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Peritoneal carcinomatosis of colorectal origin: Incidence, prognosis and treatment options

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CRC. Risk factors for developing PC have been identified: right-sided tumor, advanced T-stage, advanced N-stage, poor differentiation grade, and younger age at diagnosis. During the past decade, both chemotherapeutic and surgical treatments have achieved promising results in these patients. A chance for long-term survival or even cure may now be offered to selected patients by combining radical surgical resection with intraperitoneal instillation of heated chemotherapy. This combined procedure has become known as hyperthermic intraperitoneal chemotherapy. This editorial outlines recent advancements in the medical and surgical treatment of PC and reviews the most recent information on incidence and prognosis of this disease. Given recent progress, treatment should now be considered in every patient presenting with PC.

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Key words: Colorectal cancer; Peritoneal carcinomatosis; Hyperthermic intraperitoneal chemotherapy; Chemotherapy; Prognosis

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Abstract

Peritoneal carcinomatosis (PC) is one manifestation of metastatic colorectal cancer (CRC). Tumor growth on intestinal surfaces and associated fluid accumulation eventually result in bowel obstruction and incapacitating levels of ascites, which profoundly affect the quality of life for affected patients. PC appears resistant to traditional 5-fluorouracil-based chemotherapy, and surgery was formerly reserved for palliative purposes only. In the absence of effective treatment, the historical prognosis for these patients was extremely poor, with an invariably fatal outcome. These poor outcomes likely explain why PC secondary to CRC has received little attention from oncologic researchers. Thus, data are lacking regarding incidence, clinical disease course, and accurate treatment evaluation for patients with PC. Recently, population-based studies have revealed that PC occurs relatively frequently among patients with

INTRODUCTION

Peritoneal carcinomatosis (PC) secondary to colorectal

cancer (CRC) is characterized by the development of solid tumor deposits on the peritoneal surface^[1]. Cell shedding from the primary tumor is thought to be responsible for these peritoneal deposits, which may occur spontaneously or as a result of spillage during surgical procedures. Attachment of tumor cells to peritoneal mesothelial cells involves neoangiogenesis and is mediated by several growth factors^[2]. Tumor implantation and growth may lead to invasion of any organ or structure that is covered by peritoneum.

Common sites for peritoneal implants are the omentum, mesentery, bowel surface, pouch of Douglas, right paracolic gutter, and diaphragm^[3,4]. Patients initially present with nonspecific symptoms such as abdominal discomfort, nausea, weight loss, cachexia, and fatigue; however, these symptoms are often indistinguishable from more general features of malignant disease. The tumor growth on intestinal surfaces and associated fluid accumulation eventually result in signs of bowel obstruction^[5] and incapacitating volumes of ascites^[6,7]. Previously, providers were reluctant to treat these patients because of their extremely poor prognosis and invariably fatal outcomes^[8-10]. Therefore, PC resulting from CRC had received little attention from oncologic researchers. Hence, data was lacking regarding incidence, clinical disease course, and accurate treatment evaluation for patients with PC.

Since the 1980s, PC research has attracted renewed interest because of the observation that a subgroup of patients presents solely with peritoneal tumor implants without systemic metastases^[11,12]. This finding spurred the development of aggressive surgical treatment modalities which combined radical cytoreductive surgery with intraperitoneal chemotherapy^[13-17]. With this approach, prolonged overall survival and even disease cure have been reported^[18,19]. Here, we will discuss the recent advances in the characterization and treatment of these patients.

INCIDENCE OF PC

The extent of peritoneal disease is frequently underestimated by imaging modalities^[27], and the presence of peritoneal involvement often remains unknown until a laparotomy is performed. This uncertainty in preoperative diagnosis results from the low sensitivity and specificity of imaging techniques such as abdominopelvic ultrasound and computed tomography. The small size of tumor deposits (typically less than 1 cm) negatively affects sensitivity^[27-30]. Furthermore, peritoneal spread of tumor cells characteristically follows the anatomic outline of normal abdominal structures, making radiologic detection more challenging.

Underestimation of PC due to poor preoperative imaging diagnostics, combined with the aforementioned lack of interest, likely explains the virtual absence of data on PC incidence. Most available data were retrieved from single hospital-based studies that reported inci-

dence of PC encountered during laparotomy. In the largest study, which included 2756 patients with CRC, 214 (8%) patients were diagnosed with synchronous PC and 135 (5%) with metachronous disease^[10]. Two older studies, also single hospital-based, reported that 10% to 15% of patients with colon cancer presented with PC^[9,31]. Recently, two population-based studies reported the incidence of synchronous PC in The Netherlands (4.8%) and in Sweden (4.3%)^[32,33]. Risk factors for developing PC include right-sided tumor, advanced T-stage, advanced N-stage, poor differentiation grade, and younger age at diagnosis.

In clinical studies, metachronous PC is reported in 4% to 12% of patients following curative resection for colon cancer and in 2% to 19% of patients following curative resection for rectal cancer^[34]. In patients undergoing repeat procedures for CRC following primary curative resection, 21% to 44% of patients are diagnosed with peritoneal tumor deposits^[35,36]. In autopsy studies, PC is found in up to 40% of patients who die from colorectal carcinoma^[37,38]. On a population level, 4.2% of Swedish patients with CRC developed metachronous PC following initial treatment^[33]. Risk factors for developing metachronous PC are similar to those for synchronous PC, but also include initial emergency procedures and non-radical initial tumor resection^[33].

TREATMENT

Systemic treatment

Few studies have been published describing the effectiveness of systemic chemotherapy in patients with PC. Due to an inability to accurately measure tumor load and treatment response, patients with peritoneal tumors usually do not meet the inclusion criteria for randomized trials^[39]. The few studies describing chemotherapeutic treatment response focus on systemic 5-fluorouracil (5-FU) and leucovorin using retrospective analysis. The results invariably show a disappointing response to systemic treatment and a poor prognosis compared to other metastatic sites. A French prospective multicenter study of 118 patients with PC of colorectal origin showed a median survival of only 5.2 mo^[40]. In a large series of CRC patients, which included 392 patients with peritoneal involvement, Jayne *et al.*^[10] showed a median survival of 7 mo. Chu *et al.*^[9] reported a median survival of 6 mo in a series of 45 patients who were treated primarily with 5-FU and leucovorin. A sub-analysis by Köhne *et al.*^[41] of patients with PC treated with 5-FU-based therapy showed a median survival of 7.7 mo. Slightly better results were reported by Bloemendaal *et al.*^[8], who described 50 patients with PC but without hematogenous metastases who were treated with systemic chemotherapy and palliative surgery. Their overall median survival was 12.6 mo, with a 2-year survival rate of approximately 18%^[8,24]. However, selection bias probably explains these findings, because these patients were initially referred for hyperthermic intraperitoneal chemoperfusion (HIPEC)-

treatment but eventually randomized to the study control group. It is conceivable that these patients were healthier and their PC disease was more limited compared to the average PC patient.

A few studies have aimed to describe the effect of newer chemotherapeutic combinations, such as oxaliplatin plus irinotecan^[8,42,43]. Results are conflicting and require careful interpretation. Many of these studies were performed to compare systemic treatment with surgical treatment. Selection bias may play a role in these studies because only patients in good condition with limited disease and without systemic metastases were eligible. Nevertheless, the median survival of 23 mo, as described by Elias *et al*^[18] and obtained with modern systemic chemotherapy, is remarkable and dispels the notion that PC is chemotherapy-resistant. A similar conclusion may be drawn from the only population-based study to investigate this topic thus far. From 1995 to 2008, the administration of chemotherapy to patients with PC gradually increased, from 16% to 46% ($P = 0.001$), with the treatment rate rising to 64% for younger patients^[41]. However, a survival benefit was only apparent after 2005 when modern chemotherapy schedules were introduced^[44].

Introduction of targeted therapies, including monoclonal antibodies specifically targeted to epidermal growth factor receptor and vascular endothelial growth factor, has resulted in a significantly increased survival among patients with metastasized CRC^[41,45,46]. Although these agents are now routinely included in the treatment of patients with stage IV disease, only one small retrospective study has evaluated the effect of adding targeted therapies in patients with PC; a survival of 22.4 mo was observed when biologicals were added to first line of treatment in this patient group^[47].

Cytoreductive surgery and intraperitoneal chemotherapy

The observation that some patients present with PC in the absence of systemic metastases has led to the hypothesis that PC results from locoregional spread rather than systemic metastasis. This belief has encouraged surgical oncologists to examine possibilities for locoregional therapies. In the 1980s and 1990s, physicians and researchers developed new treatment strategies consisting of aggressive cytoreductive surgery plus intraperitoneal chemotherapy, often combined with hyperthermia.

Surgical procedures invariably start with a careful and systematic abdominal exploration and registration of the extent of peritoneal disease. The abdomen is divided in 13 regions and for each region, the number and size of tumor deposits are assessed and recorded. The sum of these scores represents the peritoneal cancer index (PCI), which ranges from 0 to 39. For PC resulting from CRC, a PCI score of 15 or more is generally accepted as exclusion criterion for HIPEC. In The Netherlands, the simplified PCI (sPCI) is commonly used to describe the PC involvement of 9 regions of the abdomen^[24]. The PCI and sPCI are well-known predictive outcome indices for

patients undergoing cytoreductive surgery and perioperative chemotherapy^[48,49].

During cytoreductive surgery, surgeons attempt to remove all visible tumor deposits from the peritoneal surface. To achieve a radical resection, resection of grossly involved organs may be required. Additionally, peritonectomy may be performed^[15]. Resection completeness is recorded using the completeness of cytoreduction score (CCR). A CCR-0 score indicates that no macroscopic peritoneal tumor remains following cytoreduction. A CCR-1 score occurs when tumor nodules less than 2.5 mm persist following cytoreduction. Residual disease measuring 2.5 mm to 2.5 cm is scored as CCR-2. A CCR-3 score indicates the presence of tumor nodules greater than 2.5 cm, or a confluence of unresectable tumor nodules at any site within the abdomen or pelvis^[50]. Alternatively, the R1-R2a-R2b scoring system classifies R1 as no macroscopic residual tumor, R2a as macroscopic residual disease less than 2.5 mm, and R2b as tumor deposits greater than 2.5 mm^[24]. Treatment outcomes are poorer in the presence of residual tumor following optimal cytoreduction, particularly for residual tumor diameters exceeding 2.5 mm. It is hypothesized that 2.5 mm is the maximum penetration depth of chemotherapeutic agents^[48].

After macroscopically complete cytoreduction, intraperitoneal chemotherapy is administered to eradicate microscopic disease. This chemotherapy can be administered immediately following surgery in the operating room, usually in combination with HIPEC or on postoperative days 1 to 5 (early postoperative intraperitoneal chemotherapy). HIPEC perfusion may be performed with a closed abdomen, or with an open “coliseum technique”. Chemotherapeutic agents and doses vary widely between centers worldwide, with mitomycin C and oxaliplatin being the most frequently used agents.

Only one completed phase III randomized trial investigating the outcome of surgical intraperitoneal treatment has been published to date. Verwaal *et al*^[24,25,51] reported a significant increase in median overall survival among patients treated with cytoreductive surgery and HIPEC, as compared to patients receiving standard palliative care using systemic 5-FU and leucovorin. The promising outcomes observed in this study have convinced many surgeons to accept this technique as standard of care for selected patients with PC, and HIPEC treatment is now offered in specialized centers all over the world. However, this study was heavily criticized for not including a control group of patients receiving cytoreductive surgery only. Therefore, it remains unclear whether cytoreductive surgery and HIPEC are both required to improve survival, or if the observed benefit was due to a single component^[52]. Ideally, these questions should be addressed in randomized trials. However, this type of study has proven difficult to implement, as demonstrated by a phase III trial in France that failed to enroll enough patients due to patient dissatisfaction

with randomization^[53]. Recently, investigations of cytoreductive surgery and HIPEC in PC animal models have provided a sound scientific rationale for the application of intraperitoneal chemotherapy in conjunction with cytoreductive surgery^[54-56], although the additional value of hyperthermia is questionable^[54].

The best available clinical evidence now comes from multi-institutional registries^[18-23]. These data require careful interpretation, as surgeon experience, technique and perioperative care differs widely between institutions^[57]. However, reported median survival rates of up to 63 mo following cytoreductive surgery and HIPEC with limited postoperative morbidity and mortality^[58,59] suggest that treatment should be considered in all patients with PC secondary to CRC.

In conclusion, PC of colorectal origin was formerly considered untreatable, with an extremely poor prognosis. Recent treatment advances using modern systemic chemotherapy or cytoreductive surgery combined with HIPEC have improved patient outcomes. In our opinion, treatment should be considered for every patient presenting with PC due to CRC. However, further understanding of PC pathogenesis, optimal diagnostics and treatment requires ongoing experimental and clinical research.

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Diagnosis of Zollinger-Ellison syndrome: Increasingly difficult

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Abstract

In the present paper the increasing difficulty of diagnosis of Zollinger-Ellison syndrome (ZES) due to issues raised in two recent papers is discussed. These issues involve the difficulty and need to withdraw patients suspected of ZES from treatment with Proton Pump Inhibitors (omeprazole, esomeprazole, lansoprazole, rabeprazole, pantoprazole) and the unreliability of many gastrin radioimmunoassays. The clinical context of each of these important issues is reviewed and the conclusions in these articles commented from the perspective of clinical management.

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Key words: Zollinger-Ellison syndrome; Gastrinoma; Hypergastrinemia; Secretin test; Serum gastrin; Gastrin; Neuroendocrine tumor

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INVITED COMMENTARY ON HOT ARTICLES

Two recent papers by Poitras *et al*^[1] and Rehfeld *et al*^[2] call attention to two areas that are making it more difficult to diagnosis Zollinger-Ellison syndrome (ZES). In this short review after listing the papers and their abstracts, the importance of these two issues will be briefly commented on.

