and the other was fixed in Bouin's fixative for immunohistochemical staining.

### Immunohistochemical staining

The fixed stomach was embedded in paraffin, sliced into 4-µm-thick sections, and mounted on glass slides. The immunohistochemistry was performed with a PowerVision two-step immunohistochemistry detection kit. The sections were stained with 3,3'-diaminobenzidine (DAB), then counterstained using hematoxylin. The sections were examined with a microscope (Model IX71; Olympus, Tokyo, Japan). Gastric mucosal cells with brown granules visible in the cytoplasm or nucleus were considered positive. The number of positive cells per section was counted in 10 random lower-power (× 10) fields, and the percentage



of positive cells (positive cells/total cells  $\times$  100%) was calculated. Three non-consecutive sections were selected from each specimen and those indexes were averaged.

### Western blotting

The frozen gastric mucosa was homogenized with a Teflon glass homogenizer in 1:10 (w/v) ice-cold homogenization buffer consisting of 50 mmol/L 3-(N-morpholino) propanesulfonic acid (MOPS, pH 7.4), 50 mmol/L NaF, 20 mmol/L sodium pyrophosphate (NaPPi), 20 mmol/L b-glycerophosphate, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L phenylmethylsulphonyl fluoride, 10 mg/mL leupeptin, 10 mg/mL aprotinin and 10 mg/mL pepstatin A. The homogenate was centrifuged at 800 g for 15 min at 4°C, and the supernatant was retained as cytoplasmic parts. Protein concentrations were determined by Coomassie brilliant blue protein assay. The proteins were heated at 100°C for 5 min with loading buffer containing 0.125 mol/L Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 10% mercaptoethanol and 0.002% bromophenol blue, then separated by 10% SDS-PAGE. The proteins were isolated by 12.5% SDS-PAGE and transferred to a nitrocellulose membrane. The blots were incubated with 4% bovine serum albumin in TBST (10 mmol/L Tris, pH 7.5; 150 mmol/L NaCl, 0.05% Tween-20) at 4°C for 6 h and probed with primary antibodies (anti-Bcl-2 polyclonal antibody 1:500, anti-Bax polyclonal antibody 1:400) at 4°C overnight. Membranes were rinsed and incubated with secondary antibody for 2 h and were detected with an NBT/BCIP assay kit. After immunoblotting, the bands were scanned and analyzed by Image J software. The optical density (OD) of the band in each lane was expressed as the fold change versus the OD of the sham control.

### Statistical analysis

All results are presented as mean  $\pm$  SD. Comparisons between two groups were made with Student's *t* test; multiple-group analyses were made by one-way ANOVA. Statistical analyses were performed with SPSS for Windows version 11.5. *P* < 0.05 was considered statistically significant.

## RESULTS

### Quantitative changes in Bcl-2 and Bax positive cells of the gastric mucosa for different reperfusion durations after ischemia

Immunohistochemical staining clearly showed that Bcl-2 and Bax were expressed and limited to the cytosol of the cells in the gastric mucosa (Figure 1). Bcl-2 expressing cells were found predominantly in the lower part of the gastric gland, and were present only in gland cells of the stomach fundus (Figure 1A-D). Bax positive cellular distribution was similar to that of Bcl-2, with prominent expression in the base, but staining for Bax was also noticeable in the cells of the pit. Bax appeared to be absent from the middle part of the gastric gland, and weak expression of Bax was detected in most cells of the gastric mucosa (Figure 1E-H). The control sample (sham-operated) obtained prior to the ischemic period showed a

normal appearance of Bcl-2 (Figure 1A and B) and Bax (Figure 1E and F), and there were significant differences between the GI-R (Figure 1C, D, G and H) and control groups (Figure 1A and E) in the quantities of Bcl-2 and Bax immunoreactive cells.

The percentage of Bcl-2 and Bax positive cells in various groups is shown in Figure 2. Bcl-2 and Bax positive cells decreased in the early phase of reperfusion, with a nadir (10.02%  $\pm$  1.21%) at 1 h of reperfusion, then increased significantly after reperfusion for 3 h, with a peak (29.76%  $\pm$  3.32%) at 24 h of reperfusion, and returned to near the base level (20.47%  $\pm$  2.97%) at 72 h of reperfusion. The opposite pattern was observed for Bax positive cells. The percentage of Bax positive cells increased in the initial stages of reperfusion, reached the highest Bax positive cell count (49.34%  $\pm$  3.83%) at 1 h of reperfusion, then decreased gradually, with its nadir (13.36%  $\pm$  3.05%) at 24 h of reperfusion, and recovered to base level (24.94%  $\pm$  2.83%) at 72 h of reperfusion.

### Protein expression of Bcl-2 and Bax in gastric mucosa at different reperfusion durations after ischemia

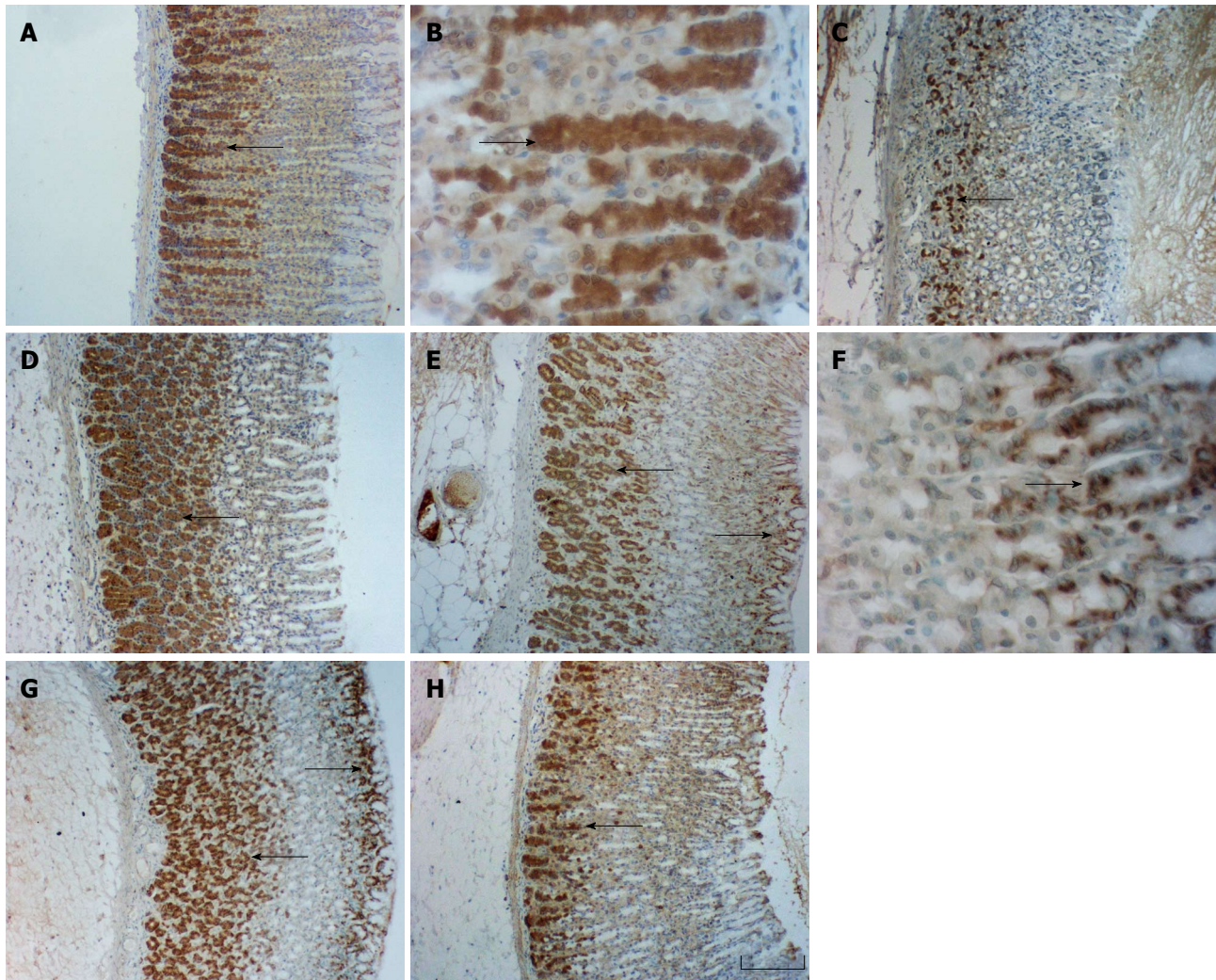
Figure 3 shows the Bcl-2 and Bax protein levels in the gastric mucosa in different groups of the study. After reperfusion, expression of Bcl-2 protein was significantly lower than that of the controls, and the lowest level was observed at 1 h of reperfusion (0.59% of sham group, *P* < 0.05). A peak of Bcl-2 protein expression was displayed in the 24 h reperfusion group (1.36 fold *vs* sham group, *P* < 0.05). The Bax protein level increased in the early stage of reperfusion, reached its peak (1.62-fold *vs* sham group, *P* < 0.05) at 1 h of reperfusion, and then decreased gradually to its lowest levels (0.57% of sham group, *P* < 0.05) at 24 h of reperfusion. At 72 h of reperfusion, Bcl-2 and Bax protein levels were the same as that of the control group.

### Effects of PD98059 on expression of Bcl-2 and Bax

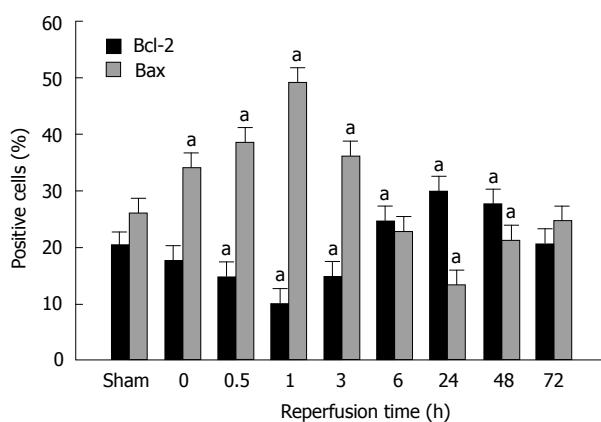
PD98059 is a specific upstream inhibitor of ERK1/2. By immunohistochemical assay, we found PD98059 had significant effects on Bcl-2 and Bax expression in GI-R. Compared with the control group (R3h group), the PD98059 + R3h group showed a fall in the number of Bcl-2 positive cells (0.58% of R3h group, *P* < 0.05) but an increase in Bax positive cells (1.31-fold *vs* R3h group, *P* < 0.05) (Figure 4). To ascertain the effect of PD98059 on expression levels of Bcl-2 and Bax protein, western blotting was performed. In the PD98059 + R3h group, gastric mucosal Bcl-2 protein level was 74% of that in the R3h group (*P* < 0.05), whereas Bax protein level was 1.35-fold more than that in the R3h group (*P* < 0.05) (Figure 5).

## DISCUSSION

Previous studies have shown that many stress conditions, such as hemorrhagic shock, burns, sepsis, major surgery, ischemia and trauma can lead to GI-R injury. In recent years, studies on GI-R injury have revealed that reactive



**Figure 1** Histological exhibition of Bcl-2 and Bax positive cells in the gastric mucosa at different reperfusion times after ischemia, by immunohistochemical staining in rats. The Bcl-2 and Bax positive cells were respectively probed with anti-Bcl-2 and anti-Bax polyclonal antibodies in rat gastric mucosa. Nuclear counterstaining was performed with hematoxylin. The examples of immunoreactive cells are those with dark brown staining in their cytosol (arrows). A and B: Bcl-2, control; C: Bcl-2, GI-R at 1 h after reperfusion; D: Bcl-2, GI-R at 24 h after reperfusion; E and F: Bax, control; G: Bax, GI-R at 1 h after reperfusion; H: Bax, GI-R at 24 h after reperfusion. Images were obtained at  $\times 100$  (A, C, D, E, G and H, Bar 100  $\mu\text{m}$ ) and  $\times 400$  (B and F, Bar 400  $\mu\text{m}$ ).

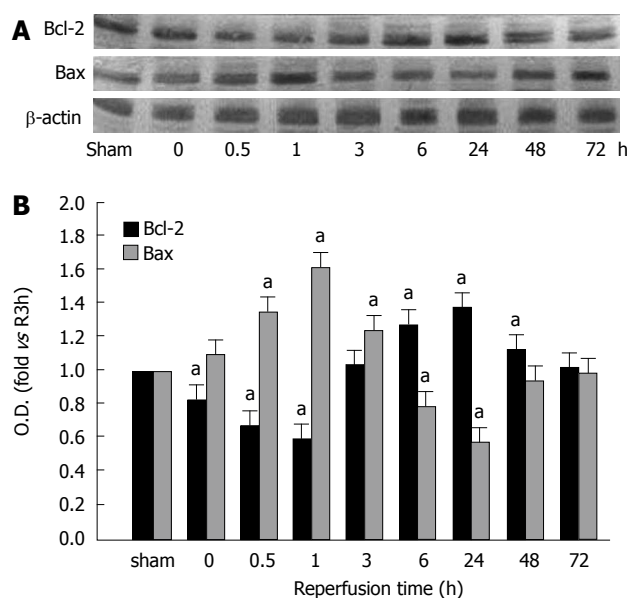


**Figure 2** Quantitative changes in Bcl-2 and Bax positive cells in rat gastric mucosa for different reperfusion times after GI-R. Reperfusion was maintained for 0, 0.5, 1, 3, 6, 24, 48 and 72 h after 30 min of ischemia. Sham: sham-operated. Values are percentage of positive cells (positive cells/total cells) counted in 10 microscopic fields. Each column represents mean  $\pm$  SD,  $n = 6$ . <sup>a</sup> $P < 0.05$  vs sham.

oxygen species, endothelin, microvascular dysfunction, polymorphonuclear leukocyte infiltration, nitric oxide release, gastric acid secretion and decreased prostaglandin concentrations may play a role in the pathogenesis of gastric mucosal injury induced by GI-R<sup>[1,5,19-25]</sup>. Although the gastric mucosa is vulnerable to damage by various factors, it can quickly repair the damage<sup>[26]</sup>. Mucosal integrity is maintained by a balance between proliferation and apoptosis of the gastric mucosal cells. To understand better the causes of gastric lesions, it is important to study the imbalance between proliferation and apoptosis<sup>[27,28]</sup>.

In previous experiments<sup>[7]</sup>, we have shown the changed courses of gastric mucosal injury and repair induced by GI-R, and the role of ERK1/2 in this process. Our results indicated clearly that the gastric mucosal injury induced by GI-R was mainly the result of reperfusion. The serious gastric mucosal lesions occurred in the initial stages of reperfusion and the aggravating processes of mucosal lesions were at 1 h after reperfusion, which were main-

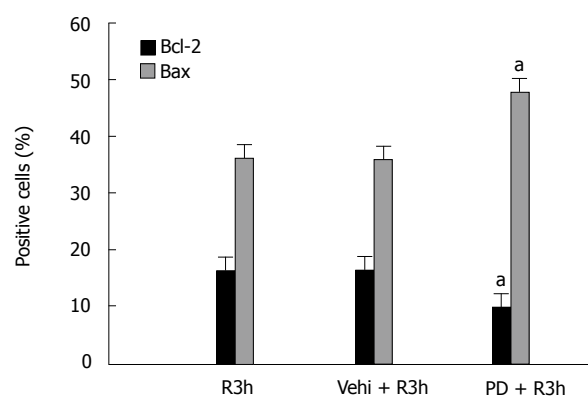




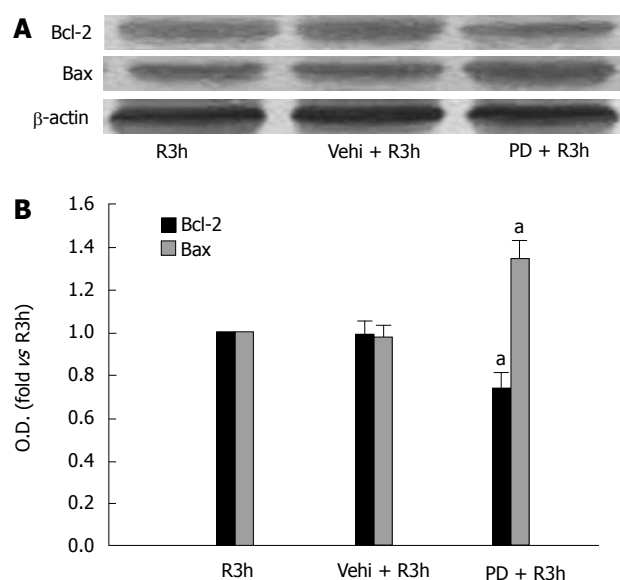
**Figure 3** Expression of Bcl-2 and Bax proteins in cytoplasm extracts from rat gastric mucosa for different reperfusion durations after gastric ischemia-reperfusion. Extracts were obtained from sham-operated rats or from gastric ischemia-reperfusion rats with different reperfusion durations (0, 0.5, 1, 3, 6, 24, 48, or 72 h) after 30 min of ischemia, and were analyzed respectively by western blotting with anti-Bcl-2 and anti-Bax antibodies. A: Representative blots corresponding to expression levels of Bcl-2 and Bax proteins; B: Semi-quantitative analysis of the levels of Bcl-2 and Bax. Sham: Sham-operated. Values are means  $\pm$  SD,  $n = 6$ . <sup>a</sup> $P < 0.05$  vs sham.

tained about 3 h after reperfusion. The gastric mucosal repairs started after 3 h of reperfusion, and the complete recovery took almost 3 d. Based on these facts, it indicated that gastric mucosa has an amazing self-repairing ability. ERK1/2 are important members of the mitogen-activated protein kinase family. The activation of ERK1/2 participates in the regulation of cellular injury and repair in many tissues. Our researches have also shown that the p-ERK1/2 protein level decreased at 0.5 h after reperfusion began, and then gradually increased, reaching its peak after 3 h of reperfusion. Inhibition of the activation of ERK1/2 aggravated the gastric mucosal injury, with apoptosis increased and proliferation reduced in the gastric mucosal cells at the same duration of reperfusion. Therefore, activated ERK1/2 inhibited apoptosis and promoted proliferation in gastric mucosal cells.

Apoptosis and proliferation are fundamental mechanisms for cell death and survival and differentiation in the gastric mucosa. The status of the Bcl-2 family proteins determines whether a cell will live or die through the regulation of cytochrome c release from the mitochondria<sup>[29,30]</sup>. Bcl-2 protein mainly inhibits apoptosis and facilitates cellular survival and differentiation, whereas overexpression of Bax protein induces apoptosis and inhibits the effect of Bcl-2<sup>[31-33]</sup>. Our data showed that Bcl-2 expression decreased significantly after the start of the reperfusion, reaching its nadir at 1 h, before increasing gradually to a peak after 24 h of reperfusion. The pattern of change in Bax expression was opposite to that of Bcl-2 expression. Bax expression increased at first, reaching its maximum



**Figure 4** Effects of PD98059 (ERK1/2 inhibitor) on quantitative changes of Bcl-2 and Bax positive cells in rat gastric mucosa after gastric ischemia-reperfusion. R3h: Reperfusion for 3 h after 30 min of ischemia; Vehi + R3h: Vehicle + R3h; PD + R3h: PD98059 + R3h; Sham: Sham-operated. Values are percentage of positive cells (positive cells/total cells) counted in 10 microscopic fields. Each column represents mean  $\pm$  SD,  $n = 6$ . <sup>a</sup> $P < 0.05$  vs R3h.



**Figure 5** Effects of PD98059 (ERK1/2 inhibitor) on expression of Bcl-2 and Bax proteins in cytoplasm extracts from rat gastric mucosa after gastric ischemia-reperfusion. R3h: Reperfusion for 3 h after 30 min of ischemia; Vehi + R3h: Vehicle + R3h; PD + R3h: PD98059 + R3h. Extracts were obtained for analysis by western blotting with anti-Bcl-2 and anti-Bax antibodies. A: Representative blots corresponding to expression levels of Bcl-2 and Bax proteins; B: Semi-quantitative analysis of the levels of Bcl-2 and Bax. Values are means  $\pm$  SD,  $n = 6$ . <sup>a</sup> $P < 0.05$  vs R3h.

after 1 h of reperfusion, and then decreased. Bcl-2 and Bax recovered gradually to base level at 72 h of reperfusion. PD98059, a specific upstream inhibitor of ERK1/2, downregulated expression of Bcl-2 and upregulated expression of Bax in GI-R. These results suggest that the course of expression of Bcl-2 and Bax were closely correlated with p-ERK1/2. Activation of ERK1/2 causes upregulation of Bcl-2 and downregulation of Bax.

In conclusion, Bcl-2 and Bax played a pivotal role in GI-R injury and repair by activation of ERK1/2. Bcl-2 was involved in recovery of GI-R-mediated gastric mucosa injury by promoting cellular proliferation, and Bax



was involved in gastric mucosal injury induced by GI-R by promoting apoptosis.

## COMMENTS

### Background

It is well known that many hemorrhagic and stress conditions lead to gastric ischemia-reperfusion (GI-R) injury. Gastric ulceration is very prevalent in humans and is usually preceded by burns, sepsis, major surgery, ischemia, trauma and other heterogeneous forms of stress. Erosions in the gastric mucosa can be demonstrated in as many as 75%-100% of patients within 24 h of admission to the intensive care unit (ICU). Clinically apparent gastrointestinal bleeding can occur in as many as 25% of ICU patients.

### Research frontiers

In recent years, studies on GI-R injury have focused on its pathogenic and underlying molecular mechanism. In recent years, studies on GI-R injury have revealed that reactive oxygen species, endothelin, microvascular dysfunction, polymorphonuclear leukocyte infiltration, nitric oxide release, gastric acid secretion and decreased prostaglandin concentrations during reperfusion may play a role in the pathogenesis of gastric mucosal injury induced by GI-R. Mucosal integrity is maintained by the equilibrium between proliferation and apoptosis of the gastric mucosal cells. To understand better the pathogenesis of gastric lesions, it is of great importance to study the imbalance between proliferation and apoptosis.

### Innovations and breakthroughs

This is believed to be the first study to investigate changes in expression of Bcl-2 and Bax at different times of reperfusion after gastric ischemia, and whether extracellular signal-regulated kinase 1/2 activation was involved in this process.

### Applications

Not only does our study provide insights into the mechanism of gastric mucosal tissue injury and repair; it also provides information that could potentially guide development of a new therapeutic strategy.

### Peer review

The quality of the paper is excellent and deserves a fast publication, considering the contribution importance.

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## Intrahepatic natural killer T cell populations are increased in human hepatic steatosis

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study. Subjects with moderate or severe steatosis had a higher percentage of intrahepatic CD3+/CD56+ NKT cells (38.6%) than did patients with mild steatosis (24.1%,  $P = 0.05$ ) or those without steatosis (21.5%,  $P = 0.03$ ). Patients with moderate to severe steatosis also had a higher percentage of NKT cells in the blood (12.3%) as compared to patients with mild steatosis (2.5%  $P = 0.02$ ) and those without steatosis (5.1%,  $P = 0.05$ ).

**CONCLUSION:** NKT cells are significantly increased in the liver and blood of patients with moderate to severe steatosis and support the role of NKT cells in NAFLD.

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**Key words:** Nonalcoholic fatty liver disease; Natural killer T cells; Natural killer T-like cells; Lymphocytes; Hepatic steatosis

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### Abstract

**AIM:** To determine if natural killer T cell (NKT) populations are affected in nonalcoholic fatty liver disease (NAFLD).

**METHODS:** Patients undergoing bariatric surgery underwent liver biopsy and blood sampling during surgery. The biopsy was assessed for steatosis and immunocyte infiltration. Intrahepatic lymphocytes (IHLs) were isolated from the remainder of the liver biopsy, and peripheral blood mononuclear cells (PBMCs) were isolated from the blood. Expression of surface proteins on both IHLs and PBMCs were quantified using flow cytometry.

**RESULTS:** Twenty-seven subjects participated in this

### INTRODUCTION

With the epidemic of obesity burgeoning across much of the world, nonalcoholic fatty liver disease (NAFLD) has become an increasingly pressing problem. The prevalence of NAFLD in western countries is as high as 17%-33%<sup>[1]</sup>, and the more severe form of NAFLD, non-alcoholic steatohepatitis (NASH), will progress to cirrhosis in 20% of patients<sup>[1]</sup>. Due to its increasing prevalence, NAFLD has become the third leading indication for liver transplantation<sup>[2]</sup>.



The etiopathogenesis of NAFLD/NASH involves a number of environmental, genetic, and inflammatory influences. However, many of the factors that play a role in the development of NAFLD and NASH remain unknown. What is clear is that NASH is associated with hepatic infiltration of inflammatory cells, resulting in hepatocyte injury and hepatocyte death. The prevailing two-hit theory, posited by Day *et al*<sup>[3]</sup>, proposes an initial hit whereby obesity is associated with hepatic accumulation of free fatty acids and triglycerides, followed by a second hit, whereby oxidative stress, mitochondrial dysfunction, elaboration of pro-inflammatory cytokines and inflammatory cell infiltration leads to development of NASH.

Natural killer T (NKT) cells are a highly conserved subset of lymphocytes with properties of both T cells (CD3+ expression) and NK cells (CD56+ and CD161+ expression)<sup>[4]</sup> and have been implicated in NAFLD. NKT cells are concentrated in the liver where they serve an important role in innate immunity. In the murine liver, NKT cells comprise 30%-50% of all hepatic lymphocytes<sup>[5]</sup>. These cells can be directly cytotoxic *via* FasL-dependent and perforin-mediated mechanisms, but also produce an array of cytokines that direct cytokine secretion by other cells within their microenvironment<sup>[5]</sup>. These functions may be responsible for cell death seen in NAFLD. NKT cells are believed to be primarily stimulated by various glycolipids, which are presented by CD1d, an MHC-like molecule on antigen presenting cells, such as Kupffer cells, to the NKT cells' invariant T cell receptor<sup>[6]</sup>. The role of NKT cells in immunity has yet to be fully elucidated and there have been many proposed functions for this unique cell, ranging from antitumor activity to autoimmune diseases<sup>[7]</sup>. In addition, murine models of obesity and fatty liver disease, using leptin-deficient, *ob/ob* mice, have suggested that NAFLD is associated with depletion of NKT cells<sup>[8]</sup>. The loss of CD4-expressing NKT cells is particularly intriguing as this cell subset is believed to primarily secrete Th2-type cytokines, including IL-4 and IL-13<sup>[9]</sup>. This loss of Th2 cytokines might tip the inflammatory milieu of the liver into a pro-inflammatory Th1 state, leading to excessive production of TNF- $\alpha$  and IFN- $\gamma$ . The increase in pro-inflammatory cytokines likely plays a role in hepatic oxidative stress and recruitment of additional inflammatory cells into the liver, resulting in NASH<sup>[10]</sup>. The transfer of NKT lymphocytes back into leptin deficient mice has been shown to reduce hepatic steatosis and improves glucose intolerance<sup>[11]</sup>. In addition, inducing expansion of the NKT cell population, by norepinephrine injection or by stimulation with glucocerebroside, has also been shown to reduce hepatotoxicity and improve hepatic fat content in murine models<sup>[12,13]</sup>.

While murine models of NAFLD clearly support a pivotal role of NKT cells in pathogenesis, data on the role of NKT cells in human NAFLD is limited. Xu and colleagues found that peripheral blood NKT cells are depleted in patients with clinically diagnosed NAFLD<sup>[14]</sup>. Three other studies evaluated intrahepatic NKT cells and had differing results. The study by Kremer *et al*<sup>[15]</sup> found that NKT cells

are depleted with increased steatosis, whereas the one by Tajiri and colleagues found an increase in NKT cells with steatosis<sup>[16]</sup>. Finally a study by Syn *et al*<sup>[17]</sup> also found an increase in NKT cells with steatosis. In this study, we sought to further investigate the changes in lymphocyte populations that occur in NAFLD.

## MATERIALS AND METHODS

### Patients and lymphocyte isolation

From January to November 2007, peripheral blood and hepatic tissue were collected from obese subjects undergoing laparoscopic gastric banding surgery. Patients were excluded if they were under the age of 18, infected with hepatitis B virus, hepatitis C virus, HIV, were known to have pre-existing hepatic disease, or found to have any non-NAFLD pathological processes found on histological examination of the liver biopsy material. Patients were also excluded if they had a known history of excessive alcohol ingestion. All enrolled subjects signed an informed consent form that was approved by the institutional review board of NYU Langone Medical Center.

Immediately prior to surgery, 10 mL of blood was obtained from each subject by venipuncture. During the surgery a 2 cm<sup>3</sup> liver scissor biopsy was obtained. The liver biopsy sample was placed in 15cc of sterile RPMI 1640 (Mediatech Inc, Herndon, VA) and was transported to the laboratory with the blood sample for lymphocyte isolation. An additional portion of the biopsy was evaluated by a single hepatopathologist who made the diagnosis of NAFLD and NASH using the staging system proposed by Brunt *et al*<sup>[18]</sup>. Mild steatosis was defined as steatosis involving up to 33% of hepatocytes, moderate steatosis involved 33%-66% of hepatocytes, and severe steatosis involved greater than 66% of hepatocytes. Steatohepatitis was defined by a number of features including steatosis, ballooning, and acinar and portal inflammation.

Once transported to the laboratory, the liver biopsy sample was washed in sterile phosphate-buffered saline (PBS) and was minced to 1 mm<sup>3</sup> pieces in a petri dish with 30 mL of RPMI 1640 containing 0.5 mg/mL collagenase type II (Clostridiopeptidase A), 0.02 mg/mL DNase I, 100 U/mL penicillin, 100 mg/mL streptomycin and 2 mmol/L L-glutamine (all from Sigma-Aldrich, St. Louis, MO) and 10% fetal calf serum (FCS) (Invitrogen, Carlsbad CA). The minced liver was incubated in this digestion solution at 37°C for 30 min after which it was strained through a 70 mm disposable plastic strainer. Immediately after isolation, cells were washed and re-suspended in PBS. The cell solution was then pipetted onto a 30%-70% percoll (Sigma-Aldrich, St. Louis MO) gradient and was centrifuged at 2000 r/min for 30 min. The isolated lymphocytes were removed from the gradient, washed with PBS, resuspended in 1 mL PBS/10% FCS containing 2% formaldehyde (Sigma-Aldrich, St. Louis MO) and placed at 4°C. Peripheral blood mononuclear cells (PBMCs) were prepared by centrifugation on a Ficoll-Hypaque density gradient (Mediatech, Herndon, VA).

Table 1 Patient characteristics

	No. steatosis ( <i>n</i> = 10)	Mild steatosis ( <i>n</i> = 11)	Moderate- severe steatosis ( <i>n</i> = 6)	Reference values
Age	36.3 ± 13.1	44.5 ± 14.4	48.3 ± 11.3	N/A
Gender (% female)	90%	73%	66%	N/A
Body mass index (kg/m <sup>2</sup> )	42.4 ± 3.2	44.2 ± 6.2	41.9 ± 4.3	18.5-24.9
Aspartate transaminase (U/L)	28.8 ± 13.2	42.8 ± 34.2	47.0 ± 26.9	0-40
Alanine transaminase (U/L)	36.1 ± 18.7	50.1 ± 48.2	75.8 ± 40.1	0-45
Alkaline phosphatase (IU/L)	79.2 ± 18.0	89.5 ± 16.6	82.5 ± 12.7	20-140

All results except gender presented as mean ± standard deviation.

### Flow cytometric analysis of lymphocyte populations

Cell surface expression of lymphocyte antigens was identified by monoclonal antibody staining of freshly isolated IHLs and PBMCs, followed by flow cytometry using a BD LSR II (Becton Dickinson Immunocytometry Systems (BDIS), Mountain View CA) flow cytometer with analysis using CellQuest<sup>®</sup> software (BDIS, Mountain View CA). Monoclonal antibodies used in this study included anti-human CD3 (clone UCHT1) (BDIS, Mountain View, CA), anti-human CD4 (clone RPA T4) (Pharmingen, San Diego, CA), anti-human CD8 (clone RPA T8) (Pharmingen, San Diego, CA), anti-human CD56 (clone NCAM16.2) (Pharmingen, San Diego, CA), anti-human CD161 (clone DX12) (Pharmingen, San Diego, CA), anti-human  $\alpha$ 24 (clone C15) (Immunotech, Fullerton, CA), and the appropriate isotype controls. During flow cytometry, lymphocytes, initially identified by their forward and side scatter characteristics, were subject to phenotypic analysis. Dead cells were excluded from analysis using 7-aminoactinomycin D (Calbiochem, La Jolla, CA).

### ELISA for quantification of IFN- $\gamma$ and IL-4 secretion

IFN- $\gamma$  and IL-4 secretion by intrahepatic and peripheral blood lymphocytes was determined by ELISA (BD Pharmingen) after culture for 12 h. For these assays,  $1 \times 10^5$  lymphocytes derived from the liver or blood were co-cultured with monocyte-derived macrophages in the presence of alpha-galactosyl ceramide at 10  $\mu$ g/mL in a 96-well flat-bottom plate.

### Statistical analysis

Values are expressed as mean ± SD. Statistical comparisons were made between PBMCs and IHLs from individuals using a paired *t*-test. Statistical comparisons were made between subjects without hepatic steatosis, those with mild steatosis and those with moderate-to-severe steatosis using a two-sample, unequal variance *t*-test. All reported *P* values were two-sided at the 0.05 significance level using SPSS<sup>™</sup> 11.0 for Windows software (SPSS, Chicago, IL).

## RESULTS

### Subject cohort characteristics

Table 1 describes the clinical characteristics of the 27 patients enrolled in this study. Ten of the twenty-seven subjects (37%) had normal liver biopsies, without steatosis, while 11 of 27 (41%) had mild hepatic steatosis and 6 of 27 (22%) had moderate-severe hepatic steatosis. Of the patients with mild steatosis, 10 had increased hepatic lymphocyte infiltration, but were not felt to be severe enough to merit a diagnosis of NASH. One of the six subjects with moderate-severe steatosis had grade 3 steatohepatitis with grade 1 fibrosis. Seventy-eight percent of the subjects were female, their mean age was 42 years, and their mean body mass index (BMI) was 42.9. There were no significant differences between patient cohorts with regard to age, gender, BMI, or serum levels of liver-associated enzymes.

### Moderate-to-severe hepatic steatosis is associated with increased percentages of intrahepatic and blood NKT cells

Using the most common phenotypic definition of NKT cells, we sought to compare the percentage of CD3+/CD56+ in the liver and the periphery of subjects without hepatic steatosis with those with moderate-to-severe steatosis. As shown in Table 2, the liver and blood of subjects with steatosis had significant increases in the percentage of NKT cells. CD3+/CD56+ NKT cells comprised  $38.6\% \pm 10.5\%$  of all intrahepatic T cells of subjects with moderate-to-severe steatosis, compared to  $21.5\% \pm 14.3\%$  T cells in the liver of subjects without any steatosis (*P* = 0.03). The percentage of CD3+/CD56+ T cells in the liver of subjects with mild steatosis ( $24.1\% \pm 12.4\%$ ) was intermediate between that of normal and moderate-to-severe steatosis, and was significantly lower than that of the subjects with moderate-to-severe steatosis (*P* = 0.05) with a correlation of 0.93. While in all three subject cohorts, the percentage of CD3+/CD56+ cells was significantly lower in the blood compared to the liver, the percentage PBMC CD3+/CD56+ NKT cells of subjects with moderate-to-severe steatosis ( $12.3\% \pm 5.6\%$ ) was significantly greater than both subjects with no steatosis ( $5.1\% \pm 5.5\%$ , *P* = 0.05) and those with mild steatosis ( $2.5\% \pm 1.5\%$ , *P* = 0.02).

