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EDITING
Editorial Board of *World Journal of Gastroenterology,* 77 Shuangta Xijie, Taiyuan 030001,
Shanxi Province, China
Telephone: +86-351-4078656
E-mail: wjg@wjgnet.com

PUBLISHING
Editorial Department of *World Journal of Gastroenterology,* 77 Shuangta Xijie, Taiyuan 030001,
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EDITORIAL

Endoscopic mucosal resection of early gastric cancer: Experiences in Korea

Jun Haeng Lee, Jae J Kim

Jun Haeng Lee, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, South Korea
Jae J Kim, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, South Korea
Correspondence to: Jae J Kim, Division of Gastroenterology, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Irwon-dong, Gangnam-gu, Seoul 135-710, South Korea. jjkim@smc.samsung.co.kr
Telephone: +82-2-34103409 Fax: +82-2-34103849
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Abstract

Endoscopic mucosal resection (EMR) has been established as one of the treatment options for early gastric cancer (EGC). However, there are many uncertain areas such as indications of EMR, best treatment methods, management of complications and follow-up methods after the procedure. Most studies on this topic have been carried out by researchers in Japan. In Korea, gastric cancer is the most common malignant disease, and the second leading cause of cancer death. In these days, EMR for EGC is widely performed in many centers in Korea. In this review, we will provide an overview of the techniques and outcomes of EMR in Korea.

Key words: Endoscopic mucosal resection; Early gastric cancer; Indication; Complication; Prognosis

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INTRODUCTION

Early gastric cancer (EGC) is defined as gastric carcinoma confined to the mucosa or submucosa regardless of the presence of regional lymph node metastases[1]. The detection rate of EGC has been steadily increasing because of technical advances and awareness of benefit from early diagnosis, especially in eastern countries. Patients who undergo resection for EGC have an excellent prognosis, with a 5-year survival rate of over 90%[2]. However, the quality of life after conventional surgical resection of gastric cancer is substantially impaired. Therefore, less invasive treatment options for EGC have been developed. Endoscopic mucosal resection (EMR) was first introduced as a treatment modality for EGC in 1984. Since then, various accessories and techniques of EMR have been developed. Early data suggest that EMR provides a survival rate of 90% comparable to that of surgery if the technique is applied with the appropriate indication[3,4]. In addition, morbidity and mortality associated with surgery can be avoided and specimens for accurate pathologic staging can be obtained. Therefore, EMR is currently accepted as a standard treatment for selected cases with EGC[5-7]. Most studies on this topic were performed in Japan, where the incidence of early gastric cancer is very high. Recently, experiences of EMR for EGC in other countries are increasingly reported[7-10]. In this review, we will provide an overview of the techniques and outcomes of EMR in Korea.

INDICATIONS OF EMR

With technical advances of EMR, the size of a lesion which can be resected en bloc is becoming larger[6]. Care must be given because EMR has a very important limitation that lymph nodes cannot be dissected. Data from Korea and Japan have shown that the incidence of lymph node metastasis in intramucosal EGC is about 2%-3% and the risks increase up to 20% when submucosal invasions are present[6,11-13]. Because results of long-term controlled trials are not available, the current indications of EMR are based on the detailed analysis of pathology results from surgically resected gastric cancers. Regarding this issue, inter-observer and/or inter-institutional variation in the pathology report of surgical and EMR specimen may be a great problem[14]. In addition, pathology reports before and after EMR may be different. For example, pathology specimen of EMR for gastric dysplasia in some cases may show gastric cancers[15]. It is important to standardize the pathology report to compare surgery or EMR results from different institutions. In Korea, the Gastrointestinal Pathology Study Group of the Korean Society of Pathologists developed a standardized reporting format for gastric cancer[16].

The ideal candidates for EMR are EGC patients who have no risk of lymph node metastasis. The problem is that there is no method that can definitely evaluate

the status of lymph node without surgical dissection. Ideally, endoscopic ultrasound (EUS) should be useful for selecting patients without lymph node metastasis. However, clinical studies evaluating the role of EUS before EMR for EGC have shown unsatisfactory results[17,18]. Although standard surgery for gastric cancer is one of the most safe abdominal surgical procedures, the mortality rate is around 0.5%-1.0% in eastern countries[19]. This rate was considered when selecting candidates for EMR, because the risk of lymph node metastasis in patients with EMR should be lower than the surgical risk. From analysis of surgical data, patients with EGC who had minimal risk of lymph node metastasis could be identified and indications of EMR could be established. The current accepted indications of EMR for the treatment of EGC are as follows: (1) differentiated (well- and/or moderately differentiated adenocarcinoma and/or papillary carcinoma) type confined to the mucosa; (2) smaller than 2 cm for superficially elevated type lesions; (3) smaller than 1 cm for the flat and depressed type lesions; (4) without ulcer or ulcer scar; and (5) without venous or lymphatic involvement[6].

Recently, based on some clinical observation and surgical data, expanded criteria for EMR have been proposed[6,20,21]. One report in which EMR indications included EGC lesions as large as 3 cm showed the disease free survival rates of 98% during a median follow-up of 38 mo when complete resections were performed[4]. Recent large surgical data from Gotoda *et al* also provided supporting evidence for expanded criteria. In differentiated mucosal cancer which size was 3 cm or smaller, no lymph node metastasis was observed irrespective of the lesion ulceration; in differentiated mucosal cancer without ulceration, no patient had nodal metastasis regardless of tumor size; and, finally, in differentiated minute submucosal cancer (SM1), no nodal metastasis was found if tumor size was no more than 3 cm[21]. In some institutions, EMR for selected cases with poorly differentiated type of EGC has been tried[22]. However, a long-term follow-up seems to be necessary to make a firm conclusion. With the recent technical advancement, endoscopic treatment of recurred gastric cancer after EMR has also been tried in some institutions[23].

Regarding the indications of EMR for EGC, we have a relatively conservative position for adopting expanded criteria because of the following reasons: (1) long-term outcome data for expanded criteria are still insufficient; (2) distinction between SM1 (upper one-third of the submucosal layer) and SM2 (middle one-third of the submucosal layer) in pathologic specimen is very subjective; and (3) the thickness of resected submucosal layer is not constant.

TECHNIQUES OF EMR

The instruments and methods of EMR in Korea are basically the same as in Japan. The most advanced technique of EMR is endoscopic submucosal dissection (ESD)[24], which is also widely performed in Korea. Detailed description of the technical aspects is out of the scope of this review. However, some comments seem to be necessary.

The practice of EMR requires application of additional techniques to standard endoscopy. Chromoscopy is important for delineating the border and assessing the depth of the lesion. Most commonly used stain is indigo carmine solution. During the indigo carmine chromoendoscopy before EMR, some additional lesions of a few millimeters may be found. However, most of the additionally detected lesions are non-neoplastic[25]. Therefore, routine chromoendoscopy for normal-looking mucosa seems to be unnecessary. Narrow band imaging (NBI) with magnification is a relatively new technique to evaluate the mucosal surface in detail[26]. However, it is not certain whether application of NBI before EMR helps increase the rate of complete resection.

Ideally, endoscopic ultrasonography (EUS) should be used in order to further ascertain the depth of invasion before EMR. Accuracy of endoscopic ultrasonography for predicting the depth of gastric cancers was reported to be 70%-80%[18,27,28]. This kind of accuracy range cannot be considered to be sensitive enough to select cancers limited in the mucosal layer. In the EUS evaluation of EGC, overstaging of mucosal cancer as submucosal cancer is reported to be quite common[18]. The overstaging can lead to unnecessary surgery which may have a great impact on the patient's quality of life. In contrast, EUS has some limitation in the detection of microinvasion into the submucosal layer. Until now, the use of EUS before EMR seems to be operator-dependent.

The list of EMR methods is quite long[29], but the basic steps are in common: (1) delineation of the lateral margin with or without chromoendoscopy, (2) marking using brief burst of electrocautery or argon plasma coagulation, (3) submucosal injection to lift the lesion, and (4) resection of the lesion. Before the development of ESD, EMR with circumferential precutting (EMR-P) was the best method to cut larger lesions in one piece[30]. Recently, ESD has become the most commonly used method to resect large lesions[31,32]. ESD was originally developed in Japan, but it is also being performed in other parts of the world[10,33,34]. In Japan, the development of ESD was largely based on new devices like IT-knife, Flex-knife, Hook-knife, triangle-tipped knife, and narrow calibered transparent hood. In Korea, new types of knives such as Fixed flexible snare and Endo FK were also developed.

COMPLICATIONS OF EMR

The complications of EMR include pain, bleeding, perforation, and EMR-induced ulcer. Pain after resection is typically mild and dull in nature. Pain can be controlled using a standard dose of proton pump inhibitor (PPI) twice a day with or without analgesics. Bleeding is the most common complication and most bleeding occurs during the procedure or within 24 h[9]. Bleeding can be successfully treated in most cases through coagulation of the bleeding vessels, or placement of metallic clips.

EMR-induced ulcer is reported to heal faster and to recur less often than noniatrogenic gastric ulcer and usually treated with antisecretory agents. One study showed that all ESD-induced ulcers healed within 8 wk regardless of

size and location using standard doses of PPI for 8 wk[35]. However, there is no consensus regarding the duration of PPI therapy for these ulcers. Recently, our institution reported that 1 wk omeprazole therapy is equivalent to 4 wk therapy in terms of EMR-induced ulcer healing rate and ulcer-related symptoms[36]. It is not certain whether administration of anti-acids before EMR reduces the complications of EMR. Watanabe *et al*[37] conducted a randomized clinical study in which EMR was performed with or without 1 wk of preoperative PPI administration. Artificial ulcers created by EMR healed more rapidly in patients who received preoperative PPI. They concluded that preoperative administration of PPI before EMR is useful for controlling and preventing bleeding, and for facilitating the healing of artificial ulcers[37]. Further studies are necessary to determine the optimal medical treatment in terms of selection of drugs, duration of treatment, mode of administration and the necessity of pre-EMR medication.

The rate of perforation by EMR is about 1%-7%[5,38]. Immediately recognized small perforations can be successfully treated non-surgically with a combination of endoscopic clipping, nasogastric suction, and broad-spectrum antibiotics[38]. Large perforations require immediate surgery. However, management of patients with microperforation (case with free air on chest X-ray after EMR without recognizing perforation during the procedure) is not well established. In our institution, we experienced 13 cases of microperforations during last two and a half years[39]. Among them, 11 cases were successfully treated only with fasting, nasogastric tube drainage, and broad-spectrum antibiotics. About two thirds of the patients with microperforation (7/11) experienced abdominal pain that required short-term intermittent intravenous analgesics. No additional endoscopic treatment was needed[39]. In order to detect microperforation, we recommend to check chest X-ray immediately after EMR.

ADDITIONAL TREATMENT AFTER EMR

Histological examination after EMR is very important to determine the completeness of resection. Radical surgery or additional endoscopic treatment may be recommended for patients with incomplete resection.

Histologically, positive resection margin can be divided into two types; positive lateral resection margin and positive vertical resection margin. If only the lateral margin is positive, additional endoscopic treatment may be tried. However, great care must be given to the positive vertical resection margin. When the vertical margin is positive for malignant cells, the depth of invasion cannot be determined. In EMR, the depth of resection is usually the mid-submucosal layer. So, the positive vertical resection margin usually means that the depth of tumor invasion is SM2 or more. Chung *et al*[40] reported their result of surgery after incomplete endoscopic resection for EGC. In 10 patients with positive resection margin in EMR specimens, there were 2 cases with lymph node metastasis[40]. In our opinion, radical surgery is mandatory when the vertical resection margin of the EMR specimen is positive.

EMR is usually attempted when the cancer is thought to be limited to the mucosal layer. However, about 10%-20% of EMR specimens show evidence of submucosal invasion[4]. There is no consensus on the necessity of additional surgery for these cases. The safest way is to do radical surgery in every patient with submucosal invasion. However, careful observation is a possible option for cases with minute submucosal invasion in SM1 layer with negative vertical margin[5]. The cutoff value of minute submucosal invasion in SM1 layer is usually 500 μm. Recently, Cho *et al*[41] raised a concern about this cutoff value. When a 2 cm × 2 cm piece of porcine gastric wall was stretched into 3 cm × 3 cm, the thickness of submucosal layer decreased from 500 μm to 200 μm. This was exactly the same in the human gastric mucosa. When a 2.5 cm × 1.0 cm piece of human gastric antral wall was stretched into 3.0 cm × 1.0 cm, the depth of submucosa decreased from 620-650 μm to 250-300 μm[41].

OUTCOMES OF EMR

The best way of evaluating the efficacy of a new treatment is a long-term, large-scaled randomized controlled trial. However, the excellent prognosis after surgical treatment (especially in cases indicated for EMR) makes controlled trial almost impossible. So, the best feasible evidence of the efficacy of EMR is a long-term clinical follow-up data.

Earlier experiences of EMR for EGC from 12 major institutions in Japan were reported by Kojima *et al*[42] in 1998. The lift-and-cut, EMR using a cap (EMR-C), and EMR with ligation (EMR-L) techniques were most commonly used. En bloc resection rate was 75.8%, and complete resection rate was 73.9%. The follow-up period was from 4 mo to 11 years. Recurrence rate after histopathologically documented eradication was 1.9% and recurred lesions were treated with endoscopic retreatment or surgery. The disease-specific survival rate was 99.1%.

The most commonly referenced result of endoscopic mucosal resection of EMR was published in 2001 by Ono[4]. Four hundred and seventy nine cancers in 445 patients were treated by EMR from 1987 to 1998, but submucosal invasion was found on subsequent pathological examination in 74 tumors. Sixty nine percent of intramucosal cancers (278/405) were resected with a clear margin. Local recurrence in the stomach occurred in 17 lesions followed conservatively, in one lesion treated endoscopically, and in five lesions with complete resection. There were no gastric cancer related deaths during a median follow up period of 38 (3-120) mo[4].

Recently, Oda *et al* reported the outcomes of ESD for EGC using IT knife, which was superior to that of other conventional methods[43]. They used expanded criteria, suggested by Gotoda *et al* as mentioned above[5,6]. *En bloc* resection rate was up to 98%, and complete resection rate was 83%[43].

In Korea, a multi-center, retrospective study has been performed[34]. From January 2000 to December 2002, 514 EGCs in 506 patients were treated by EMR in 13 institutions. The most commonly used technique was circumferential precutting followed by snare resection

(EMR-P, *n* = 269, 52.3%). Complete resection and incomplete resection after EMR were confirmed in 399 lesions (77.6%) and 103 lesions (20.0%), respectively. For completely resected mucosal cancers (*n* = 399), the median duration of follow-up was 23.5 (range, 5-70) mo. In this group, local recurrence was detected in 24 cases (6.0%) with a median interval between EMR and recurrence of 17.9 (range, 3.5-51.7) mo. There were 3 cases with perforation and 71 cases with bleeding. There was no death related with recurrence of gastric cancer during the overall median follow-up period of 39 mo[34].

In our institution, 283 patients with EGC have been treated by EMR from January 2000 to June 2005[39]. The median age of the patients was 64 (range 26-85) years. The male to female ratio was 3.2:1. The methods of EMR were mainly snare resection after circumferential precutting (EMR-P, *n* = 162) and ESD (*n* = 91). The criteria for curative resection were en bloc resection or complete resection in piecemeal resection, well or moderately differentiated histology, free of tumor in resected margin, intramucosal lesion, and no vascular or lymphatic invasion. Additional treatments, usually surgery, were recommended for cases with non-curative or non-evaluable results. The median duration of follow-up was 21 (range 3-66) mo. The mean size of cancerous lesion was 1.38 cm. The overall rate of curative resection was 72.1%. The rate of curative resection was highest with ESD (80.2%), followed by EMR-P (70.3%). Submucosal invasion was found in 44 cases (15.5%). In patients with curative resection, local recurrence at EMR site was found in only one case (0.5%). In 51 cases who underwent surgical resection due to non-curative or non-evaluable resection, residual cancer was found in 13 cases (25.0%). Among 28 patients, who were followed up without surgery after non-curative or non-evaluable results, there were 13 recurrences (12 local recurrences and 1 hepatic metastasis) after a median follow-up of 7 mo. Five patients died during the follow-up period, but there was no death related to gastric cancer[39].

Jung *et al*[44] showed comparable results to ours. In that study, 341 EGC patients were treated by EMR-P. Complete resection rate was 84.5%. During 47 mo of follow-up, 1.4% of patients underwent recurrence after complete resection. Recurrence rate after incomplete or non-evaluable resection without additional treatment was 9.1%. The median duration between EMR and local recurrence was 21 mo. The overall 5-year survival rate after EMR was 90% and no death from gastric cancer occurred during follow-up[44].

Rye *et al*[45] reported their follow-up results after EMR for gastric adenoma or EGC. More than 80% of cases were treated with endoscopic incision and submucosal dissection (EISD). The recurrence rate was 9.6% (4/41) in patients treated with conventional EMR, and 3.5% (8/230) in patients treated with EISD[45].

Lee *et al*[41] reported a prospective randomized controlled trial of EMR evaluating the efficacy of a fibrinogen mixture as a submucosal injection solution. No significant differences were observed between the 2 groups (the fibrinogen mixture group versus the normal saline group) in the rates of en bloc resection (80.6% *vs* 88.9%), complete resection rate (86.1% *vs* 80.6%), and recurrence rate (3% *vs* 6.1%)[41].

Youn *et al*[46] reported their clinical outcomes of EMR for EGC. The overall complete resection rate was 84.6% (126/149) while complete resection rate of 93.5% was achieved in mucosal cancers (115/123). The success of complete resection was significantly affected by endoscopic gross type (depressed lesion), the degree of differentiation, and the depth of invasion, independently. There were 5 cases of local recurrence with no disease-related or treatment-related mortality during the follow-up period[46].

FUTURE PERSPECTIVES

Recent data suggest that EMR provides comparable results to surgery for selected cases of EGC. In addition, limitations in EMR have been reducing with the technical advancement. However, to treat more EGCs with EMR, some efforts need to be made: (1) More long-term follow-up data are necessary to support the role of EMR in EGC treatment. Multicenter prospective studies should be performed in many countries. (2) The technical details of EMR need to be standardized, so that more endoscopists can perform EMR with an acceptable level of technical skills. Teaching systems by experts may help trainees to challenge EMR more easily; (3) Standardization of the pathological interpretation of resected specimen is necessary, so that the results from various institutions can be shared and compared. With these efforts, EMR will become safer and more reliable methods for EGC treatment.

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EDITORIAL

Role of endoscopic ultrasound in diagnosis and therapy of pancreatic adenocarcinoma

Joseph Boujaoude

Joseph Boujaoude, Department of Gastroenterology, Hotel-Dieu de France hospital, Saint-Joseph University, Beirut, Lebanon
Correspondence to: Joseph Boujaoude, Department of Gastroenterology, Hotel-Dieu de France hospital, Saint-Joseph University, Beirut, Lebanon. jbouj@hotmail.com
Telephone: +961-3-704890 Fax: +961-1-615295
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Abstract

Since its advent more than 20 years ago, endoscopic ultrasound (EUS) has undergone evolution from an experimental to a diagnostic instrument and is now established as a therapeutic tool for endoscopists. Endoscopic ultrasound cannot accurately distinguish benign from malignant changes in the primary lesion or lymph node on imaging alone. With the introduction of the curved linear array echoendoscope in the 1990s, the indications for EUS have expanded. The curved linear array echoendoscope enables the visualization of a needle as it exits from the biopsy channel in the same plane of ultrasound imaging in real time. This allows the endoscopist to perform a whole range of interventional applications ranging from fine needle aspiration (FNA) of lesions surrounding the gastrointestinal tract to celiac plexus block and drainage of pancreatic pseudocyst. This article reviews the current role of EUS and EUS-FNA in diagnosis, staging and interventional application of solid pancreatic cancer.

Key words: Endoscopic ultrasound; Diagnosis; Therapy; Pancreas; Adenocarcinoma

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INTRODUCTION

Since its advent more than 20 years ago, endoscopic ultrasound (EUS) has undergone evolution from an experimental to a diagnostic instrument and is now established as a therapeutic tool for endoscopists. Endoscopic ultrasound cannot accurately distinguish benign from malignant changes in the primary lesion or lymph node on imaging alone. With the introduction of the curved linear array echoendoscope in the 1990s, the indications for EUS have expanded. The curved linear array echoendoscope enables the visualization of a needle as it exits from the biopsy channel in the same plane of ultrasound imaging in real time. This allows the endoscopist to perform a whole range of interventional applications ranging from fine needle aspiration (FNA) of lesions surrounding the gastrointestinal tract to celiac plexus block and drainage of pancreatic pseudocyst. This article reviews the current role of EUS and EUS-FNA in diagnosis, staging and interventional application of solid pancreatic cancer.

EUS-GUIDE FNA/BIOPSY

With the advent of EUS-FNA, it becomes a viable and useful alternative procedure for acquiring a tissue diagnosis to confirm the presence of pancreatic cancer. The feasibility varies from 90% to 98% and the efficiency in terms of collecting analyzable biopsy specimens varies from 80% to 95%. For the diagnosis of pancreatic adenocarcinomas, the sensitivity of EUS-FNA varies from 75% to > 90%, the specificity being 82%-100%, with a mean accuracy of 85%[1-9]. What is the technique of choice to obtain cytologic and/or histologic material from a mass suspected to be pancreatic cancer? To respond to this important issue, we must discuss it in different clinical scenarios.

Unresectable pancreatic tumor

CT scan or magnetic resonance imaging (MRI) is a high-resolution, noninvasive, cross-sectional imaging modality. It is a very accurate technique in the diagnosis and predicting resectability when a pancreatic mass or cancer is suspected. It is generally the first test ordered in such cases[10,11]. If a pancreatic mass is clearly unresectable based on CT or MRI results, either percutaneous image-guided or EUS-guided FNA can be performed for a tissue diagnosis to confirm the presence of cancer and to offer chemotherapy or radiation.

EUS-FNA with failed alternative biopsy techniques: There is strong support for the use of EUS-FNA in pancreatic masses when other biopsy techniques have failed. In fact, in virtually all series of EUS-FNA, failure

of another biopsy technique is a common indication for EUS-FNA and yet, in these series, they were still capable of obtaining a definite cytologic diagnosis in 80% to 95% of cases[12,13].

EUS-FNA for lesions not visible or accessible to other imaging modalities: At times, small pancreatic masses may not be detectable, even on a multidectector CT scan[14]. The study by Hoewhat *et al*[15] contained 6 patients in whom CT or US could not discern small pancreatic lesions. Then EUS-FNA is clearly the preferred sampling technique if a pathologic specimen is indicated. A recently published retrospective study of 1000 cases of pancreatic FNA also found that EUS-FNA was more accurate than percutaneous techniques for masses < 3 cm[16].

EUS-guided FNA is the only preoperative procedure which can demonstrate invasion of lymph nodes located in the celiac, lumboaortic, retroduodenopancreatic or superior mesenteric regions[7]. Aspiration of ascitic fluid with a cytological study done by EUS can validate a carcinomatosis that could not be revealed using conventional imaging[17]. Recently, Tenberge *et al*[18] demonstrated that small metastases of the left liver lobe could be found and were easily accessible to biopsy by means of EUS. The finding of such lesions modifies considerably the management of supposed resectable cancer.

EUS-FNA when alternative techniques are possible: When a pathology specimen is truly the only reason for EUS-FNA, published trials directly comparing EUS-FNA to alternative sampling techniques such as CT or TUS-guided FNA/biopsy or endoscopic retrograde cholangiopancreatography (ERCP) are extremely rare[15,19,20]. In a retrospective review of CT-FNA *vs* EUS-FNA for pancreatic masses, Qian and Hecht reported a sensitivity of 71% for CT-FNA and only 42% for EUS-FNA for pancreatic malignancies. Recently, Horwhat *et al* present the unique randomized, prospective cross-trial of EUS-FNA *vs* CT- or US-FNA for diagnosing cancer in pancreatic mass lesions. There was no significant difference in the sensitivity or accuracy of CT/US-FNA and EUS-FNA, although a trend was not observed for increased sensitivity of EUS-FNA.

Multiple factors favoring EUS-FNA over transcutaneous FNA of pancreatic cancer are as follows: (1) Decision analysis models have been used for the impact of EUS-FNA in patients with pancreatic cancer because of the similarities in sensitivities and specificities of the various biopsy techniques. EUS-FNA as the primary diagnostic modality was the most cost-effective approach[21]. Fritscher-Ravens *et al*[22] showed that EUS-FNA in pancreatic cancer changed the surgical approach in 21% of patients and the therapeutic approach in 44%. (2) A factor favoring EUS-FNS over transcutaneous FNA of pancreatic cancer is the possible risk of needle tract seeding. In a large series of percutaneous or CT-FNA of abdominal lesions and masses, seeding in pancreatic cancer occurred most commonly in the skin, or with EUS-FNA the skin is not traversed[23]. (3) Other advantages of EUS-FNA may be a short needle track. Indeed, the aspiration needle travels from the gut lumen to the lesion, a pathway that usually does not cross peritoneal or pleural surfaces and the complete needle tract is included in the resected specimen. The exception to this is in EUS-FNA of liver lesions and of pancreatic body/tail masses where the lesser sac of the peritoneum is breached. A case of gastric wall seeding after EUS-FNA of a pancreatic tail adenocarcinoma was reported recently was reported[24]. Micames *et al*[25] with their retrospective, non-randomized series comparing CT-FNA with EUS-FNA of pancreatic masses showed that there were significantly more peritoneal failures after neoadjuvant chemoradiation in patients having had CT biopsy (16.3%) *vs* EUS-FNA (2.2%).

Because of its advantages in imaging pancreatic neoplasms, high diagnostic yields, and the concern over needle-tract seeding with transcutaneous aspiration, the 6th edition of the handbook on cancer staging by the American Joint Committee on Cancer recommended EUS-FNA as the preferred sampling technique in pancreatic masses if it is available[26].

Equivocal resectability of pancreatic tumor

If CT or MRI results show a pancreatic mass with equivocal resectability, EUS is generally the next staging procedure. If this reveals that the mass is clearly unresectable, one can proceed with EUS-guided FNA for tissue diagnosis. If the EUS results show that the mass is potentially resectable, then EUS-FNA should be reconsidered.

Resectable pancreatic tumor

In case of a resectable tumor, a histological diagnosis is not necessary and of little use because it does not change the ultimate need for operation. However, because some institution has a protocol or policy of giving preoperative neoadjuvant chemotherapy or radiation in resectable pancreatic adenocarcinoma, tissue diagnosis would be a prerequisite for that[27]. Others argue that pre-operative diagnosis can exclude the occasional patients with unusual histology found in 5% to 10% of pancreatic tumors (lymphoma, endocrine tumors and metatstases) who would not benefit from operation[28,29]. Sometimes a patient may demand a conclusive cancer diagnosis before consenting surgery.

Differential diagnosis of solid mass within the pancreas

The presence of a solid mass within the pancreas does not necessarily imply the diagnosis of pancreatic cancer. It concerns the difficult problem of pseudotumor, chronic pancreatitis and autoimmune pancreatitis.

EUS-FNA may be problematic in case of chronic pancreatitis because differentiating well-differentiated carcinoma from inflammatory atypia can be challenging[30]. Recent reports indicate that EUS-FNA coupled with molecular analysis could improve the sensitivity (81%), the specificity (100%), and the accuracy (85%) of the diagnosis of pancreatic carcinomas in comparison with each technique alone[31].

INTERVENTIONAL APPLICATION OF EUS-FNA OF SOLID PANCREATIC CANCER

EUS-guided celiac block and neurolysis

The pancreatic nerves are autonomic and are sensitive to chemical and mechanical stimuli. They transmit visceral afferent information to celiac plexus and then centrally *via* the splanchnic nerves. The plexus is composed of two ganglia, usually located anterior and lateral to the aorta at the level of the celiac trunk.

Debilitating pain is a common symptom in patients with pancreatic cancer. Pain tends to be a difficult symptom to treat and can require high-dosage narcotics for relief with a number of associated side effects.

Celiac plexus neurolysis (CPN) refers to permanent ablation of the celiac plexus. This is done with ethanol or alcohol. Celiac plexus neurolysis by a surgical or radiographic approach has been available for many years for palliative treatment of unresectable pancreatic cancer. The procedure is carried out via a posterior approach with potentially serious complications. More recently, the development of endoscopic ultrasound using curved-array linear echoendoscope allows direct access to the celiac ganglia. Theoretically, EUS-guided celiac plexus neurolysis should be safer than the posterior technique without the need to traverse the diaphragm, spinal nerves, or spinal arteries. In addition, a short needle can be used and the injection can be carried out with real-time imaging. A meta-analysis of 24 publications and 1145 patients treated with percutaneous celiac plexus neurolysis for cancer pain found good to excellent relief in 70%-90% of the patients for up to 3 mo[32]. In 1996, Wiersema and Wiersema[33] reported the safety and efficacy of endosonographic celiac plexus neurolysis with absolute alcohol in patients with pancreatic cancer. In their series, 79%-88% of patients had persistent improvement in their pain score and 82%-91% required the same or less pain medication. Gunaratnam *et al*[34], in a prospective study of 58 patients with unresectable and painful pancreatic cancer found that 78% of the patients improved their pain score after EUS-guided celiac plexus neurolysis. Mild complications include transient diarrhea (4%-15%), transient hypotension (1%) and transient increase in pain (9%). The major complications (2.5%) include retroperitoneal bleeding and abscess formation.

Celiac plexus neurolysis with alcohol should be considered as a first-line therapy for patients with pain due to pancreatic cancer. It is important to emphasize a realistic goal, which is not to eliminate pain but to optimize oral pharmacologic therapy and to allow a dose reduction to minimize the side effects. In summary, despite the paucity of data, EUS CPN appears to be as effective and safe as other methods of CPN for providing pain relief from pancreatic cancer. The EUS approach may be the most cost effective if CPN is performed at the time of biopsy and staging.

Radiofrequency

Image-guided ablative therapies with thermal energy sources such as radiofrequency (RF), microwaves, and laser energy have received much attention as minimally invasive strategies for the management of focal malignant disease. Percutaneous RF-induced tissue coagulation has been used in early clinical trials for the management of hepatocellular carcinoma, hepatic, cerebral metastasis and benign bony lesions (osteoid osteoma). The development of endosonographically placed therapeutic devices may provide a unique alternative for the management of premalignant pancreatic lesions and potentially may offer palliative therapy for surgically unresectable malignant pancreatic tumors. The study of Goldberg *et al*[36] demonstrated the technical feasibility of EUS-guided RF ablation in the porcine pancreas. Resultant coagulation necrosis is well visualized with EUS or CT with excellent radiologic-pathologic correlation. This technique appears to be well tolerated.

Photodynamic therapy

Photodynamic therapy (PDT) has emerged as a useful method for the ablation of malignant and benign tumors of epithelial-lined and solid organs[37]. Studies of PDT in the pancreas demonstrate that photosensitizers are avidly taken up by pancreatic tissue[38]. Light exposure with resulting localized tissue necrosis has been achieved by percutaneous placement of PDT catheters into malignant pancreatic tissue.

In the study of Chan *et al*[39], EUS was used to guide placement of quartz optical fiber with light diffuser in the pancreas, liver, spleen and kidney of 3 farm swine. This study demonstrates that EUS-guided low-dose PDT ablation of pancreas is feasible and safe and it might be most applicable to small lesions in the pancreas and the liver.

EUS-guided transhepatic cholangiography

ERCP with stent placement is the procedure of choice for biliary decompression in patients with obstructive jaundice due to pancreatic cancer. However, decompression may be unsuccessful because of an anatomic variation, peripapillary diverticulum, deep tumor infiltration or insufficient drainage despite successful stent placement. Alternative approaches for accessing and draining obstructed ducts include percutaneous transhepatic (PT) cholangiography and surgery. PT drainage has a complication rate of up to 32% including fistula, cholangitis, biliary peritonitis, hematoma and liver abscesses[40]. Surgery offers long-term decompression but is associated with high morbidity and postoperative mortality rates[41]. Interventional EUS-guided cholangiography (IEUC) is a relatively new technique, permitting therapeutic biliary procedures when ERCP is not successful. EUS-guided opacification and drainage of obstructed pancreatic and biliary ducts has been described in case report[42-46]. This usually involves a direct transgastric or transduodenal approach, with stent placement through an endoscopically created fistula. Advantages of IEUC over percutaneous transhepatic (PTC) drainage include puncture of the biliary tree with real-time US when using color-doppler information. This usually involves a direct transgastric or transduodenal approach with stent placement through an endoscopically

created fistula. Although the only reported complication is bile leak, potential complications include bleeding, bowel perforation, infection and pneumoperitoneum. The extrahepatic approach has a greater chance of complication than the intrahepatic approach. Long-term follow-up and further studies comparing IEUD with PTC are required before the use of these techniques becomes widespread.

Delivery of anti-tumor agents

In 2000, Chang *et al*[47] was the first to publish a phase I clinical trial which showed that local immunotherapy, an allogenic mixed lymphocyte culture (cytoimplant), injected in 8 patients with unresectable pancreatic adenocarcinoma under EUS guidance is feasible and safe.

Hecht *et al*[48] delivered an anti-tumor viral therapy under EUS guidance, into the primary pancreatic tumor in 21 patients with locally advanced or metastatic disease. It was given in combination with gemcitabine Ⅳ. They obtained partial regression or stabilization of the disease in 10 of 21 patients.

In the United European Gastroenteorogy Week (UEGW) 2005, Farrell *et al*[49] presented their institution experience with EUS-guided delivery of TNFerade (replication deficient adenovector containing human TNFα gene, regulated by a radiation-inducible promoter Egr-1) for patients with unresectable, locally advanced adenocarcinoma of the pancreas in combination of 5 FU Ⅳ and radiation. Multiple injections within the pancreatic mass were done. Three fifth of patients subsequently underwent uncomplicated pancreatic surgical resection.

The most recent EUS-guided anti-tumor therapy involves a novel gene therapy. In this study, Chang et al delivered TNFerade percutaneously at a single site in the tumor while up to 4 injections were given by EUS-guided fine needle injections (FNI) in combination with 5 FU Ⅳ in 37 patients with unresectable pancreatic adenocarcinoma. Tumor responses and disease control were similar in the 2 groups except for site pain (35% PTA *vs* 0% EUS). The study was updated and has been presented at DDW 2006 with 50 patients[50]. Four fifth of patients were reassessed as surgically achieved pathologically negative margins and 3 patients survived greater than 24 mo.

This demonstrated that EUS-guided FNI of TNFerade with concurrent chemoradiation is feasible and generally well tolerated. TNFerade may optimize surgical and long-term outcomes. EUS may offer a safer and more accurate route of injection compared with a percutaneous approach.

CONCLUSION

In conclusion, even if new-generation high-resolution CT scans can equally assess pancreatic cancer resectability, EUS is still useful for small tumors and doubtful findings after CT scan. EUS can also image and access pancreatic lesion and lymph nodes not visible or accessible by other imaging modalities. Endoscopic ultrasound-guided intervention has opened new and exciting clinical applications in the management of pancreatic cancer including fine needle aspiration of lesion or lymph node and celiac plexus neurolysis. Recently, endoscopists can deliver anti-tumor agents under EUS in multiple sites inside pancreatic cancer which promises innovative clinical application of EUS.

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COMMENTARY

Preventing physician quality of life from impinging on patient quality of care: Weakening the weekend effect

Marc D Basson

Marc D Basson, Department of Surgery, John D Dingell VA Medical Center and Wayne State University, Detroit, Michigan, United States
Correspondence to: Marc D Basson, MD, PhD, MBA, Chief, Surgical Service (11S), John D Dingell VA Medical Center, 4646 John R. Street, Detroit, MI 48201-1932, United States. marc.basson@va.gov
Telephone: +1-313-5763598 Fax: +1-313-5761002
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Abstract

Imprecise or delayed care can reflect many factors, including straightforward difficulties in physician judgment and false negative tests. However, the movement toward decreasing physician work hours also leads to delays in care caused by inadequate staffing or inadequate communication between staffing, which must be addressed if quality of care is to remain high. The demonstration of delays in the management of anastomotic leaks over weekends or in association with false positive radiologic studies exemplifies this challenge.

Key words: Anastomosis; Communication; Contrast; Delay; Hand-off; Intestine; Staffing; Weekend

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INTRODUCTION

Doeksen and colleagues describe an anastomotic leak rate of 12.5% in a mixed series of 289 patients with handsewn or stapled colorectal anastomoses in a variety of positions[1]. Based upon a series of classical, but non-specific, warning signs, they describe onset of warning signs at a median of 4 d after surgery, and a median "delay" of 3.5 d between the first sign and eventual reoperation. They further demonstrated statistically significant increases in delay in the face of a false-negative radiologic procedure or an intervening weekend. Although Doeksen and colleagues were not able to define an impact of such delay on the patients' subsequent hospital course or mortality, their numbers are too low to expect statistical significance for such analyses, as they themselves acknowledge.

The finding of a relatively high 12.5% anastomotic leak rate seems surprising, but is difficult to interpret given the mixed nature of the series. Recently reported anastomotic leak rates range widely, varying with anatomic position, technique, and the clinical condition of the patient. It should be pointed out that the leaks reported here presumably represent clinically significant leaks. One would expect that routine surveillance of difficult anastomoses for research purposes would yield a higher number, including some leaks that are self-sealing and never become clinically apparent[2].

The reported median 4 d delay between the first sign of leak and reoperation is also of concern, but may be attributed to the relatively non-specific nature of some of the signs of leakage studied. A postoperative fever may certainly be the first sign of a leak, for instance, but may also commonly reflect a urinary tract, pulmonary, or wound infection, phlebitis, or even atelectasis. Similarly broad differential diagnoses could be generated for the authors' other signs of anastomotic leakage, including tachycardia, leukocytosis, ileus, and delayed gastric emptying. It would have been interesting in this regard to know the frequency of these putative signs of anastomotic leakage among those patients whose anastomoses did not leak. The lack of specificity of these signs does not reflect adversely on the study design, but rather suggests the difficulty of making the diagnosis of anastomotic leakage at the present time. Notably, the mean delay in operation after the development of peritoneal signs appears to have been only 0.8 d in this series, more consistent with what one would seek in clinical practice, although still longer than one might like.

It is interesting to note that time to definitive management in this series was defined as time to reoperation. In the future, it is possible that an increasing number of these patients may be managed by endoscopic means, including stenting, plugging, and/or endoluminal repair[3-14]. However, until experience with such techniques evolves further and indications and outcomes are clarified, most surgeons will still prefer the transabdominal approach with which they have the most experience. Endoscopic repair

of acute tears in healthy tissue after colonoscopy may also prove much easier than endoscopic closure or repair at an inflamed anastomosis that has been leaking for several days.

Difficulty in identifying anastomotic leaks from clinical presentation has led some surgeons to pursue radiologic studies, whether by fluorography or CT scanning, with contrast administered by mouth or by rectum. In this series, the authors used such studies in 21 patients with a startlingly high 43% apparent false negative rate. Since the series has been censored to only include patients who actually had anastomotic leaks, the actual false negative rate may be somewhat lower if there were also false positive radiologic examinations, and we cannot discern the false positive rate from this report. The series includes a mixture of various radiologic studies. There is considerable controversy over whether oral or rectal contrast with fluorography, or CT scans are preferable for the detection of anastomotic leaks, and this is likely to vary with local expertise and the site of the anastomosis[15-17].

The authors describe a striking mean 4.6 d difference in time to operation between those patients who had a false negative radiologic study and those who had a true positive study or none at all. This number may also be difficult to interpret. Since the radiologic studies were not performed on protocol, they were most likely ordered in those patients in whom the diagnosis was difficult. A positive radiologic finding would presumably have accelerated the decision to operate on such patients, while a false negative finding would have been expected to delay it. Thus, while it would be satisfying to argue for the primacy of clinical acumen by referring to the apparent delay in management of patients with false negative radiologic testing, it would be erroneous to do so without knowing how long it would have taken to operate upon these presumably more difficult patients without any radiologic testing and without considering the potentially beneficial impact of true positive radiologic testing in this setting. In addition, the utility of radiologic testing in difficult patients may be considerably higher in institutions in which the false negative rate is lower than 43%.

A more striking finding is the apparent 4 d increase in delay in operating upon patients whose postoperative course spanned a weekend. The impact of weekends on health care has previously been examined in other settings. For instance, difficulties in weekend coverage have been implicated as a primary cause of delay in obtaining accurate radiologic interpretations[18]. Others have pointed to weekend hospital staffing by cross covering physicians as contributing to lack of a plan of management and impairment for complex decision-making such as code-status decisions, and have proposed a standard form to improve communication and influence the quality of inpatient care[19].

The impact of a "weekend effect" upon clinical care has also been demonstrated. For instance, hospital discharges were significantly lower on Sundays and Mondays in a British study, suggesting that management was less efficient over the weekend, although it remained unclear to what extent this prolongation in care reflected impaired decision-making and to what extent it reflected decreased availability of required hospital services during the weekend[20]. A 1400 bed hospital in Singapore has also reported prolongation of hospital stay for patients admitted on weekends, holidays, or after hours[21], while a similar Spanish study also documented an association between increased days of hospitalization that did not meet utilization criteria and weekends[22].

Epidemiologic evidence suggests decreased compliance with guidelines for the management of acute cerebrovascular accidents over the weekend in the United Kingdom[23]. In another particularly telling study, patients admitted to the hospital over the weekend with acute myocardial infarction in the state of New Jersey in the United States were not demographically different from their weekday counterparts. However, the weekend patients were statistically significantly less likely to have invasive procedures performed over the weekend and had a significantly higher mortality rate. The effect of the weekend stay on mortality disappeared in multivariate analysis adjusted for utilization of invasive procedures, suggesting that weekend patients were more likely to die because they were less likely to have such invasive procedures. Whether this reflected the availability of staff to do these procedures or impaired decision-making by physicians not ordering the procedures could not be discerned from this epidemiologic study[24].

A University of Maryland study suggested similar increases in mortality for all patients admitted through emergency rooms on weekends compared with patients admitted on weekdays, with logistic regression suggesting that this increase in mortality correlated with longer delays between presentation to the emergency room and admission to an inpatient unit, where presumably more aggressive treatment might be administered[25]. Similar observations have been made for 48 h mortality after admission to the hospital on weekends in comparison to patients admitted on weekdays in Spain[26].

The weekend effect may not be universal, however. Several studies have failed to define a weekend effect. At the most trivial, hospital diet tray assembly appears equally accurate during the week as on weekends[27]. This may reflect the relative simplicity of the task, as well as stability of staffing. More importantly, there was no association between hospital mortality and the day of ICU admission in a study from Peking Union Medical College[28]. Similar studies on mortality after ICU admission from Saudi Arabia[29], of hospital mortality after Emergency Room admission from The Royal Infirmary of Edinburgh[30], and from the University of Michigan on mortality after trauma admissions[31] have all reported no weekend effects. In each case, authors attributed their success to consistent dedicated staffing of the appropriate hospital units.

Importantly, ICU care, emergency room admissions, and trauma management are all acute events that may lend themselves to quantization into discrete episodes of care by fresh providers. The postoperative care of a convalescing surgical patient and detection of postoperative complications may require more attention to continuity of care. Indeed, it is incontrovertible that maintaining staffing without inducing undue fatigue requires the hand-off of responsibility to new health care

providers. The recent movement in the United States to limit the work-hours of trainees in order to minimize fatigue-induced errors has led to increasing concern over errors induced by inadequate communication of patient details. A survey of United States trainees specifically identified communication during hand-offs as a target for preventing errors[32]. Various groups have proposed mechanisms to systematize such hand-offs to make sure that information is actually passed[33-37], and at least one study suggests faster hand-offs, more full information hand-off, and increased provider satisfaction using such a system[38]. However, no study has demonstrated any improvement in patient outcomes with these systems, and this remains an important target for future study. In the particular case of the postoperative patient with a potential anastomotic leak, much of the prompt suspicion and detection of anastomotic leakage rests less on the factual information of yesterday's temperature and leukocyte count than on the less tangible perception of the patient's overall condition, attitude, and exam, and changes thereof. Such information may be less easy to transmit than quantitative data. Moreover, several authors[19,37,39-41] have alluded to the potential importance of active engagement by the cross-covering caretaker in pushing forward with care plans, rather than maintaining the status quo through the night or weekend coverage. Such professional engagement is likely to be at least as important in the management of the postoperative patient.

The study by Doeksen and colleagues may be an important harbinger of things to come in the surgical arena, as surgeons' work-hours are steadily reduced around the world to minimize the (perceived or real) impact of surgeon fatigue. If we are to preserve our quality of care, it will be important to not only maintain adequate numbers of equivalently trained staff present over the weekend, but also to effectively transfer both quantitative and qualitative data, impressions, and plans, as well as the dedication to the care of our patients which is the hallmark of our profession. The effective and efficient management of this transfer may be the greatest challenge posed by work-hour restrictions, so that attention to physician quality of life does not impair that of our patients.

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TOPIC HIGHLIGHT

Yusuf Bayraktar, Professor, *Series Editor*

How helpful is capsule endoscopy to surgeons?

Osman Ersoy, Bulent Sivri, Yusuf Bayraktar

Osman Ersoy, The Department of Gastroenterology, Hacettepe University Faculty of Medicine, Ankara, Turkey
Bulent Sivri, The Department of Gastroenterology, Hacettepe University Faculty of Medicine, Ankara, Turkey
Yusuf Bayraktar, The Department of Gastroenterology, Hacettepe University Faculty of Medicine, Ankara, Turkey
Correspondence to: Yusuf Bayraktar, Hacettepe University, School of Medicine, Department of Gastroenterology, Ankara 06100, Turkey. bayrak@hacettepe.edu.tr
Telephone: +90-312-3051712 Fax: +90-312-4429429
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Abstract

Capsule endoscopy is a new technology that, for the first time, allows complete, non-invasive endoscopic imaging of the small bowel. The efficacy of capsule endoscopy in the diagnosis of suspected small bowel diseases has been established. Important applications for surgeons include observations of obscure gastrointestinal bleeding and small bowel neoplasms.

Key words: Capsule endoscopy; Surgery; Small bowel neoplasm; Obscure gastrointestinal bleeding; Angiodisplasia

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INTRODUCTION

The small intestine is the most difficult part of the gastrointestinal tract to evaluate due to its length and complex loops[1]. Capsule endoscopy (CE) has become the procedure of choice for diagnosis of occult mucosal disorders of the small intestine. First introduced at the 2000 Digestive Disease Week Conference in San Diego, California, the Given Imaging M2A capsule (Yoqneam, Israel) subsequently received approval from the US Food and Drug Administration (FDA) in mid-2001 for use in the United States. Over 10 000 examinations have been performed worldwide and the complication rate has been established as only 0.75%[1]. The major indication for capsule endoscopy is the investigation of patients with obscure gastrointestinal bleeding (OGB), however, this novel diagnostic tool is also indicated for evaluation of early Crohn's disease (CD), suspected small bowel tumor, surveillance of inherited polyposis syndromes, evaluation of abnormal small bowel imaging, evaluation of drug-induced small bowel injury, and for partially responsive celiac disease[2]. This paper reviews the current indications for CE and strategies to optimize utilization of this technology.

WHY IS IT DIFFICULT TO FIND THE SOURCE OF SMALL BOWEL LESIONS?

The small intestine begins at the pylorus and terminates at the ileocecal sphincter. The approximate length of the small intestine is about 3.7 m to 6.7 m. The major functions of the small intestine are digestion and absorption. Despite the fact that serious small bowel disease is uncommon, symptoms related to disordered function of the small bowel are quite common. Bleeding, weight loss, diarrhea and pain are among the most common reasons for patients to seek health care.

The small intestine is an uncommon source of gastrointestinal (GI) bleeding. Bleeding can manifest as iron deficiency anemia when occult and most commonly is dark red or purple when overt. Endoscopic exclusion of upper GI and colonic sources of bleeding is the single most important clue indicating a possible small bowel source. Causes of small bowel bleeding are as follows: angiodysplasia, Dieulafoy's lesions, erosions/ulcers, Crohn's disease, small bowel varices, tumors, NSAID enteropathy, radiation enteritis, small bowel diverticulosis, small bowel polyps, aortoenteric fistula, and Meckel's diverticulum[3].

Small intestinal bleeding presents a unique clinical problem that differs from upper and lower GI bleeding in many aspects. Patients with small intestinal bleeding undergo more diagnostic procedures, require more blood transfusions, have longer hospitalizations, and have higher health care expenditures than patients with upper or lower GI bleeding[4]. Since the small intestine is the most difficult segment of the GI tract to examine with endoscopy because of its distinctive anatomy, length, and location, it is difficult to find the source of small bowel lesions.

WHAT TESTS ARE PERFORMED TO DETECT SMALL BOWEL LESIONS?

The diagnostic methods for use in potential small bowel diseases are radiologic (e.g. small-bowel follow through

[SBFT] and enteroclysis or computerized tomography [CT]), endoscopic (e.g., intraoperative endoscopy, sonde [SE], push [PE], or double balloon enteroscopy [DBE]), or surgical (with or without intraoperative endoscopy).

SBFT has a low diagnostic yield (0%-5.6%) in the investigation of obscure gastrointestinal bleeding (OGIB)[5]. The diagnostic yield of enteroclysis in OGIB has been reported to be 10% to 21%[5]. Although these radiographic studies may have high specificity for bleeding site localization and potential etiology, the sensitivity is too low to make them useful as a screening test. Yet, in the absence of tests with a higher sensitivity and specificity, these insensitive tests have been used for many years by clinicians who are trying to establish a diagnosis in patients with obscure GI hemorrhage. Although enteroclysis and SBFT might show a strictures, a large masses, a large polyps, tumors and deep ulcers, aphthous ulcer and vascular ectasia in small intestinal mucosa cannot be seen.

Angiography and technetium-99m labeled red blood cell scans are performed when bleeding is active and the patient is hemodynamically stable. Both procedures can detect bleeding rates of 0.5 to 1.0 mL/min. Diagnostic yields of nuclear scanning (sulfur colloid or red blood cells) and angiography are low, even with patients who have recurrent melena or hematochezia[6-11]. In selected patients with massive bleeding, angiography may be the best test because, in addition to demonstrating the bleeding site, it offers therapeutic capability.

PE involves peroral insertion of a long endoscope directly into the jejunum. PE has been reported to be safe and has a diagnostic yield of 38% to 75%[5,12]. However, the lesion is found within reach of the gastroscope in only 28% to 75% of the patients[13,14]. With enteroscopy, the most frequently seen lesions are angiectasias, especially in the elderly[13,15], and small bowel tumors, particularly in patients younger than 50 years[16].

SE affords good visualization of the small intestine[10,11,17]. SE is both sensitive and specific in patients with OGIB. Although SE is no longer commercially available and was never widely used, visualization of the ileum or beyond was possible in 77% of 545 patients with obscure bleeding (both occult and overt) as reported by Berner *et al*[11]. For esophagogastroduodenoscopy (EGD), with PE and SE results combined, 58% (322/553) had abnormal examinations, with 40% (219/553) beyond the reach of EGD[11]. GI angiomas were diagnosed in 34.5% of the combined enteroscopies (both PE and SE), small intestinal tumors in 5.6% of patients, and small-bowel ulcers and other lesions in 3%[11]. No bleeding sites were reported in 41.7% of all enteroscopies. The procedure time of SE is 6 to 8 h, and the diagnosis is feasible in ambulatory patients when the SE is withdrawn from the most distal locale to which peristalsis has carried it. Visual diagnosis but not biopsy, treatment, or specific localization is possible.

Before the development of CE, for patients who were operative candidates and had severe recurrent OGIB, laparotomy with intraoperative enteroscopy was strongly considered early in their course. When a focal lesion instead of diffuse disease is suspected, such a combined approach affords high diagnostic and therapeutic yields. A PE is passed orally, after the surgeon completes the exploration and has dissected out any adhesions to free up the small bowel. With assistance by the surgeon, the entire small bowel can be accordioned or pleated over the enteroscope[18-24]. The small intestine is inspected on initial entry in 10-20 cm segments. Transillumination of the bowel is recommended to detect any potential bleeding sites. Mucosal trauma and contact bleeding will often result upon manipulation of the bowel over the endoscope, and these artifacts are often confused with definitive bleeding sites if the bowel is primarily examined upon withdrawal. Lesions should be marked with a suture by the surgeon for later resection. Occasionally, active bleeding or a column of blood is detected at laparotomy. The proximal margin should be marked. Intraluminally, the mucosa can be washed, blood can be suctioned with the enteroscope, and lesions may be localized, diagnosed, and/or coagulated.

DBE is an exciting new technique that allows complete visualization of the small intestine. The source of bleeding was identified in 50 (76%) of 66 patients with GI bleeding[25].

Disadvantages of conventional endoscopic techniques, such as PE and colonoscopy with ileoscopy, include limited endoscopic examination of the small bowel and sedation requirements. A complete endoscopic evaluation was previously possible only with intraoperative endoscopy, but DBE and CE can now be used for complete examination of the small bowel. However, DBE requires sedation and this procedure is more difficult than other procedures.

WHAT IS CAPSULE ENDOSCOPY?

CE is a new technology that, for the first time, allows complete, non-invasive endoscopic imaging of the small bowel. The efficacy of CE in the diagnosis of suspected small bowel diseases has been established. Current applications include OGIB, inflammatory bowel disease (IBD), small bowel neoplasms (including polyposis syndrome), malabsorption disorders (including celiac disease), iatrogenic disease (nonsteroidal anti-inflammatory drug enteropathy and radiation enteritis), and clarification of abnormal small bowel imaging. There are many emerging indications, such as in pediatrics and suspected small bowel obstruction.

The technology, possibly due to advances in miniaturization, comprises an 11-26 mm disposable video capsule propelled by peristalsis. The capsule comprises a transparent optical dome, illumination from six light-emitting diodes, a camera, silver oxide batteries, transmitter and antennae. The field of view is 140 degrees and magnification is 1:8, which is capable of visualizing intestinal villi. The capsule takes two frames per second and the battery life is approximately 8 h, allowing the acquisition of > 55000 images[26]. Images are transmitted by radio frequency to an eight-point abdominal sensory array and recorded on a digital recorder worn on a belt. The images are downloaded to a computer and viewed with dedicated software, which allows for capsule localization. The suspected blood indicator is quite good at detecting active bleeding, but not for other lesions, and does not replace careful examination of the CE video. The capsule is swallowed after an overnight fast. There is no

Table 1 Studies comparing diagnostic yields of CE to PE in obscure GI bleeding

Studies	*n*	Diagnostic yield (%)	
		CE	PE
Ell *et al*, 2002[30]	32	83	30
Lewis *et al*, 2002[37]	21	55	30
Lim *et al*, 2002[33]	20	70	45
Hartmann *et al*, 2003[36]	33	76	21
Van Gossum *et al*, 2003[45]	21	52	61
Saurin *et al*, 2003[35]	58	69	38
Mylonaki *et al*, 2003[32]	50	68	32
Ge *et al*, 2004[38]	36	65	28
Adler *et al*, 2004[34]	20	70	25
Mata *et al*, 2004[31]	42	74	19
Leighton *et al*, 2006[39]	20	50	20

consensus currently on whether a small bowel preparation or prokinetics is required.

HOW EFFECTIVE IS CAPSULE ENDOSCOPY AT DETECTING LESIONS OF THE SMALL BOWEL?

Obscure gastrointestinal bleeding

Patients with GI hemorrhage of uncertain etiology are a diagnostic and therapeutic challenge. OGIB is defined as recurrent or persistent GI bleeding despite the absence of explanatory findings at initial upper and lower endoscopy. Estimates vary in the current prevalence of obscure bleeding among all cases of GI hemorrhage, but this was probably less than 5% before the introduction of CE. OGIB can be subclassified as either overt or occult bleeding, based on whether the patient has a history of gross GI bleeding symptoms, either melena or hematochezia. Occult bleeding can be manifested by recurrent iron deficiency anemia or positive fecal occult blood test results[6]. Most often the site of hemorrhage is suspected to be the small bowel[7,8].

OGIB is the most common indication for CE. The diagnostic yield of CE for the suspected bleeding source in OGIB has been reported to be 38% to 93%[27]. In our study, this modality demonstrated the source or bleeding in 17 of the 23 patients (73.9%) with OGIB[28]. Using CE, the most commonly detected bleeding sources or clues in the small bowel included angiectasia, fresh blood, ulceration, tumor, and varices. Early studies seem to suggest that there is no significant difference in the diagnostic yield of CE in obscure-overt and obscure-occult bleeding[29].

It has been shown that CE may be superior to PE[30-39], small bowel series[40,41], enteroclysis[42], CT scan[43], and DBE[44] in identifying small bowel lesions in OGIB. Studies comparing diagnostic yields of CE to PE in OGIB are summarized in Table 1.

The commonly missed small bowel lesions by SBFT compared with CE include angiectasia, bowel ulcer and erosion. Some investigators have proposed that SBFT only be performed in a population at high-risk for capsule impaction, such as patients with Crohn's disease or in young patients suspected of small bowel tumors.

CE seemed to have a higher diagnostic yield than PE[30-39]. There is only one report in which PE had a higher diagnostic yield than CE[45]. The diagnostic yield in CE was reported to be 52% to 83% compared to 19% to 61% in PE. Small-bowel pathologies were detected using CE in 28 (80%) of the 35 patients with OGIB, compared with 21 (60%) of the 35 patients using DBE[44].

Based on the algorithm proposed by the American Gastroenterological Association in 1999[6], we also suggest this algorithm for evaluation of OGIB in the era of CE.

Small bowel tumors

Tumors of the small bowel account for 5% of all GI tract tumors and 2% of cancers, although the accuracy of those estimates is uncertain because the current methodologies for examining the small bowel have proved inadequate. The diagnosis of small bowel tumors is frequently delayed, contributing to the poor prognosis for patients with malignant tumors[46]. The diagnosis and localization of small bowel tumors has been a clinical challenge because of the inaccessibility of the small bowel to conventional diagnostic modalities. Enteroclysis has a much higher sensitivity than SBFT in detecting small bowel tumors [11]. Although CT may be useful in diagnosing extraluminal and metastatic spread of small bowel malignancies, its role in detecting small intraluminal and mucosal lesions has been limited, with a diagnostic yield as low as 20%[47].

Endoscopic evaluation of the small intestine has included SE, PE, intraoperative enteroscopy, and DBE. PE and SE in 545 patients with OGIB identified 31 (5.6%) small bowel tumors[11]. All of these procedures, however, have significant limitations, including degree of invasiveness, incomplete inspection of the small intestine, and prolonged procedure time.

de Mascarenhas-Saraiva and da Silva Araujo Lopes reported a 3.8% rate of primary tumors in the small intestine by CE[48]. The accuracy of CE in diagnosing the small bowel tumors seemed to be superior to that of other methods. In a meta-analysis, 86 of 1349 pathologies (6.4%) that were identified at CE were intestinal neoplasms[49]. Cobrin *et al* reported that 9% of OGIB were caused by small bowel tumors[46]. The types of tumor diagnosed by CE included 8 adenocarcinomas (1.4%), 10 carcinoids (1.8%), 4 GI stromal tumors (0.7%), 5 lymphomas (0.9%), 3 inflammatory polyps, 1 lymphangioma, 1 lymphangiectasia, 1 hemangioma, 1 hamartoma, and 1 tubular adenoma. Of the tumors diagnosed, 48% were malignant.