Background: General

ZES is a clinical syndrome due to the ectopic secretion of gastrin by a neuroendocrine tumor (gastrinoma), located primarily in the duodenum (60%-80%) or pancreas (10%-40%), resulting in gastric acid hypersecretion, which if left untreated results in refractory peptic ulcer disease, severe gastroesophageal reflux disease, diarrhea and finally death, primarily due to the complications of the refractory peptic ulcer disease^[3-6]. The diagnosis of ZES, like the diagnosis of other ectopic hormonal pancreatic endocrine syndromes, historically requires the demonstration of inappropriate release of the hor-

more and evidence of hormonal hypersecretion^[3,6-8]. In the case of ZES this requires the demonstration of inappropriate gastrin release by demonstrating fasting hypergastrinemia in the presence of gastric acid hypersecretion^[3,6-10]. This is clinically most frequently accomplished by demonstrating the inappropriate presence of fasting hypergastrinemia when gastric fluid is acidic with a pH ≤ 2 is present or gastric hypersecretion is present [> 15 mEq/h basal acid output (no previous gastric acid reducing surgery), > 5 mEq/h (if gastric acid reducing surgery)]^[7,8,11-14]. The combination of fasting hypergastrinemia and elevated gastric acid secretion are required for ZES diagnosis because numerous unrelated conditions can cause one or the other of these alone^[5,6,8,10,14]. The most frequent cause of fasting hypergastrinemia is physiological hypergastrinemia (also called appropriate hypergastrinemia) due to the presence of hypo/achlorhydric, which in normal individuals results in a reciprocal increase in gastrin release from the G cells of the gastric antrum causing hypergastrinemia^[7,8,10,14,15]. This is most frequently due to chronic atrophic gastritis, commonly due to the presence of a *Helicobacter pylori* (*H. pylori*) infection that spares the antrum or due to pernicious anemia^[7,8,10,14,15]. A second very frequent cause is the use of potent gastric acid anti-suppressant drugs such as proton-pump inhibitors (PPIs) (omeprazole, lansoprazole, pantoprazole, esomeprazole, rabeprazole), which is discussed in the next paragraph. Other less common causes of hypochlorhydric/achlorhydria include chronic renal failure and vagotomy, which can be distinguished by appropriate other laboratory/clinical investigations^[5,8,10,13]. Similarly, the presence of gastric acid hypersecretion without hypergastrinemia can be seen in patients with idiopathic gastric acid hypersecretion and a few other uncommon conditions (mastocytosis, basophilic granulocytic leukemia)^[8,16,17].

While the diagnosis of ZES sounds simple enough, in recent years it is becoming increasingly more difficult, due to a number of developments, and now, as pointed out in the above two papers, it is even further complicated. Let's first consider the issues that were complicating the diagnosis of ZES prior to these two papers. First, acid secretion is now rarely measured and therefore generally not available, so alternatives to the classical basal acid output assessment were proposed. These include, in addition to the presence of fasting hypergastrinemia, an endoscopic measurement of gastric acid output^[18], the use of either pH paper or a pH meter to establish the presence of an acidic pH in gastric fluid, assessment for the presence of other features of ZES such as the presence of a tumor on imaging or pathologic studies and the development of gastric fluid pH criteria, that when coupled with the presence of hypergastrinemia, support the diagnosis of ZES^[11,11,14]. Second, the widespread use of PPIs is markedly complicating the ability to diagnosis ZES, because their use interferes with both needed assessments to establish the diagnosis of ZES: the mea-

surement of fasting gastrin levels and the assessment of acid secretion^[5,10,12,14,19]. This occurs because PPIs have a very long duration of action, with their gastric acid suppressive action lasting up to one week^[13,20,21] which not only contributes to their marked effectiveness, but also makes it difficult to withdraw patients from these drugs to assess gastric secretion^[1,5,14]. Furthermore, their potent antisecretory activity can lead to fasting hypergastrinemia due to the PPIs and thus mislead one into suspecting ZES^[14,19,22]. The result of the PPIs is that they both mask the diagnosis of ZES, leading to delays in diagnosis, because they effectively control all presenting clinical symptoms, but also they can lead to a false diagnosis^[3,5,14,19,23]. False diagnoses occur because long-term use of PPIs can cause fasting hypergastrinemia in 80%-100% of patients without ZES^[24-27] with fasting gastrin values > 4 fold increased in 20%-25% of patients^[24-27] and in some cases the gastrin levels are > 10 -fold elevated into ranges that are frequently thought to reflect ZES^[25,26].

Commentary paper

In Poitras *et al.*^[1] two patients are reported in whom ZES was suspected (later proven) after presenting with severe symptoms of gastroesophageal disease, subsequently treated with PPIs with symptom improvement and when the PPIs were withdrawn, both patients developed severe complications of peptic disease (patient No. 1, esophageal stricture requiring repeated dilations; patient No. 2, intestinal perforation). It was proposed that PPI therapy should always be maintained and diagnostic evaluations be performed while taking PPIs^[1]. This is a novel recommendation and would have a marked effect on the diagnostic approach to patients suspected of having ZES. This recommended approach differs from the approach recommended in recent consensus guidelines and by most authorities in recent reviews, wherein, it is recommended that acid antisecretory drugs have to be stopped at some point to establish the diagnosis of ZES. Specifically, both the North American Neuroendocrine Tumor Network guidelines^[9] and the European Neuroendocrine Tumor Network's guidelines^[3,12], as well as a recent reviews of the diagnosis of ZES by a number of authorities^[7,10,14,22,28], all recommend that PPIs need to be generally stopped to establish the diagnosis of ZES.

Is there any additional evidence to support this novel recommended diagnostic approach for ZES in paper 1^[1]? Others have reported severe esophageal strictures in patients with ZES, since the time that antisecretory drugs were available, whose acid hypersecretion was not controlled^[29-31] and in one large study^[32], 8% (10/122) patients with ZES required repeated esophageal dilations, because of previous poor control of the acid hypersecretion. We don't find additional cases in the literature to patient No. 1 described in this report^[1], however, we have seen one patient with ZES who developed a severe long, esophageal stricture requiring a stent, because anti-secretory medications were withdrawn at an outside hos-

pital for diagnosis (unreported case). Similarly intestinal perforations have been reported in a number of patients with ZES since antisecretory drugs became available. Intestinal perforations were reported in 7% of patients with ZES in one large series (11/160 cases)^[33] and in a number of other case reports^[34,35], with the perforations occurring prior to the diagnosis in most cases. However, we have seen three cases of ZES patients who developed intestinal perforations when taken off of antisecretory drugs for diagnostic reasons^[33,34] (unpublished 2 cases). One case occurred after a patient with suspected ZES reduced the PPI dose they were taking on their own because they developed constipation while taking the PPI and then presented with a duodenal perforation after 5 d of stopping the PPI in preparation for a secretin test^[34]. Two other cases occurred prior to the use of PPIs early in our experience and we have seen no additional cases at National Institutes of Health (NIH) in the last 20 years in acid studies of more than 300 patients with ZES, the majority of who were taking PPIs prior to diagnosis. These latter results demonstrate that acid secretory studies can be safely carried out if proper precautions are taken, even if patients are taking PPIs, although it requires a center well versed in performing these studies, and a proven approach, one of which is discussed below. However, the query raised by Poitras *et al.*^[1] still remains, as to whether it is necessary to withdraw antisecretory medications in most patients with ZES to establish the diagnosis.

Unfortunately, the evidence suggests, as concluded in the accompanying editorial^[14] to paper No. 1^[1], that to clearly establish the diagnosis of ZES, some appropriate assessment of gastric acid acidity/secretion is required after withdraw of PPIs in almost every patient. What is the evidence? First, it is both fortunate and unfortunate that PPIs are so effective in patients with ZES as well as patients with idiopathic peptic disease. It is fortunate because PPIs are very effective at controlling gastric acid secretion in these patients^[5,7,36,37]. However, it is unfortunate for diagnostic purposes, because their effectiveness results in that fact that with PPIs, the usual doses used in idiopathic peptic disease are often effective also in ZES and result in hypo/achlorhydria in both ZES patients and in patients without ZES. In contrast with Histamine H₂-receptor antagonists, frequently 10-fold greater doses than used in treating patients with idiopathic peptic disease are required in ZES patients, with more frequent dosing to control the hypersecretion, however these high doses in ZES and the usual doses in patients without ZES, rarely result in hypo/achlorhydria^[5,7,36,37]. Therefore, in most patients taking PPIs where ZES is suspected, the gastric pH will not be < 2 (the range required for diagnosis of ZES)^[7,11], and therefore physiological and pathological hypergastrinemia can not be distinguished on the drug. Second, there is no feature of the clinical course that unequivocally allows one to establish the presence of ZES, with most patients currently presenting

with idiopathic peptic ulcer disease or gastroesophageal reflux disease which is indistinguishable from that seen in non-ZES patients^[7,14,35,38]. Third, when ZES is suspected, a fasting gastrin level is almost invariably the first diagnostic study performed^[7-10,12,14,39]. Unfortunately, there is no absolute level of fasting hypergastrinemia alone that can distinguish a patient with ZES from a patient without ZES^[6,39]. This will be covered in more detail below in the discussion of Rehfeld *et al.*^[2], however a few additional points will be made here. In the most common cause of fasting hypergastrinemia, chronic atrophic gastritis, fasting gastrin levels > 70 fold elevated have been reported and levels > 1000 or > 2000 ng/L are not uncommon^[40-43], which is a similar finding in patients with pernicious anemia^[44]. These values overlap with 80%-100% of patients with ZES in various series^[39]. Similarly, in patients taking PPIs without ZES, which is the also one of the most common causes of hypergastrinemia, the PPIs can lead to various degrees of hypergastrinemia in different patients. Although, as pointed out in a number of studies, PPIs frequently lead to < 3 fold increase in fasting gastrin and in some studies do not increase the value out of a normal range^[22,45,46], this finding can not be relied on in an individual patient. This conclusion is firmly supported by various studies which report 80%-100% of the patients without ZES in their studies treated with PPIs develop hypergastrinemia, 20%-25% > 4 fold elevated, and values > 1000 pg/mL are not uncommon^[24-27,47]. These levels of PPI induced increases in fasting gastrin overlap with that seen in more than 60% of patients with ZES^[6,39,48,49]. Fourth, alone, no absolute level of any other tumor marker such as a serum chromogranin A (CgA) level, can establish the diagnosis of ZES. CgA levels are elevated in 90%-100% of patients with ZES, which can be contributed to by both the gastrinoma and the gastrin induced enterochromaffin-like (ECL) cell hyperplasia which is almost always present^[50-55]. However, the main problem is that PPIs or high doses of other antisecretory drugs, can increase CgA levels in patients without ZES, within a few days of use, which is thought secondary to the PPI-induced hypergastrinemia causing gastric ECL proliferative effects^[27,56,57]. The PPI induced increases in CgA can be usually < 4 fold, but can be up to 40-fold, which overlaps with values seen in > 90% of patients with ZES, as well as seen in numerous, non-ZES conditions^[24,27,33,54,57-59]. Fifth, the recommended establishment of a diagnosis of ZES in a hypergastrinemic patient by other methods, as proposed in paper No. 1^[1], such as by attempting to establish the presence of a neuroendocrine tumor (primarily by imaging studies) or by establishing the presence of a gastrinoma, is unlikely to be successful in many patients and may lead, in fact, to false diagnoses. It is likely to be unsuccessful in many patients because < 30% of patients with ZES at presentation at the current time, have liver metastases that possibly could be biopsied and the diagnosis of a gastrinoma established by immunohistochemistry^[13,60,61]. Even this does not secure the di-

agnosis, because other pancreatic neuroendocrine tumors [non-ZES primitive neuroectodermal tumors (pNETs)] can stain occasionally positively for gastrin but not be associated with ZES or the portion of the neuroendocrine tumor biopsied may not show gastrin staining^[62-69]. Furthermore, localization of a primary gastrinoma, even with the use of increasingly sensitive methods such as somatostatin receptor imaging or endoscopic ultrasound studies, misses most small duodenal tumors, which are present in 60%-80% of patients with ZES^[70-75]. Furthermore, cross-sectional imaging studies (computed tomographic scanning, magnetic resonance imaging, trans-abdominal ultrasound) will be negative in > 60% of all patients with duodenal tumors and thus not be able to assist in suggesting the presence of a pNET in the majority of suspected cases, especially those patients being examined early in their course^[71,72,76,77]. Lastly, sensitive methods such as somatostatin receptor scintigraphy can lead to false positive localization results and in one prospective study^[78,79], 12% of all possible pancreatic endocrine tumor localizations were false positive. These include the presence of an accessory spleen, gallbladder retention, an abscess, various inflammatory processes, inadequate bowel cleansing, thyroid disease, various granulomatous lung diseases, and other neuroendocrine tumors/proliferations such as gastric ECL proliferation or other gastrointestinal-neuroendocrine tumors like gastric carcinoids tumors^[78-81]. Therefore, one cannot conclude that localization on a somatostatin receptor scintigraphy study in a patient with hypergastrinemia necessarily equates to localization of a gastrinoma and establishment of the diagnosis of ZES. Fifth, besides the assessment of fasting gastrin levels and gastric fluid pH, various gastrin provocative tests (primarily after secretin, occasionally after glucagon)^[14,48,49,82,83] are widely used in the diagnosis of ZES. The secretin test in particular has been well studied and in one recent detailed analysis of 537 patients with ZES in the literature as well as 293 NIH patients with ZES prospectively studied, the secretin test was shown to have a sensitivity of 94% with a specificity of 100% using a criterion of ≥ 120 ng/L increase post secretin^[49]. Couldn't the secretin provocative test be used while the patient is taking PPIs to circumvent the need to stop the PPI? Unfortunately, the answer is no, because a recent study^[84] reports a false positive secretin test in a patient without ZES taking PPIs. Furthermore, another study demonstrated if patients are achlorhydric, false secretin positive tests can occur^[85].

Commentary on paper

In the study by Rehfeld *et al.*^[2], Seven of 12 tested commercial kits inaccurately measure plasma concentrations of gastrin; these assays used antibodies with inappropriate specificity that were insufficiently validated. Misdiagnosis of gastrinoma based on lack of specificity of assays for gastrin results in ineffective or inappropriate therapy for patients with ZES.