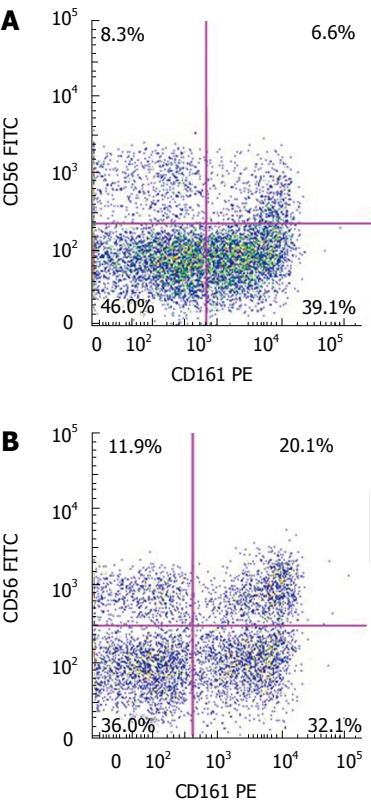
### Percentage of invariant $\alpha$ 24 NKT cells in patients with steatosis and IFN- $\gamma$ and IL-4 expression

We also analyzed invariant NKT cells; the CD-1d-reactive, glycolipid-activating NKT cells which express  $\alpha$ 24<sup>[7]</sup>. We found that a minority of CD3+/CD56+ NKT cells express  $\alpha$ 24. In addition, we did not find significant differences in expression of  $\alpha$ 24 between subjects without steatosis, those with mild or those with moderate-to-severe steatosis in the liver or blood (Table 2). When stimulated by alpha-galactosylceramide, the prototypical stimulant of  $\alpha$ 24, invariant NKT cells, hepatic-derived lymphocytes produced greater amounts of IFN- $\gamma$ , as measured

**Table 2** Percentage of CD3+ lymphocyte populations in patients with normal livers, mild hepatic steatosis, and moderate to severe steatosis *n* (%)

Lymphocyte population	N steatosis	M steatosis	MS steatosis	<i>P</i> value (N <i>vs</i> M)	<i>P</i> value (N <i>vs</i> MS)	<i>P</i> value (M <i>vs</i> MS)	Correlation coefficient
CD3+/CD4-/CD8- PBMC	8.13	3.62	22.88	0.22	0.1	0.04 <sup>a</sup>	0.73
CD3+/CD4-/CD8- IHL	12.61	9.12	26.58	0.4	0.1	0.05 <sup>a</sup>	0.76
CD3+/CD56+ PBMC	5.09	2.45	12.32	0.22	0.049 <sup>a</sup>	0.016 <sup>a</sup>	0.71
CD3+/CD56+ IHL	21.49	24.13	38.62	0.7	0.03 <sup>a</sup>	0.048 <sup>a</sup>	0.93
CD3+/CD56+/CD161+ PBMC	2.45	1.15	9.64	0.3	0.027 <sup>a</sup>	0.017 <sup>a</sup>	0.79
CD3+/CD56+/CD161+ IHL	15.50	18.90	35.81	0.6	0.006 <sup>a</sup>	0.017 <sup>a</sup>	0.93
CD3+/Vα24+ PBMC	0.60	0.53	0.57	0.48	0.23	0.14	-0.43
CD3+/Vα24+ IHL	0.43	0.42	0.76	0.9	0.37	0.36	0.85
CD3+/CD8+ IHL	55.59	49.30	26.58	0.51	0.0003 <sup>a</sup>	0.006 <sup>a</sup>	-0.95

Each percentage is the proportion of a specific CD3+ lymphocyte population out of all CD3+ lymphocytes. <sup>a</sup>*P* < 0.05. PBMC: Peripheral blood mononuclear cell; IHL: Intrahepatic lymphocyte. N: Normal; M: Mild; MS: Mod/sev.



**Figure 1** Flow cytometry of CD3+/CD56+/CD161+ intrahepatic lymphocytes in a patient with a normal liver versus a patient with moderate steatosis. Cells were initially selected via CD3+ gating prior to analysis for expression of CD56 and CD161. There are a greater percentage of natural killer T cells (CD56+/CD161+) in patients with moderate steatosis (20.1%) as compared to patients with normal livers (6.6%). A: Normal liver; B: Moderate steatosis.

by ELISA, compared to peripheral lymphocytes, though no differences were noted between patient cohorts (data not shown). In the majority of samples, IL-4 secretion remained undetectable.

**Expression of CD161+ on NKT cells is increased in patients with moderate to severe steatosis**

CD161 (NKR-P1A) is a receptor that is primarily associ-

ated with NK cells, but is also expressed on NKT cells, and may indicate an effector and memory subset of such cells<sup>[19]</sup>. We therefore assessed the expression of CD161 on the CD3+/CD56+ populations in the liver and blood (Figure 1). Again, in each cohort, there were a higher percentage of CD3+/CD56+ cells that expressed CD161 in the liver, compared to the blood (Table 2). Further, the percentage of CD161-expressing CD3+/CD56+ cells in the liver (35.8% ± 9.1%) and blood (9.6% ± 4.9%) of subjects with moderate-to-severe hepatic steatosis were significantly increased compared to those without steatosis (liver: 15.5% ± 12.6%, *P* = 0.01, blood: 2.5% ± 3.8%, *P* = 0.03) and those with mild hepatic steatosis (liver: 18.9% ± 12.5%, *P* = 0.02, blood: 1.2% ± 1.1%, *P* = 0.02).

**Moderate-to-severe steatosis alters the percentages of non NKT cell lymphocyte population**

In addition to increases in the percentages of NKT cells, other minor lymphocyte subsets were significantly affected in patients with moderate-to-severe hepatic steatosis. Intrahepatic percentages of double negative T cells (CD3+, CD4-, CD8-) were increased in the liver of subjects with moderate-severe steatosis (26.6% ± 17.0%), compared to those without steatosis [12.6% ± 10.4%, *P* = 0.05 (Table 2)].

The CD3+/CD8+ lymphocytes were the only lymphocyte population found to significantly decrease in patients with moderate-to-severe steatosis. In these patients, the percentage of CD3+/CD8+ lymphocytes (27.3% ± 9.6%) decreased significantly as compared to patients with mild steatosis (49.3% ± 10.7%, *P* < 0.001) or without steatosis (55.6% ± 14.3%, *P* < 0.001). CD3+/CD8+ lymphocytes also decreased in the peripheral blood in patients with moderate-to-severe steatosis as compared to normal livers and approached significance (17.4% ± 8.5% *vs* 26.2% ± 7.0%, *P* = 0.06).

**DISCUSSION**

NAFLD and NASH are increasing in importance throughout the world. While our immune system plays an important role in the pathogenesis of this disease, our under-



standing of the specifics of the immunopathogenesis of NAFLD is limited. Much of our information regarding NAFLD has come from murine models, and NKT cells have been shown to be a key mediator of murine fatty liver disease<sup>[12]</sup>. However, there are very few studies of intrahepatic NKT cells in humans. In this study, we sought to investigate the changes in lymphocyte populations, with a focus on NKT cells, in obese patients with histologically confirmed steatosis or steatohepatitis. We found that NKT cells, defined as CD3+/CD56+ lymphocytes, are significantly increased in patients with moderate to severe steatosis as compared to patients with no steatosis or mild steatosis. These findings differ from the numerous studies performed in mice and suggest a different role of NKT cells in fatty liver disease in humans.

There have been 4 previous studies investigating NKT cells and fatty liver disease in humans, each using different techniques and yielding different results. In a study by Xu and colleagues, the investigators found a decrease in peripheral  $\alpha$ 24+ NKT cells as compared to healthy matched non-obese controls<sup>[14]</sup>. In that study, the diagnosis of NAFLD was made on a clinical basis, as opposed to our utilization of histology, which is a more specific means of diagnosis, and IHLs were not examined. In a study by Kremer and colleagues, the investigators also found a decrease in NKT cells in patients with moderate to severe steatosis<sup>[15]</sup>. However, they defined NKT cells by expression of CD3+/CD57+, and used immunohistochemistry staining instead of flow cytometry for quantification, both of which can account for the differences in their results and ours. Finally, Tajiri and colleagues evaluated liver biopsy specimens of patients with NAFLD and performed flow cytometry on 20 of the specimens. In these 20 specimens, they found that in patients with more severe steatosis there was an increase in CD3+/CD56+ NKT cells<sup>[16]</sup>, and is in agreement with the results reported here. Finally Syn *et al*<sup>[17]</sup> studied 6 liver biopsies, 2 of which had confirmed NASH cirrhosis, and found an increase percentage of NKT cells in the livers with NASH cirrhosis compared to healthy controls and patients with other forms of hepatitis. With 27 patients enrolled in this study, this is the largest sample size to date to evaluate lymphocyte populations in patients with NAFLD. Further, we also quantified the presence of invariant NKT cells, expression of CD161 and other minor T cell populations in our biologic samples, as well as examining cytokine production.

NKT cells may play a number of immunoregulatory roles in the liver and are considered by some to be a bridge between the innate and adaptive immune systems<sup>[20]</sup>. NKT cells participate in pro-inflammatory, Th1, and anti-inflammatory Th2 mediated pathways *via* the secretion of IFN- $\gamma$  and IL-4, respectively. In murine models, it has been proposed that depletion of NKT cells shifts the hepatic immune environment toward a Th1 milieu, leading to immunocyte infiltration and development of steatohepatitis<sup>[9]</sup>. Leptin deficient mice develop steatosis and NASH, but they do not develop cirrhosis<sup>[20]</sup>. Alternatively, NKT cells, when shifting the immune environment toward a Th2

milieu may be responsible for collagen deposition in the liver. Stimulation and proliferation of NKT cells in leptin deficient mice, through adrenergic stimulation, results in hepatic collagen deposition and fibrosis secondary to IL-4 and IL-13 secretion and activation of Th2 mediated pathways<sup>[12,20]</sup>. In our study, we found an increased percentage of intrahepatic CD3+/CD56+ NKT cells in patients with moderate to severe steatosis and a low incidence of steatohepatitis, which could support a protective role of NKT cells against steatohepatitis. In addition we found a decrease in CD3+/CD8+ intrahepatic lymphocytes which may implicate NKT cells in shifting the hepatic immunoregulatory environment towards more Th2 mediated mechanisms. We were unable to identify a difference in the secretion of IFN- $\gamma$  or IL-4 by NKT cells in patients with various degrees of steatosis, although interferon, but not IL-4 production was elaborated when NKT cells were stimulated in the liver samples studied. Future studies should focus on investigating the functional role of NKT cells in human fatty liver disease.

The multiple definitions of NKT cells can lead to much confusion when discussing their role in the liver. We classified NKT cells in two different ways, both by expression of CD3+/CD56+, as well as by expression of  $\alpha$ 24+. Human NKT cells were initially described in liver donor patients by Doherty *et al*<sup>[21]</sup> as CD3+/CD56+ cells and were shown to be capable of lysing NK sensitive cells. CD3+/CD56+ lymphocytes have been analyzed for mRNA expression of  $\alpha$ 24 and approximately 5% of human hepatic CD3+/CD56+ lymphocytes expressed  $\alpha$ 24 mRNA, which encodes the TCR that recognizes CD1d ligands<sup>[21]</sup>. Thus, NKT cells are also defined functionally as  $\alpha$ 24+ lymphocytes or *via* isolation of CD3+ lymphocytes using CD1d ligands, and are classified as invariant NKT cells. The CD3+/CD56+ lymphocytes, which are also called NKT-like cells, are populations that incorporate many different type of lymphocytes such as invariant T-cells and CD161+ lymphocytes which can potentially create confusion<sup>[22,23]</sup>. Thus, although we find that this broader more diverse population (CD3+/CD56+ NKT cells) is significantly increased with greater degrees of steatosis, the more specific subgroup of invariant  $\alpha$ 24 NKT cells were unchanged. It is possible that other functional subgroups of CD3+/CD56+ lymphocytes such as CD161+ lymphocytes play a larger role in human NAFLD and NASH. This is in contrast to the murine model where there are higher percentages of invariant NKT cells normally found in the liver<sup>[24]</sup>. These findings highlight the importance of investigating the role of invariant NKT and NKT-like lymphocytes in human disease, rather than just using murine models.

The results of the study are limited by the small sample size, and impaired our ability to further characterize the role of NKT cells in NAFLD. Nevertheless the increase in NKT cells in moderate to severe steatosis was significant and correlates with other studies. Absolute lymphocyte numbers were not reported here because the values were affected by the varied size of the liver biopsy samples

taken in each patient. Thus, NKT cell percentages of total lymphocyte were reported for more precise comparison between subjects. Immunohistochemistry has not yet been performed on the liver biopsies, however we hope to conduct future studies to further elucidate the role of NKT cells in NAFLD.

In this study, we examined the change in lymphocyte populations in obese patients with NAFLD, with a focus on intrahepatic NKT cells. We found an increase in NKT cells, defined as CD3+/CD56+ and as well as CD161+ lymphocytes, in obese patients with moderate and severe steatosis. These results differ from previous murine models and some human studies. In addition we reported other changes in lymphocyte populations, such as depletion in CD3+/CD8+ lymphocytes and an increase in CD3+/CD4-/CD8- cells, which have not yet been reported in NAFLD. The results of this study highlight the importance of investigating NKT cells and other lymphocyte populations in humans with NAFLD since the pathophysiology of human NAFLD likely differs from that in murine models. Future studies to investigate the role of NKT cells in NAFLD in humans are warranted in order to elucidate the mechanisms behind the pervasive disease of NAFLD.

## COMMENTS

### Background

Non alcoholic fatty liver disease (NAFLD) is a common disease where fat infiltrates the liver, which can lead to inflammation and cirrhosis. natural killer T (NKT) cells have been implicated in the pathogenesis of NAFLD. In obese mice, NKT cells are depleted in the liver and are associated with a greater degree of steatosis. When the NKT cells are upregulated in mice, the degree of fatty infiltration diminishes. There is limited data about NKT cells in human NAFLD, and this study adds to our understanding of NKT cells in human NAFLD.

### Research frontiers

The data on intrahepatic NKT cells and its role in human steatosis has been mixed. There have been 3 studies investigating NKT cells and NAFLD. One found that NKT cells are depleted with increased steatosis whereas the others found an increase in NKT cells with steatosis. This study contains the largest sample to date which investigates NKT cells in human NAFLD.

### Innovations and breakthroughs

The authors found that NKT cells, defined as CD3+/CD56+ lymphocytes are increased in human livers with moderate and severe steatosis. In addition, they reported other changes in lymphocyte populations with steatosis, such as depletion in CD3+/CD8+ lymphocytes and an increase in CD3+/CD4-/CD8- cells, which have not yet been reported in NAFLD.

### Applications

These findings further support the role of NKT cells in NAFLD and highlight an important difference between NKT cells in the murine model of fatty liver disease and human NAFLD.

### Terminology

NKT cells are a highly conserved subset of lymphocytes with properties of both T cells (CD3+ expression) and NK cells (CD56+ and CD161+ expression). The liver contains a high percentage of these unique lymphocytes, which have been implicated in the pathogenesis of non alcoholic fatty liver disease.

### Peer review

The authors showed that patients with moderate or severe steatosis had a higher percentage of intrahepatic CD3+/CD56+ NKT cells than that with mild steatosis or without steatosis. Further, the percentage of CD3+/CD56+CD161+ cells in the liver of subjects with moderate-to-severe hepatic steatosis were significantly increased compared to those with mild hepatic steatosis or without steatosis. This is an interesting finding and may provide more information about the NKT

cells in human NAFLD because the data on NKT cells in human NAFLD is limited at present. However, there are several areas of the manuscript that the authors should expand upon that would enhance the presentation.

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## Gastrotomy closure with a new tissue anchoring device: A porcine survival study

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### Abstract

**AIM:** To evaluate the feasibility, reproducibility and efficacy of a new tissue anchoring device in a porcine survival model.

**METHODS:** Gastrotomies were performed using a needle-knife and balloon dilator in 10 female Yorkshire pigs weighing 30-35 kg. Gastric closure was attempted using a new tissue anchoring device. The tightness of the closure was confirmed by means of air insufflation and the ability to maintain gastric distension with stability in peritoneal pressure measured with a Veress needle. All animals were monitored daily for signs of peritonitis and sepsis over 14 d. During necropsy, the peritoneal cavity and the gastric access site were examined.

**RESULTS:** Transgastric access, closure and 14 d survival was achieved in all pigs. The mean closure time was  $18.1 \pm 19.2$  min and a mean of  $2.1 \pm 1$  devices were used. Supplementary clips were necessary in 2 cases. The closure time was progressively reduced ( $24.8 \pm 13.9$  min in the first 5 pigs vs  $11.4 \pm 5.9$  min in the last 5,  $P = NS$ ). At necropsy, the gastric access site was correctly closed in all cases with all brace-bars present. One device was misplaced in the mesocolon. Minimal adhesions were observed in 3 pigs and signs of mild peritonitis and adhesions in one.

**CONCLUSIONS:** The use of this new tissue anchoring device in porcine stomachs is feasible, reproducible and effective and requires a short learning curve.

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**Key words:** Gastrotomy; Closure; Suture; Survival; Porcine model; Notes

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### INTRODUCTION

Natural orifice transluminal endoscopic surgery (NOTES) has changed the approach to the peritoneum in the last

few years<sup>[1-5]</sup>. This novel technique permits access to the peritoneal organs through the mouth, rectosigmoid or vagina with diagnostic and therapeutic purposes. Numerous hybrid NOTES procedures (combining NOTES with laparoscopy) have been described in the last five years<sup>[6-9]</sup>, but it was not until 2007 that the first pure NOTES procedures in humans were reported<sup>[10-13]</sup>. Although the transluminal approach holds great potential, secure access site closure remains a critical issue<sup>[14]</sup>. In recent cases and series, endoscopic closure is substituted by use of rigid instruments, using the transvaginal access in almost all cases. However, this approach excludes the male population.

Considering the safety of laparoscopy, studies are mandatory to evaluate secure and reproducible closure methods in NOTES procedures<sup>[15]</sup>. Several closure techniques have been tested<sup>[16]</sup>, including clips<sup>[11,17-19]</sup>, septal occluders<sup>[20]</sup>, T-tags<sup>[21-23]</sup>, more complex suturing devices<sup>[24-26]</sup>, and linear endoscopic staplers<sup>[27]</sup>. T-tags have been tested recently to treat gastrogastic fistulas in humans<sup>[28]</sup>. However, most of these devices are time consuming and often difficult to implement endoscopically and the current data do not allow definitive conclusions regarding the different options<sup>[16,29]</sup>.

The aim of this study was to assess the feasibility, reproducibility and efficacy of a new tissue anchoring device as a gastric suture system in a porcine survival model.

## MATERIALS AND METHODS

### Animals

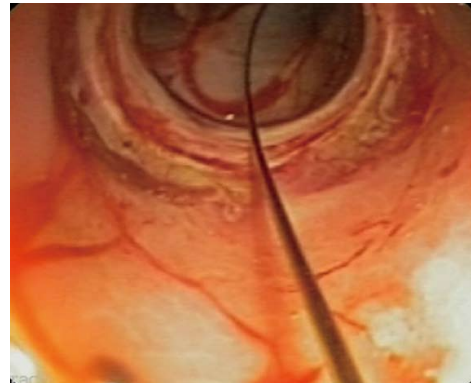
A total of ten female Yorkshire pigs weighing 30-35 kg were included in the study. Animals underwent a 3-d quarantine and acclimation period. During this period of time, veterinary personnel evaluated each animal to ensure baseline health. Animals were fed the same diet and had unlimited access to water. The study was conducted at the University of Barcelona Medical School's animal facilities. The protocol was approved by the University of Barcelona's Animal Ethics Committee.

### Preoperative care and anesthesia

Animals fasted from solids 24 h prior to the procedure. All procedures were performed with pigs under general anesthesia with endotracheal intubation and mechanical ventilation.

### Procedure

A non sterile endoscope (GIF 160, Olympus Medical Systems, Europe, Hamburg, Germany) was first inserted through the pig's mouth and the esophagus and stomach were inspected. Afterwards, gastric lavage was performed with water until the stomach was free of solid particles. An iodated solution followed by an antibiotic suspension (ceftriaxone 1 g/300 mL saline solution) was instilled and the antibiotic solution was left in the stomach for 10 min. From this point on, all the instruments used were sterile or high level disinfected. With a regular endoscope, an overtube was inserted and a double channel gastroscope (GIF 2T160, Olympus Medical Systems, Europe, Hamburg, Germany)



**Figure 1** The incision is enlarged with a balloon dilator. Through the balloon we can see peritoneal structures.

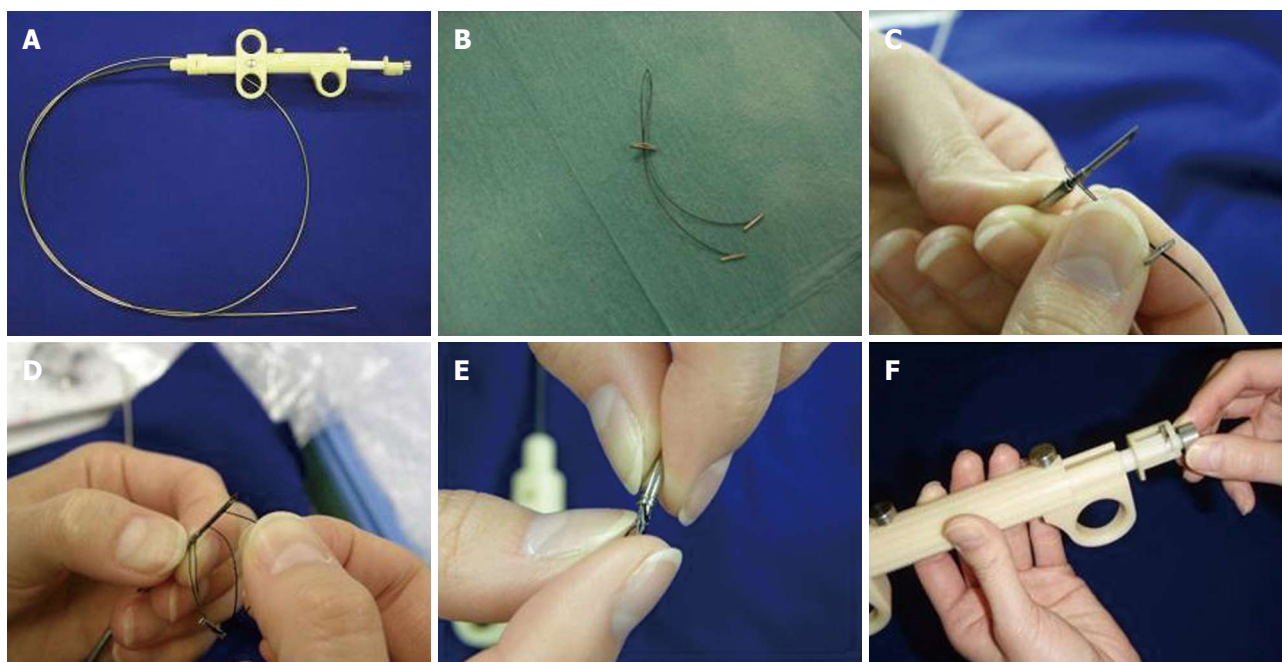
was used until the end of the peritoneoscopy. By external palpation, the anterior gastric wall was selected to perform the gastric access. A 5 mm incision was made with a needle-knife (KD-V451M, Olympus Europe, Hamburg, Germany) and it was subsequently dilated with an 18 mm balloon (CRE wire-guided balloon, Boston Scientific Microvasive, Natick, MA) (Figure 1). Then, the scope was passed through the gastric wall for a 30 min peritoneoscopy.

A Veress needle was placed at the lower left quadrant of the abdomen to control intraperitoneal pressure. To avoid respiratory compromise and impaired venous return, intraperitoneal pressures were monitored and maintained below 15 mm H<sub>2</sub>O. Pneumoperitoneum was maintained with CO<sub>2</sub> insufflation through the scope.

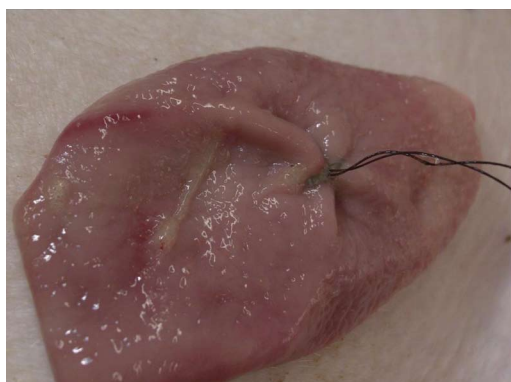
### Tissue-anchoring device

The tissue-anchoring device prototype is called brace-bar (Olympus Medical Systems, Europe, Hamburg, Germany) and is an evolution of a former prototype<sup>[21]</sup>. It consists of a single 18-gauge flexible needle catheter (Figure 2A), and a bifurcated nylon thread ("Y" shaped) with 3 small tags (2 regular tags fixed at both bifurcated distal ends and the other tag stopper at the single proximal end, which will be used for tightening) (Figure 2B). The tag stopper is movable and can be slid forward for cinching of the other tissue-anchoring tags. The proximal end of the thread is fixed to the needle with a small metallic guide (Figure 2C). Before deployment, the device has to be extracorporally loaded inside the needle catheter. The two distal tags are consecutively inserted into the needle (Figure 2D) and, finally, the needle is pulled back into the sheath inserting also the stopper tag (Figure 2E). Once inside the gastric cavity, the needle is pushed forward and the device is ready for use. The pusher button (Figure 2F) allows release of one tag at each side of the incision and the suture is tightened by pressing the tag Stopper with the needle sheath. Finally, the suture is released by extracting the metallic guide that fixed it to the needle.

The tightness of the closure was confirmed by means of air insufflation and the ability to maintain gastric distension with stability in peritoneal pressure measured with the Veress needle.



**Figure 2** Description of the tissue anchoring device. A: Single 18-gauge flexible needle catheter; B: Bifurcated nylon thread ("Y" shaped) with 3 small tags (2 regular tags fixed at both bifurcated distal ends and the other tag stopper at the single proximal end, which is used for tightening); C: The proximal end of the thread is fixed to the needle with a small metallic guide; D: The two distal tags are consecutively inserted into the needle; E: The needle is pulled back into the sheath inserting also the stopper tag; F: The pusher button allows releasing one tag at each side of the incision.



**Figure 3** The incision looks completely sealed after the insertion of one brace-bar and the stomach is able to maintain air distension.

A single channel scope (Olympus GIG 160) was only used for the suture. Endoclips were added when the sutures were not placed at the middle of the incision and one of the sides seemed not completely sealed.

### Postoperative care and necropsy

Water was immediately allowed and food was allowed after 24 h. All animals received intravenous ceftriaxone 1 g daily for 3 d and they were monitored daily for signs of peritonitis and sepsis during the next 14 d. Weight was controlled prior to surgery and necropsy. During necropsy, the peritoneal cavity and the gastric access site were examined for signs of peritonitis (exudates, abscesses) or other complications.

### Statistical analysis

Data were expressed as mean  $\pm$  SD or range. Results were analyzed using the  $\chi^2$  test with Yates correction and Fisher exact test for qualitative variables and Mann-Whitney test for quantitative parameters. A  $P$  value  $< 0.05$  was considered statistically significant. Statistical analysis was performed with SPSS Statistical Package (version 17.0, SPSS Inc., Chicago, IL).

## RESULTS

All transgastric accesses were achieved with no difficulties and a mean time of  $5.7 \pm 3.6$  min (range 2-14). No major complications occurred. Minor complications included an accidental injury to the anterior abdominal wall and 4 minor bleedings during the use of the needle-knife. A 30 min peritoneoscopy was possible in all animals.

The brace-bar was used in all cases and closure was easily achieved in  $18.1 \pm 19.2$  min. This time was reduced to  $11.4 \pm 5.9$  min when considering only the last 5 cases and was less than 9 min in the last 3 cases, whereas it was  $24.8 \pm 13.9$  min in the first 5 ( $P = 0.1$ ). Details of all procedures are described in Table 1. A total of 21 sets of sutures (mean  $2.1 \pm 1$ , range 1-4) were used to achieve closure but only 15 (mean  $1.5 \pm 0.5$ , range 1-2) could be completely tightened. Therefore, in 5 cases, the incision was closed with only 1 suture (Figure 3), but in two of them 1 and 3 clips respectively, were added.

In total, 6 sutures (29%) were ineffectively positioned. In 5 attempts one of the tags did not stay attached to the



Table 1 Summary of procedure details

Case	Number of brace-bars used	Number of brace-bars correctly placed	Cause of Misplaced brace-bar	Adjunctive method	Closure time (min)	Endoscopic view
1	2	2		Omental patch	8	Correct
2	1	1		3 endoclips	42	Correct
3	4	2	1 tag detached 1 brace-bar not tightened	None	36	Correct
4	2	2		None	19	Correct
5	2	1	1 tag detached	1 endoclip	19	Correct
6	3	2	1 tag detached	None	15	Correct
7	3	1	2 tags detached	None	20	Correct
8	2	2		None	9	Correct
9	1	1		None	7	Correct
10	1	1		None	6	Correct

Table 2 Summary of necropsy findings

Case	Weight gain (kg)	Abdominal cavity	Incision	Location of tags
1	6.85	Minimal adhesions	Totally closed	1 tag at mesocolon 3 tags at gastric serosa
2	6.90	Normal	Totally closed	2 tag stoppers at gastric mucosa 2 tags at gastric serosa.
3	5.00	Minimal adhesions	Totally closed	1 tag stopper at gastric mucosa 4 tags at gastric serosa
4	3.70	Normal	Totally closed	2 tag stoppers at gastric mucosa 4 tags inside gastric wall
5	1.90	Normal	Totally closed	1 tag at gastric serosa 1 tag inside gastric wall
6	3.74	Small clot	Totally closed	1 tag stopper at gastric mucosa 3 tags at gastric serosa 1 tag inside gastric wall
7	0.90	Normal	Totally closed	1 tag stopper at gastric mucosa 1 tag at gastric serosa 1 tag at gastric wall
8	0.00	Normal	Totally closed	1 tag stopper at gastric mucosa 1 tag at gastric serosa 3 tags at gastric wall
9	-2.78	Fibrin exudates Minimal adhesions	Totally closed	2 tag stoppers at gastric mucosa 2 tags at gastric wall
10	6.42	Minimal adhesions	Totally closed	1 tag stopper at gastric mucosa 2 tags at gastric wall 1 tag stopper at gastric mucosa

mucosa either immediately after the tag release or when tightening the suture. The remaining failure was caused by a thread rupture after steady tightening.

Immediately after gastrotomy closure, all brace-bars seemed well positioned and gastric distension with air was possible in all cases without changes in intraperitoneal pressure, suggesting the closure was correct (mean peritoneal pressure before and after the closure:  $14.3 \pm 3.3$  mmHg, range 3-15).

The mean procedure time, including gastric access creation, peritoneoscopy and gastrotomy suture, was  $63.7 \pm 18.2$  min.

All the pigs completed the 14 d follow-up period. They had a weight gain of  $3.3 \pm 3.2$  kg. At necropsy, the gastric access site was completely closed in all cases and all brace-bars were present (Table 2). The tags were usually attached

at the gastric serosa ( $n = 15$ ) or inside the gastric wall ( $n = 14$ ). In the first case, 1 tag was misplaced inside the mesocolon. In this case, an omental patch had been added to the suture pulling the omentum inside the stomach through the incision. Minimal adhesions were observed in 3 pigs and signs of mild peritonitis and adhesions in one.

## DISCUSSION

NOTES holds great appeal as a less invasive alternative to laparoscopic surgery. As NOTES heads toward human trials, it is essential that the creation and closure of transluminal incisions be performed in a safe, rapid, and reproducible manner<sup>[14-16,28]</sup>.

In this study, we assessed the feasibility of a new generation tissue anchoring device with relatively good results.

Moreover, it turned out to be easy and intuitive to use and the time for placement was short and progressively reduced. It was not necessary to use complementary clips when we gained experience with the system. One of the advantages of this device (and the main difference with the former prototype used by Sumiyama *et al.*<sup>[22]</sup>) is that it can be used with a single channel endoscope. The same needle catheter is used for releasing the tags and tightening them later without need for a different forceps grasper, and this makes the procedure shorter. Furthermore, the depth of the needle insertion is limited to 20 mm and this might decrease the risk of complications.

However, we still found some problems with the device: the needle had to be loaded extracorporally after each set of tissue anchors was applied and this prevented sequential stitching. Moreover, we observed a dysfunction of the needle after several attempts which could explain the high rate of sutures being ineffectively positioned because the tags could not be released deeply enough. We think that pre-charged and non-reusable devices might improve the procedure time and security. On the other hand, we did not drop any tags in the peritoneal cavity and, since each pair of tags is attached to a thread, we think that the possibility of dropping one in the peritoneum is extremely low.

The possibility of an inadvertent injury of organs and structures outside of the gut wall has been described as a possible limitation of T-tag based systems<sup>[30,31]</sup>. Sumiyama *et al.*<sup>[22]</sup> produced 12 gastric perforations in 6 pigs that were closed with 48 tissue anchor sets and three of the 24 used in the anterior gastric wall (12.5%) penetrated surrounding organs (2 penetrated the liver and 1 the anterior abdominal wall). However, as mentioned above, with the new brace-bar prototype the depth of the needle insertion is limited to 20 mm and this fact was crucial in the low incidence of surrounding structure injuries in our series (1 tag out of 21 sets, 4.8%). This was the first case and we tried to perform an omental patch pulling the omentum through the incision. During this maneuver, the mesocolon was probably unsafely moved towards the gastric wall causing this complication. In the remaining cases in which the omental patch was not attempted no lesions occurred at the adjacent structures. The importance of the depth of the needle to avoid complications has been demonstrated very recently by Park *et al.*<sup>[32]</sup> These authors performed needle punctures of 1-1.5 mm using a different anchor-based endoscopic system (the TAS o tissue apposition system) and they did not have any adjacent organ penetration with a 100% of closure effectiveness.

Although the ex-vivo study of Voermans *et al.*<sup>[33]</sup> suggested that t-tag based methods do not permit the serosa to serosa approach and leaking pressure is lower than with other devices, the surviving pigs showed a good post-operative course. This fact could be explained because physiological intraluminal pressures are much lower than pressures obtained in acute bursting tests and, therefore, might not be a necessary objective test for viscerotomy closure<sup>[34]</sup>. From a clinical standpoint, the critical test for a

gastric closure is animal survival without clinical signs of leakage or complications.

Previous studies have demonstrated that peritoneal contamination occurs when using transgastric access. A conservative interpretation of these findings is that the current "aseptic" technique may require further refinement, as suggested by Rolanda *et al.*<sup>[18]</sup> and Ryou *et al.*<sup>[35]</sup>. In fact, we had some difficulties in completely cleaning some of the stomachs and it was very common to notice residual liquid near the incision. Only one pig did not have a satisfactory recovery and the necropsy showed the presence of fibrin exudates in the upper abdomen. Although the incision site was seen completely sealed at necropsy, we cannot totally exclude the possibility that an initial suture failure occurred but it could be also related with a potential contamination of the abdominal cavity by the stomach content. Because in this case we used only one brace-bar, we now consider it prudent to use two devices to ensure a safer closure.

The present study has some limitations: first, the number of cases is low and we did not include a control group. Second, the 14 d survival period might be short to evaluate late complications. Finally, the use of complementary clips in two cases might modify the results of the study.

In conclusion, the use of a brace-bar in a gastric porcine model is easy, fast, and reproducible after a short learning curve and permits the use of a single channel endoscope. We believe this tissue anchoring system holds tremendous potential as a suturing method for both iatrogenic and intentional perforations of the gastric wall. Unfortunately, it is still far from a safe application in humans. Further studies and more technological improvements are still mandatory before expanding its use to humans.

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## COMMENTS

### Background

Natural orifice transluminal endoscopic surgery (NOTES) has changed the approach to the peritoneum in the last few years. Secure closure of the gastrotomy access is one of the most important issues for the development of NOTES. However, most new suturing devices are time consuming and often difficult to implement endoscopically.

### Research frontiers

T-tag based systems have already been used with variable results. The possibility of an inadvertent injury of organs and structures outside of the gut wall has been described as a possible limitation of these devices. In this study, the authors demonstrate the usefulness and safety of a new tissue-anchoring-device prototype.

### Innovations and breakthroughs

One of the advantages of this device (and the main difference with the former prototype) is that it can be used with a single channel endoscope. On the other hand, the same needle catheter is used for releasing the tags and tightening

them later without need for a different forceps grasper, and this makes the procedure shorter. Furthermore, the depth of the needle insertion is limited to 20 mm and this might decrease the risk of complications.

### Applications

Because the use of the brace-bar is easy, fast, and reproducible after a short learning curve, we believe this tissue anchoring system holds tremendous potential as a suturing method for both iatrogenic and intentional perforations of the gastric wall.

### Terminology

Natural orifice transluminal endoscopic surgery permits access to the peritoneal organs without the need of skin incisions. Tissue-anchoring devices are endoscopic suturing devices based on a nylon thread and a small tag at the distal end that are deployed within the gastric wall. When two or more of them are tight together, the margins of the incision approach and the incision is sealed.

### Peer review

This is well written and succinct with appropriate interpretation and caution in the discussion.

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## MR-arteriportography: A new technical approach for detection of liver lesions

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### Abstract

**AIM:** To evaluate the benefit and effectiveness of MR-arteriportography (MR-AP) to achieve the highest sensitivity for detection and evaluation of hepatocellular carcinoma (HCC).

**METHODS:** Twenty liver cirrhosis patients with suspected HCC were included before transarterial chemoembolization. In all patients double-enhanced Magnetic resonance imaging (MRI) was performed. A bolus of 10 mL Magnevist® was injected through a selectively placed catheter in the superior mesenteric artery and MRI of the liver was performed in arteriportographic phase. Two independent readers evaluated number, size and localization of detected lesions. Diagnostic quality was determined using a 4-point scale. Differences were analyzed for significance using a *t*-test. Interobserver variability was calculated.