Our seven patients, in whom CE was performed for OGIB, underwent surgery after CE. Four of 7 patients had been reported previously[28]. In three of 7 patients, the active bleeding (fresh blood or oozing blood) site was noted in the proximal small intestine. But the source of the bleeding in two patients was not clearly seen. The bleeding sources in all of them were identified in operation as angiodysplasia located in the proximal small intestine. In the patient who had a polyp with oozing blood, the source of bleeding was identified as follicular hyperplasia in the operation specimen (Figure 1). One patient had angiodysplasia noted without evidence of active bleeding. Because these lesions were thought to be the cause of the

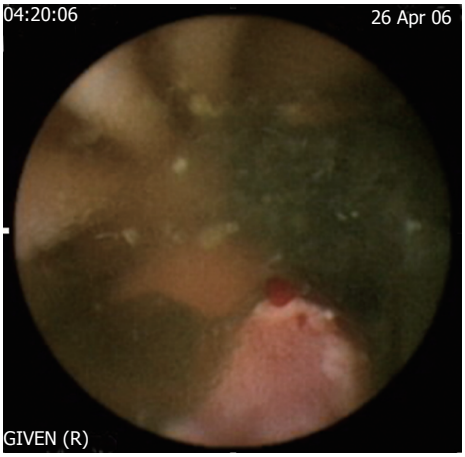

Figure 1 A polyp with oozing blood as a volcano was found by capsule endoscopy in the proximal small intestine.

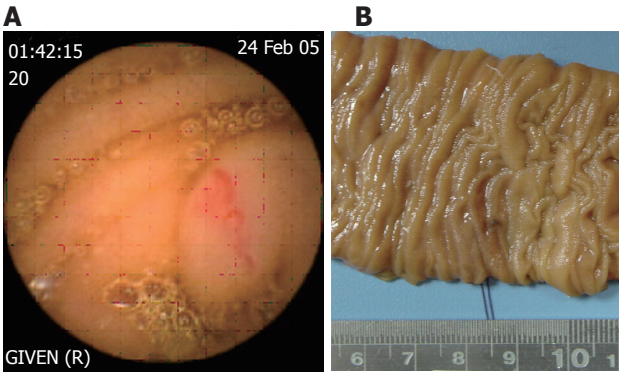

Figure 2 **A**: Angiodisplasia in proximal small intestine; **B**: Angiodisplasia was confirmed by intraoperative endoscopy in operation and was excluded with local resection.

bleeding, angiodysplasia was confirmed by intraoperative enteroscopy during operation and was excluded with local resection (Figure 2A and B).

In one patient, a vegetative mass was demonstrated in the proximal jejunum, which was missed during an abdominal CT scan small bowel series. He was operated on and diagnosed as jejunal invasion of recurrent renal cell carcinoma (Figure 3A and B).

In one patient, in whom multiple ulcers were found in the proximal intestine by CE, histopathologic examination of the biopsy specimen taken during enteroscopy demonstrated adenocarcinoma. In one patient, in whom a single ulcer was found in the proximal intestine, histopathologic examination of the biopsy specimen taken during enteroscopy demonstrated GI stromal tumor.

Although specific localization of lesions within the small intestine by CE has been reported as problematic relative to surgery or other procedures[40], localizations of small bowel lesions in all our patients were found by CE to be nearly the same as localizations during surgery.

Standard terminology and further studies to define a reference standard for diagnosis and treatment outcomes with CE will be necessary and are recommended. Although the specificity and sensitivity of CE for OGIB have been

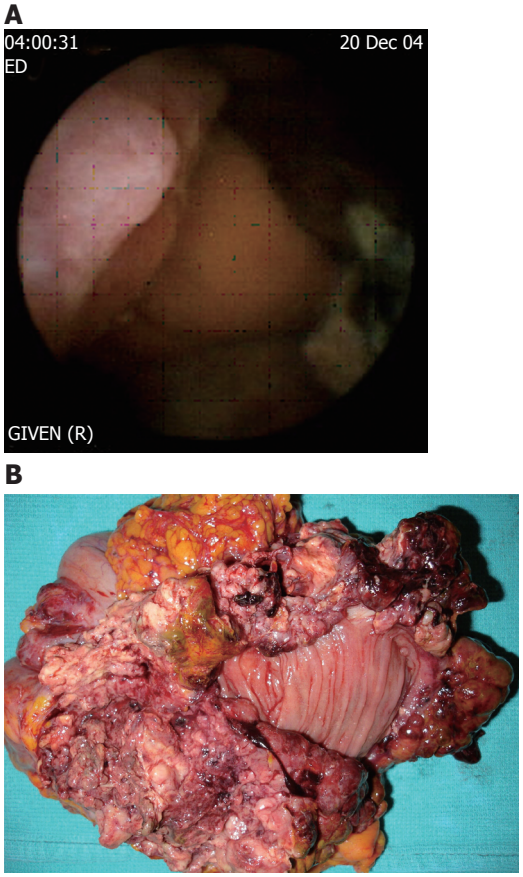

Figure 3 **A**: A vegetative mass was shown by CE; **B**: Histopathologic examination of the surgical specimen demonstrated invasion of recurrent renal cell carcinoma.

defined, these have to be established for severe obscure bleeding.

CONCLUSION

Bleeding from small bowel lesions is a rare cause of GI blood loss. Cancers, IBD and infections account for 20%-25% of all small bowel bleeding, while arteriovenous malformations account for the vast majority of causes. Endoscopic therapies are limited to the parts of the bowel within their reach and are the only minimally invasive way to apply direct treatment to bleeding sources or to take biopsies. The development of the endoscopic capsule has changed the way in which gastroenterologists will approach GI bleeding originating from small bowel lesions. With further development and innovation, capsule endoscopy will improve the management of this condition. Particularly in malignant lesions of the small bowel and in bleeding, capsule endoscopy is very helpful for surgeons before operation.

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Platelet aggregation is affected by nitrosothiols in patients with chronic hepatitis: *In vivo* and *in vitro* studies

A Federico, A Filippelli, M Falciani, C Tuccillo, A Tiso, A Floreani, R Naccarato, F Rossi, C Del Vecchio Blanco, C Loguercio

A Federico, C Tuccillo, A Tiso, C Del Vecchio Blanco, C Loguercio, Inter-University Research Centre on Foods, Nutrition and Gastrointestinal Tract, Gastroenterology School, 2nd University of Naples, Italy
A Filippelli, M Falciani, F Rossi, Department of Experimental Medicine, 2nd University of Naples, Italy
A Floreani, R Naccarato, Department of Surgical and Gastroenterological Sciences, University of Padua, Italy
Correspondence to: Alessandro Federico, Inter-University Research Centre on Foods, Nutrition and Gastrointestinal Tract, Gastroenterology School, 2nd University of Naples, Via Alcide De Gasperi 80, 84018 Scafati, Italy. alessandro.federico@unina2.it
Telephone: +39-815-666718 Fax: +39-815-666837
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Abstract

AIM: To investigate the relationship among the number of platelets and plasma levels of S-nitrosothiols (S-NO), nitrite, total non-protein SH (NPSH), glutathione (GSH), cysteine (CYS), malondialdehyde (MDA), 4-hydroxininenal (4HNE), tumor necrosis factor-alpha (TNFα) and interleukin (IL)-6 in patients with chronic hepatitis C (CH).

METHODS: *In vitro* the aggregation of platelets derived from controls and CH patients was evaluated before and after the addition of adenosine diphosphate (ADP) and collagen, both in basal conditions and after incubation with nitrosoglutathione (GSNO).

RESULTS: *In vivo*, S-NO plasma levels increased significantly in CH patients and they were significantly directly correlated with platelet numbers. Patients with platelet counts < 150 000/μL, had a smaller increase in S-NO, lower levels of GSH, CYS, NPSH, TNFα, and IL-6, and higher levels of nitrite, MDA, and 4-HNE relative to those of patients with platelet counts > 150 000/μL. *In vitro*, the ADP and collagen aggregation time was increased in platelets from patients and not from controls; in addition, platelets from CH patients but not from controls also showed a latency time after exposure to collagen.

CONCLUSION: The incubation of platelets with GSNO improved the percentage aggregation and abolished the latency time.

Key words: Liver disease; Function of platelets; Hepatitis C; Oxidative stress; Anti-aggregant

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INTRODUCTION

Thrombocytopenia is a marker of liver cirrhosis as consequence of the portal hypertension and hypersplenism[1]. However, thrombocytopenia is also frequently observed in patients with chronic hepatitis caused by hepatitis C virus (HCV), without cirrhosis, and this phenomenon appears to be related to HCV infection rather than to other causes of chronic hepatitis (CH)[2,3]. The mechanism of this alteration is unclear. The presence of antibodies to platelets has been considered an independent ("nonpathogenic") cofactor, while plasma levels of thrombopoietin have been found to be decreased, normal, or elevated in HCV-infected patients, compared to levels found in patients having other types of liver disease[1,4,5]. More recently, Fusegawa *et al*[6] demonstrated increased platelet activation in HCV-related CH patients. This observation suggests that platelet function might be altered in these patients. In various pathological situations, platelets may be subjected to external oxidative stress by exposure to radicals released by granulocytes, endothelial cells, and/or monocytes/macrophages. These cells also release increased amounts of nitric oxide (NO) through the activation of inducible nitric oxide synthase (iNOS) by proinflammatory cytokines[7]. In these conditions, also platelets may produce NO, that, in turn, modulates itself recruitment and function. Thus, platelets and their function are likely to face physiological oxidative conditions[8]. NO and NO-donors are antithrombotic and anti-aggregant agents[9], and S-nitrosothiols (S-NO) have been synthesized and proposed as a new class of drugs with these effects[10,11]. The NO produced circulates both as nitrite/nitrate and in complexes as protein and nonprotein S-nitrosothiols, with a constant exchange occurring between these substances *in vivo*[12]. The formation of

S-NO adducts, as well as the release of NO from these compounds, is strongly influenced by the bioavailability of glutathione (GSH) and cysteine (CYS) and by the redox status of plasma. Furthermore, these pathways also affect the function of platelets, as platelets very rapidly import NO from S-NO[13-16]. Figure 1 summarizes the metabolism of NO in the circulation.

On the basis of these considerations, we performed as study's hypothesis that, in absence of hypersplenism and/or portal hypertension, the thrombocytopenia frequently observed in patients with HCV-related chronic hepatitis, could depend on other factors, as an altered metabolic pathway of NO in the circulation, secondary to a possible alteration of the redox status and/or an increase of pro-inflammatory cytokines. Therefore, we evaluated, *in vivo*, in patients with HCV-related CH and a control group, (a) the levels of S-NO and their relationships with those of nitrite, nitrate, and thiols; (b) the relationships between the foregoing and plasma levels of markers of oxidative stress and proinflammatory cytokines; and (c) the relationships among all of these factors and the number of platelets. We also evaluated the relationship between S-NO and platelet function *in vitro* using platelets derived from controls and from HCV-infected patients.

MATERIALS AND METHODS

The departmental ethics committee approved the study protocol, and all individuals gave informed consent. The study population comprised 114 patients with biopsy-proven, HCV-related chronic hepatitis without cirrhosis (CH), according to Ishak's score[17]. The absence of cirrhosis was documented, other than by liver histology, also by the absence of ultrasonographic and endoscopic findings of portal hypertension and by the normality of liver function tests (albumin, prothrombin time, total bilirubin). As controls (C), we enrolled 50 healthy volunteers (no alcohol or drug users) who were negative for markers of HIV and hepatitis virus infection (HBV, HDV, and HCV). We excluded from the study subjects with alcohol intake > 30 g/d of pure ethanol, HIV or HBV positivity, decompensated diabetes, cryoglobulinaemia, and/or other associated diseases. The exclusion of HBV-positive patients was performed because we aimed to evaluate the function of platelets in HCV-positive patients, in whom a series reports have previously focused attention on a possible relationship between HCV chronic infection and thrombocytopenia[1,4-6], and also because in our clinical practice the number of HBV-positive patients is actually low. We also excluded patients with a platelet number < 100 000/μL, because this is considered a lower limit to evaluate platelet aggregation and patients treated with steroids, beta-blockers, nitro derivatives, aspirin, antioxidants, and interferon. Other drugs, such as insulin or non-absorbable disaccharides, were allowed. Table 1 summarizes the main findings regarding the enrolled subjects.

Biochemical determinations on plasma samples

From venous samples deproteinized with 10% of sulphosalicylic acid on plasma samples collected in the morning, after overnight fasting, we determined:

Total nitrite/nitrate levels: For this purpose, as suggested by the method outlined in the commercial kit (Bioxytech nitric oxide non-enzymatic assay, OXIS International, Inc., Portland, USA), we employed granular cadmium metal for the chemical reduction of nitrate to nitrite prior to evaluating nitrites. Total nitrite concentration was evaluated by the Griess method. In an acid solution, nitrite is converted to nitrous acid (HNO_2), which diazotizes sulphanilamide. This sulphanilamide-diazonium salt is then reacted with N-(1-naphthyl)ethylenediamine (NED) to produce a chromophore, which is measured at 540 nm[18,19]. Results were expressed as μmol/L.

Total nonprotein plasma SH groups (NPSH): These were determined using Ellman's reagent; absorbance was measured at 412 nm, as we have described[20]. Results were expressed as μmol/L.

Glutathione and cysteine: These two thiols are the major constituents of NPSH. We and other groups have shown that their levels in plasma are decreased in patients with liver disease. As previously reported, we measured these two thiols by Newton's method, which makes use of the stable linkage between monobromobimane (MBBR) and SH groups, which we detected by fluorescence high pressure liquid chromatography (HPLC) after derivatization of the plasma samples in the dark for 15 min[21-23]. Results were expressed as μmol/L.

S-NO: We used a less expensive and time-consuming spectrophotometric method[24,25]. Before assaying plasma samples from our study population, we added a known amount of GSNO (6.25 μg), the major constituent of plasma nitrosothiols, to a plasma sample from each of three healthy subjects and six patients (three HCV-positive and three PBC) to evaluate our ability to recover it. Recovery of the GSNO added to the plasma samples ranged from 86% to 99%. All determinations were performed in duplicate and, in some cases, on both fresh and frozen samples. No significant differences were found in the results.

Thereafter, a plasma sample (200 μL) from each of the individuals taking part in the study was added to 40 μL of 1% ammonium sulphamate (to counteract the background nitrite concentration) and then mixed with 200 μL of 0.4 mol/L HCl containing 0.3% $HgCl_2$ and 4.6% sulphanilamide to oxidize (and render undetectable) the released NO equivalent, of which > 99% was lost. Then, 300 μL of a solution of 0.4 mol/L HCl plus 0.2% mol/L-L-naphthylenediamine dihydrochloride was added. Samples were incubated for 30 min at 25℃. The amount of S-nitrosothiols was determined by visible spectrophotometry at 550 nm using GSNO (Sigma-Aldrich, Poole, UK) as a standard, since GSNO is a stable molecule and it has been demonstrated[25] that, during the assay procedures, no appreciable decomposition of the authentic GSNO occurs. Values were expressed as μmol/L.

Determination of nitrites and S-NO was performed on two different plasma samples, stored under identical conditions.

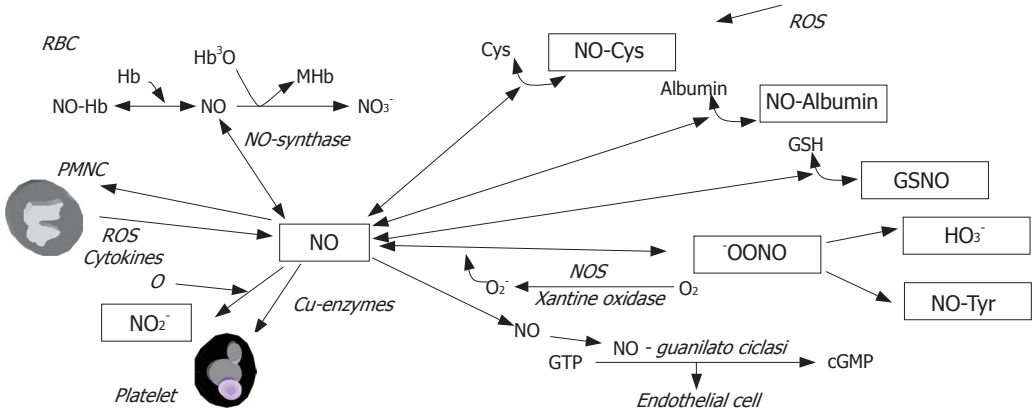

Figure 1 The metabolism of NO in the circulation: the NO produced circulates both as nitrite/nitrate and in complexes as protein and nonprotein S-nitrosothiols. The formation of S-NO adducts, as well as the release of NO from these compounds, is strongly influenced by the bioavailability of glutathione (GSH) and cysteine (CYS) and by the redox status of plasma.

Table 1 Enrolled subjects: main findings (mean ± SD)

	Controls	HCV-positive CH patients
Total number	50	114
M/F	26/24	59/55
Median age, yr (range)	53 (26-74)	54 (22-81)
ALT (IU/L, nv < 40)	12 ± 11	90.2 ± 60.4
γGT (IU/L, nv < 50)	28 ± 9	77 ± 52
Total proteins (g/dL)	6.8 ± 2.7	7.2 ± 1.2
Albumin (g/dL)	4.1 ± 1.2	3.6 ± 0.8
Total bilirubin (mg/dL)	0.8 ± 0.3	0.9 ± 0.3
Prothrombin time (s)	10.3 ± 2.1	10.5 ± 2.3
Platelets (n x 10^3/μL)	278 ± 89	159 ± 52
Ishak score for grading (median and range)	-	9 (5-11)
Ishak score for staging (median and range)	-	3 (2-3)

CH: chronic hepatitis; ALT: alanine aminotransferase; γGT: gamma-glutamyl transpeptidase.

Parameters of oxidative stress: As parameters of oxidative stress, in addition to the levels of GSH and CYS (see above), we measured those of malondialdehyde (MDA) and 4-hydroxinonenal (4-HNE), using commercial kits (Oxis International, Portland, OR, USA). Results were expressed as μmol/L.

Proinflammatory cytokines: We evaluated the plasma levels of two proinflammatory cytokines, tumor necrosis factor-alpha (TNFα) and interleukin (IL)-6, as we have previously described[26], using an immunoenzymatic reaction (ELISA test, Milenia Biotec, Germany). Values were expressed as pg/mL.

In vitro evaluation of platelet aggregation function

Whole blood (16 mL) was collected by venopuncture from each subject after overnight fasting. Platelet-rich plasma (PRP) was prepared by centrifugation of blood containing 5 mmol/L EDTA, at room temperature, at 900-1000 × g for 10 min. This sample was then transferred to the counter to determine the number of platelets/mL; another aliquot of the sample was centrifuged at 5000 × g for another 10 min to obtain plasma poor in platelets (PPP).

Specimens obtained as the result of a traumatic venopuncture were not included in the study. The pH and temperature remained within the normal range for the duration of the experiments.

Platelet number among experimental groups was normalized using a correction factor (platelet number × 350 000). Platelet aggregation was assayed by using a platelet aggregometer (Aggrecorder Ⅱ PA 3220 Menarini Diagnostici, Florence, Italy). Chart recorders graphed as a function of time. The aggregation was evaluated after the addition of common aggregating agents (3 μmol/L ADP and 1 μg/mL collagen, both in basal conditions and after incubation with nitrosoglutathione (Sigma-Aldrich, Poole, UK)[27]. Collagen type Ⅱ was from Mascia Brunelli, Italy. All *in vitro* experiments were performed within 3 h of venopuncture[8,28]. Platelet aggregation induced by collagen was measured after 3 min.

Statistical analysis

SPSS 11.0 software was used for statistical analyses. Differences between data and groups were evaluated by ANOVA and Student's t-test for paired data to evaluate levels in basal conditions and after incubation of platelet aggregations with GSNO, and for unpaired data in the other experimental conditions. For plasma data, the significance of differences was also calculated with a nonparametric Wilcoxon rank test. Correlations were calculated using Pearson's bivariate correlation test. $P \leqslant 0.05$ was considered significant.

RESULTS

Plasma values

In the first part of the present study, we compared the more recent methods employed to determine plasma levels of S-NO in humans (colorimetric, HPLC with electrochemical or spectrophotometric detection)[29,30]. No significant differences were found between the methods.

In all patients we found a significant increase in S-NO plasma levels relative to the control levels ($P < 0.01$). In contrast, plasma nitrite, NPSH, GSH, CYS, MDA,

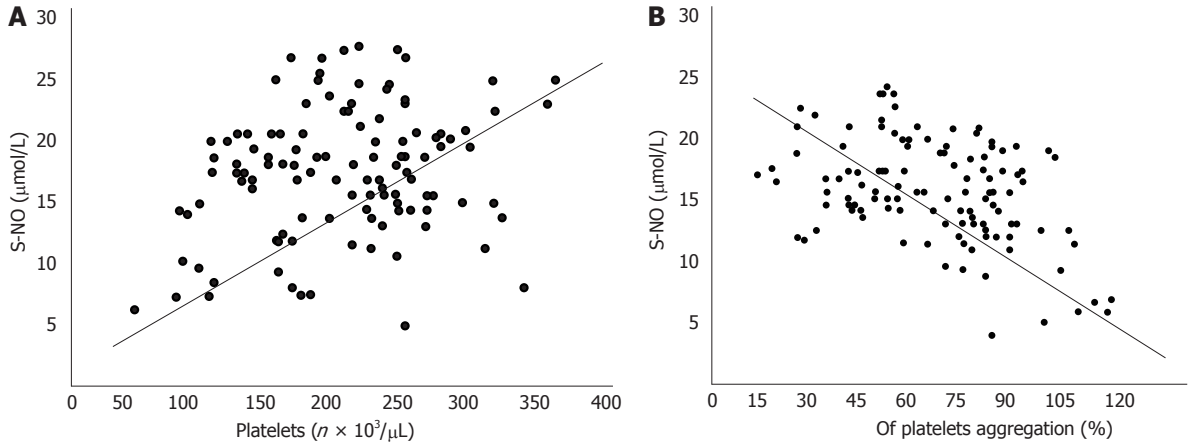

Figure 2 **A**: Correlation between S-NO and platelet number in each patient. As evident, S-NO levels were significantly directly correlated with platelet number ($r = 0.58$, $P < 0.01$). S-NO: S-nitrosothiol; **B**: Correlation between S-NO and % of platelet aggregation in each patient. As evident, S-NO levels were significantly inversely correlated with percent of platelet aggregation ($r = -0.52$, $P < 0.05$).

Table 2 Plasma parameters (mean ± SD)

	Controls	HCV-infected patients		
		CH (all patients)	CH with platelets > 150000/μL	CH with platelets < 150000/μL
Nitrite (μmol/L)	6.51 ± 4.64	7.98 ± 3.92	5.78 ± 2.03	10.64 ± 3.51[a,b]
NPSH (μmol/L)	367 ± 24	323 ± 26	372 ± 28	254 ± 21[b,d]
GSH (μmol/L)	8.1 ± 2.4	7.3 ± 3.5	8.8 ± 2.6	2.7 ± 1.0[b,d]
CYS (μmol/L)	17 ± 4.1	14 ± 5.2	16.8 ± 5.2	10 ± 2.9[b,d]
S-NO (μmol/L)	7.4 ± 1.5	27.9 ± 8.4[b]	33.8 ± 7.5[b]	21.4 ± 6.9[a,b]
MDA (μmol/L)	0.28 ± 0.05	0.25 ± 0.90	0.21 ± 0.18	0.46 ± 0.13[a, b]
4-HNE (μmol/L)	0.20 ± 0.08	0.24 ± 0.12	0.29 ± 0.11	0.59 ± 0.31[b,d]
TNFα (pg/mL)	26.7 ± 9.1	29.9 ± 5.7	36.4 ± 3.1[b]	26.2 ± 3.9[d]
IL-6 (pg/mL)	28.9 ± 7.3	31 ± 6.1	46.4 ± 3.1[b]	26.5 ± 6.4[d]
Score of fibrosis (median and range)	0	2 (1-4)	2 (1-2)	3 (2-4)[a]
HCV-RNA (× 10^6 UI/mL)	0	6.24 ± 1.38	5.48 ± 1.32	6.18 ± 2.1

[b]$P < 0.01$ *vs* controls; [a]$P < 0.05$, [d]$P < 0.01$ *vs* CH with platelet counts > 150000/μL.

4-HNE, TNF-alpha, and IL6 were similar in the two groups. S-NO levels were significantly directly correlated with platelet number ($r = 0.58$, $P < 0.01$; Figure 2A) and significantly inversely correlated with percent of platelet aggregation ($r = -0.52$, $P < 0.05$; Figure 2B). In agreement with others[31], the number of platelets was significantly inversely related to the degree of histological fibrosis ($r = -0.78$, $P < 0.01$). A significant difference was observed between plasma levels of S-NO from patients with platelet counts > 150000/μL (generally considered a mean of normal values) and those with platelet counts < 50000/μL (33.8 ± 7.5 *vs* 21.4 ± 6.9 μmol/L, respectively; $P < 0.01$). For this purpose, we further evaluated the data by dividing HCV patients into two groups on the basis of their platelet numbers. Table 2 summarizes the results. The evaluation revealed significant differences among CH patients on the basis of their platelet numbers. In fact, in patients with a high degree of histological fibrosis, in addition to the number of platelets being significantly lower than that found in patients with a low degree of fibrosis, other parameters were significantly different. Nitrite was increased and S-NO, despite always being significantly higher than in the controls ($P < 0.01$), was significantly decreased relative to its level in patients with normal numbers of platelets. GSH and CYS decreased and MDA and 4-HNE increased with the progression of liver damage and reached values significantly different from those of controls ($P < 0.01$). In contrast, proinflammatory cytokines were higher in patients with a lower degree of fibrosis ($P < 0.01$ in comparison to the others). HCV RNA levels did not differ between the two CH groups. Table 2 also shows the relationship existing among thiols in the circulation and oxidative stress. In fact, with the increase of plasma markers of oxidative stress (MDA and 4-HNE), total nitrite, NPSH, GSH and CYS decrease, while S-NO increase.

Platelet aggregation

Compared with controls, maximal aggregation induced by ADP was significantly increased in CH patients (Figure 3). Moreover, the collagen addition induced a significant increase in maximal aggregation in the platelets from CH patients (Figure 3). After incubation with GSNO in agreement with others[25,32,33], maximal aggregation induced by ADP was significantly reduced in both groups, but the reduction was much more evident in the CH patients, while maximal aggregation induced by ADP was not changed by the addition of GSNO in control group and

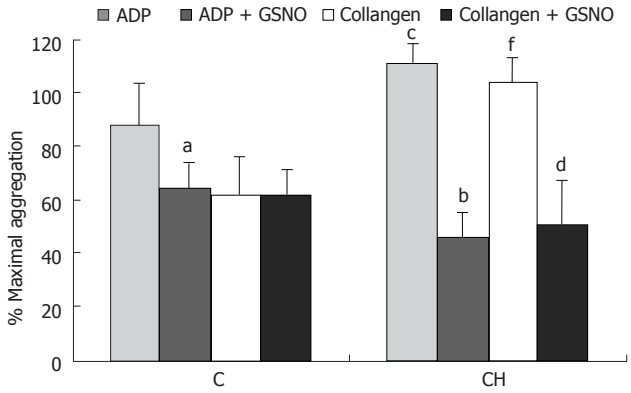

Figure 3 Percentage aggregation (mean ± SD) obtained in HCV-positive patients and controls, in basal conditions and after incubation with GSNO. Compared with controls % maximal aggregation induced by ADP was significantly increased in CH patients. Moreover, the collagen addition induced a significant increase in % maximal aggregation in the platelets from CH patients. After incubation with GSNO, % maximal aggregation induced by ADP was significantly reduced in both groups, but the reduction was much more evident in the CH patients, while % maximal aggregation induced by ADP was not changed by the addition of GSNO in control group and was significant reduced in CH patients. C: controls; CH: chronic hepatitis; GSNO: nitrosoglutathione. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* ADP; $^{d}P < 0.01$ *vs* collagen; $^{c}P < 0.05$ *vs* ADP; $^{f}P < 0.01$ *vs* collagen.

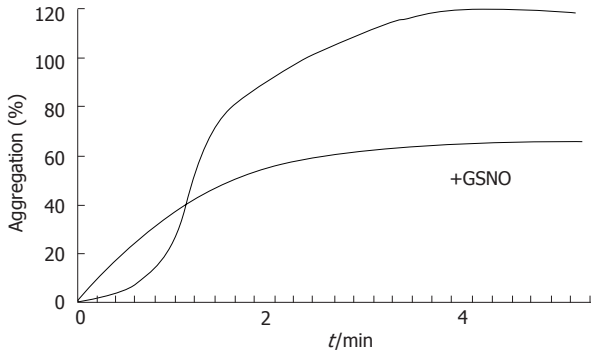

Figure 4 Aggregation curves of a single representative CH patient before and after GSNO. GSNO was able to reduce platelet % maximal aggregation and modified the curve morphology by abolishing the delay in the beginning of the aggregation. GSNO: nitrosoglutathione.

was significant reduced in CH patients (Figure 3).

Besides, the morphology of the aggregation curve induced by collagen was different in control and HCV-positive patients. In the latter a delay appeared at the beginning of the aggregation. Such a delay was completely abolished by GSNO. Figure 4 shows the aggregation curves of a single representative CH patient before and after GSNO. GSNO was able to reduce platelet maximal aggregation and modified the curve morphology by abolishing the delay in the beginning of the aggregation.

DISCUSSION

This study confirms[31] the inverse correlation between liver fibrosis and number of platelets. Thrombocytopenia in chronic hepatitis caused by HCV may be considered a consequence of portal hypertension or of myelosuppression by HCV or of reduced hepatic production of thrombopoietin[34-37]. However, our data provide additional explanations for the occurrence of thrombocytopenia in CH. In fact, we are the first to demonstrate that, as suggested by others[6], the function of platelets from patients with chronic HCV-related hepatitis may also be modified. *In vitro*, platelets from these patients display increased aggregation induced by ADP and collagen and a latency time before aggregation when compared with platelets from healthy controls. The addition of GSNO, the major constituent of circulating levels of S-NO *in vivo*, reverses these alterations, at least in part. In fact, GSNO reduces the aggregation induced by ADP in all groups, and in HCV positive patients, it not only reduces the aggregation induced by collagen but also abolishes the latency time. Our data also confirm the anti-aggregation activity of GSNO that has been documented by others *in vitro* and *in vivo*[18,25,32,33,38].

In vivo, S-NO may serve as a carrier in the mechanism of action of the endothelium-derived relaxing factor (EDRF), by stabilizing the labelled NO radical from inactivation by reactive species in the physiological milieu and by delivering NO to the heme activator site of guanyl cyclase. GSH and CYS form S-NO with anti-platelet properties associated with the stimulation of guanyl cyclase and a significant decrease in fibrinogen binding to platelets[39]. In addition, S-NO inhibits thrombin receptor-activating-peptide-induced platelet aggregation[33,40-42].

The state of NO in the circulation is dynamic, since it continuously forms S-NO adducts that are continuously exchanged with albumin or haemoglobin or released at the cellular level. The storage and biodegradability of NO in the circulation, which are strongly influenced by the redox status of plasma, significantly affect the bioavailability and effects of NO on platelets. The redox status of plasma also affects the import/export as well as the production of NO from platelets[12-14,43,44]. In fact, the dynamic process of S-thiolation in response to oxidative stress is considered an important pathway in the maintenance of the function of platelets[8,45]. S-NO may also act as antioxidant substances, as they inactivate nitrogen reactive species (nitroxyl anion, nitrosonium cation, nitrogenous oxides, peroxynitrite, *etc.*)[19]. In consideration of these complex interactions, we simultaneously evaluated plasma levels of nitrite, NPSH, GSH, CYS, and S-NO in order to have an overall picture, even if not complete, of the dynamic transport of NO in the circulation in relation to a possible alteration of the redox status.

In the present investigation, S-NO levels were increased in CH patients and directly correlated with the number of platelets. This increase is likely due to the increase in plasma levels of cytokines produced by activated neutrophils and monocytes. The activation of these circulating cells leads to an increase in the production of NO involved in exchange with NO adducts[46,47]. When we considered all patients with HCV CH together, we did not find any variation in the plasma levels of antioxidants or markers of lipid peroxidation. However, by dividing patients on the basis of their histology, we clearly documented that, in the absence of alterations in redox status, as expressed by normal plasma levels of thiols and lipid peroxidation markers, NO circulates

primarily as S-NO. In this situation, it may contribute to the maintenance of normal platelet function, probably by helping to counteract possible oxidative stress. With increased oxidative stress and/or decreased antioxidant levels, the nitrosylation pathways' activities are decreased and NO circulates as nitrite/nitrate, which are significantly increased in patients with more advanced liver fibrosis. In this condition, aggregation may be altered, resulting in the low number of platelets we recorded. In this context, the major cause of altered platelet function should be the decrease in GSH. In fact, this thiol is highly important for platelet function and when its level decreases, platelets can produce per se free radical intermediates when reacting with platelet aggregatory agents[48].

In healthy volunteers, oral administration of KNO_3 induces an increase in plasma S-NO and significantly inhibits platelet aggregation, without any effect on systemic and portal pressure[49]. Recently, S-NO have been chemically synthesized and proposed as a class of drugs that, upon decomposition, release NO and are therefore useful in patients with cardiovascular diseases[50]. They also have a detoxifying effect, as they inactivate nitrogen reactive species by continuously exchanging NO with SH groups[19].

In conclusion, our study reveals a defect in platelet aggregation in patients with HCV-related CH. This defect depends, in part, upon variations in NO-related pathways in the circulation. When the plasma redox status and bioavailability of GSH are normal, NO circulates primarily as SNO; in these conditions, the production of NO may also be increased by iNOS activated by cytokines. In this situation, S-NO could help to maintain normal platelet aggregation. Data from experiments *in vitro* confirm this hypothesis: the addition of GSNO ameliorated the aggregation defect of platelets from HCV patients. Increased oxidative stress and decreased bioavailability of GSH, as documented in patients with a high degree of fibrosis, lead to the formation of more nitrite than S-NO; all these events reduce platelet aggregation.

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BASIC RESEARCH

Experimental small bowel preservation using Polysol: A new alternative to University of Wisconsin solution, Celsior and histidine-tryptophan-ketoglutarate solution?

Lai Wei, Koichiro Hata, Benedict Marie Doorschodt, Reinhard Büttner, Thomas Minor, René H Tolba

Lai Wei, Koichiro Hata, René H Tolba, House of Experimental Therapy, University of Bonn, 53105 Bonn, Germany
Lai Wei, Institute of Organ Transplantation, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China
Benedict Marie Doorschodt, Surgical Laboratory, Academic Medical Center, 1105 AZ Amsterdam, The Netherlands
Reinhard Büttner, Institute of Pathology, University of Bonn, 53105 Bonn, Germany
Thomas Minor, Surgical Research Division, Department of Surgery, University of Bonn, 53105 Bonn, Germany
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Correspondence to: Dr. René H Tolba, House of Experimental Therapy, University of Bonn, Sigmund Freud Str. 25, 53105, Bonn, Germany. rtolba@uni-bonn.de
Telephone: +49-228-28719120 Fax: +49-228-28711657
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Abstract

AIM: To evaluate the potential of Polysol, a newly developed preservation solution, in cold storage of small bowel grafts, compared with the current standards, University of Wisconsin solution (UW), Celsior and histidine-tryptophan-ketoglutarate solution (HTK).

METHODS: Male Wistar rats were used as donors. Small bowels were retrieved, flushed and then stored in the respective 4 solutions for 18 h at 4℃. Functional integrity of the grafts was evaluated by isolated reperfusion with oxygenated Krebs-Henseleit buffer at 37℃ for 30 min in all 4 groups.

RESULTS: Polysol preservation exhibited the highest tissue ATP concentration and the lowest release of LDH. Malondialdehyde, an index for tissue lipid peroxidation, was also the lowest in Polysol. Tissue oxygen consumption was significantly higher in Polysol than in the others. Of interest, UW-storage promoted 10-fold higher apoptosis than in the others. Moreover, electron microscopy revealed that the mucosal villi/micro-villi formation and the cell organelles, including mitochondria, were both significantly better preserved in Polysol, while deleterious alterations were apparent in the others, most notably in UW. Although Celsior and HTK exhibited the better trend of results than UW in some parameters, but could not reach the over-all superiority to UW.

CONCLUSION: Cold storage using Polysol resulted in significantly better integrity and function of small bowel grafts than UW. Hence, Polysol may be a novel alternative for the small bowel preservation.

Key words: Small bowel transplantation; Ischemia/reperfusion; Cold storage; Polysol; University of Wisconsin solution; Histidine-tryptophan-ketoglutarate solution; Celsior

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INTRODUCTION

In the last decade, small bowel transplantation (SBTx) has emerged as a life-saving option for patients with intestinal failure and life threatening complications of total parenteral nutrition. Although the recent advances in immunosuppressive agents or regimens, such as Tacrolimus or anti-CD25 antibody, have brought a "break though" in organ transplantation, the patient outcome after SBTx still remains inferior to other organ transplantations, such as heart, kidney or liver[1].

Of note, the small bowel is the most perfused organ under physiological conditions, receiving up to 25% of all cardiac output, most of which (up to 90%) is consumed in the mucosa and the submucosa, to sustain its large surface area (up to 100 m^2) and its high turn over rate. This physiological feature of the gut leads, in turn, to the extreme vulnerability of the mucosal layer to ischemia during cold storage (CS). Although intestinal mucosa has high regenerative ability, Takeyoshi *et al* demonstrated that morphological recovery of the injured ileal mucosa after 24 h CS requires at least one month[2]. This prolonged damage from ischemia/reperfusion injury (IRI) surely participates not only in acute graft rejection but also in various postoperative complications, such as bacterial translocation

(BT), endotoxin absorption and long-lasting malnutrition of the recipient. Moreover, there have been lines of evidence demonstrating that the incidence of BT was not different between allogenic and isogenic transplantation, in the latter no immunological rejections occurred[3,4]. These facts clearly suggest that non-immunological factors, such as IRI, seem to play an important role in the development of BT. Taken all these together, how to protect the mucosal integrity during organ preservation still remains of primary interest to further improve the clinical outcome of SBTx[5].

Meanwhile, the current clinical standard for small bowel preservation is cold static storage using the University of Wisconsin solution (UW). Despite its overall acceptance as the first choice for solid organs, UW is unable to effectively preserve the small bowel grafts[6,7]. Small bowel preservation using UW is rather disappointing since the storage time is restricted only to 6-8 h, thus limiting the area of organ delivery. This limitation, at least in part, interferes to select the most proper recipient of HLA matching, even though more suitable candidates are out of the transportable area. Moreover, with such a relatively short storage duration, the 1-year graft survival after SBTx is still 65%[8]. Other commercially-available preservation solutions, such as Histidine-Tryptophane-Ketoglutarate (HTK) and Celsior have not been used in clinical SBTx so far, mainly due to the lack of relevant evidence for their usefulness[7,9,10]. Accordingly, there has clearly been an urgent need for developing a novel refinement in small bowel preservation as well as for a reliable comparison study of standard preservation solutions for successful SBTx.

Recently, a new preservation solution, Polysol, was developed for hypothermic machine-perfusion preservation. Polysol is a lower viscosity solution than UW, while keeping a high oncotic pressure. Furthermore, Polysol contains free radical scavengers, various kinds of amino acids, vitamins and nutrients, all of which certainly counteract the adverse effects during CS. In fact, we have recently demonstrated, using a rat model of steatotic liver preservation, that Polysol exhibited a superior quality of such "marginal" organ preservation in CS[11]. These characteristic features of Polysol have led us to hypothesize that this solution might reduce the high susceptibility of the small bowel grafts to IRI, even in simple CS.

The present study was thus designed to investigate the potential of Polysol on preserving the integrity and the function of small bowel grafts in CS, and to compare its efficacy and feasibility with the current standards, UW, Celsior and HTK.

MATERIALS AND METHODS

Animals

All animal experiments were performed in accordance with the federal German law regarding the protection of animals. This study also complied with institution guidelines as well as the criteria in "Guide for the Care and Use of Laboratory Animals" (NIH publication 86-23, revised 1985).

Male Wistar rats weighing between 250 g and 300 g were used as donors, and randomly assigned into four groups; UW, Celsior, HTK or Polysol group (n = 7 each). They all received humane care and had an acclimatization period of at least 1 wk under specific pathogen free conditions according to the FELASA (Federation of European Laboratory Animal Science Associations) recommendations.

Preservation solutions

UW (*Via* Span) was purchased from DuPont Pharma (Bad Homburg, Germany).

The HTK solution (Custodiol), from Dr. Franz Köhler Chemie GmbH (Alsbach-Hähnlein, Germany), Celsior from Genzyme (Neu-Isenburg, Germany), and Polysol from Doorzand Medical Innovations (Amsterdam, The Netherlands) were all provided free of charge for research purpose. The main components in the respective solutions are summarized in Table 1.

Retrieval of the small bowel

Prior to the surgical procedures, rats were fasted for 24 h while having free access to water, and then anesthetized by intramuscular injection of ketamine (90 mg/kg) and xylazine (10 mg/kg). The abdomen was opened by a midline incision with bilateral subcostal extensions, and the total jejunum and ileum was isolated with the vascular pedicle, as described in detail elsewhere[12]. The rats were administered heparin (1000 U/kg) before the organ retrieval. The superior mesenteric artery (SMA) was cannulated with a 20-gauge polyethylene catheter and the small bowel was immediately flushed with 10 mL of the respective solutions at 4℃. The portal vein was cannulated with a polyethylene catheter (I.D. 1.5 mm), later connected to a silicone tube to allow the collection of the venous effluent upon reperfusion. A short 14-gauge cannula was inserted into the upper jejunal lumen and secured with a circumferential tie. The intestinal lumen was first flushed with 20 mL cold normal saline solution, followed by a rinse with 15 mL of the respective preservation solutions.

Cold storage

The excised small bowel grafts were stored ischemically for 18 h in a cold bath of the respective solutions (100 mL). The temperature was kept at 4℃ constantly by an external cooling circuit (Ministat 125, Peter Huber Kältemaschinenbau GmbH, Germany).

Isolated reperfusion

The grafts were thereafter reperfused *in vitro* in a recirculating fashion at a constant flow rate of 8 mL/min for 30 min, according to the previously established techniques[12]. The grafts were weighed, and then gently reflushed with 5 mL of normal saline at 22℃ *via* SMA. They were then transferred into a temperature-controlled (37℃) organ bath, filled with the modified Krebs-Henseleit buffer (KHB). The compositions were as follows: 50 g/L dextran 78, 9.5 g/L KHB, 0.37 g/L calcium chloride, 0.06 mg/L dexamethasone, 0.07 g/L atropine, and 0.21% sodium bicarbonate. The small bowel graft was floated, but almost completely immersed in KHB at 37℃, allowing homogeneous perfusion flow and temperature. The care

Table 1 Composition of UW, celsior, HTK and polysol

Components	UW	Celsior	HTK	Polysol
Colloid (g/L)	HES (250 kDa), 50	-	-	PEG (35 kDa), 20
Na/K ratio (mmol/L)	27/125	100/15	10/15	135/5
Buffers (mmol/L)	$H_2PO_4^-$, 25	Histidine, 30	Histidine, 180	Histidine, 6.3 $H_2PO_4^-$, 21.74 HEPES, 20
Antioxidants (mmol/L)	Allopurinol, 1 Glutathion, 3	Glutathion, 3	-	Allopurinol, 1.2 Glutathion, 3 Alpha-tocopherol, 5×10^{-5} Ascorbic acid, 0.11
Nutrients (mmol/L)	-	-	Glucose, 28	Glucose, 11.1 Adenine, 5 Sodium pyruvate, 0.23
Impermeants (mmol/L)	Lactobionate, 100 Raffinose, 30	Lactobionate, 80		Raffinose, 3 Trehalose, 5.3 Na^+-Gluconate, 74.99 K^+-Gluconate, 20
Amino acids (mmol/L)	-	Glutamic acid, 20	Tryptophan, 2	various[1], 11
Vitamins (mmol/L)	-	-	-	various[2], 0.17
Ca^{2+}/Mg^{2+} (mmol/L)	-	0.25/13	0.015/4	2/4
Others (mmol/L)	Adenosine, 5	Mannitol, 60	Mannitol, 30 Ketoglutarate, 1	Adenosine, 5
pH	7.4	7.3	7.2	7.4
Osmolarity (mOsm/L)	320	320	310	320
Viscosity at 5℃ (cP)	5.7	1.3	1.8	1.8

UW: University of Wisconsin solution; HTK: histidine-tryptophan-ketoglutarate solution; HES: hydroxyethyl starch; PEG: polyethylene glycols; cP: centi-Poise. [1]The following amino acids (mmol/L) are supplemented in Polysol: alanine (1.01), arginine (1.18), asparagines (0.08), aspartic acid (0.23), cystine (0.33), cystine (0.25), glutamic acid (0.34), glutamine (0.002), glycine (0.67), isoleucine (0.38), leucine (0.57), lysine (0.48), methionine (0.30), ornithine (2.00), phenylalanine (0.30), proline (0.78), serine (0.29), threonine (0.34), tryptophan (0.88), tyrosine (0.19), and valine (0.43). [2]The following vitamins (mmol/L) are supplemented in Polysol: ascorbic acid (0.11), biotin (0.21), Ca-pantothenate (0.004), choline chloride (0.01), inositol (0.07), ergocalciferol (3×10^{-4}), folic acid (0.002), menadione (4×10^{-5}), nicotinamide (0.01), nicotinic acid (0.004), pyridoxal (0.005), riboflavin (0.003), thiamine (0.03), vitamin A (3×10^{-4}), vitamin B12 (1×10^{-4}) and vitamin E (5×10^{-5}).

was taken to avoid unnatural bending or distortion of the graft. Carbogen (95% O_2 + 5% CO_2) was used for oxygenation, and perfusate-pO_2 was continuously kept over 500 mmHg during the whole reperfusion period. The venous effluent was collected intermittently through the portal vein catheter for biochemical analysis.

Parameters

Adenosine triphosphate (ATP) concentration: At the end of reperfusion, tissue samples in all groups were snap frozen in liquid nitrogen and preserved below -80℃ until later analysis. The samples were first cut into small pieces in liquid nitrogen and weighed (wet-weight), then freeze-dried in a high vacuum system at -40℃ for six days (Beta 1-16, Martin Christ Gefriertrocknungs-Anlagen GmbH, Osterode, Germany). After tissue water was evaporated, the samples were weighed again (dry-weight). The tissue concentrations of ATP were determined by standard enzymatic tests, as described in detail elsewhere[13]. The results were ultimately corrected with the respective wet-weight per dry-weight ratio of the samples, and expressed in micromoles per gram of dry-weight.

Lactate dehydrogenase (LDH) release: Effluent activities of LDH were determined at the end of reperfusion using a commercially-available photometric kit (Boehringer, Mannheim, Germany), as an index for graft tissue damage.

Tissue lipid peroxidation: At the end of reperfusion, tissue samples in all groups were snap frozen in liquid nitrogen and preserved below -80℃. Tissue malondialdehyde (MDA) was extracted at 4℃ in 10 mL/g frozen wet-weight of 0.33 mol/L perchloric acid ($HClO_4^2$), and then neutralized with 2 mol/L potassium hydroxide (KOH). After removing precipitated $KClO_4$, the MDA concentrations were determined using a fluorometric method, as detailed elsewhere[14].

Oxygen consumption: The oxygen consumption of the small bowel tissue was determined as a parameter for the remaining metabolic activity of the grafts. Perfusate samples at 30 min reperfusion were taken both from the SMA inflow and from the portal venous effluent, and their oxygen contents were measured immediately by a pH-blood gas analyzer (ABL 5, Acid-Base Laboratory, Radiometer, Copenhagen, Denmark). The oxygen uptake was calculated from the difference between the both samples and expressed as μL O_2 per minute per gram dry-weight, according to the perfusion flow, tissue mass and the solubility coefficient (24 μL of O_2 per milliliter buffer).

Apoptosis

Perfusate samples at 15 min of reperfusion were also tested for fragmented DNA release using a Cell Death Detection ELISAPLUS Kit (Roche Molecular Biochemicals, Mannheim, Germany), according to the manufacturer's instruction.

Electron microscopy

At the end of CS, additional tissue samples (n = 3 each)

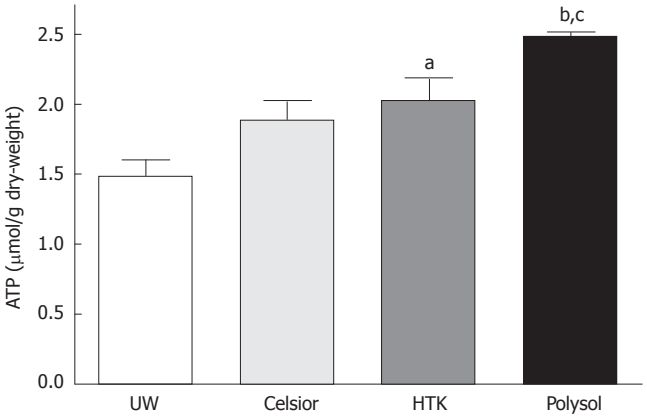

Figure 1 Tissue ATP Content as a parameter for functional recovery of the mitochondria and resultant tissue integrity after 18 h cold storage and 30 min oxygenated reperfusion. Data are expressed as micromoles per gram of tissue dry-weight (mean ± SE, $n = 6$ each). [a]$P < 0.05$ *vs* UW, [b]$P < 0.001$ *vs* UW, [c]$P < 0.05$ *vs* Celsior.

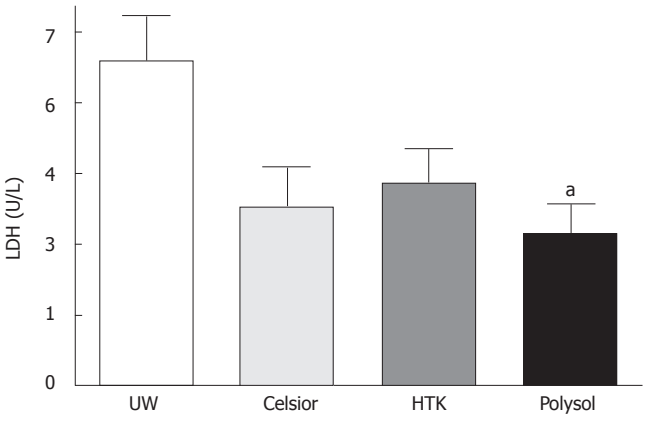

Figure 2 LDH release into the perfusate, as a parameter for general tissue damage after 18 h cold storage and 30 min oxygenated reperfusion (mean ± SE, $n = 7$ each). [a]$P < 0.05$ *vs* UW.

were perfused with glutaraldehyde and paraformaldehyde solution (2% each in phosphate-buffered saline) through SMA with a constant pressure of 70 cm H_2O, then immersed into the same solution for at least 2 d at 4℃. The samples were cut into 0.5 mm slices, post-fixed with OsO_4 (osmic acid fixative) and embedded in Epon 812 (Serva, Heidelberg, Germany). Semithin sections were then stained according to the procedure by Richardson *et al*[15]. Thin sections were thereafter stained with uranyl acetate and lead citrate, and then examined with a Phillips CM 10 electron microscope (Phillips, Eindhoven, The Netherlands).

Statistical analysis

All results are expressed as mean ± SE from the 7 independent observations, unless otherwise mentioned. Comparisons among the 4 groups were performed by one-way analysis of variance (ANOVA), followed by Tukey-Kramer's post hoc test. The differences were considered statistically significant when *P*-values were less than 0.05.

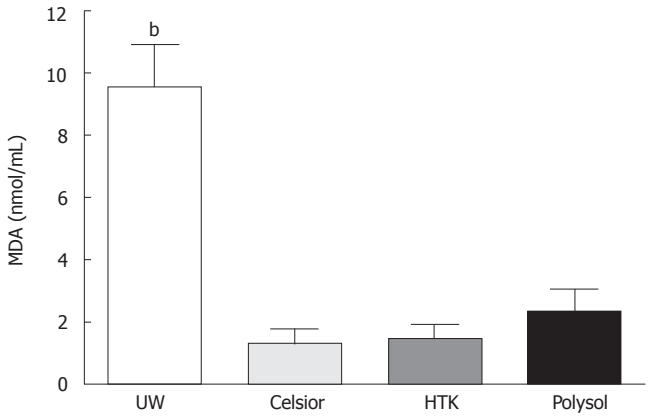

Figure 3 MDA concentrations in the graft tissues, as an index for lipid peroxidation after 18 h cold storage and 30 min oxygenated reperfusion (mean ± SE, $n = 6$ each). [b]$P < 0.001$ *vs* Celsior, HTK and Polysol.

RESULTS

Tissue ATP content

The tissue ATP concentration of the graft after CS and oxygenated reperfusion is regarded as one of the most important parameters reflecting the organ viability. We determined the tissue ATP contents at the end of reperfusion in all groups (Figure 1). The value in Polysol (2.480 ± 0.036 μmol/g dry-weight) were highest, and was significantly higher than UW (1.488 ± 0.119 μmol/g dry-weight, $P < 0.001$) and in Celsior (1.885 ± 0.143 μmol/g dry-weight, $P < 0.05$). The value in HTK (2.031 ± 0.156 μmol/g dry-weight) was also significant higher than UW ($P < 0.05$).

LDH release

After 18 h CS and 30 min oxygenated reperfusion, LDH release in UW (69 ± 9.4 U/L) was the highest, followed by HTK (43 ± 7.3 U/L) and Celsior (38 ± 8.4 U/L). The release was the lowest in Polysol (32 ± 6.5 U/L) among the 4 groups. Only the value in Polysol reached the statistically significant level versus UW ($P < 0.05$, Figure 2).

Lipid peroxidation

Tissue MDA content in UW was 9.55 ± 1.360 nmol/mL, which was significantly higher than the other 3 groups ($P < 0.001$ *vs* the others). The values in Polysol (2.34 ± 0.731 nmol/mL). HTK (1.47 ± 0.452 nmol/mL) and Celsior (1.33 ± 0.460 nmol/mL) were not significantly different (Figure 3).

Oxygen consumption

The levels of tissue oxygen uptake were not different among the 3 groups (UW: 9.65 ± 0.83, Celsior: 10.63 ± 0.80, HTK: 9.89 ± 0.46 μL/min per gram dry-weight). The value in Polysol (13.94 ± 0.51 μL/min per gram dry-weight) showed the highest among the 4 groups (Figure 4), reaching the significantly higher level *vs* Celsior, HTK ($P < 0.01$) and UW ($P < 0.001$).

Apoptosis

To quantitatively assess the magnitude of apoptosis, we

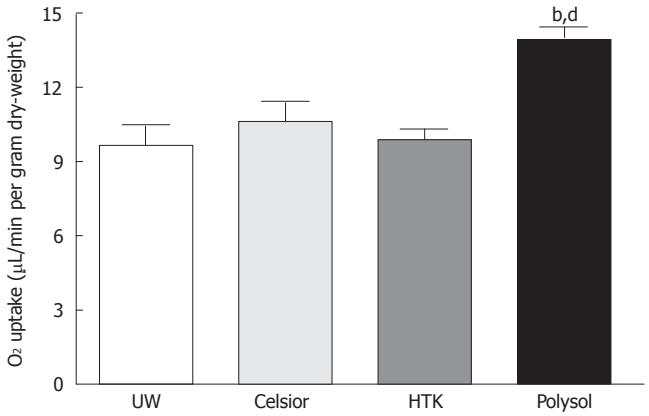

Figure 4 Tissue oxygen consumption at 30 min oxygenated reperfusion, as a parameter for the remaining metabolic activity of the grafts (mean ± SE, $n = 7$ each). $^{b}P < 0.01$ *vs* HTK and Celsior, $^{d}P < 0.001$ *vs* UW.

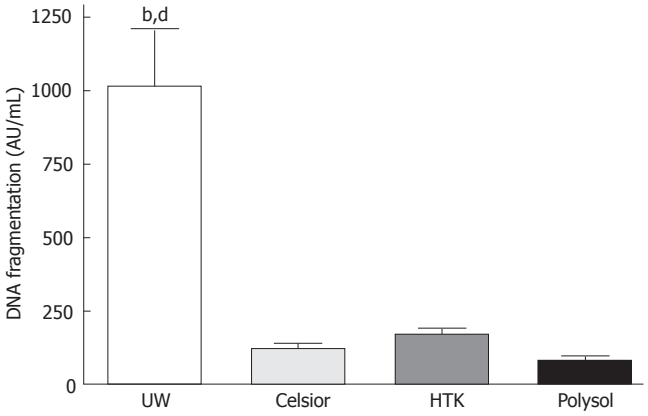

Figure 5 The release of fragmented DNA into the perfusate at 15 min of reperfusion using a Cell Death Detection ELISA kit (mean ± SE, $n = 6$ each). $^{b}P < 0.01$ *vs* HTK, $^{d}P < 0.001$ *vs* Celsior and Polysol.

performed a DNA fragmentation ELISA. The highest apoptotic cell death was detected in the UW group (1014 ± 268 AU/mL), which was significantly higher than the other 3 groups ($P < 0.01$ *vs* HTK, $P < 0.001$ *vs* Celsior and Polysol). The lowest apoptosis was detected in Polysol (81 ± 17 AU/mL), followed by Celsior (123 ± 17 AU/mL) and HTK (172 ± 20 AU/mL). Although statistically not significant to Celsior and HTK, Polysol group showed the lowest values, while 10-fold higher apoptosis was identified in UW (Figure 5).

Electron microscopy

In order to evaluate the graft tissue damage at ultrastructural level, especially focusing on the microvilli formation as well as on the cell organelles in enterocytes, we conducted electron microscopy after 18 h CS, as illustrated in Figure 6.

The microvilli formation in UW was severely damaged, showing sparse and lowered-height of microvilli (Figure 6A). In Celsior, although the microvilli density was relatively better preserved than in UW, the height of microvilli was apparently lowered to the same level in UW (Figure 6B). In HTK, the height of microvilli was better preserved than in UW and in Celsior; however, the breakdown of their apical region was diffusely noted, displaying ragged and jagged formation (Figure 6C). In contrast to the other 3 groups, such deleterious alterations were rarely visible in Polysol, indicating homogeneously preserved microvilli epithelia of the luminal surface (Figure 6D).

With regard to the damage in cell organelles, the mitochondria in UW displayed mild swelling and less electron density of their matrices, in which the cristae were hardly visible (Figure 6E). In Celsior and HTK, such detrimental alterations in mitochondria were also identified, in spite of relatively better-preserved electron density of the ultrastructures (Figure 6F and G). Unlike the other groups, the mitochondria in Polysol kept their oval shape with well-maintained cristae formation (Figure 6H). These findings are thus in good agreement with the results of tissue oxygen consumption and the resulting ATP contents, at the point of mitochondrial damage and function.