Background: An accurate assessment of fasting serum

gastrin levels (FSG) is central to the diagnosis of ZES and the diagnosis cannot be made without it. This is especially true, because this is the initial study that leads to the suspicion of ZES, in most cases^[2,7,9,12,83]. An assessment of FSG is generally used as the initial study not only because of its convenience and widespread availability, but also because it is elevated in almost all patients with ZES, except for a few specific situations. In a review of 2229 cases of ZES from the literature^[39], FSG levels were elevated in 97% of patients and in 309 patients with ZES seen at the NIH it was elevated in 99.3% of patients^[39]. These results and others^[6,86,87] demonstrate that normal FSG levels are uncommon overall in patients with ZES however, in a few small subgroups with active disease, normal values are not uncommon. This includes patients with active ZES who had had unsuccessful curative resection of a gastrinoma previously, who have ZES with multiple endocrine neoplasia type 1 (MEN1) post successful parathyroidectomy for hyperparathyroidism, in patients being treated with somatostatin analogues or occasionally in patients after various anti-tumor treatments (chemotherapy, chemo-embolization, *etc.*)^[86-96]. Although many physicians think FSG levels are massively elevated in ZES and easy to distinguish from other disorders, unfortunately, as pointed out above, this is not the case. Chronic atrophic gastritis, other hypo/achlorhydric conditions, chronic renal failure and the use of PPIs can cause FSG levels that overlap with those seen in most patients with ZES. Furthermore, in 60%-65% of ZES patients when initially diagnosed, the FSG levels are < 10-fold elevated and these levels overlap with a number of other conditions, some of which are much more frequent than ZES, which can also be associated with gastric acid hypersecretion, such as seen in ZES^[5,13,39,48,97]. This FSG level (i.e., > or < 10 \times increased) is pointed out because the existing criteria used for the diagnosis of ZES in most guidelines and reviews is divided on the basis of the elevation of FSG^[3,12,14,38,39]. If the FSG is > 10-fold elevated (usually > 1000 ng/L) and the gastric fluid pH < 2, a diagnosis of ZES is established after ruling out a possible retained antrum syndrome by history^[12,14,38,39,98]. On the other had if the FSG is < 10-fold increased and the gastric fold pH < 2, other conditions need to be excluded including *H. pylori* infection, antral G cell syndromes, gastric outlet obstruction and renal failure^[3,7,9,38,39]. In these cases a gastric analysis with determination of basal acid output, and a secretin provocative test is recommend, or if secretin is not available, a glucagon provocative test has been recommended^[7,12,49,82,87]. These latter provocative tests involve the assessment of serum gastrin before and after a secretin or glucagon injection, so an accurate assessment of serum gastrin is essential to their correct interpretation.

Specific comments: Rehfeld *et al.*^[2] report that in many cases that the gastrin laboratory assays being used to assess FSG levels are not giving accurate results. Only 5 of the 12 commercial assays examined accurately assessed

FSG levels with the others giving FSG values either too high or too low and thus their results could lead to an over- or under-diagnosis of ZES, based on the FSG levels reported^[2]. While gastrin provocative test results (secretin, glucagon) were not assessed, a similar result would be expected with these and thus the result would not be dependable in most cases. This report raises a number of problems for physicians trying to diagnose and treat patients with ZES. First, it demonstrates that FSG levels should not be compared from different laboratories using different assays unless some validation is performed. Second and more important, this report raises a real dilemma for the practicing clinician, because it raises the question of whether he can rely on FSG values reported to him by the laboratory he uses. There is no simple solution to this dilemma. The lists of laboratories assessed in this paper^[2] can be consulted to see if the one used for the blood samples sent by for the clinician's patients(s) are on this list. Because the diagnosis of ZES has such significance for any patient and alternative approach as discussed in a section below is to refer the patient to center with known expertise, or to contact them and find out which laboratory in their area they recommend to assess FSG, and confirm the results using this laboratory.

Recommended approach to diagnosis of ZES (based on points raised in papers 1, 2)

First, it is essential to realize that patients with untreated gastric acid hypersecretion with ZES can develop complications rapidly and that this needs to be adequately treated before trying to establish a diagnosis, especially by stopping PPIs. There is no urgency in establishing the diagnosis. Therefore if the patient has active peptic ulcer disease or symptoms and the diagnosis of ZES is suspected, a FSG should be drawn and the acid hypersecretion adequately controlled [our initial starting dose is equivalent to omeprazole 60 *qd*^[99,100], or if complicated disease (presence of MEN1, Billroth 2 surgery, or severe gastroesophageal reflux symptoms), we start with the equivalent of omeprazole 40 *bid*]^[37,101-103] and the patient should undergo an upper gastrointestinal endoscopy. We start with a higher PPI dose to make sure the acid hypersecretion is initially well controlled and then later it can be reduced in many patients^[37,101]. During this endoscopy, gastric pH can be measured and also the size of gastric mucosal folds noted because 92% of ZES patients have prominent gastric folds^[35]. Most patients can be satisfactorily treated by this PPI dose initially, however some require higher doses and therefore it is best to assess the control of acid hypersecretion on PPI^[37,99,104], however, only a few specialty centers have this capability, and thus most use control of symptoms to monitor effectiveness of treatment. If the patient has symptoms of gastroesophageal peptic disease or active disease on endoscopy they should be treated for 8-12 wk until symptom free and then the upper gastrointestinal endoscopy repeated

to make sure any peptic disease is resolved before attempting to establish the diagnosis by stopping PPIs. During this time the reliability of the FSG assay used needs to be explored both by reviewing the laboratories in paper No. 2^[2] and contacting some group well versed in your area with the diagnosis of ZES that uses gastrin assays regularly. If the FSG is elevated it, of course this could be due to the PPI the patient was taking when the blood sample was drawn, which is the situation in most cases, so that the diagnosis remains unclear at this point, particularly if the gastric pH was > 2. At this point many authorities recommend that consideration be given to referring the patient to a group in your area well versed in the diagnosis of ZES^[7,9,12,14]. If not possible, then after the repeat endoscopy shows healing of mucosal disease and the patient is asymptomatic, one can consider PPI withdrawal for diagnosis. Our approach is similar to outlined recently^[14] and briefly, is to carefully consult with the patient about the need to keep in close contact, then to substitute an H₂ receptor antagonist (usually ranitidine 450-600 every 4 to 6 h) for the PPI for 3-5 d and then stop the ranitidine for 24 h allowing liberal use of antacids. Then on the day of the test we measure FSG × 2, and measure gastric pH and acid output. We usually perform a secretin test at the same time if our index of suspicion is high^[49]. This circumvents the need to take the patient off of PPIs again at a later time if the secretin test is deemed necessary. If a gastric/esophageal pH probe is available the assessment of gastric pH and FSG at early times can be done with an attempt to document a pH < 2 with an elevated FSG.

One could ask at this point why go through all of this and why is it so important to establish the diagnosis of ZES correctly in most patients. The primary reason is that the diagnosis of ZES requires special treatment and the treatment must be continued life-long if the patient is not cured^[7,9,105]. If the patient is not cured, life-long PPI treatment will be required, the doses may be different than that usually used in patients with idiopathic peptic disease, periodic assessment for the presence of MEN1 will be needed because 20%-30% of ZES patients have this and its diagnosis may not be initially evident and treatment directed at the gastrinoma, which are malignant in 60%-90% of cases, must be considered^[13,35,38,106,107]. The latter include periodic assessment of tumor location/extent with imaging studies, consideration of surgical resection which is recommended in ZES patients in whom MEN1 is not present, life expectancy is good and no serious surgical contra-indications are present^[7,71,108-110]. Therefore a diagnosis of ZES markedly effects clinical management and thus needs to be well established.

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Molecular targets in the treatment of alcoholic hepatitis

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Abstract

Alcohol related costs to health and society are high. One of the most serious complications of alcohol misuse to the individual is the development of alcoholic hepatitis (AH), a clinical syndrome of jaundice and progressive inflammatory liver injury in patients with a history of recent heavy alcohol use. It has a poor outcome and few existing successful therapies. The use of glucocorticoids in patients with severe AH is still controversial and there remains a group of patients with glucocorticoid-resistant disease. However, as our understanding of the pathogenesis of the condition improves there are opportunities to develop new targeted therapies with specific actions to control liver inflammation without having a detrimental effect on the immune system as a whole. In this article we review the molecular mechanisms of AH concentrating on the activation of the innate and adaptive immune response. We consider existing treatments including glucocorticoids, anti-tumor necrosis factor therapy and pentoxifylline and their limitations. Using our knowledge of the disease pathogenesis we discuss possible novel therapeutic approaches. New targets include

pro-inflammatory cytokines such as interleukin (IL)-17, chemokines and their receptors (for example IL-8, CXCL9 and CXCR3) and augmentation of anti-inflammatory molecules such as IL-10 and IL-22. And there is also future potential to consider combination therapy to selectively modulate the immune response and gain control of disease.

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Key words: Alcoholic hepatitis; Tumour necrosis factor- α ; Pentoxifylline; Interleukins; Chemokine receptors

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INTRODUCTION

Alcohol related morbidity in developed countries is second only to tobacco use and is responsible for 2.5 million deaths globally each year^[1]. It costs 1% of the GDP of middle to high income countries^[2]. In the United Kingdom, deaths from alcohol related liver disease have increased by 36% between 2001 and 2008^[3]. Alcoholic hepatitis (AH) is a clinical syndrome characterised by jaundice and progressive inflammatory liver injury in patients with a history of recent heavy alcohol misuse. Severe cases are associated with a high mortality of around 30%-50% at 28 d^[4,5]. However, the pathogenesis of the

condition is incompletely understood and there is a lack of targeted therapy.

It is clear that alcohol acts both directly on the liver, causing cell death by toxic mediators, and *via* activation of the immune system. Over the past 2 decades considerable headway has been made into understanding the immune basis of the disease with studies on pro- and anti-inflammatory cytokines, cell trafficking to the liver and mechanisms of hepatocyte death. This work has been hampered by the absence of a suitable animal model of the disease and much of the relevant work has been inferred from other models of acute hepatic injury or through chronic ethanol feeding. Although these models share several important similarities with human AH, such as neutrophil accumulation in the hepatic ischaemia model or hepatocyte ballooning in the ethanol-fed rat model, they still have significant differences. Neither the acute hepatic ischaemia model nor the carbon tetrachloride model involves alcohol metabolism and alcohol related liver damage. The chronic ethanol feeding model does not mirror the acute phase of inflammation in AH and instead is more similar to alcoholic steatohepatitis^[6]. Therefore, at present we must interpret results from these studies with caution and focus attention on studies on human tissue.

In this review, we consider the pathogenesis of AH, focussing on the contribution of immunity (Figure 1), and relate this to the use of targeted molecular therapies. We briefly discuss the evidence for anti-tumor necrosis factor- α (TNF α) therapies and pentoxifylline, both of which have been extensively studied in AH, but do not intend this to be a definitive systematic review. For further discussion on these 2 treatments we refer the readers to recent comprehensive reviews^[7-9]. We have emphasised the molecules that we believe have the greatest future therapeutic potential.

PATHOGENESIS

Within the liver alcohol can be metabolised to acetaldehyde by three enzyme systems: alcohol dehydrogenase in the cytosol, the microsomal ethanol-oxidising system and peroxisomal catalase. These metabolic pathways lead to the production of reactive oxygen species and cause lipid peroxidation. There is a reduction in the hepatoprotective methyl donor S-adenosylmethionine and mitochondrial glutathione. Acetaldehyde forms adducts with proteins, lipids and DNA, impairing their function and promoting DNA damage (see Setshedi *et al*^[10] for a detailed review on this subject). However, it is not only the direct toxic effect of alcohol on the liver that causes damage but also activation of the immune response.

Activation of the innate immune system

Alcohol is known to change the gut microbiota^[11] and can increase numbers of both aerobic and anaerobic organisms^[12]. In addition, metabolism of alcohol by gut bacteria results in production of acetaldehyde which has

a direct effect on the gut epithelium, opening up tight junctions, increasing its permeability^[13,14]. This allows the presentation of bacteria or microbial cellular components to cells of the innate immune system. These pathogen-associated molecular patterns are recognised by toll-like receptors (TLRs) which are expressed on a wide variety of immune and stromal cells, in particular on monocytes and macrophages. Gut-derived endotoxin [or lipopolysaccharide (LPS)] crosses into the portal venous system and activates TLR4 on CD14⁺ cells. Higher levels of LPS have been demonstrated in the portal veins of patients with alcohol related cirrhosis compared to non-alcohol related cirrhosis controls^[15]. *Via* adaptor molecules MyD88 and toll/interleukin (IL)-1 receptor domain-containing adaptor inducing interferon- β , the signalling pathway converges on a few transcription regulators [nuclear factor κ B (NF- κ B), activator protein 1 (AP-1) and interferon regulatory factors], which lead to a pro-inflammatory immune response. Signalling through TLR4 plays an important role in the development of alcohol related liver damage as evidenced by TLR-mutant mice which are resistant to alcohol and have lower levels of circulating TNF α despite having higher circulating LPS^[16]. Modulation of the gut microbiota with pro- or antibiotics reduces circulating levels of LPS in mouse models^[17,18]. Activation of TLR4 on macrophages and kupffer cells (liver resident macrophages) leads to secretion of pro-inflammatory cytokines such as IL-1, IL-6 and TNF α , which act on surrounding hepatocytes and stromal cells as well as activating the adaptive immune response.

Activation of the adaptive immune system

It is clear that T-lymphocytes play a role in the development of AH with increased levels of CD4⁺ and CD8⁺ T-lymphocytes found in liver biopsy material from patients with AH compared to healthy controls^[19]. The liver has a unique dual blood supply with both portal venous and hepatic arterial blood draining into the hepatic sinusoids. Hepatic sinusoid endothelial cells (HSEC) are therefore exposed to large numbers of leukocytes and HSEC expression of adhesion molecules and chemokine receptors enables adherence of lymphocytes and transmigration into the hepatic parenchyma to the site of inflammation. Unlike other endothelial cells HSEC express low levels of selectins^[20] but high levels of intercellular adhesion molecule-1 (ICAM-1), vascular adhesion protein-1 (VAP-1) and vascular cell adhesion molecule-1 (VCAM-1)^[21]. Inhibition of ICAM-1 and VCAM-1 reduce lymphocyte adhesion in *in vitro* flow-based adhesion experiments^[21].