**RESULTS:** In all 20 patients (100%), MR-AP was feasible. Diagnostic quality was, in all cases, between 1 and

2 for both modalities and readers. MR-AP detected significantly more lesions than double-enhanced MRI (102.5 vs 61, respectively,  $P < 0.0024$ ). The inter-observer variability was 0.881 for MRI and 0.903 for MR-AP.

**CONCLUSION:** Our study confirmed that the MR-AP as an additional modality for detection of HCC is beneficial, as significantly more lesions were detected compared to MRI with liver-specific contrast.

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**Key words:** MR-arteriportography; Magnetic resonance imaging; Hepatocellular carcinoma; Liver lesions

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Rennert J, Jung EM, Schreyer AG, Hoffstetter P, Heiss P, Feuerbach S, Zorger N. MR-arteriportography: A new technical approach for detection of liver lesions. *World J Gastroenterol* 2011; 17(13): 1739-1745 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i13/1739.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i13.1739>

### INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignant tumor of the liver and often develops in patients with underlying liver cirrhosis due to excessive alcohol intake, chronic hepatitis or primary biliary cirrhosis.

In the treatment of hepatocellular carcinoma, surgical resection is considered the only potentially curative therapy. However, technical improvements in hepatic surgery have extended the indications for surgery remarkably, and also regional therapeutic procedures such as transcatheter

arterial chemoembolization (TACE)<sup>[1-3]</sup> and radiofrequency ablation (RFA)<sup>[1,4,5]</sup> have proved to be very successful. A prolonged time of survival following diagnosis is noted. Therefore, the pre-operative or pre-interventional workup of patients with suspected liver malignancy is even more important, especially concerning the evaluation and characterization of focal or diffuse lesions in the cirrhotic liver. Magnetic resonance imaging (MRI) has been used to improve identification of focal hepatic masses in a cirrhotic liver.

Dynamic MRI after a bolus injection of gadopentetate dimeglumine has been accepted as a valuable method for the detection and characterization of liver tumors<sup>[6-9]</sup>. Studies have shown that superparamagnetic iron oxide-enhanced magnetic resonance imaging (SPIO-MRI) increases sensitivity<sup>[4,10]</sup>.

In order to determine the treatment of choice for HCC, studies have shown that examinations by both computed tomography angiography (CTA) and computed tomography arteriportography (CT-AP) are indispensable because of the high sensitivity of CT-AP in detecting hepatic lesions and the capability of CTA to characterize them<sup>[11,12]</sup>. However, in contrast to its high sensitivity in detecting lesions, the specificity of CT-AP for characterizing intrahepatic lesions is low. Tumor-mimicking benign perfusion abnormalities and benign lesions (e.g. hemangiomas, arterio-venous shunts) have led to a reported incidence of false-positive lesions between 9% and 63% in primary and secondary liver lesions<sup>[13,14]</sup>.

Despite advances in CT or MRI, ultrasound (US) with or without application of contrast agents also plays a key role in the diagnostic algorithm of HCC due to its low cost, availability and non-invasiveness.

Until now, there have hardly been any studies comparing the effectiveness of MR-arteriportography (MR-AP) and contrast-enhanced MRI for diagnosis of malignant liver lesions. Thus, the purpose of this study was to combine the advantages of modern contrast-enhanced MRI with the technique of arteriportography to achieve the highest sensitivity for diagnosis of malignant liver lesions in patients suffering from HCC.

## MATERIALS AND METHODS

### Patients

Approval for this study was obtained from the institutional review board in conformity with the Declaration of Helsinki. Before the procedures were conducted, written informed consent was obtained from each patient for MRI, MR-AP and angiography after the nature of the procedure was fully explained.

During the period from February 2005 to September 2007, 20 patients [18 men, 2 women, age range from 47 to 76 years (mean age, 62 years)] with symptoms suggestive of primary malignant hepatic tumors were referred to our department. As HCC is commonly associated with liver cirrhosis, all patients had deteriorated liver but a tolerable renal function, and the cardiovascular status was stable. Twelve of our 20 patients suffered from alcohol toxic

liver cirrhosis. In 4 out of 20 patients the underlying disease was chronic hepatitis (2 patients with chronic hepatitis B, 2 patients with chronic hepatitis C). In 4 out of 20 patients, the fundamental disease could not be elicited. Concerning the severity of cirrhosis, 8 out of 20 patients were classified as Child Pugh score A, 7 out of 20 patients as Child Pugh score B and 5 out of 20 patients as Child Pugh score C.

The existence of malignant hepatic tumors was confirmed using multislice MRI and CT. MR-AP was performed to evaluate the tumor extent in order to suggest interventional therapy, surgery or chemotherapy.

Diagnosis of HCC was histologically confirmed in 15 out of 19 patients. In one patient (patient No. 16), existence of a malignant hepatic lesion was excluded histologically following liver transplantation.

In 13 out of 20 patients the  $\alpha$ -fetoprotein (AFP) level was elevated, ranging from 16.4-2513 ng/mL (mean 488.1 ng/mL). In 7 patients (including patient No. 16) AFP levels were within a normal range.

### Imaging procedures

**Angiography:** Before TACE and for MR-arteriportography, the femoral common artery was punctured under local anesthesia using the Seldinger technique and a 5-French angiographic catheter (Cobra, Cook Medical, USA) was positioned in the proximal superior mesenteric artery. A diagnostic angiography was performed to visualize the portal vein and to exclude shunts which could involve contrasting *via* the portal vein.

**MRI:** MRI was performed on a 1.5-T whole-body scanner (Magnetom Sonata, Siemens Medical Solutions, Germany) equipped with a high-performance gradient (Quantum) system (maximum gradient strength, 30 mT/m; slew rate, 125 T/ms). A combination of the standard body phased-array coil with spine array coils was used for signal reception.

**MR-AP standard protocol (Table 1):** For MR-AP, 10 mL gadopentetate dimeglumine was injected through the catheter placed in the superior mesenteric artery at a rate of 2 mL/s with a power injector (Medrad Spectris MR Injector, USA).

**MRI standard protocol (Table 2):** For MRI, 0.2 mmol/kg body weight gadopentetate dimeglumine was injected intravenously at a rate of 2 mL/s with a power injector (Medrad Spectris MR Injector). T1-weighted VIBE transversal Dynamic scans were acquired 20, 40, and 120 s after application of gadopentetate dimeglumine. T2-star-weighted Flash 2D scans and T2-weighted TSE FS scans were obtained after application of 1.4 mL Ferucarbotran (Resovist®, Bayer Schering Pharma AG, Germany).

### Image analysis

In the retrospective reviewing procedure, all images of each technique were interpreted and evaluated independently by two observers with great experience in abdominal MRI.



**Table 1** MR-arteriportography standard protocol

Scans	Plane	TE	TR	Flip angle
Unenhanced				
T2-weighted TRUFI	Coronal	1.9	3.8	71°
T2-weighted TRUFI	Transversal	1.88	3.76	71°
T1-weighted VIBE	Transversal	2.02	4.78	10°
Enhanced				
T1-weighted FLASH FS CE	Transversal	4.76	123	70°
T1-weighted VIBE dynamic <sup>1</sup>	Transversal	2.02	4.78	10°

<sup>1</sup>Dynamic scans were started immediately following application of 10 mL Gd-DTPA. MRI: Magnetic resonance imaging.

**Table 2** Magnetic resonance imaging standard protocol

Scans	Plane	TE	TR	Flip angle
Unenhanced				
T1-weighted VIBE	Transversal	1.59	4.37	10°
T2-star-weighted FLASH 2D	Transversal	10.00	169.00	90°
T2-weighted TSE FS	Transversal	105.00	2740.00	170°
T1-weighted FLASH opp	Transversal	2.71	100.00	70°
T1-weighted FLASH in	Transversal	4.76	87.00	60°
T2-weighted HASTE	Transversal	85.00	1000.00	150°
T2-weighted TRUFI	Coronal	1.83	3.65	71°
T1-weighted VIBE	Transversal	1.55	4.81	10°
Enhanced				
T1-weighted VIBE dynamic <sup>1</sup>	Transversal	1.55	4.81	10°
T1-weighted FLASH FS	Transversal	4.76	123.00	70°
T1-weighted FLASH FS	Coronal	4.76	94.00	70°
T2-star-weighted FLASH 2 <sup>2</sup>	Transversal	10.00	169.00	90°
T2-weighted TSH FS <sup>2</sup>	Transversal	105.00	2740.00	170°

<sup>1</sup>Dynamic scans were started immediately following application of Gd-DTPA (0.2 mmol/kg); <sup>2</sup>Following application of 1.4 mL Resovist.

No clinical information or patient diagnosis was given to the observers. The images from each technique were interpreted in separate sessions in a randomized sequence. In the first session, the two observers reviewed a set of images that included both unenhanced and gadopentetate dimeglumine-enhanced, as well as Resovist-enhanced, images.

In the second session, each observer reviewed a set of images (MR-AP set) that included gadopentetate dimeglumine-enhanced images after injection *via* the superior mesenteric artery.

For characterization of liver lesions, all images of each examination were reviewed together using all the sequences available. Each observer recorded the number of suspected lesions noted, their size, and the segmental location. Furthermore, the image quality of MR and MR-arteriportography was documented on a four point scale: 1-excellent, 2-minor diagnostic limitations, 3-major diagnostic limitations, 4-non-diagnostic.

### Statistical analysis

Statistical software (SPSS, version 14, Chicago, USA) was used for statistical analysis. We evaluated the differences with regard to number of lesions found using MR-arteriportography and MRI. Furthermore, the inter-observer differences in evaluation of MR-arteriportography and double-enhanced MRI were analyzed. Paired-samples

**Table 3** Number of lesions detected in MR-arteriportography and magnetic resonance imaging

Patient	Age (yr)	MR-AP number of lesions Σ 102/103		Double-enhanced MRI number of lesions Σ 60/56	
		Reader 1	Reader 2	Reader 1	Reader 2
1	62	9	9	5	4
2	47	7	8	2	2
3	70	4	5	2	2
4	74	7	8	4	3
5	51	3	3	3	3
6	76	1	2	1	1
7	70	6	6	9	8
8 <sup>1</sup>	63	-	-	(7)	(7)
9	70	4	4	2	2
10	63	9	8	2	2
11	51	9	8	6	5
12	63	10	11	3	2
13	57	6	6	2	2
14	67	5	4	4	5
15	51	4	3	3	2
16	53	0	0	1	1
17	51	2	2	2	2
18	54	5	6	3	3
19	57	9	8	4	4
20	73	2	2	3	3

<sup>1</sup>Patient No. 8 was excluded from the evaluation for diffuse infiltration of virtually all liver segments.  $P = 0.0024$  (relation of the number of lesions detected in MR-AP and MRI). MR-AP: MR-arteriportography; MRI: Magnetic resonance imaging.

$t$  tests and  $\chi^2$  test were used to compare. In paired-samples  $t$  tests,  $P < 0.05$  indicated a statistically significant difference.

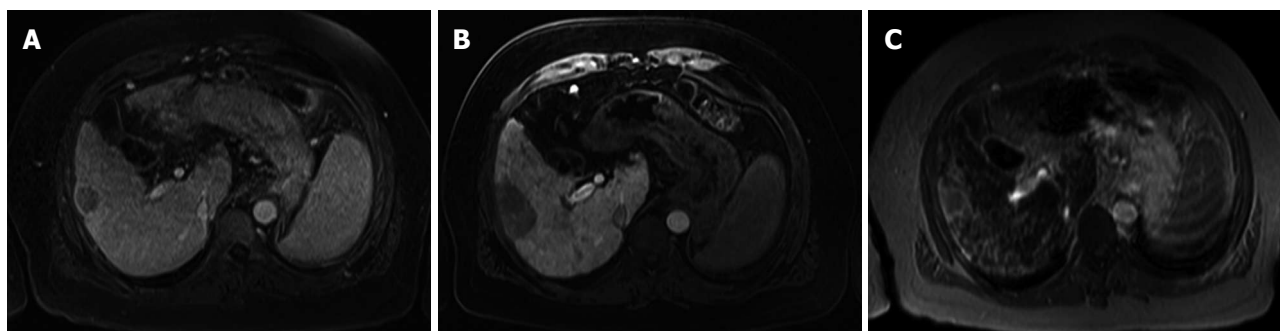
## RESULTS

Twenty patients with liver cirrhosis underwent combined MR-arteriportography and SPIO-MRI examinations. No adverse reactions were experienced by any of the patients who received Gd-DTPA and SPIO.

In all 20 patients (100%), MR-arteriportography was feasible. The image quality in both modalities (MR-AP and MRI) was excellent (1-2 in MR-arteriportography, SD: 0.4865; 1-2 in MR, SD: 0.4292). In patient No. 8, a MR-AP evaluation was not suitable due to a diffuse infiltration of virtually all liver segments, thus the patient was excluded. Altogether, 102.5 hepatic lesions were detected using MR-AP, whereas only 61 lesions could be detected using MRI (Table 3). This difference is considered to be statistically significant ( $P < 0.0024$ ).

The kappa analyses of two observers regarding the number of lesions detected by both modalities showed substantial to excellent agreement ( $\kappa = 0.903$ , 95% CI: 0.844 to 0.962 for MR-AP; and  $\kappa = 0.881$ , 95% CI: 0.795 to 0.966 for MRI). In particular,  $\kappa$  values with MR-AP imaging indicated excellent agreement.

The lesions found in all patients ranged in size from 7-120 mm (mean 28.24 mm) for MRI and from 4-120 mm (mean 24.62 mm) for MR-AP. This difference is not con-



**Figure 1** A 49-year-old man with hepatic cirrhosis due to chronic hepatitis B and C infection. Histology obtained following liver transplantation confirmed the diagnosis of a multifocal hepatocellular carcinoma. A: Magnetic resonance imaging (T1-weighted VIBE) during early venous phase shows a low signal intensity nodule with a diameter of approximately 3 cm in segment VIII/V; B: SPIO-enhanced T2-weighted fast image shows an area of increased signal intensity (segment VIII/V) within the otherwise lower but very inhomogenous signal of the liver parenchyma with profound cirrhosis; C: MR-AP (T1-weighted VIBE) during early venous phase displays an area of decreased enhancement (approx. 4.5 cm diameter) in segment VIII/V. Note the multiple smaller hypointense lesions in segments VII/VI. SPIO: Superparamagnetic iron oxide-enhanced.

sidered to be statistically significant. In MRI, 16 lesions (26.2%) were 10 mm or less in diameter. In MR-AP, 30 lesions (29.3%) were 10 mm or less in diameter.

In a total of 15 out of 19 patients, more lesions were detected by MR-AP compared to MRI with liver-specific contrast. According to the characteristic features displayed in MR-AP, all lesions were classified as malignant. Thus, as a consequence of the finding of these additional lesions, further treatment (TACE, RFA or surgery) was performed upon the patients.

In all but one patient (patient No. 16), the lesions found displayed a characteristic MRI signal, including arterial enhancement to a greater or lesser extent, with late wash out and an absent accumulation of Ferucarbotran (Resovist®). Thus, these lesions were classified as malignant. In addition, these lesions also showed characteristic features in MR-AP, such as absent enhancement of gadopentetate dimeglumine with profound demarcation compared to healthy liver tissue. Hence, the lesions were also classified as malignant.

Diagnosis of HCCs was histologically confirmed in 15 out of 19 patients. In patient No. 16, one lesion was found in segment 8 of the liver which showed a portal venous enhancement with accumulation of Ferucarbotran and no traceability in MR-AP. The lesion therefore was classified as benign, i.e. regenerated nodule that was confirmed histologically following liver transplantation (Figures 1 and 2).

## DISCUSSION

Before decisions are made as to hepatic resection of hepatocellular carcinoma or interventional treatment such as TACE, percutaneous ethanol injection therapy and radio-frequency ablation (RFA), accurate information regarding the number and localization of lesions is essential.

Because of its high sensitivity for detecting lesions, CT-AP is one of the most reliable tools for detection of liver lesions. The rationale of CT-AP is for contrast material to be delivered directly to the liver through the portal vein before it can return to the hepatic artery from the

systemic circulation, to optimize the detection of tumor lesions that do not have portal vein flow and appear as hypodense nodules. The aim of our study was to combine the advantages of arteriportal contrast and MRI to detect liver lesions in patients with HCC. MRI with liver-specific contrast agents is currently the imaging modality of choice. Most studies that have directly compared MRI with CT-AP in patients with HCC or metastases reported no significant differences in sensitivity<sup>[15-17]</sup>, but in some studies a higher specificity for MRI is reported<sup>[13]</sup>.

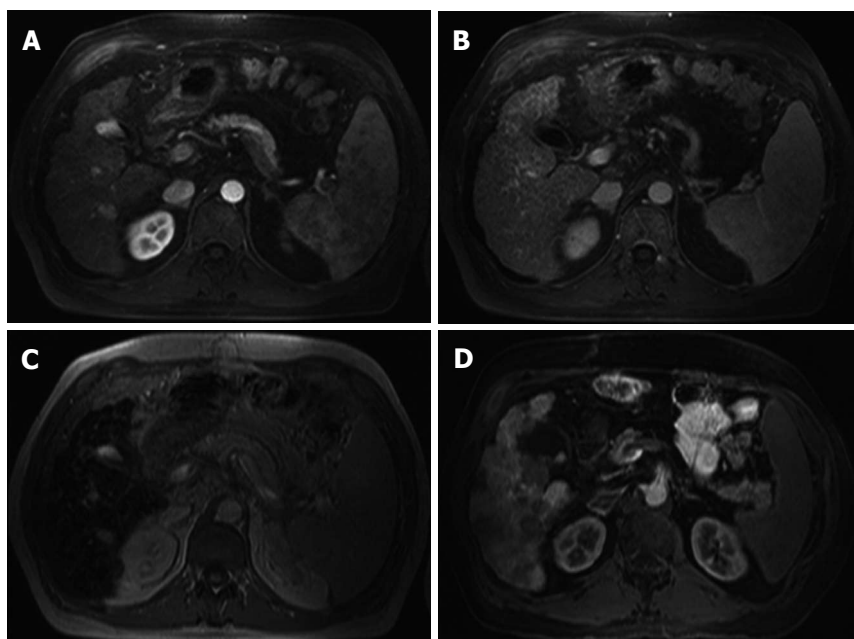
Preoperative or pre-interventional workup of patients with suspected liver malignancy is even more important, especially concerning the evaluation and characterization of a focal lesion or diffuse infiltration of the cirrhotic liver.

Previous studies have shown the usefulness of ferumoxide-enhanced MR imaging for the detection of hepatic tumors of different histological types<sup>[18,19]</sup>. After intravenous injection, the SPIO-particles are cleared by macrophages and can be identified histologically in Kupffer's cells of the liver. Poorly differentiated hepatic tumors lack Kupffer's cells; therefore, the T2 relaxation does not change after the administration of SPIO causing an increased lesion-to-liver contrast.

Studies of patients with hepatic metastases have shown that ferumoxide-enhanced MR imaging is more sensitive than unenhanced MR imaging<sup>[20,21]</sup> or contrast-enhanced CT<sup>[22,23]</sup>, and at least as accurate as CT during arteriportography<sup>[10]</sup>.

Regarding the detection of HCCs, it has been reported that the sensitivity of combined CT during arteriportography and CT hepatic arteriography is 89%-95%<sup>[24,25]</sup> in comparison to 80.4% on contrast-enhanced CT alone<sup>[26]</sup>. Furthermore, the sensitivity of MR sequences with ferumoxide enhancement was reported to be 78%-92%<sup>[18,19,24]</sup>, and that of MR-AP 94%-97%<sup>[27,28]</sup>.

Diffusion-weighted imaging (DWI) is a MRI technique that provides imaging of diffusion in biological tissues. Recent technical developments have reduced the image deformation associated with this technique and have increased the signal to-noise ratio, thus making DWI of the



**Figure 2** A 63-year-old man with hepatic cirrhosis due to a chronic hepatitis C infection. Histology obtained following liver transplantation confirmed the diagnosis of a well differentiated hepatocellular carcinoma. A: Magnetic resonance imaging (T1-weighted VIBE) during arterial phase shows a singular hyper-vascularized nodule with a diameter of approximately 2 cm in segment VII; B: T1-weighted VIBE during early venous phase shows an inhomogeneous signal of the liver parenchyma with masking of the lesion in segment VII; C: SPIO-enhanced T2-weighted fast image shows an area of increased signal intensity (segment VII) within a low signal of the liver parenchyma; D: MR-AP (T1-weighted VIBE) during early venous phase displays an area of decreased enhancement (approx. 3 cm diameter) in segment VII and various low signal lesions in segments I, IVa, VIII and VII. SPIO: Superparamagnetic iron oxide-enhanced.

body feasible<sup>[29]</sup>, especially for detection of liver malignancies<sup>[30]</sup>. Studies have reported a higher sensitivity of DWI for detection of small hepatic metastases compared to SPIO-enhanced T2-weighted images and breath-hold T2-weighted images<sup>[31,32]</sup>, particularly due to a profound signal intensity of the HCC in the cirrhotic liver. In comparison, cysts and hemangiomas, the most frequently found benign lesions of the liver, typically show low or absent signal intensities<sup>[33]</sup>.

Our study demonstrated that MR-arteriportography as an alternative method for detection of HCC is not only feasible but showed a significantly larger number of lesions than ferumoxide-enhanced MR imaging, especially in patients with hepatic cirrhosis.

Also, the lesion's size seems to play a crucial role. Out of the 41 lesions that were exclusively found using MR-AP, 21 were 10 mm or less in diameter. This confirms the high sensitivity of MR-AP, especially regarding smaller lesions. However, a definite differentiation among HCC nodules, regenerative dysplastic nodules or, for example, small arterio-venous shunts that are very common in patients with hepatocellular carcinoma is not possible.

One limitation of the study is that a histological confirmation of HCC or benign lesions could only be obtained in 16 out of 20 patients. In the other 4 patients, further treatment was based upon the combination of typical image morphology and elevated alpha fetoprotein. Another limitation is the relatively small number of patients that were examined in this study. Prospective studies are necessary in order to ascertain the diagnostic reliability of MR-AP compared to established methods.

In summary, our study confirmed that the use of MR-arteriportography as an additional modality for detection of hepatocellular carcinoma is useful. Using this technique, significantly more lesions could be detected in comparison to MRI with liver-specific contrast agent.

## COMMENTS

### Background

Hepatocellular carcinoma (HCC) is the most common primary malignant tumor of the liver and often develops in patients with underlying liver cirrhosis. In the treatment of HCC, surgical resection is considered the only potentially curative therapy; however, other regional therapeutic procedures such as transcatheter arterial embolization or radiofrequency ablations have proved to be very successful. Thus, the pre-interventional evaluation of patients with suspected liver malignancy is even more important, especially concerning the evaluation and characterization of focal or diffuse lesions in the cirrhotic liver.

### Research frontiers

MR imaging with liver-specific contrast agents has been used to improve identification of focal hepatic masses in a cirrhotic liver. Also, studies have shown that examinations by computed tomography arteriportography (CT-AP) have a very high sensitivity for detection of hepatic lesions compared to a relatively low specificity. In this study, the benefit and effectiveness of MR-arteriportography (MR-AP) in achieving the highest sensitivity for detection and evaluation of HCC was evaluated.

### Innovations and breakthroughs

Until now, there have hardly been any studies comparing the effectiveness of MR-AP and contrast-enhanced MRI for diagnosis of malignant liver lesions. This study confirmed that MR-arteriportography as an additional modality for detection of HCC is truly beneficial and may lead to change in treatment in many patients.

### Applications

The results showed that using the MR-AP approach, significantly more lesions could be detected in comparison to MRI with liver-specific contrast agent. Thus, it might play an important role for future strategy of therapeutic interventions.



# Terminology

MR-AP is an MRI procedure where the contrast agent is injected through a catheter placed in the superior mesenteric artery.

# Peer review

It is well-written and is novel work.

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## Carbachol promotes gastrointestinal function during oral resuscitation of burn shock

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### Abstract

**AIM:** To investigate the effect of carbachol on gastrointestinal function in a dog model of oral resuscitation for burn shock.

**METHODS:** Twenty Beagle dogs with intubation of the carotid artery, jugular vein and jejunum for 24 h were subjected to 35% total body surface area full-thickness burns, and were divided into three groups: no fluid resuscitation (NR,  $n = 10$ ), in which animals did not receive fluid by any means in the first 24 h post-burn; oral fluid resuscitation (OR,  $n = 8$ ), in which dogs were gavaged with glucose-electrolyte solution (GES) with volume and rate consistent with the Parkland formula; and oral fluid with carbachol group (OR/CAR,  $n = 8$ ), in which dogs were gavaged with GES containing carbachol (20  $\mu$ g/kg), with the same volume and rate as the OR group. Twenty-four hours after burns, all animals were given intravenous fluid replacement, and 72 h after injury, they received nutritional support. Hemodynamic

and gastrointestinal parameters were measured serially with animals in conscious and cooperative state.

**RESULTS:** The mean arterial pressure, cardiac output and plasma volume dropped markedly, and gastrointestinal tissue perfusion was reduced obviously after the burn injury in all the three groups. Hemodynamic parameters and gastrointestinal tissue perfusion in the OR and OR/CAR groups were promoted to pre-injury level at 48 and 72 h, respectively, while hemodynamic parameters in the NR group did not return to pre-injury level till 72 h, and gastrointestinal tissue perfusion remained lower than pre-injury level until 120 h post-burn. CO<sub>2</sub> of the gastric mucosa and intestinal mucosa blood flow of OR/CAR groups were  $56.4 \pm 4.7$  mmHg and  $157.7 \pm 17.7$  blood perfusion units (BPU) at 24 h post-burn, respectively, which were significantly superior to those in the OR group ( $65.8 \pm 5.8$  mmHg and  $127.7 \pm 11.9$  BPU, respectively, all  $P < 0.05$ ). Gastric emptying and intestinal absorption rates of GES were significantly reduced to the lowest level (52.8% and 23.7% of pre-injury levels) in the OR group at about 2 and 4 h post-burn, and did not return to 80% of pre-injury level until 24 h. In the first 24 h post-burn, the rate of gastric emptying and intestinal water absorption were elevated by a mean 15.7% and 11.5%, respectively, in the OR/CAR group compared with the OR group. At 5 days, the mortality in the NR group was 30% (3/10), 12.5% in the OR group (1/8), and none in the OR/CAR group.

**CONCLUSION:** Carbachol had a beneficial effect on oral resuscitation of burn shock by promoting gastric emptying and intestinal absorption in our canine model.

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**Key words:** Burn shock; Fluid therapy; Oral rehydration; Carbachol; Animal model; Gastric emptying; Intestinal absorption



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## INTRODUCTION

Rapid intravenous infusion of large quantity of fluids containing electrolytes and colloidal solutions remain the key measure to resuscitate hypovolemic shock as a result of a massive burn injury. This life-saving measure has unanimously been accepted worldwide. It has also been recognized that a delay in such replenishment could sometimes be fatal due to complications subsequent to delayed resuscitation of hypovolemic shock. Unfortunately, in certain cases, such as mass casualties in an incendiary bomb attack in battlefields, or a forest or prairie fire in regions with an austere environment with poor medical support and transportation facilities due to geographical barriers, not only there would be a shortage of medics to introduce an intravenous needle, but also the weight and bulk of the necessary intravenous fluids would make the treatment unrealistic. In such cases, it is our supposition that oral administration of fluids might be more practical, and it might be able to maintain the life of the victims till intravenous fluid replacement was available.

Oral fluid resuscitation has been reported with success in early clinical studies of burn care<sup>[1]</sup>. Thomas *et al*<sup>[2]</sup> have described oral fluid replacement in burn patients, and have concluded that oral resuscitation may have a slower initial onset of hemodynamic effectiveness, but after 3-4 h, it can be similarly effective. It is unanimously recognized that the main limiting factors for effective oral resuscitation of burn shock are diminution of gastric emptying capacity and intestinal absorption of fluid and electrolytes due to undermined gastrointestinal perfusion. It seems to be necessary to overcome these two hurdles before oral hydration can be successful for treatment of burn shock.

As enough data of oral resuscitation of burn shock could not be accumulated in clinical practice in ordinary situations, most investigations have been done with animals<sup>[3,4]</sup>. We found that the previous animal experiments have been limited by their short experimental duration of < 24 h post-burn, and performed under general anesthesia, which might interfere with the observation of gastric emptying and intestinal absorption of fluid and electrolytes. With these in mind, a canine model of burn shock was devised in which oral resuscitation was given in the first 24 h post-burn, followed by delayed intravenous fluid replacement, and the whole course of the experiment lasted for 120 h. Also, in this experiment, the effect of general anesthesia was eliminated, and carbachol, which is a cholinergic receptor agonist, was used in

an attempt to shorten gastric emptying time and restore intestinal peristalsis.

## MATERIALS AND METHODS

All the experimental protocols were reviewed and approved by the Committee of Scientific Research of First Affiliated Hospital of General Hospital of PLA (Beijing, China).

### Surgical preparation

Pure bred Beagle dogs (purchased from Experimental Animal Center of Academy of Military Medical Sciences of PLA, Beijing, China, License of qualification SCX 2005-0005), aged 16-20 mo, body weight 11-13 kg, were used. They were acclimatized in the animal house of our Research Laboratory for 2 wk before use. They were fasted for 24 h, and water was withheld for 4 h before the surgical preparation. Under anesthesia with 8 mg/kg ketamine (Gu-Tian Pharmacy, Fu Jian Province, China), the right carotid artery and jugular vein were individually cannulated for hemodynamic monitoring, collecting blood samples, and administration of drugs. Both cannula were led out through a subcutaneous passageway and fixed to the skin. A midline incision was made to open the peritoneal cavity to expose the proximal part of the jejunum, and small incisions were made on the jejunum 10, 20, and 50 cm distal to the Treitz ligament. A Silastic tube, 3 mm in diameter and 25 cm in length, was introduced into the jejunal lumen through each of the above openings. They were fixed with purse-string sutures, led out through a subcutaneous tunnel and fixed to the skin. A cystostomy was done for collecting urine.

### Burn protocol

Twenty-four hours after the above surgical procedures, an intravenous injection of 0.5 mL/kg propofol was given to all the animals to produce brief anesthesia for 10-15 min, which was long enough to eliminate pain during burn injury. Napalm (3%) was applied to the shaved neck and back of the dogs, and it was ignited for 30 s to produce a full-thickness burn that involved about 35% of the total body surface area (TBSA). The depth of the burn injury was verified by pathological examination.

### Experimental groups

The injured dogs were grouped into no fluid replacement (NR,  $n = 10$ ), oral fluid replacement (OR,  $n = 8$ ), and oral replacement of fluid with addition of carbachol (OR/CAR,  $n = 8$ ). For NR, the animals received no fluid replacement or any other treatment. Dogs in the OR group received intragastric, pre-warmed glucose saline solution (each 1 L containing 59.83 mmol/L NaCl, 29.76 mmol/L NaHCO<sub>3</sub>, 20.18 mmol/L KCl, and 114.94 mmol/L glucose in distilled water), and the rate of gastric infusion was consistent with that of the Parkland formula<sup>[5]</sup> (4 mL/kg for each % TBSA burn, half of the total amount given in the first 8 h). In the OR/CAR group, 20 µg/kg carbachol (Sigma, St Louis, MO, USA) was added to the glucose-saline solution. Twenty-four hours after the burn injury, all the animals in the three groups were given intravenous fluid

replenishment. Seventy-two hours after the burn injury, all the surviving animals were given 10% glucose with a mixture of 17 amino acids (Beijing Pharmaceutical, Beijing, China) and 30% fat emulsion (Hua Rui Pharmaceutical, Wu Xi, China). Also, intravenous Ringer's solution was given to make up 100-150 mL of fluid per kg body weight and maintain blood potassium level  $> 3.5$  mmol/L.

### Measurement of hemodynamics

Picco-Plus (Pulsion, Germany) was used to determine mean arterial pressure (MAP) and cardiac output (CO). Plasma volume was determined with the indigo green dilution method<sup>[6]</sup>. At each time point for determination, 12.5 mg indocyanine green (ICG) (Dan Don Pharmaceutical, Liaoning, China) was intravenously given, and 3-mL blood samples were obtained at 1, 2 and 3 min after injection. The specimens were centrifuged at 1800 *g* at 4°C, and 1 mL plasma was obtained to determine the OD value of ICG with a 723N spectrophotometer (wave length, 820 nm), and the concentration of ICG was obtained from a plotted standard curve.

### Measurement of gastrointestinal mucosal perfusion

To determine CO<sub>2</sub> partial pressure of the gastric mucosa (PgCO<sub>2</sub>), a gastric mucosa tonometer (Tonoca, Finland) was used. Intestinal mucosal blood flow (IMBF) was determined by passing a fiberoptic detector of a laser Doppler flow monitor (Peri Flus 5000 Master; Perimed, Jaarnfalla, Sweden) through the intestinal catheters, ensuring that the tips of the detectors were in contact with intestinal mucosa. The signals were transformed into blood perfusion units (BPU), which were input into a computer, and the PEIMED software package PSW 2.0 was used to plot the curves. Thirty seconds were spent for each measurement, and a stable curve that covered 10 s was taken for the calculation of the average.

### Gastric emptying rate

The gastric emptying rate was determined by using phenol red (Sigma) as a signal, according to the Scarpignato principle<sup>[7]</sup>. Before the experiment, a standard curve was plotted with ODs of different dilutions of phenol red at 500 nm. To determine gastric emptying time, 2 mL phenol red at 100 mg/L was introduced into the stomach through an indwelling gastric tube. Two milliliters of gastric juice was obtained when the dye was well mixed with the gastric juice. The amount of the dye in the gastric content was determined by spectrophotometry. Thirty minutes later, another sample of gastric content was withdrawn and phenol red was again determined. With this process, the gastric emptying time was calculated.

### Intestinal absorption rate

Intestinal absorption rate was determined using a modified Cooper method<sup>[8]</sup>. Since phenol red is a large molecular dye, it is not absorbed by the intestinal mucosa. With the absorption of fluid, the concentration of the dye increases. Thus, intestinal absorption rate of fluid could be assessed. The method used in our experiment

was as follows. Since three enteral tubes were implanted into the jejunum in equal distance, a known amount of fluid was introduced into the most proximal catheter (A) with a known velocity  $V_a$  (mL/h) using an intelligent infusion pump (ZNB-XB; Xu-li Scientific Technology Co. Beijing, China), and then specimens of intestinal fluid (1 mL) were collected from two distal catheters, where fluid velocities were  $V_b$  and  $V_c$ . By measuring the concentrations of phenol red in various specimens, the intestinal absorption rate was calculated:  $V_b = V_a \times C_a / C_b$ ,  $V_c = V_b \times C_v / C_c$ ,  $\Delta V = V_b - V_c$ .

$\Delta V$  was the absorption rate in the segment of intestine between catheters B and C. By dividing the value by the length of the intestinal segment between B and C, the intestinal absorption rate of the oral feeding fluid could be calculated, and it was expressed as mL/h.m<sup>2</sup>, i.e. the amount of orally fed liquid absorbed by unit length of the intestine during unit time.

### Statistical analysis

All data are presented as the mean  $\pm$  SD. Statistical analysis was done using SPSS 11.0 statistical software for the *F* test.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Deaths within 5 d

In the NR group, three animals died at 12, 18 and 23 h after burn injury, with a 5 d mortality of 30.0% (3/10). In the OR group, one dog died 18 h after burn injury, with a 5 d mortality of 12.5% (1/8). In the OR/CAR group, no deaths occurred within 5 d of injury, with zero 5 d mortality.

### Hemodynamic parameters

As depicted in Table 1, MAP was lowered 2 h after burn injury in all three groups (all  $P < 0.05$ ). It gradually rose after this period, and MAP in the OR and OR/CAR groups was higher than that in the NR group ( $P < 0.05$ ) 4 h post-burn, and returned to pre-injury level 8 h after injury, but it was still lower than the pre-injury level at 72 and 120 h after injury in the NR group. CO and plasma volume (PV) were lowest at 24 h post-burn in the NR group, and increased later, but never to the level before burn injury. However, CO and PV in the OR and OR/CAR groups were higher than in the NR group at 4 and 8 h post-burn (all  $P < 0.05$ ). CO and PV in the OR/CAR group were higher than in the OR group at 8 and 24 h post-burn (all  $P < 0.05$ ). However, MAP showed no significant change between the OR/CAR and OR groups.