DISCUSSION

Unlike other organs, small bowel transplantation (SBTx) has several characteristic burdens, such as rich lymphoid tissues and large mucosal surface expressing class-2 major histocompatibility antigens. Indeed, the recent achievements in immunosuppressants, including the development of Tacrolimus, had brought a new era also in the management and outcome of clinical SBTx[16]. Thus, uncontrollable immunological rejection, which had long been the main cause of patient death, was superseded by septic complications as the main cause of mortality[1,8]. These events then emphasize another aspect of high hurdles in SBTx: How to preserve the mucosal integrity and the barrier functions during/after organ preservation.

In the present study, we demonstrated that cold storage (CS) of small bowel grafts using Polysol resulted in significantly better quality of graft preservation than the current clinical standard, UW, in all the parameters we tested. Surprisingly, UW exhibited the worst trend of results in most parameters, even compared with other currently available solutions, HTK and Celsior. In particular, morphological integrity of the mucosa, both microvilli formation and cell organelles, such as mitochondria, were significantly better preserved by Polysol, whereas detrimental alterations were apparent in the UW-stored grafts. Although the present study did not elucidate the exact mechanisms of the benefits by Polysol, as well as of the worst results by UW, one possible explanation to this difference is, at least in part, in numerous amino-acid supplementations in Polysol.

Polysol contains up to 21 amino acids. During CS, these amino acids play protective roles in a variety of cellular house keeping processes by catering both for metabolic (energy production) and for synthetic (synthesis of critical molecules) aspects of intestinal metabolism[17,18]. Among them, glutamine, glutamate, and aspartate are crucial for enterocytes as major energy sources[19]. The intestinal glutamine catabolism is a multistep process, resulting in ATP production *via* the TCA cycle or *via* the provision of key elements to the nitrogen-carbon backbone of purines. Since the glucose utilization is

limited during CS, glutamine supplementation is thought to be effective to maintain cellular ATP level, thus alleviating the tissue damage[20]. Furthermore, because small intestinal mucosa becomes atrophic when the gut is deprived of glutamine, its supplementation surely counteracts the mucosal atrophy during/after cold storage, as manifested by electron microscopy. Glutamic acid also augments energy production in anaerobic conditions[12]. Moreover, glycine is expected to work against oxygen species and stabilize the tertiary protein structure of cell membranes upon reperfusion[21]. Also, glycine can restore cellular ATP levels[22]. As already reported, amino-acid based solutions could provide a better energy storage in the small bowel preservation[23,24].

Another characteristic difference is noticed in the anti-edematous ingredients (Table 1). Different from the others, Polysol contains polyethylene glycol (PEG) that enables both the required oncotic pressure and the relatively low viscosity (1.8 cP). PEG stabilizes lipid membranes and contributes to less membrane permeability[25,26], thus preventing osmotic cell swelling and vascular endothelial damage[27]. Moreover, PEG also act as a OFR scavenger, markedly reducing lipid peroxidation upon preservation/reperfusion process[27,28]. In UW, the colloid used is hydroxyethyl starch (HES), which (5% in UW) causes over 3-fold higher viscosity (5.7 cP), thereby triggering vasoconstriction and endothelial damage, in cooperation with the high potassium concentration[29,30]. Moreover, HES also causes hyperaggregation of erythrocytes, which may result in incomplete, heterogeneous blood-washout of the graft tissue. On the other hand, HTK and Celsior are crystalloid solutions with lower viscosity and lower potassium concentration (Table 1). It was already proven that HTK and Celsior were superior than UW as an initial flushing solution, not only for small bowel but for liver and heart[31,32], mainly due to the lower viscosity of 1.3-1.8 cP. The viscosity of Polysol is also 1.8 cP, and was proven to have the same efficacy to HTK as a flushing solution[33].

Among the parameters we investigated, one of the most remarkable differences is notable in the magnitude of apoptosis. Surprisingly, the apoptosis in the UW-stored grafts was almost 10-fold higher than that in Polysol. Apoptosis affects the permeability of the intestinal mucosa, impairs its barrier function after transplantation, thus leading to BT and endotoxin absorption. As previously demonstrated, apoptosis is a predominant form of cell death in intestinal IRI[34], and is an important predictive factor for primary allograft dysfunction in clinical SBTx[35]. Considering the mechanisms underlying the difference in apoptosis, we focused on the antioxidative properties in each solution. As presented in Figure 4, lipid peroxidation, caused by oxygen free radicals (OFR), in UW group was almost 5-fold higher than the other groups. Polysol has optimized OFR scavenging potential, achieved by supplementation of not only glutathione and allopurinol but also ascorbic acid (vitamin C), selenium, glycine and alpha-tocopherol (vitamin E), all of which were proven to be effective as antioxidants upon organ preservation[36-38]. Celsior and HTK, include relatively high dose of histidine (Table 1), which neutralizes reactive oxygen species and preserves high-energy phosphates[39]. Thus the enhanced antioxidative property of these 3 solutions attenuated OFR-mediated cellular damage upon reperfusion, thereby reducing apoptotic cell death.

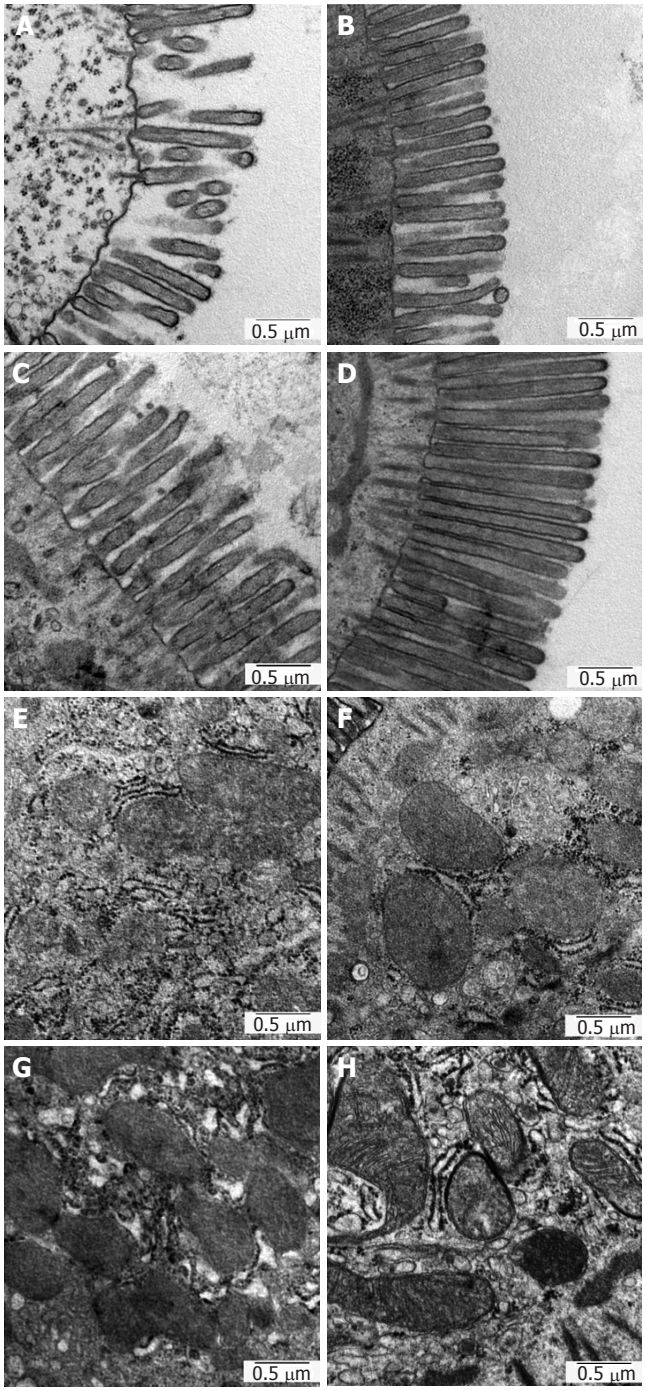

Figure 6 **A-D**: ultrastructural analysis using electron microscopy, microvilli condition after 18 h CS; **E-H**: ultrastructural analysis using electron microscopy, mitochondria in enterocytes after 18 h CS.

Although further investigations in a transplant model are of course required to confirm the findings in this study, this *ex vivo* experimental setting has several advantages to assess the quality of organ preservation. First, in contrast to a real transplant model, the results from this isolated setting are not influenced from immunological alloreactions, facilitating the precise evaluation for the organ preservation. Taken into account that intestinal mucosa is the main target from the host

immune system, because of its high expression of class-2 major histocompatibility antigens, this isolated setting is thought to be feasible to assess the preservation quality itself, without any interference from immunological reactions. Simpler procedures (just organ retrieval, without implantation) and resultant well-reproducible results are also the advantages of this method.

In summary, this is the first report demonstrating a comparison study of the currently available preservation solutions, UW, HTK, Celsior and Polysol, in the efficacy for small bowel preservation. Polysol, a newly developed preservation solution, exhibited the superior quality of small bowel preservation than the current clinical standard, UW after 18 h cold storage. HTK and Celsior also showed better potential in some parameters, however, the both could not reach the over all superiority than UW. Hence in conclusion, Polysol may be a novel suitable alternative for small bowel preservation.

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BASIC RESEARCH

Protective effects of ischemic preconditioning and application of lipoic acid prior to 90 min of hepatic ischemia in a rat model

Friedrich Duenschede, Kirsten Erbes, Nina Riegler, Patrick Ewald, Achim Kircher, Stefanie Westermann, Arno Schad, Imke Miesmer, Simon Albrecht-Schöck, Ines Gockel, Alexandra K Kiemer, Theodor Junginger

F Duenschede, K Erbes, N Riegler, P Ewald, A Kircher, S Westermann, I Miesmer, S Albrecht-Schöck, I Gockel, T Junginger, Department of General and Abdominal Surgery, University hospital Mainz, Langenbeckstr.1, D-55131 Mainz, Germany
A Schad, Institute for Pathology, University hospital Mainz, Germany
AK Kiemer, Department of Pharmacy, Pharmaceutical Biology, Saarland University, 66123 Saarbrücken, Germany
Correspondence to: Friedrich Dünschede, MD, University Hospital Mainz, Department of General and Abdominal Surgery, Langenbeckstr. 1, 55131 Mainz,
Germany. duenschede@ach.klinik.uni-mainz.de
Telephone: +49-6131-177291 Fax: +49-6131-176630
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Abstract

AIM: To compare different preconditioning strategies to protect the liver from ischemia/reperfusion injury focusing on the expression of pro- and anti-apoptotic proteins. Interventions comprised different modes of ischemic preconditioning (IP) as well as pharmacologic pretreatment by α-lipoic acid (LA).

METHODS: Several groups of rats were compared: sham operated animals, non-pretreated animals (nt), animals receiving IP (10 min of ischemia by clamping of the portal triad and 10 min of reperfusion) prior to sustained ischemia, animals receiving selective ischemic preconditioning (IPsel, 10 min of ischemia by selective clamping of the ischemic lobe and 10 min of reperfusion) prior to sustained ichemia, and animals receiving 500 μmol α-LA injected i.v. 15 min prior to the induction of 90 min of selective ischemia.

RESULTS: Cellular damage was decreased only in the LA group. TUNEL-positive hepatocytes as well as necrotic hepatocyte injury were also decreased only by LA (19 ± 2 *vs* 10 ± 1, $P < 0.05$ and 29 ± 5 *vs* 12 ± 1, $P < 0.05$). Whereas caspase 3- activities in liver tissue were unchanged, caspase 9- activity in liver tissue was decreased only by LA pretreatment (3.1 ± 0.3 *vs* 1.8 ± 0.2, $P < 0.05$). Survival rate as the endpoint of liver function was increased after IP and LA pretreatment but not after IPsel. Levels of lipid peroxidation (LPO) in liver tissue were decreased in the IP as well as in the LA group compared to the nt group. Determination of pro- and anti-apoptotic proteins showed a shift towards anti-apoptotic proteins by LA. In contrast, both our IP strategies failed to influence apototic cell death.

CONCLUSION: IP, consisting of 10 min of ischemia and 10 min of reperfusion, protects only partly against ischemia/reperfusion injury of the liver prior to 90 min of selective ischemia. IPsel did not influence ischemic tolerance of the liver. LA improved tolerance to ischemia, possibly by downregulation of pro-apoptotic Bax.

Key words: Warm liver ischemia; Liver preconditioning; Apoptosis; Lipid peroxidation; Pharmacological preconditioning

Duenschede F, Erbes K, Riegler N, Ewald P, Kircher A, Westermann S, Schad A, Miesmer I, Albrecht-Schöck S, Gockel I, Kiemer AK, Junginger T. Protective effects of ischemic preconditioning and application of lipoic acid prior to 90 min of hepatic ischemia in a rat model. *World J Gastroenterol* 2007; 13(27): 3692-3698

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INTRODUCTION

Ischemic preconditioning (IP), first described by Murry *et al*[1], has been reported to induce tolerance to ischemia/reperfusion injury (IRI) in many organs, such as brain, heart, and skeletal muscle[2]. Especially in IRI of the liver, there are numerous studies that describe the protective effects of IP[3].

With both *in vivo* and *in vitro* models, the cycle of preconditioning seems to be critical for the induction of ischemic tolerance. Hypoxic periods shorter than 5 min duration or exceeding 15 min failed to induce protection. Similarly, periods of reperfusion of shorter than 5 min or exceeding 15 min also had no protective effect[4]. Ischemic tolerance of the rat liver was demonstrated by IP consisting of 5 min of ischemia and 30 min of reperfusion[5]. IP has shown contradictory effects in numerous experimental studies using different animal species[6-8]. In humans, IP has been carried out as a protective strategy against hepatic IRI[9,10].

Apart from the beneficial effects on hepatic IRI, the mechanism by which IP confers protection remains

to be elucidated. Blocking the generation of reactive oxygen species (ROS) is considered one of the most likely mechanisms of action of IP[5,11]. IP has been shown to protect the mouse liver by inhibition of apoptosis through a caspase-dependent pathway[4].

Pro- and anti-apoptotic proteins, such as Bax and Bcl-2, play a key role in mediating apoptotic cell death in hepatic IRI. Bax was described to directly induce the release of cytochrome c from isolated mitochondria, whereas overexpression of Bcl-2 strongly attenuated the hepatic IRI by inhibition of cytochrome c release[12].

Previous studies of our group showed that LA is a fast, easy, and safe method to attenuate IRI[13]. As lipoamide, LA is a cofactor in multiple enzyme complexes that catalyze the oxidative decarboxylation of γ-keto acids, such as pyruvate, γ-ketogluterate and branched-chain γ-keto acids. It was found to be synthesized by animals and humans. Recently, it has been found that LA exerts powerful antioxidant effects. These effects have led to studies of the use of LA in a number of oxidative stress conditions[14].

Although IP is presently the best accepted strategy to attenuate IRI, IP has only rarely been compared to other interventions. A comparison of a "traditional" IP strategy (i.e. 10 min of ischemia and 10 min of reperfusion) with both a novel selective, partial clamping method and a promising pharmacologic intervention (LA) may provide insights into the mechanisms of action of IRI. Selective portal triad clamping (IPsel) has been described to minimize IRI of the liver during ablative treatment of colorectal metastases. However, studies on the influence of IPsel on IRI of the liver are rare[15].

The aim of the present study was to compare both the extent and potential mechanism of action of three different preconditioning strategies.

MATERIALS AND METHODS

Animals

Male Brown Norway rats weighing 175-200 g (Harlan, Paderborn, Germany) were used. All animals had access to water and rat chow *ad libitum* (Global Diet Harlan). The animals were maintained in accordance with the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23, revised 1985). The study was approved by our Institutional Animal Care and Use Committee.

Ischemic preconditioning

IP was carried out by placing a microvascular clamp on the portal triad. IPsel was carried out by placing a microvascular clamp on the corresponding branch of the portal vein, hepatic artery, and bile duct of the left (acute experiment) or the median (animal survival) liver lobe proximal to its origin. As a model for acute experiments, 90 min ischemia of the left liver lobe was performed by clamping the corresponding branch of the portal vein, hepatic artery, and bile duct proximal to its origin. Reperfusion was initiated by removal of the clamp. Thereafter, the abdominal cavity was closed with running sutures, and animals were allowed to awaken without resuscitation of additional fluid. The animals had free access to water and rat chow *ad libitum*. After 4 h of reperfusion, anesthetized animals were sacrificed, and liver tissue and plasma were stored for further examination.

Several experimental groups were studied: (1) sham-operated animals (n = 3); (2) 90 min ischemia without any pretreatment (n = 5), (3) 90 min ischemia with IP (10 min of ischemia by clamping of the portal and 10 min of reperfusion) prior to sustained ischemia (n = 5), (4) 90 min ischemia with IPsel of the ischemic lobe (10 min of ischemia by selective clamping of the left lobe and 10 min of reperfusion) prior to sustained ichemia (n = 5), and (5) 500 μmol LA (120 μg/mL saline) *via* the abdominal vena cava 15 min prior to sustained ischemia (n = 5). Body temperature (37 ± 1℃) was monitored and maintained by a heating lamp. The sham, untreated, IP, and IPsel groups received vehicle (1 mL).

Animal survival was investigated by resecting non-ischemic liver tissue at the time of reperfusion. After induction of anesthesia with inhaled ether, the abdominal cavity was accessed through a midline incision. Inflow occlusion of the median lobe was performed by placing a microvascular clamp on the median branch of the portal vein, hepatic artery, and bile duct. Ischemia was maintained for 90 min, immediately after which the non-ischemic liver tissue, i.e. the right, the caudate, and the left liver lobes, was resected (70% liver tissue). Thereafter, the abdominal cavity was closed with running sutures, and animals were allowed to awake without resuscitation of additional fluid.

Several experimental groups were studied: (1) control animals only treated by liver resection (n = 3); (2) 90 min of ischemia of the median liver lobe followed by liver resection (n = 5), (3) 90 min of ischemia of the median liver lobe with IP (10 min of ischemia by clamping of the portal triad and 10 min of reperfusion) prior to sustained ischemia and liver resection (n = 8), (4) 90 min ischemia of the median liver lobe with IPsel (10 min of ischemia by selective clamping of the median liver lobe and 10 min of reperfusion) prior to sustained ichemia and liver resection (n = 5), and (5) 90 min ischemia of the median liver lobe with 500 μmol LA (120 μg per 1 mL saline) administered *via* the abdominal vena cava 15 min prior to sustained ischemia and liver resection (n = 8). Body temperature was monitored and maintained at 37 ± 1.0℃ by a heating lamp. The control, untreated, IP, and IPsel groups received vehicle (1 mL).

Homogenization of liver tissue

Liver tissue was weighed prior to homogenization, and then diluted 1:5 with 0.01 mol/L PBS (phosphate buffered saline). Homogenization was carried out with a Potter tissue homogenizer. A homogeneous mixture was achieved by vortexing at 800 r/min. Next, the mixture was centrifuged at 1000 × g for 10 min and the supernatant pipetted into an Eppendorf tube and centrifuged at 10000 × g for 20 min. The clear supernatant was pipetted into a fresh Eppendorf tube and stored frozen at -20℃ until further measurement.

Measurement of alpha-glutathione-S-transferase (α-GST)

α-GST in plasma 4 h after the onset of reperfusion was

measured by absorption using a microplate reader (Bio Rad). The ELISA kit was run in accordance with the manufacturer's instructions (Biotrin, Sinsheim-Reihen, Germany).

Determination of lipid peroxidation (LPO)

LPO was determined with a standardized test (Cayman Chemical, Grünberg, Germany). Absorption measurements were done at 500 nm, using a microplate reader. The test measures the lipid hydroperoxides based on the calibration curve, utilizing the redox reactions with ferrous ions. The resulting ferric ions are detected, using thiocyanate ion as the chromogen. This procedure eliminates any interference caused by hydrogen peroxide or endogenous ferric ions in the sample, and provides a sensitive and reliable assay for lipid peroxidation.

Caspase 3- and 9- activity in liver tissue

Caspase 3- and 9- activity was measured using a colorimetric reaction (Caspase Colorimetric Assay, R&D Systems Germany, Wiesbaden) at a wavelength of 405 nm. All samples were prepared in pairs; one pair was measured with and one without substrate according to the manufacturer's instructions. The results are expressed as fold increase in caspase activity in post-ischemic versus non-ischemic sham liver tissue[16].

The protein concentration was determined by spectrophotometry with a standardized test (Protein Assay ESL, Roche Diagnostics).

Pathology and apoptosis evaluation

Formalin-fixed hepatic tissue samples were embedded in paraffin, and 5 μm sections were cut. Replicate sections were either stained with hematoxylin and eosin (H&E) for the evaluation of morphologic features of necrosis or stained with the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay for the evaluation of apoptosis.

The liver sections were fixed in freshly prepared neutral buffered formalin (PBS). Frozen sections (5 μm) of the fixed tissue were prepared and stained with the TUNEL method, using a commercial kit (Chemicon International, ApoTag peroxidase *in situ* apoptosis detection kit, Hampshire, UK). The percentage number of TUNEL-positve hepatocytes was estimated by evaluating the number of hepatocytes per high power field (hpf) compared with number of TUNEL-positive hepatocytes (× 400).

Morphological criteria of necrosis, such as shrinkage, loss of architecture, eosinophilia and karyolysis, were evaluated in serial sections stained with H&E. The percentage of necrosis was estimated by evaluating the number of microscopic fields with oncosis compared with the entire histologic section. All histologic evaluations were done in a blinded fashion.

Bax and Bcl-2 expression

The expression of Bax and Bcl-2 was assessed by RT-PCR. The expression of 18 S ribosome was used as the housekeeping gene. Total RNA was isolated using RNeasy Mini-Kits (Qiagen). After photometric determination of the amount of RNA, a first strand cDNA was synthesized from 4 μg RNA from each liver, using the following procedure: the transcription mix contained 5 μL oligo(dT) (Gibco-BRL), 3.6 μL dNTP-Mix (10 mmol/L) (Gibco-BRL), 8 μL first strain buffer, 4 μL DTT (0.1 mol/L), 1.5 μL reverse transcriptase (MLV-T, Roche Diagnostics, Mannheim, Germany) and 1.5 μL RNA free water (Qiagen). Controls were performed without the addition of reverse transcriptase. Cycle conditions were chosen as follows: 90 min at 37℃, 10 min at 94℃, and 30 s at 4℃. The cDNA was checked by amplification of 18 S robosome. Primers were designed specifically for rats with Primer 3 Software (Whitehead Institute, Boston, USA) and synthesized by MWG Biotech, Germany.

The following primers were used: Bcl-2 neu forward: 5'-TGC-AGA-GAT-GTC-CAG-TCA-GC-3' and Bcl-2 neu reverse: 5'-CAT-CCA-CAG-AGC-GAT-GTT-GT-3' (expected product 200 bp), Bax forward: 5'-ACA-GAT-CAT-GAA-GAC-AGG-GG-3' and reverse 5'-CAA-AGT-AGA-AGA-GGG-CAA-CC-3' (expected product 203 bp).

All sequences were compared with the complete rat Gene Bank library to ensure that each primer was unique for its intended target and that areas of sequence polymorphisms were avoided.

We additionally performed quantitative real-time PCR analysis of transcripts for Bax and GAPDH with predesigned and optimized TaqMan® Gene Expression Assays (Applied Biosystems) on an Applied Biosystems 7500 Real-Time PCR System, according to the manufacturer's instructions as described previously[17]. Relative quantitation was carried out using the delta-delta-CT method.

Statistical analysis

All data are expressed as mean ± SD. Statistical differences between experimental groups were calculated by GraphPad Prism Software 1994-1999 using a one-way analysis of variance (ANOVA) with subsequent post hoc Tukey-tests. Survival curves were calculated using the Kaplan and Meier method and compared by a log-rank test. Proportions were calculated using Fisher's exact test. Differences were considered statistically significant at $P < 0.05$.

RESULTS

Serum α-GST

After 90 min of ischemia and 4 h of reperfusion, α-GST release increased markedly in the untreated, IP, and IPsel groups in comparison to the sham-operated group, whereas α-GST increased only slightly after 4 h reperfusion in the LA-group. α-GST was used as a sensitive marker of hepatocellular injury for several reasons: α-GST exhibits a specific but ubiquitious hepatic distribution, has a short serum in vivo half life time (< 1 h) and is released rapidly and substantially into the circulation in large amounts after acute hepatocellular damage[18] (Figure 1).

LPO in liver tissue

LPO values obtained 4 h after reperfusion showed a

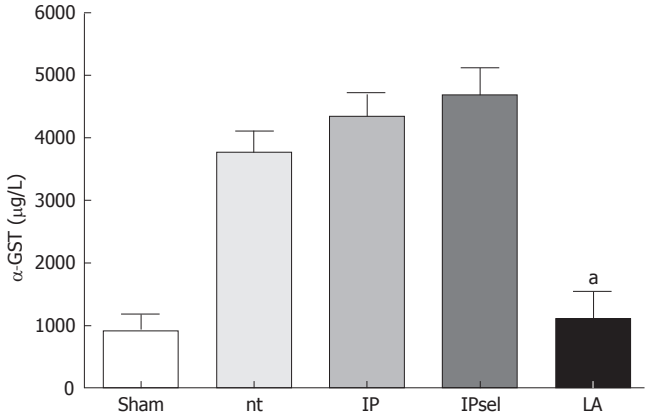

Figure 1 Plasma α-GST levels 4 h after reperfusion and 90 min of ischemia, nt: non-treated group; IP: ischemic preconditioning; IPsel: selective ischemic preconditioning, LA: lipoic acid. [a]*P* < 0.05: significantly different *vs* nt.

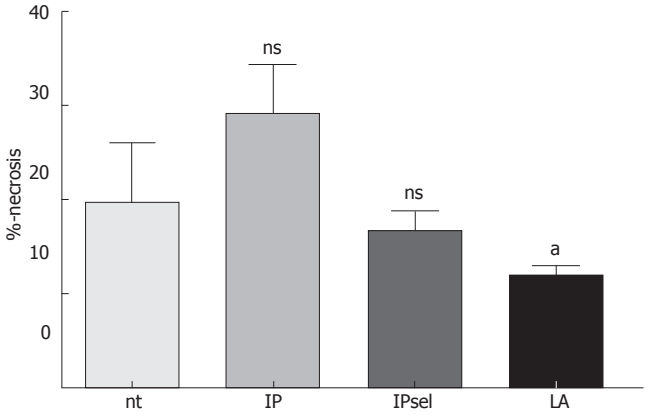

Figure 3 Percentage of necrosis as described in materials and methods after 90 min of ischemia and 4 h of reperfusion. nt: non-treated group; IP: ischemic preconditioning; IPsel: selective ischemic preconditioning; LA: lipoic acid; [a]*P* < 0.05 significantly different *vs* IP; ns: not significant *vs* nt.

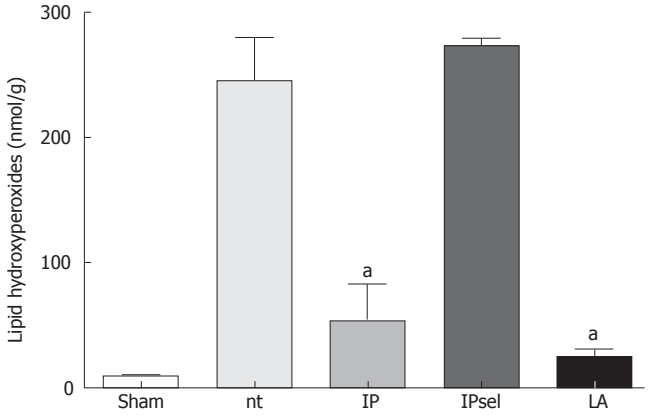

Figure 2 LPO (nmol/g) after 90 min of ischemia and 4 h of reperfusion. nt: non-treated group; IP: ischemic preconditioning; IPsel: selective ischemic preconditioning; LA: lipoic acid. [a]*P* < 0.05: significantly different *vs* nt.

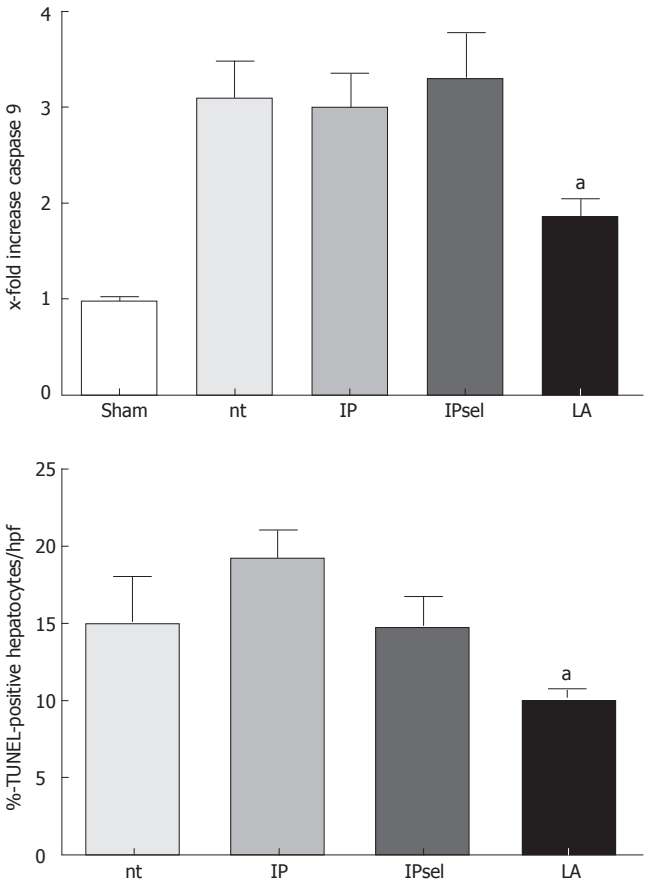

Figure 4 TUNEL-positive hepatocytes after 90 min of ischemia and 4 h of reperfusion, nt: non-treated group; IP: ischemic preconditioning; IPsel: selective ischemic preconditioning; LA: lipoic acid. [a]*P* < 0.05 significantly different *vs* IP Caspase 9 activity (x-fold increase) in liver tissue after 90 min of ischemia and 4 h of reperfusion, [a]*P* < 0.05 significantly different *vs* nt.

substantial increase in the untreated and IPsel livers compared to the sham-operated group (Figure 2). In the LA and IP groups, LPO values were decreased by about 90%.

Necrotic cell death

After 90 min of ischemia and 4 h of reperfusion there was a marked increase in oncotic hepatocytes in the untreated, IP, and IPsel groups compared to sham-operated animals (not detectable). In the LA-pretreated group, we observed a significant decrease of necrotic hepatocytes after 4 h of reperfusion (Figure 3).

Apoptoic cell death

No TUNEL-staining was observed in the livers of sham-operated animals (data not shown). In the untreated, IP, and IPsel groups, the number of TUNEL-positive cells was increased after 90 min of ischemia and 4 h of reperfusion. The LA-treated group showed a lower incidence of TUNEL-positive hepatocytes at the same time point ($P < 0.05$, Figure 4).

After 4 h of reperfusion following 90 min of ischemia, we found no measureable caspase 3- activity in the untreated group in comparison to the sham operated group (data not shown). Caspase 9- activity was increased in the non-treated group in comparison to the sham-operated group. In contrast, caspase 9- activity was

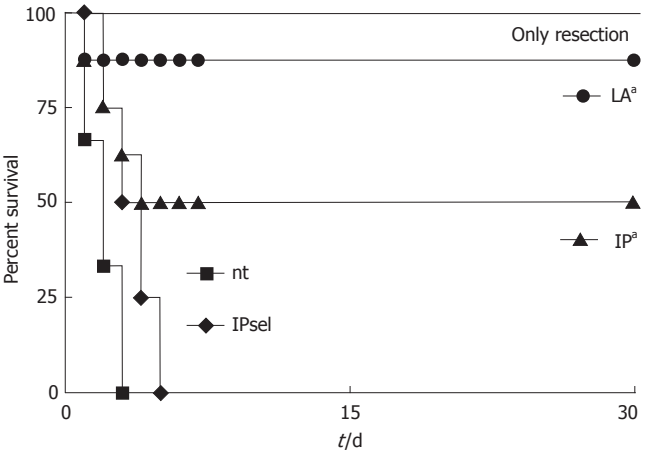

Figure 5 Kaplan and Meier survival curves after 90 min of ischemia and resection of the non-ischemic liver tissue after ischemia. nt: no treatment; IP: ischemic preconditioning; IPsel: selective ischemic preconditioning; LA: lipoic acid: 500 μmol LA 15 min prior to ischemia. [a]$P < 0.05$ logrank comparing the survival curves only resection *vs* nt.

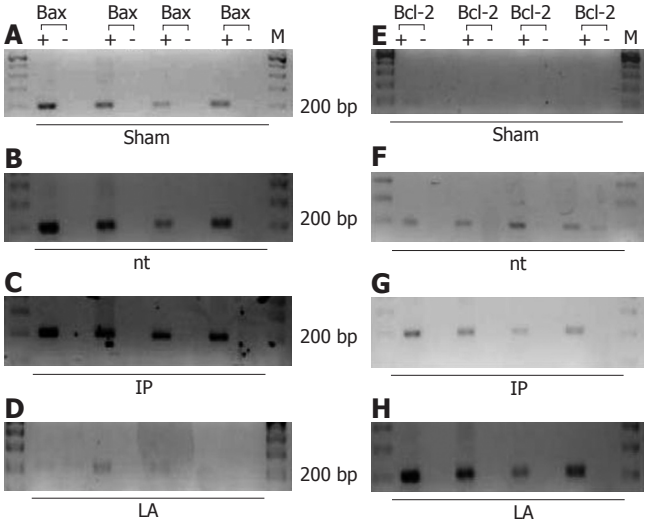

Figure 6 PCR products of Bax and Bcl-2 after 90 min of ischemia and 4 h of reperfusion. The amounts of total RNA assayed in the groups were equal. M: DNA Marker; bp: base pairs; +: sample with reverse transcriptase; -: sample without reverse transcriptase. **A,E**: sham operated animals; **B,F**: nt : non-treated group; **C,G**: IP: ischemic preconditioning; **D,H**: LA: lipoic acid. Each horizontal lane represents one different animal of the described groups ($n = 4$ each group).

decreased by LA after 4 h of reperfusion in comparison to the untreated group. IP and IPsel did not alter caspase 9-activity compared with the untreated animals.

Animal survival

We compared the survival rates of animals from the two IP-treated groups with the non-treated group and the group undergoing only liver resection (Figure 3). All animals with liver resection and without ischemia survived the 30-d monitoring period, whereas all animals of the control group died within the first three days, as did all animals of the IPsel group. In contrast, 4 of 8 animals survived in the IP group ($P < 0.05$ *vs* control group). In the LA group, 7 of 8 animals survived ($P < 0.05$ *vs* IP group; Figure 5).

Expression of Bax and Bcl-2 in liver tissue

Using RT-PCR, we saw no expression of Bcl-2 mRNA in liver of sham-operated animals, while mRNA of Bax was detectable. In the untreated group, there was expression of Bax and Bcl-2 mRNA after 90 min of ischemia and 4 h of reperfusion (Figure 6). In the IP and IPsel group (data not shown), there was expression of Bax and only weak expression of Bcl-2. After LA-pretreatment, Bcl-2 was strongly detectable at the same time point but mRNA of Bax was absent.

Data for Bax mRNA were confirmed by quantitative real time-PCR analysis showing upregulation of Bax mRNA in the untreated and IP group in comparison to the sham operated animals (2.5 ± 0.1 and 7.6 ± 2.8 versus 1.0 fold increase, $P < 0.05$). Bax expression was not different in the LA group in comparison to the sham operated animals (1.3 ± 0.1 versus 1.0 fold increase, not significant, $n = 4$ each group).

DISCUSSION

Results of the current study demonstrate that IP consisting of 10 min of ischemia and 10 min of reperfusion only partly protects the rat liver against IRI in a model of 90 min of selective ischemia. In contrast, selective IP consisting of 10 min of ischemia and reperfusion each does not attenuate liver cell injury in the same experimental setting. Pretratment with LA, however, protects strongly against IRI of the rat liver.

In general, the effects of IP segregated into early and late phases[3]. The early phase follows ischemia immediately and lasts for 2-4 h. The late phase begins 12-24 h after ischemia and lasts for about 2 d. In our current study, we investigated the early phase of IP with a short term experiment, and we compared animal survival of the groups as pivotal endpoints to study late effects.

In acordance with our previous studies, we observed necrosis and apoptosis-related cell death after 90 min of ischemia and 4 h of reperfusion. Our study suggests different protective mechanisms for IP and LA. The reduction in hepatic injury by IP is associated with attenuation of LPO. In contrast, the protective effect of LA is associated with a reduction in apoptotic and oncotic-related cell death. These latter effects are related to a shift in expression of pro- and anti-apoptic gene products towards the anti-apoptic Bcl-2[19].

The functional roles of Bax and Bcl-2 are believed to involve the mitochondrial membrane. Whereas Bax causes cytochrome c release and apoptotic cell death *via* activation of caspase 9-, Bcl-2 prevents these changes in mitochondria[20]. We demonstrated a reduction of caspase 9-activity after pretreatment with LA and downregulation of Bax, but neither caspase 9-activity nor Bax or Bcl-2 expression were altered with IP. The results of TUNEL-staining in liver tissue further underlined the attenuation of apoptotic cell death by LA.

The reduction in LPO in liver tissue under pretreatment by IP contrasts with the results of others[21], and we were unable to demonstrate a reduction of

necrosis-related cell death, measured by α-GST and the percentage of necrosis in liver tissue, in our model. These differences might be explained by the different durations of reperfusion. In our view, these results demonstrate the protective effects of IP in the early phase of IRI. ROS, mediating LPO, are generated in the early phase of reperfusion[22] and so it seems obvious that IP attenuates generation of ROS. On the other hand, IP increases animal survival in our long term study. In our view, this result may suggest the late phase protection of IP. Based on our results, the question of how IP exerts its late phase protection remains speculative. In long term experiments, it has been demonstrated that hepatoprotective effects of IP are associated with activation of nuclear factor kappa B (NF-κB), p38 MAPK and with the entry of hepatocytes into the cell cycle[23]. We speculate that these phenomena contribute to the observed late phase protection of IP.

We recently demonstrated reduction of hepatocellular injury by LA after 90 min of ischemia and 1 h of reperfusion accompained by downregulation of Bax-mRNA in our RT-PCR[19]. Anti-apoptotic action of LA in hepatocytes is mediated by inactivation of pro-apoptotic Bad[24]. Our current data add important additional information about this concept of LA as a regulator of pro and anti-apoptotic proteins by shifting the expression level towards anti-apoptotic Bcl-2.

In summary, IP consisting of 10 min of ischemia and reperfusion each decreases LPO in liver tissue accompanied by increased animal survival in a model of 90 min of selective hepatic ischemia. IPsel does not improve ischemic tolerance of the liver in our experimental setting. LA is an effective strategy against IRI of the liver and seems to fulfill its protective properties by influencing pro- and anti-apoptotic proteins towards the anti-apoptotic Bcl-2.

COMMENTS

Background

Aim of the study was to compare a fairly new method for prevention of ischemia/reperfusion inury (IRI) of the liver (lipoic acid pretreatment, LA) with the established method of ischemic preconditioning (IP).

Research frontiers

The article gives important and new information about mechanism of action of hepatic IRI and protective strategies.

Innovations and breakthroughs

We demonstrate advances of lipoic acid in comparison with IP in attenuation of IRI of the liver. Moreover, LA is not time consuming, easy to administer and without serious side effects.

Applications

The article supports LA as a new method for prevention hepatic IRI.

Peer review

The manuscript compared different preconditioning strategies to protect the liver from ischemia/reperfusion injury. It is a clearly written manuscript, with clear goals, sound experiments, and a well-balanced discussion of the discrepancies of the present results with results published previously.

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BASIC RESEARCH

Abnormal endogenous pain modulation and somatic and visceral hypersensitivity in female patients with irritable bowel syndrome

Clive H Wilder-Smith, Joan Robert-Yap

Clive H Wilder-Smith, Joan Robert-Yap, Brain-Gut Research Group, Gastroenterology Group Practice, Berne, Switzerland
Supported by the Brain-Gut Research Group, Berne, Switzerland
Correspondence to: Clive H Wilder-Smith, MD, Brain-Gut Research Group, Bubenbergplatz 11, CH-3011 Berne, Switzerland. cws@braingut.com
Telephone: +41-31-3123737 Fax: +41-31-3123770
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Abstract

AIM: To investigate the role of endogenous pain modulatory mechanisms in the central sensitization implicated by the visceral hypersensitivity demonstrated in patients with irritable bowel syndrome (IBS). Dysfunction of modulatory mechanisms would be expected to also result in changes of somatic sensory function.

METHODS: Endogenous pain modulatory mechanisms were assessed using heterotopic stimulation and somatic and visceral sensory testing in IBS. Pain intensities (visual analogue scale, VAS 0-100) during suprathreshold rectal distension with a barostat, cold pressor stimulation of the foot and during both stimuli simultaneously (heterotopic stimulation) were recorded in 40 female patients with IBS and 20 female healthy controls.

RESULTS: Rectal hypersensitivity (defined by 95% CI of controls) was seen in 21 (53%), somatic hypersensitivity in 22 (55%) and both rectal and somatic hypersensitivity in 14 of these IBS patients. Heterotopic stimulation decreased rectal pain intensity by 6 (-11 to -1) in controls, but increased rectal pain by 2 (-3 to +6) in all IBS patients ($P < 0.05$) and by 8 (-2 to +19) in IBS patients with somatic and visceral hypersensitivity ($P < 0.02$).

CONCLUSION: A majority of IBS patients had abnormal endogenous pain modulation and somatic hypersensitivity as evidence of central sensitization.

Key words: Diffuse noxious inhibitory controls; Endogenous pain modulation; Hypersensitivity; Irritable Bowel Syndrome; Quantitative sensory testing; Visceral pain; Sensitization

INTRODUCTION

Irritable bowel syndrome (IBS) is characterized by abdominal discomfort or pain accompanied by changes in gastrointestinal motility. Peripheral and central nervous system sensitization have been proposed as an underlying mechanism in IBS[1]. Persistent or altered peripheral input secondary to divers insults is likely to lead to central changes in nociception and sensory perception. Previous brain imaging studies have demonstrated differences in the central processing of visceral nociceptive input between patients with IBS and healthy controls, mainly in the centers dealing with secondary pain processing and the assigning of affective content[2,3]. Nociceptive input to the brain is subject to endogenous modulation by brainstem and cortical pathways, including the periaqueductal gray (PAG)-rostroventral medulla (RVM) network, spino-bulbo-spinal diffuse noxious inhibitory controls (DNIC) and the frontal lobe[4,5].

Functional assessment of the endogenous pain modulatory pathways has been extensively validated in humans using heterotopic stimulation ("counterirritation")[6-11]. DNIC has been shown to be abnormal in fibromyalgia and in a pilot study in IBS using heterotopic stimulation[12,13]. Altered somatic as well as visceral sensory function would be expected as a consequence of central sensitization or abnormal endogenous modulation. Visceral sensitization has been demonstrated in a majority of patients with IBS, but the studies examining somatic sensory function have yielded equivocal results[14-20].

In the current study, we investigated central sensitization in IBS by testing endogenous pain modulatory pathways and visceral and somatic sensory function in matched patients and healthy controls. Our study hypotheses were, firstly, that IBS patients demonstrate deficient endogenous pain modulation in the form of inadequate pain inhibition during heterotopic stimulation and, secondly, that IBS patients with visceral hypersensitivity are also hypersensitive to suprathreshold somatic stimulation.

MATERIALS AND METHODS

Patients

Forty female IBS patients and 20 female healthy subjects were recruited by advertisements and through the Gastrointestinal Unit. Equal numbers of diarrhea and constipation predominant IBS patients, as defined by the Rome 2 criteria, were included and none had any evidence of organic gastrointestinal pathology after gastrointestinal workup, including endoscopy, stool and blood tests, and H_2-breath test for lactose intolerance[21]. IBS patients were required to have an average abdominal pain intensity of at least 30 on the 0-100 VAS in the two weeks before study inclusion. IBS and control subjects between 18 and 60 years of age were recruited. Controls had no gastrointestinal symptoms or evidence of chronic diseases. Main exclusion criteria in all groups were bowel resections (except appendectomy), major abdominal operations, treatment with tricyclic antidepressants, selective serotonin reuptake inhibitors, gastrointestinal prokinetics, anticholinergics, antispasmodics or analgesics in the last 14 d, and chronic pain apart from IBS-especially fibromyalgia. Institutional Ethics Committee approval was given for the study and all subjects gave their written informed consent to participation. Patients were familiarized with the study procedures on a separate day before the start of the actual testing day. The same investigator performed all tests.

Rectal distension thresholds

On the morning of the study day subjects were asked to attempt defecation and a warm water enema (300 mL) was administered to empty the rectum before rectal insertion of the lubricated, flaccid and oversized 600 mL polyethylene bag on the end of a catheter. Leakage of the rectal bag was excluded by distension with air under water before insertion and after removal at the end of the study. The bag was inflated and then pulled outwards until slight resistance was felt. The catheter was taped to the buttocks, the bag deflated and subjects positioned in a relaxed supine position. The catheter was attached to a G&J Distender® barostat (Toronto, Canada) set at an inflation rate of 27 mL/s and a safety cut-off threshold of 60 mmHg. After a resting period of 20 min the minimum distending pressure was determined and the rectal pain threshold titrated using an ascending methods of limits (AML) paradigm with 5 mmHg increments of 30 s duration followed by a decrease to baseline for 30 s until the Pain threshold ("first feeling of pain") was reached. Subsequently, the following tests were performed in randomized sequence with a break of 30 min between tests.

Tonic rectal stimulation

Pain intensity was rated on a 100 mm anchored horizontal VAS after constant distension at the individually determined pain threshold pressure plus 20% for 120 s. This suprathreshold stimulation was chosen to induce moderate pain (visual analogue scale VAS score between 30 and 40, where 0 = no pain, 100 = unbearable pain) and was based on data from our own pilot studies.

Table 1 Characteristics of IBS patients and healthy controls. Means and 95% confidence intervals are shown

	IBS n = 40	Healthy controls n = 20
Age (yr)	39 (36-42)	41 (37-45)
Height (cm)	166 (164-168)	168 (163-173)
Weight (kg)	69 (65-73)	75 (67-84)
Years with IBS	6 (3-12)	Not applicable
n with diarrhea-/constipation-predominant IBS	20/20	0/0
Luteal phase/non-menstruating	19/13	11/9

Somatic stimulation: cold pressor test

Pain intensity was rated by VAS after immersion of the left foot up to the calf in a circular flow ice-water bath maintained at 4℃ for 120 s. Care was taken to position the foot comfortably in the water bath, with the calf padded by cushions.

Heterotopic stimulation

The above rectal and somatic stimuli were applied concomitantly; rectal pain was rated on the VAS after 120 s. During all tests subjects were instructed to rate only their rectal pain.

Rectal compliance was calculated from the slope of the linear portion of the volume-pressure curve from each inflation sequence.

Statistical analysis

All continuous group data were calculated as means and 95% confidence intervals. Threshold values reaching cut-off were recorded as the maximum possible value. Analysis of variance (ANOVA) testing for group differences in somatic and visceral pain ratings and for changes in ratings during heterotopic stimulation was predefined, with post-hoc testing performed by Tukey's Test in case of significance (Statistica 7.1, StatSoft Inc., Tulsa, USA). For secondary analysis IBS patients were classified as hypersensitive or non-hypersensitive based on their titrated rectal distension pressures and their somatic and rectal pain ratings using the 95% confidence intervals of healthy controls as the threshold limits for hypersensitivity, as suggested in the literature[22,23]. Correlations were assessed using multiple regression analysis with Bonferroni correction for multiple testing. A significance level of $P < 0.05$ was applied.

RESULTS

Patient characteristics are shown in Table 1. The subject groups were well matched, with no significant differences in demographics. All subjects completed the entire test series. The phase in the menstrual cycle was recorded for all subjects and there were no differences between the subject groups in the numbers of patients in the luteal phase or in those post-menopausal or post-hysterectomy.

Rectal pain thresholds and compliance

The mean pain pressure thresholds were 42 (38-46) mm

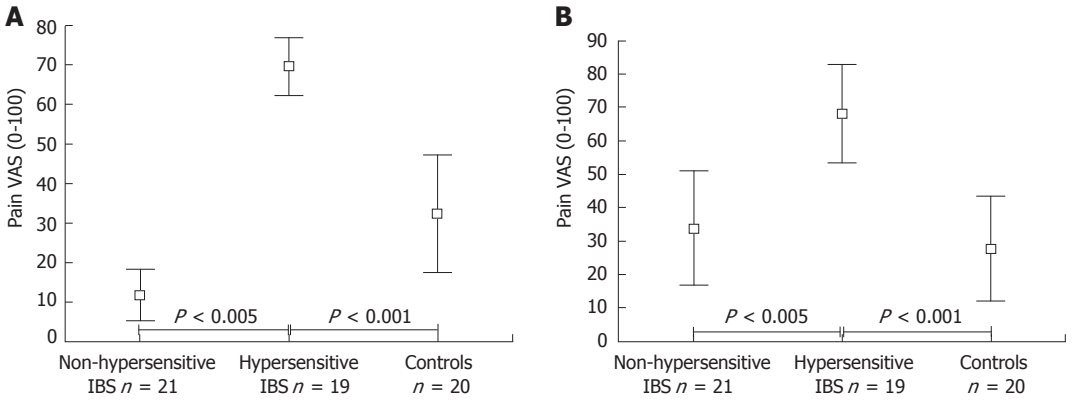

Figure 1 Pain intensity ratings (100 mm VAS) during tonic rectal (**A**) and tonic somatic (**B**) stimulation in non-hypersensitive and hypersensitive IBS patients and in healthy controls. Hypersensitivity is defined by the 95% confidence interval of healthy controls. Means (symbol), 95% confidence intervals (whisker) are shown.

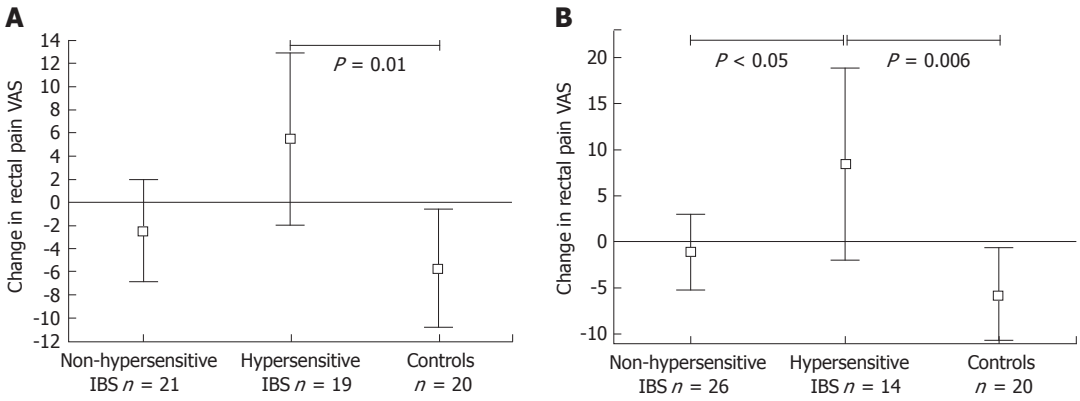

Figure 2 Change in rectal pain intensity scored on a VAS 0-100 during heterotopic stimulation in IBS subgroups and controls. Means (symbol), 95% confidence intervals (whisker) are shown in patients hypersensitive or not to tonic rectal (**A**) and both tonic rectal and somatic (**B**) stimulation. Hypersensitivity is defined by the 95% confidence interval of healthy controls.

Hg in healthy controls and 37 (34-40) mm Hg in IBS patients ($P = 0.05$). Mean rectal compliance values were 5.7 (4.6-6.9) mL/mmHg in controls and 6.9 (5.4-8.5) mL/mmHg in IBS patients, with no significant differences between IBS subgroups or to controls.

Rectal distension stimulation

Pain intensity VAS ratings during rectal tonic suprathreshold stimulation at pain threshold +20% pressures were 39 (23-54) in controls and 42 (24-52) in IBS patients (not significant). Twenty-one (53%) of all 40 IBS patients, 16 of 20 (80%) diarrhea-predominant IBS and 5 of 20 (25%) constipation-predominant IBS patients were hypersensitive compared to controls (see Methods for definition). The threshold for rectal hypersensitivity was 47 mmHg. Figure 1A illustrates the pain ratings in hypersensitive and non-hypersensitive IBS groups and in controls during tonic rectal distension. There was no difference in body weight in the hypersensitive versus non-hypersensitive groups (66 (56-76) kg and 71 (57-84) kg, respectively). Their respective rectal compliance was 4.9 (4.5-5.9) and 6.1 (4.7-7.5) (no significant difference).

Somatic stimulation

Pain intensity VAS ratings during somatic stimulation were 27 (12-43) in controls and 51 (39-64) in all IBS patients ($P = 0.02$). Premature withdrawal from the ice water due to strong pain occurred in 2 IBS patients and in one control. The maximum pain intensity score of 100 was accorded in these cases. Twenty-two of all 40 (55%) IBS patients, 14 of the 20 (70%) diarrhea-predominant and 8 of the 20 (40%) constipation-predominant IBS patients showed somatic hypersensitivity. The threshold for somatic hypersensitivity was 43 on the pain VAS.

Overlap somatic and visceral hypersensitivity

Fourteen of the 22 patients hypersensitive to the somatic stimulus were also viscerally hypersensitive. Somatic pain scores were 68 (53-83) in IBS patients hypersensitive to rectal stimulation and 32 (21-43) in IBS patients without rectal hypersensitivity ($P < 0.001$ versus controls and non-hypersensitive IBS) (Figure 1B). Visceral and somatic hypersensitivity correlated significantly in IBS patients ($r = 0.82$, $P < 0.000001$).

Heterotopic stimulation

When somatic stimulation was applied during rectal stimulation, mean group rectal pain scores *decreased* by 6 (-11 to -1) (mean change -16% from baseline) in healthy controls, *increased* by 2 (-3 to +6) (mean change +2%) in all IBS patients ($P < 0.05$ *vs* controls) and *increased* by 8 (-2 to +19) (mean change +12%) in IBS patients with both somatic and visceral hypersensitivity ($P = 0.006$ *vs* controls) (Figure 2). There was no significant correlation between somatic pain levels and the change in rectal pain scores during heterotopic stimulation. During heterotopic stimulation rectal pain changed by +2 (-4 to 9) in diarrhea-predominant IBS and by +1 (-6 to 7) in constipation-predominant IBS (not significant).

DISCUSSION

A majority of IBS patients, mainly from the IBS-D

subgroup, demonstrated either visceral or somatic hypersensitivity, with substantial but incomplete overlap of hypersensitivity to visceral and somatic stimuli. The visceral and somatic hypersensitivity states correlated significantly. While rectal hypersensitivity has previously been shown in a majority of IBS patients using various study endpoints, the few studies relating to somatic sensory dysfunction in IBS have yielded controversial results showing either somatic hypo- or hypersensitivity[14,16-20,22,24-28]. This discrepancy is probably best explained by the choice of different stimulation intensities, i.e suprathreshold versus threshold and painful versus non-painful, and to a lesser degree also by the selection of stimulation techniques, such as thermal versus pressure or electrical. In the current study we used painful, suprathreshold, tonic stimulation over a larger skin surface area as high-intensity and tonic stimulation have demonstrated sensitization consistently[29-34].

Central sensitization is a possible mechanism underlying the somatic and visceral hypersensitivity. In the current study endogenous pain modulation, one of the major mechanisms contributing to central sensitization, was shown to function as expected in healthy controls, but malfunctioned in a majority of patients with IBS. Previously, heterotopic somatic stimulation has not only been shown to reduce somatic pain intensity in healthy controls, but also discomfort or pain thresholds to gastric, duodenal and rectal distension[35,36]. Little previous data exist on endogenous pain modulation in IBS. Coffin *et al* demonstrated hyperexcitability of spinal sensory modulation in IBS using a somatic nociceptive flexion reflex (R-Ⅲ reflex) and concomitant rectal distension[35]. Wilder-Smith *et al*. in two previous studies showed abnormal endogenous pain modulation and central pain processing by functional brain MRI in IBS[13,37]. The current study extends this data by determining somatic as well as visceral sensory function in a larger and balanced group of IBS patients and correlating generalized sensory hypersensitivity with dysfunctional endogenous pain modulation. Hypersensitive IBS patients had evidence of endogenous pain facilitation rather than inhibition during heterotopic stimulation. Dysfunctional endogenous pain modulation is likely to reflect an imbalance between pain facilitatory and inhibitory systems and has been found in fibromyalgia and interstitial cystitis, both of which are associated with IBS[4,15,36,38,39].

The design of the current study with heterotopic stimulation only at a lower body site does not allow the distinction between localized, lumbosacral somatic hypersensitivity possibly involving convergence, and widespread sensitization. However, a recent study by Rodrigues *et al* clearly demonstrated uniform somatic hypersensitivity from the face to the calf in IBS, rendering localized hypersensitivity in IBS unlikely[17].

Throughout this study hypersensitivity and abnormal endogenous pain modulation were more common in diarrhea-predominant than in constipation-predominant IBS patients, although the mean group changes in rectal pain during heterotopic stimulation were similar. Differences between IBS subgroups in sensory function and in fMRI brain activity during visceral pain have been observed in earlier studies[13,40]. This study was not powered for subgroup comparison, but this comparison deserves further investigation in larger patient groups.

Dysfunctional pain modulation is an attractive mechanistic hypothesis within the biopsychophysical model of functional bowel disease, as endogenous pain modulation acts as a central filter for extraction and amplification of noxious input, providing a possible unifying concept for both the "top-down" psychological and the "bottom-up" peripheral insult etiological postulates[1,41-46]. Endogenous pain modulation plays a central role in the neuromatrix integrating cognitive, emotional, autonomic and effector responses to pain[17]. Interestingly, the activity of the pain modulatory pathways differs between men and women, possibly explaining some of the gender differences in the incidence of IBS[12,36,38]. Nonetheless, it should be pointed out approximately one quarter of IBS patients did not have evidence of any hypersensitivity with the tests applied. It would therefore at present be inappropriate to label the described sensory dysfunction as a disease marker. However, further refinement of technique and improved exclusion of other differential diagnoses may lead to better discrimination.