Liver infiltrating lymphocytes express high levels of CXCR3 and migrate towards its ligands CXCL9 and CXCL10^[22], which are produced by a variety of liver cells including hepatic stellate cells and activated myofibroblasts^[23]. High levels of TNF α found in AH stimulate production of CXCL10 from hepatocytes and HSECs increasing T cell recruitment to the liver^[24]. Data from

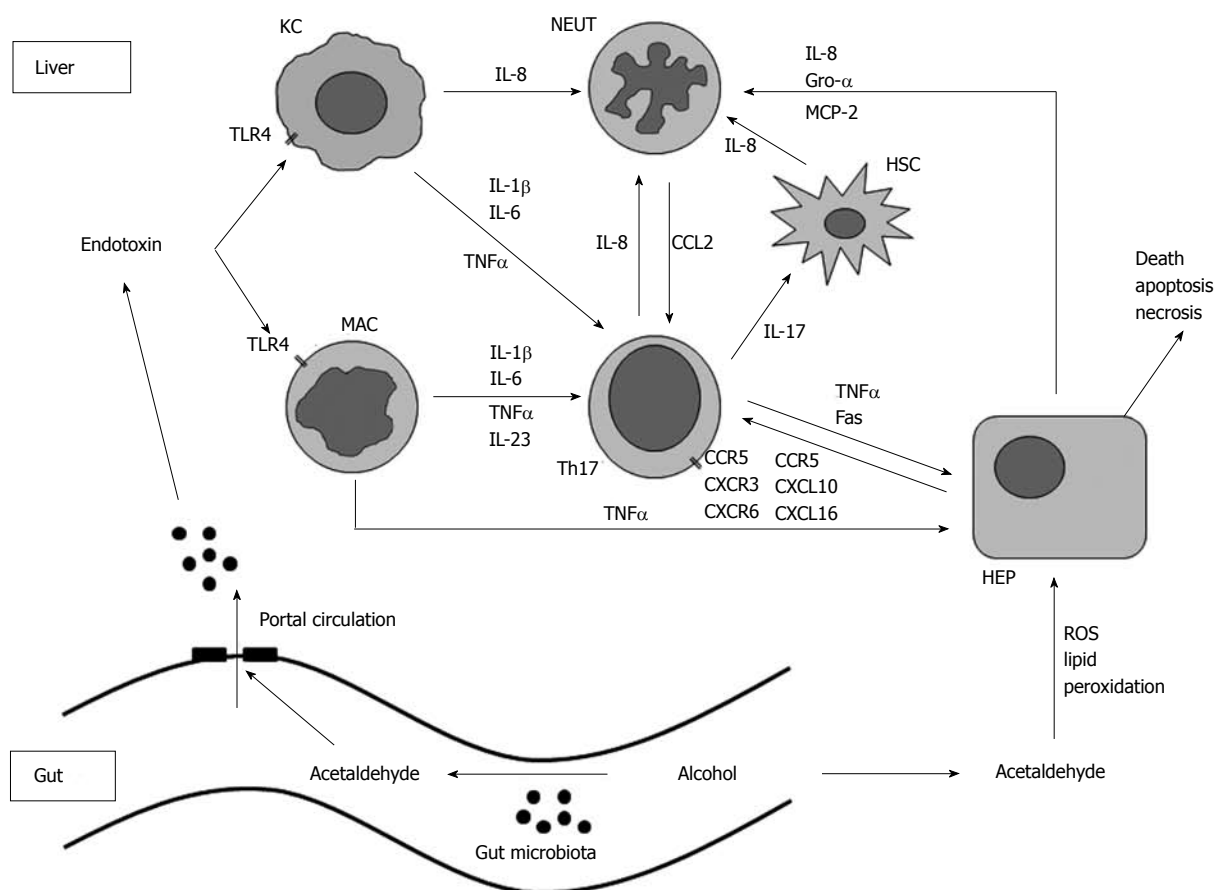


Figure 1 The effects of alcohol on the innate and adaptive immune system. Alcohol is metabolised by gut microbiota to acetaldehyde which has a direct effect on the epithelial tight junctions, making them more leaky. Endotoxin and other pathogen-associated molecular patterns enter the portal circulation and act via toll-like receptor (TLR) 4 receptors on kupffer cells (KCs) and macrophages (MAC), activating them to produce pro-inflammatory cytokines and chemokines, which act on hepatocytes, neutrophils (NEUT) and T cells. Both CD4⁺ and CD8⁺ T cells are recruited into the liver but there is a significant proportion of interleukin (IL)-17 secreting CD4⁺ Th17 cells, which in turn activate stellate cells to produce IL-8. T cells also act directly on hepatocytes via the tumor necrosis factor (TNF) receptor 1 or Fas pathways leading to apoptosis. Alcohol also has a direct effect on hepatocytes (HEP) through metabolism by alcohol dehydrogenase, the microsomal ethanol-oxidising system and peroxisomal catalase to acetaldehyde, which damages cells by the formation of reactive oxygen species (ROS) and increased lipid peroxidation. HSC: Hepatic stellate cell; MCP: Monocyte chemotactic protein.

patients with chronic hepatitis C infection also demonstrate that liver infiltrating lymphocytes express high levels of chemokine receptor CXCR3 as well as others such as CCR5 and CXCR6^[24,25].

Once T-lymphocytes have been recruited to the site of inflammation there is evidence that the CD4⁺ subset mediates hepatocyte death. Adoptively transferred liver associated CD4⁺ T cells from ethanol-fed rats demonstrated greater hepatocyte apoptosis in recipient rats compared to those transferred from non-ethanol-fed rats^[26].

Hepatocyte death can occur by necrosis or apoptosis. Necrosis is characterised by ballooning and is clearly described in cases of AH. However, apoptosis has also been identified as an important mechanism of AH. There is greater hepatocyte apoptosis in the livers of patients with AH compared to controls^[27] and the degree of apoptosis correlates with clinical measures of severity^[28]. Apoptosis may be caused by direct cytotoxic T cell activation of the Fas apoptosis pathway, which in turn can induce IL-8, a neutrophil chemotaxin. Alternatively, TNFα binding to TNF-R1, a member of the death receptor family, can initiate apoptosis. As discussed above,

TNFα is significantly elevated in severe AH suggesting it can mediate hepatocyte death by this mechanism as well as by direct toxic effects leading to necrosis.

Neutrophil recruitment

The degree of intrahepatic neutrophil infiltration correlates with severity of AH^[29] although their exact role in the pathogenesis of the disease is less well understood. Data from animal models of other acute liver injury such as ischaemia suggest that neutrophils are activated by cytokines including TNFα, IL-1β and IL-17 and are recruited to the hepatic parenchyma by CXC chemokines such as Gro-α^[29], IL-8 and monocyte chemoattractant protein 2^[30]. CXC chemokines are particularly important for neutrophil infiltration in AH^[31]. These neutrophils migrate into the parenchyma and cause hepatocyte necrosis by release of reactive oxygen species and proteases^[32].

With this understanding of the molecular mechanisms of the pathogenesis of AH, we can more effectively target therapy.

Existing therapy for severe AH

Glucocorticoids: Glucocorticoid therapy was first demonstrated to be beneficial in the treatment of patients with severe AH in 1978^[4]. Glucocorticoids are potent immunosuppressants which have wide ranging effects on a large number of cell types. Key among their actions is suppression of the pro-inflammatory transcription factors NF- κ B and AP-1^[33] resulting in lower levels of circulating TNF α and IL-8^[34].

Glucocorticoid treatment has been evaluated in many clinical trials and debate continues whether they are beneficial with conflicting results of meta-analysis^[35-37]. The largest placebo-controlled trial recruited a heterogeneous group of 90 patients (including those with moderate and severe disease as well as end stage alcoholic liver disease) and failed to show a survival benefit at 30 d^[38]. Another trial recruiting only patients with histologically proven AH showed a survival benefit with glucocorticoid treatment at 2 mo^[39] but no difference at long term follow-up at 2 years^[40]. More recent meta-analysis of individual patient data demonstrated a survival benefit with glucocorticoid treatment in patients with severe AH^[5]. However, they identify a proportion of glucocorticoid non-responders, with a significantly worse outcome, by applying the Lille model^[41] at day 7 of treatment. This highlights the fact that as in other inflammatory diseases such as asthma and rheumatoid arthritis there is a proportion of patients resistant to steroid treatment^[42]. In addition, the non-specific effect of glucocorticoids means that there is a risk of infective complications. Other therapeutic options are therefore essential.

Pentoxifylline: Pentoxifylline, a phosphodiesterase inhibitor, has a moderate effect on TNF α levels^[43-45] although circulating TNF α levels in patients on active treatment did not show any difference from those on placebo in a randomised controlled trial^[46]. The effects of pentoxifylline are likely to be less specific than inhibition of a single cytokine. In animal models of liver injury it has shown a reduction in pro-fibrogenic cytokines such as pro-collagen I and transforming growth factor beta 1^[47]. Although the exact mechanism of action of pentoxifylline in AH is not clear several trials have shown a survival benefit, predominantly through a significant reduction in mortality due to hepatorenal syndrome^[46,48]. However, a recent Cochrane systematic review was unable to draw firm conclusions about its beneficial effect due to probable bias in the design of a number of trials^[49]. A large United Kingdom multi-centre randomised placebo-controlled trial of pentoxifylline, prednisolone or combination is currently underway, which hopes to provide a definitive answer as to whether these treatments are beneficial in patients with severe AH.

Anti-oxidant therapy: Oxidative stress plays an important role in the pathogenesis of AH (Dey *et al.*^[50]). In an attempt to reduce its toxic effects, several clinical trials of anti-oxidant agents have been undertaken either in com-

bination with glucocorticoids or as a monotherapy. A recent randomized controlled trial (RCT) of 5 d of intravenous N-acetylcysteine (NAC) or placebo with 4 wk of glucocorticoids^[51] demonstrated a reduction in mortality at 1 mo in the NAC arm but failed to reach significance at 2 mo and 6 mo (the primary outcome of the trial). A lower incidence of infections was noted in the NAC treated group.

A further RCT has been reported using an anti-oxidant cocktail (including NAC and vitamin E) with or without concomitant glucocorticoids, which failed to show an improvement in 6 mo survival^[52]. A different anti-oxidant cocktail was tested against glucocorticoid therapy but a higher number of deaths at 30 d was reported in the anti-oxidant treatment arm^[53]. Vitamin E versus placebo also failed to demonstrate any benefit in outcome^[54].

Anti-TNF α therapy: Anti-TNF therapy with chimeric monoclonal antibody (infliximab) or fusion proteins (etanercept) have proved successful in other inflammatory conditions such as rheumatoid arthritis^[55] and Crohn's disease^[56]. With high levels of TNF α demonstrated in patients with severe AH^[57] and since TNF α predicts short-^[58] and long-term survival^[59], it is an appropriate target for effective therapy.

In ethanol-fed rats treated with anti-TNF antibody, there was a significant improvement in hepatic inflammation, although serum levels of TNF α remained unchanged^[60] supporting a role for this drug in human AH. An open label pilot study of a single dose of 5 mg/kg infliximab in combination with glucocorticoids found a significant reduction in Maddrey Discriminant Function^[61]. An RCT of 3 infusions of infliximab 10 mg/kg or placebo at weeks 0, 2 and 4 in combination with prednisolone was stopped early due an excess of deaths in the active treatment group due to infection^[62]. An RCT of etanercept also found that there was a significantly poorer outcome for patients on active treatment due to infection^[63]. Lower doses of infliximab or less aggressive loading regimes may reduce the incidence of infection. Alternatively, combination therapy with an anti-inflammatory treatment such as recombinant IL-10 or IL-22 (see below) may boost the immune response to infections.

Alteration of the gut microbiota

Alcohol alters the gut microbiota and together with increased intestinal permeability there is activation of the innate immune system to produce an inflammatory response in AH. No clinical trial of probiotics has yet been reported in patients with AH. However, there is evidence that changing the bowel microbiota with probiotics in patients with alcohol related cirrhosis reduces the production of pro-inflammatory cytokines^[64] and improves liver function tests in patients with alcoholic psychosis^[65]. Further investigation is required to determine whether probiotic therapy is beneficial in AH. Alteration of the gut microbiota with non-absorbable antibiotics

such as rifaximin may also be beneficial and has recently been proven effective for the prevention of hepatic encephalopathy in patients with cirrhosis^[66].

The treatments for AH discussed so far have wide-ranging effects on the immune system. These non-targeted therapies have failed to demonstrate a clear benefit in outcome. Both anti-TNF α therapy and glucocorticoids suppress the appropriate immune response to infection, resulting in a higher incidence of serious infections. Over the last decade the emphasis in treatment of inflammatory conditions has shifted to a more targeted approach to lessen the impact of inflammatory mediators, while reducing the systemic complications of treatment.

Future molecular targets

IL-10: IL-10 is a potent anti-inflammatory cytokine, suppressing the production of inflammatory cytokines (including TNF α) by T helper cells, monocytes and kupffer cells. IL-10 can also inhibit production of reactive oxygen species by neutrophils^[67]. IL-10 is upregulated in surviving patients that had a response to glucocorticoid therapy^[34]. This is consistent with studies of glucocorticoid effects on T cells in other inflammatory conditions such as asthma^[68]. Therefore increasing the levels of IL-10 in patients with significant inflammation may prove to be a useful therapeutic target.

Unfortunately, treatment with recombinant human IL-10 (rhuIL-10) in patients with Crohn's disease has proved disappointing. Although well tolerated, it failed to induce clinical remission or response in patients with mild to moderately active Crohn's disease^[69,70] and a recent systematic review suggested a lack of effect of rhuIL-10 in patients with Crohn's^[71]. One study found that administration of rhuIL-10 lead to an increased capacity of leucocytes to produce interferon-gamma (IFN γ), a potent Th1 secreted pro-inflammatory cytokine^[72], which may explain its lack of anti-inflammatory effect.

In the context of liver disease, rhuIL-10 has been trialled in chronic hepatitis C. Thirty patients with advanced fibrosis who failed treatment with antiviral therapy were treated with rhuIL-10 administered subcutaneously daily or 3 times weekly for 1 year^[73]. There was a significant improvement in serum transaminase and a reduction in inflammation scores in 13 of 28 patients and fibrosis scores in 11 of 28 patients and a 2 fold reduction in the number of hepatitis C virus (HCV) specific CD4⁺ IFN γ secreting T cells. However, there was a significant increase in HCV RNA levels which returned to baseline 6 mo after end of treatment.

A pilot open label study of rhuIL-10 in combination with glucocorticoids in 8 patients with severe AH failed to show any changes to neutrophil-derived or serum IL-8 and TNF α production^[74]. No significant differences in mortality or MDF were observed in comparison to the control group. This may be explained by the fact that although in patients with severe AH IL-10 is upregulated, it is not sufficient to reduce the levels of TNF α ^[75]. Therefore a combination of cytokine targets

to inhibit TNF α while augmenting IL-10 may be a useful approach.

IL-22, a member of the IL-10 family, shares its anti-inflammatory effects and potentiation of this molecule may be more beneficial than the use of rhuIL-10 since the IL-22 receptor is only expressed on epithelial cells such as hepatocytes. A study in a chronic ethanol-fed mouse model showed that treatment with recombinant IL-22 ameliorates liver injury and hepatic oxidative stress^[76]. In a murine model of acute hepatitis IL-22 receptor is upregulated on hepatocytes and blockade of IL-22 exacerbates disease while administration of IL-22 ameliorates it^[77]. Interestingly IL-22 is produced by Th17 cells, thought to be pathogenic (see below). Perhaps the strategy of blocking IL-17 in conjunction with augmenting IL-22 will have a more synergistic effect.