### Gastro-intestinal perfusion

As shown in Table 2, PgCO<sub>2</sub> was rapidly elevated, and IMBF sharply decreased in all three groups. They were seen to elevate gradually afterwards. In the NR group, these two values were still worsen than that before injury at 120 h post-burn (all  $P < 0.05$ ). However, in the OR and OR/CAR groups, they recovered to pre-burn levels at 72 h post-burn. From 2 h post-burn, PgCO<sub>2</sub> in the OR group was lower than that in the NR group ( $P < 0.05$ ), al-

Table 1 Effects of carbachol on hemodynamic parameters in oral resuscitation of burn shock (mean  $\pm$  SD)

Post-burn (h)	MAP (mmHg)			CO (L/min)			PV (mL/kg)		
	NR	OR	OR/CAR	NR	OR	OR/CAR	NR	OR	OR/CAR
0	135 $\pm$ 9.3	131 $\pm$ 18.7	128 $\pm$ 12.6	2.46 $\pm$ 0.13	2.43 $\pm$ 0.17	2.39 $\pm$ 0.19	49.6 $\pm$ 5.6	46.8 $\pm$ 4.2	48.3 $\pm$ 4.0
2	100 $\pm$ 7.2 <sup>a</sup>	94 $\pm$ 15.3 <sup>a</sup>	89 $\pm$ 10.0 <sup>a</sup>	1.28 $\pm$ 0.11 <sup>a</sup>	1.34 $\pm$ 0.1 <sup>a</sup>	1.30 $\pm$ 0.11 <sup>a</sup>	38.2 $\pm$ 3.8 <sup>a</sup>	40.3 $\pm$ 3.0 <sup>a</sup>	41.3 $\pm$ 3.1 <sup>a</sup>
4	108 $\pm$ 8.3 <sup>a</sup>	136 $\pm$ 18.9 <sup>b</sup>	130 $\pm$ 15.1 <sup>b</sup>	1.12 $\pm$ 0.10 <sup>a</sup>	1.54 $\pm$ 0.11 <sup>a,b</sup>	1.48 $\pm$ 0.12 <sup>a,b</sup>	32.8 $\pm$ 2.3 <sup>a</sup>	39.0 $\pm$ 3.8 <sup>a</sup>	40.8 $\pm$ 3.9 <sup>a</sup>
8	120 $\pm$ 14.3	125 $\pm$ 15.9	127 $\pm$ 14.3	1.20 $\pm$ 0.17 <sup>a</sup>	1.65 $\pm$ 0.12 <sup>a,b</sup>	1.86 $\pm$ 0.14 <sup>a,b,c</sup>	30.9 $\pm$ 3.4 <sup>a</sup>	36.2 $\pm$ 3.4 <sup>a,b</sup>	42.2 $\pm$ 3.4 <sup>a,b,c</sup>
24	124 $\pm$ 8.9	126 $\pm$ 8.8	129 $\pm$ 8.9	1.07 $\pm$ 0.17 <sup>a</sup>	1.94 $\pm$ 0.18 <sup>a,b</sup>	2.16 $\pm$ 0.15 <sup>a,b,c</sup>	30.6 $\pm$ 4.4 <sup>a</sup>	40.4 $\pm$ 3.0 <sup>a,b</sup>	45.8 $\pm$ 3.6 <sup>b,c</sup>
48	129 $\pm$ 8.7	135 $\pm$ 20.8	123 $\pm$ 15.8	1.88 $\pm$ 0.15 <sup>a</sup>	2.39 $\pm$ 0.23 <sup>b</sup>	2.49 $\pm$ 0.16 <sup>b</sup>	34.5 $\pm$ 2.4 <sup>a</sup>	43.0 $\pm$ 3.8 <sup>b</sup>	45.6 $\pm$ 3.6 <sup>b</sup>
72	115 $\pm$ 13.7 <sup>a</sup>	137 $\pm$ 11.0 <sup>b</sup>	135 $\pm$ 13.1 <sup>b</sup>	2.10 $\pm$ 0.13 <sup>a</sup>	2.34 $\pm$ 0.12 <sup>b</sup>	2.41 $\pm$ 0.20 <sup>b</sup>	35.8 $\pm$ 2.9 <sup>a</sup>	43.8 $\pm$ 3.4 <sup>b</sup>	45.8 $\pm$ 4.0 <sup>b</sup>
120	112 $\pm$ 11.4 <sup>a</sup>	134 $\pm$ 14.6 <sup>b</sup>	131 $\pm$ 14.2 <sup>b</sup>	2.15 $\pm$ 0.15 <sup>a</sup>	2.39 $\pm$ 0.15 <sup>b</sup>	2.41 $\pm$ 0.20 <sup>b</sup>	37.6 $\pm$ 3.5 <sup>a</sup>	44.7 $\pm$ 3.2 <sup>b</sup>	46.9 $\pm$ 3.4 <sup>b</sup>

Compared with that at 0 h, <sup>a</sup> $P$  < 0.05; compared with no fluid resuscitation (NR), <sup>b</sup> $P$  < 0.05; compared with oral fluid resuscitation (OR), <sup>c</sup> $P$  < 0.05. MAP: mean arterial pressure; CO: Cardiac output; PV: Plasma volume; CAR: Carbachol.

Table 2 Effect of carbachol on gastrointestinal perfusion in oral resuscitation of burn shock (mean  $\pm$  SD)

Post-burn (h)	PgCO <sub>2</sub> (mmHg)			IMBF (BPU)		
	NR	OR	OR/CAR	NR	OR	OR/CAR
0	32.2 $\pm$ 3.7	33.2 $\pm$ 6.1	33.8 $\pm$ 6.8	203.8 $\pm$ 17.6	198.3 $\pm$ 11.9	207.3 $\pm$ 13.9
2	73.1 $\pm$ 7.7 <sup>a</sup>	60.8 $\pm$ 8.2 <sup>a,b</sup>	57.4 $\pm$ 8.0 <sup>a,b</sup>	74.2 $\pm$ 10.8 <sup>a</sup>	101.2 $\pm$ 12.2 <sup>a,b</sup>	112.6 $\pm$ 10.2 <sup>a,b</sup>
4	83.1 $\pm$ 6.5 <sup>a</sup>	74.0 $\pm$ 6.5 <sup>a,b</sup>	70.0 $\pm$ 6.2 <sup>a,b</sup>	71.5 $\pm$ 15.3 <sup>a</sup>	108.8 $\pm$ 12.2 <sup>a,b</sup>	138.8 $\pm$ 14.1 <sup>a,b,c</sup>
8	86.4 $\pm$ 8.6 <sup>a</sup>	69.2 $\pm$ 6.8 <sup>a,b</sup>	68.5 $\pm$ 5.8 <sup>a,b</sup>	77.8 $\pm$ 10.0 <sup>a</sup>	114.7 $\pm$ 12.0 <sup>a,b</sup>	134.7 $\pm$ 13.9 <sup>a,b,c</sup>
24	82.5 $\pm$ 7.6 <sup>a</sup>	65.8 $\pm$ 5.8 <sup>a,b</sup>	56.4 $\pm$ 4.7 <sup>a,b,c</sup>	79.2 $\pm$ 17.3 <sup>a</sup>	127.7 $\pm$ 11.9 <sup>a,b</sup>	157.7 $\pm$ 17.7 <sup>a,b,c</sup>
48	61.5 $\pm$ 8.2 <sup>a</sup>	56.0 $\pm$ 8.4 <sup>a</sup>	57.0 $\pm$ 6.4 <sup>a</sup>	146.8 $\pm$ 13.8 <sup>a</sup>	159.3 $\pm$ 19.1 <sup>a</sup>	179.3 $\pm$ 19.1 <sup>a,b</sup>
72	56.8 $\pm$ 6.6 <sup>a</sup>	39.4 $\pm$ 8.9 <sup>b</sup>	35.4 $\pm$ 5.6 <sup>b</sup>	168.5 $\pm$ 9.7 <sup>a</sup>	180.7 $\pm$ 18.5	198.7 $\pm$ 16.5 <sup>b</sup>
120	45.8 $\pm$ 6.2 <sup>a</sup>	31.2 $\pm$ 5.0 <sup>b</sup>	34.2 $\pm$ 4.0 <sup>b</sup>	178.8 $\pm$ 16.5 <sup>a</sup>	203.5 $\pm$ 23.2 <sup>b</sup>	200.5 $\pm$ 18.2 <sup>b</sup>

Compared with that at 0 h, <sup>a</sup> $P$  < 0.05; compared with no fluid resuscitation (NR), <sup>b</sup> $P$  < 0.05; compared with oral fluid resuscitation (OR), <sup>c</sup> $P$  < 0.05. IMBF: Intestinal mucosal blood flow; CAR: Carbachol.

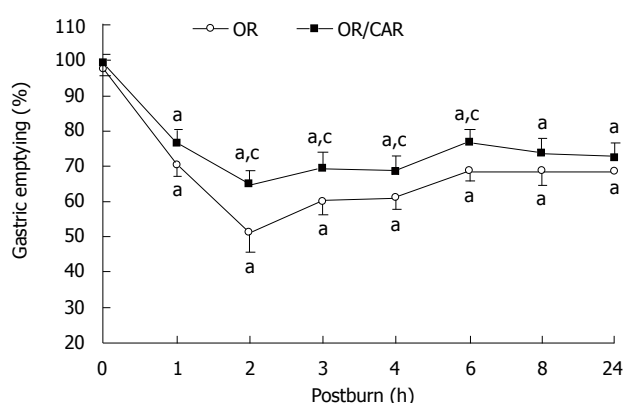


Figure 1 Carbachol promoted gastric emptying rate in oral fluid resuscitation/Carbachol group compared with those of oral fluid resuscitation group at 2, 3, 4 and 6 h after burn injury. <sup>a</sup> $P$  < 0.05 vs 0 h, <sup>b</sup> $P$  < 0.05 vs oral fluid resuscitation (OR) group (one-way ANOVA). Error bars represent mean  $\pm$  SD. CAR: Carbachol.

though it was higher than that in the OR/CAR group, but there was no significant difference ( $P$  > 0.05). IMBF was always higher in the OR and OR/CAR groups compared with the NR group (all  $P$  < 0.05), but the value was lower in the OR group compared with the OR/CAR group at 4, 8 and 24 h post-burn (all  $P$  < 0.05).

### Gastric emptying rate

Figure 1 shows that, in the OR and OR/CAR groups, gastric emptying rate was lowered, especially in the for-

mer group, with 52.8% of the normal emptying rate at 2 h, and 70.5% at 24 h post-burn. In the OR/CAR group, it was also lowered, but reached 65.2% at 2 h, and 73.0% at 24 h post-burn, and the values were all significantly higher than those in the OR group at 2, 3, 4 and 6 h post-burn (all  $P$  < 0.05). Thus, it was estimated that approximately 15.7% more gastric content was expelled from the stomach in 24 h in the OR/CAR group as compared with the OR group.

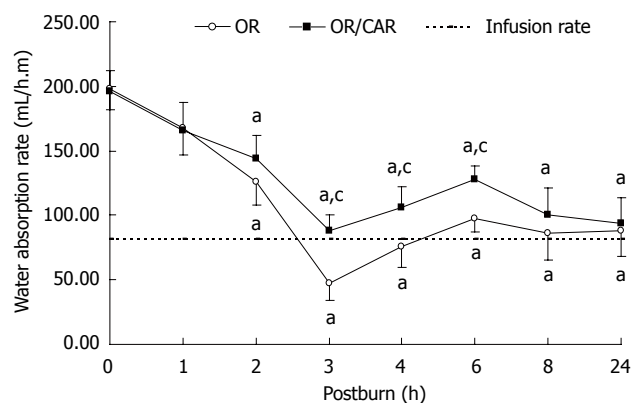
### Intestinal absorption rate

As shown in Figures 2 and 3, the rate of absorption of orally administered water and Na<sup>+</sup> was lowest at 3 h post-burn in the OR group, and the values were 23.7% and 50.3% of pre-injury levels. These absorption rates were gradually increased to 44.4% and 65.1%, respectively, at 24 h post-burn. In the OR/CAR group, all these values were higher than those in the OR group at 3, 4 and 6 h post-burn (all  $P$  < 0.05). In the first 24 h post-burn, the rate of intestinal water absorption was elevated by 11.5% in the OR/CAR group. It was estimated that within 24 h after injury, the water absorption rate at 24 h in the OR and OR/CAR groups was 110.9  $\pm$  17.1 mL/h.m and 127.8  $\pm$  17.3 mL/h.m, respectively, and they were actually higher than the pre-requisite of the Parkland formula 82.1  $\pm$  11.2 mL/h.m ( $P$  < 0.05).

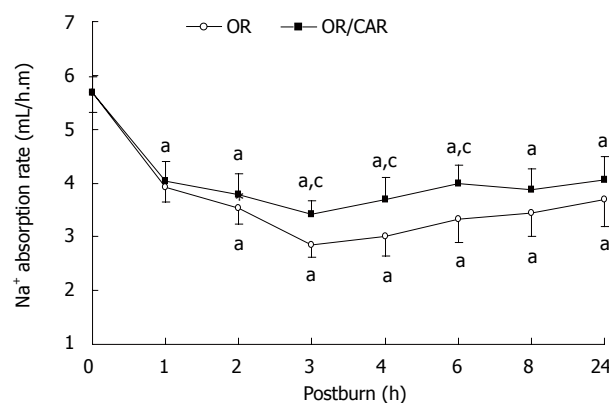
## DISCUSSION

Mass casualties from burn injury may occur in a region





**Figure 2** Carbachol significantly improved rate of water absorption of intestine in oral fluid resuscitation/Carbachol group compared with those of oral fluid resuscitation group at 3, 4 and 6 h after burn injury. <sup>a</sup> $P < 0.05$  vs 0 h, <sup>c</sup> $P < 0.05$  vs oral fluid resuscitation (OR) group (one-way ANOVA). Error bars represent mean  $\pm$  SD. CAR: Carbachol.



**Figure 3** Carbachol significantly improved rate of Na<sup>+</sup> absorption of intestine in oral fluid resuscitation/Carbachol group compared with those of oral fluid resuscitation group at 3, 4 and 6 h after burn injury. <sup>a</sup> $P < 0.05$  vs 0 h, <sup>c</sup> $P < 0.05$  vs oral fluid resuscitation (OR) group (one-way ANOVA). Error bars represent mean  $\pm$  SD. CAR: Carbachol.

where the major problems facing medical personnel are the availability and probability of fluid replacement with sterile intravenous fluids. If we resuscitate a single burn victim weighing  $> 60$  kg with a burn of 30% TBSA it would require  $> 5$ -7 kg of fluid for a medic to carry. In a very harsh environment with lack of decent medical support, resuscitation of burn shock is severely handicapped, and the life of those with extensive burn injury is jeopardized. One strategy for reducing such a hazard is to try to supplement liquid with sufficient electrolytes by mouth until intravenous infusion fluids are available<sup>[9]</sup>. Early in 1970, Monaf<sup>[10]</sup> reported a clinical trial of oral administration of hypertonic lactated saline solution to patients with burn injury of various extents, and he found that at least partial oral resuscitation of severe burns could be successful. However, oral resuscitation of burn shock has not been popular, because, ordinarily, intravenous resuscitation is almost always available, especially in cities, and even in many rural areas where medical facilities have been established. Nevertheless, in certain scenarios, such as fire disasters in cities after a strong earthquake, forest fires in mountainous terrain, or in combat zones after incendiary devices have exploded, when transportation is seriously lacking or hampered due to geographical barriers, and medical support is lacking, so that sterile intravenous fluids and trained personnel are not available, oral intake of fluids should be considered, in the hope that the victims can be tided over burn shock.

Sufficient data about oral resuscitation of burn shock can not be accumulated in clinical practice under normal circumstances, therefore, most investigations have been done with animals. In these experiments, animals were always under general anesthesia to prevent restlessness of the animals<sup>[1,3,4]</sup>. Thus, the results of oral hydration might not reflect the true states of fluid absorption because the anesthesia inevitably inhibits gastrointestinal motility and other functions. The present study was planned to measure all the physiological parameters in a conscious state. All the catheters for measuring physiological indexes were implanted 1 d before burn injury. Burn injury was

produced under brief anesthesia to eliminate mental strain and pain. Thereafter, all the measurements were made with the animals in a fully conscious state. Pure bred Beagle dogs were used because they were tame, docile and cooperative. No restriction of the body was necessary during the whole course of the experiment, which rendered all the measurements complete without any restriction of the animals or general anesthesia. Thus, all the disturbances to gastrointestinal mobility or absorption were greatly alleviated. Therefore, there was no external interference to influence the measurements during the whole course of the experiment, which lasted for 120 h, which increased the reliability of the measurements.

The degree of perfusion of the gastrointestinal tract is at present considered as an important index of circulatory shock and tissue oxygen delivery. The determination of PCO<sub>2</sub> of the gastric mucosa has been used to estimate the pH of the mucosa, and the change in pH of the mucosa reflects the condition of blood perfusion and oxygen delivery to the gastric tissue<sup>[11]</sup>. The procedure is untraumatic, and the catheter can also be used to give necessary fluids as well as a convenient tool for measuring gastric emptying rate. To determine blood perfusion of the intestinal mucosa by way of preformed fistulae has already been a standard method in monitoring the circulatory state of transplanted intestine<sup>[12]</sup>. In our experiment, a flexible fiberoptic detector of a laser Doppler flow monitor was passed through the preformed fistulae. Each reading took about 1 min only, so that it did not give any discomfort to the animal, and sequential measurements were assured. The experimental results showed that, in injured dogs without the benefit of fluid gavage, blood perfusion was rapidly diminished in the intestinal mucosa. It was also shown that this change was consistent with a decrease gastrointestinal mucosal perfusion, and its recovery lagged behind the improvement in hemodynamic parameters. Up to 120 h after injury, it was still lower than that before injury. These phenomena corroborated that which was found in human patients with hypodynamic shock.

When a solution with electrolytes and glucose is intro-

duced into the stomach, the emptying rate of the liquid from the stomach depends on the pressure gradient between the stomach and duodenum. It also is influenced by the state of blood supply to the stomach and regulatory activity of the vagus nerve (cholinergic nerve) and humoral agents (motilin)<sup>[13]</sup>. Absorption of water through the intestinal mucosa depends on translocation of  $\text{Na}^+$  ions, while the latter process could only be realized with the presence and activity of  $\text{Na}^+/\text{K}^+$ -ATPase, which is localized in the basal layer of the intestinal mucosal epithelium. Therefore, it is obvious that the rate of absorption of water by the intestinal mucosa is under the influence of the intestinal blood flow, activity of  $\text{Na}^+/\text{K}^+$ -ATPase, and aquaporin (AQP)-1 expression, along with consumption of ATP. Thus, it is evident that with ischemia and hypoxia of the intestinal mucosa during hypovolemic shock, water absorption by the intestinal mucosa is hampered.

Heavy leakage of fluid from the circulation results in a sharp decrease in ATP. This shortage in ATP is further amplified by an increased demand created by feeding and absorption of water and electrolytes. Under such conditions, the presence of glucose, which can be metabolized into lactic acid, alanine and  $\text{CO}_2$  to produce ATP under anaerobic conditions, is essential. This additional ATP is helpful for the absorption potential of the intestinal mucosa. During the process of absorption, the transport of glucose is coupled with the transport of water molecules and  $\text{Na}^+$  ions, and two  $\text{Na}^+$  ions and 223 water molecules are absorbed. Therefore, addition of glucose to an electrolyte solution is an ideal liquid for oral replacement during hypovolemic shock<sup>[14,15]</sup>.

Thomas *et al.*<sup>[2]</sup> have described an experiment to study gastric emptying with oral replacement of fluid in hypovolemic shock. They gavaged glucose-electrolyte solution (GES) into the stomach of pigs with 40% TBSA burn injury, according to the Parkland formula. They showed that the gastric emptying volume increased with an increase in the volume of gavaged fluid. However, the volume of fluid passed into the duodenum was one half of the volume required by the Parkland formula, and hemodynamic parameters did not recover to the pre-injury level. Michell *et al.*<sup>[4]</sup> have performed a study of duodenal infusion of glucose-electrolyte solution in pigs with 40% TBSA burns, and demonstrated that a total of 93% of infused solution was absorbed during the course of the 4 h experiment. However, these experiments were performed in animals under general anesthesia, and there was unavoidable impairment of gastrointestinal peristalsis and absorption ability. Therefore, the results could not be considered as reflecting the true condition of the gastrointestinal tract.

In our present study, we determined the gastric emptying and intestinal absorption rate of GES without the interfering effects of general anesthesia. We found that gastric emptying and intestinal absorption were significantly reduced to the lowest level (52.8% and 23.7% of pre-injury levels) in the OR group at about 2 and 4 h post-burn, respectively, and did not return to 80% of pre-injury level until 24 h. It was estimated that within 24 h

after injury, the water absorption rate in the OR group was  $110.9 \pm 17.1$  mL/h.m, and it was actually higher than the pre-requisite of the Parkland formula ( $82.1 \pm 11.2$  mL/h.m). This intestinal absorptive rate was similar to that of Michael *et al.*<sup>[4]</sup>. The above results suggest that, in large animals (e.g. pigs or dogs), with < 40% TBSA burns, almost all GES infused according to the Parkland formula could be fully absorbed, although intestinal absorption was inhibited due to gut hypoperfusion. Therefore, we considered that gastric emptying is the main limitation to effective gastrointestinal resuscitation.

The results of using carbachol in our experiment clearly demonstrated that this drug could improve tissue blood perfusion of the gastrointestinal tract. Thus, it was helpful in expediting gastric emptying of its contents and improving intestinal absorption of water and electrolytes. Carbachol is a cholinergic receptor agonist. It stimulates peristalsis of the gastrointestinal tract by activating M cholinergic receptors, and also activates  $\alpha 7$  subunits of cholinergic nicotinic receptors on macrophage and endothelial cells which leads to cellular deactivation and inhibition of cytokine release, thus attenuating the systemic or regional inflammatory response<sup>[16-18]</sup>. In addition, it is an antioxidant and inhibitor of apoptosis<sup>[19,20]</sup>. In our study, the addition of carbachol to the resuscitation fluid did improve blood perfusion of the gastrointestinal tissues, expedite gastric emptying, and improve intestinal absorption rate of water and electrolytes. These beneficial effects of carbachol may be attributable to the following mechanisms: (1) inhibition of release of proinflammatory cytokines alleviates the inflammatory reaction of the gastrointestinal tissue, thus resulting in reduced loss of AQP1<sup>[21,22]</sup>; (2) improvement in intestinal peristalsis and blood perfusion due to stimulation of M receptors facilitates intestinal absorption; and (3) promotion of activity of  $\text{Na}^+/\text{K}^+$ -ATPase, which is essential for absorption of water and especially  $\text{Na}^+$  by intestinal mucosa<sup>[23]</sup>. Our experiment showed that the gastric emptying time was promoted by 15.7%, and the intestinal absorption rate was increased by 11.5%.

In conclusion, our experiment has successfully reproduced a canine model of serious burn injury. In this model, we are able to study the effects of oral replenishment of GES for resuscitation of burn shock. The hemodynamic parameters, gastric emptying rate, and intestinal absorption of GES can be relatively accurately determined. The results of the experiment also show that burn shock can be ameliorated to a certain extent by administration of GES, with the addition of carbachol to enhance gastric emptying and intestinal absorption of fluids. Oral resuscitation for burn shock might be a surrogate measure where mass casualties from burn injury occur in an area where medical support is minimal and transportation is difficult. Carbachol may be beneficial because it is a cholinergic receptor agonist, and it possesses the function of enhancing gastric emptying and intestinal absorption of fluid when given by mouth. Therefore, in circumstances when intravenous replacement of fluids for resuscitation of burn shock is not fea-

sible, oral feeding of GES, with addition of carbachol, may be possible.

## COMMENTS

### Background

Rapid intravenous infusion remains unanimously the key measure to resuscitate hypovolemic shock as a result of massive burn injury. Unfortunately, in armed conflicts or massive disasters (such as forest fire, earthquake, or terrorist attack) mass casualties occur in an austere environment with poor medical support and transportation facilities. In such cases, it is our supposition that oral or gastrointestinal administration of fluids might be more practical, and could have a positive effect in maintaining the life of the victims until intravenous replacement of fluids is available.

### Research frontiers

Gastrointestinal tract ischemia and hypoxia due to massive surgical stresses such as severe trauma, extensive burns and major surgery resulting in hypovolemic shock can lead to dysfunction of gastric emptying and intestinal absorption, followed by poor transportation and absorption of oral electrolytes and nutrients in the gastrointestinal tract. Improvement of mucosal blood perfusion, and enhancement of gastrointestinal tolerance to oral rehydration fluid and enteral nutrition are not only the foci of research in the surgical and critical care fields, but they are key factors for facilitation of oral resuscitation of hypovolemic shock.

### Innovations and breakthroughs

A large animal model of severe burn injury was reproduced to investigate the feasibility of oral resuscitation of burn shock, without the disturbing effects of general anesthesia. The effects of carbachol, which is a cholinergic receptor agonist, on blood circulation, gastrointestinal perfusion, gastric emptying and intestinal absorption of fluid and electrolytes were investigated. The results indicated that oral resuscitation with the help of such a drug might be an ideal way in lieu of intravenous resuscitation for burn shock, especially in battlefields or other sites of mass casualties.

### Applications

Orally administered fluid can be considered to be a simple and effective means of replacement of body fluid that is feasible for resuscitation of hypovolemic shock, especially when there is an extreme shortage of means of medical support in an austere environment such as battlefields and disasters.

### Peer review

The manuscript is very well written and the conclusions applicable.

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## Gastroesophageal reflux in cirrhotic patients without esophageal varices

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### Abstract

**AIM:** To evaluate the esophageal motility and abnormal acid and bile reflux incidence in cirrhotic patients without esophageal varices (EV).

**METHODS:** Seventy-eight patients with liver cirrhosis without EV confirmed by upper gastroesophageal endoscopy and 30 healthy control volunteers were prospectively enrolled in this study. All the patients were evaluated using a modified protocol including Child-Pugh score, upper gastrointestinal endoscopy, esophageal manometry, simultaneous ambulatory 24-h esophageal pH and bilirubin monitoring. All the patients and volunteers accepted the manometric study.

**RESULTS:** In the liver cirrhosis group, lower esophageal sphincter pressure (LESP,  $15.32 \pm 2.91$  mmHg), peristaltic amplitude (PA,  $61.41 \pm 10.52$  mmHg), peristaltic duration (PD,  $5.32 \pm 1.22$  s), and peristaltic velocity (PV,  $5.22 \pm 1.11$  cm/s) were all significantly abnormal in comparison with those in the control group ( $P < 0.05$ ), and LESP was negatively correlated with Child-Pugh score. The incidence of reflux esophagitis (RE) and pathologic reflux was 37.18% and 55.13%, respectively

(vs control,  $P < 0.05$ ). And the incidence of isolated abnormal acid reflux, bile reflux and mixed reflux was 12.82%, 14.10% and 28.21% in patients with liver cirrhosis without EV.

**CONCLUSION:** Cirrhotic patients without EV presented esophageal motor disorders and mixed acid and bile reflux was the main pattern; the cirrhosis itself was an important causative factor.

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**Key words:** Gastroesophageal reflux disease; Liver cirrhosis; Esophageal varices; Esophageal manometry; pH; Bilirubin; Monitoring

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### INTRODUCTION

Gastroesophageal reflux disease (GERD) is one of the most common diseases in modern civilization, which greatly affects people's health and quality of life<sup>[1]</sup>. GERD is defined as reflux of gastroduodenal content to the esophagus, and includes reflux esophagitis (RE), nonerosive reflux disease (NERD) and Barrett's esophagus (BE). GERD originates from a disturbance in the structure and function of the lower esophageal sphincter (LES) barrier, and dysfunctional esophageal motility coupled with a weak LES can cause uncoordinated propulsion, regurgitation of gastric and/or duodenal contents into the esophagus<sup>[2]</sup>.

Gastroesophageal reflux consists of a broad mixture

of oral-esophageal, gastric, and duodenal secretions. It is accepted that acid reflux plays an important role in the pathogenesis of GERD<sup>[3]</sup>. But the role of non-acid reflux is still a controversy<sup>[4,5]</sup>, and some recent studies have shown that duodenogastroesophageal reflux (DGER) is another important causative factor in esophageal mucosal damage<sup>[6,7]</sup>. So the combination of esophageal pH and bilirubin monitoring is indispensable for a precise diagnostic test in acid and non-acid reflux of GERD.

GERD can be induced or aggravated under many conditions including liver diseases. It has been reported that in patients with liver cirrhosis and portal hypertension, gastroesophageal reflux occurs at a high frequency (64%)<sup>[8]</sup>. Moreover, hepatic cirrhosis has a high morbidity and mortality due to the portal hypertension with the development of esophageal varices, and the possibility of a digestive hemorrhage and worsening of hepatic insufficiency<sup>[9-11]</sup>. It is important to identify predictive or aggravating factors and if possible, to prevent these factors. Esophageal motor disorders have been found to be associated with acid gastroesophageal reflux in cirrhotic patients with esophageal varices, and functional studies have shown decreased functions of the lower esophageal sphincter with low amplitude of primary peristalsis and acid clearance, which might attribute to a mechanical effect of the presence of varices<sup>[12]</sup>. It is still unclear whether the presence of cirrhosis itself presents as a causative factor for the onset of gastroesophageal reflux, and there are few studies on the incidence of acid reflux and DGER in the cirrhotic patients without esophageal varices. Therefore, this study was designed to evaluate the esophageal motility and abnormal acid and bile reflux incidence in cirrhotic patients without esophageal varices.

## MATERIALS AND METHODS

### Patients

Seventy-eight patients with liver cirrhosis without EV confirmed by upper gastroesophageal endoscopy from March 2008 to November 2010 were prospectively enrolled to this study. All the patients were the inpatients of Beijing Tiantan Hospital, Capital Medical University. Patients with systemic diseases related to esophageal motor disorders and/or gastroesophageal reflux diseases (progressive systemic sclerosis, diabetes mellitus, neuromuscular disorders), alcohol abusers within 6 mo and chronic drug users that influence esophageal motility (such as theophylline, nitrates and calcium channel blockers) were excluded.

All the patients were evaluated by the same physician according to a modified protocol including Child-Pugh score<sup>[13]</sup>, ascites, and other complications and a reflux disease questionnaire (RDQ; AstraZeneca R and D, Wuxi, China). RDQ is a detailed questionnaire regarding the severity and frequency of four symptoms: heartburn, acid regurgitation, food regurgitation, and retrosternal pain, and each symptom is graded in severity and frequency. The diagnosis of liver cirrhosis was verified by the clinical, laboratory, radiologic and histopathological results according to the criteria of the Chinese Medical

Society for Liver Diseases<sup>[14]</sup>.

Thirty healthy volunteers (15 women) with a mean age of 33 years served as controls in this study. None of them had a history of reflux disease or of surgery in the upper gastrointestinal tract or thorax. All the volunteers accepted the manometric study without medication.

The written informed consent for the study was approved by the hospital ethics committee, and obtained from all the subjects and the procedure followed the principles of the Declaration of Helsinki.

### Methods

Upper gastrointestinal endoscopy. To exclude the EV cases, all patients received the upper gastrointestinal endoscopic examination (OlympusXQ260; Olympus, Japan). Gastric varices and/or related congestive gastropathy were also recorded. Reflux esophagitis if present was classified according to the Los Angeles classification standards. Barrett's esophagus was defined as a columnar-lined esophageal mucosa with intestinal metaplasia.

Esophageal manometry. Manometry was performed using a water-perfused manometric assembly (Medtronic, Deutschland). The manometric probe consisted of a 4.5-mm polyvinyl catheter (Medtronic) with eight measuring sites (0, 1, 2, 3, 5, 10, 15, and 20 cm). The position, length and pressure of the lower esophageal sphincter (LES) were identified by the method of stepwise retraction of the probe through gastroesophageal junction (GEJ). After correct positioning of the catheter in the esophageal lumen, patients were asked to swallow ten 5-mL boluses of water. Manometric signals were recorded on a computer for subsequent display and analysis, and the information included: the length of the LES, antegrade and retrograde peristalses, synchronous and isolated contractile waves, peristaltic amplitude (PA), peristaltic duration (PD), and peristaltic velocity (PV) of primary peristaltic wave in distal esophagus. LES disorder and esophageal body dysmotility were diagnosed according to the criteria in a previous study<sup>[15]</sup>.

Simultaneous ambulatory 24-h esophageal pH and bilirubin monitoring. After esophageal manometry, an antimony esophageal pH electrode and fiber optic probe for detecting acid and bilirubin were positioned pernasally 5 cm above the upper border of the LES and connected with an ambulatory pH recorder (Digrapper Mk III 2000, Synetice Medical, Sweden) and an ambulatory duodenogastroesophageal reflux (DGER) monitoring system (Bilitic 2000, Synetice Medical, Sweden), respectively. The method was reported previously<sup>[16]</sup>. The recorded data were analyzed using the Synectics PM Software.

In brief, the 24-h pH ambulatory recording was carried out with a portable digital system composed of a catheter with an antimony electrode and external reference electrode. Patients were instructed to keep a diary recording the time of meals, position changes, and the time and type of their symptoms, and encouraged to pursue their normal daily activities and maintain their usual diet, avoiding citric fruit and soft drinks. Proton pump inhibitor if in use, were discontinued at least 7-10 d prior to the

**Table 1** Results of esophageal manometry in liver cirrhosis patients and controls (mean  $\pm$  SD)

Group	LESP (mmHg)	PA (mmHg)	PD (s)	PV (cm/s)
Liver cirrhosis ( <i>n</i> = 78)	15.32 $\pm$ 2.91 <sup>a</sup>	61.41 $\pm$ 10.52 <sup>a</sup>	5.32 $\pm$ 1.22 <sup>a</sup>	5.22 $\pm$ 1.11 <sup>a</sup>
Child A ( <i>n</i> = 28)	16.18 $\pm$ 2.81	70.52 $\pm$ 8.93 <sup>a</sup>	3.91 $\pm$ 1.03 <sup>a</sup>	4.56 $\pm$ 1.22 <sup>a</sup>
Child B ( <i>n</i> = 27)	15.41 $\pm$ 3.13 <sup>c</sup>	67.4 $\pm$ 9.3 <sup>c</sup>	5.11 $\pm$ 1.21 <sup>c</sup>	5.10 $\pm$ 1.02 <sup>c</sup>
Child C ( <i>n</i> = 23)	14.52 $\pm$ 2.91 <sup>e</sup>	56.13 $\pm$ 10.06 <sup>e</sup>	6.02 $\pm$ 1.23 <sup>e</sup>	5.91 $\pm$ 1.01 <sup>e</sup>
Control ( <i>n</i> = 30)	16.21 $\pm$ 5.33	74.41 $\pm$ 17.53	2.70 $\pm$ 0.81	3.71 $\pm$ 1.82

<sup>a</sup>Compared with control, *P* < 0.05; <sup>c</sup>Compared with Child A, *P* < 0.05; <sup>e</sup>Compared with Child B, *P* < 0.05. LESP: Lower esophageal sphincter pressure; PA: Peristaltic amplitude; PD: Peristaltic duration; PV: Peristaltic velocity.

examination, H<sub>2</sub> blockers at least 48–72 h and prokinetics agents 24 h. An esophageal pH of less than 4 for at least 15 s was considered to be a reflux episode. Pathological acid reflux was considered if the percentage of the time with the intraesophageal pH less than 4 was greater than 4%, the number of reflux episodes was larger than 50 or the DeMeester value was higher than 14.72<sup>[17]</sup>.

The fiber optic spectrophotometer Bilitec 2000 was used to quantify DGER. The system consisted of a miniaturized probe measuring 1.5 mm in diameter that carried light signals into the esophagus and backed *via* a plastic fiberoptic bundle. Before each study, the probe was calibrated in water, and the probe tip was checked for obstruction after completion of the study.

Patients were also encouraged to maintain normal activities, sleep schedule, and to follow a particular low-fat diet containing light food elements, and not to take coffee, tea and fruit juice, in order to prevent any interference with the spectrophotometric recording. Skimmed milk and non-sparkling water were allowed. An episode of DGER was defined as an increase in esophageal bilirubin absorbance 0.14 for more than 10 s<sup>[18,19]</sup>.

### Blood sample detection

Blood samples were drawn for a complete analysis of blood cell count and levels of prothrombin, albumin, alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase, gamma glutamyl transferase, bilirubin, cholesterol, creatinine.

### Statistical analysis

Statistical analysis was performed using the statistical program SPSS 13.0 for Windows (SPSS Inc., Chicago, USA). All data were presented as mean  $\pm$  SD, and *P* values lower than 0.05 were considered statistically significant.