Attentional effects represent a potential confounding and overlapping factor in the study of descending pain modulation pathways, as cognition feeds into the same neural pathways. A recent fMRI study clearly demonstrated distinct effects due to attention and DNIC on pain pathways, with minor functional anatomical overlap[37]. Potential weaknesses of this study are, firstly, the absence of psychological and emotional correlational data in our subjects. These factors are known to influence pain perception, but are likely to exert much of their influence via the studied endogenous pain modulatory mechanisms. Secondly, the intensity of the somatic, heterotopic stimulus was not individually titrated, hence introducing a potential stimulation bias as IBS patients rated this stimulus as more intense than controls.. There was, however, no significant correlation between somatic stimulation intensity and the change in rectal pain scores during heterotopic stimulation. Additionally, recent data has confirmed that endogenous modulation effects do not depend on the intensity of the conditioning stimulus[47,48].

In conclusion, a majority of IBS patients demonstrated evidence for central sensitization, with visceral and somatic hypersensitivity associated with abnormal function of endogenous pain modulation and pain facilitation.

COMMENTS

Background

IBS is a very common syndrome characterized by abdominal discomfort or pain accompanied by changes in gastrointestinal motility. Peripheral and central nervous system sensitization have been proposed as an underlying mechanism in IBS. Previous brain imaging studies have demonstrated differences in the central processing of visceral nociceptive input between patients with IBS and healthy controls, mainly in the centers dealing with secondary pain processing and the assigning of affective content. Input regarding pain to the brain is subject to extensive endogenous modulation by brainstem and cortical pathways. Altered somatic as well as visceral sensory function would be expected as a consequence of central sensitization or abnormal endogenous modulation. We have previously shown abnormal endogenous modulation of visceral pain in IBS, but there is no data on modulation of somatic pain or sensory input.

Research frontiers

Brain imaging and new sensory testing techniques are enabling the redefining and detailed examination of brain function and pain processing in health and disease.

Innovations and breakthroughs

The above techniques are demonstrating abnormal processing of sensory and pain information in the brain in somatic and visceral pain disorders and are revealing an intricate functional integration of cognitive, emotional, homeostatic, motor and sensory brain centres. Understanding dysregulation in this neuromatrix, with a central role for endogenous modulation, is providing us with a new, holistic understanding of hitherto difficult to understand diseases, such as so-called 'functional' syndromes.

Applications

This and previous related publications demonstrate malfunction in one of the brain's central regulatory mechanisms, endogenous pain modulation, in IBS. Because of the manifold connections between many major brain centres and this modulatory network, potential exist for manipulation via several avenues, including psychological, pharmaceutical as well as physical therapy. These research data can and will be applied to other related chronic pain syndromes.

Peer review

This is a study of 20 healthy female controls, 20 IBS-D, and 20 IBS-C women to try to further examine rectal hypersensitivity and somatic hypersensativity using a cold pressor test of the foot. It's an excellent work, very timely and interesting.

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CLINICAL RESEARCH

Healing property of the *Piper betel* phenol, allylpyrocatechol against indomethacin-induced stomach ulceration and mechanism of action

S Bhattacharya, D Banerjee, AK Bauri, S Chattopadhyay, SK Bandyopadhyay

S Bhattacharya, D Banerjee, SK Bandyopadhyay, Department of Biochemistry, Dr. B.C. Roy Post Graduate Institute of Basic Medical Sciences IPGME&R, 244B, Acharya Jagadish Chandra Bose Road, Kolkata-700020, India
AK Bauri, S Chattopadhyay, Bio-Organic Division, Bhabha Atomic Research Centre, Mumbai-400085, India
Correspondence to: Professor Sandip K Bandyopadhyay, Department of Biochemistry, Dr. B.C. Roy Post Graduate Institute of Basic Medical Sciences and IPGMERR, 244B, Acharya Jagadish Chandra Bose Road, Kolkata 700020, India. s_dip2@rediffmail.com
Telephone: +91-33-22244314 Fax: +91-33-22801807
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Abstract

AIM: To evaluate the protective activity of allylpyrocatechol (APC), the major antioxidant constituent of *Piper betel*, against the indomethacin-induced stomach ulceration in the rat model and correlates with its antioxidative and mucin protecting properties.

METHODS: Male Sprague-Dawley rats were divided into five groups. Normal control rats (group Ⅰ) were given the vehicle oral dose of gum acacia in distilled water (1 mL per rat); ulcerated control and treated rats (groups Ⅱ-Ⅴ) were given a single dose of indomethacin (30 mg/kg body wt.); group Ⅱ rats were sacrificed 4 h after indomethacin administration; groups Ⅲ-Ⅴ rats were given the vehicle (1 mL per rat) or APC (2 mg/kg body wt.) or misoprostol (1.43 μg/kg body wt.) once daily by oral intubation for 7 d starting from 4 h after the indomethacin administration. After 7 d, the stomach tissues were excised for histological examination and biochemical analysis.

RESULTS: Treatment with APC (2 mg/kg body wt per day) and misoprostol (1.43 μg/kg body wt per day) for 7 d could effectively heal the stomach ulceration as revealed from the ulcer index and histopathological studies. Compared to the zero day ulcerated group, treatment with APC and misoprostol reduced the ulcer index by 93.4% and 85.4% respectively ($P < 0.05$). Both APC and misoprostol accelerated ulcer healing observed in natural recovery ($P < 0.05$), their respective healing capacities not being significantly different. The healing capacities of APC and misoprostol could be attributed to their antioxidant activity as well as the ability to enhance the mucin content of the gastric tissues. Compared to the ulcerated untreated rats, those treated with APC and misoprostol showed near normal MDA levels, while the protein levels were 86% and 78% of the normal value respectively ($P < 0.05$). Likewise, both APC and misoprostol increased the SOD, catalase, and mucin levels significantly ($P < 0.05$), the effect of APC being better.

CONCLUSION: APC can protect indomethacin-induced gastric ulceration due to its antioxidative and mucin protecting properties.

Key words: Allylpyrocatechol; Antioxidant; Histopathology; Indomethacin; Mucin; *Piper betel*; Stomach ulcer

Bhattacharya S, Banerjee D, Bauri AK, Chattopadhyay S, Bandyopadhyay SK. Healing property of the *Piper betel* phenol, allylpyrocatechol against indomethacin-induced stomach ulceration and mechanism of action. *World J Gastroenterol* 2007; 13(27): 3705-3713

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INTRODUCTION

Gastrointestinal toxicity associated with nonsteroidal anti-inflammatory drugs (NSAIDs) is an important medical problem despite recent pharmaceutical advances[1]. Besides being used as pain-killers, the NSAIDs are being increasingly used for prevention of malignancies, stroke, pre-eclampsia, Alzheimer's disease, and many other illnesses[2-4]. The percentage of gastric ulcer cases induced by NSAIDs is emerging day by day, accounting for approximately 25% of gastric ulcers[5]. In addition, various factors such as stress, hunger, *H pylori* invasion etc are also known to cause gastric ulcer. Consequently, prevention of gastrointestinal disorder continues to be of concern for both medical professionals and researchers. Various synthetic anti-ulcer drugs are presently available, and some of these like misoprostol are specifically used to prevent or treat the NSAID induced gastric ulcer. However, each of these drugs confers simpler to severe side effects such as diarrhea, itching, skin rash, dizziness, and inactivation of

some antifungal drugs (proton pump inhibitors), confusion in elderly patients, headache and antiandrogenic effect (H-2 receptor blockers), constipation, vomiting, indigestion, back pain, and dizziness (sucralfate), bleeding diathesis and abortion for pregnant women (misoprostol)[6]. Thus, there is a growing interest on non-toxic, antiulcer formulations from medicinal plants, and many taxa of medicinal plants have been assessed worldwide for their antiulcerogenic effects[7,8]. In the developing nations, this turn of events has also been prompted, in part, by the high cost of the modern antiulcer medication.

The *Piper betel* plant is found widely growing in the tropical humid climate of South East Asia, and its leaves, with a strong pungent and aromatic flavor, are widely consumed as a mouth freshener. The Indian traditional system of medicine has identified the *P. betel* leaves with digestive and pancreatic lipase stimulant activities[9,10]. Earlier, we also reported gastrocytoprotective properties of the leaf extract on experimentally induced gastric lesions[11].

The major problem with the herbal drugs is the lack of their quality control and improper authentication. Identifying the major active principle(s), and ensuring their presence in optimum quantity in the herbal preparations can minimize problems associated with their questionable quality. Very recently we have found that the extraordinary antioxidant activity of the *P. betel* leaf extract is due to its major phenolic constituent, allylpyrocatechol (APC, Figure 1)[12]. Consequently, the aim of the present study was to evaluate the healing effect of APC on indomethacin-induced acute gastric ulceration of rats and compare the activity with that of the drug, misoprostol.

MATERIALS AND METHODS

Chemicals and reagents

The *P. betel* leaves were collected from the local market and identified (collection no. 2610) by taxonomy by the Botanical Survey of India, Indian Botanical Garden, West Bengal. 2-Thiobarbituric acid (TBA), Tris, ethylen ediaminetetraacetic acid (EDTA), acetic acid, methanol and ethanol were procured from E Merck (India), while trichloroacetic acid (TCA) was from Thomas Baker, India. Alcian blue, indomethacin, dimethylaminobenzaldehyde, epinephrine, Cu (Ⅱ) sulphate, bovine serum albumin (BSA), hematoxylin, alum, mercuric oxide, eosin, Schiff's reagent and periodic acid were procured from Sigma Chemicals, St. Louis, MO (USA). Other reagents used were H_2O_2 (35%, Lancaster, England), and perchloric acid, 2,4-dinitrophenyl hydrazine (DNPH), K_2HPO_4, KH_2PO_4, KOH and HCl (all from SRL, India). Stock solutions of EDTA and H_2O_2 were prepared in triply distilled deaerated water just prior to use. Stock solutions (1% w/v) of TBA were prepared in 50 mmol/L NaOH solution and used within a week.

Isolation of APC from P. betel

APC was isolated from *P. betel* ethanol extract as in the reported procedure[12]. The air-dried leaves of the *P. betel* (250 g) were chopped into fine pieces, soaked in 95% ethanol (1 L) for two days and the supernatant decanted. The entire process was repeated three times, the combined extracts were filtered through a nylon mesh, and evaporated in vaccuo. The extract (8.0 g) was dissolved in methanol (50 mL), treated with activated charcoal (0.2 g), and the mixture warmed at -60℃. After filtration, the extract was concentrated under vacuum, and finally lyophilized to obtain a chlorophyll-free amorphous yellowish brown solid (yield: 1.23% w/w) that was stored in a vacuum dessicator.

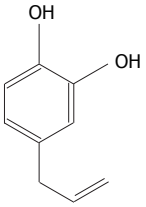

Figure 1 Chemical structure of allylpyrocatechol.

The extract (4.7 g) was subjected to column chromatography over silica gel using gradient elution. The column was eluted with 0, 2%, 5% and 10% ethyl acetate/hexane followed by 0, 5%, 10%, 15%, 50% and 100% methanol/chloroform (500 mL each), and 50 mL fractions were collected. The fraction eluting with 10% ethyl acetate/hexane furnished APC (yield: 0.9% w/w of the extract) as a light yellow oil, which was characterized using IR and 1H NMR spectroscopic data. The chemical structure of APC is shown in Figure 1.

3,4-Dihydroxyallylbenzene (Allylpyrocatechol, APC): ^{1}H NMR (CDCl3): δ 3.28 (d, J = 6.5 Hz, 2H, ArCH_2), 4.99-5.08 (m, 2H, olefin), 5.36 (broad, 2H, 2 × Ar-OH), 5.80-6.01 (m, 1H, olefin), 6.59-6.79 (m, 3H, ArH).

Preparation of the drug

A specific dry weight of APC or misoprostol was macerated with a mortar and pestle in double distilled water containing gum acacia 2% (w/w) to provide the drugs, which were administered po.

Animals

The rats were bred at Dr. B. C. Roy Post Graduate Institute of Basic Medical Sciences, Kolkata, India and BARC Laboratory Animal House Facility, Mumbai, India. These were procured after obtaining clearance from the respective Animal Ethics Committee of the two centers and were handled following international Animal Ethics Committee guidelines. Male Sprague-Dawley rats (weighing 180-200 g) were reared on a standard laboratory diet (Ralston Purina, Chicago, Illinois, USA) and given tap water. They were kept in a room where temperature (20 ± 2℃), humidity (65%-70%), and day/night cycle (12/12 h) were controlled.

Experimental protocol for indomethacin-induced ulceration and healing

Ulceration in the rats was induced with a single dose of indomethacin (30 mg/kg body wt., oral intubation) dissolved in distilled water. The rats were deprived of food but had free access to tap water 24 h before ulcer induction.

For the standardization of drug dose, APC (0.5, 1, 2,

5, and 10 mg/kg body wt.) was given once daily by oral intubation for 7 d starting from 4 h after the indomethacin administration. Five rats were taken for each dose and each experiment was repeated three times. The extent of healing was assessed from the macroscopic damage scores. The effect of the treatment with APC (2 mg/kg body wt.) for 10 d was also studied.

Based on the results of dose optimization, the histopathological, and biochemical parameters were assessed in separate experiments. For this, a total of 25 rats were randomly divided into 5 groups in each set of experiment, which was replicated three times. Group Ⅰ rats serving as the normal control received only the vehicle oral dose of gum acacia in distilled water (1 mL per rat). Ulceration was induced in the groups Ⅱ-Ⅴ rats. Group Ⅱ rats serving as the experimental control received only indomethacin and were sacrificed 4 h after indomethacin administration. Group Ⅲ-Ⅴ rats were given the vehicle (1 mL per rat) or APC (2 mg/kg body wt.) or misoprostol (1.43 μg/kg body wt.) once daily by oral intubation for 7 d starting from 4 h after the indomethacin administration. After 7 d, the rats of group Ⅰ and groups Ⅲ-Ⅴ were sacrificed under ether anesthesia, followed by cutting off the aorta abdominalis.

Macroscopic and histopathological studies

The stomach from the normal and treated groups were removed rapidly, opened along the greater curvature, and thoroughly rinsed with normal saline. After recording the ulcers produced in the stomach, a longitudinal section of the gastric tissue was taken from the anterior part of the stomach and fixed in a 10% formalin solution. After 24 h of fixation followed by embedding in a paraffin block, it was cut into sections of 5 micron onto a glass slide and stained with hematoxylin-eosin for histological assessment of the gastric mucosa. For biochemical studies, the stomach was opened along the greater curvature, and the gastric antral portion was used. The wet weight of the portion was also recorded.

Quantification of ulceration

Area of glandular portion comprising of the fundic and corpus region of each stomach was measured in square millimeters. The total eroded gastric mucosal areas (lesions) were also measured (mm^2) with a dissecting microscope under × 20 magnification. The percentages of the whole glandular area contributing to the damaged part are referred to as the ulcer indices according to a reported procedure[13]. The ulcer indices for the cold stress rats were also calculated in the same manner.

Preparation of tissue homogenate

The stomach tissue was homogenized in a 50 mmol/L phosphate saline buffer (PBS) pH 7.2 under cold condition, using a glass-teflon homogenizing tube. The homogenate was centrifuged at 2500 r/min for 10 min and the supernatant was carefully removed from the pellet and used for biochemical analyses.

Quantification of lipid, protein and DNA damages

The lipid peroxidation products were estimated[14] in terms of malondialdehyde (MDA) formation. The amounts of protein carbonyls and DNA contents were estimated following reported methods[15,16].

Assessment of enzymatic activities

The specific activity of catalase (CAT) in the tissue was estimated according to a reported method[17]. The tissue homogenate (20 μL) was added to a H_2O_2-phosphate buffer mixture (3 mL), maintaining the optical density at 240 nm to 0.500 ± 0.010 (d = 1 cm) against buffer. The rate of change of optical density at 240 nm with time was recorded for the calculation of the catalase activity.

Following a reported method[18], the tissue superoxide dismutase (SOD) activity was measured. This involves assaying the SOD-mediated inhibition of epinephrine autooxidation at an alkaline medium. For this, the absorbances of the samples at 480 nm were noted at an interval of 30 s. The enzyme activity was measured in arbitrary units, considering 50% inhibition as 1 unit of enzyme activity.

Mucin assay

Following a reported method[19], the free mucin in the gastric tissues was estimated. Briefly, the gastric tissues were incubated with a 1% buffered solution of Ab in 3% aqueous acetic acid (0.5 mL) at 37℃ for 30 min. After centrifuging, the concentration of Ab in the solution was measured from the absorbance at 615 nm.

Hexosamine assay

The hexosamine concentrations in gastric tissues were assayed according to a reported method[20] with a minor modification. The gastric tissues were hydrolyzed in acidic medium, the hydrolysate neutralized with 3 mol/L NaOH (litmus) and diluted to 10 mL with double distilled water. Acetyl acetone solution (1 mL, prepared by dissolving 1 mL of acetyl acetone in 50 mL 0.5 mol/L sodium carbonate) was added to the above solution (1 mL), which was mixed well, and heated on a boiling water bath for 15 min avoiding evaporation. After cooling, ethanol (5 mL, 95%) was added to the mixture followed by Ehrlich's reagent (1 mL, prepared by dissolving 0.8 g para-dimethylaminobenzaldehyde in 30 mL methanol and 30 mL conc. HCl). The mixture was diluted to 10 mL with 95% ethanol, allowed to stand for 30 min, and its absorbance at 530 nm was read.

Statistical analyses

All the results were expressed as mean ± SE. Student's paired *t*-test for comparison between groups and one way analysis of variance (ANOVA) for multi-sample groups at $P < 0.05$ were used to assess statistical significance in various groups of animals.

RESULTS

Effect of APC on healing indomethacin-induced gastric ulceration in rats

Oral administration of APC at different doses accelerated the rate of healing of gastric lesion induced by indomethacin. As shown in Table 1, for a 7-d treatment,

Table 1 Comparative healing capacity of different doses of APC against indomethacin-induced ulceration in rats[1]

Samples	Treatment		Ulcer index[1]
	Dose (mg/kg body wt.)	d	
Unulcerated control			0 ± 0
Ulcerated control (4 h)[2]	-	-	26.71 ± 1.62
APC treated	0.5	7	20.22 ± 1.17
APC treated	1	7	11.21 ± 0.91
APC treated	2	7	1.78 ± 0.34
APC treated	5	7	1.67 ± 0.28
APC treated	10	7	1.83 ± 0.51
APC treated	2	10	1.65 ± 0.31

[1]Stomach ulceration in rats was induced by oral administration of indomethacin (30 mg/kg body wt.). The ulcer indices values are mean ± SE (n = 15). [2]The ulcer indices were measured 4 h after indomethacin administration. For other samples the measurement was carried out after 7 d.

Table 2 Comparative healing capacity of APC and misoprostol on ulcerated rats[1]

Samples	Ulcer index[1]	Protection(%)[2]
Unulcerated control	0 ± 0	-
Ulcerated control (4 h)[3]	26.71 ± 1.62	0
Ulcerated untreated control	17.64 ± 1.24	36.08 ± 1.38
Ulcerated APC treated	1.78 ± 0.34	93.26 ± 6.77
Ulcerated misoprostol treated	3.91 ± 0.30	85.40 ± 3.51

[1]Stomach ulceration in rats was induced by oral administration of indomethacin (30 mg/kg body wt.). APC (2 mg/kg body wt.) and misoprostol (1.43 μg/kg body wt.) were used for these experiments. The ulcer indices values are mean ± SE (n = 15). [2]Considering an ulcer index of 100 for the ulcerated, untreated rats. 3The ulcer indices were measured 4 h after indomethacin administration. For other samples the measurement was carried out after 7 d.

increasing the dose of APC from 1 to 2 mg/kg body wt. led to a significantly improved ulcer healing. Increasing the doses of APC further did not provide any additional benefit. Even when the treatment with APC (2 mg/kg body wt.) was continued for 10 d, the extent of ulcer healing remained almost the same. Thus, administration of APC at a dose of 2 mg/kg body wt. for 7 d after ulcer induction was found to be optimal for effective ulcer healing. Hence all subsequent experiments were carried out with the same protocol. The dose of the positive control, misoprostol (1.43 μg/kg body wt.) was decided based on its recommended therapeutic dose for humans. Earlier misoprostol has been used at significantly higher doses with the rat models[21]. However, our studies were primarily aimed at developing anti-ulcer drugs for humans. For this it was essential to compare the ulcer-healing efficacy of APC with that of the established drug, misoprostol at the dose recommended for humans.

Treatment of rats with indomethacin (30 mg/kg body wt.) by oral intubation produced typical time-dependent acute lesions in the gastric mucosa, while the rats receiving vehicle only showed no visible mucosal lesions. Compared to the zero day ulcerated group (group Ⅱ) for which an ulcer index was taken as 100%, treatment with APC and misoprostol reduced the ulcer index by 93.4% and 85.4% respectively. The ulcer healing data of the treated groups (APC and misoprostol) were significantly different from those of both 0 and 7 d untreated experimental control rats (groups Ⅱ and Ⅲ) ($P < 0.05$). However, the difference in healing between the two treated groups (groups Ⅳ and Ⅴ) was insignificant (Table 2).

The visible morphological features of the ulcerated rat stomachs of the treated and untreated groups were compared to those with normal rats. This revealed that the zero-day ulcerated stomach had a number of blood clots in the ulcer spots and perforations. The stomach of the untreated experimental control rats (group Ⅲ) showed lesser spots, but the tissues were hyaline in nature. The result with misoprostol treatment was marginally better than that of the group Ⅲ rats. In comparison, stomachs of the APC treated rats (groups Ⅳ) were healthy, and equivalent to those of the normal control rats (group Ⅰ).

Histopathological investigation of the healing capacity of APC

The photomicrographs of rat stomachs belonging to groups Ⅰ-Ⅴ shown in Figure 2A-E gives a better comparison of the gastric lesion and its healing. Significant acute ulceration in rats was developed within 4 h of indomethacin (30 mg/kg body weight) administration as revealed from Figure 2A (normal rats) and Figure 2B (rats after 4 h of indomethacin administration). Indomethacin intake induced severe and extensive macroscopic gastric mucosal damage in the irrigated starved rats, characterized by injury in the epithelial layer of the mucosa. The lamina propria also were greatly damaged along with elongated hemorrhagic lesions confined mainly to the gastric corpus and running parallel to the long axis of the stomach that had the highest ulcer scoring rate. The disruption in the gastric mucosa was partially restored after 7 d even without any treatment, revealing autohealing. These observations matched well with the ulcer index parameter. Histology of the rat stomach of the untreated ulcerated group showed some damage in the mucosal layer with moderate infiltration in the mucous membrane (Figure 2C). However, the gastric mucosal tissues of the APC treated group (Figure 2D) showed almost normal and continuous mucosal layer and formation of the epithelial layer. The efficacy of APC was better than that of misoprostol as revealed from the Figures 2D and E.

Effect of APC on lipid peroxidation and DNA and protein contents in gastric tissues of ulcerated rats

The effects of indomethacin intake alone, and following administration of APC on the extent of lipid peroxidation (measured in terms of MDA), protein oxidation (measured in terms of total proteins and protein carbonyls), and DNA damage in the gastric tissues of rats are shown in Table 3. Indomethacin administration markedly stimulated lipid peroxidation in gastric tissues, and the MDA content was elevated by about 14 times compared to the normal rats. This was reduced by 11% after 7 d due to autohealing for the untreated control rats (group Ⅲ), although the MDA content remained significantly high (13 times) compared to that in the normal rats. Administration of APC for 7 d significantly reduced the MDA level (2.02 nmol/mg prot.)

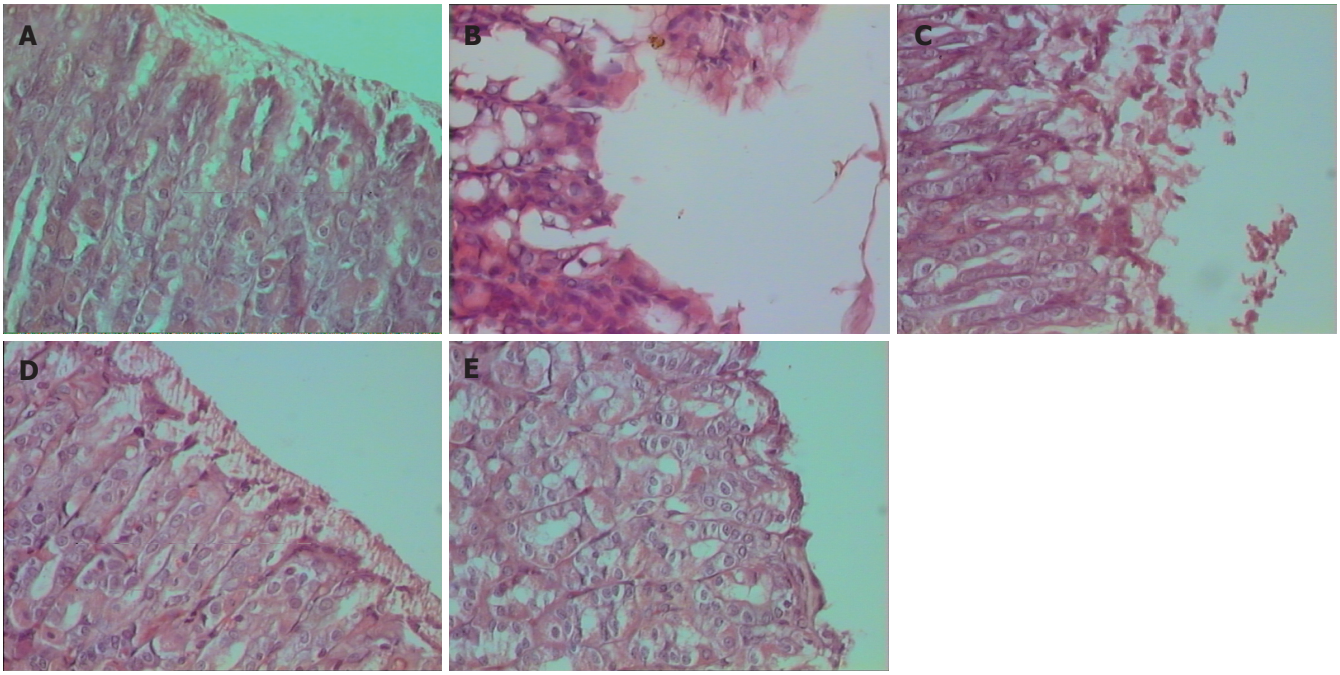

Figure 2 Histological assessment of acute gastric mucosal injury induced by indomethacin in rats and its prevention by APC (2 mg/kg body wt.) and misoprostol (1.43 μg/kg body wt.). Section of rat stomachs obtained from **A**: normal control rats; **B**: ulcerated untreated control rats 4 h after indomethacin administration; **C**: ulcerated untreated control rats 7 d after indomethacin administration; **D**: ulcerated rats treated with APC for 7 d; **E**: ulcerated rats treated with misoprostol for 7 d.

Table 3 The effect of APC and misoprostol on lipid, protein, DNA, SOD and CAT levels of ulcerated gastric tissue of rats[1]

Parameters	Group Ⅰ	Group Ⅱ[2]	Group Ⅲ	Group Ⅳ	Group Ⅴ
MDA (nmol/mg protein)	1.89 ± 0.01	27.04 ± 0.84	24.28 ± 1.12	2.02 ± 0.03	2.62 ± 0.02
Proteins (mg/mL)	16.52 ± 6.05	9.03 ± 1.28	12.55 ± 1.18	14.21 ± 1.8	12.89 ± 0.87
CO (mg/mg protein)	5.26 ± 1.34	17.29 ± 1.83	9.46 ± 2.15	6.03 ± 1.51	6.54 ± 1.12
DNA (mg/g tissue)	1.82 ± 0.22	0.81 ± 0.06	1.12 ± 0.21	3.56 ± 0.05	1.68 ± 0.23
SOD (U/min/mg protein)	22.2 ± 2.34	5.76 ± 2.32	11.58 ± 1.32	21.14 ± 1.24	16.71 ± 2.57
CAT (U/min/mg protein)	21.2 ± 1.12	9.02 ± 2.54	14.20 ± 2.84	19.04 ± 2.15	15.84 ± 2.09

[1]Stomach ulceration in rats was induced by oral administration of indomethacin (30 mg/kg body wt.). APC (2 mg/kg body wt.) and misoprostol (1.43 μg/kg body wt.) were used for these experiments. The values are mean ± SE (n = 15). [2]The assays were carried out 4 h after indomethacin administration. For other samples these were carried out after 7 d.

in gastric tissues almost to that of the normal rats (1.89 nmol/mg prot.). The effect of misoprostol was marginally less (MDA level 2.62 nmol/mg prot.) to that of APC. The data for the APC and misoprostol treatment are significant compared to that of the indomethacin induced untreated ulcerated control rats of groups Ⅱ and Ⅲ ($P < 0.05$).

Compared to the protein contents in the normal animals, the ulcerated group showed poor protein content (54% reduction), which improved for the untreated group on the 7 d and became 76% of the normal value. Treatment with APC and misoprostol increased the protein levels respectively to 86% and 78% of the normal value. The increase of the protein contents by the APC was significantly different ($P < 0.05$) compared to both ulcerated and untreated ulcerated controls (groups Ⅱ and Ⅲ). However, the data of misoprostol treatment group (group Ⅴ) was not significantly different from that due to autohealing (group Ⅲ).

The oxidative damage to tissue proteins was also evaluated by assessing the contents of protein carbonyls. The amount of protein carbonyls that increased significantly (329% compared to normal rats) due to indomethacin administration, was reduced by 45.3% due to autohealing. APC treatment restored the level of protein carbonyls to normalcy (6.03 μg/mg *vs* 5.26 μg/mg protein in normal rats), while the effect of misoprostol was less (6.54 μg/mg protein). The reduction of protein carbonyls by APC was significant ($P < 0.05$) as compared to untreated ulcerated controls (group Ⅲ).

The tissue DNA concentration was significantly (56%) reduced by indomethacin administration. During autohealing, the DNA level increased to 61.5% of the normal value. Surprisingly the APC treatment increased the DNA level by 196% compared to the normal rats, while treatment with misoprostol restored the level of DNA to normalcy (1.68 mg/g tissue for treated *vs* 1.82 mg/g tissue for normal rats). The augmentation of DNA by APC was significant ($P < 0.05$) compared to the ulcerated control (group Ⅱ) as well as the misoprostol group.

Effect of APC on the SOD and CAT levels in gastric tissues of ulcerated rats

The effect of indomethacin intake alone, and following administration of APC on the tissue levels of SOD and CAT in gastric tissues of rats are also presented in Table 3. Following indomethacin administration, the levels of these enzymes in gastric tissue were also depleted. The SOD activity decreased by 74%, while that of CAT was reduced by about 57% compared to those in normal control rats. Treatment with APC increased the SOD activity ($P < 0.05$) to near normalcy (21.1 U/min *vs* 22.2 U/min per mg protein in normal rats), while the CAT level showed 90% recovery ($P < 0.05$). The effect of misoprostol was significantly less than that of APC ($P < 0.05$) restoring the SOD and CAT levels to 75% and 74% of the normal values. In comparison, the autohealing restored the SOD and CAT levels to 52% and 67% of the normal values only. The data for the APC-treated rats were significantly different ($P < 0.05$) from those of the untreated 0 and 7 d groups.

Effect of APC on hexosamine and mucin contents of ulcerated gastric mucosa

Alcian blue assay: Indomethacin administration to rats significantly decreased the secretion of mucin (43.5%, $P < 0.05$) and mucosal glycoproteins (76.4% decrease, $P < 0.05$) in the ulcerated rats of group Ⅱ compared to those in unulcerated rats (group I). Treatment with APC enhanced the tissue mucin level to that in normal unulcerated rats, while the mucosal glycoprotein content was also increased to 65.4% of the normal value. The augmentation of mucin level by APC was significant ($P < 0.05$) as compared to ulcerated controls (group Ⅱ). Misoprostol also increased the mucin secretion and mucosal glycoproteins in the ulcerated rats to 94.2% and 89.9% of the respective normal values. The results are summarized in Table 4.

PAS staining: The photomicrographs of groups Ⅰ-Ⅴ rat stomachs after PAS staining are shown in Figure 3A-E. The indomethacin-induced stomach ulceration led to a drastic reduction in the mucin content that was partially restored due to autohealing. However, treatment with APC improved the mucin content in the stomach tissues (Figure 3D) further, bringing to a normal level (Figure 3A) along with considerable restoration of stomach morphology and epithelium lining. In comparison, the effect of misoprostol was not significantly different from that due to autohealing.

Table 4 The effect of APC and misoprostol on mucin and hexacosamine levels of ulcerated rats[1]

Samples	Hexosamine (mg/mg protein)	Mucin[2]
Unulcerated control	61.27 ± 2.28	4.62 ± 2.24
Ulcerated control[3]	14.48 ± 1.62	2.60 ± 4.14
Ulcerated APC treated	40.05 ± 3.78	4.53 ± 1.38
Ulcerated misoprostol treated	55.10 ± 1.21	4.35 ± 0.68

[1]Stomach ulceration in rats was induced by oral administration of indomethacin (30 mg/kg body wt.). APC (2 mg/kg body wt.) and misoprostol (1.43 μg/kg body wt.) were used for these experiments. The values are mean ± SE ($n = 15$). [2]Ab binding proteins. [3]The measurement was carried out 4 h after indomethacin administration. For other samples the measurement was done after 7 d.

DISCUSSION

Oxygen free radicals are known to play a role in the induction and pathogenesis of gastroduodenal injury[22]. Extensive research has proven that antioxidants might be effective not only in protecting against gastric mucosal injury, but also inhibiting progression of a gastric ulcer. Ulcer progression is caused by free radical-induced chain processes. Consequently, its arrest by radical scavengers helps in faster healing. Indomethacin is known to induce the reactive oxygen metabolites in animal models, which may contribute to mucosal injury[23]. The cytoprotective role of antioxidants in the prevention and healing of gastric lesions has been widely investigated in a number of studies[24,25].

The notion that APC has shown a powerful antioxidant potential in various *in vitro* models[26] warranted our attention to address its possible protective effects against indomethacin-induced gastric lesions in rats. Earlier, the cytoprotective and healing properties of *P. betel* crude extract against indomethacin as the gastric mucosal irritant was established by us[11]. The aim of the present study was, therefore, to study the healing effect of its principal antioxidant constituent, APC on indomethacin-induced acute stomach ulceration of rats.

Administration of indomethacin to rats induced marked damage to the gastric mucosa as evident by macroscopic and histopathological examinations. This led to elongated hemorrhagic lesions, confined to the glandular portion, with the highest subjective ulcer-scoring rate.

In the ulcerated control animals that were given the vehicle only during the seven-day period of treatment, the ulcer craters also receded through the process of autohealing, reducing the ulcer index by 36.2% for the group Ⅲ rats even without any drug treatment. The present study demonstrated that APC had a potent healing effect on indomethacin-induced gastric lesions in rats. The rate of healing in the APC-treated animals was significantly faster than that found in the case of autohealing. The histopathological observations revealed that the ulceration was acute explaining the autohealing observed in the untreated control rats.

Tissue damage is always associated with the loss/reduction of DNA content and loss or impairment of protein synthesis[27] due to excess generation of free radicals. These free radicals also damage the cellular antioxidant enzymes such as CAT, SOD and others, acting as the first line of cellular defense against oxidative injury. This might lead to aggravated tissue damage during stomach ulceration[28]. Our results revealed that the indomethacin-induced stomach ulceration was accompanied with a severe oxidative stress in gastric tissue causing damages to key biomolecules such as lipids, proteins and DNA. This was apparent from the stimulated lipid and protein oxidation leading to increased accumulation of MDA and

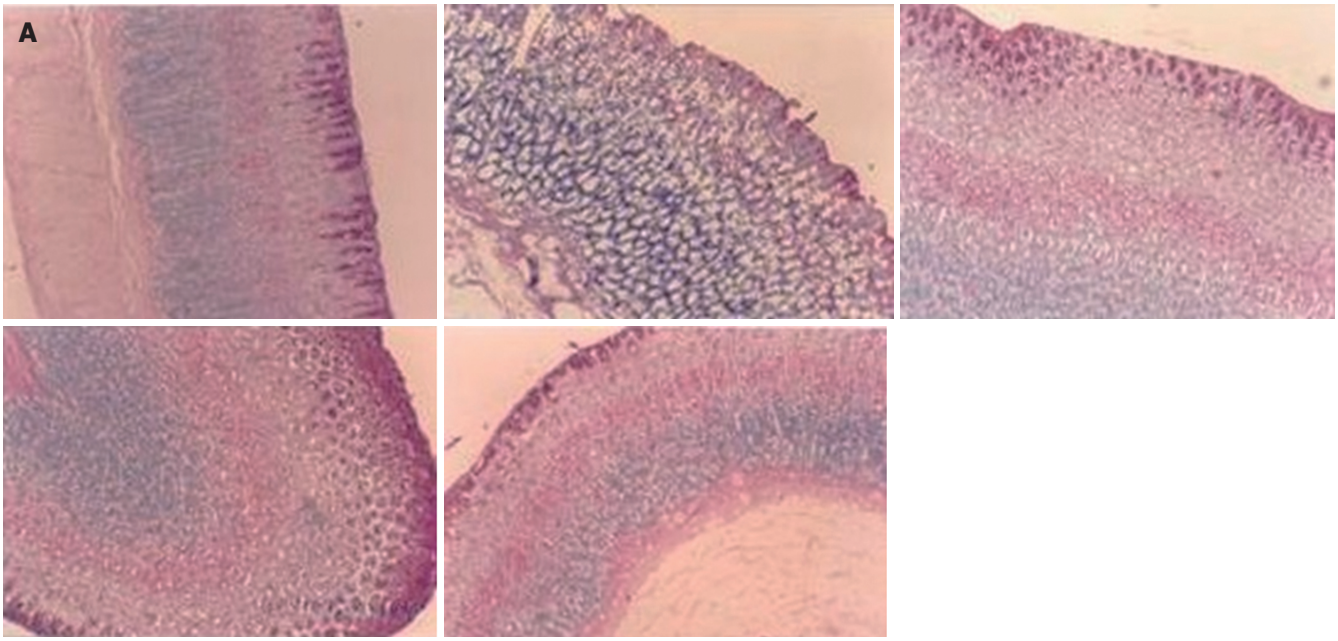

Figure 3 Depletion of mucin level in rat stomachs due to indomethacin-induced acute gastric ulceration and its prevention by APC (2 mg/kg body wt.) and misoprostol (1.43 μg/kg body wt.) as revealed by PAS staining. Section of rat stomach obtained from **A**: normal control rats; **B**: ulcerated untreated control rats 4 h after indomethacin administration; **C**: ulcerated untreated control rats 7 d after indomethacin administration; **D**: ulcerated rats treated with APC for 7 d; **E**: ulcerated rats treated with misoprotsol for 7 d.

protein carbonyls, as well as reduction in the tissue protein and DNA contents. These findings are in tune with many previous reports. APC provided a marked suppression of oxidative damage due to its excellent radical scavenging capacity and brought most of these parameters to normal levels, than observed in natural recovery.

Accumulation of the reactive products is known to markedly alter the antioxidant enzymes leading to enhanced oxidative damage during stomach ulceration[28]. In the present study, the gastric activities of SOD and CAT were found to be reduced notably following indomethacin intake. Treatment with APC reversed these oxidative changes with concomitant increase in SOD and CAT levels, thereby suppressing most of the biochemical adverse effects induced by indomethacin. This might decrease the ulcer progression and promote healing of gastric lesions induced by acute intake of indomethacin.

Ulcer-healing is a complex process involving a combination of wound retraction and re-epithelization[29]. Release of preformed mucus also plays a role in promoting epithelial recovery after acute injury by forming a mucoid cap beneath which re-epithelization occurs[30]. Besides providing significant buffering capacity for the neutralization of luminal acid, the mucus can offer protection against the endogenous aggressors like pepsin and oxidants produced in the gastric lumen, as well as against exogenous damaging agents, such as NSAIDs.

The macromolecular glycoprotein, hexosamine constitutes the major fraction of gastric mucin and is accepted to be an ideal index of gastric mucus production[31]. The NSAID-produced mucosal hemorrhagic ulcer may be due to a decrease of gastric mucus production[32]. Thus, drugs that arrest ulcer progression by antioxidant action, and also increase the synthesis and secretion of gastric mucus would accelerate gastric ulcer healing.

In this study, the decreased mucin secretion in the indomethacin-administered rats indicated reduced ability of the mucosal membrane to protect the mucosa from physical damage and back diffusion of hydrogen ions. The decrease in the glycoprotein content of the gastric mucosa further proved the decreased ability of the gastric mucosa to withstand the offensive onslaught. Mucosal damage can be easily produced by the generation of exogenous and endogenous active oxygen and free radicals[33]. An increase in mucus production usually assist the healing process by protecting the ulcer crater against irritant stomach secretions (HCl and pepsin) thereby enhancing the rate of the local healing process. Treatment with APC and misoprostol significantly accelerated the ulcer healing process, which is associated with an increase in the mucus layer in the gastric mucosa. Apparently, the free radicals scavenging property of APC might be contributing in protecting the oxidative damage to gastric mucosa.

It is well known that PAS stain is the best method to identify gastric metaplasia of duodenal mucosa. Metaplastic gastric type epithelium in the duodenum has been regarded as an adaptive defensive response to mucosal injury of any kind, including acid and/or pepsin[34]. The presence and extent of gastric metaplasia have been correlated with acid secretory capacity in men[35], and they have been induced in experimental animals by stimulation of excessive acid secretion. In contrast to the untreated controls in which the autohealing process can be attributed to other mechanisms, the healing action of APC is evidently related to its ability to improve mucus production. All these indicated that enhancement of the mucus modulation by APC play a significant role in its ulcer healing effect.

Given that some drugs can show mild-to-severe side

effects even after short-term intake, we also evaluated the possible toxic effects of APC up to a dose of 25 mg/kg body wt. with both mice and rats. There was no observable physical sign change and the animals had normal food and water as well as stool during the experimental period. These findings suggested that APC given at the current dose does not have any potential side effects in the animals. The non-toxicity of APC was expected considering that it is the major constituent of *P. betel* leaves that is freely consumed in India and South-East Asian countries.

In conclusion, the present study established that the APC, the major constituent of *P. betel* leaves can heal indomethacin-induced stomach ulceration in rats by its antioxidant action and ability to form mucus. Apparently, by scavenging free radicals, APC might be protecting the gastric mucosa, and in turn, the stomach epithelium from the oxidative damage. This accelerates healing of gastric ulcers. Earlier, our group has also reported the cytoprotective activity of the crude ethanol extract of *P. betel* leaves against indomethacin-induced stomach ulceration. These results, taken together along with the non-toxicity of APC indicate its potential as an anti-ulcerogenic drug for further investigations. Comparison of its efficacy with that of misoprostol further confirmed the findings.

Ulceration due to NSAID is also believed to occur because of non-selective inhibition of cyclooxygenases that hampers the release of mucus due to reduction in prostaglandin synthesis. The relationship between prostaglandins and leukotrienes, the products of prostaglandin H synthase (PGHS) and 5-lipoxygenase, respectively, seems to be an important factor in gastric ulcers. Many phenolic compounds stimulate prostaglandin synthesis by acting as reducing substrates for the oxidized intermediates of PGHS, thereby accelerating the peroxidase cycle and by functioning as electron-donating co-substrates for the peroxidase component of PGHS. In this way, they can modulate the PGHS and 5-lipoxygenase pathways of arachidonic acid[36]. Thus, it would be of interest to study the effect of APC on the PG-dependent pathway of healing gastric ulcer. Investigation in this regard is currently in progress in our laboratory and the results will be reported later.

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CLINICAL RESEARCH

Evaluation of clinical relevance of examining K-ras, p16 and p53 mutations along with allelic losses at 9p and 18q in EUS-guided fine needle aspiration samples of patients with chronic pancreatitis and pancreatic cancer

C Salek, L Benesova, M Zavoral, V Nosek, L Kasperova, M Ryska, R Strnad, E Traboulsi, M Minarik

C Salek, M Zavoral, V Nosek, Department of Gastroenterology, Central Military Hospital and 1st Medical Faculty of Charles University, Prague, Czech Republic
C Salek, L Benesova, L Kasperova, M Minarik, Laboratory for Molecular Genetics and Oncology, Genomac International Ltd., Prague, Czech Republic
C Salek, M Zavoral, Department of Gastroenterology, Institute for Postgraduate Medical Education, Prague, Czech Republic
M Ryska, R Strnad, Department of Surgery, Central Military Hospital and 2nd Medical Faculty of Charles University, Prague, Czech Republic
E Traboulsi, Department of Pathology, Central Military Hospital, Prague, Czech Republic
Supported by Internal Grant Agency of the Ministry of Health, Czech Republic, No. 8027-3
Correspondence to: Dr. Marek Minarik, Laboratory for Molecular Genetics and Oncology, Genomac International Ltd., Bavorska 856, 15541 Prague 5, Czech Republic. mminarik@email.com
Telephone: +420-224-458048 Fax: +420-224-458021
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Abstract

AIM: To establish an optimum combination of molecular markers resulting in best overall diagnostic sensitivity and specificity for evaluation of suspicious pancreatic mass.

METHODS: Endoscopic ultrasound (EUS)-guided fine needle aspiration cytology (FNA) was performed on 101 consecutive patients (63 males, 38 females, 60 ± 12 years; 81 with subsequently diagnosed pancreatic cancer, 20 with chronic pancreatitis) with focal pancreatic mass. Samples were evaluated on-site by an experienced cytopathologist. DNA was extracted from Giemsa stained cells selected by laser microdissection and the presence of K-ras, p53 and p16 somatic mutations was tested by cycling-gradient capillary electrophoresis (CGCE) and single-strand conformation polymorphism (SSCP) techniques. In addition, allelic losses of tumor suppressor genes p16 (INK4, CDKN2A) and DPC4 (MADH4, SMAD4) were detected by monitoring the loss of heterozygosity (LOH) at 9p and 18q, respectively.

RESULTS: Sensitivity and specificity of EUS-guided FNA were 75% and 85%, positive and negative predictive value reached 100%. The remaining 26% samples were assigned as inconclusive. Testing of molecular markers revealed sensitivity and specificity of 70% and 100% for K-ras mutations ($P < 0.001$), 24% and 90% for p53 mutations (NS), 13% and 100% for p16 mutations (NS), 85% and 64% for allelic losses at 9p ($P < 0.001$) and 78% and 57% for allelic losses at 18q ($P < 0.05$). When tests for different molecular markers were combined, the best results were obtained with K-ras + LOH at 9p (92% and 64%, $P < 0.001$), K-ras + LOH at 18q (92% and 57%, $P < 0.001$), and K-ras + LOH 9q + LOH 18q (96% and 43%, $P < 0.001$). When the molecular markers were used as complements to FNA cytology to evaluate inconclusive samples only, the overall sensitivity of cancer detection was 100% in all patients enrolled in the study.

CONCLUSION: EUS-guided FNA cytology combined with screening of K-ras mutations and allelic losses of tumor suppressors p16 and DPC4 represents a very sensitive approach in screening for pancreatic malignancy. Molecular markers may find its use particularly in cases where FNA cytology has been inconclusive.

Key words: Pancreatic cancer; Chronic pancreatitis; Endoscopic ultrasound-guided fine-needle aspiration; Molecular markers; Loss of heterozygosity

Salek C, Benesova L, Zavoral M, Nosek V, Kasperova L, Ryska M, Strnad R, Traboulsi E, Minarik M. Evaluation of clinical relevance of examining K-ras, p16 and p53 mutations along with allelic losses at 9p and 18q in EUS-guided fine needle aspiration samples of patients with chronic pancreatitis and pancreatic cancer. *World J Gastroenterol* 2007; 13(27): 3714-3720

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INTRODUCTION

Enormous progress in diagnostic and therapeutic approaches in the last decade had very limited impact on generally poor survival rates of patients diagnosed with

pancreatic cancer (PC)[1]. Mortality of the disease is almost at the level of its incidence as the majority of cases are diagnosed in advanced, not resectable stage[2]. Since the fundamental molecular-genetic mechanisms of PC have already been recognized, there is a great expectation that molecular tests could substantially assist in early diagnosis as well as open new therapeutic possibilities for this serious disease[3,4].

The development of pancreatic cancer follows a distinct path from normal ductal epithelia, pancreatic intraepithelial neoplasia (PanIN Ⅰ-Ⅲ) up to the carcinoma[5,6]. This path is accompanied by sequential accumulation of genetic defects (mostly point mutations, gene amplifications and allelic deletions). Activation of K-ras oncogene by somatic point substitution is seen as an initial event in pancreatic carcinogenesis[7]. This alteration can be detected already in PanIN-1A lesions as well as in chronic pancreatitis (CP) and therefore represents an independent risk factor for pancreatic cancer. In advanced pancreatic cancer, K-ras mutations are found in close to 90% of cases, therefore considered as a potential molecular marker for early detection of developing cancer. Following the initial K-ras activation, a number of other genetic abnormalities take place. PanIN-1A and PanIN-1B phases are characterized by overexpression of Her-2/neu oncogene, which is found in 50% of pancreatic neoplasms. Increased Her-2/neu expression, however, is a result of higher transcription rate rather than gene amplification, rendering Her-2/neu an unusable therapeutic target[8]. Aside from the above oncogenes, there are a number of tumor suppressor genes affected by genetic alterations during the transformation process. Among them, p16 tumor-suppressor (also referred to as CDKN2 or INK4), located at chromosome 9p21 is inactivated already during transition from PanIN-1B to PanIN-2 phases[9]. Furthermore, loss of another important tumor-suppressor gene, SMAD4 (known also as deleted in pancreatic carcinoma, DPC4), located at chromosome 18q21 has also been observed[10]. Consequently, the p16 and DPC4 are inactivated in almost 95% (55% respectively) of cases of invasive pancreatic cancers, therefore, potentially useable as molecular markers. All of the genetic mutation events adversely affect control of the cell cycle, thus enabling defected cells to proliferate. Oncogene K-ras encodes for GTP-binding protein responsible for signaling in the MAP-kinase pathway of intracellular signal transduction[11]. Tumor suppressor gene p53 is translated into a product that regulates transcription of other regulatory proteins, such as p21, inhibitory protein of cyclinD/CDK2 family[12]. The product of p16 tumor suppressor binds to the complex of cyclinD/CDK4 or CDK6, and thus regulates progression of cell cycle at the G1 control point[13]. Finally, the DPC4 tumor suppressor is a member of the SMAD protein family which plays a crucial role in intracellular signaling of TGF-beta[14].

Current diagnostic approaches mostly rely on evaluation of morphological changes in pancreatic tissue in combination with histology/cytology examination of samples obtained by fine-needle aspiration (FNA)[15]. EUS-guided FNA typically delivers sensitivity of 80% and specificity of 99%, while its positive and negative predictive values are at 99%, and 73% levels[16]. In order to increase diagnostic sensitivity of the FNA cytology, several papers have demonstrated detection of somatic aberrations as potential markers for early pancreatic cancer in DNA material from pancreatic juice, pancreatic ductal brushings, perioperative or percutaneous biopsies, plasma, duodenal aspirate, bile or stool. Among the various molecular markers in pancreatic cancer, K-ras is the most frequently studied. Its prevalence is estimated to reach 90%-95%. The reported rates of positivity, however, depend on experimental method of K-ras mutation detection as well as on the source material in which presence of K-ras mutations is to be detected. The capture rates range from 78%-100% in pancreatic tissue[17], 61%-89% in pancreatic juice[18,19], 72%-83% in pancreatic ductal brushing[20,21], 35% in plasma[22], 33% in bile[23], and 25% in duodenal aspirate[24]. Detection of K-ras in stool gives better sensitivity than in bile, however specificity drops significantly[25]. Acceptable specificity was reported only in pancreatic ductal brushings and pancreatic juice (77%-100%).

Because of the high sensitivity of genetic testing in pancreatic juice, numerous mutations in other genes have been reported in this material. Sensitivity and specificity of genetic tests in pancreatic juice is 40%-89% and 33%-96% for K-ras[26,27], 11%-43% and 70%-100% for p16[18,28,29], 14%-47% and 88%-100% for p53[25,30], 36%-70% and 39%-100% for DPC4[18,31]. The combination of several molecular markers in pancreatic juice is believed to improve sensitivity of genetic testing, giving best results for combination K-ras plus p53 which resulted in 100% sensitivity[30]. In contrast to pancreatic juice analysis, there are only a limited number of publications on frequency of gene mutations in EUS-guided FNA samples. Takahashi's study which included 62 consecutive patients with focal pancreatic mass is the largest. The authors screened for K-ras mutations and gave 74% sensitivity with 100% specificity[32].

From the original PC progression model it is clear that the pancreatic malignant conversion comes from a combination of multiple genetic events rather than originating from a single mutation[3,5]. Given the inherent heterogeneity of the carcinogenic pathways, simultaneous examination of multiple markers should lead to improved testing efficacy. The aim of the presented work was to evaluate a possibility of examining several somatic genetic events as potential molecular markers for early detection of pancreatic cancer in risk groups, such as in chronic pancreatitis patients. The main emphasis was on finding an ideal combination of markers resulting in optimum results when used in combination with commonly used cytology readings.

MATERIALS AND METHODS

Subjects

A total of 106 consecutive patients with focal pancreatic mass undergoing EUS-guided fine needle aspiration (FNA) were enrolled into the study. Patients were divided into pancreatic cancer group and the control group of patients with chronic pancreatitis based on histology of

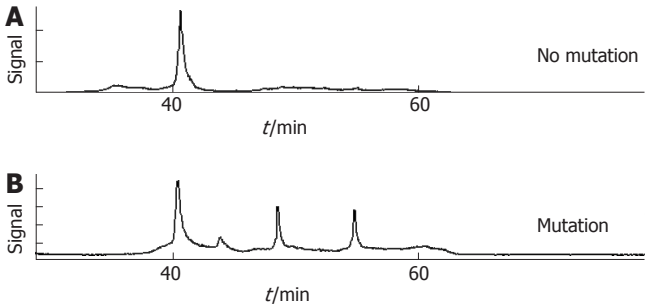

Figure 1 An example of the K-ras mutation analysis by CGCE method. **A**: sample without mutation; **B**: sample with K-ras mutation in codon 12.

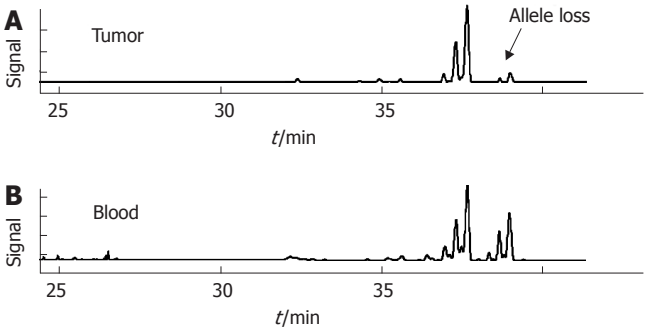

Figure 2 An example of LOH at chromosome 9p, analysis by CGCE method. **A**: tumor sample with allelic loss; **B**: blood sample with both alleles present.

surgical specimen or long-term follow-up. Five patients were excluded due to other diagnosis (adenoma, malignant fibrous histiocytoma, endocrine tumor, cholangiogenic carcinoma), or for malignant duplicity (patient with a breast carcinoma).

Of 101 patients in the final group a total of 63 (62%) were males and 38 (38%) females. The mean age in the group was 60 ± 12 years, range 32-84 years (+2.01/-2.21 standard deviation). There were 81 (80%) patients with pancreatic cancer, and 20 (20%) patients with chronic pancreatitis. All patients signed informed consent with participation in the study as well as with genetic analysis of their tissue material.

Methods

EUS was performed by a single experienced endoscopist using GFUM-20 radial and GFUCT-140 linear array scanning echoendoscopes (Olympus Europe). Quality of FNA samples was evaluated by an on-site cytologist after quick staining by hematoxylin-eosin. Definitive FNA diagnosis was stated by a single pathologist, blinded to the EUS, after staining additional slides by Giemsa. The same samples were subsequently submitted for genetic analysis. Furthermore, in a subset of 18 patients the genetic analysis of FNA samples were extended to genetic analysis of tissue acquired during subsequent perioperation biopsy. Laser microdissection of Giemsa-positive cells was performed on a P.A.L.M. Microlaser instrument (Carl Zeiss, Germany). Normally, between 100 and 200 cells were dissected from each slide. Genomic DNA was extracted from the dissected cells by a standard spin-column extraction protocol using the GMC tissue DNA isolation kit (Genomac, Czech Republic).

Presence of somatic point mutations in codons 12 and 13 of K-ras and in exons 5-8 of p53 was detected by cycling-gradient capillary electrophoresis (CGCE), a high-sensitivity mutation detection technique based on heteroduplex analysis in a temperature gradient[33]. The experimental details of the K-ras and p53 mutation assay were described previously[34,35]. Briefly, a PCR amplification of the target sequence containing the mutation hotspots was performed with one of the primers fluorescently labeled and the other primer extended by a 40 bp artificial high-melting domain (GC-clamp). Following PCR, the 140 bp fragment was heated and slowly cooled to allow formation of homo- and heteroduplex forms upon re-annealing of wildtype and mutant sequences. The resulting double-stranded fragments were subjected to capillary-electrophoretic separation at a cycling temporal temperature gradient. A typical result is shown in Figure 1.

Mutations in the p16 gene were analyzed by standard single-strand conformation polymorphism (SSCP) using amplification conditions previously described in literature[36,37] followed by capillary electrophoresis analysis of the resulting fragments in a non-denaturing gel matrix (GMC-SSCP, Genomac, Czech Republic).

Allelic losses at chromosomal positions 9p and 18q were monitored by the loss of heterozygosity analysis (LOH) using a total of 3 microsatellite (STR) markers for 9p (D9S157, D9S171 and D9S1748) and 2 markers for 18q (D18S363, D18S474)[38,39]. Detected LOH at chromosomal site 9p is shown in Figure 2.

All capillary electrophoretic experiments including previously described temperature-gradient, SSCP and fragment analysis were performed on a capillary-array DNA sequencer (MegaBACE™ 1000, GE Healthcare, Piscataway, NJ) equipped with Caddy™ 1000 robotic sample loader (Watrex Praha, Prague, Czech Republic) for unattended overnight operation.

Statistical analysis

Analysis was based on two-way and multiway contingency tables, sensitivity and specificity of tests and on a 95% confidence interval of relative frequencies with use of BMDP PC90 and MedCalc software.

RESULTS

The data in the study represent patients collected within a 2-year period from 2003 to 2005. Due to the dismal nature of the disease the total project time period exceeded by far the mean survival rate of pancreatic cancer patients enrolled in this study. The ultimate diagnosis of the malignant disease could, therefore, be unequivocally assigned based on clinical follow-up. During the final statistical analysis and evaluation, sensitivity and specificity of various diagnostic approaches performed during the patients dispensation could later be accurately determined.