IL-8: Inhibition of neutrophil mediated hepatic damage may also prove to be an important therapy. Levels of IL-8 gene expression and serum protein are elevated in patients with AH and higher levels are associated with a poorer outcome^[31]. IL-8 gene expression is also related to neutrophil hepatic infiltration as well as increased portal pressure^[31]. IL-8 antagonism may reduce neutrophil recruitment into the liver without impairing the neutrophils' function in host defence and elimination of pathogens.

Hepatocyte growth factor: Hepatocyte growth factor (HGF) is thought to play an important role in the regeneration of the liver after an injury such as AH. It reduces hepatocyte apoptosis^[78] as well as reducing ethanol-related oxidative damage^[79]. Higher levels of HGF correlate with greater hepatocyte proliferation in AH^[80] and are associated with better outcome^[81]. Treatment of alcohol fed mice with recombinant HGF reduced the development of fatty liver^[82]. Interestingly, HGF is produced by neutrophils and the degree of neutrophil infiltration in the liver correlates with levels of HGF^[83]. Therefore a strategy of blocking neutrophil trafficking to the liver by IL-8 antagonism may also have a detrimental effect by reducing the liver's regenerative capacity. Combination of IL-8 antibodies with recombinant HGF or HGF gene therapy may reduce the inflammatory effects of neutrophils while maintaining the beneficial effects on hepatocyte proliferation.

T cells: T cells play a role in the recruitment of neutrophils and the perpetuation of inflammation in AH. Reduction in T cell proliferation through targeting IL-2, a T cell proliferation and survival factor, may reduce the number of pro-inflammatory cells within the inflamed liver. Inhibition of IL-2 receptor alpha (CD25) with a chimeric monoclonal antibody reverses glucocorticoid resistance *in vitro* in peripheral blood mononuclear cells from patients with AH^[42]. This monoclonal antibody (basiliximab) is licensed for use in acute cellular rejection of cadaveric renal transplants. Promising results

Table 1 Summary of mechanism of action and existing evidence about potential future molecular targets for the treatment of severe alcoholic hepatitis

Treatment	Mechanism of action	Clinical evidence
rhu IL-10	Suppression of pro-inflammatory cytokines, e.g., TNF α	Phase 2 study in severe AH with concomitant glucocorticoids showed no significant difference in mortality or levels of IL-8 and TNF α ^[74]
rhu IL-22	Suppression of reactive oxygen species Increased levels of anti-inflammatory IL-22 Reduced oxidative stress	Reduced liver injury in ethanol-fed rat model ^[76] and acute hepatitis mouse model ^[77] . No studies in human liver disease yet reported
Anti-IL-8 antibody	Reduced neutrophil recruitment to liver	Phase 2 studies in COPD demonstrated reduced dyspnea ^[103] . No studies in human liver disease have yet been reported
rhu hepatocyte growth factor	Reduced hepatocyte apoptosis	HGF levels correlate with outcome in severe AH ^[81] . Well tolerated in a small phase 1/2 trial in fulminant hepatitis but no clear clinical benefit was seen ^[104]
Basiliximab (anti-CD25 antibody)	Reduced T cell proliferation and subsequent pro-inflammatory cytokine production	Reverses <i>in vitro</i> glucocorticoid resistance in patients with severe AH ^[42] . No clinical trials yet conducted in liver disease
Secukinumab (anti-IL-17A antibody)	Reduced pro-inflammatory IL-17A	Well tolerated in patients with rheumatoid arthritis, psoriasis or uveitis ^[93] . No clinical benefit in Crohn's disease ^[94] . No clinical trial yet reported in liver disease
Anti-CXCL10 antibody	Reduced Th1 cell recruitment to liver	CXCR3 (receptor for CXCL10) is elevated in patients with chronic hepatitis C infection ^[95] . Phase 2 clinical trial is underway in patients with PBC

rhu: Recombinant human; AH: Alcoholic hepatitis; TNF α : Tumor necrosis factor- α ; HGF: Hepatocyte growth factor; PBC: Primary biliary cirrhosis; IL: Interleukin; COPD: Chronic obstructive pulmonary disease.

from an open label study in patients with glucocorticoid resistant ulcerative colitis showed a benefit^[84] but has not been borne out in a double-blind randomised controlled trial^[85]. The immunogenicity of the drug together with a high achievement of clinical response and remission in the placebo arm, may have contributed to the lack of benefit over placebo in this study. However, given in a single dose (shown to be less immunogenic in renal transplant patients^[86]) basiliximab may prove to be a useful adjunct to glucocorticoid therapy in patients who do not respond to this therapy.

Th17 cells: Recently, T cells producing IL-17 (known as Th17 cells) have been ascribed a central role in the pathogenesis of many inflammatory and autoimmune conditions^[87] and are present in chronically inflamed tissues^[88]. The generation of Th17 cells in humans requires IL-1 β and IL-6 with IL-23 as a survival factor^[89]. IL-17 can itself act as a neutrophil chemotaxin but can also stimulate production of other chemotaxins such as IL-8 and CXCL1^[90]. To date, 6 Th17 cytokines have been described (known as IL-17A-F)^[91], the most studied being IL-17A.

Serum levels of IL-17 are higher in patients with AH compared to healthy and HCV controls^[92]. From the same study liver biopsy material from patients with AH showed significantly higher numbers of infiltrating IL-17⁺ cells compared with alcohol related cirrhosis samples and the number of infiltrating cells correlated with the Maddrey Discriminant Function. The increased levels of IL-17 within the liver are likely to act on hepatic stellate cells, which when stimulated with IL-17 increase chemotaxis of neutrophils^[92].

Phase 1 trials of humanised anti-IL-17A monoclonal

antibody (secukinumab) have proved successful in the treatment of rheumatoid arthritis, psoriasis and uveitis^[93] with phase 2 and 3 trials in rheumatoid arthritis ongoing. Disappointingly, small benefit was seen over placebo in patients with active Crohn's disease^[94], which may be due to low concentration of the drug at the site of inflammation or presence of other Th17 produced cytokines such as IL-17F. To date no studies of secukinumab have been reported in patients with liver disease.

Lymphocyte trafficking to the liver

A number of chemokines and receptors have been identified as playing a role in neutrophil, macrophage or lymphocyte trafficking to the liver. Targeting these receptors or blocking their ligands could limit liver infiltration and subsequent damage leaving cells to perform other functions such as clearance of infection in the periphery. Several possible targets are appealing: CXCR3 and its ligand CXCL10 appears to be important in liver disease. CXCR3 is expressed on Th1 cells and intrahepatic CXCR3 is upregulated in patients with chronic hepatitis C infection^[24,95]. Although no studies have yet been performed in AH, a monoclonal antibody to CXCL10 is currently under evaluation in patients with primary biliary cirrhosis, Crohn's disease and ulcerative colitis. However, this approach may have its limitations; a murine model of IFN γ mediated hepatitis [by Concanavalin A (ConA) challenge] demonstrated that CXCR3^{-/-} mice developed a more severe hepatitis and failed to induce tolerance *via* conversion to an IL-10 secreting regulatory T cell (Treg) phenotype on re-challenge with ConA^[96]. This suggests that CXCR3 is required for liver homing of lymphocytes in inflammation with subsequent development of an anti-inflammatory IL-10 secreting

Treg phenotype to limit hepatic inflammation. Therefore inhibition of the initial accumulation of T cells may lead to alteration of hepatoprotective downstream events. Combination therapy with anti-CXCL10 and rhuIL-10 or IL-22 may be an option to maintain the protective effects of IL-10 secreting Tregs.

VAP-1 is another potential target; VAP-1 is elevated in patients with inflammatory liver conditions^[97] but also correlates with disease severity in AH^[98]. Blockade of this molecule reduces peripheral blood and liver derived lymphocyte migration across HSECs^[99].

Selective inhibition of T cell subsets, such as Th17 cells, from infiltrating the liver may reduce the most pro-inflammatory cell population from reaching the liver and causing more damage. However, the migratory fate of Th17 cells in humans is still poorly understood. Two chemokines, CCR4 and CCR6, have been identified as playing a role of Th17 migration in inflammatory disease^[100,101] but these are also expressed on a variety of other T cell subsets^[102]. Ongoing work is hoping to elucidate this pathway.

In conclusion, broad spectrum therapy for the treatment of severe AH has not been demonstrated to be clearly beneficial mainly due to development of infective complications. With our increasing understanding of the pathophysiology of the disease together with its immunological mechanisms, we have the opportunity to develop targeted molecular approaches (Table 1). However, work on AH remains challenging in the absence of an appropriate animal model. Many of the pre-clinical studies use chronic ethanol feeding murine models or we must infer results from non-alcoholic models of acute liver injury such as ischaemic or carbon tetrachloride induced hepatitis. This deficit must be addressed so we can further our understanding of the pathogenesis of this disease. Furthermore, clinical studies in AH lag far behind other inflammatory and autoimmune conditions such as rheumatoid arthritis and Crohn's disease. Learning from these studies we have seen that selected cytokine inhibition has rarely been successful (with TNF α being a notable exception). We therefore would strongly endorse future assessment of combination therapy such as recombinant IL-10 or IL-22 with anti-IL-17, HGF with anti-IL-8, glucocorticoids with IL-10, IL-22 or anti-CD25 or selective inhibition of chemokines and cytokines within the liver.

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Checkmate to liver biopsy in chronic hepatitis C?

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Abstract

Liver biopsy (LB) has traditionally been considered the gold standard for pretreatment evaluation of liver fibrosis in patients with chronic hepatitis C (CHC). However, LB is an invasive procedure with several shortcomings (intra- and interobserver variability of histopathological interpretation, sampling errors, high cost) and the risk of rare but potentially life-threatening complications. In addition, LB is poorly accepted by patients and it is not suitable for repeated evaluation. Furthermore, the prevalence of CHC makes LB unrealistic to be performed in all patients with this disease who are candidates for antiviral therapy. The above-mentioned drawbacks of LB have led to the development of non-invasive methods for the assessment of liver fibrosis. Several noninvasive methods, ranging from serum marker assays to advanced imaging techniques, have proved to be excellent tools for the evaluation of liver fibrosis in patients with CHC, whereas the value of LB as a gold standard for staging fibrosis prior to antiviral therapy has become questionable for clinicians. Despite significant resistance from those in favor of LB, noninvasive methods for pretreatment assessment of liver fibrosis in patients with CHC have become part of routine clinical practice. With protease inhibitors-based

triple therapy already available and substantial improvement in sustained virological response, the time has come to move forward to noninvasiveness, with no risks for the patient and, thus, no need for LB in the assessment of liver fibrosis in the decision making for antiviral therapy in CHC.

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Key words: Liver biopsy; Fibrosis; Noninvasive methods

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INTRODUCTION

Chronic hepatitis C (CHC) is a major public health concern, with around 180 million individuals affected worldwide^[1]. Liver fibrosis and its end-point cirrhosis are the main causes of morbidity and mortality in patients with CHC^[2]. Information on the stage of liver fibrosis is useful in patients with CHC not only for estimation of prognosis, but also for indication of antiviral therapy. Early international guidelines, consensus statements and expert panel opinions on the management of CHC unanimously recommended that decisions on treatment should be made only after performing a liver biopsy (LB) for pretreatment evaluation of the disease^[3-5]. Consequently, antiviral treatment for patients with CHC has been indicated only for those with moderate to severe

stages of fibrosis (Metavir F2, F3 or F4), while patients with no or minimal fibrosis (Metavir F0, F1) have not been treated^[6]. The rationale of such a strategy was to treat all patients with advanced fibrosis to halt disease progression and prevent complications, rather than those with no or minimal fibrosis who may await better treatments considering the slowly progressing natural history of CHC^[7]. The recommendations mentioned above led to the routine performance of LB in nearly all patients who were newly diagnosed with CHC and potential candidates for antiviral therapy. More recent guidelines^[8] still recommend LB in making treatment decisions, although it has been recognized that it is not necessary in patients with genotype 2 or 3, who can have as high as a 80% sustained virological response (SVR) rate.

For several decades, LB has been widely regarded as the gold standard for the staging of liver fibrosis^[9]. However, LB is an invasive procedure and it is sometimes associated with rare but severe complications^[10]. In addition, LB has several drawbacks (intra- and interobserver variability in histopathological interpretation, sampling errors, variable accessibility, high cost) which raises questions about its value for pretreatment assessment of liver fibrosis in patients with CHC^[11,12]. Nowadays, many clinicians no longer cite LB as the gold standard but, at best, it can only be considered an imperfect standard for the staging of liver fibrosis^[13]. It was this context that, in recent years, triggered a huge interest in the noninvasive assessment of liver fibrosis in patients with CHC. The introduction of a noninvasive methodology for the assessment of liver fibrosis as an alternative to LB in patients with CHC represents a major advancement in clinical hepatology^[14]. Many of the noninvasive methods demonstrated accuracy to a considerable degree in identifying significant fibrosis, particularly cirrhosis, and consequently, noninvasive assessment of fibrosis is already a reality in patients with CHC^[15]. Obviously, with the recent therapeutic development in CHC and reliable noninvasive diagnostic procedures available, LB has lost both its monopoly in the pretreatment assessment of fibrosis and the influence on decision making for antiviral therapy in patients with CHC.