## RESULTS

### Patient characteristics

Seventy-eight patients met the inclusion criteria, 40 males (51.28%) and 38 females (48.72%), with a mean age of 56.41  $\pm$  9.72 years (range, 18–75 years). Twenty-eight patients were classified as Child A, 27 as Child B and 23 as Child C patients. Typical symptoms of gastroesophageal reflux disease were present in 25 (32.05%) patients. The RDQ scores were significantly higher in liver cirrhosis group (11.32  $\pm$  3.14) than in control group (6.25  $\pm$  3.31)

(*P* < 0.01). There were no statistical differences of RDQ scores among the liver cirrhosis subgroups, and no relationship between Child-Pugh score and abnormal reflux (*P* > 0.05).

### Esophageal manometry

In the liver cirrhosis group, LESP (15.32  $\pm$  2.91 mm Hg), PA (61.41  $\pm$  10.52 mmHg), PD (5.32  $\pm$  1.22 s), and PV (5.22  $\pm$  1.11 cm/s) were all significantly abnormal in comparison with those in the control group (*P* < 0.05) (Table 1). The results showed a gradual decrease of LESP and PA, also an extension of PD and PV in the liver cirrhosis group from Child A to Child C. LESP was negatively correlated with Child-Pugh score (*P* < 0.01, *r* = -0.625).

### 24-h esophageal pH monitoring

The results demonstrated a stepwise increase of pathologic esophageal pH-metry in liver cirrhosis patients, and an increase of acid reflux episodes and percentage of a pH < 4 in the upright, supine and total phases of measurement (*P* < 0.05) (Table 2).

### 24-h esophageal bilirubin monitoring

The results showed a significant stepwise increase of pathologic esophageal bilirubin-metry in liver cirrhosis patients, along with significant increases of bile reflux episodes and percentage of absorbance > 0.14 in the upright, supine, and total phases of measurement (*P* < 0.05) (Table 3).

### Incidence of RE and abnormal reflux

The incidence of RE and pathologic reflux was 37.18% and 55.13% in patients with liver cirrhosis, respectively, which were all higher than those in the control group (*P* < 0.05) (Table 4 and Figure 1). The incidence of isolated abnormal acid reflux, bile reflux and mixed reflux was 12.82%, 14.10% and 28.21% in patients with liver cirrhosis, respectively (Table 5). And the incidence of BE was 5.13% (4/78) in patients with liver cirrhosis, and none was found in the control group.

## DISCUSSION

As a complication of chronic liver disease, GERD in cirrhotic patients with EV accounted for about 20%, which mainly belongs to a dyskinetic type<sup>[20]</sup>. Previous studies found that esophageal varices played an important role



Table 2 Results of ambulatory 24-h esophageal pH monitoring in liver cirrhosis patients and controls (mean ± SD)

Group	Number of acid reflux episodes	Number of acid reflux episodes lasting ≥ 5 min	Mean time pH < 4 (%)		
			Total	Upright	Supine
Liver cirrhosis (n = 78)	61.17 ± 33.35 <sup>a</sup>	15.25 ± 5.73 <sup>a</sup>	10.34 ± 4.45 <sup>a</sup>	5.22 ± 2.71 <sup>a</sup>	9.56 ± 3.42 <sup>a</sup>
Child A (n = 28)	51.24 ± 20.54 <sup>a</sup>	10.66 ± 7.28 <sup>a</sup>	8.11 ± 2.32 <sup>a</sup>	4.48 ± 1.76 <sup>a</sup>	7.32 ± 5.44 <sup>a</sup>
Child B (n = 27)	60.35 ± 18.66 <sup>c</sup>	12.35 ± 9.83 <sup>c</sup>	10.51 ± 1.62 <sup>c</sup>	5.64 ± 1.31 <sup>c</sup>	9.14 ± 4.37 <sup>c</sup>
Child C (n = 23)	73.52 ± 28.63 <sup>e</sup>	17.34 ± 12.46 <sup>e</sup>	12.34 ± 2.15 <sup>e</sup>	6.79 ± 1.51 <sup>e</sup>	11.56 ± 5.43 <sup>e</sup>
Child D (n = 30)	39.62 ± 29.32	4.81 ± 2.04	2.35 ± 1.53	3.58 ± 1.34	8.69 ± 3.45

<sup>a</sup>Compared with control, *P* < 0.05; <sup>c</sup>Compared with Child A, *P* < 0.05; <sup>e</sup>Compared with Child B, *P* < 0.05.

Table 3 Results of ambulatory 24-h esophageal bilirubin monitoring in liver cirrhosis patients and controls (mean ± SD)

Group	Number of bile reflux episodes	Number of bile reflux episodes lasting ≥ 5 min	Mean time Abs > 0.14 (%)		
			Total	Upright	Supine
Liver cirrhosis (n = 78)	36.53 ± 9.31 <sup>a</sup>	4.09 ± 1.15 <sup>a</sup>	6.73 ± 1.15 <sup>a</sup>	3.32 ± 1.05 <sup>a</sup>	4.37 ± 1.44 <sup>a</sup>
Child A (n = 28)	27.32 ± 10.31 <sup>a</sup>	3.85 ± 1.34 <sup>a</sup>	5.12 ± 1.45 <sup>a</sup>	3.15 ± 0.92 <sup>a</sup>	4.12 ± 0.97 <sup>a</sup>
Child B (n = 27)	39.46 ± 18.31 <sup>c</sup>	4.11 ± 1.65 <sup>c</sup>	6.54 ± 1.21 <sup>c</sup>	3.37 ± 1.13 <sup>c</sup>	5.04 ± 1.11 <sup>c</sup>
Child C (n = 23)	48.54 ± 26.41 <sup>e</sup>	4.23 ± 2.14 <sup>e</sup>	7.32 ± 1.34 <sup>e</sup>	4.28 ± 1.22 <sup>e</sup>	5.52 ± 1.12 <sup>e</sup>
Control (n = 30)	12.76 ± 6.97	2.15 ± 1.36	1.98 ± 0.86	1.03 ± 0.23	0.83 ± 0.62

<sup>a</sup>Compared with control, *P* < 0.05; <sup>c</sup>Compared with Child A, *P* < 0.05; <sup>e</sup>Compared with Child B, *P* < 0.05. Abs: Absorbance.

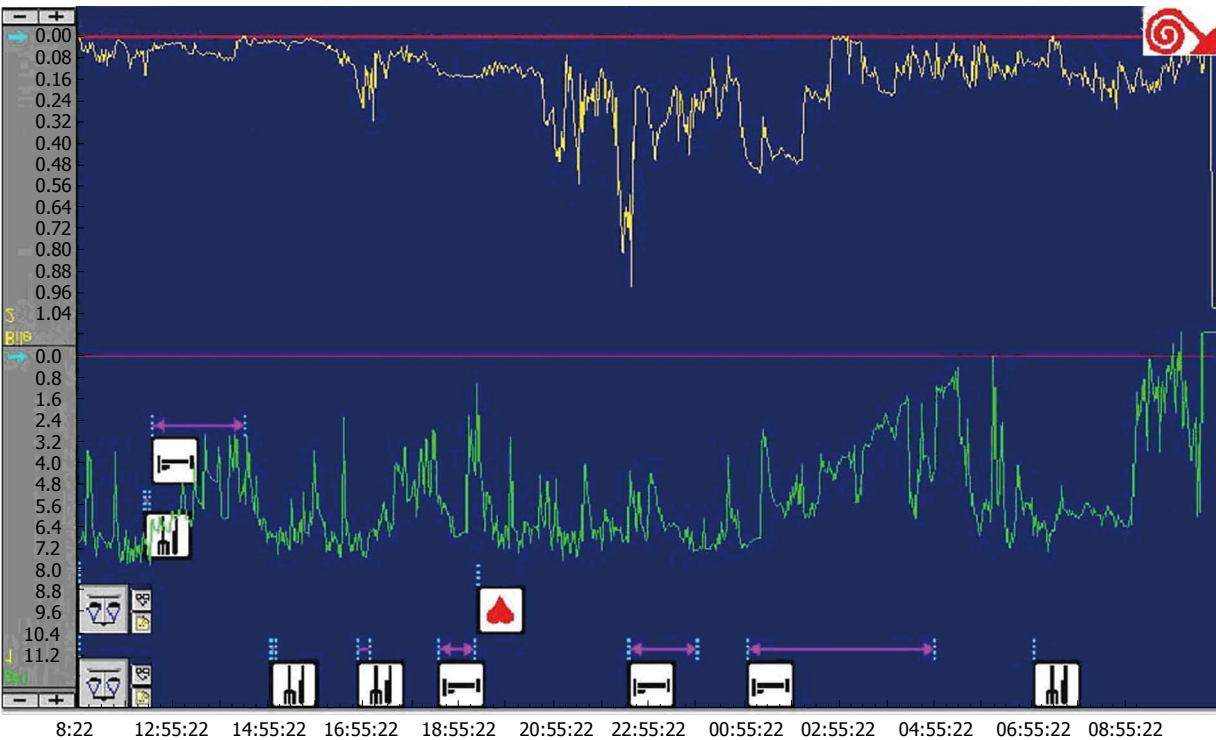


Figure 1 Mixed abnormal acid and bilirubin reflux curves in a typical Child C patient.

Table 4 Relationship between liver function classification of cirrhotic patients and gastroesophageal reflux disease n (%)

Group	RE	Abnormal reflux
Child A (n = 28)	8 (28.57)	12 (42.86)
Child B (n = 27)	11 (40.74)	15 (55.56)
Child C (n = 23)	10 (43.48)	16 (69.57)
Total (n = 78)	29 (37.18)	43 (55.13)

RE: Reflux esophagitis.

in the development of esophageal motor disorders and abnormal gastroesophageal reflux in these patients, who presented obvious esophageal motor and motility disorders<sup>[21,22]</sup>. The most prevalent disorder was the inefficient esophageal motility, along with abnormal PA, PD and PV<sup>[12,21-23]</sup>. Some studies found that motor disorders existed in the esophageal body in these cirrhotic patients with EV, as compared with the cirrhotic patients without varices and control group<sup>[24]</sup>. Thus, it seemed that EV itself, inde-

Table 5 Abnormal reflux in liver cirrhosis patients

Group	Isolated abnormal acid reflux	Isolated abnormal bile reflux	Mixed abnormal reflux	No abnormal reflux
Child A ( <i>n</i> = 28)	4	3	5	16
Child B ( <i>n</i> = 27)	3	4	8	12
Child C ( <i>n</i> = 23)	3	4	9	7
Total ( <i>n</i> = 78)	10 (12.82%)	11 (14.10%)	22 (28.21%)	35 (44.87%)

pendent of the cirrhosis, delayed esophageal clearance and increased the contact time between acid and mucosa.

In this study, LESP, PA, PD and PV in cirrhotic patients without esophageal varices were significantly abnormal as compared with those in the control group. LESP was markedly lower in patients with severe liver function damage, and negatively correlated with Child-Pugh score ( $P < 0.01$ ,  $r = -0.625$ ). The results showed that cirrhosis itself was another important factor for the esophageal motor disorder.

The incidence of esophageal acid reflux among cirrhotic patients with EV has also been studied in the last decades using pH-metry recording. It has been postulated that acid reflux may contribute to esophagitis and variceal bleeding in cirrhotic patients, and it occurs at a high frequency (64%) in patients with liver cirrhosis and portal hypertension, irrespective of the etiology of cirrhosis and the grade of esophageal varices<sup>[8]</sup>. The results indicated that there was a correlation between typical gastroesophageal reflux disease and abnormal reflux, but no relationship between ascites, variceal size, congestive gastropathy and Child-pugh score and abnormal reflux.

The high incidence of RE in patients with severe chronic liver disease was also demonstrated, and asymptomatic RE was more common in cirrhotic and liver failure patients<sup>[25,26]</sup>. In the present study, abnormal reflux and RE were demonstrated in 55.13% and 37.18% of the cirrhotic patients without esophageal varices, and the more severe liver function damage, the more abnormal parameters of acid and bilirubin reflux. In the mean time, typical symptoms of gastroesophageal reflux disease were presented in only 32.05% of the cirrhotic patients in this study, and abnormal reflux was found in 62% of the patients in the night possibly due to the lowered esophageal defenses during this period, with reduction of saliva production, swallowing and esophageal clearance.

GERD may occur in acid, bile or a mixed form, and DGER is considered as an independent risk factor for complicated GERD. However, few studies have reported the incidence of DGER among cirrhotic patients. Patients with Barrett's esophagus had significantly higher levels of DGER than patients with uncomplicated GERD, and bile reflux either alone or mixed with acid reflux contributed obviously to the severity of erosive and non-erosive reflux disease<sup>[6]</sup>. Moreover, DGER in acid medium was more injurious to the esophagus than DGER in alkaline pH<sup>[7]</sup>. We studied for the first time the incidence of BE and DGER in cirrhotic patients without esophageal varices. We found that the mixed acid and bile reflux was the predominant pattern of reflux in GERD patients, and the reflux incidence was also higher in Child B or C group than in Child

A group. A stepwise increase of mixed reflux was demonstrated along with the severity of liver function damage. Four BE patients (2 with mixed abnormal reflux, 2 with DGER) were found in Child C group.

The causes and the mechanism of liver cirrhosis in patients with abnormal GERD have not been fully elucidated. In this study, we demonstrated an obvious esophageal motility disorder and abnormal gastroesophageal reflux in cirrhotic patients without esophageal varices, and abnormalities of esophageal motility and reflux parameter were correlated with the severity of liver function damage. It seemed that not only mechanical effect (EV), but also neural and humoral factor are related to the high incidence of GERD in patients with liver cirrhosis. The progress of liver dysfunction decreased the incidence of LESP, worsened the esophageal motility and the reflux in the cirrhotic patients. In some studies, the levels of plasma vasoactive peptides and neurotensin were markedly higher in patients with liver cirrhosis than in the normal population, which were also known to lower the pressure of the LES, facilitating the reflux of the stomach content<sup>[21,27]</sup>.

The importance of nitrous oxide (NO) in the exacerbation of portal hypertension in liver cirrhosis was also reported<sup>[28,29]</sup>. This substance can be found in large amounts in the systemic circulation of cirrhotic patients, and NO concentration increased significantly in patients with liver disease, which was closely related to the transient LES relaxation, suggesting that NO played an important role in the process of GERD. Whether the excessive NO in cirrhotic patients could exacerbate these manifestations, needs to be further confirmed.

We found that gastric half-emptying of liquid food was delayed in patients with liver cirrhosis, and the function of gastric emptying was also influenced by the damaged liver function<sup>[30]</sup>. Ascites induced an increase in intra-abdominal pressure, compressing the stomach and the stomach content reflux<sup>[31]</sup>.

In summary, the majority of cirrhotic patients without EV presented esophageal motor disorders; mixed acid and bile reflux was the main pattern of reflux in GERD patients; and the presence of cirrhosis itself was an important causative factor for the onset of gastroesophageal reflux. Further researches on the functional and humoral factors and mechanism of GERD in liver diseases will gain a broad attention and interest in this field.

## COMMENTS

### Background

Gastroesophageal reflux disease (GERD) is one of the most common diseases

in modern civilization, and it has been reported that gastroesophageal reflux disease occurs at a high frequency in patients with liver cirrhosis.

### Research frontiers

The relationship between esophageal motor disorders and acid gastroesophageal reflux in cirrhotic patients with esophageal varices has been reported. This study was designed to evaluate the esophageal motility and abnormal acid and bile reflux incidence in cirrhotic patients without esophageal varices.

### Innovations and breakthroughs

This study showed that the presence of cirrhosis itself was an important causative factor for the onset of gastroesophageal reflux in patients with liver cirrhosis without varices. It is the first research on the incidence of Barrett's esophagus and DGER in cirrhotic patients without esophageal varices.

### Applications

This study helped better understand the mechanism of GERD in patients with liver cirrhosis, and contributed to the diagnosis and treatment of liver cirrhosis and its complications in clinical practice.

### Peer review

This is an interesting study on GERD in patients with liver cirrhosis, but without esophageal varices. Since it has before been thought that esophageal varices somehow have something to do with the increased frequency of GERD in patients with liver disease, the authors have made an interesting contribution to the literature by showing that reflux symptoms and pathologic esophageal motility changes are more common in patients with cirrhosis but without varices.

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## Association between polymorphism rs6983267 and gastric cancer risk in Chinese population

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### Abstract

**AIM:** To explore the association between single nucleotide polymorphisms (SNPs) at 8q24 and gastric cancer risk.

**METHODS:** A case-control investigation including 212 gastric cancer patients and 377 healthy controls was conducted. The genotypes of SNPs (rs6983267, rs7008482 and rs10808555) were examined and established through polymerase chain reaction-restriction

fragment length polymorphism (PCR-RFLP). Multivariate logistic regression models were used to evaluate the association between SNPs and gastric cancer.

**RESULTS:** The genotype frequencies of rs6983267 in gastric cancer patients were obviously different from those in the control ( $P = 0.005$ ). GT genotype of rs6983267 was associated with an increased risk of gastric cancer compared with GG genotype (adjusted odds ratio = 2.01, 95% confidence interval: 1.28-3.14). Further stratified analysis indicated that rs6983267 GT genotype facilitated the risk of gastric cancer of non-cardiac and intestinal type (OR: 2.638, 95% CI: 1.464-4.753; OR: 1.916, 95% CI: 1.166-3.150, respectively).

**CONCLUSION:** This study demonstrates for the first time that rs6983267 is involved in susceptibility to gastric cancer, although further large-sample investigations are still needed.

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**Key words:** Gastric cancer; Genetic susceptibility; Single nucleotide polymorphism; MYC; 8q24

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### INTRODUCTION

Gastric cancer is the second most common cause of death

from cancer in the world<sup>[1]</sup> and the incidence rate was 16.2 per 100 000<sup>[2]</sup>. Despite of a marked decrease in gastric cancer mortality rate in many countries, there is a higher prevalence of gastric cancer in the Chinese population than in other races. No doubt, either a high absolute number or a high mortality of gastric cancer has become a key public health issue in China.

Although numerous biological and epidemiological studies have shown risk factors for gastric cancer, the available knowledge is still insufficient to reveal the exact mechanism of gastric cancer. Current researches have shown that both genetic and environmental factors play an important role in gastric carcinogenesis<sup>[3,4]</sup> and genetic susceptibility accounts for 35% of disease etiology<sup>[5]</sup>. Recently, the association between variants at 8q24 and breast, prostate and colorectal cancers has been discovered and confirmed by several research groups<sup>[6-15]</sup>, which suggested a complex contribution of polymorphisms at 8q24 to the formation of multiple adenomas. However, whether these common variants in 8q24 are also associated with the risk of gastric cancer has so far not been published.

In the present study, we conducted a case-control association study to evaluate the effect of rs6983267, rs7008482 and rs10808555 in 8q24 in the risk of gastric cancer in the Chinese population.

## MATERIALS AND METHODS

### Subjects

A total number of 216 cases and 400 controls were enrolled from January 2009 to January 2010 in Tongji Hospital, Shanghai. Among the 1360 subjects who were invited to take part in this study, only 45% individuals agreed to participate and donated 3 mL venous blood sample. All the gastric cancer cases had been checked by the gastroscopy and diagnosed by the specialized physician. The exclusion criteria of cases included: (1) Having a history of any other cancers or any metastasized cancer (carcinomas were not originally from stomach); and (2) Having undergone radiotherapy or chemotherapy. Controls were randomly selected among the first-visit outpatients who were confirmed to have no cancer or a prior history of neoplasm. Available baseline characteristics, including age, gender, race, tumor location, histological type, were recorded. All the subjects were genetically unrelated ethnic Han Chinese. This study was approved by the institutional review board of Tongji University School of Medicine. Written informed consent was obtained from all participants.

### Genotyping

According to the manufacturer's protocol, we used Flexi Gene DNA Kit (Qiagen, Hilden, Germany) to extract genomic DNA from peripheral blood leukocytes of the subjects and stored extracted DNA at -20°C. Unique primer sequences were designed in the website of primer3 (<http://frodo.wi.mit.edu/primer3/input.htm>) and primer sequences for rs6983267, rs7008482 and rs10808555 were as follows: 5'-ATGAAGGCGTTCGTCCTCAAATGA-3'

(forward) and 5'-TTGGCTGGCACTGTCTGTATA-3' (reverse); 5'-CCAAGCAGAGAGGAACCAACT-3' (forward) and 5'-GCCACCCCTTTATTCTCCAACC-3' (reverse); 5'-ATATGGTCCCTGCCCTCAAG-3' (forward) and 5'-CACTGTGCTAAAGGAATCAGCAA-3' (reverse), respectively. Polymorphisms genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Amplification reactions were carried out in a total volume of 15 µL containing 0.3 mmol/L of each deoxynucleoside triphosphate, 10 mmol/L Tris-HCl, 50 mmol/L KCl, 2 mmol/L MgCl<sub>2</sub>, 20% Q solution (Qiagen, Hilden, Germany), 0.16 µmol/L of each primer, 10 ng genomic DNA, and 1 U Taq (TaKaRa, Otsu, Shiga, Japan). Cycling conditions were: 94°C for 3 min, followed by 10 cycles of 94°C for 30 s, 64°C for 30 s with a 0.5°C decrement of the annealing temperature per cycle and 72°C for 30-45 s, followed by 30 cycles of 94°C for 30 s, 59°C for 30 s and 72°C for 30 s, followed by 72°C for 8 min. PCR products were digested overnight at 37°C with a predicted restriction enzyme, Ts-p45I (Fermentas, Vilnius, Lithuania) for rs6983267, CviQI (Fermentas, Vilnius, Lithuania) for rs7008482, Eco130I (Fermentas, Vilnius, Lithuania) for rs10808555 and were analyzed on 3% agarose with ethidium bromide staining. Three sorts of PCR products were digested into 3 different types of fragments. For rs6983267, the G allele resulted in two fragments of 198-bp and 344-bp, and the C allele produced one fragment of 498-bp. For rs7008482, the G allele resulted in two fragments of 270-bp and 131-bp, while the T allele produced one fragment of 401-bp. For rs10808555, the G allele digested into two fragments of 193-bp and 110-bp, and the A allele generated one fragment of 303-bp.

All the samples were assayed blindly without knowing the case or control status. After genotyping was performed, two research assistants read the gel pictures independently. When they failed to reach a consensus on the tested genotypes (< 1%), they would repeat the genotyping again so as to achieve a final consensus. To ensure the genotyping accuracy, randomly selected PCR products were reevaluated by DNA sequencing<sup>[16]</sup>. In addition, 5% of all samples were randomly selected and genotyped in duplicate, and the results were 100% concordant.

### Statistical analysis

Hardy-Weinberg equilibrium was tested using the two-sided  $\chi^2$  test.  $\chi^2$  test was used to compare genotype frequency and demographic distributions between cases and controls. Multivariate unconditional logistic regression was used to calculate odds ratios (OR) and 95% confidence intervals (CIs) for the association between genotypes and gastric cancer, adjusting for age and gender. The co-dominant and the dominant models were used for the analysis. In the co-dominant model, each SNP was separated into three categories, 1 for each genotype, with one genotype chosen as the reference group. For the dominant model, each SNP was modeled as a dichotomous variable with

Table 1 Characteristics of cases and control

Variables	Gastric cancer <i>n</i> (%)	Control <i>n</i> (%)	<i>P</i> value <sup>2</sup>
Overall	212	377	
Sex			0.063
Male	152 (71.7)	242 (64.2)	
Female	60 (28.3)	135 (35.8)	
Age			0.670
Mean ± SD (yr)	62.47 ± 11.6	62.89 ± 11.3	
Histological types			
Intestinal	155 (73.1)		
Diffuse	44 (20.8)		
Mixed	13 (6.1)		
Tumor location <sup>1</sup>			
Cardia	74 (38.5)		
Noncardia	118 (61.5)		

<sup>1</sup>The number of subjects in cases for tumor location (*n* = 192) was less than the total number (*n* = 212) because some information was not obtained;

<sup>2</sup>Two-sided  $\chi^2$  test for the frequency distribution of variants between gastric cancer cases and controls.

1 genotype chosen as the reference group, and the other two genotypes combined into one category. All tests were two-sided and *P* values < 0.05 were considered statistically significant. All statistical analyses were performed using SPSS 16.0 software package (SPSS, Chicago, IL)

## RESULTS

### Demography

Among 216 gastric cancer cases and 400 controls, 4 cases and 23 controls were dropped out due to poor-quality genomic DNA. Of the 212 cases of gastric cancer, 155 were intestinal type, 44 diffuse type and 13 mixed-type. The mean age of cases was 62 years and the mean age of controls was 63 years. The characteristics of the cases and controls are summarized in Table 1. There was no significant difference between the groups with respect to the age and gender distributions.

### SNPs and gastric cancer risk

Among the controls, the genotype distributions of rs6983267 and rs7008482 were in Hardy-Weinberg equilibrium (*P* > 0.1 and *P* > 0.9, respectively), but rs10808555 did not fit Hardy-Weinberg equilibrium (*P* < 0.05). The genotype frequencies of rs6983267 were obviously different between gastric cancer patients and control ( $\chi^2 = 10.8$ , *P* = 0.005). Analysis under both co-dominant model and dominant model showed that only rs6983267 was significantly associated with gastric cancer risk, after adjustment for age and gender (Table 2). In the co-dominant model, rs6983267 GT genotype was associated with approximately 2 times higher odds of gastric cancer risk (OR: 2.01, 95% CI: 1.28-3.15) compared with the GG genotype. In the dominant model, combined genotypes (GT + TT) of rs6983267 were significantly associated with increased risk of gastric cancer in comparison with GG genotype (OR: 1.82, 95% CI: 1.18-2.81). However, the genotype frequencies of rs7008482 were similar between gastric cancer

Table 2 Association between variation in single nucleotide polymorphisms rs6983267 and rs7008482 and risk of gastric cancer

Genotype	Control <i>n</i> (%)	Cases <i>n</i> (%)	<i>P</i> value
rs6983267			
GT/TT	268 (72.8)	166 (83.0)	0.007
TT	72 (19.6)	32 (16.0)	0.369
GT	196 (53.3)	134 (67.0)	0.002
GG	100 (27.2)	34 (17.0)	
rs7008482			
GT/GG	224 (61.0)	142 (67.0)	0.138
GG	52 (14.2)	38 (17.9)	0.108
GT	172 (46.9)	104 (49.1)	0.250
TT	143 (39.0)	70 (33.0)	

patients and controls (*P* > 0.05).

Furthermore, we evaluated the contributions of SNPs to subgroups according to age, gender, different histological types and tumor locations (Table 3). In the subgroup aged ≤ 60 years, rs6983267 GT genotype markedly increased the risk of gastric cancer referring to GG genotype (OR: 3.21, 95% CI: 1.52-6.68), but in the subgroup aged > 60 years, no significant difference was found (*P* = 0.137). In addition, rs6983267 GT genotype was significantly associated with augmentation of gastric cancer risk in both male and female. As to the histological types and tumor sites, rs6983267 GT heterozygote had a significantly increased risk for non-cardiac gastric cancer (OR: 2.64, 95% CI: 1.46-4.75) and intestinal-type gastric cancer (OR: 1.92, 95% CI: 1.17-3.15) in contrast with GG genotype. Further analysis in Table 4 demonstrated that rs6983267 GT genotype increased the risk of an intestinal-type gastric adenocarcinoma from non-cardiac region. For rs7008482, only GG genotype was associated with significantly increasing risk of gastric cancer compared with TT genotype in male subgroup (OR: 1.88, 95% CI: 1.01-3.47) (Table 3).

## DISCUSSION

This is the first study to discover the association between rs6983267 at 8q24 and the susceptibility of gastric cancer, although other previous studies had reported that rs6983267 was associated with the risk of colorectal cancer and prostate cancer<sup>[13,17]</sup>. Our observation and analysis indicated that compared with GG genotype of rs6983267, GT genotype and combined genotypes (GT + TT) were both markedly associated with the increasing risk for gastric cancer. And further stratified investigation confirmed that rs6983267 GT genotype facilitated the risk of non-cardiac and intestinal-type gastric cancer. Therefore, rs6983267 is a novel gastric cancer associated polymorphism in 8q24 in Chinese Han population.

Rs6983267 resides at 8q24, proximal to a processed pseudogene, *POU5F1P1*, which is a retrotransposed copy of the POU-domain transcription factor Oct4<sup>[18]</sup>. At least one mouse *Oct4* pseudogene has been shown to mediate stem cell regulatory function<sup>[19]</sup>, suggesting that *Oct4*



Table 3 Association between rs6983267 and rs7008482 polymorphism and clinicopathological features of gastric cancer

	rs6983267							rs7008482						
	GG		GT		TT			TT		GT		GG		
	HC/GC	HC/GC	OR (95% CI)	P value	HC/GC	OR (95% CI)		HC/GC	HC/GC	OR (95% CI)	P value	HC/GC	OR (95% CI)	P value
Age (yr)														
≤ 60	46/11	76/59	3.21 (1.52-6.77)	0.002	29/13	1.86 (0.73-4.72)	0.191	66/30	71/46	1.42 (0.80-2.51)	0.230	15/11	1.61 (0.66-3.92)	0.295
> 60	54/23	120/75	1.54 (0.87-2.74)	0.137	43/19	1.05 (0.50-2.16)	0.901	77/40	101/58	1.15 (0.69-1.90)	0.592	37/27	1.44 (0.77-2.70)	0.258
Sex														
Male	60/26	125/94	1.77 (1.04-3.02)	0.036	48/23	1.10 (0.56-2.17)	0.783	98/46	107/77	1.52 (0.96-2.41)	0.072	32/29	1.88 (1.01-3.47)	0.045
Female	40/8	71/40	3.68 (1.49-9.06)	0.005	24/9	2.22 (0.73-6.74)	0.160	45/24	65/27	0.83 (0.42-1.65)	0.599	20/9	0.98 (0.38-2.54)	0.967
Histological types														
Intestinal	100/26	196/98	1.92 (1.17-3.15)	0.010	72/23	1.20 (0.63-2.28)	0.571	143/52	172/73	1.17 (0.77-1.78)	0.047	52/30	1.56 (0.89-2.72)	0.118
Diffuse	100/7	196/25	1.93 (0.80-4.66)	0.144	72/8	1.74 (0.60-5.09)	0.309	143/15	172/23	1.36 (0.68-2.71)	0.390	52/6	1.26 (0.46-3.47)	0.651
Mixed	100/1	196/11	5.56 (0.71-44.52)	0.103	72/1	1.37 (0.08-21.06)	0.827	143/3	172/8	2.29 (0.63-9.43)	0.228	52/2	1.95 (0.34-13.47)	0.474
Location														
Cardia	100/16	196/40	1.26 (0.67-2.38)	0.469	72/14	1.18 (0.54-2.58)	0.681	143/25	172/35	1.18 (0.67-2.07)	0.574	52/14	1.52 (0.73-3.18)	0.263
Noncardia	100/16	196/82	2.64 (1.46-4.75)	0.001	72/12	1.06 (0.47-2.38)	0.888	14/39	172/59	1.28 (0.81-2.04)	0.295	52/20	1.47 (0.78-2.77)	0.228

Multivariate unconditional logistic regression was used to calculate odds ratios (OR) and 95% confidence intervals (CIs) for the association between genotypes and gastric cancer, adjusting for age and gender. HC: Health control; GC: Gastric cancer.

Table 4 Stratified analysis of rs6983267 genotypes and gastric cancer

	Control n (%)	Intestinal case		Diffuse case	
		n (%)	OR (95% CI)	n (%)	OR (95% CI)
Cardia					
TT	72 (19.6)	12 (22.2)	1.243 (0.53-2.90)	1 (8.3)	0.44 (0.04-4.31)
GT	196 (53.3)	29 (53.7)	1.131 (0.56-2.28)	8 (66.7)	1.36 (0.35-5.28)
GG	100 (27.1)	13 (24.1)		3 (25)	
Noncardia					
TT	72 (19.6)	8 (9.6)	0.99 (0.38-2.59)	4 (16.7)	1.91 (0.44-8.25)
GT	196 (53.3)	64 (77.1)	2.95 (1.49-5.86)	16 (66.6)	2.56 (0.80-8.17)
GG	100 (27.1)	11 (13.3)		4 (16.7)	

pseudogene may exert influence in regulating stem cell proliferation<sup>[7]</sup>. *Oci4* also plays a critical role in maintaining stem cell pluripotency<sup>[20]</sup>, self-renewal, and lineage commitment<sup>[21]</sup>. *Oci4* has been found to promote tumor growth in a dose-dependent manner<sup>[22]</sup> and epithelial dysplasia by interfering with progenitor cell differentiation<sup>[23]</sup>. Although the expression of many *Oci4* pseudogenes in poorly differentiated tumors<sup>[24]</sup> has been observed, the related molecular mechanism in cancer is unknown.

On the other hand, rs6983267 is located in the region which is 335 kb away from the nearest gene, *MYC*. *MYC* is able to increase the growth and proliferation of normal gastric cells<sup>[25]</sup>, and may enhance the canceration of gastric epithelial cells by regulating a variety of genes related to proliferation, differentiation<sup>[26]</sup>, and apoptosis<sup>[27]</sup>.

*MYC* overexpression has been described in over 40% of gastric cancer (in both intestinal- and diffuse-type gastric adenocarcinoma)<sup>[28]</sup>. Overexpression of *MYC* gene can influence some biological characteristics of normal gastric cells, directly regulate the genes involved in cell cycle regulation<sup>[29]</sup>, such as *cyclin A*, *cyclin B* and *cdk4*<sup>[30]</sup>, and accelerate cancerous growth ultimately. The promotion of the growth and proliferation of these cells helps tumor cells maintain malignant phenotype. Moreover, the therapeutic medicine inhibits gastric cancer cell growth by suppressing *MYC* gene expressions, which consistently confirms the crucial function of *MYC* in gastric cancer cell growth<sup>[31]</sup>. The region harboring rs6983267 is a transcriptional enhancer and differentially binds transcription factor 7-like 2 (TCF7L2) due to rs6983267, leading to a different physi-

cal interaction with *MYC*<sup>[32]</sup>. Given that the cancer risk-associated SNP enhances the expression of *MYC* through increased distal enhancer activity<sup>[32,33]</sup>, it is reasonable to speculate that rs6983267 may alter expression of *MYC* through modifying regulatory sequences in this region. Despite the research progress, further studies are needed about the concrete molecular mechanisms of the joint effect between *MYC* and rs6983267 polymorphism.

Previous studies have demonstrated that rs6983267 is possibly related to some kinds of malignant tumor. In the present study we found that rs6983267 is a novel gastric cancer related polymorphism. Stratified analysis indicated that the associations between rs6983267 GT genotype and gastric cancer tended to vary with tumor sites and histological types. Rs6983267 GT genotype was associated with both intestinal and non-cardiac type of gastric cancer but not associated with the diffuse and cardiac type, and increased the risk of intestinal type among the non-cardiac gastric cancer, which suggested that rs6983267 GT genotype is more important in modulating the intestinal and non-cardiac type of gastric cancer. However, TT genotype in rs6983267 tended to be a protective factor in intestinal type among the non-cardiac gastric cancer although this was not significant in the association analysis. This phenomenon could be explained, because distinct clinical, epidemiological and molecular features have been noted among tumors arising from cardia or non-cardia, and among intestinal or diffuse histological subtypes<sup>[34]</sup>. For instance, the loss of p16 and smad4 protein expression and the positive *EBstein-Barr virus* (EBV) status are more frequent in cardiac carcinomas than that in non-cardiac carcinomas reported by Kim *et al*<sup>[35]</sup>. Lu *et al*<sup>[36]</sup> reported that intestinal-type gastric cancer predominates in high-risk geographic areas, especially in Japan, Korea and China, whereas the diffuse-type gastric cancer has a uniform geographic distribution. The observed differences between gastric cancers in tumor location and histological types suggest that they are distinct diseases with different etiologies<sup>[37]</sup>. Thus, various genetic factors, including rs6983267, may be involved in different subtypes of gastric cancer (cardiac or non-cardiac; intestinal or diffuse). Another plausible explanation for this situation may be the genetic heterogeneity which may limit the ability to detect an association between TT genotype and gastric cancer. Other variants, which have a strong association with risk of gastric cancer, including as yet undiscovered susceptibility genes, may affect the outcome of this research. Moreover, the limited sample size may be not sufficient to generate this association. Overall, the genetic susceptibility and environmental factors have been proposed to play an important role in the etiology of gastric cancer, and different subtypes of gastric cancer may have diverse biological mechanisms.

Apart from the discovery in rs6983267, this study failed to demonstrate the association between rs7008482 and the risk of gastric cancer, although rs7008482 was reported to be associated with prostate and colorectal cancer<sup>[10,11]</sup>. Nevertheless, positive association between rs7008482 GG genotype and risk of gastric cancer in male subgroup has been

shown. Rs7008482 lies within an intronic region of the *NSMCE2* (also called *MMS21*) gene, and MMS21 protein is a SUMO ligase which is required for DNA replication, recombination and repair<sup>[11]</sup>. Considering the function of *NSMCE2* gene and MMS21 protein, we wonder whether the limited sample size hampered the detection of association between rs7008482 and gastric cancer risk. Therefore, further studies are still needed to confirm it.