Endoscopic ultrasonography (EUS)

EUS is considered the most sensitive method for visualizing

Table 1 Tests for K-ras, p53 and p16 mutations, and LOH at chromosome 9p (site of p16 gene) and LOH at chromosome 18q (site of DPC4 gene) *n* (%)

		Chronic panceratitis	Pancreatic cancer	Total	Sensitivity (95% CI)	Specificity (95% CI)	*P*	Youden's index
K-ras	Negative	20 (100)	24 (29.6)	44 (43.6)	70%	100%	< 0.001	70%
	Positive	0 (0)	57 (70.4)	57 (56.4)	(60%-80%)			
	Total	20 (100)	81 (100)	101 (100)				
p53	Negative	18 (90)	62 (76.5)	80 (79.2)	24%	90%	0.18 (NS)	14%
	Positive	2 (10)	19 (23.5)	21 (20.8)	(14%-34%)	(85%-95%)		
	Total	20 (100)	81 (100)	101 (100)				
p16	Negative	20 (100)	70 (87.5)	90 (90)	13%	100%	0.096 (NS)	13%
	Positive	0 (0)	10 (12.5)	10 (10)	(7%-19%)			
	Total	20 (100)	80 (100)	100 (100)				
LOH 9p	Negative	9 (64.3)	8 (15.4)	17 (25.8)	85%	64%	< 0.001	49%
	Positive	5 (35.7)	44 (84.6)	49 (74.2)	(75%-95%)	(53%-75%)		
	Total	14 (100)	52 (100)	66 (100)				
LOH 18q	Negative	8 (57.1)	11 (22.4)	19 (30.2)	78%	57%	< 0.05	35%
	Positive	6 (42.9)	38 (77.6)	44 (69.8)	(67%-89%)	(45%-69%)		
	Total	14 (100)	49 (100)	63 (100)				

focal pancreatic lesions and staging of locoregional progression of the pancreatic disease[40]. All patients in our group were subjected to EUS for initial evaluation of pancreatic lesions. The EUS differentiation between the malignant or benign nature of the lesions resulted in 79% sensitivity and 77% specificity. The overall rate of false negatives was 5% and false positives 4%. In 11% of cases the endoscopist was not able to reliably state the diagnosis.

Fine-needle aspiration cytology (FNA-cytology)

EUS-guided FNA cytology has been adopted as a routine method for all patients admitted to our gastroenterology department with a suspicion of pancreatic cancer. The main benefit of this safe method is in its sensitivity. At the same time, the acquired morphological information (TN staging) removes a need for additional diagnostic testing and/or surgery, surpassing CT or MR imaging[41]. Following the initial test for specimen quality by a "quick-test" using hematoxylin-eosin staining immediately following the puncture, samples were stained by Giemsa and thoroughly evaluated by an experienced cytologist. In the present study the overall sensitivity and specificity for FNA cytology evaluation was 75% and 85% respectively; the positive and negative predictive value reached 100% with no malignant specimens assigned as benign or vice versa. In FNA testing, however, 26% of smears were assigned as inconclusive. This mostly owing to the fact that the cellular atypia found in ductal epithelia did not allow clear differentiation between both diagnoses.

Histology of surgical resection tissue

Finally, in a subset of 18 patients, surgery was performed and collected pancreatic tissue was evaluated by a pathologist. Histological evaluation of surgical resection resulted ultimately in 100% specificity, but 95% sensitivity due to the fact that one resection sample was falsely evaluated as cancer-negative.

Molecular marker examination

Activating mutations in the K-ras oncogene were found in 57 out of the total 101 samples. Comparison with the final diagnosis revealed that all K-ras positives were subsequently confirmed with malignancy, while none of the chronic pancreatitis samples exhibited K-ras mutation. Hence, the resulting specificity was 100% and the sensitivity 70% with 95% CI (60%-80%). There were 24 (30%) cancerous specimens without K-ras mutation. Detecting mutations in tumor suppressor gene p53 uncovered only 19 positive cases (total of 101 cases) with a sensitivity of 24% with 95% CI (14%-32%) and specificity of 90% with 95% CI (85%-95%). Similarly, low mutation rates were obtained for p16 gene with 10 of 100 cases leading to only 13% sensitivity (95% CI 5%-9%), specificity was 100%. Forty-four of a total of 66 samples exhibited allelic loss at 9p with a sensitivity of 85% with 95% CI (75%-94%) and specificity of 64% with 95% CI (53%-75%). Although 9p harbors p16 tumor-suppressor gene, no correlation was found between occurrence of p16 mutations and 9p allelic deletions. A combination of the two tests (p16 mutations and losses at 9p) yielded overall sensitivity of 84% with a specificity of 64%, 95% CI (75%-94%) and (53%-75%) respectively. Sole LOH test at chromosomal position 18q, corresponding to a loss of tumor suppressor gene DPC4, was detected in 38 of 63 cases with a sensitivity of 78% (95% CI 66%-89%) and specificity of 57% (95% CI 45%-69%) (Table 1).

When combining tests for independent molecular markers (Table 2), the best results were obtained with a combination of K-ras and LOH 9p. Sensitivity and specificity of this combination were 92% with 95% CI (86%-99%) and 64% with 95% CI (53%-75%), respectively. Another promising combination was K-ras and LOH 18q resulting in sensitivity of 92% with 95% CI (85%-99%) and a specificity of 57% with 95% CI (45%-69%). By combining two markers with high specificity K-ras and low-sensitivity p53 reasonable values were obtained: sensitivity of 74% with 95% CI (65%-83%) and specificity of 90% with 95% CI (85%-95%). This, however, does not significantly improve the sole K-ras test showing 70% sensitivity and 100% specificity as noted above. Similarly,

Table 2 Combination of test for various molecular markers

Combination of tests			95% CI	*P*	Youden's index
K-ras + LOH 9p	Sensitivity	92%	85%-99%	< 0.001	57%
	Specificity	64%	53%-75%		
K-ras + LOH 18q	Sensitivity	92%	85%-99%	< 0.001	49%
	Specificity	57%	45%-69%		
LOH 9p + LOH 18q	Sensitivity	92%	85%-99%	< 0.01	34%
	Specificity	43%	31%-55%		
p16 + LOH 9p	Sensitivity	84%	74%-94%	< 0.001	49%
	Specificity	64%	53%-75%		
K-ras + p53	Sensitivity	74%	65%-83%	< 0.001	64%
	Specificity	90%	85%-95%		
K-ras + LOH 9p + LOH 18q	Sensitivity	96%	92%-100%	< 0.001	39%
	Specificity	43%	31%-55%		
K-ras + p16 + LOH 9p	Sensitivity	92%	85%-99%	< 0.001	57%
	Specificity	64%	53%-75%		
p53 + LOH 9p + LOH 18q	Sensitivity	92%	85%-99%	< 0.001	35%
	Specificity	43%	31%-55%		

a combination of the LOH tests performed on the two chromosomal loci (9p and 18q) resulted in the sensitivity of 92% with 95% CI (84%-99%) and specificity of 43% with 95% CI (31%-55%), which does not surpass the combination of the relatively simpler K-ras mutation test with either of the individual LOH tests.

If three markers are taken into account, a combination of K-ras with both LOH (18q and 9p) show the highest sensitivity of 96% with 95% CI (91%-100%) and a specificity of 43% with CI (31%-55%). The combination of p53 with LOH 18q and 9p gives comparable results: sensitivity of 92% with 95% CI (85%-99%) and specificity of 43% with 95% CI (31%-55%). Combination of K-ras with both methods for detecting genetic variations in p16 (SSCP and LOH 9p) results in a sensitivity of 92% with 95% CI (85%-99%) and specificity of 64% with 95% CI (53%-75%).

Finally, fidelity of genetic testing in FNA-cytology smears versus resection tissue was evaluated in case of 18 patients where both sample types were obtained. There was no discrepancy in K-ras, p53 or p16 mutation rates as the results were identical in both sample types. On the contrary, however, FNA-cytology specimens proved to be more suitable for detection of allelic losses at 9p by the LOH test ($P < 0.001$); higher sensitivity of FNA specimens for detection of LOH 18q was close to statistical significance ($P < 0.10$).

DISCUSSION

EUS-guided FNA-cytology is widely regarded as the "golden standard" in morphological diagnosis of pancreatic neoplasms. In agreement with this common perception, our own experience also confirms a high diagnostic value of the technique with positive and negative predictive value reaching 100% over the course of the presented study. These results mirror high efficacy of the protocol if FNA biopsy is first evaluated on-site by the cytologist, and then conclusively interpreted by a skilled pathologist with proper experience in pancreatic cytology. This encouraging result, however, is reduced by the fact that in addition to the unequivocally assigned samples a remaining total of 26% of FNA smears are marked as inconclusive. This mirrors the fact that distinction between reactive changes in chronic pancreatitis and well differentiated adenocarcinoma may be problematic and cause under diagnosis of pancreatic cancer[42]. Hence, the resulting 74% success rate of FNA-cytology clearly opens a need for additional diagnostic tools.

Molecular diagnosis of early pancreatic cancer has been studied for several years. Although many molecular markers have been identified, it is evident that diagnostic and/or screening should be based on a set of tests rather than relying on one universal molecular indicator. In our study, we have obtained reproducible results indicating a notable capture rate of pancreatic cancers by using a combination of highly specific multiple markers. As shown in Table 1, the test for K-ras mutation exhibited the highest possible specificity. Our finding is in agreement with reports of K-ras testing in pancreatic juice (sensitivity of 40%-89% and specificity 33%-96%). With regard to K-ras testing in FNA samples, our sensitivity was similar to a recent study (70% *vs* 74%), moreover, at the same time we have confirmed 100% specificity of K-ras testing in FNA reported in the same study[32]. The fidelity of the K-ras test in our work was followed by LOH analysis for the detection of allelic losses at 9p and 18q chromosomal positions. Satisfactory sensitivity with relatively low specificity of all above mentioned genetic tests make them suitable for screening purposes rather than for differential diagnosis of pancreatic masses.

As expected, the LOH analysis greatly profited from laser microdissection of tumor cells from FNA-cytology specimens. In comparison to manual dissection from resected tissue, the sensitivity for detection of allelic loss was higher for laser-microdissected FNA samples. Low diagnostic value of p53 and p16 point mutations is in agreement with the overall limits of sensitivity and specificity intervals for these markers being previously tested in pancreatic juice[18,25,28-30]. Similarly in FNA samples, p53 or p16 mutations seem suitable for differential diagnosis or screening in FNA samples.

Based on the observations from this study, a diagnostic algorithm reflecting the most efficient approach to distinguish pancreatic cancer from chronic pancreatitis in FNA samples can be constructed (Figure 3). As the EUS-guided FNA-cytology still has the highest diagnostic relevance reaching 100% both predictive values while showing acceptable sensitivity and specificity, it should always remain a preferred method of choice for examination of a focal pancreatic mass (Figure 3, step 1). Only a subset of FNA-inconclusive samples should be further examined by genetic analyses. The size of such a sample set will undoubtedly depend on the pathologist's level of expertise. Of the various markers, K-ras is a prime candidate for first-level genetic analysis as the K-ras positivity showed to reliably differentiate patients with malignancy (Figure 3, step 2). Because of a lower sensitivity of the K-ras test, samples negative for K-ras mutation should, consequently, be examined for allelic losses by LOH tests. Due to its higher sensitivity, LOH

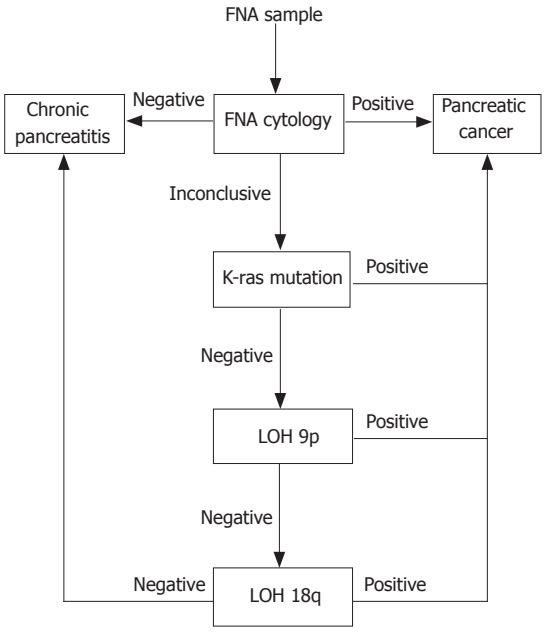

Figure 3 Four-step diagnostic algoritm evaluating FNA cytology and genetic changes for differentiation of benign and malignant lesions of the pancreas.

on the chromosome 9p, should be tested first (Figure 3, step 3), followed by a final testing of the LOH 18q performed on the remaining samples showing negativity for all previous tests (Figure 3, step 4). Such a set of four subsequent testing steps delivers satisfactory results. When using to process data acquired in our study, malignancy was correctly assigned to all patients with ultimately confirmed cancer status with no false negatives. One patient with chronic pancreatitis was incorrectly assigned with pancreatic cancer, a false positive, due to positivity of both 9p and 18q LOH tests.

In conclusion, the most sensitive genetic test for screening for malignancy in EUS-guided FNA samples from pancreatic mass seems a combination of K-ras mutation analysis with detection of p16 gene loss by LOH at 9p. Combination of K-ras with LOH analysis at both p16 and DPC4 genes further improves the sensitivity to 96%. The best compromise of sensitivity and specificity according to the Youden's index is single K-ras (70%) or combination of K-ras with LOH 9p (57%). Based on our observations it seems that due to relatively high specificity of the used markers, malignancy is usually indicated already by a single positive test. Therefore, only negative samples are subsequently tested by further markers, increasing the cost effectiveness of such diagnostic testing.

COMMENTS

Background

According to the numerous papers published in recent years, molecular diagnostics do not exceed the capabilities and limitations of conventional histological or cytological procedures. However, its contribution to the diagnostics in cases where morphology is not conclusive was not taken into account.

Research frontiers

The development of a diagnostic approach to pancreatic cancer relying on the analysis of genetic markers is a hotspot for scholars for the past decade. As monitoring of singular molecular markers has failed in this endeavor up to date, the focus has shifted to expression profiling, proteomics, and development of diagnostic arrays.

Innovations and breakthroughs

The study applies genetic methods previously tested in pancreatic juice directly on DNA extracted from tumour cells won by EUS-guided FNA. It was performed in a relatively large group of patients (n = 101) and monitors changes in four genetic loci (K-ras, p16, p53 and DPC4). No previous study describing loss of heterozygosity at 9p and 18q in pancreatic FNA samples was published up to date.

Applications

A sequential four-step diagnostic algorithm combining EUS-guided FNA and various genetic tests is suggested for differentiating between benign and malignant focal pancreatic masses. Its potential to become a part of a screening program for pancreatic cancer in high risk groups of patients is to be evaluated.

Terminology

Endoscopic ultrasound navigated fine needle aspiration (EUS-guided FNA) is a safe and highly sensitive method for getting pancreatic tissue samples by transgastric or transduodenal puncture. Fine needles (17-19 Gauche) allow obtaining high quality material for cytology examination which, however, is not generally sufficient for biopsy samples. Cycling gradient capillary electrophoresis (CGCE) is a high-sensitivity mutation detection technique. PCR fragments are heated and slowly cooled so that homo- and heteroduplex forms arise upon re-annealing of wildtype and mutant sequences. The resulting double-stranded fragments are separated according to different velocity in capillary electrophoresis at cycling temperature gradient.

Peer review

The authors evaluated clinical relevance of multiple genetic alterations in EUS-guided fine needle aspiration samples of patients with chronic pancreatitis and pancreatic cancer. The data are very informative because the experiments are well designed and performed.

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RAPID COMMUNICATION

Factors determining delay in relaparotomy for anastomotic leakage after colorectal resection

A Doeksen, PJ Tanis, BC Vrouenraets, JJB Lanschot van, WF Tets van

A Doeksen, PJ Tanis, BC Vrouenraets, WF Tets van, Department of Surgery, Sint Lucas Andreas Hospital, Jan Tooropstraat 164, 1006 AE Amsterdam, The Netherlands
A Doeksen, PJ Tanis, JJB Lanschot van, Department of Surgery, Academic Medical Center, Meibergdreef 9, 1100 DD Amsterdam, The Netherlands
Correspondence to: PJ Tanis, MD, PhD, Department of Surgery, Academic Medical Center, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands. p_tanis@hotmail.com
Telephone: +31-20-5669111 Fax: +31-20-6914858
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Abstract

AIM: To analyze the time interval ('delay') between the first occurrence of clinical parameters associated with anastomotic leakage after colorectal resection and subsequent relaparotomy.

METHODS: In 36 out of 289 consecutive patients with colorectal anastomosis, leakage was confirmed at relaparotomy. The medical records of these patients were retrospectively analysed and type and time of appearance of clinical parameters suggestive of anastomotic leakage were recorded. These parameters included heart rate, body temperature, local or generalized peritoneal reaction, leucocytosis, ileus and delayed gastric emptying. Factors influencing delay of relaparotomy and consequences of delayed recognition and treatment were determined.

RESULTS: First documentation of at least one of the predefined parameters for anastomotic leakage was after a median interval of 4 ± 1.7 d after the operation. The median number of days between first parameter(s) associated with leakage and relaparotomy was 3.5 ± 5.7 d. The time interval between the first signs of leakage and relaparotomy was significantly longer when a weekend was included (4.2 d *vs* 2.4 d, $P = 0.021$) or radiological evaluation proved to be false-negative (8.1 d *vs* 3.5 d, $P = 0.007$). No significant association between delay and number of additional relaparotomies, hospital stay or mortality could be demonstrated.

CONCLUSION: An intervening weekend and negative diagnostic imaging reports may contribute to a delay in diagnosis and relaparotomy for anastomotic leakage. That delay was more than two days in two-thirds of the patients.

INTRODUCTION

Anastomotic leakage after colorectal resection is an adverse event with a tremendous impact on morbidity, mortality and quality of life. Mortality rates of more than 30% in patients who developed anastomotic leakage have been reported in the literature[1-6]. Clinically symptomatic leakage often requires one or more operative reinterventions with frequent need for intensive care admission and prolonged hospital stay. When a stoma is constructed at reexploration, this is meant to be temporary but often appears to be permanent. In those patients whose bowel continuity is restored, late functional consequences may be encountered[7].

Many studies have concentrated on risk factors for anastomotic leakage, including comorbidity and surgical technique, trying to find ways to prevent leakage in high-risk groups[2,4,6,8-11]. When leakage occurs, it seems important to detect this complication at an early moment to minimize associated morbidity and mortality[12]. However, the clinical diagnosis of anastomotic leakage is often difficult and it may only become evident after several days of close observation[13]. Little is known about the incidence and consequences of a delay in the diagnosis and subsequent treatment of anastomotic leakage after colorectal resection. Therefore, we retrospectively determined time intervals between first clinical signs and relaparotomy and assessed risk factors and consequences of a delay in recognition and treatment of anastomotic leakage.

MATERIALS AND METHODS

Between January 2000 and July 2003, 289 consecutive patients underwent an ileocolic, colo-colonic or colorectal

anastomosis at the Sint Lucas Andreas Hospital, a non-university teaching hospital in Amsterdam, the Netherlands. There were 158 females and 131 males with a mean age of 69 (range 20-96) years. In 15 patients (5%), the anastomosis was performed to restore colonic continuity after previous colostomy, while in the remaining patients the anastomosis was constructed immediately following bowel resection. Ileocolonic resection was performed in 27 patients (9%), right hemicolectomy in 94 (33%), transverse colonic resection in 10 (3%), left hemicolectomy in 20 (7%), sigmoidal resection in 72 (25%) and subtotal or total colectomy in 7 patients (3%). A low anterior resection was performed in 44 patients (15%). Patients electively planned for colonic or rectal resection were admitted to the hospital one day before surgery. Bowel preparation was given to patients undergoing left-sided resections and consisted of oral phosphate solution. In addition, one enema was given the morning of surgery to patients who underwent low anterior resection. Antibiotic prophylaxis consisted of a cephalosporin and metronidazol and was given in a single dose during induction of anesthesia. Operations were performed by consultant surgeons in 184 patients (64%) and by trainees under supervision in 105 patients (36%). Type of anastomosis (e.g. end-to-end or end-to-side) depended on the preference of the individual surgeon. Hand-sewn anastomoses were performed using a one layer continuous suture of propylene 3/0 in 209 patients. Stapled anastomoses were performed in 80 patients (28%). Postoperative oral intake was gradually restarted depending on nausea, bowel movements, gastric tube production (if applied), and passage of flatus or stools. No fast-track recovery programs were used during the study period. Patient's temperature, blood pressure, and heart rate were routinely recorded three times daily. The patients were seen by the attending doctor at least once daily during morning rounds, even during the weekends. Radiological examination of the anastomosis by contrast radiography or computed tomography (CT) was not performed on a routine basis, but only when leakage was suspected on clinical grounds.

For the purpose of this study, simple clinical parameters suggestive of anastomotic leakage were identified from the literature[1,14,15] and retrospectively collected from the records of patients who developed anastomotic leakage confirmed at relaparotomy. These parameters included tachycardia (heart rate > 100 beats per minute), fever (body temperature > 38℃), local or generalized peritoneal reaction during physical examination, leucocytosis (> 10×10^3/mL), prolonged adynamic ileus (> 2 d) as demonstrated by symptoms and signs during physical examination or plane abdominal radiography, and delayed gastric emptying (increased gastric tube production of more than 200 mL per day or vomiting necessitating tube reinsertion). In addition, the postoperative day of first appearance of any of these parameters was scored, as well as the first day the attending doctor recognized these signs, resulting in a description in the patient's files. Delay until relaparotomy was calculated from the day of first retrospective presence of clinical parameters associated with leakage and from the day the possibility of anastomotic leakage was explicitly suggested in the medical records by the attending doctor. The following factors were tested for their association with delay of relaparotomy for anastomotic leakage: age, sex, body mass index, site of anastomosis, radiological examination, and presence of a weekend in the period between first appearance of clinical parameter(s) and relaparotomy. To determine the influence of a weekend on the delay of relaparotomy, patients with a delay of more than seven days were excluded. Consequences of a delay for number of relaparotomies, hospital stay, and in-hospital mortality were assessed.

Table 1 Patient and treatment characteristics of 36 patients with anastomotic leakage confirmed by relaparotomy

Characteristic	*n* (%)
Gender	
Male	21 (58)
Female	15 (42)
Mean age (range) (yr)	67 (26-87)
Mean Quetelet index (range)	25 (17-43)
American Society of Anesthesiology score	
1	14 (39)
2	16 (44)
3	6 (17)
Comorbidity	
Laparotomy in medical history	14 (39)
Diabetes mellitus	6 (17)
Cardiovascular disease	17 (47)
Preoperative radiotherapy	4 (11)
Chronic Obstructive Pulmonary Disease	8 (22)
Type of operation	
Ileocolonic resection	5 (14)
Right hemicolectomy	3 (8)
Transverse colonic resection	2 (6)
Left hemicolectomy	1 (3)
Sigmoidal resection	14 (39)
Subtotal colectomy	3 (8)
Anterior resection	7 (19)
Restoring continuity after colostomy	1 (3)

Statistical analysis

Univariate analyses using the Mann-Whitney test, F test and χ^2-test were performed to compare data of two groups. Spearman's correlation coefficient was used to determine the correlation between two continuous variables. Significance was set at $P \leqslant 0.05$ (two-sided). Statistical analyses were performed with Statistical Package for the Social Sciences software (SPSS, Chicago, IL, USA).

RESULTS

Anastomotic leakage was confirmed during relaparotomy in 36 patients. Patient and treatment characteristics of the 36 patients are displayed in Table 1. Symptomatic anastomotic leakage occurred despite the presence of a diverting ileostomy in three patients after low anterior resection. In three patients, anastomotic leakage was not confirmed during first relaparotomy, but only after repeated laparotomy at three ('second look' at day one, 'third look' at day three), 24 and 28 d after the initial operation, respectively. Apart from irrigation of the contaminated abdominal cavity, the operative procedure for leakage consisted of breakdown of the anastomosis

Table 2 Incidence and median postoperative day of first occurrence of simple clinical parameters in 36 patients with anastomotic leakage confirmed at relaparotomy

Variable	Incidence (%)	Median postoperative day (SD)
Tachycardia (> 100 beats/min)	61	4 (2.6)
Fever (> 38℃)	67	5 (2.4)
Peritoneal reaction	28	6 (3.7)
Leucocytosis (> 10×10^3/mL)	72	6 (2.5)
Adynamic ileus	47	6 (4.6)
Delayed gastric emptying	67	4 (2.0)

SD: standard deviation.

Table 3 Median time intervals between first occurrence of clinical parameters or the attending doctor's suggestion of anastomotic leakage in the medical record and relaparotomy

Signs/symptoms First occurrence of:	Median delay of relaparotomy (d)	SD	Range
Tachycardia	2.0	7.2	0-29
Fever	2.8	5.7	0-29
Peritoneal reaction	0.8	2.8	0-8
Leucocytosis	2.0	6.1	0-29
Ileus	1.5	2.4	0-9
Delayed gastric emptying	2.0	5.2	0-25
At least one parameter	3.5	5.7	0-29
At least two parameters	2.5	5.4	0-12
At least three parameters	1.8	5.7	0-8
Doctor's suggestion of leakage in medical record	1.0	5.1	0-29

and construction of a colostomy in 21 patients (58%), a diverting loop-ileostomy in twelve (33%), abscess drainage in two (6%) and no additional intervention in one patient (3%).

For the 36 patients with leakage confirmed at relaparotomy, the incidence and median postoperative day of first occurrence of the simple clinical parameters are displayed in Table 2. The first appearance of at least one of these signs was after a median interval of 4 (± 1.7; range 1-8) d after the operation. This interval was 5 ± 2.3 (range 2-12) d and 5.5 ± 2.8(range 2-12) d for at least two and three signs, respectively. Relaparotomy for anastomotic leakage was performed after a median interval of 7 d after initial surgery (± 4.1; range 3-24) d. The median number of days between the first occurrence of each specific parameter, at least one parameter, at least two parameters and at least three parameters associated with leakage and relaparotomy are displayed in Table 3. The median time interval between the presence of at least one positive parameter and relaparotomy ('the delay') was 3.5 d; 23 relaparotomies for anastomotic leakage (64%) were performed after a delay of more than two days. The median number of days between the attending doctor's suggestion of anastomotic leakage in the medical records and relaparotomy was one day.

A negative result of either contrast study or CT scanning in nine patients resulted in a significantly longer delay of relaparotomy as shown in Table 4. If a weekend (Saturday and/or Sunday) was included in the time

Table 4 Risk factors for prolonged delay of relaparotomy because of anastomotic leakage

Variable		*n*	Mean delay (d)	*P*
Age (yr)	< 70	19	4.5	0.71
	⩾ 70	17	4.9	
Sex	Male	21	4.5	0.95
	Female	15	4.9	
Body mass index (kg/m^2)	< 25	18[1]	4.6	0.29
	⩾ 25	14	5.3	
Site of anastomosis	Left	25	4.8	0.29
	Right	11	4.3	
Radiological examination performed	Yes	21	6.0	0.051
	No	15	2.7	
Outcome of radiological examination	FN	9	8.1	0.007
	TP/NP	27	3.5	
Weekend included in period between first clinical parameter and relaparotomy	Yes	14[2]	4.2	0.021
	No	18	2.4	

FN: false-negative; TP: true-positive; NP: not performed. [1]Four missing values, [2]Four patients excluded with delay of more than seven days. Significance of differences in delay between subgroups is determined using the Mann-Whitney test.

interval between the first positive parameter suggestive of leakage and relaparotomy, delay of relaparotomy was also significantly longer in comparison with patients in whom observation and decision to reoperate did not take place during a weekend. No other factors determining the length of the delay could be demonstrated (Table 4).

After the first relaparotomy for anastomotic leakage, one additional laparotomy was performed in eight patients (22%) and more than one relaparotomy in another 10 patients (28%). Although the patients who needed at least one additional relaparotomy did have a longer delay between the first appearance of a clinical parameter and the first relaparotomy in comparison with patients who did not need additional relaparotomies [6.0 (± 7.6) d *vs* 3.3 (± 2.4) d], this difference did not reach statistical significance ($P = 0.52$). Patients with anastomotic leakage were admitted to the hospital for a mean period of 59 (range 7-259) d. There was no significant correlation between the delay of relaparotomy and duration of hospital stay (Spearman's correlation coefficient 0.16, $P = 0.34$). Overall in-hospital mortality was 36% (13 of 36 patients). Delay of relaparotomy for anastomotic leakage was not significantly longer in patients who died postoperatively (5.5 ± 5.6 d *vs* 4.2 ± 5.8 d for patients who did not have a delay, $P = 0.54$).

DISCUSSION

Two thirds of relaparotomies were performed more than two days from the first appearance of at least one positive parameter suggestive of anastomotic leakage with a median delay of 3.5 d. This is similar to the median delay of 4 d in a series of 22 patients with clinical symptomatic leakage as reported by Sutton *et al*[13]. Even if at least three positive parameters were present, it took a median number of 1.8 d until relaparotomy for anastomotic leakage was performed in our series. In a study by Alves *et al*[14], the risk of leakage increased to 67% if three or more signs associated with anastomotic failure were present. A remarkable finding

was the increase in delay when signs and symptoms suggestive of leakage appeared just before or during a weekend. During weekends, all patients are seen by a staff surgeon and a surgical resident during morning rounds on Saturday as well as on Sunday. The higher work load, the absence of the attending surgeon who initially performed the anastomosis, and the absence of a plenary discussion of clinical problems by the entire surgical staff during weekends may explain this disturbing finding.

The routine use of radiographic imaging in diagnosing anastomotic leakage is surrounded by controversies. We found that a negative result of either contrast study or CT scanning in nine patients resulted in a significantly longer delay of relaparotomy. This observation opens the discussion whether to perform radiographic imaging before relaparotomy. Nicksa *et al*[16] retrospectively studied 36 patients who were reoperated for anastomotic leakage and found that 3 of the 18 contrast enemas (17%) and 14 of the 27 CT scans (52%) were false-negative. Another study described 16 patients with a clinical anastomotic leakage, in whom four imaging studies (25%) were initially misinterpreted[17]. A similar sensitivity was reported by Akyol *et al* in a series of 233 patients who underwent left sided colonic or colorectal anastomoses. The false-negative percentage of a routine water soluble contrast enema in the early postoperative period was 22% (11 of 51 patients with anastomotic leakage)[18]. None of these studies describe the impact of imaging on the delay of relaparotomy.

But what does eventually lead to the decision to perform a relaparotomy? Is it one specific parameter that has more impact than some others or is it a specific combination of positive parameters? Comparing the delay after each individual parameter, the presence of peritoneal reaction is the only parameter that resulted in surgical intervention within 24 h in most cases. It is unclear whether this symptom is so important in surgical decision making or it is just a relatively late sign which in combination with other earlier positive parameters makes relaparotomy inevitable. The difficulty in clinical decision making is calculating the pre-test chance of an event (i.e. anastomotic leakage) based on a number of predictive factors. In addition, a cut-off point has to be determined at which the optimum is reached in terms of benefit of an intervention on the one hand and unnecessary harm on the other. It would seem that watchful waiting as long as it is not associated with significant morbidity and mortality would be preferable to early re-laparotomy and a higher negative re-exploration rate. The question is at what point the morbidity of waiting outweighs the morbidity of operating. Known risk factors, such as the level of anastomosis, chronic obstructive pulmonary disease, obesity, the use of steroids, poor nutritional state or the need for blood transfusion increase the chance of anastomotic leakage beforehand[2,4,6,9,10]. The finding of adynamic ileus, fever or leucocytosis in high-risk patients will further increase the pre-test chance and may facilitate the decision to reoperate in these patients. However, one should take into account the risk of false-positivity of these clinical parameters which may result in a false-negative reintervention. The complete diagnostic evaluation of the clinical parameters identified from the literature (including sensitivity, specificity and positive/negative predictive value) was beyond the scope of the present study. In the previously mentioned study by Alves *et al*[14], clinical parameters suggestive of anastomotic leakage were analyzed in 655 patients who underwent colorectal resection. They found a significantly higher number of patients with fever on day two, absence of bowel action on day four, diarrhea before day seven, collection of more than 400 mL of fluid through abdominal drains from day zero to three, renal failure on day three and leucocytosis after day seven in the group in which anastomotic leakage occurred compared with the uncomplicated group. No other studies on the incidence and timing of these signs and symptoms have been published to our knowledge. Ultimately, a prospective analysis should be performed of all known risk factors and clinical parameters in order to construct a decision model that can help the surgeon to make a weighed choice for the individual patient.

What can minimize the delay in diagnosis and treatment of anastomotic leakage besides simple clinical parameters? Radiological examination of the anastomosis can be misleading[19]. Negative contrast studies and/or CT scanning undoubtedly result in a longer delay before surgical reintervention. Currently, we prospectively collect data about the additional value of radiological imaging of the anastomosis. A few investigational studies have focused on biochemical analysis of effluents of abdominal drains in patients who underwent colorectal anastomosis. Positive correlations with anastomotic leakage were found for lysozyme activity level and endotoxins[20,21]. The value of these findings in daily clinical practice, however, is probably limited.

The finding that patients who ultimately died in the hospital did not have had a longer delay of relaparotomy is comparable with observations that were done by Alves *et al*[14]. In that study, a non-significantly higher mortality rate was seen in patients who were reoperated on or after day five compared to those reoperated before day five. The absence of a significant association between delay of relaparotomy for anastomotic leakage and mortality is probably just a reflection of the small number of patients in both studies. It is our opinion that delay of relaparotomy in a patient with peritonitis should have an impact on outcome and that a more aggressive approach probably reduces morbidity and mortality.

In conclusion, although positive clinical parameters associated with anastomotic leakage were observed relatively early in the postoperative period, the final decision to perform a relaparotomy took a median of 3.5 extra days. The surgical team must be vigilant in the clinical observation of patients in the immediate postoperative period, also on weekends and review carefully the interpretation of diagnostic imaging of the anastomosis. Especially patients at an increased risk of anastomotic leakage due to comorbidity, septic conditions, technical difficulties and level of anastomosis deserve a close clinical observation with appropriately timed surgical reintervention.

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RAPID COMMUNICATION

Association of ezrin expression in intestinal and diffuse gastric carcinoma with clinicopathological parameters and tumor type

Nebil Bal, Sedat Yildirim, Tarik Z Nursal, Filiz Bolat, Fazilet Kayaselçuk

Nebil Bal, Filiz Bolat, Fazilet Kayaselçuk, Department of Pathology, Başkent University Faculty of Medicine, Turkey
Sedat Yildirim, Tarik Z Nursal, Department of General Surgery, Başkent University Faculty of Medicine, Turkey
Correspondence to: Dr. Fazilet Kayaselcuk, Baskent University, Adana Research and Medical Center, Pathology Department, Yuregir, Adana 01250, Turkey. faziletks@yahoo.com
Telephone: +90-322-3272727-1157
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Abstract

AIM: To investigate the correlation between ezrin expression and types of gastric carcinoma and clinicopathological variables.

METHODS: We examined ezrin protein expression in 75 gastric carcinoma (53 intestinal types of adenocarcinoma, 22 diffuse types of carcinoma) tissues by immunohistochemistry. The results were compared with clinicopathological parameters such as tumor type, grade of tumor, clinical stage, presence of metastatic lymph node, and depth of invasion.

RESULTS: Ezrin immunostaining was positive in 43 cases (81.1%) of intestinal type and in 9 (40.9%) cases of diffuse type adenocarcinomas ($P < 0.001$). In gastric carcinomas, the expression of ezrin protein correlated with the status of *H pylori* and survival. There was no correlation between expression of ezrin with TNM stage and histological grade of gastric carcinomas ($P > 0.05$).

CONCLUSION: The low expression of ezrin implicates the loss of adhesion in diffuse carcinomas. Furthermore, overexpression of ezrin in carcinomas with *H pylori* infection may be a genuine specific pathway in which *H pylori* may cause/initiate gastric carcinoma.

Key words: Ezrin; Gastric carcinoma; *H pylori*; Metastasis

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INTRODUCTION

Ezrin, a member of the ezrin-radixin-moesin (ERM) family of species-conserved protein in the band 4.1 superfamily, is a membrane cytoskeleton linker and is involved in cellular functions, including epithelial cell morphogenesis and adhesion. The proteins ezrin, moesin, and radixin act as linkers between the plasma membrane and the actin cytoskeleton. The inactivation of ezrin causes a massive cell retraction and leads to the destruction of both cell-cell and cell-substrate adhesion, whereas the overexpression of ezrin in insect cells results in enhanced cell adhesion[1-3]. Pujuguet *et al* showed that ezrin plays a role in the transition from polarized epithelial cell form to a more spreading form by regulating the transport of E-cadherin to the plasma membrane[4]. This mechanism between ezrin and E-cadherin may be important in its emerging role in tumor progression.

ERM proteins have a cytoskeletal linking function to connect the actin filaments to cell adhesion molecules such as CD43, CD44, ICAM 1, ICAM 2, modulating cell morphology, motility and adhesion[5]. The binding of ezrin to cell surface adhesion molecules is involved in cell migration and metastasis, because high levels of CD44 are associated with invasive and potentially metastatic tumor cells[5]. Ezrin is expressed at high levels in small intestine, stomach, pancreas and kidney, at intermediate levels in spleen, thymus, lymph nodes, at low levels in heart, brain and testis. In muscle and liver, expression of ezrin is not detected[6].

Human gastric carcinomas have been classified by Lauren into two major groups, intestinal (well differantiated) and diffuse (poorly differentiated) types. Intestinal type adenocarcinoma has better prognosis than diffuse type[7]. In the diffuse carcinoma, tumor cells are scattered in a fibrous stroma with loss of tight intracellular adhesions between cancer cells[7]. Although histopathological diagnosis is valuable in clinical medicine, the Lauren classification has been widely used because it separates two biological entities that are different in epidemiology, pathogenesis, behavior and carcinogenesis[8].

With the advent of new molecular technologies, information about grade of malignancy, prognosis and differential diagnosis can now be obtained[8-10]. Although ezrin is detected in many malignant cell types, such as adenocarcinoma of pancreas, renal cell carcinoma, and osteosarcoma, to the best of our knowledge we could

not identify any information regarding ezrin and gastric carcinoma[5,6,11,12]. In this study, we aimed to document the relation of ezrin expression to the clinical outcome and the histological parameters in carcinomas of stomach.

MATERIALS AND METHODS

For immunohistochemical analysis, 53 intestinal type adenocarcinoma and 22 diffuse type carcinoma tissue samples were selected from pathological archives. All patients with gastric carcinoma were treated surgically, diagnosed histologically, and followed up in the Department of General Surgery. Surgical staging was determined in accordance with the TNM criteria, 10 (13.3%) cases were classified as stage 1, 9 (12%) as stage 2, 23 (30.7%) as stage 3, and 33 (44.0%) as stage 4. The histological grade of each gastric cancer sample was determined according to the accepted criteria, resulting in 4 cases of grade I (well differentiated), 25 grade Ⅱ (moderately differentiated) and 46 grade Ⅲ (poorly differentiated).

The samples were fixed in 10% neutrally buffered formalin and paraffin embedded. For immunohistochemistry, serial sections of 5 μm thickness were cut from the paraffin blocks. The sections were deparaffinized with xylene and rehydrated with ethanol. Non-enzymatic antigen retrieval was performed on each slide and washed with phosphate-buffered saline (PBS). Immunohistochemical staining was performed manually using the standard avidin-biotin peroxidase complex technique with DAKO (LSAB kit, DAKO, Denmark). The primary antibody for ezrin (mouse mAbIgG1, clone 3C12, Neomarkers) was applied to the slides.

Histological and immunohistochemical evaluation was performed by one pathologist. Each stained slide was assessed and given a score, in which the intensity of the staining (no staining = 0, weak staining = 1, medium staining = 2, and strong staining = 3) and the percent of stained cells (0% = 0, 1%-24% = 1, 25%-49% = 2, 50%-74% = 3 and greater than 75% = 4) were multiplied. With the applied system, the maximum score was 12 (over 75% of the cells showing strong staining) and the minimum score was 0 (negative staining).

For detection of *H pylori* (*H pylori*), all the selected slides were Giemsa stained. Level of *H pylori* was assessed as 0-3 (no: 0, mild: 1, moderate: 2, severe: 3) according to the Sydney system.

SPSS for Windows version 11.0 was used for statistical analyses. Normality was checked for continuous variables. Data were expressed as mean ± SD (standard deviation), median minimum-maximum, *n* (number of cases) and percent (%). The Mann Whitney-U, *t* test and Spearman's correlation tests were used where appropriate. Bonferroni's correction was applied ($P < 0.05/n$; where n = number of comparisons) when multiple comparisons were made. *P* value less than 0.05 or 0.02 was considered as significant in difference.

RESULTS

Ezrin immunreactivity and clinical parameters

Positive staining for ezrin was observed in the epithelium of the gastric glands and tumor cells and in the cytoplasm of the cells. In noncancerous, normal gastric mucosa, glandular epithelium showed positive staining for ezrin. Ezrin immunostaining was positive in 43 cases (81.1%) of intestinal type and in 9 (40.9%) cases of diffuse type adenocarcinomas ($P < 0.001$) (Figures 1-3). Furthermore, the mean weighted ezrin score in the intestinal type adenocarcinoma was higher than that of the diffuse type (4.98 ± 3.841 *vs* 2.05 ± 3.00 respectively, $P = 0.001$).

As the gastric carcinomas were assessed as a whole group, there was no correlation between ezrin expression and TNM stage and histological grade in gastric carcinomas ($P > 0.05$) (Table 1). The expression of ezrin was positively correlated with the presence of *H pylori* and

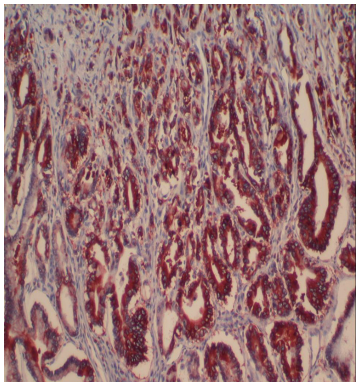

Figure 1 Strong immunoreactivity of ezrin in grade Ⅱ intestinal type gastric adenocarcinoma cells (IHC, x 200).

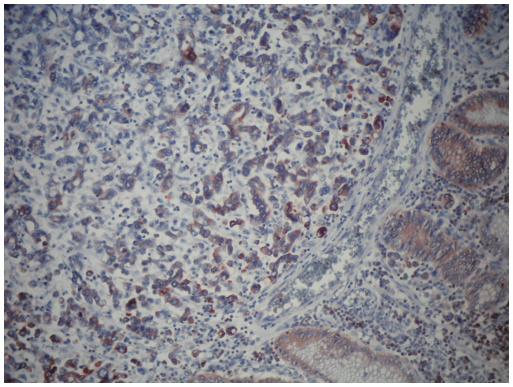

Figure 2 Cytoplasmic immunpositivity of ezrin in poorly differentiated gastric adenocarcinoma (IHC, x 200).

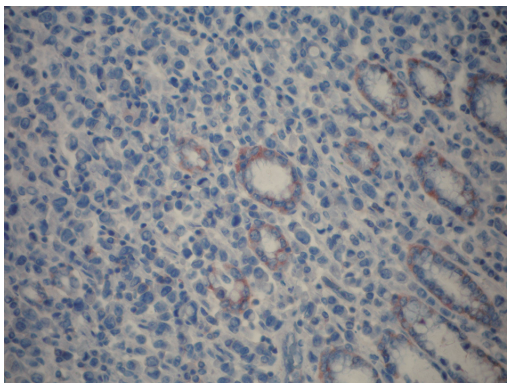

Figure 3 Negative staining in atypical cells for ezrin in diffuse type gastric carcinoma. Positive staining in normal gastric glands (IHC, x 200).

Table 1 Ezrin expression in intestinal and diffuse type gastric adenocarcinoma and its correlation with *Hp* infection, overall survival and histologic grade

	İntestinal type adenocarcinoma		Diffuse type adenocarcinoma		Gastric adenocarcinoma (All groups)		İntestinal type adenocarcinoma			Diffuse type adenocarcinoma
	Hp present	Hp absent	Hp present	Hp absent	Alive	Death	Grade 1	Grade 2	Grade 3	Grade 3
Ezrin negative	4	6	3	10	5	15	0	6	4	13
Ezrin positive	26	17	4	5	15	18	4	19	20	9
P	< 0.05				< 0.05		> 0.05			

Table 2 Ezrin expression in intestinal and diffuse type gastric adenocarcinoma and its correlation with lymph node metastasis and distant metastasis

	Intestinal type adenocarcinoma		Diffuse type adenocarcinoma		Intestinal type adenocarcinoma		Diffuse type adenocarcinoma	
	With lymph node metastasis	Without lymph node metastasis	With lymph node metastasis	Without lymph node metastasis	With distant metastasis	Without distant metastasis	With distant metastasis	Without distant metastasis
Ezrin negative	9	1	12	1	1	8	1	12
Ezrin positive	31	12	8	1	11	31	2	7
P	> 0.05				> 0.05			

overall survival ($P < 0.05$) (Table 1). There was a negative correlation between ezrin and lymph node metastasis (Table 2), lymphovascular space invasion, and perineural invasion in all gastric carcinomas, but was not significant statistically ($P > 0.05$), while no association with depth of invasion, localization of tumor, and diameter of tumor, and distant metastasis ($P > 0.05$), (Table 2).

In diffuse carcinoma ($n = 22$), ezrin expression was not associated with histological and clinical parameters while ezrin expression in intestinal type adenocarcinomas ($n = 53$) has a negative correlation ($P < 0.05$) with the size of tumor, but not with other parameters.

In this study, standard histological parameters such as grade, depth of tumor, metastatic lymph node status, lymphovascular space and perineural invasion, and diameter of tumor were significantly correlated with stage of tumor ($P = 0.001$, $P = 0.007$, $P < 0.001$, $P < 0.001$, $P < 0.001$, $P < 0.001$). The type of tumor showed no correlation with stage, depth of tumor, metastatic lymph node status, lymphovascular space and perineural invasion. The size of the tumor was significantly correlated with stage of tumor.

Sixty patients (80%) had lymph node metastasis and lymphovascular invasion and 15 patients (20%) had distant metastases such as liver and omentum. Fifty-three patients with gastric carcinoma were followed up for a mean (SD) duration of 12.37 mo. The median survival time was 18 mo in the group without ezrin expression, and 24 mo in the group with ezrin expression ($P > 0.05$). In diffuse and intestinal type carcinomas, the expression of ezrin was not correlated with the overall survival.

DISCUSSION

Despite identification of the new key regulatory molecules in metastasis and growth of tumor, metastasis is still an extremely complex and unclear process. To metastasize successfully into a clinically relevant mass, tumor cells must overcome a series of challenges. These include invasion into the surrounding tissue, extravasation into the lymphovascular space, arrive at a distant side, and intravasation into a new environment. In all of these stages, various regulatory molecules have to be expressed in a coordinated pattern. Ezrin is known to be a component of cell-surface structures that, together with ERM proteins, are involved in cell adhesion to the extracellular matrix, as well as in cell-cell interactions, receptor tyrosine-kinase signaling, signal transduction and interactions with the Akt-mediated cellular apoptotic mechanism. One of the functions of ezrin is to participate in the formation of cell-surface complexes, such as E-cadherin, integrin that mediates cell-cell and cell-extracellular matrix attachments[1-4].

Many molecular genetic studies in gastric carcinogenesis have shown that the development of intestinal and diffuse carcinoma follow two different pathways. For instance, microsatellite instability was found to be 64% in diffuse type, but only 17% in intestinal type carcinoma[9,10]. Loss of heterozygosity, mutation of APC gene and DCC gene are frequently observed in cancer of intestinal type , but seldom found in diffuse type. Similarly, the cadherin gene plays an important role in the carcinogenesis of diffuse type carcinoma which is characterized by invasion and high metastatic potential. In addition, the abnormal transcription of CD44 has found both types, although deletion of cadherin gene is 50% in intestinal type carcinoma[8].

Moilanen *et al* reported that healthy ovarian epithelium showed strong ezrin immunreactivity, but weak or negative expression of ezrin in ovarian carcinoma was associated with shorter survival, histological grade, and advanced age of the patient[12]. Controversially, in other studies, glial tumor, uveal malignant melanoma, and pancreatic carcinoma cells have shown high ezrin expression[5,6,11,13]. In human pancreatic adenocarcinoma cells, increased ezrin expression correlated with high metastatic potential. In this study, we observed strong ezrin immunoreactivity in normal, noncancerous gastric mucosa, but in the diffuse

carcinoma group, the immunoreactivity of ezrin decreased as compared with normal mucosa. Ezrin expression showed inverse correlation with presence of metastatic lymph nodes, and lymphovascular space invasion in gastric carcinomas. Similar to our findings, the ezrin expression in human colon cancer was reported to decrease compared to normal tissues[14]. These seemingly contradictory results indicate that the expression of ezrin in various tumor types may be associated with different cell functions of ezrin. The previous studies implicated that the inactivation of ezrin caused a massive cell retraction, suggesting a constant exchange between the soluble and membrane-skeleton-associated ezrin, which drives cell spreading[15]. The suppression of ezrin led to the destruction of both cell-cell and cell-substrate adhesion whereas the overexpression of ezrin in insect cells enhanced cell adhesion[16,17]. Reduced cell-cell adhesion may be responsible for increased invasiveness and metastasis in malignant tumors. Considering the marked loss of adhesion of histologically diffuse carcinomas when compared to the intestinal carcinomas, the parallel loss of ezrin immunoreactivity may be meaningful perhaps causal in this regard.

Lim *et al* demonstrated that *H pylori* infection increased the cell adhesion-related gene expression in gastric epithelial AGS cells and ezrin expression induced by *H pylori* infection[18]. The expression of ezrin and possibly by other ERM proteins contributes to enhancement of cell-cell or cell-extracellular matrix adhesion of gastric epithelial cells. Lim *et al* suggested that the differential expression of ezrin in *H Pylori* infection may play an important role in gastric carcinogenesis, including cell proliferation and cell adhesion[18]. In the present study, we also found a positive correlation between *H pylori* status and expression of ezrin.

Generally, intestinal type gastric carcinomas have a better prognosis than their diffuse type counterparts. Environmental factors are believed to play a greater role in tumorigenesis in intestinal type carcinomas. *H Pylori* with its propensity resulting in atrophic gastritis is hypothesized to play a role in tumorigenesis of the intestinal type gastric cancer. However, there is a lack of information on the specific pathways and processes on *H pylori*-induced carcinogenesis. In this study, we have shown that *H pylori* infected gastric carcinomas have a greater expression of ezrin in the cells. If one takes into account of the ezrin's role in numerous critical pathways of cellular adhesion and proliferation, this may be a genuine specific pathway in which *H pylori* may cause/initiate gastric carcinoma.

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RAPID COMMUNICATION

Significant increase in HBV, HCV, HIV and syphilis infections among blood donors in West Bengal, Eastern India 2004-2005: Exploratory screening reveals high frequency of occult HBV infection

Prasun Bhattacharya, Partha Kumar Chandra, Sibnarayan Datta, Arup Banerjee, Subhashish Chakraborty, Krishnan Rajendran, Subir Kumar Basu, Sujit Kumar Bhattacharya, Runu Chakravarty

Prasun Bhattacharya, Subhashish Chakraborty, Subir Kumar Basu, Institute of Blood Transfusion Medicine and Immunohematology, Kolkata, India
Partha Kumar Chandra, Sibnarayan Datta, Arup Banerjee, Runu Chakravarty, ICMR Virus Unit, Kolkata, India
Krishnan Rajendran, Sujit Kumar Bhattacharya, National Institute of Cholera and Enteric Diseases, Kolkata, India
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Correspondence to: Runu Chakravarty, Indian Council of Medical Research Virus Unit, GB 4, 1st Floor, ID & BG Hospital Campus, Kolkata-700010, India. runugc@yahoo.com.uk
Telephone: +33-23-537425/4 Fax: +33-23-537425
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Abstract

AIM: To evaluate the prevalence of markers of hepatitis B virus (HBV) and hepatitis C virus (HCV) and human immunodeficiency virus (HIV) among blood donors in Kolkata, Eastern India for two consecutive years and to conduct a pilot study to explore the presence of HBV DNA among hepatitis B surface antigen (HBsAg) negative but anti-HBc positive blood donors.

METHODS: Seroprevalence of HBsAg, anti-HCV and anti-HIV was studied among 113 051 and 106 695 voluntary blood donors screened in 2004 and 2005, respectively. Moreover, a pilot study on 1027 HBsAg negative donors was carried out for evaluating the presence of HBV DNA by PCR on HBsAg negative/anti-HBc positive donors.

RESULTS: A statistically significant increase in the prevalence of HBV (1448 *vs* 1768, $P < 0.001$), HIV (262 *vs* 374, $P < 0.001$), HCV (314 *vs* 372, $P = 0.003$) and syphilis (772 *vs* 853, $P = 0.001$) infections was noted among blood donors of Kolkata West Bengal in 2005 as compared to 2004. Moreover, the exploratory study on 1027 HBsAg negative donors revealed that 188 (18.3%) of them were anti-HBc positive out of which 21% were positive for HBV DNA.

CONCLUSION: The findings of this study underscore the significantly increasing endemicity of hepatitis viruses, syphilis and HIV among the voluntary blood donors of our community. The pilot study indicates a high rate of prevalence of HBV DNA among HBsAg negative/anti-HBc positive donors and thus emphasizes the need for a more sensitive and stringent screening algorithm for blood donations.

Key words: Hepatitis B virus; Human immunodeficiency virus; Hepatitis C virus; Blood donation; Occult HBV infection

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INTRODUCTION

Hepatitis B is one of most common infectious diseases of the world and has infected 2 billion people worldwide; including an estimated 400 million chronically infected cases[1]. Individuals with chronic infection have a high risk of developing liver cirrhosis and hepatocellular carcinoma. Hepatitis C virus (HCV) infection is another common chronic blood borne infection with an estimated 3.9 million persons infected by the virus and a high rate of development of liver cirrhosis. Infection by hepatitis B virus (HBV) and HCV causes serious mortality, morbidity and financial burden and are thus a major global health problem[2]. In addition, prevalence of human immunodeficiency virus (HIV), is increasing in the world. India, with an estimated 5.7 million cases of HIV

infection, is the second highest pool of these patients in the world.

Evaluation of data on the prevalence of these transfusion transmitted infections (TTIs), namely HBV, HCV, HIV and syphilis, among blood and plasma donors permits an assessment of the occurrence of infections in the blood donor population and consequently the safety of the collected donations. It also gives an idea of the epidemiology of these diseases in the community.

Among these infections HBV is more infective than the other viruses[1]. Detection of hepatitis B surface antigen (HBsAg) in blood is diagnostic for infection with HBV and in the blood banks screening for HBsAg is carried out routinely to detect HBV infection. Occult HBV infection is defined as the presence of HBV DNA in blood or liver tissues in patients negative for HBsAg but who may or may not be positive for HBV antibodies[1].

Thus some HBsAg negative individuals, positive for antibodies against HBV core antigen (anti-HBc) and/or HBsAg (anti-HBs) continue to be positive for HBV DNA. Due to limitations in current blood screening practices in developing countries, donation by such individuals is a potential source of HBV transmission to the recipients[3-7]. Such occult hepatitis B infection may be detected in (1) Individuals with resolving HBV infection positive for both antiHBc and anti-HBs (2) "anti-HBc-only" carriers in a window period of infection who are seronegative for HBsAg (3) carriers in whom HBsAg is not detectable due to presence of escape mutants[5]. High frequencies of HBV DNA positivity (10% to 40%) have been described among anti-HBc only sera[3]. Routine anti-HBc screening of individual blood donations and nucleic acid amplification testing (NAT) by pooling of sera is done in some countries to exclude these donations[8-10]. In India, detection of HBV infection among blood donors is carried out by HBsAg screening while detection of anti-HBc is rarely done[11].

In this study we aimed to assess the prevalence and trends of the transfusion-transmitted infections (TTIs) in two consecutive years, 2004 and 2005, among blood donors of West Bengal. Moreover, in a pilot study, we also explored the prevalence of occult HBV infection among 1027 randomly selected HBsAg negative donor samples, without sera pooling.

MATERIALS AND METHODS

The Institute of Blood Transfusion Medicine and Immunohematology (IBTMI), is located in Kolkata (Calcutta). It is under the Department of Health and Family Welfare, Ministry of Health, West Bengal. It is the leading organization that coordinates blood transfusion services throughout the state of West Bengal. The state has a population of 80 176 197 according to the 2001 census. The majority of contributors to this blood bank are voluntary donors. The voluntary donations primarily were obtained from blood donation camps, mostly organized by clubs, colleges, political parties, religious organizations *etc*.

The screening of blood donations for HBsAg, anti-HIV, syphilis and anti-HCV is mandatory in IBTMI. Blood donations from individuals who are found to be positive for any of the above infections previously were deferred at IBTMI. Moreover, the donors were pre-counseled regarding their health status and also required to fill out a donor screening registration form as part of a routine blood donation screening procedure. During the 2-year period, blood samples from 1027 HBsAg negative blood donors (564 in the year 2004 and 463 in the year 2005) were chosen at random, at various periods of time for the pilot study. Informed consent was obtained from all the donors. The study abided by the rules of the Ethical Committee of IBTMI.

All the donor samples were examined in IBTMI, using commercial ELISA, HBsAg and anti-HCV (Span diagnostics, India), anti-HIV (General Biologicals, Taiwan) and RPR (Rapid Plasma Reagin) for Syphilis (Span diagnostics, India). Anti-HBc and anti-HBs (Organon Teknika, Boxtel, The Netherlands) was detected in a subset of 1027 donors for exploratory screening at ICMR Virus Unit. All anti-HBc positive samples were retested in duplicate for HBsAg as well as for anti-HBc. Only repeat HBsAg negative/anti-HBc positive samples were considered to be positive for anti-HBc. HBV DNA was detected by in-house nested PCR, amplifying two different regions of the HBV genome as described earlier[12].

Table 1 Prevalence of TTIs among the blood donors in the years 2004 and 2005

	Total No of samples studied		No of samples reactive (%)		Statistical significance	
	2004	2005	2004	2005	RR (95% CI)	*P* values
HBsAg	113 051	106 695	1448 (1.28)	1768 (1.66)	1.29 (1.21-1.39)	< 0.001
Anti-HCV	113 051	106 695	314 (0.28)	372 (0.35)	1.26 (1.08-1.46)	0.003
Anti-HIV	113 051	106 695	262 (0.23)	374 (0.35)	1.51 (1.29-1.77)	< 0.001
Rapid plasma reagin (Syphilis)	113 051	106 695	772 (0.68)	853 (0.80)	1.17 (1.06-1.29)	0.001

RESULTS

A total of 219 746 blood units were collected at IBTMI, from January 2004 to December 2005, 94.6% of which were collected from voluntary donors. Analysis of the prevalence of TTIs among them revealed a statistically significant increase in the occurrence of all the blood borne infections in the year 2005 as compared to 2004 (Table 1); frequency of co-infection of these viruses was negligible. Furthermore, relative risk (RR) of HIV infection was 1.51 (95% confidence interval, $P < 0.001$). In 2005 the prevalence rates (per 100 000 donations) were 350 for HIV, 350 for HCV, 1660 for HBV and 800 for syphilis.