CASE AGAINST LB

For the last 50 years, LB has been considered the gold standard for the staging of liver fibrosis in spite of its several shortcomings: intra- and interobserver variability in histopathological interpretation^[16,17], sampling errors^[18,19], and potentially life-threatening complications^[20,21]. In clinical practice, we frequently encounter the intra- and interobserver variability in the staging of liver fibrosis^[16,17]. Diagnostic errors made by nonspecialist pathologists were reported in > 25% of patients undergoing LB in academic centers^[22,23]. According to a recent study^[24], community pathologists understaged liver fibrosis in > 70% of cases with CHC. Several studies have shown that sampling errors occur when the LB specimen size is too small for an accurate estimation of fibrosis^[18,19]. Both the

length and the diameter of the biopsy core may affect the accuracy of fibrosis stage evaluation in patients with CHC^[25,26]. Obviously, the shorter and thinner the samples are, the greater is the number of misclassifications of liver fibrosis. There is some controversy among pathologists in defining an adequate LB sample for an accurate staging of liver fibrosis. Some investigators^[27] suggest that a sample of at least 15 mm in length and containing more than five portal tracts is adequate, while others recommend biopsy samples of 20 mm containing at least 11 portal tracts^[26] or even larger samples, up to 25 mm^[18]. Bigger is better^[28], but at the price of an increased risk of severe complications^[10,18]. However, it should be noted that, in clinical practice, few LB specimens reach an adequate length of 20 mm^[29]. Furthermore, LB only samples an extremely small part of the whole organ (1/50 000) and therefore, there is a risk in the evaluation of lesions that are heterogeneously distributed throughout the entire liver^[21]. LB may underestimate the amount of fibrosis, and cirrhosis could be missed in 10%-30% of cases^[30]. Studies concerning fibrosis staging have also shown differences in one third of cases with CHC between LB samples obtained from the right and left lobes of the liver during laparoscopy^[19]. Data on LB complications are heterogenous and contain wide variations in reported rate from one study to another^[10,20,21,31-34]. Major complications include bleeding and bile peritonitis, with a reported mortality rate ranging from 0.03% to 0.1%^[10,20,31,32,34]. It is worthwhile mentioning that both the transjugular route and ultrasound guidance approaches to LB do not significantly reduce the rate of major complications^[35,36]. Complication rates are higher when LB is performed by less-experienced physicians^[31,37]. In addition, LB is costly, variably available, poorly accepted by patients, and not suitable for repeated evaluation. The cost of an LB in the United States, United Kingdom and Australia varies between 1000 and 2000 USD, and it could go over 3000 USD if complications occur^[12,38-40]. LB is not welcomed by patients and it may be refused by more than half of those with CHC^[41]. LB is inappropriate for a dynamic evaluation of liver fibrosis over time, and recommendation to repeat biopsy every 3-5 years to follow up disease progression is certainly unrealistic, mainly due to patient nonadherence^[40]. LB is contraindicated in the presence of coagulopathy and thrombocytopenia. Last but not least, the prevalence of CHC makes LB impossible in all patients with CHC who are candidates for antiviral therapy. It is these drawbacks of LB that have led to the development of noninvasive methods for the assessment of liver fibrosis in patients with CHC and, hopefully, to a major change in hepatology practice.

Nevertheless, LB has some well-recognized advantages for assessing fibrosis in CHC, such as direct measuring of liver fibrosis, well-established staging system, and evaluation of associated lesions (steatosis, iron deposition, inflammation, alcoholic liver disease, nonalcoholic fatty liver disease, metabolic syndrome), although these diagnostic advantages are counterbalanced by the aforementioned disadvantages.

CASE IN FAVOR OF NONINVASIVE METHODS

Noninvasive methods for detecting liver fibrosis may be divided in two main groups: serum markers of fibrosis and transient elastography (Fibroscan).

Serum markers for liver fibrosis are commonly divided into direct serum markers, which are directly linked to the modifications in extracellular matrix turnover produced by hepatic stellate cells during the process of fibrogenesis in the liver, and indirect serum markers which reflect alterations of the hepatic functions. The direct markers include glycoproteins (hyaluronate, laminin, YKL-40), collagen family (procollagen III, type IV collagen), collagenases and their inhibitors (matrix metalloproteinases, tissue inhibitory metalloproteinase-1), and they are not routinely available in most clinical laboratories. The indirect markers are biochemical parameters determined in routine blood tests [platelet count, prothrombin time, aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio]. Serum markers for liver fibrosis may be used singly^[42-45] or combining panels of direct or indirect serum markers and demographic parameters^[46-55], with the aim of increasing the accuracy of single parameters. Some of them are patent-protected and commercially available: FibroTest® (Biopredictive, Paris, France) licensed under the name of Fibrosure® in the United States (LabCorp, Burlington, NC, United States)^[51], Fibrometer® (BioLiveScale, Angers, France)^[52], Hepascore (PathWest, University of Western Australia, Australia)^[53], ELF® (Enhanced Liver Fibrosis Test, iQur Ltd, Southampton, United Kingdom)^[54], and FibroSpect II® (Prometheus Laboratory Inc. San Diego, Ca, United States)^[55]. Among these, Fibrotest [α -2-macroglobulin, γ -glutamyl transpeptidase (GT), apolipoprotein A1, haptoglobin, total bilirubin, age, sex] is the most widely used and was validated by several studies on patients with CHC^[56-63]. The reported accuracy of Fibrotest for significant fibrosis/cirrhosis expressed as area under receiving operating characteristic curve (AUROC) ranges from 0.74% to 0.87%^[46,51]. To improve the performance of Fibrotest, its combination with Fibroscan has been suggested; with such a combination, one study reported AUROC of 0.88 for at least F2 (stage in the Metavir scoring system) and 0.95 for F3 or F4^[56]. The sensitivity and specificity of serum-marker-based tests could also be improved by combining them using sequential algorithms. Thus, Sebastiani *et al*^[64] combined AST/platelets ratio (APRI) with Fibrotest - a combination known as sequential algorithm for fibrosis evaluation biopsy - and found it to have an accuracy of 92.5% in the detection of fibrosis in CHC, obviating 81.5% of liver biopsies. APRI has a slightly lower performance than Fibrotest, with an accuracy between 60% and 82% for significant fibrosis and 60% and 88% for cirrhosis^[46,64], but it is a simple cost-free readily available test in all hospital settings. Both Fibrometer (platelet count, hyaluronate, AST, α -2-macroglobulin, international normalized ratio, urea, age) and Hepascore (bilirubin, γ GT, α -2-macroglobulin, hyaluronic acid, age, sex) showed good

performance for detection of significant fibrosis^[52,53,65].

There are several advantages of serum markers such as high applicability, with no risk for the patient and no contraindication; they can be performed and repeated in outpatient clinics; widespread availability; and inter-laboratory reproducibility^[66]. However, there are some limitations of serum markers: none is liver specific; results are unreliable in comorbidities (hemolysis, Gilbert syndrome, rheumatoid arthritis); and they have poor performance in the diagnosis of intermediate stages of liver fibrosis^[66]. Nevertheless, it is important to note that the performance of each noninvasive marker is evaluated against LB which is an imperfect gold standard, and the apparent failure of noninvasive markers to make an accurate distinction between different stages of intermediate fibrosis could be the consequence of misclassifications from biopsy^[67,68].

Transient elastography (Fibroscan®, Echosens, Paris, France) measures liver stiffness in a volume at least 100 times greater than a standard LB sample, and therefore, may be more representative of the entire liver. Fibroscan is composed of an ultrasound transducer probe mounted on the axis of a vibrator; vibration is transmitted to induce an elastic shear wave that propagates through the liver. Pulse-echo ultrasound acquisition is used to measure the velocity of the shear wave, which is directly related to liver stiffness: the stiffer the liver, the faster the shear wave propagates. Results are expressed in kPa, and values range from 2.5 kPa to 75 kPa, with normal values < 5.5 kPa^[69]. According to several studies, a cutoff value of 7.2-8.7 kPa defines significant fibrosis, and cirrhosis is diagnosed by a cutoff value of 12.5-14.5 kPa^[70,71]. Fibroscan seems to be a reliable method for the diagnosis of significant fibrosis (AUROC 0.84) and cirrhosis (AUROC 0.95)^[72,73]. Its combination with serum-based tests (Fibrotest, Fibrometer) increases the performance (but also the costs) for the diagnosis of significant fibrosis^[56,71,72]. Among noninvasive methods for diagnosis of cirrhosis, Fibroscan has the highest level of performance^[62,72,73], and its combination with serum markers does not increase accuracy^[63,72].

Fibroscan has several advantages: it is painless; quick (< 5 min); highly reproducible, with results immediately available; inexpensive; and easy to perform in the outpatient clinic and at the bedside^[66]. In addition, Fibroscan can be repeated for longitudinal disease monitoring, which is difficult, if not impossible, with LB. In cirrhotic patients, Fibroscan values correlate with portal pressure (based on the hepatic venous pressure gradient measurement), which is a reliable predictor of clinical outcomes^[74-77], disease severity^[78], and the risk of hepatocellular carcinoma^[79]. Finally, Fibroscan and serum markers are well accepted by patients, therefore, they could be used as screening methods for the detection of liver fibrosis/cirrhosis in at-risk groups^[80] and even in general population^[81], while LB is unacceptable for screening purposes. Fibroscan measurement failure and unreliable results are due to limited operator experience^[82], narrowed intercostal spaces^[82], and obesity^[82,83], although

this last problem seems to be overcome by a new specially designed probe^[84-86]. Results are influenced by ALT flares^[87,88], extrahepatic cholestasis^[89,90], and congestive heart failure^[91].

DISCUSSION

In the past, expert consensus guidelines on the management of CHC unanimously recommended routine LB before initiation of antiviral therapy^[3-5,92,93]. Based on LB findings, treatment has often been advocated only for patients with at least moderate to severe stages of fibrosis (Metavir F2, F3 or F4), and withheld for those with no or minimal fibrosis (F0, F1)^[6,93]. As a consequence, tens of thousands of patients were most likely denied proper antiviral therapy. More recent guidelines^[8,94] recommend LB only in patients with CHC genotype 1 (SVR rate < 50%) in treatment decision making, and consider it unnecessary in those with genotype 2 or 3 who may have an SVR rate as high as 80%. The primary endpoint of antiviral therapy for CHC is achieving SVR - defined as undetectable serum HCV RNA at 24 wk after discontinuation of therapy. Viral eradication prevents disease progression, improves survival, and reduces health care costs associated with the management of complications. Thus, if viral clearance is the aim of antiviral therapy in CHC, then to what degree does an exact histopathological fibrosis stage established through biopsy still matter? With the new protease inhibitor (PI)-based triple therapy (addition of telaprevir or boceprevir to pegylated interferon and ribavirin) available and SVR rates approaching 75% in patients with CHC genotype 1^[95,96], it is clear that LB has lost its importance in the recommendation of antiviral therapy.

During the past 10 years, an intensive debate has taken place between those in favor of LB and those who promote noninvasive methods for pretreatment assessment of liver fibrosis in patients with CHC. There is extensive literature showing the pros and cons of LB or noninvasive methods. As in chess, winning does not come easy for a supporter of noninvasive methods against a supporter of LB with a firmly rooted preference. Step by step, those in favor of non-invasive methods have gained ground, waiting for the final move: checkmate! Today, several noninvasive methods, ranging from serum marker assays to advanced imaging techniques, have proved to be excellent tools for the evaluation of liver fibrosis in patients with CHC. According to the latest European Association of the Study of the Liver clinical practice guidelines^[97] and United Kingdom consensus guidelines^[98] recommendations, noninvasive methods can be used instead of LB in patients with CHC to assess liver disease severity prior to antiviral therapy. It is therefore surprising that many experts in the field of hepatology and the most recent American Association for the Study of Liver Diseases 2011 practice guidelines^[99] favor LB before therapy initiation, despite substantial improvement in treatment success rate for genotype 1 patients with PI-based triple therapy. The

main reason against noninvasive methods for evaluation of liver fibrosis is their apparent failure to make an accurate distinction between different stages of intermediate fibrosis. It is important to note that the performance of each noninvasive method was evaluated in all studies by calculating the AUROC using LB as a reference standard. As LB is an imperfect standard, a perfect noninvasive method will never reach the maximum value (1.0)^[100], and therefore, noninvasive methods are as inaccurate as LB for the assessment of fibrosis stage. Thus, the failure of noninvasive methods to discriminate between different stages of intermediate fibrosis could be the consequence of classification errors from histopathological findings of biopsy^[67,68]. For clinicians, it is more important to know if their patients have no/mild or advanced fibrosis/cirrhosis, rather than the exact pathological scoring system through LB, and this could be easily achieved by means of noninvasive methods. Taking into account that all recent international guidelines^[97-99] recommend treatment with PI-based triple therapy in all patients with CHC genotype 1, provided that they have no contraindications to peg-interferon and ribavirin, the need to stage liver fibrosis accurately is decreasing in treatment decisions.

The final move - checkmate to LB - is, therefore, possible once the rate of SVR has reached 75% with PI-based triple therapy for patients with CHC genotype 1. Consequently, it is clear that in the era of PI-based triple therapy and other new potent direct-acting agents in the pipeline, the information obtainable from LB has little, if any, influence on treatment decisions. It should be underlined that in this article, checkmate to LB in patients with CHC refers strictly to cases with no need for this invasive and risky procedure in therapeutic decision making. With PI-based triple therapy already available in many countries, and an allocation system probably based mainly on medical need (therapy for those likely to develop complications in the next few years), noninvasive methods with the highest accuracy for detecting severe fibrosis/cirrhosis used as an alternative to LB for pretreatment assessment of liver fibrosis in patients with CHC are now part of routine clinical practice. Fibroscan or any patented biomarkers (Fibrotest, Fibrometer and Hepascore) have recently been recommended for first-line staging of liver fibrosis^[101] before deciding on antiviral therapy. However, the adoption rates of noninvasive methods by hepatologists differ from country to country. In France, a survey of 546 hepatologists revealed that 81% of them used noninvasive methods^[102], while in the United States, despite the aforementioned shortcomings of LB, there is still significant resistance to accepting noninvasive methods as an alternative to biopsy. We believe that sooner or later this will change, and the requirement of LB prior to starting antiviral therapy in patients with CHC will be reassessed.

In conclusion, in the era of PI-based triple therapy and other new potent direct-acting agents on the horizon that can achieve SVR rates approaching 100%, the time has come to move forward to risk-free noninvasive

methods for the patient, leaving LB behind in the evaluation of liver fibrosis in decision making for CHC antiviral therapy. In other words, checkmate to LB?

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Treatment of locally advanced rectal cancer: Controversies and questions

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Abstract

Rectal cancers extending through the rectal wall, or involving locoregional lymph nodes (T3/4 or N1/2), have been more difficult to cure. The confines of the bony pelvis and the necessity of preserving the autonomic nerves makes surgical extirpation challenging, which accounts for the high rates of local and distant relapse in this setting. Combined multimodality treatment for rectal cancer stage II and III was recommended from National Institute of Health consensus. Neoadjuvant chemoradiation using fluoropyrimidine-based regimen prior to surgical resection has emerged as the standard of care in the United States. Optimal time of surgery after neoadjuvant treatment remained unclear and prospective randomized controlled trial is ongoing. Traditionally, 6-8 wk waiting period was commonly used. The accuracy of studies attempting to determine tumor complete response remains problematic. Currently, surgery remains the standard of care for rectal cancer

patients following neoadjuvant chemoradiation, whereas observational management is still investigational. In this article, we outline trends and controversies associated with optimal pre-treatment staging, neoadjuvant therapies, surgery, and adjuvant therapy.