In conclusion, our data demonstrated for the first time that rs6983267 may predispose to the susceptibility of gastric cancer, especially the intestinal and non-cardiac type. However, as the sample size of the present study is relatively small, additional tests of variant at 8q24 for its association with gastric cancer in a larger population, and functional studies of *MYC* and other nearby genes will be required to fully understand the mechanisms of the cancer-specific risk at 8q24.

## COMMENTS

### Background

Gastric cancer (GC) is one of the most common cancers, and the second most frequent cause of cancer-related deaths in the world. Epidemiological studies have shown that genetic factors play a crucial role in gastric carcinogenesis. Recently, common polymorphisms located at chromosome 8q24 have been identified to increase the tumor risk. The authors investigated the associations between rs6983267 polymorphisms and GC risk.

### Research frontiers

Chromosome 8q24 is an established risk locus for many common epithelial cancers. Polymorphism rs6983267 is a susceptibility marker for prostate and colon cancers, and perhaps also ovarian and other cancers. The relationship between rs6983267 polymorphism and GC needs to be addressed.

### Innovations and breakthroughs

To our knowledge, this is the first study of GC risk variant at 8q24 in a Chinese population. Polymorphism rs6983267 was found to be associated with an increased risk of GC, which had been not reported before. The result of stratified analysis according to histological types confirms the contribution of rs6983267 in non-cardiac and intestinal type of gastric carcinogenesis.

### Applications

These findings might be of value in the explanation of gastric carcinogenesis. They could be used for further investigations about the association between genetic predisposition and the risk of GC at 8q24.

### Terminology

*MYC* is an oncogene, the protein encoded by this gene is a multifunctional, nuclear phosphoprotein that plays a role in cell cycle progression, apoptosis and proliferations. Single nucleotide polymorphism is a DNA sequence variation occurring when a single nucleotide-A, T, C, or G-in the genome (or other shared sequence) differs between members of a species or paired chromosomes in an individual.

### Peer review

The authors investigated the association of the 3 single nucleotide polymorphisms (SNPs) (rs6983267, rs7008482 and rs10808555) with the risk of gastric cancer by a case-control study, and found that GT genotype of rs6983267 was associated with an increased risk of gastric cancer compared with GG genotype (AOR = 2.01, 95% CI: 1.28-3.14). After stratification, rs6983267 GT genotype was associated the risk of non-cardiac and intestinal type of gastric cancer. This study provides some new SNP for the evaluation of gastric cancer risk.

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## EUS for choosing best endoscopic treatment of mesenchymal tumors of upper gastrointestinal tract

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were treated by endoloop, 10 stromal tumors were treated by endoscopic mucosal resection and 8 stromal tumors were treated by endoscopic submucosal dissection. Complete resection of the lesion was achieved in all cases. Of the mesenchymal tumors, 90.38% diagnosed by EUS were also identified by pathohistology. All wounds were closed up nicely and no recurrence was found in the follow-up after 2 mo.

**CONCLUSION:** EUS is an effective means of diagnosis for upper GIMTs and is an important tool in choosing the endoscopic therapy for GIMTs, by which the lesions can be treated safely and effectively.

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**Key words:** Leiomyoma; Stromal tumor; Endoscopic ultrasonography; Endoscopic therapy

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### Abstract

**AIM:** To evaluate the value of endoscopic ultrasonography (EUS) in the choice of endoscopic therapy strategies for mesenchymal tumors of the upper gastrointestinal tract.

**METHODS:** From July 2004 to September 2010, 1050 patients with upper gastrointestinal mesenchymal tumors (GIMTs) were diagnosed using EUS. Among them, 201 patients underwent different endoscopic therapies based on the deriving layers, growth patterns and lesion sizes.

**RESULTS:** Using EUS, we found 543 leiomyomas and 507 stromal tumors. One hundred and thirty-three leiomyomas and 24 stromal tumors were treated by snare electrosection, 6 leiomyomas and 20 stromal tumors

### INTRODUCTION

Gastrointestinal mesenchymal tumors (GIMTs) originate from mesenchymal cells other than epithelial cells or lymphocytes. They are further classified as stromal tumors, leiomyomas, leiomyosarcomas, neural tumors, fibroblast tumors or liparomphalus. Clinically, mesenchymal tumors are usually incidentally discovered as subepithelial bulges during routine endoscopic examinations for unrelated conditions. The classification and management of these

lesions can be challenging. In recent years, with the wide use of endoscopic ultrasound (EUS) to clarify the nature and origin of the subepithelial tumor, great progress has been made in diagnosis and treatment of GIMTs<sup>[1,2]</sup>. Importantly, under the guidance of the EUS, GIMTs can be removed by appropriate endoscopic treatment without severe complications<sup>[2-4]</sup>.

From July 2004 to September 2010, we analyzed 1050 patients with GIMTs diagnosed by EUS in our hospital. Of these patients, 201 underwent different endoscopic therapies based on the EUS results. Our aim in this retrospective study was to evaluate the value of EUS in the choice of endoscopic therapy strategies for mesenchymal tumors of the upper gastrointestinal tract.

## MATERIALS AND METHODS

### Patients

The medical records of 1050 patients with upper GIMTs diagnosed by EUS examination in the First Affiliated Hospital of Zhejiang University were retrospectively reviewed. All these patients with submucosal protruding lesions in the upper gastrointestinal tract by routine endoscopy were examined by EUS. There were 499 men and 551 women, with a mean age of 52.6 years (range, 19-86 years). Of these patients, 201 patients underwent endoscopic therapy in the First Affiliated Hospital of Zhejiang University, Beilun Zongrui Hospital, the Traditional Chinese Medical Hospital of Ninghai and Jinhua Wenrong Hospital, respectively.

### Methods

A two-channel endoscope (GIF-2T240, Olympus, Tokyo, Japan) and a 12 MHz probe (GF-UM 2R, Olympus, Tokyo, Japan) were used for the ultrasonographic study. Scanning of the tumor was performed after filling the upper gastrointestinal tract with 100-500 mL of deaerated water. Diagnosis was made according to the layer of origin, size, nature, internal echo pattern, outer margin and grow pattern of the lesion. Following the EUS procedure, if the lesion was identified as an intramural lesion  $\leq 2.5$  cm, endoscopic treatment was performed. A lesion  $> 2.5$  cm in size and suspected to be malignant was suggested for surgery. A large proportion of patients were followed up with EUS, because their poor conditions were unsuited for the therapy or the lesion was too small.

An Olympus GIF-XQ240/260 gastroscope (Tokyo, Japan) was used for the resection when it was indicated. Informed consent was given by each patient before the endoscopic therapy. Four different resection techniques were used: snare electrosection, endoloop, endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD).

EMR procedure: Epinephrine (0.001%) was injected into the submucosal layer to lift the lesion, and then a conventional electrosurgical snare (FD-IU, Olympus, Tokyo, Japan) and an electrosurgical unit (VIO 200D,

ERBE, Tübingen, Germany) were used for removal of the overlying mucosa and resection of the tumor.

ESD procedure: the surrounding area of the lesion was marked with argon plasma coagulation (APC 300A, ERBE, Tübingen, Germany). Normal saline solution with 0.002% indigo carmine and 0.001% epinephrine was injected into the submucosal layer to lift the lesion. An initial incision was made outside the marking dots with a hook-knife (KD-620LR, Olympus, Tokyo, Japan). The submucosal resection under the lesion was done with insulation-tipped (IT) electrosurgical knife (KD-610L, Olympus, Tokyo, Japan). Finally, a snare was used to remove the surrounding tissues. Bleeding and visible vessels in the resection area were closed using hemoclips (HX-201YR-135, Olympus, Tokyo, Japan).

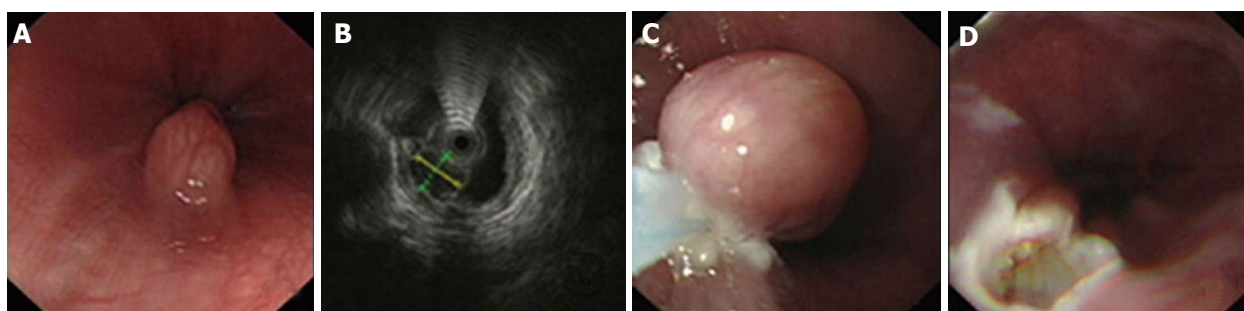
Postoperative EUS examination was made to check whether the lesions were completely removed except those with endoloop ligation. The specimens were sent for pathologic study, some of which were assayed by immunohistochemistry. All 201 patients were examined two months later with EUS.

## RESULTS

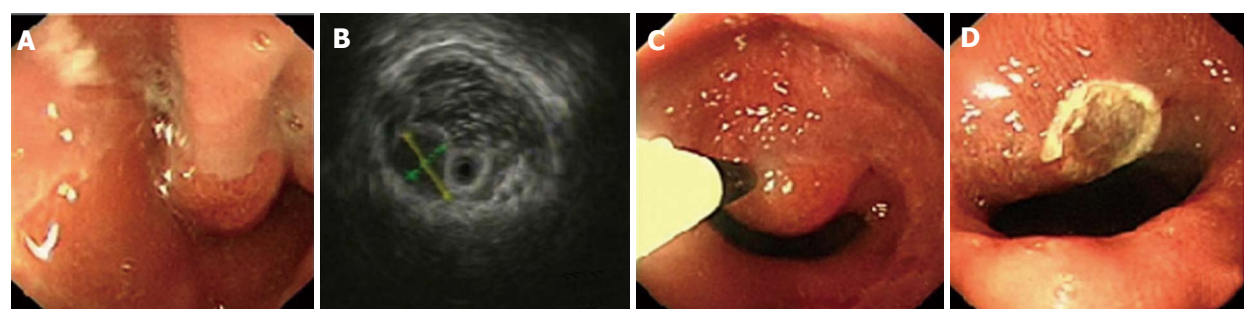
Using EUS, we identified 1050 patients with GIMTs, 543 of them had leiomyomas. Five hundred and twenty lesions were in the esophagus, 22 in the stomach, and 1 in the duodenum. The mean maximal tumor diameter was 3.3 cm. Five hundred and seven cases were stromal tumors. Forty-nine lesions were in the esophagus, 428 were in the stomach, and 30 were in the duodenum. The mean maximal tumor diameter was 6.6 cm. EUS features of leiomyomas and stromal tumors were characteristic with regular borders, a hypoechoic mass with homogeneous or heterogeneous echo patterns (Figures 1-4). The echogenicity of leiomyomas was slightly lower than the normal proper muscle layer, while that of stromal tumors was slightly higher. Malignant stromal tumors often appeared as a heterogeneous mass with irregular borders.

One hundred and thirty-nine leiomyomas and 62 stromal tumors underwent endoscopic therapy after EUS examination. No obvious malignant signs were seen in these lesions. The location, origin level and removal methods of the lesions are shown in Table 1. The mean maximal tumor diameter was 2.5 cm. For a tumor protruding into the cavity, if it originated from the muscularis mucosa or from submucosa  $\leq 1$  cm, snare electrosection was directly used. If the lesion originating from submucosa was flat and  $> 1$  cm, electrosection would be expected to fail, and other treatments, such as endoloop, EMR or ESD, were used. For a lesion originating from muscularis propria but not growing outward, endoloop or ESD was used. Among these patients, 133 leiomyomas and 24 stromal tumors were treated by snare electrosection, 6 leiomyomas and 20 stromal tumors were treated by endoloop, 10 stromal tumors were treated by EMR and 8 stromal tumors were treated by ESD (Figures 1-4). Complete resection of

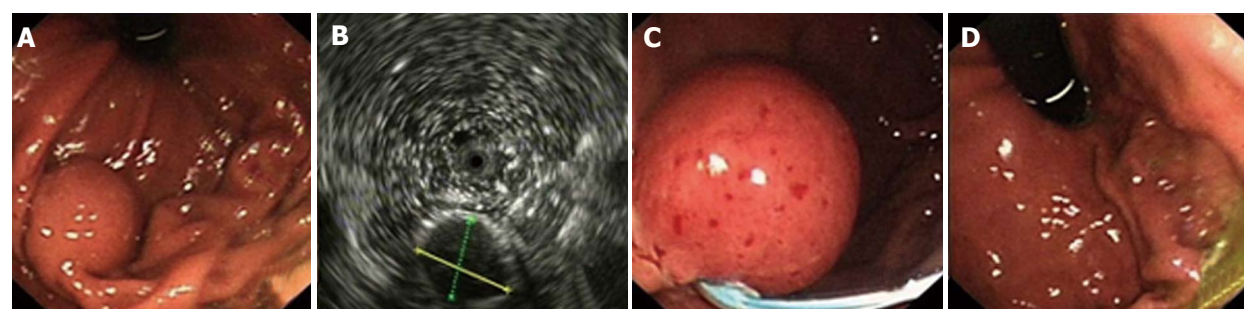




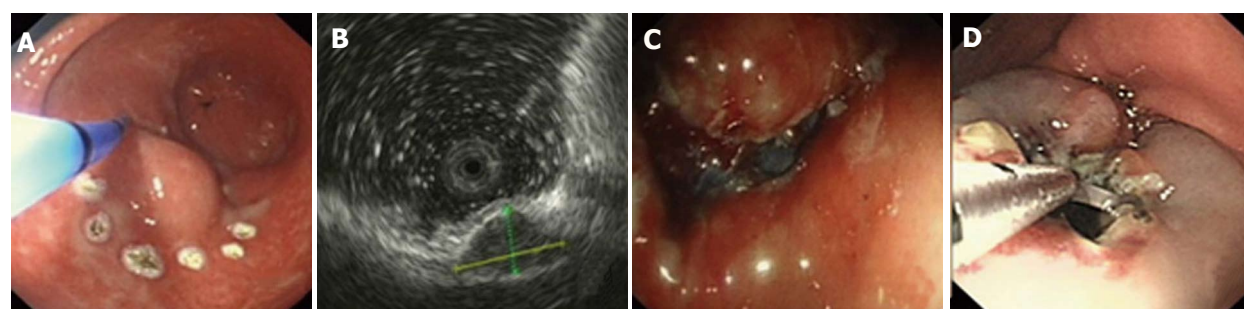
**Figure 1** An esophageal leiomyoma treated by snare electrosection. A: An elevated lesion in the lower esophagus; B: A homogeneous, hypoechoic mass ( $1.0 \times 0.7 \text{ cm}^2$ ) with a regular border originated from muscularis mucosa, which was diagnosed as a leiomyoma by endoscopic ultrasonography; C: The tumor was snared at the base, and then it was resected by snare electrosection; D: Postoperative wounds.



**Figure 2** A gastric stromal tumor treated by endoscopic mucosal resection. A: An elevated lesion in the cardia; B: A homogeneous, hypoechoic mass ( $1.4 \times 0.8 \text{ cm}^2$ ) with a regular border originating from submucosa, which was diagnosed as a stromal tumor by endoscopic ultrasonography; C: Epinephrine (0.001%) was injected into the submucosa to lift the lesion; D: Postoperative wounds.



**Figure 3** A gastric stromal tumor treated by endoloop. A: An elevated lesion in gastric fundus; B: A homogeneous, hypoechoic mass ( $2.0 \times 1.5 \text{ cm}^2$ ) with a regular border originating from muscularis propria, which was diagnosed as a stromal tumor by endoscopic ultrasonography; C: Endoscopic ligation with an endoloop; D: Endoscopic view of an ulcer scar without tumor recurrence at the ligation site 2 mo later.



**Figure 4** A gastric stromal tumor treated by endoscopic submucosal dissection. A: An elevated lesion in the gastric antrum; B: A homogeneous, hypoechoic mass ( $2.0 \times 1.2 \text{ cm}^2$ ) with a regular border originating from muscularis propria, which was diagnosed as a stromal tumor by endoscopic ultrasonography; C: The surrounding area of the lesion was marked with argon plasma coagulation. After normal saline solution with 0.002% indigo carmine and 0.001% epinephrine was injected into the submucosal layer to lift the lesion, an initial incision was made outside the marking dots with hook-knife. Submucosal dissection under the lesion was performed with an IT knife; D: The tumor was dissected and the postoperative wounds were closed using hemoclip.

Table 1 Location, origin and treatment of 201 gastrointestinal mesenchymal tumors

Diagnosis by EUS	Location		Layer of origin			Treatment			
	Esophagus	Stomach	Muscularis mucosa	Submucosa	Muscularis propria	Snare electrosection	Endoloop	EMR	ESD
Leiomyoma	134	5	121	15	3	133	6	0	0
Stromal tumor	22	40	18	19	25	24	20	10	8
Total	156	45	139	34	28	157	26	10	8

EUS: Endoscopic ultrasonography; EMR: Endoscopic mucosal resection; ESD: Endoscopic submucosal dissection.

the lesion was achieved in all cases. No residual lesion was detected by postoperative EUS examination except for those by endoloop ligation. None of the patients suffered from severe hemorrhage or resection-related perforation. Postoperative histological results showed that 141 of 156 patients were in agreement with the preoperative diagnosis of EUS. All the specimens tested had complete envelope and negative resection margin in pathology. Wounds were closed up nicely in all patients when rechecked after two months. No residual lesion was detected by EUS examination and pathology demonstrated negative results at the same time.

## DISCUSSION

Leiomyomas and stromal tumors are the most common GIMTs of the upper gastrointestinal tract. Many lesions are subepithelial, and they are often difficult to diagnose by general endoscopy. Some also need to be identified with extrinsic compression. EUS can reliably characterize the nature, size, and layer of origin of lesions, and accurately differentiate intramural from extramural, leading to a diagnosis<sup>[5]</sup>. Features of leiomyomas and stromal tumors seen with EUS often include: a round shape, and a homogeneous, hypoechoic mass with regular borders<sup>[6]</sup>. A marginal halo, hyperechoic spots and higher echogenicity as compared with the normal muscle layer is seen more frequently in stromal tumors than in the leiomyomas<sup>[7]</sup>. Malignant stromal tumors are characterized by large size (> 5 cm), irregular borders, and echogenic foci<sup>[8,9]</sup>.

In this study, we identified 1050 patients with GIMTs using EUS. There were 543 leiomyomas and 507 stromal tumors. The majority of leiomyomas were located in the esophagus while most stromal tumors were located in the stomach, which is in accordance with other studies<sup>[6-10]</sup>. For these mesenchymal tumors, 90.38% diagnosed by EUS were also identified by pathohistology. Among these, 5 retention cysts, 4 stromal tumors with leiomyoma differentiation, and 1 hyperplastic polyp were diagnosed as leiomyoma, and 3 leiomyoma and 2 hyperplastic polyps were diagnosed as stromal tumors by EUS. Submucosal retention cysts are small and often filled with thick fluid, and thus the ultrasonographic image is of a hypoechoic mass that may be confused with mesenchymal tumors. Stromal tumors with leiomyoma differentiation are also difficult to discriminate by routine pathology and should be identified by immuno-

histochemistry. Samples from EUS-guided fine-needle aspiration biopsies can be sent for cytological, pathological and immunohistochemical assays which may enable clinicians to make more accurate diagnoses than using EUS examination alone<sup>[6-11]</sup>.

In the past, conventional endoscopy could not accurately determine the location and categorization of subepithelial lesions. Therefore, GIMTs were usually treated by surgery. The introduction of EUS has solved these problems and it has played an important role in the choice of endoscopic therapy for mesenchymal tumors. Based on EUS images, we treated 201 GIMTs with different endoscopic therapies, including snare electrosection, endoloop, EMR and ESD. Complete resection of the lesions was achieved in all cases. None of the patients suffered from severe hemorrhage or resection-related perforation. All wounds were closed up nicely and no recurrence was found in the follow-up after 2 mo.

Electrosection is the most common endoscopic treatment, and its value for the treatment of gastrointestinal submucosal tumors has been recognized<sup>[12,13]</sup>. It is mainly used for protuberant lesions (especially the pedunculated ones). In this study, 157 GIMTs arising from non-muscularis propria, with a diameter of  $\leq 1$  cm, were treated by snare electrosection after EUS examination. It is reported that serious complications rarely occurred when electrosection is used to cut non-muscularis propria tumors with a diameter  $\leq 3$  cm<sup>[3]</sup>. Tumors originating from muscularis propria are associated with an increased risk of perforation and hemorrhage complications during endoscopic treatment, and snare electrosection was not used in these cases.

Compared with ordinary snare removal, EMR is more suitable for the treatment of flat lesions generally confined to  $< 2$  cm<sup>[14]</sup>. In this study, 10 flat lesions were treated by EMR. We injected 0.001% epinephrine into the submucosal layer to lift the lesion and made it easy to snare. Furthermore, this may provide a buffer to protect the inherent muscle function, which could reduce the bleeding and perforation risk during the process of muscle removal. Examination by EUS before surgery to determine the size and depth of lesions could help determine the injection site and the resection scope.

Endoloop ligation of tumors at the base, blocking blood supply and causing tumor necrosis, could significantly reduce the risk of hemorrhage and perforation<sup>[15,16]</sup>. But, the procedure is not suitable for large lesions. Incomplete ligation might leave residual tumors,

while ligation could increase the risk of hemorrhage and perforation. Therefore, the range and depth of ligation should be strictly controlled according to the results of EUS during surgery. In the past, the majority of tumors studied have been only the muscularis mucosa and submucosa<sup>[3]</sup>. Recently, it was reported that endoloop could remove tumors arising from muscularis propria safely and effectively<sup>[15,17]</sup>. In this study, we also used endoloop removal of lesions arising from muscularis propria, without hemorrhage or perforation. The tumor from the muscularis propria can grow inside or outside the cavity, therefore, preoperative EUS for defining the tumor growth pattern is very important to determine whether the lesion can be safely and completely removed.

ESD should be performed using a high-frequency electric knife to dissect the subepithelial tumor, which is more suitable for treatment of large and flat lesions. Tumors derived from the muscularis mucosa and submucosa can be completely dissected<sup>[18,19]</sup>. It is difficult to dissect lesions from the muscularis propria because of the increased risk of hemorrhage and perforation. In Lee *et al.*'s<sup>[20]</sup> study, among 12 cases of gastrointestinal submucosal muscle tumors arising from muscularis propria treated by ESD, 9 tumors were completely dissected. The size of these tumors ranged from 0.6 to 4 cm (average, 2 cm). In this study, 8 stromal tumors arising from submucosa or muscularis propria were treated safely by ESD. All of them were dissected once and clipping was used to close deep wounds to reduce hemorrhage and perforation risk.

Our clinical practice demonstrates that endoscopic treatment can be applied to GIMTs arising from muscularis mucosa, submucosa and muscularis propria. Based on the results of the EUS procedure, lesions > 2.5 cm in size and suspected to be malignant should be considered for surgery. Moreover, if the tumor grew outside the cavity, endoscopic treatment should be aborted as well. We also suggested a follow-up with EUS for the few patients who are not indicated for the endoscopic therapy or whose tumor is too small.

In conclusion, EUS can help determine the origin, size, shape, nature and growth pattern of lesions, with a high diagnostic accuracy for upper GIMTs. Preoperative EUS examination is important for choosing the type of endoscopic therapy for mesenchymal tumors, by which the lesions can be treated safely and effectively.

## COMMENTS

### Background

Clinically, gastrointestinal mesenchymal tumors (GIMTs) are usually incidentally discovered as subepithelial bulges during routine endoscopy for unrelated conditions. The classification and management of these lesions can be challenging.

### Research frontiers

With the wide use of endoscopic ultrasonography (EUS) to clarify the nature and origin of the subepithelial tumor, great progress has been made in diagnosis and treatment of GIMTs. However, the value of EUS in the choice of endoscopic treatment strategies for GIMTs has not been well established.

### Innovations and breakthroughs

This study indicated that EUS could help determine the origin, size, shape,

nature and growth pattern of lesions, with a high diagnostic accuracy for upper GIMTs. Under the guidance of the EUS, GIMTs could be removed by appropriate endoscopic treatment, such as snare electrosection, endoloop, endoscopic mucosal resection (EMR) and endoscopic submucosal dissection (ESD) without severe complications.

### Applications

The results of this study demonstrate that EUS is an effective means of diagnosis for upper GIMTs. Preoperative EUS examination is important for choosing the type of endoscopic therapy for mesenchymal tumors. The study will guide the clinical application of EUS in the endoscopic therapy for upper GIMTs.

### Terminology

GIMTs are tumors which originate from mesenchymal cells other than epithelial cells or lymphocytes. They are further classified as stromal tumors, leiomyomas, leiomyosarcomas, neural tumors, fibroblast tumors or liparomphalus. EMR is a minimally invasive technique for resection of a lesion that requires the separation of the submucosa by injecting a fluid agent. ESD is a new endoscopic method using special knife for complete en bloc resection of early gastrointestinal neoplasms.

### Peer review

This is a well written paper which describes the experience of the authors in the EUS diagnosis and subsequent endoscopic treatment of gastrointestinal mesenchymal tumors. The pictures well support the authors' findings and conclusions.

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## $\beta$ -catenin accumulation in nuclei of hepatocellular carcinoma cells up-regulates glutathione-s-transferase M3 mRNA

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**RESULTS:** Two up-regulated genes, glutamine synthetase and glutathione-s-transferase M3 (GSTM3), were identified in radiation-induced mouse HCC cells. Influence of  $\beta$ -catenin accumulation in nuclei of HCC cells on up-regulation of GSTM3 mRNA was investigated. The nearby upstream domain of GSTM3 contained the  $\beta$ -catenin/Tcf-Lef consensus binding site sequences [5'-(A/T)(A/T)CAAAG-3'], and the total GST activity ratio was considerably higher in B6C3F1 mouse HCC cells with  $\beta$ -catenin accumulation in nuclei of HCC cells than in those without  $\beta$ -catenin accumulation ( $0.353 \pm 0.117$  vs  $0.071 \pm 0.064$ ,  $P < 0.001$ ). The TWS119 (a distinct GSK-3 $\beta$  inhibitor)-induced total GST activity was significantly higher in HepG2 cells with  $\beta$ -catenin accumulation than in those without  $\beta$ -catenin accumulation in nuclei of HCC cells. Additionally, the GSTM3 mRNA level was significantly higher at 24 h than at 12 h in TWS119-treated HepG2 cells.

**CONCLUSION:**  $\beta$ -catenin accumulation increases GST activity in nuclei of HCC cells, and GSTM3 may be a novel target gene of the  $\beta$ -catenin/Tcf-Lef complex.

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**Key words:**  $\beta$ -catenin accumulation; Differential display analysis; Glutathione-s-transferase M3; Hepatocellular carcinoma; Radiation

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### Abstract

**AIM:** To identify the differentially over-expressed genes associated with  $\beta$ -catenin accumulation in nuclei of hepatocellular carcinoma (HCC) cells.

**METHODS:** Differentially expressed genes were identified in radiation-induced B6C3 F1 mouse HCC cells by mRNA differential display, Northern blot and RT-PCR, respectively. Total glutathione-s-transferase (GST) activity was measured by GST activity assay and  $\beta$ -catenin localization was detected with immunostaining in radiation-induced mouse HCC cells and in HepG2 cell lines.

Li YS, Liu M, Nakata Y, Tang HB.  $\beta$ -catenin accumulation in nuclei of hepatocellular carcinoma cells up-regulates glutathione-s-transferase M3 mRNA. *World J Gastroenterol* 2011; 17(13): 1772-1778 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i13/1772.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i13.1772>

## INTRODUCTION

Hepatocellular carcinoma (HCC) is a primary cancer of the liver chronically injured by infection, metabolic disease or various drugs<sup>[1]</sup>. As generally observed in other carcinomas, HCC is attributed to accumulated genetic alterations, including (1) Activation of oncogenes N-ras, H-ras, and K-ras, c-erbA, c-met, RB and c-myc<sup>[2-5]</sup>; (2) Transcriptional activation of c-jun and nuclear factor  $\kappa$ B by hepatitis B virus factors<sup>[6]</sup>; (3) Repression or mutation of the p53 anti-oncogene<sup>[7]</sup>; and (4) Accumulation of  $\beta$ -catenin<sup>[8]</sup>. Although the genetic events responsible for either HCC initiation or progression are not clear, they involve at least three carcinogenesis pathways: the p53, RB and Wnt/ $\beta$ -catenin signaling pathways<sup>[11-10]</sup>.

$\beta$ -catenin is an essential downstream effector of the canonical Wnt signaling pathway<sup>[11,12]</sup>. Approximately 20% of HCC cells display  $\beta$ -catenin aberrant activation<sup>[9,13]</sup>. In the normal steady state,  $\beta$ -catenin is continuously phosphorylated at serine and threonine residues by glycogen synthase kinase 3 $\beta$  (GSK-3 $\beta$ ) in a complex with adenomatous polyposis coli (APC)-axin/conductin and is quickly degraded through the ubiquitin/proteasome pathway. In mice, liver-specific deletion of APC induces  $\beta$ -catenin stabilization and increases the number of HCC cells. Although the activation of  $\beta$ -catenin is likely an initiating or contributory factor for HCC, more fundamental information is required for a better understanding of the detailed genetic mechanism underlying HCC associated with  $\beta$ -catenin.

To uncover the detailed genetic mechanisms underlying HCC in the present study, several genes in mouse cancerous liver tissue samples were identified to disclose more of the genes that play a very important role in the regulation of cell proliferation and the development of HCC. We identified several cDNA fragments that were differentially expressed in radiation-induced mouse HCC and compared with those in matched nontumorous liver tissue. Samples using a differential display technique<sup>[14]</sup>. We determined whether the nearby upstream domain of those genes contain the  $\beta$ -catenin/Tcf-Lef consensus binding site sequences. The influence of  $\beta$ -catenin accumulation in nuclei of HCC cells on activation of protein encoded by the gene containing  $\beta$ -catenin/Tcf-Lef consensus binding site sequences was further investigated.

## MATERIALS AND METHODS

### Sample preparation

Surgically resected HCC and adjacent nontumorous tissue samples were taken from the livers of 18-mo-old mice irradiated by 3.5 Gy 60Co  $\gamma$ -ray for 1 wk immediately after they were born. B6C3F1 mouse HCC and matched nontumorous liver tissue samples were obtained immediately under the same conditions for measurement of total GST activity<sup>[15]</sup>, isolation of total RNA<sup>[16]</sup>, and immunohistochemical expression of  $\beta$ -catenin and hematoxylin-eosin (HE) staining. Histological analysis of HCC tissue samples from mice was carried out according to the general rules for clinical and pathological study of primary liver cancer.

Table 1 Primer sets used in mRNA differential display analysis

Anchor primers	Arbitrary primers
T12MG (10 $\mu$ mol/L)	AP-10 (2 $\mu$ mol/L), 5'-TAGCAAGTGC-3'
T12MA (10 $\mu$ mol/L)	AP-11 (2 $\mu$ mol/L), 5'-CAGACCGTTC-3'
T12MT (10 $\mu$ mol/L)	AP-12 (2 $\mu$ mol/L), 5'-TGCTGACCTG-3'
T12MC (10 $\mu$ mol/L)	AP-14 (2 $\mu$ mol/L), 5'-AATGGGCTGA-3'

M represents a degenerated mixture of dA, dG and dC. The 16 different primer sets used for PCR amplification were randomly combined from the four arbitrary primers and the four anchor primers.

On the other hand, HepG2 cells obtained from China Center for Type Culture Collection were maintained in DMEM supplemented with 10% fetal calf serum (Sigma, Louis, MO), 200 mmol/L L-glutamine and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) at 37°C in a water-saturated atmosphere containing 5% CO<sub>2</sub>. Cell cultures were allowed to reach 90% confluence. The cells were then treated with or without 1  $\mu$ mol/L of 4, 6-disubstituted pyrrolopyrimidine (TWS119, Cayman Chemical, Ann Arbor, MI) and incubated at 37°C for 24 h. Finally, total RNA or cytoplasmic proteins, including total GST proteins, were extracted from surgically resected frozen tissue samples or HepG2 cells by homogenization with a Vibra cell sonicator (Sonics and Materials, Inc., Danbury, CT) under a regularity condition.

### mRNA differential display analysis and DNA sequencing

mRNA differential display analysis was performed as previously described<sup>[14]</sup> with a RNAmapper kit A (GenHunter, Nashville, TN) (Table 1). Total RNA (0.4  $\mu$ g) extracted from radiation-induced mouse HCC and matched nontumorous liver tissue samples was reverse-transcribed with different combinations of arbitrary and anchor primers (Table 1) for initial cDNA synthesis. The thermal cycler parameters were as follows: 1 cycle at 94°C for 4 min, followed by 40 cycles at 94°C for 30 s, at 40°C for 2 min and at 72°C for 30 s. Amplified subpopulations were distributed on a 6% DNA sequencing gel. The bands of interest were cut out from the polyacrylamide gel, and cDNA fragments were re-amplified using the same pair of primers and the same cycle parameters as described above. The re-amplified cDNA fragments were purified from 2% agarose gels and subcloned into pCRII-TOPO vectors using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA). Clones were selected by the same size of bands cut from the polyacrylamide gel as described above, followed by inverse hybridization and DNA sequencing. DNA sequences were compared with those in GenBank by the Blast Service provided by NIH (Bethesda, MD).

### Northern blot analysis

Total RNA extraction (10  $\mu$ g) was denatured and electrophoresed in a 1.2% agarose gel containing 0.66 mol/L formaldehyde and then transferred onto a Hybond-nylon membrane (Amersham Biosciences, Buckingham, England). The membranes were UV cross-linked, pre-hybrid-

ized and hybridized. The respective cDNA fragments obtained from the differential display reaction were used as a probe for Northern blot analysis. Probes were [ $\alpha$ - $^{32}$ P] dCTP (110 TBq/mmol) labeled using the Megaprime™ DNA labeling kit (Amersham, UK), pre-hybridized to filters at 42°C for 30 min, and then hybridized to the filters overnight at 42°C. The filters were washed twice at 55°C in 1 × SSC, 0.1% SDS for 15 min, and then exposed to X-ray film for 24–72 h at -80°C.

### Immune staining

Immunofluorescence and immunocytochemistry analyses of  $\beta$ -catenin localization were performed, respectively, with mouse monoclonal anti- $\beta$ -catenin antibody diluted at 1:500 for immunofluorescence, and diluted at 1:100 for immunocytochemistry (Sigma, Louis, MO) on 5- $\mu$ m paraffin-embedded sections and paraformaldehyde-fixed HepG2 cell sections that were differentially resected from 24 radiation-induced B6C3F1 mouse HCC and adjacent nontumorous liver tissue samples<sup>[17,18]</sup>. The tissue sections were then incubated for 1 h at room temperature with Alexa Fluor 546 goat anti-mouse IgG diluted at 1:1000 (Molecular Probes, Eugene, OR, USA), washed three times with PBS and visualized under a Nikon Eclipse Ti fluorescent microscope (Japan). The HepG2 cell sections were further treated with a Histofine simple stain rat MAX-PO (MULTI) kit (Nichirei, Tokyo, Japan), stained brown with a DAB substrate kit (Nichirei, Tokyo, Japan) and blue with hematoxylin. Negative controls were stained with the omitted primary antibody. Omission of the primary antibody resulted in no staining of the cells.

### GST activity assay

Total GST activity was assayed as previously described<sup>[15]</sup>. Total GST activity with aromatic substrates was determined by monitoring changes in absorbance with a microplate reader (Infinite M200, Tecan, Switzerland). A complete assay mixture without total GST was used as a control. HepG2 cells, HCC cells and matched nontumorous liver tissue cells were broken for 20 s at 4°C, respectively, in 1 mL 0.1 mol/L potassium phosphate buffer (pH 6.5) using a sonicator. Samples were collected into different tubes containing EDTA, centrifuged at 10000 r/min for 1 h at 4°C, and the supernatant was stored at -20°C. GST activity was assayed with 5  $\mu$ g protein in duplicate with 1 mmol/L 1-chloro-2, 4-dinitrobenzene and glutathione (Sigma, Louis, MO) and used without further purification in a total volume of 1 mL. Optical density of GST was measured within at least 3 min after incubation at 25°C for 15 min at a wavelength of 340 nm ( $\epsilon$  = 9.6 mmol/L per cm). The activity of GST was expressed as a unity of nmol mg per min.