Out of the 1027 HBsAg negative blood samples screened, 18.3% were found to be anti-HBc positive (Table 2). Notably, 21.3% of the anti-HBc positive samples were HBV DNA positive by PCR. Among the 'anti-HBc only' subgroup, a marginal increase in the proportion of HBV DNA positive samples was noted in 2005 as

Table 2 Detection of HBV DNA in anti-HBc positive blood donors

Samples collected in the year	Total number of HBsAg negative samples collected	No of Anti HBc positive samples (irrespective of anti HBs status) (%)		No of 'Anti HBc only' samples (anti HBs negative) (%)	
		Total	Presence of HBV DNA	Total	Presence of HBV DNA
2004	564	95 (16.8)	23 (24.2)	36 (6.4)	11 (30.5)
2005	463	93 (20.1)	17 (18.3)	25 (5.4)	8 (32.0)
Total	1027	188 (18.3)	40 (21.3)	61 (5.9)	19 (31.1)

compared to 2004 (Table 2). None of the HBV DNA positive samples were co-infected with HCV or HIV.

DISCUSSION

Our study was aimed at analyzing two blood transfusion related issues-one was to assess the trends in TTIs in two consecutive years and the other was to evaluate the prevalence of occult HBV infection among HBsAg negative donors in a pilot study.

We examined the occurrence of HBV, HCV, HIV and syphilis infections among blood donors in Kolkata, West Bengal by serological methods and compared the results to assess the trends in two consecutive years, 2004 and 2005. 94.6% of the blood donors have donated voluntarily in the blood donation camps organized by different clubs, religious organizations, offices, political parties, *etc*. Therefore, the prevalence of viral carrier rates in blood donors is similar to that of the general population. Thus, the data highlighting the increase of TTIs among the blood donors is of concern. It indicates that the occurrence of these infections among the voluntary donors should be monitored carefully and the possible causes evaluated. The Report of National AIDS Control Organization[13], shows a considerable increase in HIV prevalence in the year 2005 among antenatal clinics (0.5% to 0.84%) and sexually transmitted diseases clinics (0.88% to 2.16%) from the state of West Bengal, whereas adult HIV prevalence in the rest of the country was comparable to the previous years. Simultaneous increase of HBV, syphilis and HIV infections indicate that sexual transmission might be a possible route.

It is generally accepted that the diagnosis of infection by HBV is based on the presence of the HBsAg in the bloodstream[14]. However, screening of blood bank donors for HBsAg does not totally eliminate the risk of HBV infection through blood transfusion[15,16], since the absence of this marker in the serum does not exclude the presence of HBV DNA[17-20]. It is possible that, donors with occult HBV infection, who lacked detectable HBsAg but whose exposure to HBV infection was indicated by a positive anti-HBc and HBV DNA, are a potential source of HBV infection[21-23]. Our pilot study revealed that 188 of 1027 (18.3%) HBsAg negative blood donors were anti-HBc positive and thus were exposed to HBV infection. Occult HBV infection was observed in approximately 21% of those anti-HBc positive donors subjected to exploratory screening (Table 2). Thus these donors have the potential to transmit HBV contaminated blood through the public blood supply. Recent reports actually documented transmission of occult HBV by transfusion[24]. Moreover, in the 'anti-HBc only' group 31% were positive for HBV DNA (Table 2). The infectivity of anti-HBc positive/HBV DNA positive blood components is reported to be low[25]. In contrast, 'anti-HBc only' blood products, which are HBV DNA positive, are more prone to transmit HBV infection[7]. Presence of occult HBV infection has also been reported in blood donors from other Asian countries; HBV DNA was detected among 16 of 131(12.2%) anti-HBc positive donors in Iran, 7 of 250 (2.8%) in Lebanon and 5 of 167 (2.9%) in Pakistan[26-28]. A previous study from Delhi, Northern India showed prevalence of HBV DNA to be 25% among the 'anti-HBc only' donors; another study from Chandigarh in Northwestern India showed 0% prevalence while the present study has revealed 31% prevalence from West Bengal, Eastern India[29,30].

Our study raises serious concerns regarding the safety of the blood supply in our community, even after donor screening for HBsAg. In India transfusion associated HBV is estimated to be approximately 50% or more in multiply transfused patients and approximately 1.5% in post surgical recipients[31]. Thus the absence of HBsAg in the blood of apparently healthy individuals may not be sufficient to ensure lack of circulating HBV. Blood containing anti-HBc with or without detectable presence of HBsAg might be infectious, therefore routine blood donor screening for anti-HBc has been implemented in some countries resulting in a decrease in the risk of post-transfusion HBV infection[2]. The trends in the two years in IBTMI, suggest that routine anti-HBc screening of blood donations could possibly prevent some transfusion-transmitted HBV infections in this population. However, the usefulness of screening for anti-HBc as an additional screening test to improve the safety of the blood supply in India deserves further analysis. A national study, including a statistically significant number of blood donors from different blood donation centers across the country, should determine whether screening for anti-HBc in addition to HBsAg detection and introduction of PCR based screenings like NAT should also be considered for the Indian blood donors.

Our study underscores the increasing endemicity of TTIs in our community and the need for a sensitive screening algorithm of blood donations to improve blood safety.

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COMMENTS

Background

Transfusion of blood and blood product is a life saving measurement and benefits numerous patients worldwide. At the same time blood transfusion is an important mode of transmission of infection to the recipients.

Research frontiers

For evaluation of safety of blood transfusion in West Bengal, Eastern India, both

the markers of transfusion transmitted infections (TTIs) in human immunodeficiency virus, hepatitis B virus, hepatitis C virus and syphilis virus infection among the donors and the prevalence of occult hepatitis B virus infection by the presence of HBV DNA in absence of HBsAg were studied.

Innovations and breakthrough

There is an increasing prevalence of TTIs in Kolkata, West Bengal, India in the two years. An exploratory study highlights a high rate of occult HBV infection among the blood donors.

Applications

High prevalence of occult infection indicates a need to reconsider the current policy of blood donor screening.

Peer review

This is an important and timely paper documenting the prevalence of viral hepatitis and HIV in the West Bengali blood donor population, written by an authoritative Indian group from the major city, Kolkata. It deserves publication.

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wjg@wjgnet.com

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RAPID COMMUNICATION

Comparison of early pre-cutting *vs* standard technique for biliary cannulation in endoscopic retrograde cholangiopancreatography: A personal experience

Kannikar Laohavichitra, Thawatchai Akaraviputh, Asada Methasate, Somchai Leelakusolvong, Udom Kachintorn

Kannikar Laohavichitra, Thawatchai Akaraviputh, Asada Methasate, Department of Surgery, Siriraj GI Endoscopy center, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand
Somchai Leelakusolvong, Udom Kachintorn, Siriraj GI Endoscopy center, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand
Correspondence to: Thawatchai Akaraviputh, MD, Department of Surgery, Siriraj GI Endoscopy center, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand. sitak@mahidol.ac.th
Telephone: +66-2-4198005 Fax: +66-2-4121370
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Abstract

AIM: To compare the results and complications of early pre-cutting technique with standard technique.

METHODS: From January 2003 to December 2004, a total of 416 consecutive therapeutic biliary ERCP procedures were performed by one endoscopist (T.A.). Data were retrospectively collected according to procedure indication and results. Of these, 293 procedures (70.4%) were done with standard technique (group A) and 123 procedures (29.6%) with early pre-cutting technique in case of difficult cannulation (group B). The results and complications of ERCP were compared.

RESULTS: Success rate of first attempt cannulation was 98.0% in group A and 87.8% in group B. The overall incidence of post-ERCP pancreatitis, hemorrhage, perforation and cholangitis was 0%, 0.2%, 0.5% and 0.5%, respectively. Morbidity rate was not significantly different. No procedure-related mortality was occurred.

CONCLUSION: For an experienced hand, the early pre-cutting technique for biliary cannulation is safe and effective as standard technique.

Key words: Pre-cutting; Endoscopic retrograde cholan giopancreatography; Biliary cannulation; Complication; Pancreatitis

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INTRODUCTION

Pre-cutting technique is useful in allowing biliary access when standard cannulation techniques with double lumens sphincterotome failed[1-4]. It is known to significantly increase not only the rate of selective biliary cannulation but also the complication rate. This may be because of the multiple attempts to achieve cannulation by using sphincterotome catheter before pre-cutting and causing excess edema and papillary trauma. One earlier study has described conducting a needle knife cut to achieve biliary cannulation in difficult cases, a so called early pre-cutting technique, is more safe and effective compared with performing the pre-cut late in the procedure[5]. However, the data of this technique is still limited. The aim of this retrospective study was to compare the results and complications of early pre-cutting with the standard biliary cannulation technique for ERCP in the hands of an experienced endoscopist.

MATERIALS AND METHODS

At Siriraj GI Endoscopy Center, Faculty of Medicine, Siriraj hospital, Mahidol University, Bangkok, Thailand, 416 consecutive therapeutic Endoscopic retrograde cholangiopancreatography (ERCP) procedures from January 2003 to December 2004 were included in the study. These were performed by one experienced endoscopist (> 1000 career ERCPs, with an ongoing workload of > 200 ERCPs each annually: T.A.). They were classified into two groups (A and B) by the technique of biliary cannulation.

All cases were commenced with a standard double-lumen sphincterotome (Ultratome XL; Boston Scientific, Natrick, USA) preloaded with contrast. If required, a guidewire (0.035" Jackwire; Boston Scientific, Miami, USA) was used to aid biliary cannulation (Standard cannulation technique: group A). If biliary access failed after 10 min or if more than 3 pancreatic injections with contrast were

Table 1 Characteristics of patients in 416 ERCP procedures

	All (*n* = 416)	Group A (*n* = 293)	Group B (*n* = 123)
Age (yr) (mean)	60. 6	61.2	59.5
Gender: Male	214	153	61
Female	202	140	62
Procedure: Elective	399	278	121
Emergency	17	15	2
Anesthesia: TIVA	410	287	123
GA	3	3	0
Indications (%)			
Cholelithiasis	203 (48.8)	140 (47. 8)	62 (50.4)
Biliary stricture			
Malignant	150 (36.1)	99 (33.8)	52 (42.3)
Benign	22 (5.3)	21 (7.2)	3 (2.4)
GS pancreatitis	4 (1.0)	4 (1.4)	1 (0.8)
Bile leakage	3 (0.7)	3 (1.0)	2 (1.6)
SOD	2 (0.5)	2 (0.7)	0
Miscellaneous	32 (7.7)	24 (8.2)	3 (2.4)
Factors effect to cannulation			
Juxtaduodenal diverticulum			
Previous operation	66	46 (15.7)	20 (16.3)
HPB surgery	73	47 (16.0)	26 (21.1)
Billroth Ⅱ gastrectomy	4	3 (1.0)	1 (0.8)

TIVA: Transintravenous anesthesia; GA: General anesthesia; SOD: Sphincter of Oddi dysfunction.

made (whichever occurred first), the early pre-cutting technique with needle knife catheter (MicroKnife XL, Boston Scientific, Natrick, USA) (group B) was used.

In group A, diathermy was applied with the Endocut mode in the ERBE system (120 W cut; 15 W coagulation) (ERBE USA, Atlanta, GA), which adjusts the amount of cutting and coagulating current automatically, depending on the tissue resistance. Group B was performed with pure cutting mode in the same system.

Sedation for the procedures consisted of a combination of propofol or fentanyl and midazolam with buscopan as needed for duodenal relaxation. In left lateral position, all patients underwent continuous cardiopulmonary monitoring throughout the procedure by an anesthesiologist. If the patient conditions were not appropriated for sedation we used the general anesthesia for the procedures. All procedures were done using an Olympus video duodenoscope (TJF160R, Olympus Corporation, Tokyo, Japan).

After completion of the ERCP, admission into the inpatient hospital service was arranged to rule out post-ERCP complications. Follow-up was determined by a retrospective review of the patients' computerized medical records. Complications were defined using criteria as Cotton *et al*[6] described. The statistical software package SPSS for Windows Version 11 (SPSS Inc, Chicago, IL) was used to analyze the data. A significance level of 5% was used throughout.

RESULTS

469 ERCP procedures were performed between January 2003 and December 2004. 53 diagnostic ERCP procedures without endoscopic sphincterotomy or biliary access were excluded. Of this 416 procedures (214 men, 202 women; mean age 60.56 years, range 14-97 years) included in the study, 293 procedures were classified in group A and 123 procedures were in group B.

Table 2 Overall complications of therapeutic ERCP *n* (%)

Complications	Total	Group A	Group B
Cholangitis	2 (0.5)	1 (0.3)	1 (0.8)
Perforation	2 (0.5)	1 (0.3)	1 (0.8)
Bleeding	1 (0.2)	-	1 (0.8)
Pancreatitis	-	-	-
Hypoxia	1 (0.2)	1 (0.3)	-
Total	6 (1.4)	3 (1.0)	3 (2.4)

The characteristic of the group A and group B populations were compared. There were no statistically significant difference between the two groups in age, gender, setting of the procedure, and choice of anesthesia. Indication for therapeutic ERCP and the factors that were claimed as affecting the difficulty of biliary cannulation were compared which there were no statistically difference also (Table 1).

The results of first attempt cannulation

The overall success rate of biliary cannulation was 95%. The success rate of the group A was 98% and group B was 87.8%. The difference result of success rate of first attempt cannulation were statistically compared between the two groups by used Chi-Square Tests found that the lower success rate of group B was statistically significant.

Complications

Of the 416 procedures, overall complications occurred in 6 procedures (1.4%) (Table 2). The overall incidence of hemorrhage, perforation and cholangitis was 0.2%, 0.5% and 0.5%, respectively. None of the study patients developed post-ERCP pancreatitis. One anesthetic complication (hypoxia) was occurred (0.2%). No procedure-related mortality was occurred. The complications of two groups were compared by using Chi-Square Test which there was no statistically significant difference.

DISCUSSION

At early period, the use of precut papillotomy as alternative method for achieve deep cannulation of the bile duct was not recommended for inexperienced endoscopists because it was claimed to increase post-ERCP complications. The procedure should be reserved for used by experts in high risk patients with strong indication for sphincterotomy when other standard techniques have been exhausted. The device that is commonly used to perform precut is the needle knife, first described in 1977 by Osnes and Kahrs[7]. Since then, several authors have reported the successful application of this technique, especially for a suprapapillarily prominent CBD[8-11]. There were many studies of precut sphincterotomy, even retrospective and prospective review supporting of precut sphincterotomy was the procedure that can increase the success rate of biliary cannulation[12-19]. Each study had difference designs, number of cases, number of endoscopist, indications

of therapeutic ERCP which can results the difference outcome. In this study, all of the ERCP procedures were performed by single experience endoscopist (TA) and the success rate and complications of early pre-cutting technique were compared to standard technique.

In this study, early-precutting with the needle knife was needed in the care of 123 of 416 (29.6%) patients among whom standard technique of achieving CBD access had failed. We made subgroup analysis in success rate of cannulation between subgroup A (precutting technique excluded patients with the prior attempted bile duct cannulation: 165 patients) and group B. There is no statistical different between these two groups. Rollhauser *et al*[15] published their experience with 68 needle knife papillotomy procedures. They found an improvement in successful cannulation from 68% to 98%, suggesting that the success rate improved with endoscopist's experience. In our study, the success rate of 1st attempted CBD cannulation after precutting (group B) was 87.8%. This success rate is comparable to that of other studies, which ranged from 66%-99%[20-22].

Complications can be attributed to several independent patient- and technique-related risk factors, including the endoscopist's skill[15]. The incidence of clinical pancreatitis following precutting was reported 2.1%-3.8%[20,23,24]. It has often been postulated that relatively high postprecut pancreatitis rates are related to the prolonged period of cannulation attempts that occurs before precut is finally performed[25]. These repeated cannulation attempts also mean that precut is often performed under difficult conditions, with a papilla that is already edematous and distorted. The relatively low rates of successful biliary cannulation immediately after precut in several reports may reflect this problem. In this study, there was no post-ERCP complications. This may result from our policy that precut sphincterotomy is routinely performed after 3 PD injection/cannulation and the high success rate of immediate CBD cannulation after precutting.

Bleeding as a result of early-precutting with a needle knife occurred 0.8% of our patients similar to those found in several investigators, such as O'Connor *et al*[26] (1.2%), and Huibregtse *et al* (1.5%)[1]. In the present study, the rate of perforation and cholangitis in the group treated using the standard approach was 0.3% and not significantly higher than that of the group treated primarily with precut (0.8%). Because of very low event of overall complication (1.4%) and no statistically significant differences between the two groups, it is unnecessary to find the predictor or confounding factors of the complication.

In summary, the early pre-cutting technique for biliary cannulation when cannulation of the CBD when performed by standard methods is not possible, is safe and effective by the experienced endoscopist.

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RAPID COMMUNICATION

Is prophylactic placement of drains necessary after subtotal gastrectomy?

Manoj Kumar, Seung Bong Yang, Vijay Kumar Jaiswal, Jay N Shah, Manish Shreshtha, Rajesh Gongal

Manoj Kumar, Seung Bong Yang, Vijay Kumar Jaiswal, Jay N Shah, Manish Shreshtha, Rajesh Gongal, Department of Surgery, Patan Hospital, Kathmandu, Nepal
Correspondence to: Dr. Seung Bong Yang, Department of Surgery, Patan Hospital, PO Box 252, Kathmandu, Nepal. yang971@hotmail.com
Telephone: +977-1-5523855
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Abstract

AIM: To determine the evidence-based values of prophylactic drainage in gastric cancer surgery.

METHODS: One hundred and eight patients, who underwent subtotal gastrectomy with D1 or D2 lymph node dissection for gastric cancer between January 2001 and December 2005, were divided into drain group or no-drain group. Surgical outcome and post-operative complications within four weeks were compared between the two groups.

RESULTS: No significant differences were observed between the drain group and no-drain group in terms of operating time (171 ± 42 min *vs* 156 ± 39 min), number of post-operative days until passage of flatus (3.7 ± 0.5 d *vs* 3.5 ± 1.0 d), number of post-operative days until initiation of soft diet (4.9 ± 0.7 d *vs* 4.8 ± 0.8 d), length of post-operative hospital stay (9.3 ± 2.2 d *vs* 8.4 ± 2.4 d), mortality rate (5.4% *vs* 3.8%), and overall post-operative complication rate (21.4% *vs* 19.2%).

CONCLUSION: Prophylactic drainage placement is not necessary after subtotal gastrectomy for gastric cancer since it does not offer additional benefits for the patients.

Key words: Prophylactic drainage; Subtotal gastrectomy; Gastric cancer; Post-operative complications; Operative outcome

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INTRODUCTION

Prophylactic drainage of the peritoneal cavity after gastrointestinal (GI) surgery has been widely practiced since the mid-1800 s, with the dictum of Lawson Tait, the 19th-century British surgeon, "When in doubt, drain," well known to all surgical trainees. During the last two centuries, prophylactic drains have been employed to remove intra-peritoneal collections, such as ascites, blood, bile, chyle, and pancreatic or intestinal juice. In addition, prophylactic drains had their signal function to detect early complications, such as postoperative hemorrhage and anastomotic leakage[1]. Thus, prophylactic drainage gained wide acceptance as a useful method to prevent complications after GI surgery. However, surgically placed drains are not without risk: they have been associated with increased rates of intra-abdominal and wound infection, increased abdominal pain, decreased pulmonary function, and prolonged hospital stay, organ damage, and some other discomforts to the patients[2-9]. Advances in surgical techniques, anesthesia, and peri-operative patient care have consistently decreased postoperative complication rates after gastric cancer surgery, especially in better GI centers[10,11].

Sims was the first surgeon who used prophylactic drains after gynecologic operations in the last quarter of the 19th century[12]. Since that time, surgeons have routinely used prophylactic drainage of the peritoneal cavity after abdominal surgery. Theodor Billroth was convinced that prophylactic drainage of the peritoneal cavity saved many lives after GI surgery[13]. However, some other contemporaries believed that drainage of the peritoneal cavity is impossible and, therefore, prophylactic drainage is useless[14,15].

Unfortunately, the principle of drainage is not based on any scientific data, and, in general, the prophylactic value of drains in abdominal surgery remains controversial. During the last three decades, surgeons have made efforts to investigate the value of prophylactic drainage after abdominal surgery in controlled randomized clinical trials (RCTs)[16]. Despite evidence-based data questioning prophylactic drainage in many instances, most surgeons around the world continued to use drainage on a routine basis untill now. To the best of our knowledge, there is little information regarding the scientific evidences of prophylactic drainage placement in gastric cancer surgery. In this study, we, therefore, aimed at assessing the value of prophylactic drainage placement in gastric cancer surgery.

MATERIALS AND METHODS

Patients

One hundred and eight patients (69 males and 39 females; mean age: 55.62 ± 15.67 years), who underwent subtotal gastrectomy, regardless whether it was radical or palliative, or D1 or D2 lymph node dissection, at Surgical Department, Patan Hospital, Kathmandu (Tertiary Care Hospital) between January 2001 and December 2005 were enrolled in this study. In the drain group (n = 56), a tube drain was routinely placed in the right upper quadrant, while not in the no-drain group (n = 52) (Table 1). During the post-operative and four-week follow-up period, surgical outcomes and post-operative complications were compared between the drain group and no-drain group (Table 2).

Operative techniques

All surgical procedures were performed by consultant surgeons in Surgical Department, Patan Hospital, following the standard guidelines of gastric cancer surgery[17]. The operative protocols generally consisted of radical or palliative subtotal gastrectomy (resection of 70%-85% of the stomach) with D1 or D2 lymph node dissection, and a distal tumor-free margin of greater than 2 cm and a proximal tumor-free margin of greater than 6 cm. In the drain group, a single tube drain (28-F) was placed in the right upper quadrant *via* the foramen of Winslow below the hepatoduodenal ligament.

Post-operative care

Post-operative pain control was achieved by intramuscular administration of diclofenac (75 mg, bid), and/or morphine (5-7.5 mg), phenergan (25 mg) as necessary, followed by oral analgesics when the patients tolerated liquid. Drains were generally removed when the output was ascitic or serosanguinous and less than 50 mL in 24 h. Patients were allowed to sip water generally from the second or third post-operative day. Liquid diet was started after confirmation of bowel sound with passage of flatus and advanced to soft diet when the patients tolerated the liquid diet for at least 12 h. Patients were discharged from the hospital after tolerating a soft diet for at least 2 d.

Assessment of surgical outcome

Surgical outcomes were evaluated in terms of operative time, number of post-operative days until passage of flatus, number of post-operative days until initiation of soft diet, length of post-operative hospital stay, post-operative complications and mortality. Post-operative complications were defined as any adverse event that required surgical or medical intervention within four weeks of surgery, and mainly included wound infection, wound dehiscence, pulmonary infection (pneumonia), drain-related complications, fever, abdominal distention and frequent vomiting. Surgical outcomes were compared between the two groups.

Statistical analysis

Data were expressed as mean ± SD. All statistical analyses were performed using the SPSS version 13 software (SPSS Inc., Chicago, IL). Comparisons between the two groups were performed using Student's t test for continuous variables and the Chi-square test for discrete variables. A P value less than 0.05 was considered statistically significant.

Table 1 Demographics and clinical characteristics of the patients *n* (%)

Characteristics	Drain group (n = 56)	No-drain group (n = 52)	P value
Age (yr)	54.34 ± 11.23	57.54 ± 13.45	0.859
Sex (male:female)	36:20	33:19	0.864
Tumor stage			0.468
ⅠA	4 (7.14)	3 (5.76)	
ⅠB	6 (10.71)	4 (9.61)	
Ⅱ	11 (19.64)	13 (25.00)	
ⅢA	11 (19.64)	10 (19.23)	
ⅢB	9 (16.07)	10 (19.23)	
Ⅳ	15 (26.78)	12 (23.07)	
Operation type			0.284
Radical	38 (67.85)	37 (71.15)	
Palliative	18 (32.14)	15 (28.84)	
LN dissection			0.352
D1	20 (35.71)	17 (32.69)	
D2	36 (64.28)	35 (67.31)	

Table 2 Comparison of surgical outcomes between the two groups

Surgical outcomes	Drain group (n = 56)	No-drain group (n = 52)	P value
Operating time (min)	171.4 ± 42	155.6 ± 39	0.096
Passage of flatus (POD)	3.67 ± 0.57	3.52 ± 0.95	0.495
Initiation of soft diet (POD)	4.87 ± 0.72	4.82 ± 0.84	0.314
Hospital stay (POD)	9.32 ± 2.21	8.39 ± 2.35	0.402
Complications, n (%)			0.324
None	44 (78.57)	42 (80.76)	
Wound infection	4 (7.14)	4 (7.69)	
Pulmonary infection	7 (12.50)	6 (11.53)	
Wound dehiscence	2 (3.57)	2 (3.84)	
Fever	7 (12.50)	6 (11.53)	
Anastomotic leak	1 (1.78)	1 (1.92)	
Others[1]	8 (10.71)	6 (7.69)	
Drain-related complications	4 (7.14)	0	
Hospital mortality, n (%)	3 (5.35)	2 (3.84)	0.284

POD: Post-operative days. [1]Abdominal distention, nausea, vomiting.

RESULTS

Characteristics of patients

A total of 108 patients (69 males and 39 females; mean age: 55.62 ± 15.67 years, range: 30-80 years) were included in this study. There was no significant difference in the mean age of patients between the two groups (P = 0.859) (Table 1). In addition, no obvious differences were observed between the both groups in terms of tumor aggressiveness (tumor stage), surgical procedures and extent of lymph node dissection (radical or palliative, D1 or D2 lymph node dissection) (Table 1). Stage Ⅲ was found to be the most frequent gastric tumor (37.0%,

40/108), followed by stage Ⅳ (25.0%, 27/108), stage Ⅱ (22.2%, 24/108) and stage I (15.7%, 17/108), indicating that a majority of the patients had advanced cancer at the time of operation (Table 1).

Surgical outcomes

Drains were removed at an average of 5.4 (range: 3-9) d after surgery. The average amount of output from the drains was 325 mL (range: 100-700 mL; 60 mL/d) which was mostly ascitic or serosanguinous fluid (Table 2). Although the no-drain group had less operating time (156 ± 39 min *vs* 171 ± 41 min, $P = 0.096$) and post-operative hospital stay (8.4 ± 2.4 d *vs* 9.3 ± 2.2 d, $P = 0.402$) compared to the drain group, the data did not reach statistical significance. In addition, no significant differences were observed between the two groups in terms of number of post-operative days until passage of flatus, number of post-operative days until initiation of soft diet, wound infection rate, wound dehiscence rate, pulmonary infection rate, fever, abdominal distension, ascites, and vomiting. Similarly, there was no significant difference in post-operative in-hospital mortality rate between the two groups (5.4% *vs* 3.8%, $P = 0.284$). However, there were four drain-related complications (i.e., omentum coming out through the drain site after removal of the drain, continuous leakage from the drain site for more than 3 d, drain site infection). There was one anastomotic leakage in each group, which was diagnosed clinically and with the aid of ultrasound.

DISCUSSION

Our data clearly demonstrate that prophylactic drain placement is not beneficial or may even add to morbidity or cost of procedure or time and resource consumption for drain care after subtotal gastrectomy with D1 or D2 dissection. Various studies on the use of prophylactic drains in other abdominal surgery, such as hepatic resection, pancreatoduodenectomy, colorectal surgery have not advocated for prophylactic use of drains except some special conditions, because post-operative complications, such as subcutaneous abscess at the drain site, subcutaneous drain tract tumor recurrence, intra-abdominal abscess, collection, or fistula, have been reported to be caused by drains[8,17-19]. Prophylactic drainage after gastric surgery is a common practice in many institutions. Surprisingly, there lack of adequate studies on the value of prophylactic drainage after gastric surgery, despite gastric surgery constitutes a significant part of GI surgery. Thus, for subtotal gastrectomies, the value of prophylactic drainage remains unclear, and there is little information regarding the evidence-based recommendations for prophylactic drainage in these procedures. Therefore, we aimed at highlighting evidence-based values of prophylactic drainage after subtotal gastrectomy for gastric cancer.

Despite the controversies whether the post-operative complications after gastric cancer surgery are indeed associated with the extent of lymph node dissection, the current incidence of severe post-operative complications, such as anastomotic leakage, is extremely low[10]. Similarly, we found a low incidence of post-operative complications in our study, showing no significant difference in the incidence of severe post-operative complications between the drain group and no-drain group, which is in agreement with a recent study by Kim *et al*[20]. In contrast, the majority of the patients in this study had advanced cancer at the time of surgery. In this study, there were a total of five post-operative in-hospital mortalities. Interestingly, all cases, irrespective of their age, had advanced gastric cancer (stage Ⅳ = 4 and stage Ⅲ = 1), suggesting that tumor aggressiveness, not age, might be associated with post-operative in-hospital mortality.

Some surgeons experienced a high risk of pancreas-related complications after gastrectomy with D2 or more extended lymph node dissection, thereby suggesting prophylactic drainage placement in gastric cancer surgery to avoid a re-operation[21]. Moreover, some surgeons believe that prophylactic use of drains gives early information about anastomotic leakage, intra-abdominal bleeding, *etc.* However, some authors believed that drainage of the peritoneal cavity is impossible and, therefore, prophylactic drainage is useless[14,15]. In our series of patients, we found a very low incidence of anastomotic leakage (1.8%, 2/108); one in each group, which was suspected clinically and confirmed after re-exploration. Besides drainage output, anastomotic leakage can be diagnosed by radiological and clinical findings, such as features of peritonitis. It has been reported that interventional radiology-guided drainage has remarkably reduced the number of laparotomies for surgical complications, thereby supporting abdominal surgery without the prophylactic use of drains[22].

In this study, we found no obvious differences in number of post-operative days until passage of flatus and until initiation of soft diet, and length of post-operative hospital stay between the two groups, which are in agreement with a previous study[20]. Moreover, we did not observe any significant difference in operating time between the two groups, which is in contrast with a prospective study by Kim *et al*[20], who reported significantly longer operating time in the drain group. A recent study demonstrated that morbidity and postoperative hospital stay were statistically higher in the drain group of patients with total gastrectomy[8].

It is important to note that there have been reported data showing drain-related complications, such as fistula, drain site infection and pain, in abdominal surgery[17,18]. Similarly, we also found some drain-related complications, such as omentum came out through the drain site after removal of the drain, and continuous ascitic fluid leakage from the drain site for more than 3 d.

Several well-constructed, prospective studies failed to show any benefit from surgically placed closed suction drainage[8,9]. After a variety of intra-abdominal procedures, such as colorectal resection[7,23,24], closure of perforated duodenal ulceration[6], open or laparoscopic cholecystectomy[4,25], radical hysterectomy and pelvic lymphadenectomy[26], or retroperitoneal lymphadenectomy[5], there appears to be no statistical difference in the rate of complications between patients who are drained and those who are

not, suggesting at best that routine placement of intraperitoneal drains is unnecessary. In fact, many of the studies imply that peritoneal drainage may be associated with adverse effects[8,9].

In conclusion, based on these results, our study suggests that prophylactic drainage placement after subtotal gastrectomy is not necessary since it does not offer additional benefits for the patients undergoing subtotal gastrectomy regardless of D1 or D2 lymph node dissection and radical or palliative resection.

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RAPID COMMUNICATION

Cross-reactivity of anti-*H pylori* antibodies with membrane antigens of human erythrocytes

Feng-Hua Guo, Xiao-Mei Yan, Chun-Xiang Fan, Fei Zhao, Yuan Hu, Di Xiao, Xun Zeng, Mao-Jun Zhang, Li-Hua He, Fan-Ling Meng, Jian-Zhong Zhang

Feng-Hua Guo, Xiao-Mei Yan, Chun-Xiang Fan, Fei Zhao, Yuan Hu, Di Xiao, Xun Zeng, Mao-Jun Zhang, Li-Hua He, Fan-Ling Meng, Jian-Zhong Zhang, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, PO Box 5, Changping, Beijing 102206, China
Supported by the National Natural Science Foundation of China, No. 30370078
Correspondence to: Jian-Zhong Zhang, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, PO Box 5, Changping, Beijing 102206, China. helico99@sina.com
Telephone: +86-10-61739456 Fax: +86-10-61739456
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Abstract

AIM: To investigate whether anti-*H pylori* antibodies have cross-reaction with antigens of erythrocyte membrane.

METHODS: Blood samples were collected from 14 volunteers (8 positive and 6 negative for *H pylori* detected by ^{13}C-urea breath test) of the general population. Erythrocyte membrane proteins of the subjects were examined by Western blot using anti-*H pylori* serum. The proteins related to the positive bands were identified by mass spectrum analysis.

RESULTS: Anti-*H pylori* antibodies had cross-reaction with the proteins of about 50 kDa of erythrocyte membranes in all samples independent of *H pylori* infection. One protein in the positive band was identified as Chain S, the crystal structure of the cytoplasmic domain of human erythrocyte Band-3 protein.

CONCLUSION: Anti-*H pylori* antibodies cross-react with some antigens of human erythrocyte membrane, which may provide a clue for the relationship between *H pylori* infection and vascular disorders.

Key words: *H pylori*; Antibodies; Erythrocyte; Cross-reactivity

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INTRODUCTION

H pylori, first isolated by Marshall and Warren[1], a gram-negative spiral bacterium, colonizing in gastric mucosa, is notorious for causing chronic infections and has been linked to various gastric diseases such as chronic gastritis, peptic ulcer, gastric mucosa-associated lymphoid tissue lymphoma and gastric cancer[2-4]. In recent years, infection by *H pylori* has been linked to extradigestive pathologies including ischemic cardiac and cerebral diseases. Many seroepidemiological studies revealed the relationship between *H pylori* and vascular disorders[5,6] even though the prevalence of positive findings varied widely between studies and not all studies reported positive results[7-9]. However, the exact nature of the association is not completely elucidated.

Several investigations revealed that heat shock proteins (HSPs) of *H pylori* are extremely homologous with HSPs of humans[10], the O-side chain of the lipopolysaccharide (LPS) of a number of *H pylori* strains is structurally similar to the Lewis histo-blood group antigens[11], anti-CagA antibodies cross-reacted with antigens of blood vessels[12]. All these imply that autoimmunity might take part in pathomechanisms of *H pylori*.

The changes of erythrocytes affect the whole blood viscosity, which contributes importantly to thrombosis and atherosclerosis (AS). Our previous studies found that anti-*H pylori* serum reacted with parts of erythrocytes and endothelial cells of heart valves using immunohistochemical method[13,14]. But it remains unknown which antigen resulted in these positive reactions. The present study was aimed to investigate whether the proteins of erythrocyte membrane cross-react with anti-*H pylori* by Western blot assay and to identify the special proteins by mass-spectrum assay in an effort to provide a clue for pathogenic link between *H pylori* infection and vascular disorders.

MATERIALS AND METHODS

Blood samples

Fresh blood samples were collected from 14 subjects from the general population whose results of ^{13}C-urea

Table 1 General data about the subjects

	Subject No.													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Gender	F	F	M	M	F	M	M	F	F	M	M	M	F	F
Age (yr)	27	45	44	44	31	49	32	42	47	42	34	45	28	30
^{13}C -UBT (DOB)	40.93	+	8.48	+	+	8.81	11.23	+	10.33	23.08	+	+	25.10	38.71

F: female; M: male; +: UBT positive.

breath test (^{13}C-UBT) were supplied by Chinese People's Liberation Army General Hospital. The kit for ^{13}C-UBT was provided by Altachem Pharma Ltd. Current infection of *H pylori* was confirmed by a value of ^{13}C-UBT greater than 4. General data about the subjects are shown in Table 1. Informed consents were obtained from all the volunteers before ^{13}C-UBT and blood sampling.

Extraction of erythrocyte membrane proteins

Fresh blood collected from the subjects were mixed with heparin as anti-coagulant. The erythrocytes were separated by centrifugation at 1230 × *g* and were lysed with deionized water and then centrifuged at 12000 × *g* for 20 min at 4℃. The pellets were washed in three volumes of cold phosphate buffer at 5 mmol/L, pH 8.0, containing 1 mmol/L EDTA and 1 mmol/L PMSF (Sigma) 6 times until the membranes were white and then were resuspended in the same buffer and centrifuged at 30000 × *g* for 1 h at 4℃. The pellets were frozen at -80℃ and dried at -56℃ in cold vacuum. The membranes were resuspended in the 2-DE lysis buffer cocktail consisting of 7 mol/L urea, 2 mol/L thiourea, 10 g/L DTT, and 40 g/L CHAPS at 4℃ for 2 h, then ultrasonicated on ice. The concentration of proteins in each sample was 6-12 g/L determined by Bradford protein assay[15]. The whole proteins of *H pylori* NCTC11637 were extracted as positive. All reagents in 2-DE lysis buffer were bought from Amersham.

Reactivity of anti-H pylori serum with erythrocyte membrane proteins by Western blot

SDS-PAGE was performed using a Bio-Rad Mini-Protean 3 electrophoresis cell. Approximately 120 μg of membrane proteins were parallelly loaded into two wells of 10% SDS-polyacrylamide minigel, 60 μg per well. Thirty μg of whole proteins of *H pylori* NCTC11637 as positive control and 5 μL prestained molecular weight standards marker (Fermantas) were also respectively loaded in two wells per gel.

Proteins were transferred to a PVDF membrane (Amersham) using Bio-Rad Semi-Dry transfer unit. Blocking was performed overnight at 4℃ in blocking buffer (TBS containing 50 g/L BSA). The membrane was bisected and one part was incubated with the primary antibody, rabbit anti-*H pylori* NCTC11637 serum (from immunized rabbits with *H pylori* NCTC11637, the animals were provided by Vital River Laboratories Co. Ltd. and raised by the Department of Laboratory Animal Science, Peking University Health Science Center) for 2 h at room temperate (RT). To exclude the color reaction resulting from the direct conjugation of the second antibody and the normal serum with the proteins on PVDF membranes, the normal serum (pre-immunization serum) of the same rabbits was used as control for another part of membranes with the same samples. Other steps were performed according to the Western blot assay. The second antibody, goat anti-rabbit IgG AP conjugate and AP substrates were from Vector.

Excision of protein bands and in-gel reduction, alkylation and trypsin digestion of proteins

The blots incubated in anti-*H pylori* serum were compared with the others of the same sample incubated in normal serum to find out the different reacted bands. The samples were chosen according to different bands and SDS-PAGE was performed and the gel was stained with Coomassie blue-R250 dye. The bands in the SDS-PAGE gel in accordance with different reacted ones in Western blot were excised, and in-gel reduction, alkylation and trypsin digestion was performed according to EMBL protocol (http://www.proteomics.com.cn/paper/InGel.html). Briefly, after a washing step, gel particles were reduced with DTT and alkylated with iodoacetamide. A second washing was performed before overnight digestion with 3 μL (40 mg/L) trypsin (Sigma). The resulting peptides were extracted with 500 mL/L ACN and 50 mL/L TFA and dried in a cold vacuum.

Mass spectrometric (MS) analyses of tryptic peptides and identification of proteins

The digested samples were mixed with a saturated matrix solution (1:1) (α-cyano-4-hydroxycinnamic acid prepared in 500 mL/L acetonitrile and 1 mL/L formic acid). All mass spectra were obtained on a 4700 Proteomics analyzer with TOF/TOF optics (Applied Biosystems, Foster City, CA, USA) in the positive ion reflector mode with a mass accuracy of about 50 ppm. The MALDI tandem mass spectrometer used a 200 Hz frequency-tripled Nd:YAG laser operating at a wavelength of 355 nm. MS spectra were obtained between *Mr* 800 and 4000 with ca. 1000 laser shots. MS/MS spectra were acquired with 2000 laser shots using air as the collision gas. The singly charged peaks were analyzed using an interpretation method present in instrument software, where the five most intense peaks were selected and MS/MS spectra were generated automatically, excluding those from the matrix, due to trypsin autolysis peaks. Spectra were processed and analyzed by the Global Protein Server Workstation (Applied Biosystems, Foster City, CA, USA), which uses internal Mascot v2.0 software(Matrix Science, UK) for searching the peptide mass fingerprints and MS/MS data. Searches

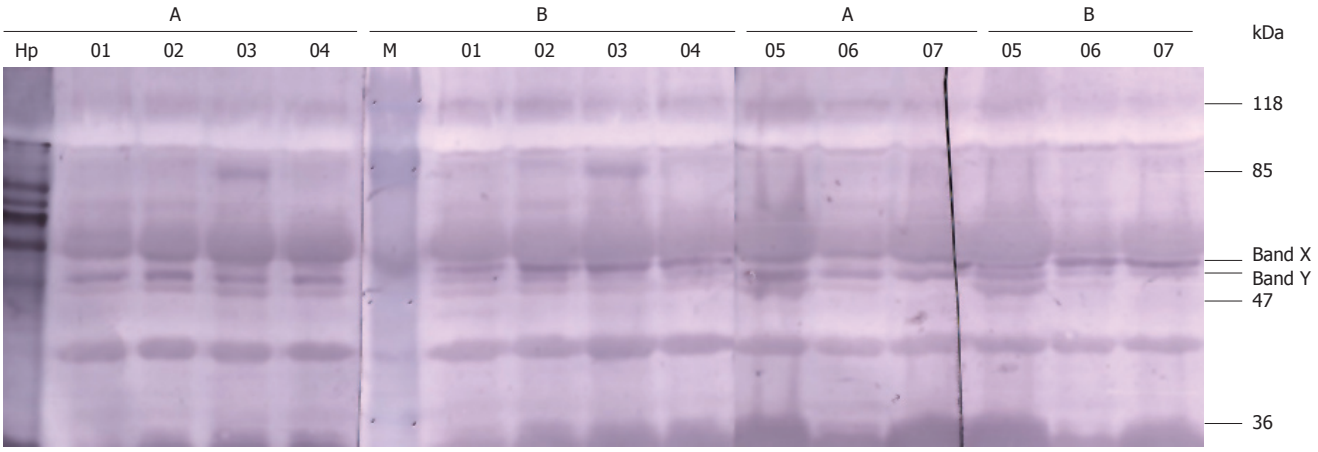

Figure 1 Anti-*H pylori* serum immunoreacted with erythrocyte membrane proteins in Western blot. **A**: incubated with anti-*H pylori* serum of rabbit; **B**: incubated with normal serum of rabbit. 01-07: the number of erythrocyte membrane protein samples. Hp: *H pylori* NCTC11637; M: prestained molecular weight standards marker.

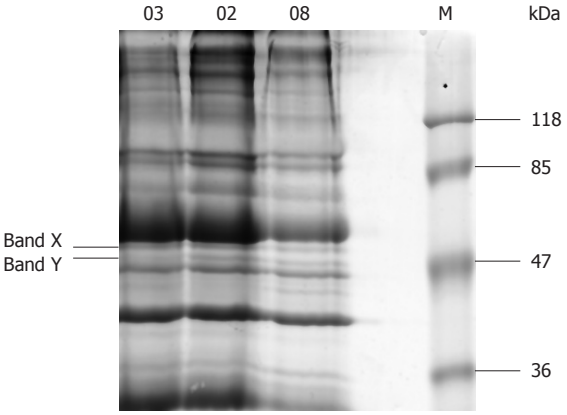

Figure 2 SDS-PAGE of erythrocyte membrane proteins. 03,02,08: the code of erythrocyte membrane protein samples. M: prestained molecular weight standards marker.

were performed against the NCBI non-redundant protein database (updated 18 November 2005). Identifications with a GPS confidence interval of greater than 95% were accepted.

RESULTS

Reactivities of anti-H pylori serum with erythrocyte membrane proteins

Both normal rabbit serum and anti-*H pylori* serum showed immunoreactivities with the membrane proteins of about 110 kDa, 55 kDa, 51kDa, 50 kDa, 40 kDa and 27 kDa of all erythrocytes. However, anti-*H pylori* serum specially recognized antigens of about 50 kDa (marked as band Y in Figure 1) from erythrocytes compared with the normal serum. Remarkably, this feature existed not only in *H pylori* $^{+}$ subjects (No. 01, 03, 06, 07, 09, 10, 13, 14) but also in *H pylori* $^{-}$ subjects (No. 02, 04, 05, 08, 11, 12). The immunoreactivity of another band (marked as band X in Figure 1) with anti-*H pylori* serum was weaker than that with normal serum.

Identification of specific proteins

There were 17-18 bands in the SDS-PAGE 10% gel of erythrocyte membrane protein sample (Figure 2). The special band of about 50 kDa and another one closely above it (respectively marked as band Y and band X in Figure 2) corresponding to the specially reacted bands in Western blot were faintly stained. Five proteins were identified in the two bands, 4 in band X and 1 in band Y (Table 2).

DISCUSSION

The pathogenesis of ischemic vascular diseases is multifactorial. AS and thrombosis, the principle basis of ischemic vascular disease, determine the occurrence of ischemic events. However, many AS patients lack traditional risk factors, suggesting that other mechanisms may be involved in the AS development[16,17]. In recent years, more attention has been paid to the relationship between infection and ischemic diseases[16,18,19].

Several studies indicated the association between *H pylori* infection and ischemic vascular disease especially when the CagA^{+} strain was involved[5,6], although the results are currently being debated[7-9]. By now, most studies have been based on seroepidemiology and nonspecific systemic inflammation. The exact mechanisms by which *H pylori* infection contributes to the progression of vascular disorders have not been elucidated.

The molecular mimicry between elements of *H pylori* and those of host cells[10,11] provides clues for autoimmunity as one of the candidate pathopoiesis. Franceschi and his colleagues[12] reported that anti-CagA antibodies cross-reacted with antigens of both normal and atherosclerotic blood vessels by immunohistochemistry and anti-CagA antibodies also specifically immunoprecipitated two antigens of 160 and 180 kDa from both normal and atherosclerotic artery lysates. The authors speculated that the immunoprecipitated proteins were not CagA of *H pylori* but vascular elements because the two antigens were different from CagA (about 116-140 kDa) in molecular weight. The reactivity detected in vessels with anti-CagA antibodies was caused by the mimicking vascular antigens. We think this speculation reasonable. However, the two antigens were not identified. Moreover,

Table 2 List of proteins identified from the special bands in Figure 2

Band code	Protein name (source)	Accession No.	Protein score	Protein acore CI%	Protein MW	Protein PI
X	Flotillin 1 (Sus scrofa)	gi|41529176	88	99.560	47 325.6	7.66
	Flotillin 1 (Macaca mulatta)	gi|55700801	87	99.433	47 383.6	6.71
	Flotillin 1 variant (Homo sapiens)	gi|62896619	87	99.378	47 324.6	8.18
	Predicted: similar to flotillin-1 isoform 5 (Canis familiaris)	gi|73972134	84	98.640	34 070.7	8.52
Y	Chain S, crystal structure of the cytoplasmic domain of human erythrocyte Band-3 protein (Homo sapiens)	gi|14277742	93	99.861	42 509.3	4.49

the difficulty in obtaining vascular tissue makes the investigation in the relationship between vascular endothelium and *H pylori* infection unfruitful.

Erythrocyte is one of most important factors affecting hemodynamics. Its membranes can be easily isolated in large quantities and many blood group antigens are expressed not only on the surface of blood cells but also on vascular endothelial cells. Thus, we chose erythrocyte to investigate the cross-reaction of human plasma membrane and anti-*H pylori* antibodies. Our previous study showed that anti-*H pylori* serum reacted with erythrocytes by immunohistochemical method[13]. But we did not know which elements resulted in the immunoreaction and whether the elements belong to erythrocytes or to *H pylori*. In the present investigation, antigens of about 50 kDa from erythrocyte membrane strongly immunoreacted with anti-*H pylori* serum rather than normal serum in all 14 samples (Figure 1). This feature did not depend on current infection of *H pylori*. Therefore, we speculate the reacted antigens are not elements of *H pylori* but the mimicking erythrocyte antigens. The results of mass spectrum assay confirmed our speculation. One protein was identified as Chain S, the crystal structure of the cytoplasmic domain of human erythrocyte Band-3 protein (*Mr* 42.5 kDa) in the special band (band Y in Figure 2).

Band 3 protein is the most abundant transmembrane protein to maintain the normal metabolism and function of human erythrocyte. This protein of about 95-100 kDa has two domains. The N-terminal domain of about 40 kDa is located within the cytoplasm and participates in signal transmission across membranes and other functions such as growth, differentiation and interaction of cellules, while the C-terminal of 55 kDa domain is membrane-associated and mediates the exchange transportation of anions Cl^-/HCO_3^- across the erythrocyte membrane[20,21]. In this study, the two antigens of 160 and 180 kDa mimicking with CagA were not found possibly because of the diversity of erythrocytes and vascular cells.

We consider that antibodies against *H pylori* may not contact with cytoplasmic domain of Band 3 of normal erythrocyte. However, oxygen free radicals and systemic inflammation caused by acute or chronic infection could damage erythrocyte membrane leading to the decrease of erythrocyte deformability, increase of erythrocyte fragility and elevation of erythrocyte aggregation index. Some authors reported these changes in several ischemic cardiac disease patients with *H pylori* infection[22]. The impaired erythrocytes might be easier to be disrupted, inducing internal antigens (including the cytoplasmic domain of Band 3 protein) to be exposed to circulating antibodies. Then anti-*H pylori* antibodies could bind the exposed antigens and cause inflammatory cell activation, which might be associated with the changes of hemorheology and hemodynamics, plaque aggregation, thrombus formation and atherogenesis leading to ischemic events.

In band X (Figure 2), 4 proteins were identified, which were considered to be flotillin 1 variants according to their resource and molecular weight. The reason why the reaction of the band X incubated with normal serum was stronger than with anti-*H pylori* serum is being investigated.

The protein that cross-reacted with anti-*H pylori* antibodies probably is another one that we could not identify due to its trace quantity and the limit of separation ability of SDS-PAGE. Nevertheless, our study provides an experimental evidence of molecular mimicry between *H pylori* antigens and erythrocyte membrane proteins. The results support the hypothesis that autoimmunity induced by *H pylori* infection plays an important role not only in vascular disorders but also in various extragastric diseases.

COMMENTS

Background

The pathogenesis of ischemic vascular diseases is multifactorial. The conventional risk factors do not fully account for the risk of these diseases. In recent years, more attention has been paid to the relationship between infection and ischemic diseases. Several studies indicated the association between *H pylori* infection and vascular disorders. However, the exact nature of the association is not completely elucidated.

Research frontiers

The molecular mimicry between elements of *H pylori* and those of host cells provides clues for autoimmunity as one of candidate pathopoiesis. Autoimmunity has become one of the hot spots of studies in recent years. Some studies have found that anti-*H pylori* antibodies reacted with endothelial cells and erythrocytes.

Innovations and breakthroughs

This study choose erythrocyte, which is easily to be isolated in large quantities, to investigate the cross-reaction of human plasma membrane and anti-*H pylori* antibodies and found anti-*H pylori* antibodies cross-reacted with the proteins of about 50 kDa of erythrocyte membranes in Western blot. The protein was identified by mass spectroscopy.

Applications

Erythrocyte is one of most important factors affecting hemodynamics. Many blood group antigens are expressed not only on the surface of blood cells but also on vascular endothelial cells. The materials selecting and the results of this study provide a new clue and experimental evidence for autoimmunity as one of the potential pathopoiesis of *H pylori* infection in vascular disorders.

Peer review

This study looks at the cross-reaction of human plasma membrane and anti-*H pylori* antibodies. Although the contribution of the cross-reaction to the relationship

between *H pylori* infection and vascular disorders is not clear, this study provides some interesting observations and a new clue for autoimmunity as one of the potential pathopoiesis of *H pylori* infection.

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RAPID COMMUNICATION

Novel mutations and sequence variants in exons 3-9 of human T Cell Factor-4 gene in sporadic rectal cancer patients stratified by microsatellite instability

Wen-Jian Meng, Ling Wang, Chao Tian, Yong-Yang Yu, Bing Zhou, Jun Gu, Qing-Jie Xia, Xiao-Feng Sun, Yuan Li, Rong Wang, Xue-Lian Zheng, Zong-Guang Zhou

Wen-Jian Meng, Chao Tian, Yong-Yang Yu, Zong-Guang Zhou, Department of Gastrointestinal Surgery, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China
Ling Wang, Bing Zhou, Jun Gu, Yuan Li, Rong Wang, Xue-Lian Zheng, Zong-Guang Zhou, Division of Digestive Surgery and Organ-Microcirculation, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China
Qing-Jie Xia, Ophthalmologic Surgery Laboratory, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China
Qing-Jie Xia, Zong-Guang Zhou, National Key Laboratory of Biotherapy of West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China
Xiao-Feng Sun, Department of Oncology, Linköping University, Linköping, Sweden
Supported by the National Natural Science Foundation of China, No. 39925032
Correspondence to: Zong-Guang Zhou, Department of Gastrointestinal Surgery, West China Hospital, Sichuan University, 37 Guo Xue Xiang, Chengdu 610041, Sichuan Province, China. zhou767@21cn.com
Telephone: +86-28-85164035 Fax: +86-28-85164035
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Abstract

AIM: To establish the role of human T Cell Factor-4 (hTCF-4) gene exons 3-9 mutation status in association with sporadic rectal cancer with microsatellite instability (MSI).

METHODS: Microsatellite markers were genotyped in 93 sporadic rectal cancer patients. Eleven cases were found to be high-frequency MSI (MSI-H). Sequence analysis of the coding region of the exons 3-9 of hTCF-4 gene was carried out for the 11 MSI-H cases and 10 controls (5 microsatellite stability (MSS) cases and 5 cases with normal mucosa). The sequencing and MSI identification were used.

RESULTS: Several novel mutations and variants were revealed. In exon 4, one is a 4-position continuous alteration which caused amino acid change from Q131T and S132I (391insA, 392 G > A, 393 A > G and 395delC) and another nucleotide deletion (395delC) is present in MSI-H cases (5/10 and 4/10, respectively) but completely absent in the controls.

CONCLUSION: Novel mutations in exon 4 of hTCF-4 gene were revealed in this study, which might be of importance in the pathogenesis of sporadic rectal cancer patients with MSI-H.

Key words: hTCF-4; Sporadic rectal cancer; Microsatellite instability; Mutation analysis

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INTRODUCTION

Colorectal cancer is the second leading cause of cancer related deaths in the Western world. It is the third leading cause of cancer deaths and tends to increase in China. The mechanism of the malignant transformation of colorectal cells is far from being clearly understood. Involvement of the Wnt signaling pathway, also called the APC/β-catenin/TCF pathway, has been demonstrated notably in colorectal cancers. Wnts act by stabilizing cellular levels of the transcriptional coactivator β-catenin, which forms complexes with sequence-specific DNA binding T cell factor/lymphocyte enhanced factor (TCF/LEF) transcription factors. In the absence of nuclear β-catenin, TCF/LEFs act as transcriptional repressors by recruiting proteins such as Groucho/Transducin-like enhancer-of-split (TLE), and COOH-terminal-binding protein (CtBP). Upon Wnt activation, the binding of β-catenin to TCFs generates a bipartite transcription factor, in which TCFs provides the DNA binding domain and the C terminus of β-catenin provides the transactivation domain, therefore inducing a transcriptional switch. However, the molecular basis of the switch from transcriptional repression to activation during Wnt signaling is still not clear.

TCF-4 (also known as TCF7L2) is a sequence-specific transcriptional factor, whose functions of the downstream

effectors of Wnt/β-catenin signals are similar to the other human TCF proteins (TCF-1, TCF-2/LEF-1, and TCF-3) TCF-4 is the most intensively expressed member of the TCF/LEF gene family in normal colonic tissues. In the TCF-4 gene, three major domains are present: β-catenin-binding domain in exon 1, the DNA-binding HMG boxes in exon 10 and 11, and the COOH-terminal-binding domain in exon 17[1,2]. The human TCF-4 (hTCF-4) gene was reported to be one of the targets of microsatellite instability (MSI) in colorectal cancers. Recently, in colorectal cancer with high-frequency MSI (MSI-H), frequent frameshift mutations involved in hTCF-4 exon 17 have been found to modulate transcriptional activity through the truncation at the COOH-terminal region[3,4]. However, Ruckert *et al*[5] found that mutations in the A_9 repeat of hTCF-4 exon 17 do not contribute to tumorigenesis. The hTCF-4 protein has been shown to repress transcription by recruiting corepressor proteins not only CtBP, but also Groucho/TLE[6]. A major advance in explaining how TCFs repress transcription followed the discovery that Groucho/TLE is a specific binding partner of TCFs. Binding occurs between the Q-domain of Groucho and a conserved region in TCFs between the β-catenin binding domain and the HMG box[7]. The conserved region was encoded by exons 3-9. Therefore, whether mutations of the region interfere with binding to Groucho/TLE family proteins remains to be elucidated. To gain insight into the molecular basis of rectal cancer, we have screened hTCF-4 mutations in human rectal tumors in exons 3-9. The up-to-date methods of sequencing and MSI identification were used. The data showed that novel mutations in exon 4 of hTCF-4 gene might be of importance in the pathogenesis of sporadic rectal cancer patients with MSI-H.

MATERIALS AND METHODS

Patients

One hundred and two patients from Chinese Han population who underwent surgical resection for sporadic rectal cancer at West China Hospital of Sichuan University (Sichuan, China) from 2002 to 2005 were included in the study. All cases were deemed sporadic, based on the absence of relevant family histories as recorded prospectively at initial patient interview. Patients who were suspected clinically to have HNPCC fulfilling the Amsterdam Ⅰ/Ⅱ[8] or Bethesda criteria[9] were excluded. Patients treated by preoperative radiotherapy or chemotherapy were also excluded. Of 102 cases, 9 were excluded because of tissue unavailability, resulting in a total of 93 eligible cases. Tumor histotype and grading were defined according to the recommendations of the World Health Organization for the histological typing of colorectal cancer[10]. All cases were staged using the 6th edition of the American Joint Committee on Cancer (AJCC) Staging Manual[11]. Informed consent was provided by all subjects, and approval was obtained from the Regional Ethics Committee.

Nucleic acid extraction

Samples of tumor tissues and of normal mucosa were frozen immediately after surgery in liquid nitrogen and stored at -80℃ until analysis. Before DNA extraction, the presence of adequate neoplastic material (at least 60%-70% of tumor cells) was verified by microscopic examination. Genomic DNAs of tumor and corresponding normal mucosa were extracted by a standard phenol-chloroform procedure. Total RNA was extracted from each subject using Trizol (Invitrogen Co., Ltd) method. Total RNA of each sample was dissolved in RNase-free water and stored in the refrigerator at -80℃ before use. The concentration of DNA and RNA was determined by spectrophotometer, and their integrity was checked by each gel electrophoresis.

Detection of MSI

Primers used to amplify simple sequence repeat markers were obtained from Life Technologies (TaKaRa, Dalian, China). MSI was studied at five loci recommended by a National Cancer Institute workshop on MSI (BAT25, BAT26, D2S123, D5S346 and D17S250)[12]. All primer sequences were as reported in Genome DataBase (GDB; http://www.gdb.org). Lesions were characterized as MSI-H if they manifested instability at two or more of the five loci in tumor DNA, and as microsatellite stability (MSS) if showing no instability at any loci when compared with normal DNA. For each marker analysis, PCR was carried out as previously described[13]. Electrophoreses of the amplified PCR products were performed in 2% agarose gels containing goldview in Tris-Borate EDTA buffer. Gels were examined using an UV transilluminator and verified as a single band. Electrophoresis of PCR products was separated on a 6% denaturing polyacrylamide gel containing 8 M urea. Gels were stained with silver salts according to Sanguinetti *et al*[14]. Experiments to ascertain MSI were done independently two to three times for each genetic locus. Through detection of MSI, 11 cancers demonstrating MSI-H and 10 controls (5 MSS and 5 with normal mucosa) were included for mutation analysis.