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Key words: Rectal cancer; Neoadjuvant chemoradiation; Response; Treatment; Staging; Recurrence

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INTRODUCTION

The mainstay of treatment for colorectal cancer is surgery. Complete, margin-negative resection confers the greatest chance for cure. However, chemotherapy and radiation have important roles in assuring long-term, recurrence-free survival. Colon cancer and extraperitoneal rectal cancers (lesions above the anterior peritoneal reflection, or 12-15 cm from the anal verge) are generally treated with surgery followed by adjuvant chemotherapy; these patients are believed to be at high risk for harboring micrometastasis and, therefore, of developing of recurrent disease. Mid to low rectal cancers lying below

the anterior peritoneal reflection and extending through the rectal wall, or involving locoregional lymph nodes (T3/4 or N1/2), have historically been more difficult to cure. The confines of the bony pelvis and the necessity of preserving the autonomic nerves makes surgical extirpation challenging, which accounts for the high rates of local and distant relapse in this setting. The Gastrointestinal tumor study group and National surgical adjuvant breast and bowel project trials demonstrated that chemoradiotherapy following surgical resection could reduce local recurrence from 55% to 33%, with significantly prolonged disease-free survival (DFS)^[1,2]. This was the basis for the National Institute of Health consensus statement in 1990 recommending combined modality therapy for Stage II and III rectal cancer^[3]. In the 20 years since, there have been considerable advances in treatment.

We will outline current trends and controversies in the treatment of locally advanced rectal cancer. The topics to be covered include staging, neoadjuvant therapies, surgery, and adjuvant therapy.

OPTIMAL CLINICAL STAGING

Pre-treatment evaluation, including accurate staging, is critical when planning treatment for rectal cancer patients. The mainstay of treatment is surgery, which runs the gamut from local excision to radical resection with or without chemoradiation. Accurate staging is the first crucial, necessary step in optimizing therapy, maintaining quality of life, and limiting over- and under-treatment.

Most rectal cancers are diagnosed at colonoscopy, usually after patients present with asymptomatic anemia or change in bowel habits. Histologic confirmation is important, as benign stricture and inflammatory conditions may mimic malignancy. Full colonoscopy is necessary in order to identify synchronous polyps and malignancies, which occur in about 30% and 3%-5% of cases, respectively^[4,5]. If the colon cannot be fully evaluated preoperatively due to colonic obstruction, it is recommended at 3-6 mo postresection^[6].

Once a patient is diagnosed with rectal cancer, the most commonly ordered imaging study is computed tomography (CT). When properly performed with oral and intravenous contrast, CT provides important information regarding extent of disease. Although it is not a very accurate imaging modality for determining the degree of primary tumor penetration into the rectal wall or involvement of locoregional lymph nodes, CT scanning of the chest, abdomen and pelvis accurately identifies distant metastatic disease in the lung, liver, pelvic and periaortic lymph nodes.

Staging of the primary rectal lesion begins with a detailed digital rectal examination (DRE). This confers important information about the primary lesion, including its location with respect to the top of the anorectal ring (external sphincter complex) as well as its mobility. The degree of tumor fixity in the pelvis corresponds to depth

of penetration through the rectal wall. Mobile lesions are often limited to the mucosa, submucosa or muscularis propria, whereas tethered lesions are likely to extend into the perirectal fat (mesorectum). A fixed lesion may signify extension of tumor into surrounding anatomic structures such as the seminal vesicles, prostate or vagina (T4 lesions). Rigid proctosigmoidoscopy provides the best estimate of tumor distance from the anal verge.

Endorectal ultrasonography (ERUS) and magnetic resonance imaging (MRI) are the most accurate tools for assessing T and N stage. ERUS is advantageous in imaging small, superficial tumors, whereas MRI is useful in evaluating bulky tumors that extend to the circumferential margin (CRM)^[7]. Based on the results of large meta-analyses, the accuracy of ERUS in determining T and N stage is 87% and 74%, respectively^[8]. The accuracy of MRI in determining T and N stage is 71%-91% and 45%-79%, respectively^[9], similar to that of ERUS. Introduction of endoluminal coil MRI improves imaging resolution of the rectal wall. However, the true strength of MRI lies in phased-array MRI, which facilitates accurate assessment of tumor encroachment of the CRM, or invasion into surrounding anatomic structures^[10]. Both MRI and ERUS are less accurate in assessing locoregional lymph node metastasis. Morphology, such as irregular border or heterogeneous intensity, is often more predictive than lymph node size^[11]. It is important to remember that 18% of nodal metastases occur in lymph nodes measuring less than 5 mm^[12]. Rather than being competing modalities, however, ERUS and MRI are often complimentary in the process of rectal cancer staging. ERUS is particularly useful in the staging of early rectal cancers; it also has the advantage of low cost, and can be performed quickly in the outpatient office. Phased-array MRI is much more expensive and not as readily available; however, it affords a comparatively greater field of vision, enhancing the ability to identify at-risk CRM and invasion of adjacent organs.

CHEMORADIATION: POSTOPERATIVE VS PREOPERATIVE

A paradigm shift in preoperative radiotherapy (RT) was introduced by the Swedish Rectal Cancer Trial in 1997. This study randomized 1168 patients to receive either one week of RT followed by surgery, or surgery alone. Compared with surgery alone, patients who received preoperative RT had reduced local recurrence (11% *vs* 27%, $P < 0.001$) and prolonged survival [5-year overall survival (OS) of 58% *vs* 48%, $P = 0.004$]^[13]. At a median follow-up of 13 years, the benefits in terms of local recurrence (9% *vs* 26%, $P < 0.001$) and OS (38% *vs* 30%, $P = 0.008$) remained significant in patients who received preoperative RT^[14]; however, these patients did experience more gastrointestinal complications and had a higher rate of hospitalization over the 6-mo period following surgery^[15].

Total mesorectal excision (TME) involves sharp dis-

section along the embryonic planes between the visceral and parietal layers of the endopelvic fascia. This ensures complete removal of locoregional lymph nodes contained within the mesorectum, while preserving the autonomic nerves and limiting blood loss. Multiple retrospective and cohort studies have shown that TME is associated with lower rates of local recurrence compared to the less optimal blunt surgical technique. The Dutch TME trial (published in 2003) was the first to compare the results of TME with and without preoperative short-course RT. Of 1861 accrued patients, 924 and 937 were randomized to receive either preoperative radiation followed by TME, or TME alone. Local recurrence was significantly lower in patients, who received preoperative RT plus TME (2.4% *vs* 8.2%, $P < 0.001$), but there was no difference in OS^[16]. Long-term follow-up showed lower recurrence rates in the preoperative RT arm, especially in the subgroups of patients with nodal involvement, patients with tumor located between 5-10 cm from the anal verge, and patients with free CRMs^[17].

While preoperative short-course radiotherapy consisting of 25 Gy in 5 fractions has been the favored treatment in Northern Europe and Scandinavia, in North America and in some European countries long-course chemotherapy has been the treatment of choice, based in part on the results of the Gastrointestinal Tumor Study Group and National Surgical Adjuvant Breast and Bowel Project (NSABP) trials. With theoretical advantages of better tolerance and increased efficacy, many centers moved toward preoperative neoadjuvant chemoradiation: 50.4 Gy delivered in 28 fractions, and concurrent fluoropyrimidine-based chemotherapy. The majority of patients receiving chemoradiotherapy obtain tumor downstaging (in which the final pathologic stage at time of surgery is lower than the initial clinical stage at time of presentation)^[18]. Indeed, as many as 15%-20% of patients will have a complete pathologic response to treatment, with no viable tumor cells noted in the resected rectum. Tumor downsizing may facilitate complete tumor resection and, in the setting of low-lying tumors, may alter the surgical plan by making a sphincter-saving procedure possible^[19,20].

Although two prospective, randomized controlled trials comparing preoperative and postoperative chemoradiation failed to accrue; NSABP R03^[21] and the Intergroup 1047 trial^[22] in the United States, the German Rectal Cancer Study Group successfully completed such a trial. The German CAO/ARO/AIO 94 trial^[19] compared preoperative and postoperative long-course chemoradiation for T3 or T4 and/or node-positive rectal cancer. Chemoradiotherapy consisted of 50.4 Gy in 28 fractions with concurrent infusional fluorouracil (1000 mg/m² per day for 5 d in the first and fifth week of radiation). Four hundred twenty-one and 402 patients were randomly allocated to receive preoperative and postoperative chemoradiotherapy, respectively. There was 6% local recurrence in the preoperative group and 13% in the postoperative group ($P = 0.006$). Grade 3 or higher

acute and long-term toxicity occurred significantly less frequently in patients who received neoadjuvant chemoradiation ($P = 0.001$ and $P = 0.01$, respectively). However, the rates of sphincter preservation, DFS and OS did not differ between the two groups.

Comparison of long- and short-course radiotherapy was the aim of a Polish randomized study^[23,24] of patients with T3/4 mid to low rectal cancer. The results demonstrated higher rates of complete pathologic response in the group of patients receiving long-course chemoradiotherapy: 16% and 1% had complete pathologic response in the long-course and short-course chemoradiotherapy groups, respectively. Although rates of sphincter preservation were similar in both study groups, patients receiving long-course chemoradiotherapy had a 4% rate of positive CRM at time of surgery, compared with 13% in the short-course group ($P = 0.017$). However, there was no significant difference in local recurrence, DFS or OS.

The recently reported MRC CR07 and NCIC-CTG C016 multi-center randomized study^[25] of 1350 patients compared the outcomes of preoperative short-course RT *vs* initial surgery followed by selective postoperative chemoradiation in patients with positive CRM. The primary outcome was local recurrence. This study demonstrated a significant decrease in local recurrence in patients receiving preoperative short-course RT (hazard ratio 0.39, $P < 0.0001$), which was associated with a 6% absolute improvement in DFS at 3 years ($P = 0.03$). Again, these data demonstrate the superiority of preoperative chemoradiotherapy.

The above data indicate that both preoperative long-course and short-course radiotherapy followed by proper TME provide excellent local control. The advantages of long-course chemoradiotherapy include tumor downsizing and downstaging, which may alter the surgical treatment plan in favor of a sphincter-preserving procedure. Long-course chemoradiation is associated with higher acute toxicity than short-course (18.2% *vs* 3.2%, respectively)^[23]. On the other hand, short-course RT may lead to more long-term complications secondary to higher dose per fraction. In the United States, long-course chemoradiotherapy consisting of 5040 cGy, delivered concurrently with 5-fluorouracil (5FU) chemotherapy, is the most common regimen^[6].

With improvements in surgical technique, including TME, the utility of chemoradiation in early T3 lesions without at-risk CRM has been called into question. A study of 95 T3N0 rectal cancer patients undergoing TME demonstrated less than 10% local recurrence rate without adjuvant therapy^[26]. A pooled analysis of 3791 patients from several randomized studies showed no difference, in terms of OS, in T3N0 rectal cancer patients who received surgery plus chemotherapy with, or without, radiation (85% *vs* 74%-80%, respectively). Moreover, local relapse was similar among stage II patients undergoing surgery alone (14%) or surgery plus radiotherapy (12%)^[27]. It has been suggested that patients at lower

risk of local recurrence, e.g., those with proximal T3N0 lesions with clear margin, may be adequately treated by surgery and only adjuvant chemotherapy. However, correctly assessing T and N stage remains problematic. Two studies have reported the limitations of ERUS or MRI in determining accurate nodal stage, with false negative rates of up to 22%-28%^[28,29]. Therefore, preoperative chemoradiation for clinical T3N0 rectal cancer patients should be considered by weighing the risk of unnecessary treatment against the possibility that the patient may ultimately require postoperative chemoradiation, which is associated with lower local control and higher toxicity than preoperative chemoradiation. Currently, preoperative chemotherapy remains the standard of treatment for T3N0 patients based on the principle that overtreatment is less harmful than undertreatment.

MAXIMIZING RESPONSE TO NEOADJUVANT CHEMORADIATION

Fluoropyrimidine-based chemotherapy synergizes with long-course radiotherapy. A 2 × 2 study of the European Organization for Research and Treatment of Cancer (EORTC) 22921 trial^[30] assessed the efficacy of adding chemotherapy to RT, with or without adjuvant (postoperative) chemotherapy. The addition of chemotherapy at some point in the treatment regimen, either preoperatively or postoperatively, conferred a significant benefit in terms of local control. RT is fundamental to neoadjuvant treatment of rectal cancer, resulting in increased pathological response rate and better local control. However, it has no effect on distant metastasis. These findings imply that fluorouracil acts as a potent radiation sensitizer but has no significant eradicating impact on micrometastatic disease. In an attempt to improve response rates and reduce distant metastasis, new preoperative strategies are being investigated; these include combinations of new chemotherapeutic agents used concurrently with RT as well as induction chemotherapy. Currently efforts are being made to integrate novel agents in combination with RT, using the pathologic complete response (pCR) rate as a surrogate endpoint. Pathological CR has demonstrated correlation with clinical outcomes, i.e., relapse-free survival, DFS and OS^[31,32].

Capecitabine, an oral pro-drug of fluoropyrimidine designed to enable selective 5FU activation in tumor tissue, has been studied as an agent used concurrently with RT. Several phase I / II studies have revealed a pCR rate of 7%-31% with acceptable toxicities^[33-36]. Two randomized controlled trials showed capecitabine to be non-inferior to 5FU in the perioperative treatment of stage II -III rectal cancer. The NSABP R-04^[37] compared the efficacy of continuous infusion 5FU to capecitabine delivered concurrently with preoperative RT, with or without oxaliplatin, in 1608 patients. The rates of pCR were 19% and 22% in patients who received 5FU and capecitabine, respectively ($P = 0.12$). Long-term outcomes of DFS and OS are awaited. Another phase III trial from Ger-

many^[38] demonstrated that capecitabine, used in the neoadjuvant or adjuvant setting, is not inferior to 5FU in terms of 5-year OS (capecitabine 76%; 5FU 67%, $P = 0.053$). The use of oral capecitabine (825 mg/m² taken twice daily) throughout the course of radiation has been an alternative option; it is not inferior to infusional fluoropyrimidine in terms of efficacy or toxicity, and has the potential advantage of convenience for the patient.