### Real-time PCR for detection of glutathione-s-transferase M3 mRNA expression

Total RNA harvested from HepG2 cells was subjected to reverse transcription into cDNA using a Superscript kit (Life Technologies, Gaithersburg, MD) according to

its manufacturer's protocol. Thereafter, 2  $\mu$ g of cDNA samples was used immediately in measurement of GSTM3 mRNA level by real-time PCR with iQ SYBR Green Supermix (Bio-Rad, Tokyo, Japan), forward primer (5'-GCTCCTGGAGTTCACGGATA-3'), and reverse primer (5'-GCTCCTGGAGTTCACGGATA-3') on a DNA engine Opticon 2 real-time PCR detection system (Bio-Rad, Tokyo, Japan). The thermal cycler parameters were as follows: 1 cycle at 95°C for 3 min, followed by 40 cycles at 95°C for 15 s, at 60°C for 30 s and at 72°C for 30 s<sup>[19]</sup>. A  $\beta$ -actin control was run simultaneously with the same reaction recipe listed in the instruction manual for the iQ SYBR Green Supermix (Bio-Rad, Tokyo, Japan). All data were normalized to  $\beta$ -actin mRNA levels to account for any variation in RNA concentrations between the samples obtained from three separate experiments.

### Statistical analysis

The data are presented as mean  $\pm$  SE. Statistical analyses were performed among the three groups by a one-way analysis of variance followed by Bonferroni's test, and between two groups by the unpaired Student's *t*-test. *P* < 0.05 was considered statistically significant.

## RESULTS

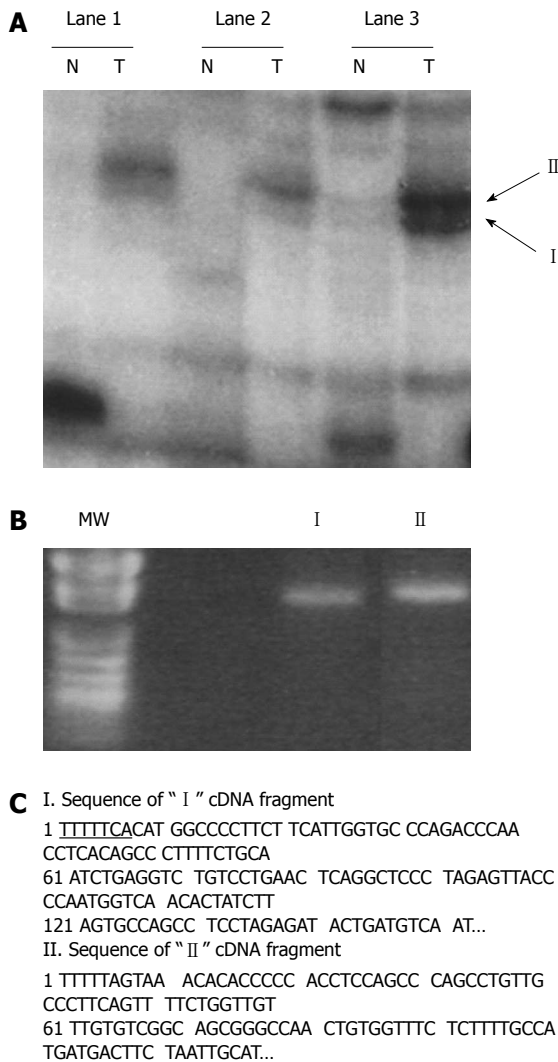
### Identification of differentially expressed genes in mouse HCC and matched nontumorous liver tissue samples

Differential display analysis is a powerful tool for the comparison of differential gene expressions between two or more mRNA populations<sup>[14]</sup>. Using this method, we compared the mRNA expression patterns of B6C3F1 mouse HCC and matched nontumorous liver tissue samples. Representative differentially displayed autoradiographs of "I and II" cDNAs are shown in Figure 1A. The interesting "I and II" cDNA fragments were successfully recovered from the dried DNA sequencing gel, re-amplified (Figure 1B), purified, and subcloned into the TA cloning site of pCRII-TOPO vector. Its nucleotide sequences were compared with those in GenBank (Figure 1C). Concurrently, the purified products of "I and II" fragments from 2% agarose gels were used as probes for reverse Northern blotting. The Northern blotting patterns of "I and II" fragments differentially expressed in mouse HCC cells are shown in Figure 2A. In comparison with a nucleotide sequence in GenBank, "I and II" nucleotide sequences were identified as the up-regulated gene coding products, such as glutathione-s-transferase M3 (GSTM3) and glutamine synthetase (GLNS) in radiation-induced B6C3 F1 mouse HCC cells.

### Dependence of mouse GSTM3 activity on $\beta$ -catenin in B6C3F1 mouse HCC

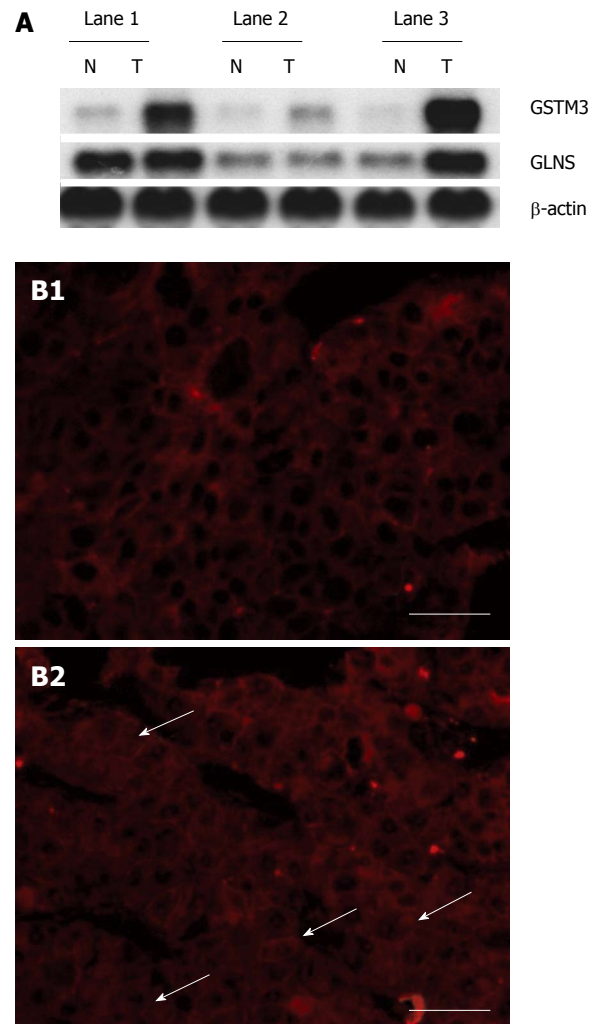
It has been reported that nuclear translocation of  $\beta$ -catenin may represent an early event in liver carcinogenesis<sup>[20]</sup>. Therefore, we are interested in the relation between  $\beta$ -catenin and the discovered gene described above. Analysis of mRNA expression showed that the expression level





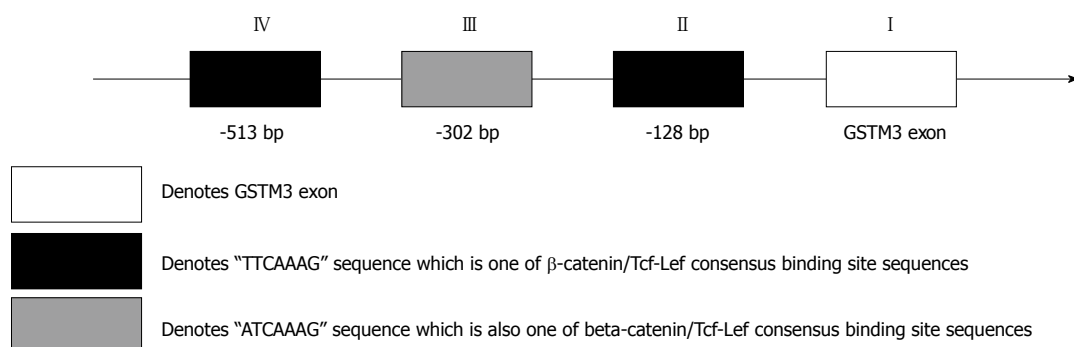
**Figure 1** Identification of differentially displayed cDNA fragments from paired hepatocellular carcinoma cells (T) and nontumorous liver tissues (N). A: Differentially displayed PCR products ("I" and "II") amplified with AP-10 primer and  $T_{12}$ MA. Lanes 1-3 denote the three B6C3 F1 mouse samples, respectively; B: Recovered "I" and "II" from the dried DNA sequencing gel reamplified by PCR. MW lane shows the pUC118 DNA fragments cut by HapII (a restriction enzyme) as molecular weight markers; C: Nucleotide sequences of the bands shown in A. Flanking sequences of  $T_{12}$ MA primers are underlined. The two insert-containing fragments were sequenced and identified as gene fragments of GLNS and GSTM3 in comparison with those of nucleotide in GenBank.

of GSTM3 mRNA was significantly higher in cell nuclei of B6C3F1 mouse HCC cells with  $\beta$ -catenin accumulation than in those without  $\beta$ -catenin accumulation (Figure 2B), suggesting that  $\beta$ -catenin can increase the GSTM3 activity. To confirm whether  $\beta$ -catenin increases the GSTM3 activity, the gene near the upstream domain of individual mouse GSTM3 containing the  $\beta$ -catenin/Tcf-Lef consensus binding site sequence [5'-(A/T)(A/T) CAAAG-3'<sup>[21]</sup>] was detected by searching it in GenBank. On the other hand, whether the increased GSTM3 activity in mouse HCC cells is correlated with  $\beta$ -catenin accumulation in nuclei of HCC cells was also assayed (Figure 3), showing that the nearby upstream domain of mouse GSTM3 contains  $\beta$ -catenin/Tcf-Lef consensus binding site sequences [5'-(A/T)(A/T) CAAAG-3']. Because the GSTM3 activity



**Figure 2** Levels of glutamine synthetase and glutathione-s-transferase M3 mRNA and expression of  $\beta$ -catenin. A: T denotes surgically resected B6C3F1 mouse hepatocellular carcinoma (HCC) tissue samples and N denotes matched nontumorous liver tissue samples; B: Representative immunofluorescence photomicrographs for  $\beta$ -catenin (red photomicrographs) in HCC tissue samples. B1 denotes negative  $\beta$ -catenin staining in nuclei of HCC cells, B2 denotes positive  $\beta$ -catenin staining in nuclei of HCC cells, and white arrows indicate  $\beta$ -catenin detected in nuclei of HCC cells. Bars: 50  $\mu$ m. GLNS: Glutamine synthetase; GSTM3: Glutathione-s-transferase M3.

level was rather variable in normal mouse tissue samples, it was difficult to estimate the  $\beta$ -catenin dependence on GSTM3 activity using the absolute GST activity level. Therefore, we analyzed the increased total GST activity in HCC tissue samples relative to that of normal tissue samples by the ratio of (T-N)/N, where T and N denote the total GST activity in HCC and normal tissue samples, respectively. On the other hand, to see the directly significant relation between total GST activity and  $\beta$ -catenin accumulation in nuclei of HCC cells, the average total GST activity level was also measured in B6C3F1 mouse HCC and matched nontumorous liver tissue samples with or without  $\beta$ -catenin accumulation (Table 2). It can be clearly seen from Table 2 that the total GST activity ratio was considerably higher in B6C3F1 mouse HCC tissue samples with  $\beta$ -catenin accumulation than in those without  $\beta$ -catenin accumulation in nuclei of HCC cells. The



**Figure 3** Identification of  $\beta$ -catenin/Tcf-Lef consensus binding sites with three  $\beta$ -catenin/Tcf-Lef consensus binding site sequences [5'-(A/T)(A/T) CAAAG-3'] located at the nearby upstream domain of mouse GSTM3 by searching GenBank.

**Table 2** Total glutathione-s-transferase activity in B6C3F1 mice hepatocellular carcinoma cells (T) and matched nontumorous (N) liver tissue samples

Samples	Total GST activity (nmol/mg per min)		Values of GST activity ratio (T-N)/N
	T	N	
(+)	3122 $\pm$ 189 (n = 13)	2447 $\pm$ 180 (n = 13)	0.353 $\pm$ 0.117 <sup>a</sup>
(-)	2644 $\pm$ 199 (n = 11)	2523 $\pm$ 205 (n = 11)	0.071 $\pm$ 0.064

[(T-N)/N] indicates the glutathione-s-transferase (GST) activity values for the samples with and without  $\beta$ -catenin accumulation in hepatocellular carcinoma (HCC) cell nuclei. "(+)" or "(-)" denotes the samples from B6C3F1 mice with or without  $\beta$ -catenin accumulation in nuclei of HCC cells. <sup>a</sup> $P < 0.001$  vs negative  $\beta$ -catenin staining group.

averaged GST activity ratio was also significantly higher in HCC tissue samples with  $\beta$ -catenin accumulation than in those without  $\beta$ -catenin accumulation ( $0.353 \pm 0.117$  vs  $0.071 \pm 0.064$ ,  $P < 0.001$ ), suggesting that the GST activity ratio is significantly different (Table 2).

#### Similarly increased GST activity and $\beta$ -catenin accumulation in nuclei of HepG2 cells

To further elucidate the above findings, we mimicked the canonical Wnt pathway in cultured HepG2 cells using TWS119 (an inhibitor of GSK-3), which led to phosphorylation, nuclear translocation, and abnormal accumulation of  $\beta$ -catenin in nuclei of HepG2 cells. We treated the cultured HepG2 cells with or without 1  $\mu$ mol/L TWS119 at 37°C for 24 h, measured the total GST activity in these cells, and then analyzed the relation<sup>[15]</sup> between GST-GSH protein complex concentration and time (at least 15 min) in "control" and "TWS119" groups using a microplate reader at a wavelength of 340 nm. Concurrently, the  $\beta$ -catenin accumulation in HepG2 cells was detected with immunocytochemical staining with a rabbit polyclonal antibody against  $\beta$ -catenin as previously described<sup>[17]</sup>. Thereafter, we comprised the linear function of total GST-GSH protein complex concentration between the two groups. It can be clearly seen from Figure 4A that the total GST activity ratio was considerably higher in the "TWS119" group with abnormal nuclear accumulation of  $\beta$ -catenin in

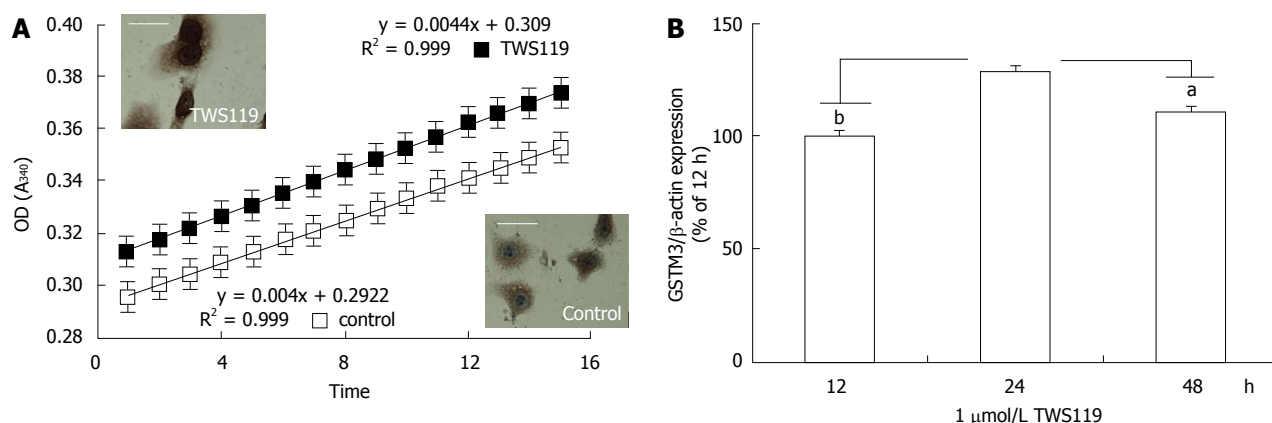
HepG2 cells than in the "control" group with low  $\beta$ -catenin nuclear accumulation. Additionally, both the GSTM3 mRNA expression level and the GST activity were significantly higher in HepG2 cells and controls after treatment with 1  $\mu$ mol/L TWS119 for 24 h ( $100\% \pm 5\%$  vs  $137\% \pm 7\%$ ,  $P < 0.05$ , Figure 4B), supporting that  $\beta$ -catenin nuclear accumulation in nuclei of HCC cells up-regulates the GSTM3 mRNA expression and the GSTM3 activity.

## DISCUSSION

The experimental results of this study demonstrate that mRNAs originally identified from gene expression profiles are differentially expressed in mouse HCC cells. The expression levels of GSTM3 and GLNS mRNAs were higher in mouse HCC tissue samples than in matched nontumorous liver tissue samples. However, the detailed genetic mechanism underlying HCC remains unknown.

GSTM3 is a GST Mu-class subunit. Little is known about the role of GSTM3 in metabolism of harmful agents, except for its overlapping substrate specificity to GSTM1<sup>[22]</sup>. As one of the primary phase II detoxification enzymes, GST can be divided into four classes, namely Alpha, Mu, Pi and Theta<sup>[23]</sup>, which protect against the oxidative stress of their products<sup>[24]</sup>. GST is a potentially important enzyme that regulates the susceptibility to cancer because of its ability to metabolize reactive electrophilic intermediates to usually less reactive and more water soluble glutathione conjugates<sup>[25]</sup>. It was reported that the GST activity, as an indicator of resistance to chemotherapy, is high in human cancer, because GST increases the formation of drug glutathione (GSH) conjugates<sup>[26]</sup>. In this study, the total GST activity was assayed to understand why the GSTM3 mRNA expressions are up-regulated in HCC cells. Based upon these observations, the results of this study showing the up-regulated expression of GSTM3 mRNA in B6C3F1 mouse HCC cells suggest that the increased GSTM3 mRNA expression is a significant phenomenon in cellular detoxification, namely enhancing the metastatic potential in HCC cells.

It was reported that  $\beta$ -catenin plays an important role in cell-cell adhesion<sup>[27]</sup> and in Wnt signaling pathway<sup>[28,29]</sup>.  $\beta$ -catenin can enter the nuclei of HCC cells by binding the



**Figure 4** Total glutathione-s-transferase activity (A) and glutathione-s-transferase M3 mRNA expression (B) in HepG2 cells. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs TWS119 at 24 h by one-way analysis of variance followed by Bonferroni's test.

Tcf-Lef family of DNA binding proteins, and regulate the transcription of target genes (for example, c-myc, gastrin, cyclin D1 and PPAR are identified as target genes of the  $\beta$ -catenin/Tcf-Lef complex)<sup>[30-33]</sup>. In the present study, the GSTM3 mRNA level was higher in B6C3F1 mouse HCC cells with  $\beta$ -catenin accumulation than in those without  $\beta$ -catenin accumulation. To our knowledge, no similar observation has been reported. The total GST activity was much higher in B6C3F1 mouse HCC cells with  $\beta$ -catenin accumulation than in normal tissue samples without  $\beta$ -catenin accumulation (Table 2). The averaged GST activity ratio was significantly higher in HCC cells with  $\beta$ -catenin accumulation than in those without  $\beta$ -catenin accumulation ( $0.747 \pm 0.360$  vs  $0.071 \pm 0.213$ ,  $P < 0.001$ ), suggesting that the GST activity ratio is significantly different (Table 2). Furthermore, by searching the GenBank, we found that the upstream region of the GSTM3 gene contained three  $\beta$ -catenin/Tcf-Lef consensus binding site sequences in mouse GST polymorphisms. The canonical Wnt pathway in cultured HepG2 cells was further mimicked using TWS119, a GSK-3 $\beta$  inhibitor, which caused abnormal  $\beta$ -catenin accumulation. As a result, TWS119-induced  $\beta$ -catenin accumulation enhanced the GST activity and the GSTM3 mRNA expression in HepG2 cells, suggesting that  $\beta$ -catenin accumulation in nuclei of HCC cells can increase the activity of mouse GSTM3, one of the enzymes responsible for the metabolism of a variety of xenobiotics and carcinogens, and that mouse GSTM3 may be a novel downstream target gene of the  $\beta$ -catenin/Tcf-Lef complex in mouse HCC.

It was reported that GLNS can catalyze the synthesis of glutamine<sup>[34]</sup>, a major energy source of cells (an important ATP source), and is a precursor for the synthesis of nucleotides and numerous amino acids, and up-regulated in a subset of human HCC<sup>[35]</sup>. In this study, at 3 wk after tumor implantation, the glutamine synthetase activity in rats increased by 34%, which is consistent with the reported findings<sup>[36]</sup>. However, further study is needed to observe the possible pharmacological action (s) of  $\beta$ -catenin in the up-regulated expression of GLNS mRNA.

In conclusion, GSTM3 and GLNS genes are differen-

tially expressed in mouse HCC cells. The expression level of GSTM3 mRNA and total GST activity are higher in B6C3F1 mouse HCC cells with  $\beta$ -catenin accumulation than in those without  $\beta$ -catenin accumulation, indicating that GSTM3 may be a novel target gene for the  $\beta$ -catenin/Tcf-Lef complex in mouse HCC.

## COMMENTS

### Background

Hepatocellular carcinoma (HCC) is a primary cancer of the liver. However, the genetic events responsible for HCC initiation and progression are not clear. Since approximately 20% of HCC display  $\beta$ -catenin aberrant activation, Wnt/ $\beta$ -catenin signaling pathways may be involved in HCC occurrence.

### Research frontiers

Recent data show that  $\beta$ -catenin may be an initiating or contributory factor for HCC. In this study, the authors demonstrated that glutathione-s-transferase M3 (GSTM3) might be a novel target gene of the  $\beta$ -catenin/Tcf-Lef complex in mouse HCC.

### Innovations and breakthroughs

The authors identified two up-regulated genes, glutamine synthetase (GLNS) and GSTM3, in nuclei of radiation-induced mouse HCC cells with  $\beta$ -catenin accumulation. Three  $\beta$ -catenin/Tcf-Lef consensus binding site sequences were observed in mouse glutathione-s-transferase (GST) polymorphisms. GST activity and GSTM3 mRNA levels were induced in cultured HepG2 cells by TWS119 (an inhibitor of GSK-3 $\beta$ ). To our knowledge, no similar observation has been reported.

### Applications

By demonstrating that GSTM3 may be a novel target gene of the  $\beta$ -catenin/Tcf-Lef complex in mouse HCC, this study may represent a future strategy for therapeutic intervention in patients with HCC.

### Terminology

$\beta$ -catenin plays an important role in cell-cell adhesion and in Wnt signaling pathway, can enter nuclei by binding to the Tcf-Lef family of DNA binding proteins and regulate the transcription of target genes.

### Peer review

This paper reports the results of investigations on some differentially over-expressed genes associated with  $\beta$ -catenin accumulation in nuclei of HCC cells, showing that GSTM3 may be a novel target gene of the  $\beta$ -catenin/Tcf-Lef complex in mouse HCC using mRNA differential display, Northern blot analysis, immunostaining and RT-PCR techniques, respectively. It is worthy of publication.

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## Nutrition support in surgical patients with colorectal cancer

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### Abstract

**AIM:** To review the application of nutrition support in patients after surgery for colorectal cancer, and to propose appropriate nutrition strategies.

**METHODS:** A total of 202 consecutive surgical patients admitted to our hospital with a diagnosis of colon cancer or rectal cancer from January 2010 to July 2010, meeting the requirements of Nutrition Risk Screening 2002, were enrolled in our study. Laboratory tests were performed to analyze the nutrition status of each patient, and the clinical outcome variables, including postoperative complications, hospital stay, cost of hospitalization and postoperative outcome, were analyzed.

**RESULTS:** The "non-risk" patients who did not receive postoperative nutrition support had a higher rate of postoperative complications than patients who received postoperative nutrition support ( $2.40 \pm 1.51$  vs  $1.23 \pm 0.60$ ,  $P = 0.000$ ), and had a longer postoperative hospital stay ( $23.00 \pm 15.84$  d vs  $15.27 \pm 5.89$  d,  $P = 0.009$ ). There was higher cost of hospitalization for patients who received preoperative total parenteral nutrition (TPN)

than for patients who did not receive preoperative TPN ( $62\,713.50 \pm 5070.66$  RMB Yuan vs  $43\,178.00 \pm 3596.68$  RMB Yuan,  $P = 0.014$ ). Applying postoperative enteral nutrition significantly shortened postoperative fasting time ( $5.16 \pm 1.21$  d vs  $6.40 \pm 1.84$  d,  $P = 0.001$ ) and postoperative hospital stay ( $11.92 \pm 4.34$  d vs  $15.77 \pm 6.03$  d,  $P = 0.002$ ). The patients who received postoperative TPN for no less than 7 d had increased serum glucose levels ( $7.59 \pm 3.57$  mmol/L vs  $6.48 \pm 1.32$  mmol/L,  $P = 0.006$ ) and cost of hospitalization ( $47\,724.14 \pm 16\,945.17$  Yuan vs  $38\,598.73 \pm 8349.79$  Yuan,  $P = 0.000$ ). The patients who received postoperative omega-3 fatty acids had a higher rate of postoperative complications than the patients who did not ( $1.33 \pm 0.64$  vs  $1.13 \pm 0.49$ ,  $P = 0.041$ ). High level of serum glucose was associated with a high risk of postoperative complications of infection.

**CONCLUSION:** Appropriate and moderate nutritional intervention can improve the postoperative outcome of colorectal cancer patients.

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**Key words:** Nutritional support; Nutrition assessment; Colorectal cancer; Surgery; Prognosis

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### INTRODUCTION

Colorectal cancer is the fourth most common cancer in men and the third most common cancer in women

worldwide<sup>[1]</sup>. It is also a significant cause of morbidity and mortality throughout the world<sup>[2]</sup>. Malnutrition is common in patients presenting for surgical management of colorectal cancer, and multiple factors, such as tumor location, tumor type, tumor stage, and preoperative radiation or chemotherapy, may predispose the patients to malnutrition<sup>[3]</sup>. Postoperative outcomes, including incidence of complications, morbidity and survival, are usually better in the patients who are in a good nutritional condition<sup>[4]</sup>. Comprehensive clinical application of nutrition support in colorectal cancer patients appears to be necessary.

Unfortunately, malnutrition has remained a troublesome problem because of lack of nutrition support routines and a discrepancy between clinical practice and guidelines regarding nutrition support<sup>[5]</sup>.

Currently, international guidelines on nutrition support have been established, such as the European Society for Clinical Nutrition and Metabolism (ESPEN) guidelines and the American Society for Parenteral and Enteral Nutrition (ASPEN) guidelines. Both are the authoritative guidelines at present, and should be followed and used in clinical practice as appropriate to the specific medical condition. However, since fewer than one sixth of the recommendations in the current guidelines are Grade A, and more than 50% are Grade C<sup>[6]</sup>, more and better controlled trials are needed in the specific fields.

We carried out a retrospective study to evaluate the nutritional risk of colorectal cancer patients who underwent elective surgery, and assessed the nutrition support process by analyzing the postoperative clinical outcomes and comparing with the international recommendations or guidelines. In particular, we investigated the current status of nutrition support for patients undergoing surgery for colorectal cancer, and determined the requirements of feasible and appropriate nutrition support strategies for such patients.

## MATERIALS AND METHODS

### Case selection

We reviewed a total of 220 consecutive patients admitted to our hospital with a diagnosis of colon cancer or rectal cancer from January 2010 to July 2010, and excluded 18 patients, including one with hydroperitoneum according to the exclusion criteria of the Nutrition Risk Screening (NRS) 2002<sup>[7]</sup>, and 17 who had received non-surgical treatment. The remaining 202 patients were enrolled in this study.

### Methods

In order to evaluate the clinical effect of different nutritional strategies in colorectal cancer patients with different nutritional status, we stratified the patients into five groups.

In Group A, to evaluate the effect of NRS score, we excluded the patients who received preoperative nutritional support, and divided the remaining 199 patients into a “non-risk” group ( $n = 148$ ) whose NRS score was 0-2, and an “at-risk” group ( $n = 51$ ) whose NRS score was  $\geq 3$ . We further divided the two groups into two subgroups, a

nutrition support group (NS) who received postoperative nutrition support and a non-nutrition support group (NNS) who did not receive postoperative nutrition support (Table 1). Diagnosis and tumor stage were used to illustrate preoperative health status. The tumor stage was determined according to the 7th edition of the AJCC cancer staging manual (American Joint Committee on Cancer)<sup>[8]</sup>. Complications, postoperative hospital stay, cost of hospitalization and postoperative outcomes were used to assess the clinical effect of postoperative nutritional intervention. In addition, we graded complications as none = 1, infection = 2, fistula = 3, others = 4, and postoperative outcome as recovery = 1, no recovery = 2, death = 3, for statistical evaluation of the results.

Group B consisted of five patients whose NRS score was  $> 4$ , the clinical effect of preoperative TPN in patients with severe malnutrition was evaluated. They were divided into two groups: group 1 ( $n = 2$ ) who received preoperative TPN and group 2 ( $n = 3$ ) who did not (Table 2). Diagnosis, tumor stage, and preoperative albumin, potassium, and sodium levels reflected the preoperative nutrition status. Postoperative enteral nutrition (EN), postoperative TPN and postoperative TPN duration were indicative of the postoperative nutritional intervention. Postoperative serum glucose level was fluctuated according to the proportion of insulin in TPN. Postoperative day 1 (POD1) albumin, potassium and sodium, and POD5 albumin, potassium and sodium levels reflected the postoperative nutritional status. Complications, postoperative hospital stay, cost of hospitalization, and postoperative outcomes were used to assess clinical outcome.

In Group C, the application of postoperative EN in colorectal cancer patients was assessed. Patients who received preoperative nutrition support were excluded, and the remaining 199 patients were divided into two groups: group 1 ( $n = 25$ ) who received postoperative EN and group 2 ( $n = 174$ ) who did not (Table 3). Diagnosis, tumor stage and NRS 2002 score reflected the preoperative nutrition status. As an interferential factor in this group, postoperative TPN was used in the statistical analysis to identify the effect of postoperative EN. Postoperative fasting time, occurrence of complications, postoperative hospital stay, cost of hospitalization, and postoperative outcome indicated clinical outcome.

In Group D, to determine the effect of postoperative TPN duration, we excluded the patients who received preoperative nutrition support and postoperative EN, and included the remaining 174 patients who received postoperative TPN only, dividing them into two groups: group 1 ( $n = 66$ ) with postoperative TPN duration of no less than 7 d, and group 2 ( $n = 108$ ) with a duration of less than 7 d (Table 4). Diagnosis, tumor stage, NRS 2002 score, preoperative albumin, potassium and sodium levels gave an indication of preoperative nutrition status. POD1 albumin, potassium and sodium, and POD5 albumin, potassium and sodium reflected postoperative nutritional status. Complications, postoperative hospital stay, cost of hospitalization and postoperative outcome



Table 1 Nutrition risk screening

	Non-risk		<i>P</i>	At-risk		<i>P</i>
	NS	NNS		NS	NNS	
Patient number	143	5		49	2	
Diagnosis (colon / rectal cancer)	50/93	1/4		24/25	2/0	
Gender (male/female)	63/80	2/3	0.893	30/19	2/0	0.088
Tumor stage <sup>1</sup>			0.066			0.358
Complications <sup>2</sup>	1.23 ± 0.60	2.40 ± 1.51	0.000	1.20 ± 0.45	1.50 ± 0.70	0.348
None (= 1)						
Infection (= 2)						
Fistula (= 3)						
Others (= 4)						
Postoperative hospital stay (d)	15.27 ± 5.89	23.00 ± 15.84	0.009	14.55 ± 4.11	14.50 ± 2.12	0.986
Cost of hospitalization (RMB Yuan)	43469.88 ± 9961.67	35825.00 ± 16271.94	0.301	41802.97 ± 13300.99	33845.80 ± 8374.80	0.187
Postoperative outcome <sup>2</sup>	1.02 ± 0.16	1.00 ± 0.00	0.707	1.10 ± 0.30	1.50 ± 0.70	0.090

<sup>1</sup>The tumor stage of the patients was judged according to the 7th edition of American Joint Committee on Cancer staging manual<sup>[8]</sup>; <sup>2</sup>Complications are defined as none = 1, infection = 2, fistula = 3, others = 4, and postoperative outcome as recovery = 1, no recovery = 2, death = 3, for easier statistical presentation of the results. NS: Nutrition support; NNS: Non-nutrition.

Table 2 Preoperative total parenteral nutrition in malnourished patients

	Group 1	Group 2	<i>P</i>
Patient number	2	3	
Diagnosis (colon/rectal cancer)	2/0	1/2	
Gender (male/female)	2/0	1/2	0.219
Tumor stage	1	2	
Preoperative albumin (g/L)	36.30 ± 5.65	32.83 ± 9.00	0.669
Preoperative potassium (mmol/L)	4.53 ± 0.36	3.75 ± 0.92	0.352
Preoperative sodium (mmol/L)	138.45 ± 2.05	142.13 ± 3.40	0.274
Postoperative EN	2	0	
Postoperative TPN	2	2	
Postoperative TPN duration (d)	7.00 ± 1.41	3.66 ± 3.2	0.276
POD1 <sup>a</sup> serum glucose (mmol/L)	6.91 ± 1.11	9.05 ± 3.65	0.498
POD1 albumin (g/L)	27.80 ± 3.11	26.13 ± 3.19	0.605
POD1 potassium (mmol/L)	4.49 ± 0.36	4.45 ± 0.78	0.953
POD1 sodium (mmol/L)	135.8 ± 3.11	137.66 ± 2.51	0.508
POD5 <sup>a</sup> serum glucose (mmol/L)	6.29 ± 0.24	6.39 ± 2.88	0.968
POD5 albumin (g/L)	28.65 ± 5.16	32.33 ± 4.67	0.466
POD5 potassium (mmol/L)	4.67 ± 0.11	4.42 ± 0.12	0.115
POD5 sodium (mmol/L)	135.95 ± 1.34	135.93 ± 2.72	0.994
Complications			0.445
Postoperative hospital stay(d)	10.50 ± 4.94	13.00 ± 2.64	0.500
Cost of hospitalization (RMB)	62713.50 ± 5070.66	43178.00 ± 3596.68	0.014
Postoperative outcome			0.495

<sup>a</sup>POD1: Postoperative day 1; POD5: Postoperative day 5; EN: Enteral nutrition; TPN: Total parenteral nutrition.

were used to assess the clinical outcome. In addition, the comparison of preoperative serum glucose, POD1 serum glucose, and POD5 serum glucose reflected the contribution of postoperative TPN duration to postoperative serum glucose.

Group E excluded the patients who received preoperative nutrition support or postoperative EN, and included the remaining 167 patients who received postoperative TPN. This group was subdivided into two groups: group 1 (*n* = 102), those who received postoperative application of omega-3 fatty acids, and group 2 (*n* = 65), those who

Table 3 Postoperative enteral nutrition and clinical outcome

	Group 1	Group 2	<i>P</i>
Patient number	25	174	
Diagnosis (colon / rectal cancer)	8/17	66/108	0.568
Gender (male/female)	9/16	91/83	0.129
Tumor stage			0.777
NRS 2002 score	1.88 ± 0.88	1.96 ± 1.01	0.689
Postoperative fasting time (d)	5.16 ± 1.21	6.40 ± 1.84	0.001
Postoperative TPN	27	167	0.996
Complications	1.04 ± 0.20	1.29 ± 0.66	0.060
None (= 1)			
Infection (= 2)			
Fistula (= 3)			
Others (= 4)			
Postoperative hospital stay (d)	11.92 ± 4.34	15.77 ± 6.03	0.002
Cost of hospitalization (RMB)	44210.88 ± 7635.85	42060.09 ± 13066.15	0.752
Postoperative outcome	1.00 ± 0.00	1.06 ± 0.27	0.214
Recovery (= 1)			
Unrecovery (= 2)			
Dead (= 3)			

Group 1: Patients who received enteral nutrition postoperatively; Group 2: Patients who did not receive enteral nutrition postoperatively. TPN: Total parenteral nutrition; NRS: Nutrition risk screening.

did not, as shown in Table 5. Diagnosis, tumor stage, and NRS 2002 score were indicative of preoperative nutrition status. The total lymphocyte count reflected the immune status. Complications, postoperative hospital stay, cost of hospitalization, and postoperative outcome were used to assess the clinical outcome.

We also analyzed the relationship between postoperative day 5 serum glucose levels and postoperative complications of infection (Figure 1).