Amplifications of exons 3-9 of hTCF-4

Screening for hTCF-4 exons 3-9 mutations was performed by RT-PCR of total RNA. Two-step methods were adopted. Reverse transcription conditions for all PCRs were optimized on iCycler iQ (Bio-Rad, USA) and the optimum annealing temperature was 53.1℃. The following iCycler iQ run protocol was used: denaturation program (95℃, 5 min), amplification programs repeated 50 times (95℃ for 20 s, 53.1℃ for 30 s and 72℃ for 30 s). The first half of the exons 3-9 was amplified using F1 (5'CGCCAACGACGAACTGAT3') and R1 (5'GCACCACTGGCACTTTGT3') primers, whereas the second half was amplified using F2 (5' ATGAAATGGCCACTGCTTGA3') and R2 (5' CCTTTTTGGAGTCCTGATGCT3') primers. The annealing temperature was 56℃ and 58℃, respectively. Presence of all sequence variants was confirmed by performing three independent PCR reactions and subsequent DNA sequencing. In addition, amplification for hTCF-4 exon 4 was performed by PCR of genomic DNA. The primers and conditions were used according to Duval *et al*[15]. For PCR reaction, a tube without template DNA served as a negative control.

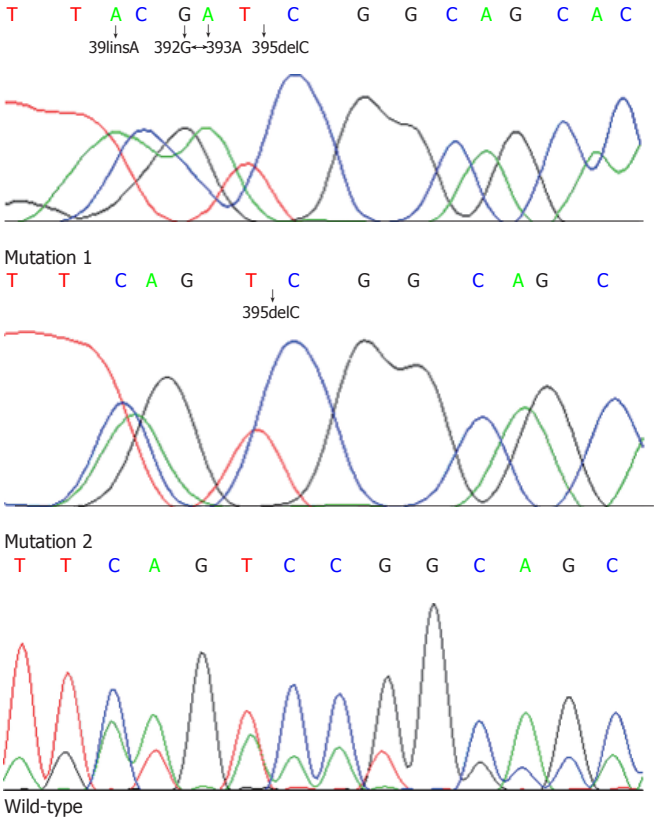

Figure 1 Sequencing analysis of mutated hTCF-4 exon 4. Sequence chromatograms at the beginning of the exon 4: mutation 1 (391insA, 392 G > A, 393 A > G and 395delC) representing an amino acid change of Q131T and S132I and mutation 2 (395delC) altering reading frame and stopping after incorporating 22 amino acid. The arrow indicates the location of changes.

Direct DNA sequencing

Electrophoreses of the amplified products were performed in 2% agarose gels containing goldview in Tris-Borate EDTA buffer. Amplicons were purified by solution extraction and bidirectional sequencing using an ABI Prism 377 DNA sequencer.

In silico analysis

To determine the potential deleterious effect of the amino acid changes, we used SIFT (http://blocks.fhcrc. org/sift/SIFT.html). The SIFT software uses the protein sequence similarity of different species and the hydrophobic characteristics of amino acids to calculate the probability of the deleterious effect of specific amino acid variants[16]. Scores lower than 0.05 suggest a potential pathogenic amino acid substitution.

RESULTS

A total of 11 cases demonstrated MSI-H using markers recommended by a National Cancer Institute workshop by PCR amplification from 93 sporadic rectal cancer patients in our study. Sequencing data collection and analysis were successfully performed for the hTCF-4 gene (exons 3-9) in these MSI-H cases (n = 10) and controls (n = 10) except for one MSI-H case. This study revealed several novel mutations and sequence variants between exons 3-9. The sequence at the beginning of exon 4 showed a TACGATCG repeat, which was present in 5 of MSI-H cases but not in the controls, as shown in Figure 1 and Table 1. Sequencing of exon 4 revealed a deletion of cytosine at 395 (395delC) which was only found in 4 MSI-H cases without above-mentioned mutation. Genetic and clinical details of these 4 cases are given in Tables 1 and 2, respectively.

In addition, there was a missense mutation (450 G > C) in exon 4 in one MSI-H case, resulting in transition of Glutamine to Histidine (Q150H) when translated. Finally, a change of 868 G to A, leading to a V290M amino acid change, was observed in exon 8 in two MSI-H cases. These novel variants were not present in any of the controls either. SIFT software suggested no potential deleterious effect of the two amino acid changes. No mutation was observed in other exons. The mutation (C to T) at the nucleotide 35 of exon 4 detected by DGGE only in the SW48 cell line was not observed in our study[15].

Table 1 Sequence analysis of hTCF-4 exons 3-9 in sporadic rectal cancer patients with MSI-H and controls

Exon	Nucleotide change	Amino acid change	Mutation type	Frequency	
				Patients (n = 10)	Controls (n = 10)
4	4-position continuous alteration[1]	Q131T	Missense	5	0
4	395delC	S132I	Frameshift	4	0
4	450 G > C	Q150H	Missense	1	0
8	868 G > A	V290M	Missense	2	0

[1]4-position continuous alteration refers to the mutation (391insA, 392 G > A, 393 A > G and 395delC).

Table 2 Clinical details of 10 MSI-H rectal cancer patients

Case No.	Age (yr)	Sex	Size (mm)	Pathology	TNM stage	CEA (ng/mL)
1	54	F	20	Mucinous	I	1.55
2	28	F	50	Mucinous	IV	310.5
3	68	F	60	Mucinous	II	3.47
4	49	F	40	Mucinous	II	1.6
5	51	M	40	Unmucinous	II	10.11
6	36	M	100	Mucinous	II	6.37
7	71	F	40	Unmucinous	I	3.53
8	47	M	40	Unmucinous	III	4.96
9	54	M	80	Unmucinous	II	4.3
10	54	M	80	Unmucinous	III	5.8

DISCUSSION

A link was previously established between hTCFs and Wnt signaling, a pathway that plays a crucial role in many developmental processes as well as in human carcinogenesis[1]. Although it is well established that the formation of nuclear β-catenin/TCF complexes plays a pivotal role in the activation of Wnt target genes, the exact mechanisms of transcriptional activation and regulation are still under investigation[17,18]. Duval *et al*[2] reported frequent frameshift alterations in an A_9 coding repeat localized in exon 17 of hTCF-4 in MSI-H colorectal cancers and

the main consequence of such a mutation was to change hTCF-4 transactivating properties by modifying the respective proportions of the different isoforms containing CtBP binding domains. However, Ruckert *et al*[5] found that the mutations do not contribute to tumorigenesis. Thus, the question is if mutations of the Groucho/TLE binding domain encoded by exons 3-9 interfere with binding to Groucho/TLE family proteins and remove the repressive effect of Groucho/TLE proteins. To our knowledge, there has been no report on it.

Sequencing data collection and analysis were successfully performed for the hTCF-4 gene (exons 3-9) in these MSI-H cases (n = 10) and controls (n = 10) except for one MSI-H case. This study revealed several novel mutations and sequence variants between exons 3-9. The sequence at the beginning of exon 4 showed a TACGATCG repeat which did not match perfectly to the TCAGTCCG repeat in the previously published hTCF-4 mRNA sequence[15]. Although an explanation regarding the apparent discrepancy is not forthcoming, the determinacy of this sequence variant may be supported by the following: Firstly, the sequence was confirmed by repetition of three independent PCR and sequencing reactions, including sequencing in reverse direction. Secondly, the sequence variant was present in 5 MSI-H cases but not in the controls. Sequence alignments have shown that although the 4-position continuous alteration (391insA, 392 G > A, 393 A > G and 395delC) did not alter the whole reading frame, the change from a Serine to a highly hydrophobic Isoleucine (S132I) is likely to have any functional relevance.

Another finding in this study was that a novel mutation could be implicated in the rectal cancer pathogenesis. Sequencing of exon 4 revealed a deletion of cytosine at 395 (395delC) only in 4 MSI-H cases without above-mentioned mutation. The absence of this 394delC in the controls suggests a potential pathogenic effect. The 394delC altered reading frame and 22 amino-acid peptides are encoded instead of a full-length protein. Therefore, the mutation in hTCF-4 exon 4 may modulate the switch status through the truncation between the β-catenin binding domain and the HMG box DNA-binding region (Groucho/TLE binding domain) of the hTCF-4 protein, which suggests this mechanism could be of functional significance for regulating hTCF-4 transcriptional activity. Except for this mutation associated with female and mucinous caricinoma patients ($P < 0.05$) (data not shown), no other clinicopathological features (such as age at diagnosis, tumor size, TNM stage, level of preoperative serum CEA and differentiation) could be identified in these 4 patients associated with mutation 395delC. We infer that the upstream of exon 4 is an unstable area so that it is subjected to mutations, which may be one of the mechanisms of the tendency to be truncated in colorectal tumors. This indicates that these novel mutations in exon 4 of hTCF-4 gene revealed in the study, might be of importance in the pathogenesis of sporadic rectal cancer patients with MSI-H.

In conclusion, this study provides important evidence for novel mutations and sequence variants in association with the pathogenesis of sporadic rectal cancer with MSI-H. These sequence variants in Chinese Han population indicates the diversity of human library and the complexity of rectal carcinogenesis. Further confirmatory studies on the spectrum, prevalence rates, and functional effect of sequence variants in the exons 3-9 of hTCF-4 gene, in particular exon 4, in other populations should further demonstrate the true contribution of this gene to colorectal carcinogenesis.

ACKNOWLEDGMENTS

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COMMENTS

Background

A link was previously established between hTCFs and Wnt signaling which plays a crucial role in many developmental processes as well as in human carcinogenesis. Although it is well established that the formation of nuclear β-catenin/TCF complexes plays a pivotal role in the activation of Wnt target genes, the exact mechanisms of transcriptional activation and regulation are still under investigation. Recently, in colorectal cancer with MSI-H, frequent frameshift mutations involved in hTCF-4 exon 17 have been reported to modulate transcriptional activity through the truncation at the COOH-terminal region. However, Ruckert et al. found that the mutations do not contribute to tumorigenesis. Thus, the question is if mutations of the Groucho/TLE binding domain encoded by exons 3-9 interfere with binding to Groucho/TLE family proteins and remove the repressive effect of Groucho/TLE proteins. We have screened for hTCF-4 mutations in human rectal tumors in exons 3-9.

Research frontiers

The mechanism of colorectal cancer is far from being clearly understood. Involvement of the Wnt signaling pathway has been demonstrated notably in colorectal cancers. Wnts act by stabilizing cellular levels of the transcriptional coactivator β-catenin, which forms complexes with sequence-specific DNA binding TCF/LEF transcription factors. However, the molecular basis of the switch from transcriptional repression to activation during Wnt signaling is not clear. TCF-4 is sequence-specific HMG box transcriptional factors that function as the downstream effectors of Wnt/β-catenin signals. TCF-4 is the most intensively expressed member of the TCF/LEF gene family in normal colonic tissue. The hTCF-4 gene was reported to be one of the targets of MSI in colorectal cancers. Recently, in colorectal cancer with MSI-H, frequent frameshift mutations involved in hTCF-4 exon 17 have been found to modulate transcriptional activity through the truncation at the COOH-terminal region. However, Ruckert *et al* found that mutations in the A_9 repeat of hTCF-4 exon 17 do not contribute to tumorigenesis. The hTCF-4 protein has been shown to repress transcription by recruiting corepressor proteins not only CtBP, but also Groucho/TLE. A major advance in explaining how TCFs repress transcription followed the discovery that Groucho/TLE is a specific binding partner of TCFs. Binding occurs in a conserved region in TCFs encoded by exons 3-9. Therefore, whether mutations of the region interfere with binding to Groucho/TLE family proteins remain to be elucidated.

Innovations and breakthroughs

The mutation in hTCF-4 exon 4 may modulate the switch status through the truncation Groucho/TLE binding domain of the hTCF-4 protein, which suggest this mechanism could be of functional significance for regulating hTCF-4 transcriptional activity. We infer that the upstream of exon 4 is an unstable area so that it is subjected to mutations, which may be one of the mechanisms of the tendency to be truncated in colorectal tumors. This indicates that these novel mutations in exon 4 of hTCF-4 gene revealed in the study, might be of importance in the pathogenesis of sporadic rectal cancer patients with MSI-H.

Applications

This present study provides an important evidence for novel mutations and sequence variants in association with the pathogenesis of sporadic rectal cancer with MSI-H. These sequence variants in Chinese Han population indicate the diversity of human library and the complexity of rectal carcinogensis.

Peer review

This is an interesting study investigating the possible association between TCF-4 exon 3-9 mutations and MSI high rectal cancer. It is a study reporting on previously uninvestigated area. The main conclusion of the authors was that new mutations discovered in the study might be of importance in the pathogenesis of sporadic rectal cancer patients with MSI-H.

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RAPID COMMUNICATION

Donor safety in adult living donor liver transplantation using the right lobe: Single center experience in China

Fu-Gui Li, Lu-Nan Yan, Yong Zeng, Jia-Yin Yang, Qi-Yuan Lin, Xiao-Zhong Jiang, Bin Liu

Fu-Gui Li, Lu-Nan Yan, Yong Zeng, Jia-Yin Yang, Qi-Yuan Lin, Xiao-Zhong Jiang, Bin Liu, The Center of Liver Transplantation, the Second Department of General Surgery, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China
Correspondence to: Lu-Nan Yan, The Center of Liver Transplantation, the Second Department of General Surgery, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China. lfg1616@163.com
Telephone: +86-28-66866019 Fax: +86-28-84388728
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Abstract

AIM: To evaluate the safety of donors in adult living donor liver transplantation (LDLT) using the right lobe in a single liver transplantation center in China.

METHODS: We investigated retrospectively 52 living donor liver resections performed from October 2003 to July 2006. All patients were evaluated by blood tests and abdominal CT. The mean donor age was 28.2 ± 7.4 years. Residual liver volume was 42.1% ± 4.7%. Mean operative time was 420 ± 76.2 min; mean ICU stay, less than 36 h; mean hospital stay, 16.4 ± 8.6 d; and mean follow-up period, 6 mo.

RESULTS: There was no mortality. The overall complication rate was 40% (21 donors). Major complications included biliary leak in two, and pneumonia in 2 donors. Minor complications included mild pleural effusion in 12 donors, transient ascites in 6, mild depression in 4, intra-abdominal collections in 2, and wound infections in 1 donor. Residual liver volume did not affect the complication rate. None required re-operation. Return to pre-donation activity occurred within 5-8 wk.

CONCLUSION: Right hemi-hepatectomy can be performed safely with minimal risk in cases of careful donor selection. Major complications occurred in only 7.7% of our series.

Key words: Safety; Donor; Liver transplantation; Complication

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INTRODUCTION

Living donor liver transplantation (LDLT) is an acceptable modality to treat end-stage liver disease and offers hope to patients with end-stage liver disease in areas where the waiting mortality is high and the availability of deceased donor organs falls short of the population. Regardless of the potential benefit that LDLT offers to the critically ill patients with end-stage liver disease, donor safety is a prime concern[1]. Furthermore, the graft mass cannot satisfy the demand in adult patients requiring use of the right lobe[2]. This donor risk was especially emphasized by discouraging episodes of donor mortality in North America, Europe, South America, and East Asia[3-7]. A few donors had to undergo liver transplantation due to hepatic failure following liver donation[3,4]. The advance of LDLT using right lobe grafts has raised special concerns about the safety of living liver donors. In 2003, our team started a new program using the right lobe in living donor transplantation. The aim of this study was to retrospectively review our experience with donor hepatectomy using the right lobe, specifically in the context of preserving donor safety at a single center in China.

MATERIALS AND METHODS

From October 2003 to July 2006, 52 donor operations for adult LDLT using the right lobe were performed at the Department of General Surgery, West China Hospital, Sichuan University of China. We investigated retrospectively the 52 living donor liver resections. All patients were evaluated by blood tests and abdominal CT. Donors' age ranged from 17 to 52 years, the mean age was 28.2 ± 7.4 years, 29 were men and 23 were women. In relation to the recipient, there were 22 sons, 12 spouses, 8 brothers or sisters, and 10 other relatives.

Preoperative donor evaluation included computed tomography with volumetry and magnetic resonance imaging with angiography and cholangiography. The criteria for donor selection included ABO blood type compatability, acceptable ranges of liver function tests,

reasonable liver volumes, age < 50 years, and fatty change < 30% by liver biopsy. The transection line was demarcated on the liver surface by temporary occlusion of right hepatic artery and portal vein. Inflow vascular occlusion was not used during liver transection. Vascular and biliary stumps were closed using a Prolene or an interrupted suture. Liver volume was evaluated with CT volumetry during the preoperative period and at 3 mo postoperatively. Intraoperative liver biopsy was performed routinely by one hepatic pathologist to check the percent of fatty change. We reviewed the donor characteristics, operative findings, and postoperative results, including the peak value of liver enzymes (AST, ALT and bilirubin). Findings were correlated with donor age (< 30 years and < 40 years), percent of fatty change in donor liver (no change, < 10%, and < 30%) size of remnant left lobe volume (< 35%, < 40%, and > 40%) and regeneration activity, as evaluated by CT volumetry at 3 mo postoperatively.

Operative technique

The donor procedure involved several steps. First, cholecystectomy and intraoperative cholangiography were performed to delineate the biliary anatomy. Next, the right hepatic artery and right portal vein were dissected. Intraoperative ultrasound was then performed to define the hepatic venous drainage of the right liver lobe. In most of our donors, the middle hepatic vein was preserved to avoid outflow obstruction to the remaining donor segment 4. The right hepatic vein was then isolated and the attachments between the right lobe and the diaphragm were divided to expose the inferior right hepatic veins (IRHVs), which drains the right lobe directly into the inferior vena cava. All IRHVs of more than 5 mm diameter were preserved for subsequent anastomosis to the recipient inferior vena cava. The right bile duct was cut sharply. The hepatic parenchyma was divided along Cantlie's line 1 cm to the right of the main stem of the middle hepatic vein using electrocautery and a Cavitron ultra-sonic aspirator. After the right lobe was completely separated, vascular clamps were applied to the right portal vein, right hepatic vein, and IRHVs. The lobe was removed, transferred to a back table, and flushed with a heparinized solution. Abdominal closure was performed in standard fashion.

RESULTS

All donors survived the procedure. Fifty-two right lobectomies required 316-576 (420 ± 76.2) min. The transfusions during operation ranged from 0 to 6 (mean 1.29 ± 1.21) U. The mean length of stay in the intensive care unit was less than 36 h, and the mean hospital stay was 16.4 ± 8.6 (range, 10 to 48) d. The total volume of the donor liver ranged from 976 to 1816 (mean 1106 ± 201) mL, including 382-925 (mean 526 ± 146) mL in the volume of the left lobe, and the ratio of the left lobe to the whole liver ranged from 32.3% to 46.2% (mean 38.6% ± 4.8%).

In the immediate postoperative period, all donors exhibited transient liver enzyme elevation, hyperbilirubinemia and hypoalbuminemia. The liver profiles normalized after a mean of 12 d. Prothrombin time was prolonged in the early postoperative period, but in most cases this was normalized within 14 d.

The postoperative peak values of liver enzymes increased based on the severity of fatty changes, especially between the groups with < 10% or > 10% fatty change, but the differences were not statistically significant. According to remnant left liver volume, there was statistically significant difference between the group with < 35% and the group > 35% postoperative liver enzymes ($P < 0.05$), but there was no significant difference among the groups with > 35%. The volume of the remnant liver is an important factor that influences the postoperative liver enzymes.

Computed tomography with volumetry was performed preoperatively and at 3 months postoperatively. The regeneration of the remnant liver (percent) was calculated as the liver volume on postoperative mo 3 versus preoperative liver volume × 100. The mean regeneration of the remnant left lobe at 3 mo postoperatively was 208% ± 41% (148%-312%) compared with the preoperative liver volume.

The mean follow-up time for the 52 cases was 6 mo. There was no donor mortality, and overall complication rate was 40% ($n = 21$). Four donors (7.7%) developed early postoperative major complications, including biliary leakage(two cases), and pneumonia (two cases). Bile leakage from the stump of the duct in one patient was treated with continuous drainage and healed spontaneously. The other patient with bile leakage was successfully managed with endoscopic retrograde cholangiopancreatography (ERCP) and placement of a biliary stent, which extended the patient's hospital stay to 36 d. The stent was removed 6 wk later and no further interventions were needed. Both cases of pneumonia were successfully treated with antibiotics. The minor complications included mild pleural effusion (12 donors, 23%), transient self-limited ascites (6 donors, 11.5%), mild depression in (4 donors, 7.7%), intra-abdominal collections (2 donors, 3.8%)and wound infection (1 donor, 2%). The pleural effusion was on the right side in most cases. Three donors had to be readmitted 1 mo after the operation for aspiration of a purulent subphrenic collection. The most common problem, especially for young donors who cared about their looks, was scar formation[8]. At 1-year follow-up, prominent hypertrophic scar was observed in about 5% of donors, but no keloid has ever been detected. No one received wound revision for cosmetic purposes. All donors returned to their predonation daily activities within 5-8 wk, and no liver impairment was noted during follow-up.

DISCUSSION

Selection and evaluation of a living liver donor for adult recipients is a complex process that involves optimizing graft size in relation to the safety of donors and recipients, technical details of liver procurement, and ethical problems of using nonrelated live donors. As in most countries,

including the United States and Japan, no legal restrictions exist for living donation, local ethics committees confirm whether the candidates are appropriate potential donors. Voluntarism is the primary selection criterion and medical evaluation can only be started after confirmation of the voluntary nature of the donation.

Volumetric study using computed tomography scans is mandatory. For patients with advanced liver disease, a graft volume of greater than 40% of the recipient standard liver volume is necessary[9], while for the living donor the remnant liver mass must be more than 30% of the whole liver[10]. Selection of right lobe graft should be very prudently considered if the right liver appears to be 65% of the whole liver volume[11]. The term "standard liver volume" has become a key concept in LDLT[12]. Estimated liver volume on computed tomography in healthy volunteers is proportional to body surface area and is calculated using the following formula: liver volume (mL) = 706.2 × body surface area (m^2) + 2.4.

In living donor liver transplantation using the right lobe, donor safety must always be the primary consideration. We reviewed the peak value of liver enzymes as parameters of donor risk and considered several factors, including donor age, degree of fatty change, and volume of remnant liver as factors that influence the value of liver enzymes. Among the factors, the volume of remnant liver was most important. Several investigators have suggested that individuals with normal liver function tolerate resection of up to 60% or 70% of a nontumorous liver[13].

Our data indicate that the peak value of liver enzymes in donors with < 35% of the liver as a remnant were significantly higher than the group with > 35%. These values could induce risks to the donor. With regard to the safety margin, a remnant liver volume of 30% of the total is probably the lowest limit.

During screening donor evaluation, many candidates have some degree of fatty liver. We select donors whose livers have < 30% fatty change. Our data suggest that, even in this acceptable limit of fatty change, the postoperative peak value of liver enzymes increased according to the degree of fatty change, especially in cases of > 10% fatty change. Although this factor is not significant itself, it is problematic when combined with other risk factors.

The recovery of the donor liver depends on the regenerative activity of the remnant liver. Regeneration after resection usually starts in the immediate postoperative period, and occurs mainly within 2 wk after operation. The liver mass of small-for-size grafts increased more rapidly to meet the metabolic demands of greater relative body size. We observed liver regeneration at postoperative month 3. Our data indicate that regeneration of liver volume at 3 mo postoperatively is about twofold greater than the preoperative value, and the regenerative activity was increased among the groups with smaller remnants.

The most important complication during donor operation is biliary injury. However, there was no biliary injury in our data. The precise biliary anatomy and meticulous hilar dissection could prevent such injury. Ischemia due to excessive dissection of the right hepatic artery is probably responsible for biliary stricture. We recommend dissection of the right hepatic artery to a lesser degree, confining the exposure to the right side of common hepatic artery.

In conclusion, our single-center experience showed that life-threatening complications of the right hemi-hepatectomy donor operation could be avoided or overcome only through the strict selection of living donors, intensive postoperative surveillance, and timely feedback of surgical techniques. In LDLT, the physical and psychologic sacrifice by the donor is significant and is associated with high expectations regarding a good outcome for themselves and the recipient[14]. There can be significant risks to the donor, including the risk of death and substantial morbidity, that must be taken into account before patients, physicians, and transplant programs embark on LDLT. Universally acceptable criteria for donor selection should be established to prevent immoderate procurement of liver graft. Right hemi-hepatectomy can be performed safely with minimal risk in cases of careful donor selection such that the remnant liver volume exceeds 30% of the total liver volume while showing minimal fatty change. Major complications occurred in only 7.7% of our series.

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CASE REPORT

An unreported complication of acute pancreatitis

G Muthukumarasamy, V Shanmugam, SR Yule, R Ravindran

G Muthukumarasamy, R Ravindran, Department of Surgery, Aberdeen University and Aberdeen Royal Infirmary, Foresterhill, Aberdeen, Scotland, AB25 2ZN, United Kingdom
V Shanmugam, Department of Surgery, Lincoln County Hospital, Lincoln, England, LN2 5QY, United Kingdom
SR Yule, Department of Radiology, Aberdeen University and Aberdeen Royal Infirmary, Foresterhill, Aberdeen, Scotland, AB25 2ZN, United Kingdom
Correspondence to: Mr Girivasan Muthukumarasamy, Clinical Research Fellow, Department of Surgery, Polwarth Building, University of Aberdeen, Aberdeen,
United Kingdom. sur197@abdn.ac.uk
Telephone: +44-7966-228305
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Abstract

Acute pancreatitis constitutes 3% of all admissions with abdominal pain. There are reports of osteal fat necrosis leading to periosteal reactions and osteolytic lesions following severe pancreatitis, particularly in long bones. A 54-year-old man was admitted to our hospital with acute pancretitis, who later developed spinal discitis secondary to necrotizing pancreatitis. He was treated conservatively with antibiotics and after a month he recovered completely without any neurological deficit. This case is reported for its unusual and unreported spinal complications after acute pancreatitis.

Key words: Acute pancreatitis; Spinal osteomyelitis; Lumbar discitis; Fat necrosis; Necrotizing pancreatitis

Muthukumarasamy G, Shanmugam V, Yule SR, Ravindran R. An unreported complication of acute pancreatitis. *World J Gastroenterol* 2007; 13(27): 3756-3757

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INTRODUCTION

Acute pancreatitis constitutes 3% of all admissions with abdominal pain[1]. The majority of cases are mild and self-limiting. However, severe pancreatitis is associated with high morbidity and mortality and affects almost all the organs in the body. The involvement of the skeletal system has been reported after an attack of acute pancreatitis[2,3]. There are reports of osteal fat necrosis leading to periosteal reactions and osteolytic lesions following pancreatitis, particularly common in long bones[2,4]. Nevertheless, pyogenic spinal osteomyelitis in association with pancreatitis is rarely reported[5]. We present one of the rare complications of acute necrotizing pancreatitis, the spinal discitis.

CASE REPORT

A 54-year-old male chronic alcoholic patient with vomiting and abdominal pain was referred by his general practitioner (GP) to the general surgical emergency ward. The referring doctor recorded a pulse rate of 120/min and blood pressure of 220/130 mmHg. There was no relevant past medical history except recent diagnosis of reflux oesophagitis. The patient was not on any regular medications. Clinical examination revealed diffuse abdominal tenderness and guarding (serum amylase, 1150 U/L; CRP, 180 mg/L). A provisional diagnosis of acute alcoholic pancreatitis was made and managed symptomatically. Three days later, the patient discharged himself against medical advice.

The next day, he was re-referred by his GP with abdominal pain, vomiting, diarrhoea, and malena. His vitals showed tachycardia and tachypnoea. He was flushed and his abdomen distended with diffuse tenderness and guarding. Glasgow severity score for pancreatitis was 3 at this point. Computerized tomography (CT) scan of the abdomen showed extensive pancreatic necrosis with peri-pancreatic fluid collection (15 cm × 13.3 cm) and bilateral pleural effusions. There were no radiological signs of peri-pancreatic infection. Two weeks later, he developed vomiting with pyrexia; white cell count (20.9×10^9/L) and C-reactive protein were elevated (239 mg/L), and haemoglobin dropped from 157 to 91 mg/L. Abdomen was more distended with diffuse tenderness. Ultrasound guided fine needle aspiration of peri-pancreatic collection revealed gram-positive cocci on microscopic examination. Laparotomy, necrosectomy and debridement of peri-pancreatic area were performed and the abdominal wound closed 48 h later, after lateral relaxing skin incision. Post-operatively, the patient was managed in the intensive therapy unit (ITU) for cardiac and respiratory support. His postoperative recovery was eventful; complicated by renal failure (requiring renal support), left sided cerebral infarct, respiratory failure, candidaemia, staphylococcal septicaemia and pulmonary embolism from ilio-femoral deep venous thrombosis (optease vena cava filter deployed). Persistent pyrexia and imaging evidence of recurrent pancreatic abscess lead to open drainage through a left lumbar incision. Nearly a month later he developed

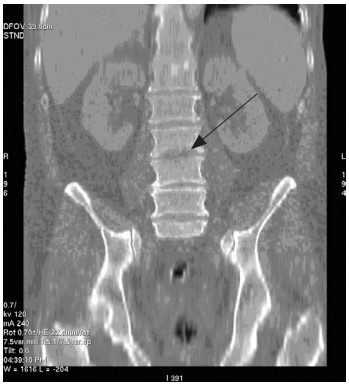

Figure 1 Coronal reconstruction through lumbar spine showing changes in the inter-vertebral disc ($L_{2/3}$) with erosion of vertebral end-plates.

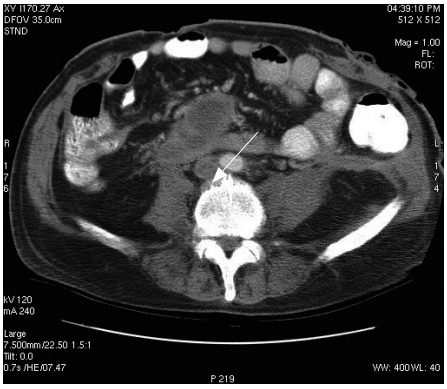

Figure 2 Transverse section through lumbar spine ($L_{2/3}$) showing discitis with soft tissue collection in front of the vertebra, in addition to residual pancreatic collection and retroperitoneal fat-stranding.

back pain. Clinical examination did not reveal any obvious spinal pathology. X-ray of the lumbar spine demonstrated loss of lordosis and decreased inter-vertebral disc heights at $L_{3/4}$ and $L_{4/5}$ with associated sclerosis and osteophytosis. However, there was no collapse of vertebral bodies. A radiological diagnosis of degenerative change was made, and managed symptomatically after appropriate orthopaedic advice. Follow-up CT scan of the abdomen after a month demonstrated irregularity of the end plates of $L_{2/3}$ with a small but definite fluid collection surrounding the anterior aspect of this inter-vertebral disc, raising the strong suspicion of infective discitis (Figures 1 and 2). The patient was managed conservatively with antibiotics without any residual deformity.

Recent radioactive bone scan revealed no evidence of active infection. On clinical examination, he completely recovered (without neurological deficit) except for occasional back pain and an incisional hernia.

DISCUSSION

Metastatic fat necrosis following an episode of pancreatitis or pancreatic carcinoma in adults is a well-known entity and reported to manifest in long bones, pericardium, mediastinal fat and subcutaneous tissue[2,4,6]. Two types of bone lesions were described. The first type produces multiple osteolytic lesions and periosteal reaction with intramedullary fat necrosis; the second type is the result of bone infarction. Neuer *et al*[7] reported a case of osteolytic lesions in a 3-year-old child after traumatic pancreatitis. However, the exact mechanism for these bony lesions is unclear. Circulating pancreatic enzymes (trypsin, elastase, lipase and collagenase) have been implicated as the causation for the intramedullary fat necrosis[2]. Perry, from his rat experiment, demonstrated lymphatic transport of free enzymes resulted in metastatic fat necrosis[3]. This was hypothesized as the mechanism for metastatic ischaemic lesions noticed in pancreatitis. Shinowara *et al*[8] had proposed the possibility of ischaemic necrosis caused by occlusion of end arteries due to intravascular thrombosis in pancreatitis. Another possible explanation, particularly for spinal osteomyelitis, is that the inflamed pancreas might produce local tissue ischaemia, predisposing to lower thoracic or upper lumbar osteomyelitis due to the close proximity of pancreas to these structures[9].

So far, there has been no reported evidence of spinal discitis following an episode of pancreatitis. Glassman *et al*[5] have published a case of pyogenic osteomyelitis associated with a previous history of pancreatitis. However, no concurrent spinal disease and pancreatitis was established. They raised the suspicion of bacterial septicaemia, as a complication of pancreatitis, and as the cause of spinal osteomyelitis. In our report, the patient was proved to have definite clinical, radiological and bacteriological evidence of pancreatitis and septicaemia. This correlates well with pancreatitis as the cause for spinal disease. Though the mechanism of involvement of spinal vertebrae in our patients was not clear, the different possibilities including direct spread of infection or chemical soft tissue destruction (because of the closeness of lesion to pancreas) or from metastatic involvement (septicaemia), can be considered. This case was presented for its unusual and unreported spinal complications after acute pancreatitis.

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CASE REPORT

Diagnostic approach using endosonography guided fine needle aspiration for lymphadenopathy in primary sclerosing cholangitis

Shintaro Tsukinaga, Hiroo Imazu, Yujiro Uchiyama, Hiroshi Kakutani, Akira Kuramoti, Masayuki Kato, Keisuke Kanazawa, Tsuyoshi Kobayashi, Yasuyuki Searashi, Hisao Tajiri

Shintaro Tsukinaga, Hiroo Imazu, Yujiro Uchiyama, Hiroshi Kakutani, Akira Kuramoti, Masayuki Kato, Keisuke Kanazawa, Department of Endoscopy, Jikei University School of Medicine, Tokyo, Japan
Tsuyoshi Kobayashi, Yasuyuki Searashi, Hisao Tajiri, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Jikei University School of Medcine, Japan
Correspondence to: Hiroo Imazu, MD, Department of Endoscopy, Jikei University School of Medicine, 3-25-8 Nishi-shinbashi, Minatoku, Tokyo 105-8461, Japan. himazu21@aol.com
Telephone: +81-3-34331111-3181 Fax: +81-3-3459452
Received: 2007-03-18 Accepted: 2007-04-16

Abstract

We report a case of primary sclerosing cholangitis (PSC) with benign lyphadenopathy which was diagnosed with endosonography guided fine needle aspiration (EUS-FNA). A 65-year-old woman was admitted to Jikei University Hospital with severe jaundice. Although endoscopic retrograde cholangiography and liver biopsy revealed the findings consistent with PSC, abdominal computed tomography revealed numerous large perihepatic lymph nodes with a maximum diameter of more than 3 cm. Therefore, EUS-FNA was done in order to exclude malignant lymphadenopathy, and adequate specimens obtained by EUS-FNA showed reactive hyperplasia of lymphnode. The patients were scheduled to undergo liver transplantation.

Key words: Endosonography guided fine needle aspiration; Primary sclerosing cholangitis; Lymphadenopathy

Tsukinaga S, Imazu H, Uchiyama Y, Kakutani H, Kuramoti A, Kato M, Kanazawa K, Kobayashi T, Searashi Y, Tajiri H. Diagnostic approach using endosonography guided fine needle aspiration for lymphadenopathy in primary sclerosing cholangitis. *World J Gastroenterol* 2007; 13(27): 3758-3759

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INTRODUCTION

Primary sclerosing cholangitis (PSC) is a disease of unknown etiology that is associated with inflammatory bowel disease and with an increased risk of developing cholangiocarcinoma[1]. Although the course of PSC varies from one patient to another, the disease is generally progressive and usually leads to the development of liver cirrhosis. Advanced PSC is therefore considered to be an indication for liver transplantation. It has been reported that perihepatic lymphadenopathy occurs in approximately 70% of patients with PSC[2,3]. Although most of such lymphadenopathy is benign, it can also be caused by concomitant cholangiocarcinoma[2]. It is very important to detect malignant lymphadenopathy in PSC patients, because the existence of a malignant tumor, especially cholangiocarcinoma, has an adverse effect on survival after liver transplantation[1,2].

CASE REPORT

A 65-year-old woman was admitted to Jikei University Hospital (Tokyo, Japan) with severe jaundice. Laboratory tests revealed a serum albumin level of 2.0 mg/dL, while aspartate aminotransferase was 124 IU/L, alanine aminotransferase was 76 IU/L, alkaline phosphatase was 1373 IU/L, and CA19-9 was 114 U/L. Her total bilirubin level was 9.7 mg/dL, with a direct bilirubin level of 5.8 mg/dL. Abdominal computed tomography revealed numerous large perihepatic lymph nodes with a maximum diameter of more than 3 cm, although no mass lesion was seen (Figure 1A). Endoscopic retrograde cholangiography (ERC) showed a bile duct stricture associated with a diverticulum-like out pouching (Figure 1B), and brushing cytology was Class Ⅱ. A diagnosis of PSC was made on the basis of liver biopsy and typical ERC findings, but the possibility of concomitant cholangiocarcinoma was also considered because of her lymphadenopathy and severe bile duct stricture. Therefore, EUS-FNA was conducted to obtain biopsy specimens of the enlarged lymph nodes and to investigate the presence of concomitant cholangiocarcinoma. A curvilinear echoendoscope (GF UCT240-AL5, Olympus Medical Systems, Tokyo, Japan) was advanced into the duodenum, and biopsy of enlarged lymph nodes was done through the duodenal wall using a 22-gauge echo-tipped needle (Willson-Cook, Winston) (Figure 2A). Adequate biopsy specimens were obtained for histological examination, which revealed reactive hyperplasia of the enlarged lymph nodes with

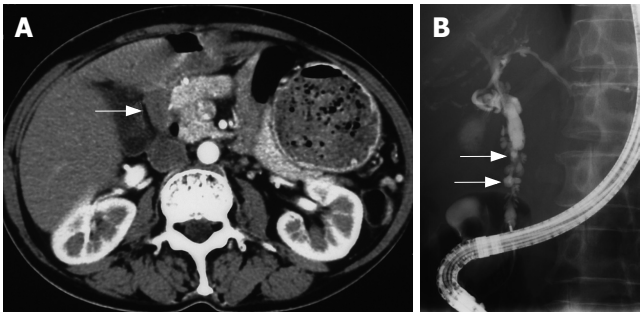

Figure 1 **A**: Abdominal computed tomography reveals markedly enlarged lymph nodes (arrow); **B**: Endoscopic retrograde cholangiography shows a bile duct stricture with a diverticulum-like outpouching (arrows).

infiltration of lymphocytes (Figure 2B). Since concomitant cholangiocarcinoma was excluded by this histological finding and brushing cytology under ERC, she was scheduled to undergo liver transplantation. Although she is waiting for liver transplantation for six months at present, malignant lesion including cholangiocarcinoma and additional lymphadenopathy has never been seen.

DISCUSSION

EUS-FNA has emerged as an effective method for obtaining tissue samples from the perigastric and periduodenal organs, such as the pancreas, and for the investigation of peri-intestinal and celiac lymphadenopathy[4]. Although a high sensitivity and specificity of EUS-FNA without any severe complications have been shown for the diagnosis of various diseases[4], there have been few information about its use for lymphadenopathy in patients with PSC. In our present patient with PSC, EUS-FNA was both convenient and effective for making a diagnosis of perihepatic lymphadenopathy. We recommend this diagnostic approach to lymphadenopathy in patients with PSC, especially prior to liver transplantation, to exclude concomitant malignancy. However, further studies will be needed to confirm the clinical value of EUS-FNA for patients with PSC.

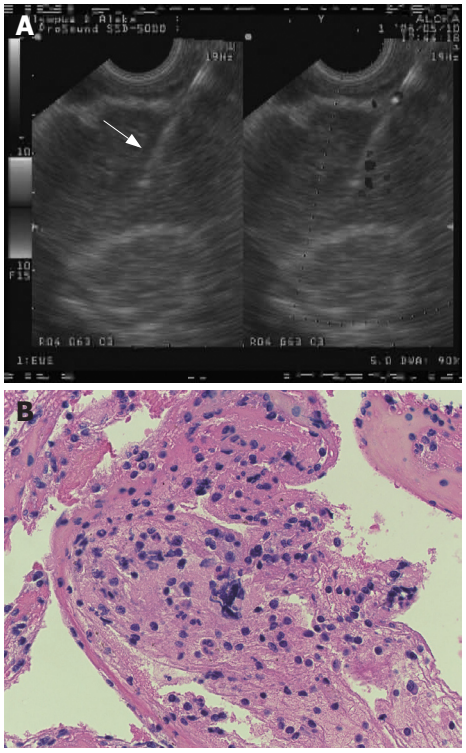

Figure 2 **A**: Endosonographic findings during EUS-FNA. The arrow indicates the biopsy needle inside an enlarged lymph node; **B**: Histological features the specimen obtained by EUS-FNA.

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CASE REPORT

Eosinophilic cholecystitis along with pericarditis caused by *Ascaris lumbricoides*: A case report

Kosuke Kaji, Hitoshi Yoshiji, Masahide Yoshikawa, Masaharu Yamazaki, Yasuhide Ikenaka, Ryuichi Noguchi, Masayoshi Sawai, Masatoshi Ishikawa, Tsuyoshi Mashitani, Mitsuteru Kitade, Hideto Kawaratani, Masahito Uemura, Junichi Yamao, Masao Fujimoto, Akira Mitoro, Masahisa Toyohara, Motoyuki Yoshida, Hiroshi Fukui

Kosuke Kaji, Hitoshi Yoshiji, Masaharu Yamazaki, Yasuhide Ikenaka, Ryuichi Noguchi, Masayoshi Sawai, Masatoshi Ishikawa, Tsuyoshi Mashitani, Mitsuteru Kitade, Hideto Kawaratani, Masahito Uemura, Junichi Yamao, Masao Fujimoto, Akira Mitoro, Masahisa Toyohara, Motoyuki Yoshida, Hiroshi Fukui, Third Department of Internal Medicine, Nara Medical University, Nara, Japan
Masahide Yoshikawa, Department of Parasitology, Nara Medical University, Nara, Japan
Correspondence to: Hitoshi Yoshiji, MD, PhD, Third Department of Internal Medicine, Nara Medical University, Shijo-cho 840, Kashihara, Nara 634-8522, Japan. yoshijih@naramed-u.ac.jp
Telephone: +81-744-22-30513415 Fax: +81-744-247122
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Abstract

Although the etiology of eosinophilic cholecystitis is still obscure, the postulated causes include allergies, parasites, hypereosinophilic syndrome, and eosinophilic gastroenteritis. It is sometimes accompanied by several complications, but a simultaneous onset with pericarditis is very rares. A 28-year-old woman complained of acute right hypocondrial pain and dyspnea associated with systemic eruption. Several imaging modalities revealed acute cholecystitis and pericarditis with massive pericardial effusion. A marked peripheral blood eosinophilia was observed, and the eruption was diagnosed as urticaria. Her serum had a high titer of antibody against *Ascaris lumbricoides*. Treatment with albendazole drastically improved all clinical manifestations along with normalization of the imaging features and eosinophilia. We report herein a rare case of simultaneous onset of acute cholecystitis and pericarditis associated with a marked eosinophilia caused by parasitic infection.

Key words: Cholecystitis; Pericarditis; Ascaris; Parasite; Eosinophilia; Albendazole

Kaji K, Yoshiji H, Yoshikawa M, Yamazaki M, Ikenaka Y, Noguchi R, Sawai M, Ishikawa M, Mashitani T, Kitade M, Kawaratani H, Uemura M, Yamao J, Fujimoto M, Mitoro A, Toyohara M, Yoshida M, Fukui H. Eosinophilic cholecystitis along with pericarditis caused by *Ascaris lumbricoides*: A case report. *World J Gastroenterol* 2007; 13(27): 3760-3762

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INTRODUCTION

Eosinophilic cholecystitis is an infrequent form of cholecystitis[1,2]. In the clinical practice, eosinophilic cholecystitis is usually unsuspected and is clinically indistinguishable from the predominant form of calculous cholecystitis[2,3]. Although the etiology of eosinophilic cholecystitis is still obscure, the postulated causes include allergies, parasites, hypereosinophilic syndrome, eosinophilic gastroenteritis, and local reaction to gallstones[4]. It sometimes shows a marked peripheral blood eosinophilia. Peripheral eosinophilia indicates that eosinophilic cholecystitis is likely to be a manifestation of a systemic hypereosinophilic disorder[4]. A marked peripheral blood eosinophilia is associated with several biological conditions, such as allergy, hypersensitivity diseases, parasitic infections, connective tissue disorders, blood dyscrasias, malignancies, and immunodeficiency status[5,6]. Eosinophilic infiltration can be associated with disorders of the lung, heart, gastrointestinal tract, biliary tract, and gall bladder[6]. Although eosinophilic cholecystitis sometimes accompanies other organ dysfunction, close temporal association with pericarditis has been very rarely reported so far.

Ascariasis, a helminthic infection of man, is the most common parasitic infection of the gastrointestinal tract, but invasion of worms into the gall bladder is rare (2.1% of the hepatobiliary ascariasis in endemic areas)[7,8]. Regarding the imaging modalities available for diagnosis of biliary ascariasis, recent studies have shown that abdominal ultrasonography (US) and magnetic resonance cholangiopancreatography (MRCP) are very useful, sensitive, safe, and non-invasive[8-10]. Herein, we report a case that showed simultaneous onset of acute cholecystitis and pericarditis (diagnosed by abdominal US and MRCP) along with a marked eosinophilia caused by *Ascaris lumbricoides* infection.

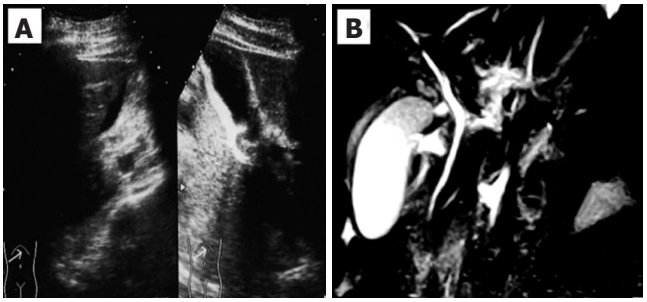

Figure 1 A: Abdominal ultrasonography (US) revealed a marked gall bladder swelling and wall thickness without stones or debris, indicating an acute cholecystitis; **B**: Magnetic resonance cholangiopancreatography (MRCP) revealed a similar gall bladder thickness as well as free space around the gall bladder, suggesting exudate associated with severe inflammation.

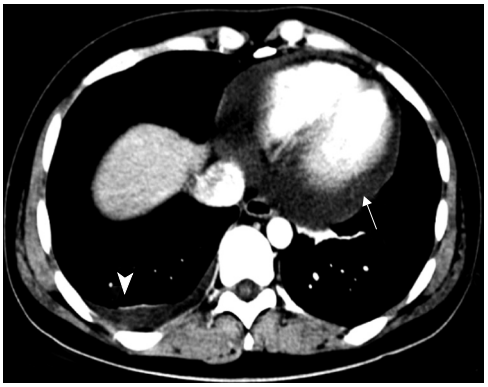

Figure 2 A chest computed tomography (CT) scan showing massive pericardial effusion (arrow) and right pleural effusion (triangle).

CASE REPORT

A 28-year-old woman was admitted into our hospital complaining of acute right hypocondrial pain and dyspnea associated with systemic eruption. She did not have specific past medical history including allergic reactions. Physical examination demonstrated a severe right hypocondrial tenderness, high-grade fever, and systemic eruption. The systemic eruption mainly consisted of wheal, and was diagnosed as urticaria. Laboratory examination revealed mild elevation of transaminases, biliary enzymes, and a marked eosinophilia along with elevation of IgE. Abdominal US revealed that a marked gall bladder swelling and wall thickness without stones or debris, indicating an acute cholecystitis (Figure 1A). Subsequently, MRCP was carried out for further confirmation of the US diagnosis. MRCP revealed similar findings as well as a free space around the gall bladder, suggesting exudates associated with severe inflammation (Figure 1B). Furthermore, chest computerized tomography (CT) scanning, in turn, revealed a massive pericardial effusion and moderate right pleural effusion (Figure 2). Echocardiography confirmed a massive pericardial effusion without asynergy or valvular disorder. Although we could not detect any worm lying either in the bile duct or gall bladder, we suspected parasitic infection because of marked eosinophilia and IgE elevation along with systemic urticaria [31% of leukocyte (7500/μL) and 246.6 U/mL, respectively]. Her serum had a high titer of antibody against Ascaris suum and Toxocara canis (pig ascaris and dog ascaris, respectively) by enzyme-linked immunosorbent assay (ELISA) (generously measured by Dr. Hiromatsu; Department of Parasitology, Miyazaki University School of Medicine) (150-fold and 100-fold higher as compared with the control serum, respectively). Treatment with albendazole (600 mg/d) drastically improved all clinical manifestations along with normalization of the imaging findings, eosinophilia, and IgE elevation within a week after the treatment. Abdominal US revealed disappearance of the gall bladder swelling and wall thickness after only four days treatment with albenzasole (Figure 3). The free space around the gall bladder vanished, too. She discharged the hospital four-week after the treatment with albendazole.

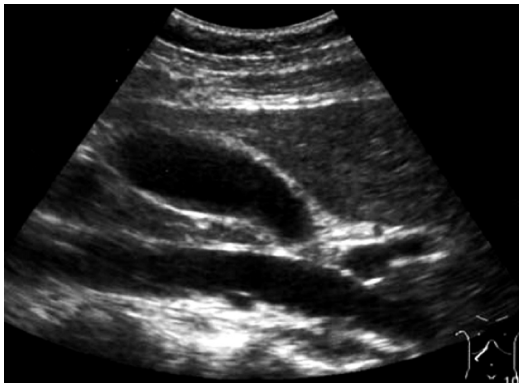

Figure 3 After treatment with albendazole, abdominal US revealed disappearance of the gall bladder swelling and wall thickness.

DISCUSSION

Eosinophilic cholecystitis is an infrequent form of cholecystitis that was first described by Albot in 1949[1]. The physical and laboratory findings are not specific. Although the etiology of eosinophilic cholecystitis is still uncertain, the postulated causes include parasites, gallstones, and allergic hypersensitivity response to drugs such as phenytoin, erythromycin, cephalosporin, and herbal medications[4,11]. Eosinophilic cholecystitis may develop with several diseases, such as eosinophilic gastroenteritis, eosinophilic pancreatitis, and idiopathic hypereosinophilic syndrome (HES)[6,12,13]. In the literature, we found only one report on a case of eosinophilic cholecystitis that developed almost simultaneously with eosinophilic appendicitis and eosinophilic pericarditis[4]. Our case is very rare because of the simultaneous onset of eosinophilic cholecystitis and pericarditis caused by parasite; namely, *Ascaris lumbricoides*.

Unlike eosinophilic gastritis, most patients with eosinophilic cholecystitis have no history of allergy as in our case[14]. Peripheral hypereosinophilia is not a constant finding, either, although the characteristic histologic feature of eosinophilic cholecystitis is transmural inflammatory infiltration of the gall bladder wall that is composed of more than 90% eosinophils[2]. In the current case, we did not have any histological evidence of eosinophilic cholecystitis since the patient did not

undergo cholecystectomy. This is the main negative point of the final diagnosis of eosinophilic cholecystitis in our case. However, the patient exerted a marked peripheral eosinophilia and elevation of IgE along with confirmation of acute cholecystitis by several imaging modalities. It has been reported that a marked peripheral eosinophilia usually correlates with eosinophilic infiltration in the gall bladder wall[2,4,14], indicating that, at least, there was some infiltration of eosinophils in the gall bladder in our case. After treatment with albendazole, eosinophilia, IgE elevation, and all clinical manifestations drastically improved along with normalization of the imaging features.

Ascariasis is the most common helminthic infection, which occurs commonly in the tropical and/or developing countries, partly due to the poor sanitary and hygienic conditions that help maintain the infection cycles[15]. In addition to these areas, ascaris infection is sometimes observed even in the industrial countries because raw food may be contaminated with worms. From the duodenum, the worms can enter the biliary tract resulting in right hypocondrial pain. Our patient had eaten fresh vegetables on the day before admission and had severe right hypocondrial pain at admission.

Abdominal US is now recognized as a very useful modality for diagnosis of biliary ascariasis[10]. It can detect adult worms in the biliary tract as a longitudinal structure with inner parallel linear bands and undulating movements inside the gall bladder[8]. MRCP has been the latest innovation for examination of the biliary tract. MRCP has the advantage of providing three-dimensional images of the biliary tract without the risks of contrast allergy and invasiveness. On MRCP imaging, adult worms may be detected with round shape lying obliquely in the bile duct[9]. These modalities sometimes can monitor the slow movement of the worms inside the bile duct and gall bladder. Since we could not detect the worm with either modality, these movements could not be followed up in our case. Probably, only the larva of *Ascaris lumbricoides* migrated into the biliary tract and gall bladder, which were too small to be detected with these modalities in the current case.

In conclusion, herein we reported a rare case of simultaneous onset of acute cholecystitis and pericarditis associated with a marked eosinophilia caused by *Ascaris lumbricoides*. Since the physical findings of eosinophilic cholecystitis are indistinguishable from manifestations of the common acute cholecystitis, physicians should be aware of biliary ascariasis in patients with manifestations of acute cholecystitis, and should search for worms by abdominal US and MRCP.

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CASE REPORT

Endoscopic resection of carcinoid of the minor duodenal papilla

Takao Itoi, Atsushi Sofuni, Fumihide Itokawa, Takayoshi Tsuchiya, Toshio Kurihara, Fuminori Moriyasu

Takao Itoi, Atsushi Sofuni, Fumihide Itokawa, Takayoshi Tsuchiya, Toshio Kurihara, Fuminori Moriyasu, Department of Gastroenterology and Hepatology, Tokyo Medical University, Tokyo, Japan
Correspondence to: Takao Itoi, MD, PhD, Department of Gastroenterology and Hepatology, Tokyo Medical University Shinjuku-ku, Nishishinjuku 6-7-1, Tokyo, Japan. itoi@tokyo-med.ac.jp
Telephone: +81-3-33426111 Fax: +81-3-53816654
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Abstract

We encountered a 65-year-old man with a carcinoid tumor of the minor duodenal papilla. Since he had liver cirrhosis and completely refused surgery, we performed an endoscopic snare papillectomy. The papillectomy was performed successfully without procedure-related complication. The specimens revealed a carcinoid tumor showing that the margin of the tumor was positive. One week later, upper GI endoscopy was performed and the biopsy specimens obtained from base of ulcer showed no neoplastic cells. We performed a duodenoscopy and CT 3, 6 and 18 mo later, and there was no macroscopic or microscopic evidence of tumor recurrence after more than 4 years.

Key words: Endoscopic papillectomy; Carcinoid tumor; Minor duodenal papilla; Papilla of Vater tumor; Duodenal papilla

Itoi T, Sofuni A, Itokawa F, Tsuchiya T, Kurihara T, Moriyasu F. Endoscopic resection of carcinoid of the minor duodenal papilla. *World J Gastroenterol* 2007; 13(27): 3763-3764

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INTRODUCTION

Recently, endoscopic papillectomy for not only adenoma, but also carcinoid of the major duodenal papilla is being increasingly performed as a minimally invasive alternative to conventional surgery[1-5]. However, it is controversial among endoscopists whether this technique can be adaptable for lesions of the minor duodenal papilla. We describe the successful endoscopic papillectomy of carcinoid of minor duodenal papilla and discuss the applications of the procedure.

CASE REPORT

A 65-year-old asymptomatic man underwent esophagogastroduodenal (EGD) endoscopy screening, which revealed a slightly swollen and yellowish minor duodenal papilla. A biopsy specimen from the lesion revealed a carcinoid tumor. EUS demonstrated a 12 mm × 11 mm hypoechoic mass located in the submucosa (Figure 1). CT revealed a slightly contrast-enhanced duodenal tumor without metastasis to the regional lymph nodes or liver. ERCP findings were normal, demonstrated dominant pancreatic drainage via the ventral duct and no effacement of the dorsal duct by tumor. The routine laboratory tests were normal, including tumor markers. Since he had liver cirrhosis and completely refused surgery, we then performed an endoscopic snare papillectomy after obtaining appropriate written informed consent. Snare excision was performed with a polypectomy snare forceps (SD-5U-1, 6U-1, Olympus Medical Systems, Tokyo, Japan) and a generator with an automatically controlled cut-out system (ICC200, Erbe Elektromedizin GmbH, Tubingen, Germany). The papillectomy was performed successfully without any procedure-related complications (Figure 2A and B). The specimens revealed a carcinoid tumor and showed that the margin of the tumor was positive (Figure 3A). One week later, EGD was performed and the biopsy specimens obtained from the base of ulcer showed no neoplastic cells (Figure 3B). We performed a duodenoscopy and CT 3, 6 and 18 mo later (Figure 4), and there was no macroscopic or microscopic evidence of tumor recurrence more than 4 years.

DISCUSSION

Carcinoid tumor of the gastrointestinal tract has a relatively high occurrence rate and often shows invasive growth[6]. Although Noda *et al* reported that, microscopically, in the duodenal papilla, especially the minor duodenal papilla, carcinoids and endocrine cell micronests seemed to occur more frequently than generally thought[7], clinically, carcinoid tumors of the major or minor duodenal papilla are rare[6-8]. No definitive statement can be made regarding the optimal treatment of carcinoid of the major duodenal papilla, given the small number of cases in the literature. Hatzitheoklitos *et al* reported that carcinoid of the papilla of less than 10 mm in size

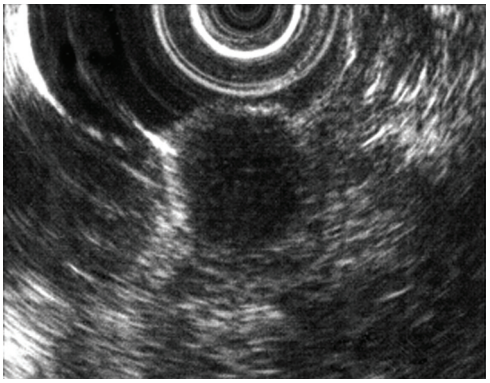

Figure 1 EUS revealed localized hypoechoic mass below the mucosal layer.