### Statistical analysis

Analyses were performed using SPSS statistical software

Table 4 Postoperative total parenteral nutrition duration

	Group 1	Group 2	P
Patient number	66	108	
Diagnosis (colon/rectal cancer)	31/35	35/73	
Gender (male/female)	32/34	59/49	0.434
Tumor stage			0.493
NRS 2002 score	1.92 ± 0.94	1.99 ± 1.05	0.676
Preoperative serum glucose (mmol/L)	6.10 ± 1.86	5.75 ± 1.17	0.134
Preoperative albumin (g/L)	38.47 ± 4.44	39.51 ± 6.37	0.249
Preoperative potassium (mmol/L)	3.98 ± 0.38	6.57 ± 1.84	0.270
Preoperative sodium (mmol/L)	140.98 ± 3.23	139.58 ± 14.1	0.435
POD1 serum glucose (mmol/L)	8.59 ± 3.39	7.37 ± 2.06	0.100
POD1 albumin (g/L)	32.24 ± 3.65	35.49 ± 4.11	0.725
POD1 potassium (mmol/L)	4.05 ± 0.44	3.99 ± 0.45	0.424
POD1 sodium (mmol/L)	136.35 ± 3.59	135.72 ± 14.50	0.763
POD5 serum glucose (mmol/L)	7.59 ± 3.57	6.48 ± 1.32	0.006
POD5 albumin (g/L)	31.79 ± 3.53	39.91 ± 3.66	0.063
POD5 potassium (mmol/L)	4.21 ± 0.50	4.16 ± 0.53	0.553
POD5 sodium (mmol/L)	136.5 ± 18.60	137.84 ± 2.81	0.348
Complications			0.533
Postoperative hospital stay (d)	15.59 ± 5.32	15.87 ± 6.45	0.761
Cost of hospitalization (RMB)	47724.14 ± 16945.17	38598.73 ± 8349.79	0.000
Postoperative outcome			0.166

Group 1: The duration of postoperative total parenteral nutrition (TPN) was not less than 7 d; Group 2: The duration of postoperative TPN was less than 7 d. NRS: Nutrition Risk Screening.

(SPSS for Windows Ver. 11.5). Results of different groups were compared using descriptive statistics (mean ± SD).  $P \leq 0.05$  was considered statistically significant.

## RESULTS

Nutrition risk screening is a necessary and effective tool to identify the nutritional status of colorectal cancer patients, and to aid in providing the appropriate nutrition intervention. As Table 1 shows, the “non-risk” patients who did not receive postoperative nutrition support had a higher rate of postoperative complications than those who received postoperative nutrition support ( $2.40 \pm 1.51$  vs  $1.23 \pm 0.60$ ,  $P = 0.000$ ), and also had a longer postoperative hospital stay ( $23.00 \pm 15.84$  vs  $15.27 \pm 5.89$ ,  $P = 0.009$ ), which indicated that postoperative nutrition support may be necessary for “non-risk” patients. Postoperative nutrition support or not did not show a significant difference in the outcome of “at-risk” patients, though postoperative nutrition support tended to improve the postoperative outcome ( $1.10 \pm 0.30$  vs  $1.50 \pm 0.70$ ,  $P = 0.090$ ), thus moderate nutrition support is allowable for “at-risk” patients.

Table 2 shows that the cost of hospitalization for malnourished patients who received preoperative TPN was significantly higher than in patients who did not ( $62\,713.50 \pm 5070.66$  RMB Yuan vs  $43\,178.00 \pm 3596.68$  RMB Yuan,  $P = 0.014$ ) with no significant difference in the outcome.

Postoperative EN markedly improved postoperative recovery course, including a reduction in postoperative fasting time ( $5.16 \pm 1.21$  d vs  $6.40 \pm 1.84$  d,  $P = 0.001$ ) and postoperative hospital stay ( $11.92 \pm 4.34$  d vs  $15.77$

Table 5 Postoperative administration of omega-3 fatty acids

	Group 1	Group 2	P
Patient number	102	65	
Diagnosis (colon / rectal cancer)	42/60	40/25	
Gender (male/female)	49/53	45/38	0.089
Tumor stage			0.317
NRS 2002 score	1.94 ± 0.87	2.00 ± 1.15	0.710
Preoperative total lymphocyte count	1.80 ± 0.63	1.93 ± 0.59	0.186
POD1 total lymphocyte count	0.99 ± 0.34	1.11 ± 0.40	0.067
POD5 total lymphocyte count	1.26 ± 0.59	1.29 ± 0.35	0.660
Complications	1.33 ± 0.64	1.13 ± 0.49	0.041
None (= 1)			
Infection (= 2)			
Fistula (= 3)			
Others (= 4)			
Postoperative hospital stay (d)	16.04 ± 5.81	14.81 ± 4.29	0.159
Cost of hospitalization (RMB)	43936.75 ± 14260.31	39938.89 ± 10741.40	0.055
Postoperative outcome	1.09 ± 0.33	1.06 ± 0.27	0.055
Recovery (= 1)			
Unrecovery (= 2)			
Dead (= 3)			

Group 1: Patients who received omega-3 fatty acids; Group 2: Patients who did not receive omega-3 fatty acids.

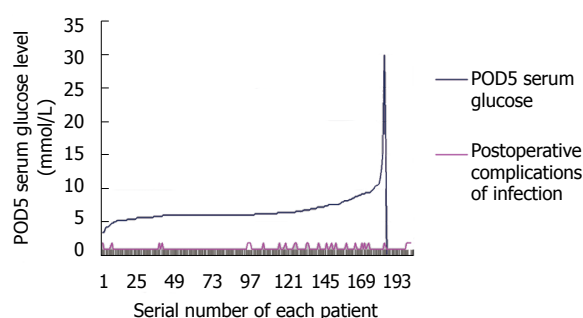


Figure 1 Relationship between postoperative serum glucose level and complications of infection. Abscissa: the serial number of each patient, arranged according to the Postoperative day 5 serum glucose level; Ordinate: numerical value; The red line shows the incidence of postoperative complications of infection.

$\pm 6.03$  d,  $P = 0.002$ ), as shown in Table 3.

Longer postoperative TPN was not associated with better clinical outcome (Table 4). The patients who received postoperative TPN for no less than 7 d had increased POD5 serum glucose ( $7.59 \pm 3.57$  mmol/L vs  $6.48 \pm 1.32$  mmol/L,  $P = 0.006$ ) and cost of hospitalization ( $47\,724.14 \pm 16\,945.17$  Yuan vs  $38\,598.73 \pm 8\,349.79$  Yuan,  $P = 0.000$ ), compared to those with less than 7 d postoperative TPN, suggesting that less than 7 d nutrition support for postoperative colorectal cancer patients is adequate.

More postoperative complications occurred in the patients with postoperative administration of omega-3 fatty acid ( $1.33 \pm 0.64$  vs  $1.13 \pm 0.49$ ,  $P = 0.041$ ) than in patients who did not receive the fatty acid (Table 5).

Postoperative complications were positively correlated with the postoperative serum glucose level, a high postoperative serum glucose level being associated with a higher risk of complications of infection (Figure 1).

## DISCUSSION

Malnutrition is common in patients with colorectal cancer, and in our study, 52 (25.7%) of the 202 cases had a NRS score of more than 3, and had a high nutrition risk<sup>[7]</sup>. Poor nutrition status impacts on the recovery of physical performance status in cancer patients after treatment<sup>[9]</sup>. It was reported that about 20% of cancer patients died of malnutrition or related complications rather than the malignant disease itself<sup>[10]</sup>. Malnutrition is often neglected in our daily clinical practice, and can also induce many clinical problems, including impaired wound-healing, immunocompromization, diminished cardiac and respiratory function, and a host of other complications that can lead to longer hospitalization and a higher mortality rate<sup>[11]</sup>. Although provision of nutrition support to cancer patients may cause tumors to grow more quickly, nutrition support is recommended when the nutrition status is so compromised that patients are at a high risk of complications, or cannot comply with the oncologic therapy as reported in the clinical practice ESPEN guidelines<sup>[12]</sup>. Thus perioperative nutrition support is beneficial for moderately or severely malnourished gastrointestinal cancer patients<sup>[13]</sup>. The implementation of nutrition support guidelines has facilitated many appropriate nutritional support procedures for colorectal cancer patients<sup>[4,6,14-16]</sup>.

### **Preoperative nutrition risk screening can identify nutritional risks**

Patients with cancer are at a risk of malnutrition, and nutrition screening should be performed to identify those who require nutrition support<sup>[17]</sup>. When a patient is admitted to our ward, knowledge of the nutritional status, which is a clinical predictor of postoperative mortality and morbidity in surgery for colorectal cancer<sup>[18-20]</sup>, is essential, not only for screening malnourished or non-malnourished patients, but also for multimodal oncological treatment<sup>[21]</sup>. There are various kinds of screening methods, including NRS 2002, which is a rapid screening tool recommended by ESPEN<sup>[7]</sup>, and has been proven to be an appropriate scoring system for predicting unfavorable clinical outcomes<sup>[22]</sup>. ASPEN suggested using a subjective global assessment (SGA) as a screening tool<sup>[23]</sup>, and was shown to be a reliable assessment tool which could predict hospital stay and medical expenditure of surgical gastrointestinal cancer patients<sup>[24,25]</sup>. We believe that the assessment of nutritional status requires a multidimensional approach, which includes different clinical indices and various nutritional parameters, so it is better to use both SGA and NRS 2002 to predict the clinical outcome<sup>[26]</sup>. Our study indicated that, for “non-risk” colorectal patients, postoperative nutrition support is necessary to avoid postoperative complications and shorten postoperative hospital stay. Although postoperative nutrition support to “at-risk” colorectal patients showed no significant advantage, in our opinion, moderate nutrition support is allowable, as no harm or economic burden was incurred. Further prospective studies are necessary to confirm this.

### **Preoperative TPN is not always necessary**

The goal of preoperative nutrition support is to minimize negative protein balance by avoiding starvation, to maintain muscle, immune and cognitive functions, and to enhance postoperative recovery, as the ESPEN guidelines indicated<sup>[27]</sup>. Preoperative parenteral nutrition is indicated in severely undernourished patients in whom enteral nutrition cannot be adequately administered either orally or enterally. Conversely, its use in well-nourished patients has no benefit but increased morbidity. In our study, preoperative nutrition support in severely malnourished colorectal cancer patients only increased the economic burden, with little beneficial effect. This is in agreement with the ASPEN guidelines, which recently recommended that nutrition support should not be used routinely in patients undergoing major cancer surgeries<sup>[17]</sup>. Because of the limited sample size, further prospective studies with a larger sample size should be carried out.

Gunerhan's study<sup>[28]</sup> recently showed that preoperative immunonutrition resulted in a significant increase in serum prealbumin levels, but it did not significantly alter the T lymphocyte subpopulation count, the rate of postoperative complications and the hospitalization duration, thus preoperative immunonutrition should not be provided routinely. None of our patients received preoperative immunonutrition.

### **Postoperative EN can shorten the fasting time and hospital stay**

Previously, many colorectal doctors believed that nutrients in the gut disrupted anastomoses, so they preferred delaying the EN postoperatively, and administered TPN instead to avoid anastomotic leak, which requires substantial use of hospital resources<sup>[29]</sup>. However, Seidner<sup>[11]</sup> emphasized that there were no significant differences in morbidity and mortality between patients who received EN or TPN, and recommended the guideline: if the gut works, use it. The available evidence lends support to the use of enteral over parenteral feeding in inpatients with functioning gastrointestinal tracts<sup>[30]</sup>. The application of EN can reverse the loss of gut mucosal integrity resulting from surgical trauma<sup>[31]</sup>, and early nutrition support (EEN) is associated with a decreased infection risk, a decreased mortality, a reduced hospital stay, an increase in collagen deposition at anastomosis and wound strength, and a clear trend of a reduction in anastomotic breakdown<sup>[32,33]</sup>. In addition, EEN can reduce the use of nasogastric tubes, which may delay the return of bowel function and increase pulmonary complications<sup>[34,35]</sup>. Osland<sup>[3]</sup> even suggested adopting EEN as a standard of care in cancer patients undergoing gastrointestinal resections. As Table 3 shows, postoperative EN in colorectal cancer patients can significantly shorten the postoperative fasting time and postoperative hospital stay, and there is a tendency to reduce postoperative complications ( $P = 0.060$ ). Although it remains to be determined how much should be provided initially, underfeeding with a small amount of nutrients, which “bathe” the gut mucosa, makes EEN necessary or desirable.



The risk of overfeeding should not be neglected, as it can overwhelm the digestive and absorptive capacity of the gastrointestinal tract, and lead to occurrence of some clinical complications, such as gastric distention, nausea, and diarrhea<sup>[32]</sup>.

### **Postoperative TPN can offer a smooth postoperative recovery**

Parenteral nutrition (PN) has been widely used in clinical practice, and a safe PN system must be developed which minimizes procedural incidents and maximizes the ability to meet individual patient requirements<sup>[36]</sup>. Thus, it is desirable to provide, devise, or make available customized PN formulations for individuals who have complex requirements secondary to disease or underlying illness, or when otherwise warranted by routine monitoring of electrolytes, organ function, growth, and development. Not only fat and carbohydrates, but also a full range of vitamins and trace elements should be important components of the TPN bag, and optimal nitrogen-sparing can be achieved when all components of the PN mix are administered simultaneously over 24 h. However, when early oral food intake or EN is combined with PN, intravenous supplementation with vitamins appears to be unnecessary<sup>[27]</sup>.

Should colorectal cancer patients be administered postoperative TPN? Planas<sup>[4]</sup> recommended that such patients having elective surgery should not be given postoperative PN routinely. Seidner<sup>[11]</sup> implied that administering PN in disregard of the patient's nutritional status could do more harm than good, and suggested that postoperative TPN should be reserved for patients who have a prolonged postoperative ileus, generally more than 7-10 d, and for those who are severely malnourished and whose feeding cannot be started within 3-5 d. According to the ESPEN guidelines<sup>[27]</sup>, postoperative PN is recommended in patients who cannot meet their caloric requirements within 7-10 d both orally or enterally, and in patients who require postoperative artificial nutrition, enteral feeding or a combination of enteral and supplementary parenteral feeding.

In our study, most colorectal cancer patients could resume feeding 5-8 d postoperatively (Table 3), so postoperative TPN may be beneficial during the period of postoperative fasting. Should we give the patients TPN for 7 d or more, or is less than 7 d adequate? The results in Table 4 indicate that a longer duration of TPN incurs high hospitalization costs and induces hyperglycemia, which is associated with a higher rate of postoperative complications (Figure 1), thus less than 7 d' postoperative TPN appears to be appropriate.

PN can be delivered through short-term, non-tunneled central venous catheters, and the appropriate choice, insertion, and monitoring of the venous access are of paramount importance to avoid a catheter-related bloodstream infection, an important and still very common complication of PN<sup>[37]</sup>. Such infections can be reduced by adopting cost-effective, evidence-based interventions, including specific training of staff, an adequate handwashing, the correct type

of device and site of insertion, the use of maximal barrier protection during insertion, and removal of central lines as soon as they are no longer necessary.

### **Postoperative application of omega-3 fatty acids**

There is controversy as to whether visceral proteins should be used to assess nutrient status in hospitalized patients. Seidner<sup>[11]</sup> suggested that visceral proteins can be used in the hospital setting, because they can identify patients at risk of a poor outcome who may benefit from nutrition support. In addition, the total lymphocyte count can be used to assess a patient's immune function, which has been shown to correlate with the degree of visceral protein depletion and clinical outcome. Therefore, total lymphocyte counts were used in our study to assess the effect of the omega-3 fatty acids.

Postoperative supplementation of omega-3 fatty acids by TPN has been reported to have a favorable effect in the outcomes of colorectal cancer patients undergoing radical resection, by lowering the magnitude of the inflammatory response and modulating the immune response<sup>[38,39]</sup>. In contrast, the application of omega-3 fatty acids showed no significant benefit in our study, and indeed there was a trend of an increased risk of postoperative complications, an increased economic burden, and a poorer postoperative outcome. Further prospective research is necessary with a larger sample to assess the functional benefit or otherwise of omega-3 fatty acids in the postoperative setting.

Currently, many barriers, including low priority of nutritional support, no routine or established procedures in many medical centers, insufficient knowledge of nutritional support, lack of qualified and optional nutritional menus for the patients, and lack of leadership support from the medical team, make the nutritional therapy difficult to carry out in many hospitals<sup>[40]</sup>. A greater effort should be made in the nutritional assessment of patients.

In conclusion, nutrition support is an important therapy for colorectal cancer patients, and appropriate and moderate nutritional intervention can significantly improve the postoperative recovery course, relieve the patient's suffering, and reduce the medical cost of the patients. Clinicians must be aware of nutrition support principles and methods in order to administer appropriate nutrition support and avoid blind nutrition administration.

## **ACKNOWLEDGMENTS**

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## **COMMENTS**

### **Backgrounds**

Nutrition support has been widely used in the area of surgery, where the benefit on patients' prognosis is evident. Colorectal cancer is the fourth most common cancer in men and the third most common cancer in women worldwide, and is also a significant cause of morbidity and mortality throughout the world, thus an appropriate and feasible nutrition support strategy is necessary and beneficial for patients' prognosis.

## Research frontiers

Nutritional support is widely used in postoperative colorectal cancer patients, but the role of nutrients has not been clearly defined. This study investigated the effect of nutrition support on the outcomes of patients with different nutritional status.

## Innovations and breakthroughs

The authors found that appropriate and moderate nutritional intervention can significantly improve the postoperative outcome of the patients with colorectal cancer.

## Applications

The study provides a reference for daily clinical practice and future research. A prospective, multicenter, randomized, controlled trial with a larger sample is necessary to validate the statistical results and diminish bias.

## Peer review

Although this is a retrospective review, I believe it will be of interest to the readers. And, it does add something to the literature.

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## Application of a wire-guided side-viewing duodenoscope in total esophagectomy with colonic interposition

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### Abstract

Therapeutic endoscopic retrograde cholangiopancreatography (ERCP) is the mainstay treatment for bile duct disease. The procedure is difficult per se, especially when a side-viewing duodenoscope is used, and when the patient has altered anatomical features, such as colonic interposition. Currently, there is no consensus on the standard approach for therapeutic ERCP in patients with total esophagectomy and colonic interposition. We describe a novel treatment design that involves the use of a side-viewing duodenoscope to perform therapeutic ERCP in patients with total esophagectomy and colonic interposition. A gastroscope was initially introduced into the interposed colon and a radio-opaque standard guidewire was advanced to a distance beyond the papilla of Vater, before the gastroscope was withdrawn. A side-viewing duodenoscope was then introduced along the guidewire under fluoroscopic guidance. After cannulation into the papilla of Vater, endoscopic retrograde chol-

angiography (ERC) revealed a filling defect (maximum diameter: 15 cm) at the distal portion of the common bile duct (CBD). This defect was determined to be a stone, which was successfully retrieved by a Dormia basket after complete sphincterotomy. With this treatment design, it is possible to perform therapeutic ERCP in patients with colonic interposition, thereby precluding the need for percutaneous drainage or surgery.

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**Key words:** Wire-guided; Duodenoscope; Endoscopic retrograde cholangiopancreatography; Esophagectomy; Interposition of colon

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Yii CY, Chou JW, Peng YC, Chow WK. Application of a wire-guided side-viewing duodenoscope in total esophagectomy with colonic interposition. *World J Gastroenterol* 2011; 17(13): 1787-1790 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v17/i13/1787.htm> DOI: <http://dx.doi.org/10.3748/wjg.v17.i13.1787>

### INTRODUCTION

The application of a side-viewing duodenoscope in total esophagectomy with colonic interposition is technically difficult, because of the altered structure of the colon and the redundancy of the endoscopic route. We report a wire-guided treatment designed to overcome this pitfall by introducing a side-viewing duodenoscope along a radio-opaque standard guidewire to facilitate therapeutic ERCP in patients undergoing esophagectomy with colonic interposition. The use of this treatment method ensured the safety of wire-guided therapeutic ERCP in patients undergoing total esophagectomy with colonic interposition.

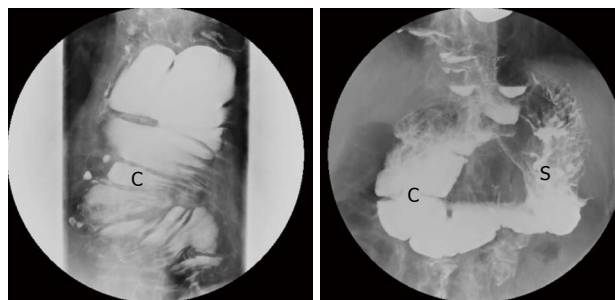
## CASE REPORT

An 87-year-old man was referred to our hospital, a tertiary referral medical center, for the management of episodic fever, chills, and right upper quadrant abdominal pain, which had been occurring intermittently for two months. He had undergone total esophagectomy with colonic interposition 17 years ago for the treatment of intractable esophageal ulcers with massive bleeding (Figure 1). He denied having passed tea-colored urine or clay-colored stool. Abdominal ultrasonography revealed dilatation of the common hepatic duct (CHD) and common bile duct (CBD; diameter: 1.45 cm). Magnetic resonance cholangiopancreatography (MRCP) showed the presence of a stone impacted at the distal portion of the CBD (Figure 2). The patient was intravenously administered midazolam (3 mg), pethidine (50 mg), and butylscopolamine (20 mg), and ERCP was performed with the patient in the left lateral position. A forward-viewing gastroscope (GIF-Q260, Olympus) was initially introduced; it was advanced through the interposed colonic segment, gastric remnant, and duodenum to reach the papilla of Vater. A radio-opaque standard guidewire (THSF-35-480, Wilson-Cook) was inserted deep into the small intestine, up to a distance beyond the papilla of Vater, via the accessory channel (Figure 3). The gastroscope was then withdrawn over-the-wire. Under fluoroscopic guidance, and with the patient in the left-lateral position, a side-viewing duodenoscope (IJF-240, Olympus) was introduced carefully along the guidewire until it reached the papilla of Vater. After cannulation with an ERCP catheter (StarTip cannula, PR-106Q-1, Olympus) as usual, cholangiography showed a filling defect (diameter, 1.5 cm) in the distal portion of the CBD; the lesion was determined to be a CBD stone (Figure 4). Complete sphincterotomy with a traction sphincterotome was performed (Figure 5). The pigmented stone was successfully retrieved using a Dormia basket (Figure 6). Subsequent balloon-occlusion cholangiography showed complete clearance of the CBD. The patient was followed up in the outpatient department and remains well.

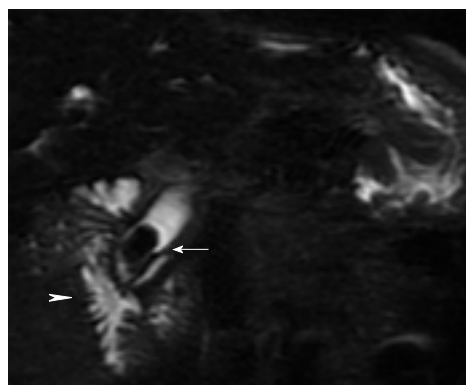
## DISCUSSION

The colon has been used as an esophageal substitute since 1911. It has been proven to be superior to other substitutes, such as the stomach and small intestine, because of its length, acid resistance, and richness of vascular supply. It affords good overall satisfaction and allows maintenance of a wider surgical resection margin in patients with cancers of the gastroesophageal junction. The disadvantages of its application include prolonged operation time, extensive preoperative preparation, and the late redundancy of colonic grafts<sup>[1,2]</sup>.

Therapeutic ERCP with the application of a side-viewing duodenoscope is widely used in the management of pancreatic or hepatobiliary diseases, such as biliary stones<sup>[3]</sup>. Technically, it is difficult to advance a side-viewing duodenoscope through the colon because the duodenoscope affords visualization of only areas to the sides of the scope, and because of the presence of colonic interhaustral folds,



**Figure 1** Esophagography showing the interposition of the colon (C) and the gastric remnant (S).

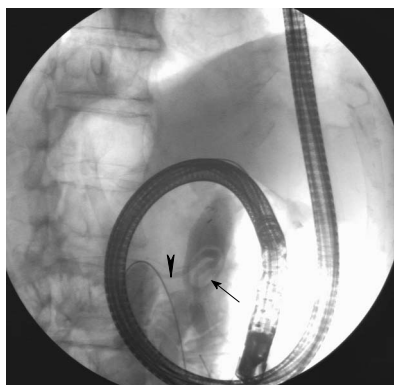


**Figure 2** Magnetic resonance cholangiopancreatography showing a stone in the distal common bile duct (arrow). The arrowhead shows the second portion of the duodenum.

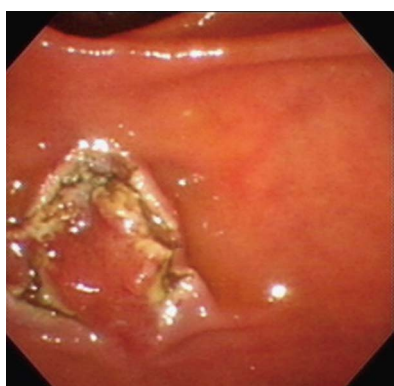


**Figure 3** The radio-opaque standard guidewire (arrowhead) was inserted through the working channel of the gastroscope.

the angulation of the colon, and the redundancy of the colonic graft<sup>[4]</sup>. To date, several techniques have been described for using the side-viewing duodenoscope to visualize the colon. Dafnis reported the successful application of a unique technique for approaching an inaccessible colonic polyp at the splenic flexure using an overtube to advance the side-viewing duodenoscope<sup>[5]</sup>. Another report of a case series on the management of inaccessible colonic polyps, advocated the technique of slightly bending the tip of the side-viewing duodenoscope, thereby providing a sloped-forward view for performing polypectomy<sup>[6]</sup>. We believe that the use of a wire-guided side-viewing duodenoscope



**Figure 4** With the patient in the left-lateral position, endoscopic retrograde cholangiopancreatography showed a filling defect in the distal part of the common bile duct (arrow). The arrowhead shows the pancreatic duct.

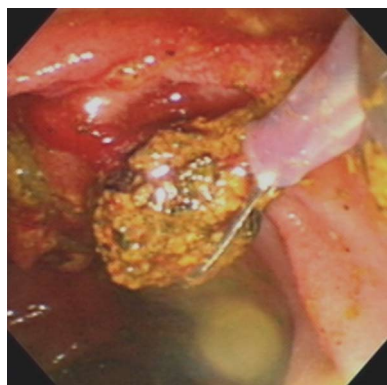


**Figure 5** Complete sphincterotomy.

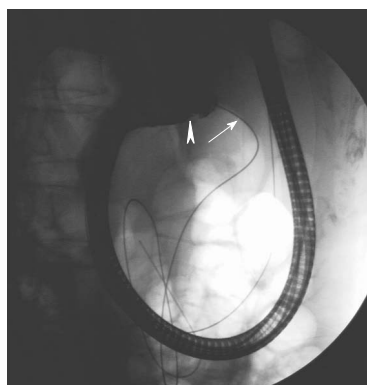
might represent a safe technique for approaching inaccessible colonic polyps.

In the present case, our most important concern was the smooth advancement of the duodenoscope through the colonic graft. To address this concern, we inserted a radio-opaque guidewire to serve as a roadmap. Fry *et al.*<sup>[7]</sup> reported an over-the-wire method by using a Super-Stiff Amplatz guidewire, which was actually designed for cardiac catheterization, to intubate the duodenum with a side-viewing duodenoscope in a patient with large paraesophageal hernia. The reason we chose the standard guidewire, instead of a Super-Stiff Amplatz guidewire, was because it is entirely radio-opaque. It facilitated the localization and visualization of the tip of the duodenoscope under close fluoroscopic guidance. Despite this, the duodenoscope did, at one point, move away from the appropriate path in the gastrointestinal tract, during the procedure. When the graft lumen could not be visualized on the endoscopic screen, we pushed the duodenoscope forward once its axis was the same as that of the wire, as determined by fluoroscopy; the scope was advanced in this manner until the graft lumen could be seen (Figure 7). The duodenoscope was advanced through the graft, and the CBD stone was eventually retrieved.

Manipulation of the guidewire is an art. One of its principles is to avoid looping, especially in a spacious cavity, such as the stomach. In our experience, we have observed



**Figure 6** The pigment stone retrieved by a Dormia basket.



**Figure 7** The duodenoscope (arrowhead) was pushed along the guidewire (arrow) at the same axis under fluoroscopic guidance.

that the looping of the guidewire may cause the failure of esophageal or duodenal metallic stent implantation in patients with malignant obstruction. The looping of the guidewire could render it difficult to introduce the scope further. To avoid this looping, we advanced the tip of the guidewire to a distance beyond the papilla of Vater, instead of stopping within the stomach.

Some experienced endoscopists prefer to backload the guidewire through the working channel of the duodenoscope. However, we think that this is not feasible because the side-viewing characteristic, with its acute angle of elevation. Backloading would render it difficult to insert the duodenoscope and would increase the number of loops formed. Furthermore, the double-balloon enteroscope could not be applied in our case because it is a forward-viewing scope and lacks the angle of elevation required to support the use of ERCP accessories.

Another technique that could have been considered in the present case would be the direct introduction of the side-viewing duodenoscope without the initial use of the forward-viewing gastroscope; however, this would have made it difficult to clearly visualize the lumen, especially as this patient had undergone colonic interposition. Such an approach would be accompanied by a high risk of perforation. The successful application of our technique for performing therapeutic ERCP is proof of the feasibility of this technique. To the best of our knowledge, this is the

first report on the use of this novel technique for treating a CBD stone in a patient with esophagectomy and colonic interposition.

In conclusion, in cases with rare clinical presentations, it is necessary to carefully and accurately estimate possible hindrances and develop appropriate solutions to successfully overcome them.

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## Meetings

### Events Calendar 2011

January 14-15, 2011  
AGA Clinical Congress of  
Gastroenterology and Hepatology:  
Best Practices in 2011 Miami, FL  
33101, United States

January 20-22, 2011  
Gastrointestinal Cancers Symposium  
2011, San Francisco, CA 94143,  
United States

January 27-28, 2011  
Falk Workshop, Liver and  
Immunology, Medical University,  
Franz-Josef-Strauss-Allee 11, 93053  
Regensburg, Germany

January 28-29, 2011  
9. Gastro Forum München, Munich,  
Germany

February 4-5, 2011  
13th Duesseldorf International  
Endoscopy Symposium,  
Duesseldorf, Germany

February 13-27, 2011  
Gastroenterology: New Zealand  
CME Cruise Conference, Sydney,  
NSW, Australia

February 17-20, 2011  
APASL 2011-The 21st Conference of  
the Asian Pacific Association for the  
Study of the Liver  
Bangkok, Thailand

February 22, 2011-March 04, 2011  
Canadian Digestive Diseases Week  
2011, Vancouver, BC, Canada

February 24-26, 2011  
Inflammatory Bowel Diseases  
2011-6th Congress of the European  
Crohn's and Colitis Organisation,  
Dublin, Ireland

February 24-26, 2011  
2nd International Congress on  
Abdominal Obesity, Buenos Aires,  
Brazil

February 24-26, 2011  
International Colorectal Disease  
Symposium 2011, Hong Kong, China

February 26-March 1, 2011  
Canadian Digestive Diseases Week,

Westin Bayshore, Vancouver, British  
Columbia, Canada

February 28-March 1, 2011  
Childhood & Adolescent Obesity:  
A whole-system strategic approach,  
Abu Dhabi, United Arab Emirates

March 3-5, 2011  
42nd Annual Topics in Internal  
Medicine, Gainesville, FL 32614,  
United States

March 7-11, 2011  
Infectious Diseases: Adult Issues  
in the Outpatient and Inpatient  
Settings, Sarasota, FL 34234,  
United States

March 14-17, 2011  
British Society of Gastroenterology  
Annual Meeting 2011, Birmingham,  
England, United Kingdom

March 17-19, 2011  
41. Kongress der Deutschen  
Gesellschaft für Endoskopie und  
Bildgebende Verfahren e.V., Munich,  
Germany

March 17-20, 2011  
Mayo Clinic Gastroenterology &  
Hepatology 2011, Jacksonville, FL  
34234, United States

March 18, 2011  
UC Davis Health Informatics:  
Change Management and Health  
Informatics, The Keys to Health  
Reform, Sacramento, CA 94143,  
United States

March 25-27, 2011  
MedicReS IC 2011 Good Medical  
Research, Istanbul, Turkey

March 26-27, 2011  
26th Annual New Treatments in  
Chronic Liver Disease, San Diego,  
CA 94143, United States

April 6-7, 2011  
IBS-A Global Perspective, Pfister  
Hotel, 424 East Wisconsin Avenue,  
Milwaukee, WI 53202, United States

April 7-9, 2011  
International and Interdisciplinary  
Conference Excellence in Female  
Surgery, Florence, Italy

April 15-16, 2011  
Falk Symposium 177, Endoscopy  
Live Berlin 2011 Intestinal Disease  
Meeting, Stauffenbergstr. 26, 10785  
Berlin, Germany

April 18-22, 2011  
Pediatric Emergency Medicine:  
Detection, Diagnosis and Developing  
Treatment Plans, Sarasota, FL 34234,  
United States

April 20-23, 2011  
9th International Gastric Cancer  
Congress, COEX, World Trade  
Center, Samseong-dong, Gangnam-  
gu, Seoul 135-731, South Korea

April 25-27, 2011  
The Second International Conference  
of the Saudi Society of Pediatric  
Gastroenterology, Hepatology &  
Nutrition, Riyadh, Saudi Arabia

April 25-29, 2011  
Neurology Updates for Primary  
Care, Sarasota, FL 34230-6947,  
United States

April 28-30, 2011  
4th Central European Congress of  
Surgery, Budapest, Hungary

May 7-10, 2011  
Digestive Disease Week, Chicago, IL  
60446, United States

May 12-13, 2011  
2nd National Conference Clinical  
Advances in Cystic Fibrosis, London,  
England, United Kingdom

May 19-22, 2011  
1st World Congress on Controversies  
in the Management of Viral Hepatitis  
(C-Hep), Palau de Congressos de  
Catalunya, Av. Diagonal, 661-671  
Barcelona 08028, Spain

May 21-24, 2011  
22nd European Society of  
Gastrointestinal and Abdominal  
Radiology Annual Meeting and  
Postgraduate Course, Venice, Italy

May 25-28, 2011  
4th Congress of the Gastroenterology  
Association of Bosnia and  
Herzegovina with international  
participation, Hotel Holiday Inn,  
Sarajevo, Bosnia and Herzegovina

June 11-12, 2011  
The International Digestive Disease  
Forum 2011, Hong Kong, China

June 13-16, 2011  
Surgery and Disillusion XXIV  
SPIGC, II ESYS, Napoli, Italy

June 14-16, 2011  
International Scientific Conference

on Probiotics and Prebiotics-  
IPC2011, Kosice, Slovakia

June 22-25, 2011  
ESMO Conference: 13th World  
Congress on Gastrointestinal Cancer,  
Barcelona, Spain

June 29-2, 2011  
XI Congreso Interamericano  
de Pediatría 'Monterrey 2011',  
Monterrey, Mexico

September 2-3, 2011 Falk Symposium  
178, Diverticular Disease, A Fresh  
Approach to a Neglected Disease,  
Gürzenich Cologne, Martinstr. 29-37,  
50667 Cologne, Germany

September 10-11, 2011  
New Advances in Inflammatory  
Bowel Disease, La Jolla, CA 92093,  
United States

September 10-14, 2011  
ICE 2011-International Congress of  
Endoscopy, Los Angeles Convention  
Center, 1201 South Figueroa Street  
Los Angeles, CA 90015,  
United States

September 30-October 1, 2011  
Falk Symposium 179, Revisiting  
IBD Management: Dogmas to be  
Challenged, Sheraton Brussels  
Hotel, Place Rogier 3, 1210 Brussels,  
Belgium

October 19-29, 2011  
Cardiology & Gastroenterology |  
Tahiti 10 night CME Cruise, Papeete,  
French Polynesia

October 22-26, 2011  
19th United European  
Gastroenterology Week, Stockholm,  
Sweden

October 28-November 2, 2011  
ACG Annual Scientific Meeting &  
Postgraduate Course, Washington,  
DC 20001, United States

November 11-12, 2011  
Falk Symposium 180, IBD 2011:  
Progress and Future for Lifelong  
Management, ANA Interconti Hotel,  
1-12-33 Akasaka, Minato-ku, Tokyo  
107-0052, Japan

December 1-4, 2011  
2011 Advances in Inflammatory  
Bowel Diseases/Crohn's & Colitis  
Foundation's Clinical & Research  
Conference, Hollywood, FL 34234,  
United States



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- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.00000035706.28494.09]

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- 5 **Vallancien G**, Emberton M, Harving N, van Moorseelaar RJ, Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

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- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

### Books

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

*Chapter in a book (list all authors)*

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

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- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

**Electronic journal** (list all authors)

- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

**Patent** (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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