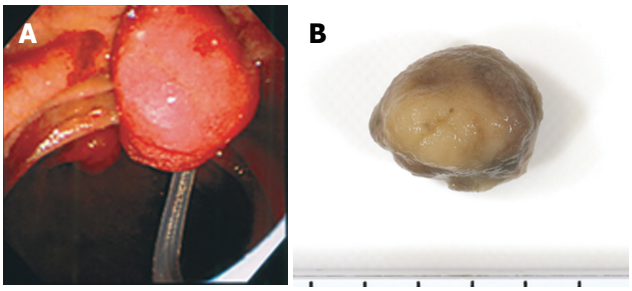

Figure 2 **A**: Endoscopic snare papillectomy of minor papilla was performed; **B**: Macroscopically, the tumor was yellowish and hard.

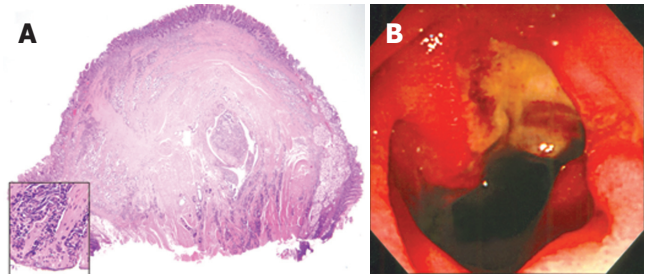

Figure 3 **A**: Histologically, the specimens showed invasive carcinoid tumor cells and the resected cut-end margin showed cancer cells; **B**: Duodenoscop revealed ulcer formation after the papillectomy.

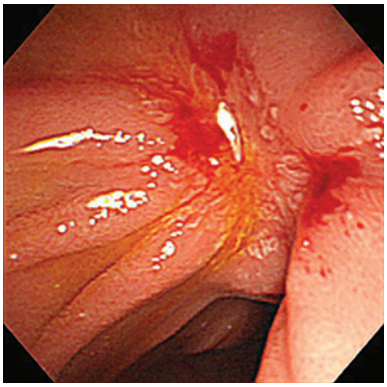

Figure 4 There was no evidence of recurrence 18 mo after the papillectomy.

rarely metastasizes (2%), whereas 20% of tumors 10 to 20 mm and 75% of tumors greater than 20 mm in diameter metastatisize[9]. Radical surgery, such as local excision or pancreaticoduodenectomy, has generally been preferred as the treatment of choice for carcinoid tumors of the papilla[6,8,9].

Endoscopic snare papillectomy has been accepted as a safe and feasible treatment for adenoma of the major duodenal papilla because of lower operative mortality and morbidity, provided that certain criteria are strictly observed[1-4]. Furthermore, recently, endoscopic papillectomy has been performed for not only adenoma of major duodenal papilla but also ampullary adenoma with intraductal extension and invasive carcinoma[3,10] or carcinoid tumor[3,5]. To the best of our knowledge, this is first report of a successful papillectomy for carcinoid tumor of the minor duodenal papilla. The growth pattern of carcinoids, however, is essentially frequently submucosal invasive. We think that there were two fortunate points in this case. One was that the growth pattern of this tumor showed mainly protrusion to the duodenal lumen, and the other was that follow-up biopsy was negative, perhaps because the cauterizing effect resulted in elimination any remaining carcinoid tumor cells despite the positive cut margin.

In conclusion, although further studies with long-term follow-up are needed to determine the ultimate outcome, this case, with a 4-year disease-free follow-up period, suggests that endoscopic resection of carcinoid of the minor duodenal papilla is one option when surgery cannot be applied in patients with minor duodenal papilla tumors.

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CASE REPORT

Inflammatory fibroid polyp occurring in the transverse colon diagnosed by endoscopic biopsy

Shoji Hirasaki, Minoru Matsubara, Fusao Ikeda, Hideaki Taniguchi, Seiyuu Suzuki

Shoji Hirasaki, Minoru Matsubara, Fusao Ikeda, Hideaki Taniguchi, Seiyuu Suzuki, Second Department of Internal Medicine, Sumitomo Besshi Hospital 3-1 Ohji-cho, Niihama 792-8543, Japan
Correspondence to: Dr. Shoji Hirasaki, Second Department of Internal Medicine, Sumitomo Besshi Hospital, 3-1 Ohji-cho, Niihama 792-8543, Japan. shoji_hirasaki@ni.sbh.gr.jp
Telephone: +81-897-377111 Fax: +81-897-377121
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Abstract

A case of an inflammatory fibroid polyp occurring in the transverse colon and diagnosed by endoscopic biopsy is reported. The patient was an 82-year-old man who visited our hospital for further evaluation of occult blood in stool. The Colonoscopy revealed a small, red, and peduncular polyp, about 6 mm in diameter, in the transverse colon. Histological examination of the biopsy specimen obtained from the polyp revealed proliferation of fibroblasts and infiltration of inflammatory cells such as plasma cells and eosinophils. This polyp was diagnosed as an inflammatory fibroid polyp, which can appear in many different locations throughout gastrointestinal tract, though still rare in the transverse colon.

Key words: Inflammatory fibroid polyp; Colonoscopy; Inflammation; Type Ⅱ pit pattern

Hirasaki S, Matsubara M, Ikeda F, Taniguchi H, Suzuki S. Inflammatory fibroid polyp occurring in the transverse colon diagnosed by endoscopic biopsy. *World J Gastroenterol* 2007; 13(27): 3765-3766

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INTRODUCTION

Inflammatory fibroid polyp (IFP) is a relatively rare disorder, which is thought to be clinically and histologically benign, and was first described as "polypoid fibroma" in 1920 by Konjetzny[1]. It can appear in many different locations throughout the gastrointestinal tract. The most common site is the gastric antrum (in about 70% of cases), followed by the small bowel (in about 20% of cases)[2]; however, it rarely occurs in the esophagus and large intestine[3]. The tumor appears as a peduncular polyp, and is often an incidental finding on a colorectal examination for underlying disease. There have been few cases of colorectal IFP diagnosed by histological examination obtained by endoscopic biopies[4,5]. Herein we report a man diagnosed with IFP arising in the transverse colon and diagnosed by endoscopic biopsy.

CASE REPORT

An 82-year-old man visited our hospital for further evaluation of fecal occult blood noted in a yearly physical check-up in 2003. He had a past history of an appendectomy from over 50 years ago. No specific family history was identified. Routine hematological examinations and biochemical tests were within normal limits. The colonoscopy revealed a small, red, hard, and peduncular polyp, about 6 mm in diameter in the transverse colon (Figure 1). With conventional colonoscopy, the lesion showed type Ⅱ pit pattern even though the magnifying colonoscopy was not performed, and we speculated that this polyp was non-neoplastic. This polyp was suspected to be a hyperplastic polyp or hamartoma from endoscopic findings. Histological examinations of the biopsy specimen revealed proliferation of fibroblasts and infiltration of inflammatory cells such as plasma cells and eosinophils. The peduncular polyp was diagnosed as an inflammatory fibroid polyp occurring in the transverse colon. The patient rejected a follow-up colonoscopy because of his advanced age.

DISCUSSION

IFP is a rare submucosal lesion of the gastrointestinal tract that usually appears as a smooth sessile or pedunculated polyp and follows a benign course. The pathogenesis of IFP remains unknown. Some authors have proposed that IFP is caused by an allergic reaction to inflammatory stimuli such as bacterial, chemical, traumatic stimuli, etc, or is a reactive lesion of fibroblastic or myofibroblastic nature[4]. Others have proposed that IFP is neurogenic in nature[4,5]. A familial relationship has been described, with multiple recurrent lesions affecting three successive generations[6].

It may occur anywhere throughout the gastrointestinal tract, and the most common site is the gastric antrum,

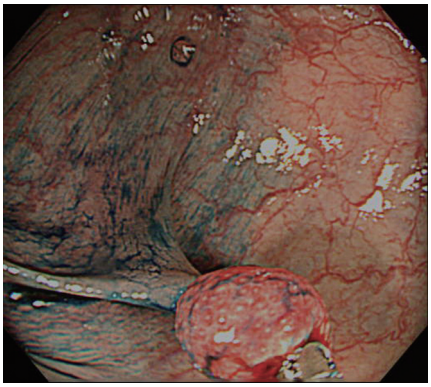

Figure 1 Endoscopic appearance of the transverse colon. A red, hard, and peduncular polyp, about 6 mm in diameter was seen. The lesion showed type Ⅱ pit pattern. Endoscopic biopsy was performed to confirm the histological diagnosis.

followed by the small bowel, and rarely the colon; however, recent advances in diagnostic techniques, especially the widespread use of colonoscopy for colorectal tumors, have enabled us to identify small and asymptomatic polyps, and reports of IFP of the colon have been increasing.

According to Nakase *et al*[7], a review of Japanese literature, revealed 25 cases of IFP in the large intestine in Japan up to 2000. They described that the macroscopic appearance is the pedunculated type in 68% of cases, and the sessile type in 32%. In that report, the sites of IFP in the large intestine were studied and 8 of 25 cases (32%) had lesions in the transverse colon and 13 of 25 patients (52%) had lesions in the ascending colon or cecum. Thus, IFPs of the large intestine are predominantly in the proximal colon. IFPs in the gastrointestinal tract are usually asymptomatic and are often detected incidentally on barium enema or endoscopy[7]. Another review of Western literature revealed that the main clinical features of colonic IFPs are abdominal pain (54%), bloody stools (33%), weight loss (21%), diarrhea, and anemia (17%)[2].

There are no reports describing the pit pattern of the mucosa covering colonic IFP. Two kinds of endoscopic findings of the surface of colonic IFP are described as follows: (1) covered with normal colonic mucosa like submucosal tumors[7], and (2) irregular surface redness. In the present case, hyperplastic changes in the normal colonic mucosa covering IFP might cause type Ⅱ pit pattern. We speculated that an endoscopic diagnosis of colonic IFPs could seldom be made by surface observation because most fibroids are located in the submucosa of colonic IFPs. The final diagnosis of colonic IFP depends upon pathological findings. However, further studies on the endoscopic findings of the surface of colonic IFP observed by magnifying colonoscopy are certainly required.

As to therapy, IFP of the large intestine can best be removed endoscopically, because it is thought to be clinically and histologically benign. But only 6 of 25 patients (17%) with IFP of the large intestine in Japan were treated with poplypectomy or EMR[7]. If we could confirm the histological diagnosis of small colonic IFP by endoscopic biopsy, as in the present case, endoscopic resection of IFP might be unnecessary because IFP is a submucosal lesion of the gastrointestinal tract that follows a benign course. Nakase *et al*[7] described that 18 of 25 patients with IFP of the large intestine (72%) in Japan were treated with surgical resection. We think that the ratio of patients with colonic IFP who undergo surgical resection will decrease and endoscopic resection will increase in the future because of recent advances in diagnostic technologies to include improved endoscopic images.

In conclusion, we reported a case of inflammatory fibroid polyp occurring in the transverse colon that was diagnosed by endoscopic biopsy. IFP should generally be taken into consideration as a differential diagnosis of peduncular polyp of the colon. IFP of the large intestine is not fatal and patients remain asymptomatic in their daily lives except for gastrointestinal bleeding or bowel obstruction; therefore, it is likely that, in the future,there will be many latent patients with colorectal IFP who might be incidentally discovered.

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CASE REPORT

Combination of thrombolytic therapy and angioplastic stent insertion in a patient with Budd-Chiari syndrome

Fatemi Reza, Daryani E Naser, Ganaati Hossein, Zahmatkesh Mehrdad

Fatemi Reza, Daryani E Naser, Ganaati Hossein, Zahmatkesh Mehrdad, Gastrointestinal & Liver Diseases Research Center, Semnan University of Medical Sciences, 17th Shahrivar Ave., Semnan, Iran
Supported by Semnan University of Medical Sciences
Correspondence to: Fatemi Reza, MD, Gastrointestinal & Liver Diseases Research Center, Fatemieh Hospital, 17th Shahrivar Ave., Semnan, Iran. nedafat20@yahoo.com
Telephone: +98-231-3328017 Fax: +98-231-3334610
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Abstract

A 31-year-old female who had well-established polycythemia vera one year before, presented with the sudden onset. She had severe ascites and hepatic encephalopathy 12 d prior to admission. Real-time ultrasonography revealed a supra hepatic thrombosis extending toward the inferior vena cava (IVC). Thrombolytic therapy with systemic streptokinase (250 000 IU loading + 100 000 IU/h infusion) was started. At the end of 72 h infusion, the patient's general condition improved. A color Doppler ultrasonography then showed complete and partial resolution of the thrombosis in the supra hepatic vein and IVC, respectively. Despite this good response, 12 d later, the symptoms recurred. Venography detected complete obstruction of the IVC. Percutanous balloon angioplasty with stent insertion was performed successfully and the patient was discharged without any evidence of liver disease. A combination of systemic streptokinase and radiological intervention was effective in our patient.

Key words: Hepatic vein thrombosis; Anticoagulants; Thrombolytic therapy; Stents

Reza F, Naser DE, Hossein G, Mehrdad Z. Combination of thrombolytic therapy and angioplastic stent insertion in a patient with Budd-Chiari syndrome. *World J Gastroenterol* 2007; 13(27): 3767-3769

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INTRODUCTION

Budd-Chiari syndrome (BCS) is a rare entity caused by the obstruction, usually thrombotic in origin, of the hepatic venous outflow[1]. It may be a result of various etiologies including myeloproliferative disorders (polycythemia rubra vera, chronic myelogenous leukemia, essential thrombocythemia, agnogenic myeloid metaplasia), malignancies, infections and benign hepatic lesions, oral contraceptives, pregnancy and other hypercoagulability, states. It usually involves one, two or all the three major hepatic veins with or without extension of the thrombus into the inferior vena cava (IVC). The syndrome may presents with acute (sometimes fulminant), subacute or chronic signs and symptoms of abdominal pain, hepatomegaly, ascites, the lower extremities edema, and venous collateral formation. Without treatment, patients often die primarily of progressive liver failure from chronic hepatic congestion[2].

So far, different therapeutic modalities have been suggested. These include medical treatments (supportive measures, anticoagulants, and thrombolytics), radiological procedures e.g., angioplasty, trans-jugular intrahepatic portacaval shunt (TIPS) and stenting, and surgical interventions (shunting procedures and liver transplantation). There are only few reports of success following conventional treatments such as anticoagulants and diuretics. Radiological procedures and surgical interventions are the most effective treatments recommended for BCS. These include angioplasty, stenting, open endvenectomy[3], shunt and TIPS operations, and more recently, liver transplantation[2].

Fibrinolytic therapy should be restricted to acute or subacute conditions when the formed thrombus is young enough to be resolved by thrombolytic therapy[1].

In this report, we present a patient with BCS who was treated successfully with a combination of thrombolytic therapy and angioplasty with stent insertion.

CASE REPORT

A 31-year-old woman, with a one-year history of polycythemia vera was admitted to our center for a progressive abdominal distension, right upper quadrant abdominal pain, nausea and vomiting for 12-15 d. On physical examination, a massive ascites, pitting edema of the lower extremities and hepatosplenomegaly were evident. Based on the laboratory findings (Table 1), the patient was diagnosed as having stage Ⅱ hepatic encephalopathy. Real-time ultrasonography, color Doppler and Venography of abdominal veins showed a thrombosis in the hepatic vein and IVC, 5 cm proximal to the hepatic vein junction, deteriorating the blood flow of the portal

Table 1 Changes in laboratory findings within 10 d

	Day of submission			
	1st	2nd	4th	10th
WBC (/mm^3)	18000	16500	14300	8000
Hb (g/dL)	19	16	12	11.5
Plt (/mm^3)	421000	410000	380000	275000
Cr (mg/dL)	2	1.8	1.8	1.3
BUN (mg/dL)	29	25	27	18
ALT (U/L)	101	84	61	22
AST (U/L)	165	127	87	39
ALP (U/L)	315	229	218	-
BLT (T) (mg/dL)	3.7	-	-	1.8
BLT (D) (mg/dL)	1.9	-	-	0.45
PT[1] (s)	20	19	15	22[2]
PTT (s)	55	50	49	45

[1]Mean normal PT was 13 s; [2]Patient received warfarin.

Figure 1 Venography of complete obstruction of IVC.

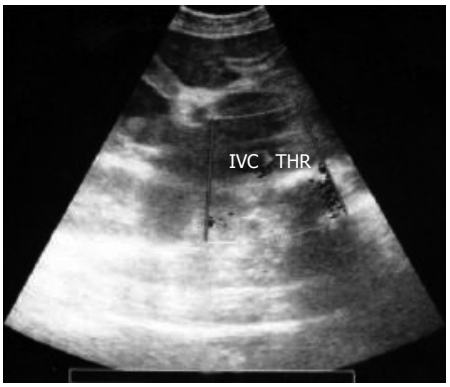

Figure 2 Location of venous thrombosis in IVC on the ultrasound.

vein and IVC (Figures 1 and 2). The renal vein was normal. The hepatic and splenic longitudinal diameters were 20 and 16 cm, respectively. The urinary output was slightly low < 400 mL/24 h. All these findings were clearly in favor of an acute BCS. The initial treatment was systemic streptokinase at a loading dose of 250000 IU for 30 min, followed by a maintenance dose of 100000 IU/h for 72 h. She also received the standard treatment for encephalopathy. Streptokinase therapy increased the urinary flow to 35 mL/h and reduced both the ascites and the liver size. After four days, the hepatic encephalopathy subsided completely.

During the thrombolytic therapy, the serum fibrinogen

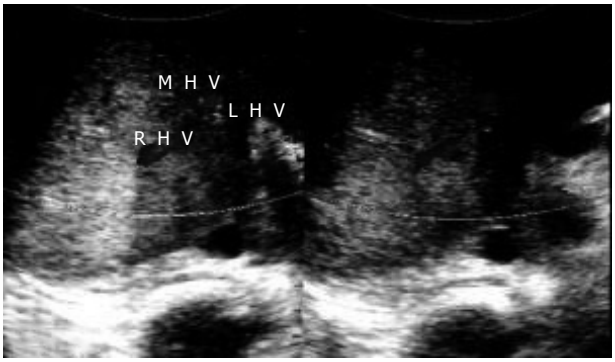

Figure 3 Post-stenting sonography, showing the right, left and middle hepatic veins.

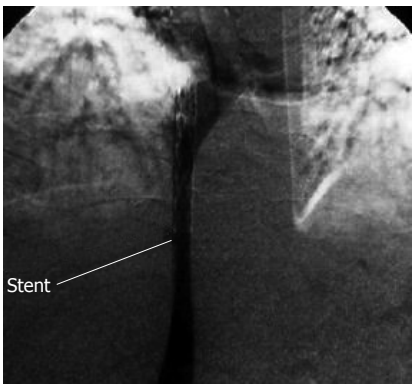

Figure 4 Post-stenting venography.

level was measured every 12 h, which ranged between 160 and 200 mg/dL. After completion of thrombolytic therapy (on the third day), Doppler ultrasonography showed an acceptable patency of both IVC and hepatic vein due to complete resolution of the thrombi (Figure 3). Thereafter, the standard anticoagulant therapy was started. The patient was heparinized first and followed by administration of warfarin for attaining a target international normalization ratio (INR) of 2 to 3. To maintain a hemoglobin level < 12 g/dL, 1000 mg/d hydroxyurea along with repeated aggressive phlebotomy was administered from the first day. The patient's weight and abdominal distension gradually reduced until the 15th d after admission.

After two weeks, the abdominal pain and a progressive ascites recurred unexpectedly. Venography showed a 5-cm thrombus located in the IVC, proximal to the hepatic vein. The hepatic venous flow was minimal, yet no thrombosis was observed. After percutanous transluminal balloon angioplasty (PTA) was performed, the blood flow of the IVC was restored. However, over the next 48 h, the flow decreased and recurrent stenosis made it necessary to repeat PTA with insertion of a wall stent in the IVC (Figure 4). Two wall stents were placed in the IVC. Color Doppler ultrasonography after stenting showed a complete flow with good patency of both the hepatic vein and IVC. The patient was kept under close observation during the early post-intervention period. Over the next 48 h, the patient's symptoms subsided, the

urinary output increased, and the abdominal girth returned to normal. The anticoagulant therapy was instituted. ASA 325 mg/d was added after the stent insertion. Five days after the intervention, the laboratory tests revealed WBC: 11000/mm^3, Hb: 12.5 g/dL, serum creatinine: 1.2 mg/dL, BUN: 23 mg/dL, ALT: 19 IU/L, AST; 25 IU/L, a total serum billirubin level; 0.6 (direct = 0.2) mg/dL, PTT: 40 s, and INR: 2 (PT = 20 s). After one year at follow-up, the patient's status was satisfactory and the hepatic vein and IVC blood flow was complete as assessed by color Doppler ultrasonography at each visit.

DISCUSSION

Budd-Chiari syndrome can be defined as any patho-physiologic process resulting in interruption or diminution of the normal blood flow out of the liver. If left untreated, it is almost always lethal of progressive liver failure due to chronic hepatic congestion. Medical treatment with diuretics controls the ascites. However, it just provides symptomatic relief. Radiological interventions and surgical approaches to address the underlying etiology are the best treatment to restore the hepatic venous flow[2]. A variety of non-medical approaches have been proposed. Currently, invasive methods like meso-caval or meso-arterial shunting, angioplasty, either alone or in combination with stenting and TIPS, are accepted as standard treatments for BCS. They, however, cannot be employed under certain circumstances, such as severe hepatocellular damage, seriously ill patients or those with extensive thrombosis.

Angioplasty with or without stent insertion is the treatment of choice for many acute cases of BCS involving the IVC. In this technique, the length of thrombosis is an important limiting factor[4]. Due to recurrent stenosis following percutaneous balloon angioplasty, it is necessary to utilize wall stents in many cases. However, further evaluations are required to watch for the intimal fibrosis and subsequent obliteration of the wall stent.

While the conventional anti-coagulation therapy has been poorly effective with the 5-year venous patency of only 10%, the thrombolytic therapy with rTPA, urokinase, or streptokinase is recently reported to be promisingl[5-7]. Sholar *et al* reported two cases of BCS due to paroxysmal nocturnal hemoglobinuria that were successfully treated with streptokinase[8]. In another report, seven patients with hepatic vein and vena caval thrombosis plus disturbed hepatic function were treated with streptokinase. The treatment was successful in four patients with no recurrence[9]. The successful treatment of two children with BCS by this method has also been reported[10]. Although, thrombolytic therapy is used mainly in acute phase of the disease, it may be used as a temporary treatment either to improve the condition of seriously ill patients awaiting surgery, or to reduce the length of the thrombus while preparing the patient for angioplasty and stent insertion. In conclusion, a combination of thrombolytic therapy and angioplasty with wall stent insertion might be beneficial to the patients in the acute phase of BCS. Nevertheless, more investigations are required to evaluate its definite effectiveness and safety.

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CASE REPORT

Double common bile duct: A case report

Srdjan P Djuranovic, Milenko B Ugljesic, Nenad S Mijalkovic, Viktorija A Korneti, Nada V Kovacevic, Tamara M Alempijevic, Slaven V Radulovic, Dragan V Tomic, Milan M Spuran

Srdjan P Djuranovic, Milenko B Ugljesic, Nenad S Mijalkovic, Viktorija A Korneti, Nada V Kovacevic, Tamara M Alempijevic, Dragan V Tomic, Milan M Spuran, Clinic for Gastroenterology and Hepatology, Clinical Center of Serbia, Belgrade, Serbia
Slaven V Radulovic, 1st Surgical Clinic, Clinical Center of Serbia, Belgrade, Serbia
Correspondence to: Srdjan P Djuranovic, MD, PhD, Clinic for Gastroenterology and Hepatology, Clinical Center of Serbia, K.Todorovića St. No 2, 11000 Belgrade, Serbia. gastroendo@beotel.yu
Telephone: +381-11-3615587 Fax: +381-11-3615587
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Abstract

Double common bile duct (DCBD) is a rare congenital anomaly in which two common bile ducts exist. One usually has normal drainage into the papilla duodeni major and the other usually named accessory common bile duct (ACBD) opens in different parts of upper gastrointestinal tract (stomach, duodenum, ductus pancreaticus or septum). This anomaly is of great importance since it is often associated with biliary lithiasis, choledochal cyst, anomalous pancreaticobiliary junction (APBJ) and upper gastrointestinal tract malignancies. We recently recognized a rare case of DCBD associated with APBJ with lithiasis in better developed common bile duct. The opening site of ACBD was in the pancreatic duct. The anomaly was suspected by transabdominal ultrasonography and finally confirmed by endoscopic retrograde cholangiopancreatography (ERCP) followed by endoscopic sphincterotomy and stone extraction. According to the literature, the existence of DCBD with the opening of ACBD in the pancreatic duct is most frequently associated with APBJ and gallbladder carcinoma. In case of DCBD, the opening site of ACBD is of greatest clinical importance because of its close implications with concomitant pathology. The adequate diagnosis of this rare anomaly is significant since the operative complications may occur in cases with DCBD which is not recognized prior to surgical treatment.

Key words: Double common bile duct; Accessory common bile duct; Anomalous pancreaticobiliary junction

Djuranovic SP, Ugljesic MB, Mijalkovic NS, Korneti VA, Kovacevic NV, Alempijevic TM, Radulovic SV, Tomic DV, Spuran MM. Double common bile duct: A report of a case. *World J Gastroenterol* 2007; 13(27): 3770-3772

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INTRODUCTION

Double common bile duct (DCBD) is a rare congenital anomaly in which two common bile ducts exist. One usually has normal drainage into the duodenum and the other usually named accessory common bile duct (ACBD) opens in different parts of upper gastrointestinal tract (stomach, duodenum, ductus pancreaticus or septum). Bile from different parts of the liver is drained through the ACBD. This anomaly is of great importance since it is often associated with lithiasis in the ectopic bile duct, choledochal cyst, anomalous pancreaticobiliary junction (APBJ) and upper gastrointestinal tract malignancies. Anomalous pancreaticobiliary junction is a congenital anomaly in which the pancreatic and biliary ducts are joined outside the duodenal wall forming a long common channel[1]. It is well known that APBJ is frequently associated with congenital choledochal cyst and cancer of the biliary system regardless of DCBD existence[2].

We recently recognized a rare case of DCBD associated with APBJ and lithiasis in better developed common bile duct.

CASE REPORT

A 65-year-old woman underwent cholecystectomy due to calculosis in 1980. In 1992 she had choledocholythiasis and underwent ERCP with endoscopic sphincterotomy followed by the calculus extraction. After that she had no problems for several years and in 1998 she was referred again to the regional hospital and ERCP, additional sphincterotomy and calculus extraction were performed again.

In November 2005, she was referred to our hospital because of pain in the right upper abdomen, accompanied by nausea, chills and mild fever for four weeks before admission. Her physical examination revealed only mild tenderness in the right upper abdomen. Routine laboratory analysis showed slightly elevated alkaline phosphatase, normal blood cell counts, normal renal and hepatic function and normal serum and urine amylase levels. Transabdominal ultrasonography disclosed the absence of the gallbladder due to previous cholecystectomy, the dilatation of the common bile duct of 16 mm and clearly visible choledocholithiais of 15 mm in its lumen. It is an interesting finding that, among the four parallel ducts,

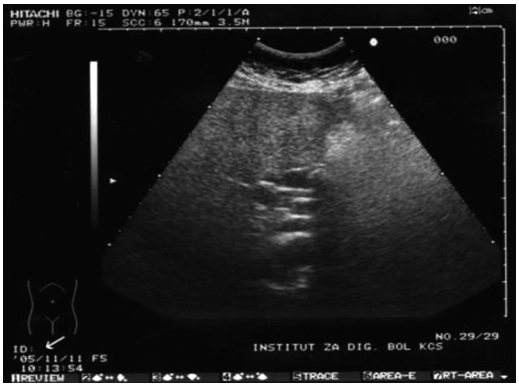

Figure 1 Transabdominal ultrasonography showing four parallel ducts.

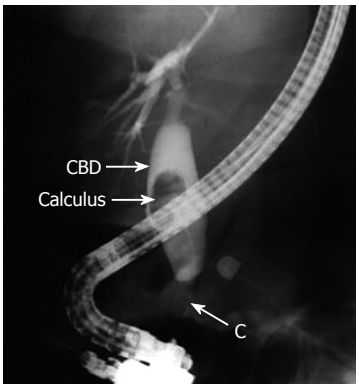

Figure 2 ERCP showing right CBD with calculus in its lumen (CBD: common bile duct; C: canula).

the most anterior was the presumed ductus choledochus, the second one probably ACBD, the third one hepatic artery and the last one the portal vein (Figure 1). Endoscopic ultrasonography demonstrated dilated ductus choledochus with calculus in its lumen and one bile duct without calculosis adjacent to the main duct. On ERCP, we saw two separate openings in the zone of the earlier sphincterotomized papilla duodeni major. After canulating the upper opening, the contrast medium was introduced into the dilated bile duct with big calculus in its lumen. We observed intrahepatic bile ducts of the right liver lobe, but could not fill the intrahepatic bile ducts of the left liver lobe (Figure 2). After that we canulated the lower opening on the papilla duodeni major, the contrast filled pancreatic duct, and the APBJ was revealed. We noticed that very close to the distal end of the pancreatic duct there was a communication with large bile duct and the contrast filled the previously missing part of the left liver lobe intrahepatic bile ducts (Figure 3). We performed endoscopic sphincterotomy of the upper opening and extracted the calculus from the right common bile duct. No complications occurred after the procedure and the patient was without symptoms and laboratory abnormalities on the control examination six and twelve months later.

DISCUSSION

It seems that double common bile duct is a very rare anomaly in western world, since Teilum[3] identified only 24

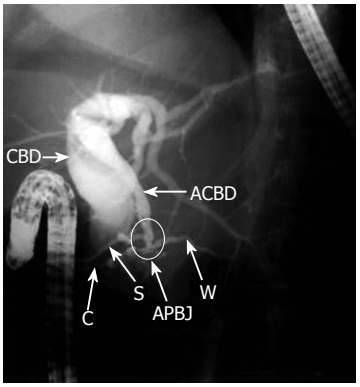

Figure 3 ERCP showing left CBD (CBD: common bile duct; ACBD: accessory common bile duct; APBJ: anomalous pancreaticobiliary junction; W: main pancreatic duct-Santorini; C: canula).

cases in western literature until 1986. On the other hand, Yamashita reviewed Japanese literature from 1968 to 2002 and found 47 patients with this anomaly[4].

The origin of this rare anomaly could be related to a random subdivision of the hepatic diverticulum during the first week of embryogenesis[5]. The embryonic development of the liver, gallbladder system and biliary tree starts around the third week of gestation, when the primordial liver, designated as the hepatic diverticulum, is formed as an outgrowth of the endodermis in the distal part of the anterior intestine. As the hepatic diverticulum grows, its cells penetrate the mesenchyma of the ventral mesogastrium, dividing into a ventral and a dorsal bud. The primitive gallbladder is formed from the ventral bud (pars cystica). The dorsal bud (pars hepatica) divides in turn to the left and right liver lobe. As the liver and biliary tree develop inseparably, the stem of the hepatic primordium becomes the bile duct[6]. The definite lumen of the bile tree is developed by recanalisation of the epithelium. Another important feature of the bile duct development is rotation of the primitive duodenum along its longer axis, which brings the bile duct dorsal to the upper limb of duodenum. The development of double common bile duct can be ascribed to disturbances in recanalisation of the hepatic primordium[7].

If we define the ACBD as the channel of the aberrant common bile duct which did not open into the major duodenal papilla, it can open into various parts of the digestive tract, all portions of the duodenum (including the site just above the major duodenal papilla), pancreatic duct, or it can only be presented by a septum in the common bile duct. Goor and Ebert[8] made an effort to embriologically classify different types of DCBD according to the anatomic appearance of the anomaly. Saito *et al*[9] modified the classification based on Goor and Ebert's morphological grouping which consisted of 4 different types of DCBD regardless of the site of the ACBD opening. On the other hand, the most important clinical feature of the DCBD seems to be the site of opening of the ACBD. Yamashita *et al*[4] highlighted the clinical importance of the opening site of the ACBD rather than its anatomic appearance. According to that gastric and biliary system cancer, APBJ, choledochal cysts and biliary lithiasis are the most serious complications of this condition. Gastric cancer is a possible complication usually found in patients with ACBD opening in the

stomach, while gallbladder cancer and ampullary cancer usually occur in patients with ACBD openings in the second portion of the duodenum and pancreatic duct. Most of the cases of biliary system cancers are associated with APBJ. Approximately one third of the patients with DCBD have choledocholithiasis and 10% have choledochal cysts [4].

Our patient had the ACBD opening in the pancreatic duct. According to the literature, this opening site of the ACBD is most frequently associated with APBJ and gallbladder carcinoma. Our patient underwent cholecystectomy 25 years ago due to lithiasis but not to carcinoma. At the same time, she had APBJ and choledocholithiasis of the better developed common bile duct. We decided not to give surgical treatment due to patient's age and general condition, but performed endoscopic sphincterotomy with successful stone extraction.

Treatment options of DCBD depend on the coexistence of APBJ and concomitant gastric or biliary system cancer. In cases without cancer, the resection of ACBD is recommended for surgical treatment. When APBJ is present, the separation of the flow of bile and pancreatic juice into the gastrointestinal tract should also be performed to prevent cancer in the biliary system[10].

Precise preoperative recognition of this anomaly is rare but very important. Preoperative adequate diagnosis of biliary tree anomalies prevents surgeons from impairing the anomalous bile duct who discovered these anomalies at operation accidentally. Our case is interesting that transabdominal ultrasonography displayed a clinical suspicion of DCBD. Magnetic resonance cholangiography could also reveal the existence of this anomaly, especially when the confluence of the right and left hepatic ducts were not clearly shown.[11] In such a situation careful surgical dissection should be performed followed by intraoperative cholangiography in order to avoid unnecessary injury of the bile duct.

Long-term results after treatment of DCBD depend mostly on concomitant cancer, because biliary system cancers have bad prognosis. APBJ will also influence the outcome because it is closely associated with biliary system cancer.

In conclusion, a case of DCBD associated with APBJ and choledocholithiasis was reported. In case of DCBD the opening site of ACBD is of greatest clinical importance because of its close implications with concomitant pathology. The adequate diagnosis of this rare anomaly is stressed since the possible operative complications may occur when DCBD is not recognized prior to surgical treatment.

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CASE REPORT

Malignant lymphoma of spleen presenting as acute pancreatitis: A case report

Chao-Ming Wu, Lung-Chih Cheng, Gin-Ho Lo, Kwok-Hung Lai, Chia-Ling Cheng, Wen-Cheng Pan

Chao-Ming Wu, Chia-Ling Cheng, Wen-Cheng Pan, Department of Gastroenterology, Tian-Sheng Memorial Hospital, Taiwan 813, China
Chao-Ming Wu, Lung-Chih Cheng, Gin-Ho Lo, Division of Gastroenterology, Department of Medicine, Kaohsiung Veterans General Hospital, Taiwan 813, China
Kwok-Hung Lai, Deputy Superintedent Kaohsiung Veterans General Hospital, Taiwan 813, China
Correspondence to: Gin-Ho Lo, Division of Gastroenterology, Department of Medicine, Kaohsiung Veterans General Hospital, 386 Ta-Chung 1st Rd kaohsiung, Taiwan 813, China. ghl@isca.vghks.gov.tw

Abstract

This is a case report of a patient who presented with acute pancreatitis without the common causes. A pancreatic biopsy revealed large B cell lymphoma. Spleen lymphoma with pancreatic involvement inducing acute pancreatitis, which is a rare disorder, was diagnosed. Here we also review the few similar cases reported in the literature.

Key words: Primary lymphoma of spleen; Large B cell lymphoma; Acute pancreatitis; Splenomegaly; Idiopathic pancreatitis

Wu CM, Cheng LC, Lo GH, Lai KH, Cheng CL, Pan WC. Malignant lymphoma of spleen presenting as acute pancreatitis: A case report. *World J Gastroenterol* 2007; 13(27): 3773-3775

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INTRODUCTION

The spleen is involved in 30%-40% of non-Hodgkin's lymphoma cases and primary spleen lymphoma (PSL) is a rare disorder, with an incidence of less than 1%[1]. Most patients have only nonspecific symptoms, such as fatigue, weight loss, and fever of unknown source, so it is therefore difficult to diagnose at an early stage. The prognosis for patients with PSL is related to the stage of the disease and pathological cell type involved. Here we report a case of malignant lymphoma of the spleen with invasion to the pancreatic tail with presentation as acute pancreatitis. We also review the literature on PSL.

CASE REPORT

A 68-year-old female patient presented with complaints of abdominal pain for 3 d and weight loss of about 6 kilograms in one month. Symptoms also included anorexia, nausea, and postprandial vomiting. Pain was localized to the right upper quadrant and was not related to meal or bowel habit changes. The pain could be relieved by assuming a knee-chest position and a left side decubitus posture. The symptoms did not include cough and fever. The patient had a history of type 2 diabetes mellitus with oral hypoglycemic agent control for more than 5 years. She denied any other systemic disease and did not have a habit of smoking or alcohol abuse. The patient was admitted to the hospital after being seen in the emergency room.

Physical examination showed anemic conjunctiva, no icteric sclera, and mild right upper quadrant tenderness. Neither a mass nor lymph nodes were palpable. The spleen was palpable about 8 cm below the costal margin. Laboratory tests showed the following: white blood cell count, 6300/cumm; hemoglobin, 12.0 g%; platelets, 101 000/cumm; segment, 60%; lymphocytes, 30%; monocytes, 10%; sugar, 155 mg/dL. Liver panel results, including total bilirubin, alanine aminotransferase, asparate aminotransferase, gamma glutamyltransferase, and lactic dehydrogenase, were within the normal range. Amylase was 410 U/L (< 180 U/L) and lipase was 715 U/L (< 160 U/L). An abdominal computed tomography (CT) scan showed a mass lesion of about 11.2 cm at the pancreatic tail and the spleen with multiple lymphadenopathy around the tumor. Splenic vein obliteration was also noted as well as edematous change over the peripancreatic area (Figure 1A-C). A panendoscopy examination showed esophageal and gastric varices. Our initial impression was tumor invasion resulting in acute pancreatitis.

Tumor markers, including carcinoembryonic antigen (CEA), alfafetal protein (AFP), and C19-9, were within the normal range. Sono-guided biopsy over the pancreatic tumor was carried out. Pathological examination showed diffuse large B-cell lymphoma. Cytokeratin was negative, LCA was positive, CD20 was positive, and CD3 was negative (Figure 2A-C). The brain CT scan was negative. Chest imaging showed right pleural effusion and a pig-tail tube was inserted. Analysis of pleural fluid showed no malignant cells.

The patient fasted with fluid hydration and electrolytes for 3 d. Subsequently, the abdominal pain subsided

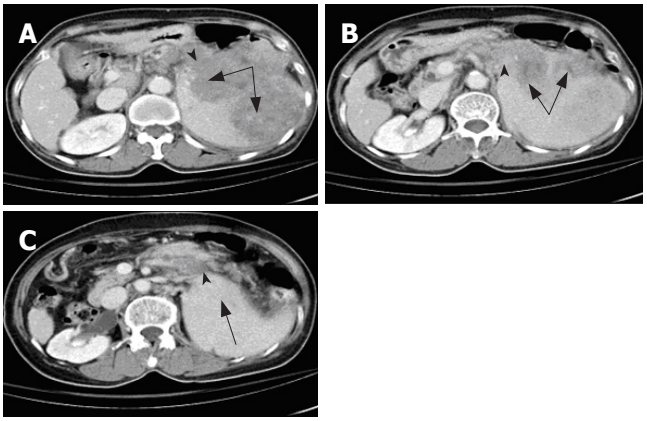

Figure 1 **A**: Arrow head-pancreatic tail with edematous and swelling changes, with the lesion adhered to the main tumor over the spleen. Long arrows-hypodense tumor mass occupying the spleen hilum; **B**: Arrow head-more involvement in the pancreatic tail. Long arrow-the main tumor with mild necrotic change over the spleen; **C**: Arrow head-the pancreatic tail is still enlarged, with swelling and little fluid acumination. Long arrow-tumor occupying the upper pole of the spleen.

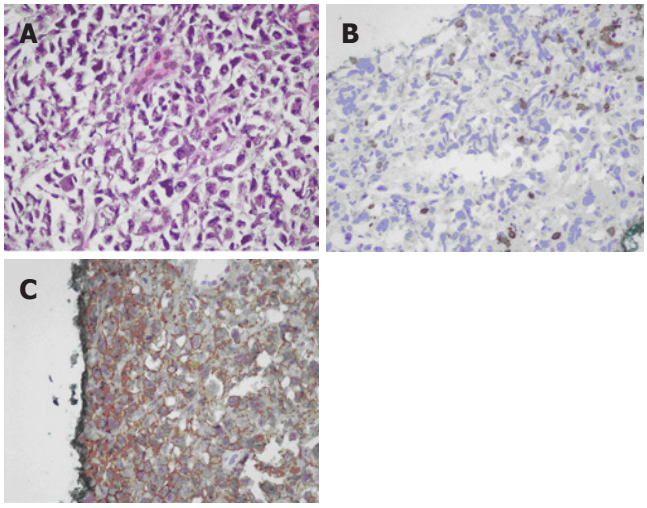

Figure 2 **A**: Pancreatic tail biopsy showing diffuse large lymphoid cells with highly polymorphic nuclei; **B**: The neoplastic cells are negative for T cell-associated markers, CD3; **C**: Large lymphoid cells stained by CD20.

and a biopsy of the pancreas was performed. After the procedure, the patient's condition remained stable. Our initial treatment plan was chemotherapy. Unfortunately, fever and a urinary tract infection developed on the 7th day after admission. Cefazolin, 1 gram every 8 h, and Gentamycin, 80 mg every 12 h, were administered. However, sepsis with respiratory failure developed, the chemotherapy was put on hold and the patient died after 12 d of hospitalization.

DISCUSSION

Acute pancreatitis is a disorder with numerous causes and an obscure pathogenesis. Bile duct stones and alcohol abuse together account for about 80% of acute pancreatitis. Other causes are various toxins and drugs, obstructions, such as malignancy, fibrotic sphinctor of Oddi, metabolic abnormalities, trauma, ischemia, infection, and autoimmune diseases. In 10% of the cases of acute pancreatitis no underlying cause can be identified, and these cases are described as idiopathic pancreatitis. Occult microlithiasis may be the cause of two-thirds of the cases of idiopathic acute pancreatitis[2].

Acute pancreatitis related to tumor obstruction is not unusual and primary pancreatic carcinoma (about 3% of all patients) and common bile duct cancer are the main etiologies. A case of carcinoid tumor of the pancreas with obstructive pancreatitis has been reported. Acute pancreatitis caused by metastatic carcinoma is uncommon, with broncogenic carcinoma as the main metastasis-induced pancreatitis tumor[3,4]. Metastasis-induced acute pancreatitis typically has occurred in patients known to have advanced brochogenic carcinoma[5]. Our patient presented with typical abdominal pain and high levels of amylase and lipase. We excluded other etiologies of pancreatitis, and therefore spleen lymphoma with pancreatic involvement was the main etiology of the observed acute pancreatitis.

Das Guta *et al* stated that clinical presentation of these symptoms must indicate splenomegaly without any evidence of disease elsewhere. They emphasized that the liver biopsy specimen, as well as para-aortic and mesenteric lymph nodes, should be free of malignant lymphoma[5]. Sharin *et al* reported on splenomegaly without significant lymphoadenopathy and no hepatomegaly or peripheral blood involvement[6]. Catherian *et al* defined malignant lymphoma with primary presentation in the spleen as splenomegaly without peripheral lymphadenopathy, pathological involvement of spleen with or without involvement of regional lymph nodes, bone marrow or liver[8]. Therefore, our patient could be diagnosed as having primary malignant spleen lymphoma due to the main involvement of the spleen without peripheral blood involvement, according to the definition of Catherian *et al*.

The spleen is involved in 30%-40% of the cases of non-Hodgkin's lymphoma and PSL has a reported incidence of less than 1%. In published reports, the incidence of diffuse large cell lymphoma in PSL varies from 22.4% to 33.3%[1]. Large B cell lymphoma, presenting with a tumor mass, is associated with a relatively favorable clinical course and the clinical presentation of a tumor confined to the spleen and the hilar lymph node is associated with lower aggravates[7]. The most common presenting symptoms in malignant lymphoma of the spleen are fever, malaise and weight loss.

There are some reports that revealed large B cell lymphoma in the spleen in patients with hepatitis C virus infection[5]. The prevalence of HCV infection (51.7%) in the examined splenic diffuse large B cell lymphoma cases was significant ($P < 0.05$)[1,10,11]. Saadoun *et al* demonstrated that treatment with interferon and ribavirin led to a complete virological response and hematological remission as well as the disappearance of its clinical symptoms. However, there were no studies indicating whether aggressive treatment of HCV infection by pegylated interferon is effective in treating or preventing lymphoma.

The spleen can accommodate different types of large B-cell lymphomas, which need to be distinguished

to establish a precise prognosis and the most suitable treatment[5]. Large B-cell lymphoma in the spleen as a tumor mass has a relatively favorable clinical course[12]. Only a few case reports have been published that included pancreas involvement.

In the case discussed in this report, the patient presented with non-specific symptoms, such as body weight loss and fatigue, followed by acute abdomen pain and symptoms of pancreatitis. The tumor was large enough to detect in the imaging studies, such as sonography or CT scan. A main problem is detecting the tumor as soon as possible because prognosis is related to tumor stage. Therefore acute pancreatitis with unknown etiology needs more detailed study to exclude primary pancreatic or metastasis, especially with those showing previous warning symptoms, such as fatigue, malaise or unknown cause of fever. If we could detect tumors earlier, prognoses would be better. Even if tumors are hard to remove surgically, chemotherapy should be effective.

The experience of our patient also warned us that these patients have weakened immunity and sepsis may be a major cause of mortality. If there is clinical suspicion of sepsis, antibiotics are indicated.

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CASE REPORT

Congenital bronchoesophageal fistula in an adult: A case report

Lei Su, Xiu-Qin Wei, Xiu-Yi Zhi, Qing-Sheng Xu, Ting Ma

Lei Su, Xiu-Yi Zhi, Qing-Sheng Xu, Ting Ma, Department of Thoracic Surgery, Xuan Wu Hospital of Capital University of Medical Sciences, Beijing 100053, China
Xiu-Qin Wei, Digestive Disease Center, Xuan Wu Hospital of Capital University of Medical Science, Beijing 100053, China
Correspondence to: Dr. Lei Su, Department of Thoracic Surgery, Xuan Wu Hospital of Capital University of Medical Science, No.45, Changchun Street, Beijing 100053, China. drsulei@yahoo.com.cn
Telephone: +86-10-83198646 Fax: +86-10-63027064
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Abstract

Bronchoesophageal fistulas are usually diagnosed in the neonatal period. As such, the condition is rare in adults. We present a case of a congenital bronchoesophageal fistula in a 62-year-old man with the complaint of severe bouts of cough and choking after swallowing liquid. His workup included a barium esophagogram that revealed a fistula between the esophagus and a right lower lobe bronchus. The diagnosis should be considered in certain individuals with suggestive symptomatology and unexplained respiratory pathology. The fistula was divided and resected, The patient had an uneventful recovery.

Key words: Bronchoesophageal fistula; Congenital; Diagnosis; Treatment

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INTRODUCTION

Congenital bronchoesophageal fistula (BEF) is usually associated with esophageal atresia and is readily diagnosed in infancy. But if it is not associated with esophageal atresia, it may persist until adulthood. We present a case of a 62-year-old man with congenital BEF. The patient was treated surgically, with ligation and resection of the fistula. The patient had an uneventful recovery and tolerated a regular diet without coughing at the time of his discharge.

CASE REPORT

A 62-year-old man was admitted to our hospital with the complaint of severe bouts of cough and choking after swallowing liquid. The patient had done well until 6 mo prior to the admission. The increased symptoms of cough developed. Laryngoscopy showed normal motion of the vocal cords. Bronchofiberscopy revealed no abnormalities. Esophagoscopy showed a depressed lesion that looked as if it was papilla or a dimple without inflammation at a distance of 30 cm from the incisors in the middle intrathoracic esophagus, but the fistula opening was invisible. The barium esophagram demonstrated a fistulous connection between the esophagus and a right lower lobe bronchus (Figure 1). He had no significant medical or surgical history and did not smoke or drink alcohol. His physical exam was unremarkable, as was his admission chest radiograpy, abdominal films, and results of laboratory tests.

The patient underwent a right posterior lateral thoracostomy. The fistula was identified between the middle intrathoracic esophagus and the right intermediate bronchi. The diameter of the fistula in the patient was 8 mm, with a length of 2.40 cm (Figure 2). There was no evidence of inflammation or adherent lymph nodes around the fistula. The tract was divided and resected, and covered with fibrin glue. Pathological examination of the resected specimen revealed that the fistula was lined by benign squamous epithelium with the muscularis mucosa, and there was no evidence of malignancy, infection, or chronic inflammation. The postoperative course was uneventful. No cough and dysphagia after swallowing liquid in the 7th day after operation.

DISCUSSION

Congenital BEF or tracheoesophageal fistula (TEF) were first reported by Negus in 1929[1]. These congenital fistulas are still rare in adults[2-5]. Diagnosis can be difficult because of the nonspecific nature of the symptoms. Benign bronchoesophageal fistulas can remain undiagnosed for years. Bouts of coughing when swallowing liquids (Ohno's sign) are reported to be pathognomonic for this condition and present in 65% of cases[3,4]. The duration of symptoms has been reported to vary from 6 mo to 50 years before diagnosis[2,4,6,7]. One review reported a patient's condition diagnosed at the age of 83[2]. The congenital nature of the fistula is suggested by the absence of adherent lymph nodes[2] and past or present surrounding inflammation[4], by

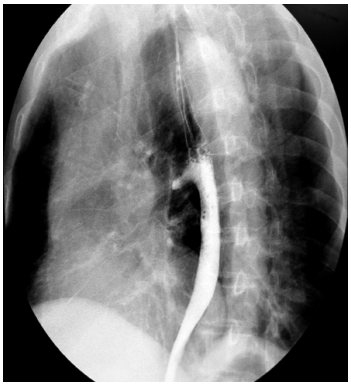

Figure 1 Barium esophagogram demonstrating the downward direction of the fistula from the esophagus to the right lower lobe bronchus.

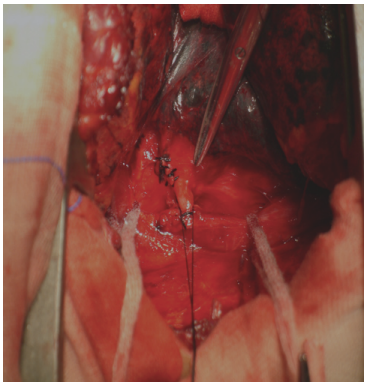

Figure 2 Operation demonstrating the fistula tract exiting between the esophagus and the bronchus.

the presence of a mucosa and definitive muscularis mucosa within the fistulous tract[5], and by the patient's history[8]. Conventional barium esophagography is considered to be the most sensitive test for diagnosing tracheoesophageal or bronchoesophageal fistula[4-7,9,10]. Esophagoscopy and bronchoscopy may not always demonstrate the fistulous orifice, but these procedures may help us chose modus operandi[2,4,6,7]. CT scanning may be utilized to rule out the presence of a neoplasm and adenopathy and to define the extent of coexisting pulmonary disease, which may need resection[5,7,11].

Braimbridge and Keith[8] classified congenital bronchoesophageal fistulas into four types. In type Ⅰ, a fistula is associated with an esophageal diverticulum. Type Ⅱ consists of a short tract running directly from the esophagus to the bronchus. The type Ⅲ fistula communicates between the esophagus and a cyst in the lung lobe, and type Ⅳ involves a fistula between the esophagus and a sequestered pulmonary segment. Type Ⅱ is the most prevalent and comprises almost 90% of all cases in some series[2]. Our patient had a simple type Ⅱ fistula.

The insidious nature of such a fistula may become life threatening, with repeated infection leading to pneumonia, bronchiectasis and abscess formation[5,7,12]. Despite the benign nature of this anomaly, if left untreated, it may lead to fatal complications.

For most cases of fistula formation, surgical management *via* thoracotomy is the traditional treatment[2,4-7]. The fistula is exposed and divided, and both the defects in the bronchus and the esophagus are repaired with interposition of viable tissue(e.g., pleural or muscular flap) between the suture lines[4,7,10,12]. Pulmonary resection is often needed in patients with coexistent pulmonary disease. The prognosis after surgical repair is excellent. Obliteration of the esophageal orifice with silver nitrate or biological glue is reserved for the patient who cannot tolerate thoracotomy.

Congenital BEF is a rare anomaly in adults. Barium swallow was the most useful diagnostic test. Once a bronchoesophageal fistula is recognized, surgery is the treatment of choice.

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Chief of Surgery, John D. Dingell VA Medical Center, 4646 John R. Street, Detroit, MI 48301, United States

Peter Draganov, PhD
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Institut de Malalties Digestives i Metaboliques Hospital Clinic, Villaroel 170, Esc 7-4, Barcelona 08036, Spain

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Laura E Matarese, MS, RD, LDN, FADA, CNSD
Institute, UPMC Montefiore, 7 South, 3459 Fifth Avenue, Pittsburgh, PA 15213, United States

Ton Lisman, PhD
Thrombosis and Haemostasis Laboratory, Department of Haematology G.03.550, University Medical Centre, Heidelberglaan 100, 3584 CX Utrecht,
The Netherlands

Christina Surawicz, MD
Christina Surawicz, Harborview Medical Center, 325 9th Ave, #359773, Seattle WA 98104, United States

Jacqueline L Wolf, MD
Division of Gastroenterology, Beth Israel Deaconess Medical Center, 330 Brookline Ave, Rabb 437, Boston, MA 02215, United States

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Department of Medicine, National University Hospital, 119074, Singapore

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Department of General Medicine, Hiroshima University Hospital, 1-2-3 Kasumi, Minami-ku, Hiroshima 7348551, Japan

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Department of Radiology, Meram Medical Faculty, Selcuk University, Konya 42080, Turkey

Reinhard Buettner, Professor
Institute of Pathology, University Hospital Bonn, Sigmund-Freud-Str. 25, D-53127 Bonn, Germany

Hiroki Sasaki, PhD
Genetics Division, National Cancer Center Research Institute, 1-1, Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan

Toru Ishikawa, MD
Department of Gastroenterology, Saiseikai Niigata Second Hospital, Teraji 280-7, Niigata, Niigata 950-1104, Japan

Simon D Taylor-Robinson, MD
Department of Medicine A, Imperial College London, Hammersmith Hospital, Du Cane Road, London W12 0HS, United Kingdom

Christoph F Dietrich, MD
Innere Medizin 2, Caritas-Krankenhaus, Uhlandstr. 7, Bad Mergentheim97980, Germany

Masayoshi Ito, MD
Department of Endoscopy, Yotsuya Medical Cube, 5-5-27-701 Kitashinagawa, Shinagawa-ku, Tokyo 1410001, Japan

Jose Sahel, Professor
Hepato-gastroenterology, Hospital sainti Marevenite, I270 Boolevard AE Sainti Margrenise, Marseille 13009, France

Frank Ivor Tovey, OBE, ChM, FRCS
Honorary Research Felllow, Department of Surgery, University College London, London, United Kingdom

Luigi Bonavina, Professor
Department of Surgery, Policlinico San Donato, University of Milano, via Morandi 30, Milano 20097, Italy

Özlem Yilmaz, PhD, Associate Professor of Microbiology
Dokuz Eylul University, School of Medicine, Department of Microbiology and Clinical Microbiology, Inciralti 35340, Izmir, Turkey

Dino Vaira, Professor
Department of Internal Medicine and Gastroent, University of Bologna, S.Orsola-Malpighi Hospital - Nuove Patologie, Pad. 5 - via Massarenti 9, Bologna 40138, Italy

Bruno Annibale, Professor
Digestive and Liver Disease Unit, University " La Sapienza" II School of Medicine, Via di Grottarossa 1035, Roma 00189, Italy

Xupeng Ge, MD, PhD
Transplant Biology Research Center, Massachusetts General Hospital, Harvard Medical School, MGH East, Building 149-5102, 13th street, Boston MA 02129, United States

Olivier Detry, PhD
Department of Abdominal Surgery and Transplantation, University of Liège, CHU Sart Tilman B35, B-4000 Liège, Belgium

Minoti Vivek Apte, Associate Professor
Pancreatic Research Group, South Western Sydney Clinical School, The University of New South Wales. Liverpool, NSW 2170, Australia

Nageshwar Duvvuru Reddy, Professor
Asian Institute of Gastroenterology, 6-3-652, Somajiguda, Hyderabad - 500 082, India

Richard A Kozarek, MD
Department of Gastroenterology, Virginia Mason Medical Center, 1100 Ninth Avenue, PO Box 900, Seattle 98111-0900, United States

Pietro Invernizzi, PhD
Division of Internal Medicine, Department of Medicine, Surgery, Dentistry, San Paolo School of Medicine, University of Milan, Via Di Rudinfi 8, 20142 Milan, Italy

Xian-Ming Chen, MD, Associate Professor
Department of Medical Microbiology and Immunology, Creighton University, 2500 California Plaza, Omaha NE 68178, United States

Naohiko Harada, PhD
Department of Gastroenterology, Fukuoka Higashi Medical Center, Chidori 1-1-1, Koga, Fukuoka 811-3195, Japan

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Meeting Falk Research Workshop: Morphogenesis and Cancerogenesis of the Liver
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Meeting Canadian Digestive Diseases Week (CDDW)
16-20 February 2007
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Meeting Falk Symposium 158: Intestinal Inflammation and Colorectal Cancer
23-24 March 2007
Sevilla
symposia@falkfoundation.de

Meeting BSG Annual Meeting
26-29 March 2007
Glasgow
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NEXT 6 MONTHS

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Meeting Falk Symposium 159: IBD 2007 - Achievements in Research and Clinical Practice
4-5 May 2007
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Meeting European Society for Paediatric Gastroenterology, Hepatology and Nutrition Congress 2007
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Digestive Disease Week
19-24 May 2007
Washington Convention Center, Washington DC

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Meeting ESGAR 2007 18th Annual Meeting and Postgraduate Course
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Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar 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]

No author given

6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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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]

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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 billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

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Author(s) and editor(s)

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Conference proceedings

13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

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Electronic journal (list all authors)

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/eid.htm

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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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