The results of two phase III studies studying combined oxaliplatin and fluoropyrimidine used concurrently with radiotherapy have also been reported. The ACCORD12 trial compared CRT using capecitabine + 45 Gy RT to capecitabine + oxaliplatin (CAPEOX) + 50 Gy RT^[39]. This study showed pCR rates of 13.8% and 18.8% in the capecitabine/RT and CAPEOX/RT groups, respectively ($P = 0.11$). Another phase III study from Italy investigated the efficacy of adding weekly oxaliplatin to protracted infusion of 5FU, concurrently with radiation, in locally advanced rectal cancer patients^[40]. There was no difference in the pCR rate (16% in the 5FU group *vs* 15% in the 5FU + oxaliplatin group, $P = 0.98$). Interestingly, fewer patients in the 5FU + oxaliplatin arm were found to have metastatic disease after neoadjuvant chemoradiation (0.5% *vs* 3%, $P = 0.014$); this data may imply that oxaliplatin is potentially efficacious in controlling micrometastasis. Another trial of NSABP R-04^[37], reported at the 2011 American Society of Clinical Oncology Annual Meeting, demonstrated that oxaliplatin does not improve pCR rates (19.1% *vs* 20.9%, $P = 0.46$), but significantly increases toxicity. However, neither randomized controlled trial showed any improvement in acute endpoints, i.e., pCR. At this time, single agent fluoropyrimidine, either infusional 5FU or capecitabine used concurrently with pelvic radiation, remains the standard of care in stage II and III rectal cancer.

Attempts have been made to integrate molecularly targeted therapy with preoperative combined modalities, in order to improve the efficacy of standard neoadjuvant chemoradiation. The addition of cetuximab to capecitabine was evaluated in a phase I / II trial in which only 5% of patients reached pCR; however, there was no unexpected toxicity^[41]. Another study from Germany also reported a suboptimal pCR rate (9%) after preoperative cetuximab + capecitabine, oxaliplatin and radiotherapy^[42]. Data on preoperative bevacizumab are limited to a few phase I studies. However, in contemplating treatment with bevacizumab, the known adverse effects of this monoclonal antibody (such as bowel perforation, bleeding, and impaired surgical wound healing) must be considered.

Induction chemotherapy followed by neoadjuvant chemoradiation was considered a potential approach to controlling micrometastasis. A phase II United Kingdom trial, using this new strategy of neoadjuvant chemotherapy with CAPEOX prior to CRT and surgery, has been reported^[43]. One hundred five individuals, identified by high-resolution MRI as poor-risk rectal cancer patients, were enrolled. The definition of "poor-risk"

entailed one of the following features: CRM threatened or involved by tumor, low-lying tumor at or below the levators, T3 tumor with radial margin > 5 mm, T4 lesion, or N2 disease. The results showed the feasibility of this approach, with overall rates of 5-year and DFS 75% and 68%, respectively. In the setting of locally advanced rectal tumors, these are very impressive statistics.

A Spanish trial^[44] was conducted to compare this new strategy with the more conventional approach of neoadjuvant chemoradiation followed by surgery and adjuvant chemotherapy. One hundred eight patients were randomized to receive 4 cycles of CAPEOX for the induction period, followed by neoadjuvant chemoradiation and then surgery; or else to receive neoadjuvant chemoradiation followed by surgery, and subsequently 4 cycles of adjuvant CAPEOX. There was no difference in pCR rate (13% *vs* 14%). Significantly less toxicity and a greater ability to tolerate chemotherapy throughout the schedule were found in patients who received induction chemotherapy. Another phase II study from Memorial Sloan-Kettering Cancer Center^[45] was conducted to determine the efficacy of induction chemotherapy, followed by chemoradiotherapy and then surgery, for stage III or potentially resectable stage IV rectal cancer patients. Thirty-four patients were enrolled. Seventeen of 27 patients who ultimately underwent TME had > 90% pathological tumor response. At a median follow-up of 25 mo, no local recurrence was identified. Three-year DFS and OS were 63% and 100%, respectively, in patients with resected stage III rectal cancer. This study showed a promising clinical outcome with tolerable toxicity. We believe that this approach will become one of the mainstays of treatment for patients with locally advanced rectal cancer in the future, but the data must be borne out in larger phase III studies.

PREDICTORS OF RESPONSE TO NEOADJUVANT THERAPY

Several parameters associated with tumor response to neoadjuvant chemoradiation have been identified. Some studies have concluded that lower pre-treatment carcinoembryonic antigen (CEA) level is associated with better response to treatment^[46-50]. The cut-off CEA levels associated with response rate were 2.5^[46], 3^[47], and 5 ng/mL^[48-50]. However, because the pre-treatment CEA level in a majority of patients is normal and the cut-off level inconclusive, the applicability of CEA in predicting treatment response remains unclear.

Location of tumor, including tumor distance from the anal verge and the extent of luminal involvement by tumor, has also been examined. Bulky T3 lesions are less likely to show significant downstaging^[51]. In a study of 247 patients, T3 tumors with > 2.5 mm extension into the mesorectum demonstrated a lesser response to therapy, with a low degree of downstaging^[51]. It cannot be determined if this is merely attributable to tumor volume or to actual tumor biology. Predictably, this study

also noted that poorly differentiated histology and metastatic disease were associated with limited response to treatment. Similarly, other reports have found that there is comparatively less downsizing in lesions with > 60% circumferential involvement. Interestingly, in this study lesions located higher than 5 cm from the anal verge were more likely to show a significant response to neoadjuvant chemoradiation^[46].

In the past decade, many studies have attempted to identify correlations between immunohistochemical biomarkers such as Ki-67, p53, p21, bax/bcl2, epidermal growth factor receptor, thymidylate synthase, and tumor response after neoadjuvant chemoradiation. Currently, however, these studies remain inconclusive^[52-54].

RESTAGING AFTER NEOADJUVANT CHEMORADIATION

Identifying the 15%-20% of patients who achieve a complete response to neoadjuvant therapy remains a challenge. Because of their fundamental roles in the neoadjuvant chemoradiation setting, DRE, endoscopy, ERUS, CT, MRI and positron emission tomography (PET) have been proposed as investigational tools to determine tumor response to treatment. The problem is that none of these modalities are capable of accurately predicting pCR^[55], the reason being that fibrosis and inflammation caused by radiation have a deleterious affect on accuracy. A study from our institution^[56] demonstrated that DRE is an inaccurate method for determining response to treatment after neoadjuvant chemoradiation; the overall concordance between DRE and pathologic response following neoadjuvant chemoradiotherapy was only 22%. A study from Italy showed that only half of patients who were defined as having complete response on endoscopic biopsy had true pCR according to pathology from surgical resection^[55].

The accuracy of ERUS and MRI in the setting of post-neoadjuvant chemoradiation is also limited. A prospective study compared the accuracy of CT, MRI and ERUS *vs* pathologic assessment in determining clinical T and N stage in 90 patients receiving long-course neoadjuvant chemoradiation. The accuracy of these imaging modalities in determining T stage was low (37% by CT, 34% by MRI, and 27% by ERUS); most of this was due to over-staging. The rate of accuracy in nodal staging was 62% by CT, 68% by MRI and 65% by ERUS^[57].

Because of the limited accuracy of all existing imaging modalities in staging rectal cancer post-neoadjuvant chemoradiation, several groups have investigated novel imaging methods. Diffusion-weighted MRI is a functional MRI imaging technique with better accuracy than that of conventional MRI^[58-60]. However, the role of this emerging method is still investigational, and more studies with larger numbers of patients are awaited. In the meantime, repeat CT of the chest, abdomen and pelvis is useful in identifying development of interval metastatic disease, the presence of which could alter the surgical plan.

PET is a functional imaging study. Theoretically, the change of activity in the tumor should relate to the treatment response. Support to this idea, there are some studies demonstrated the relative change of maximum standardized uptake value relate to tumor response^[61-63]. On the contrary, the data from another study demonstrated 63% sensitivity and 74.4% specificity of PET to predict patients with complete response^[64]. While, PET is sometimes utilized in the process of restaging, the current data on the efficacy of this modality is inconsistent and limited; thus we do not routinely use PET as a restaging tool.

OPTIMAL TIMING OF SURGERY AFTER NEOADJUVANT THERAPY

In an attempt to increase response to chemoradiation, some groups have investigated lengthening the interval between chemoradiotherapy and surgery. Traditionally, surgery has been recommended 6-8 wk after neoadjuvant chemoradiation^[65-69] to allow for tumor regression without extensive fibrosis. Indeed, several studies have shown that extending the period between chemoradiation and surgery may increase rates of complete pathologic response without increasing perioperative complications^[63-65]. However, the effect of a longer interval on long-term outcome has yet to be defined. A well-designed multicenter prospective trial of increasing the interval between neoadjuvant chemoradiation and surgery is ongoing^[70]. It is unclear if this will impact outcome; nevertheless, it may be of significance in the development of nonoperative strategies (see below). Some have also proposed adding chemotherapy to the waiting period, in an attempt to treat potential micrometastatic disease^[71,72]. Habr-Gama *et al.*^[71] have reported the preliminary results of a study adding chemotherapy in the interval between neoadjuvant chemoradiation and surgery, which demonstrate an increased rate of complete clinical response.

MINIMALLY INVASIVE SURGERY FOR LOCALLY ADVANCED RECTAL CANCER

Laparoscopic surgery for rectal cancer has been proven feasible, but it is not yet a standard treatment in the United States. The United Kingdom Medical Research Council Trial of Conventional *vs* Laparoscopic-Assisted Surgery in Colorectal Cancer trial^[73] demonstrated equivalent long-term recurrence and survival results in rectal cancer patients who did not receive neoadjuvant treatment. The Comparison of Open *vs* Laparoscopic surgery for Mid and Low Rectal Cancer after Neoadjuvant Chemoradiotherapy trial^[74] is a prospective randomized trial that includes 340 patients with locally advanced rectal cancer receiving neoadjuvant chemoradiotherapy, randomized to laparoscopic and open groups in a ratio of 1:1. The conversion rate is only 1.2% (2 in 170 patients).

From the short-term results, operative time is significantly longer in the laparoscopic group, but estimated blood loss was significantly less. The rate of positive circumferential resection margin did not differ significantly between the two groups; nor did distal resection margin. The long-term oncological outcomes are awaited.

PREDICTORS OF RECURRENCE AND SURVIVAL

Several factors are reportedly predictive of recurrence and survival. Some of these features are the same in patients who receive or do not receive neoadjuvant chemoradiation. Some differ.

Post neoadjuvant chemoradiation CEA level

It has been proposed that post-neoadjuvant chemoradiation CEA levels < 2.5 ng/mL^[75] and 5 ng/mL^[76] are associated with significantly better DFS and OS. Another study, however, discerned no correlation^[77]. Moreover, most studies focusing on correlations between predictors and long-term outcomes do not focus on CEA levels^[77-79]. Thus, application of this parameter in attempting to predict recurrence and survival remains inconclusive.

Tumor response and nodal status after neoadjuvant chemoradiation

The pathologic response of tumor to treatment is one of the most significant prognostic factors in rectal cancer^[18,80]. In terms of recurrence and survival, pCR is most strongly correlated with excellent outcome^[80-85]. Several tumor regression grading systems classify tumor response following neoadjuvant chemoradiation in patients with pathologic partial response. All of these grading systems are consistent and demonstrate a strong association between pathologic response and outcome^[18,32,79,83]. In the setting of neoadjuvant chemoradiation, preoperative treatment reduces lymph node yield at the time of surgery. However, the ratio of positive lymph nodes to total lymph nodes is prognostic, and may in fact be a more useful prognostic factor than total number of lymph nodes culled following neoadjuvant chemoradiation^[83]. Several studies have associated positive pathological lymph node (ypN) status with poor prognosis^[30,77-83].

Distal resection margin

Historically, the standard guidelines have recommended a distal resection margin (DRM) of 4-5 cm from the distal edge of tumor^[6,86]. However, several reports have found that a DRM < 2 cm does not increase recurrence rates^[87,88] or negatively impact survival^[88]. In the setting of low rectal cancer located < 5 cm from the anal verge, 1-2 cm may be acceptable^[6]. Especially following preoperative treatment with neoadjuvant chemoradiation, the 1-2 cm DRM rule seems to be less important than obtaining a clear resection margin^[89,90].

CRM

The standard cut-off point of the CRM is still a matter of controversy. Most published studies have used $<$ or $=$ 1 mm^[91-93], 2 mm^[94-96], or 5 mm^[7,97] as acceptable cut-off points in chemoradiotherapy-naïve patients. All reported significantly higher recurrence rates and shorter survival in patients with CRM $<$ or $=$ 1 mm^[91-93], 2 mm^[94-96], or 5 mm, respectively^[7,98]. Therefore, in patients who have not received neoadjuvant chemoradiation and whose pathology indicates a close CRM (less than 1-5 mm), adjuvant chemoradiation should be strongly considered regardless of other pathological results^[90,98]. In patients who have already received neoadjuvant chemoradiation, adjuvant chemotherapy should be considered^[95-97].

Perineural invasion and lymphovascular invasion

These are poor prognostic factors in colon and rectal cancer in general. Even with neoadjuvant chemoradiotherapy, Perineural invasion and lymphovascular invasion remain predictors of poor outcome^[20,97].

Acellular mucin pools

Acellular mucin pools (AMP) has been found in 11%-27% of surgical cases after neoadjuvant chemoradiation^[99,100]. The question is whether AMP after neoadjuvant chemoradiation should be considered residual disease or not. Several reports have concluded that AMP does not have a significant impact on outcome^[99-101]. The College of American Pathologists has recommended that AMP not be interpreted as residual disease^[102].

POSTOPERATIVE ADJUVANT CHEMOTHERAPY: IS IT NECESSARY?

There is a high risk of local recurrence and distant metastasis in stage II and III rectal cancer patients treated by surgery alone. The role of adjuvant chemotherapy is to eradicate micrometastatic disease. Patients with T3 or node-positive disease who are initially treated by transabdominal resection should receive six months of adjuvant therapy, consisting of a "sandwich regimen" of two-month fluoropyrimidine-based chemotherapy followed by concurrent chemotherapy and radiation, followed by another 2 mo of chemotherapy.

Previously, the standard adjuvant chemotherapy regimen for rectal cancer was 5FU with or without leucovorin. The use of combination oxaliplatin, 5FU and leucovorin (FOLFOX4) or capecitabine in adjuvant treatment has been extrapolated from data available for colon cancer. According to the MOSAIC trial^[103], FOLFOX4 compared to 5FU + leucovorin improved DFS and OS in stage III colon cancer patients. The X-ACT study^[104] showed that, in terms of DFS and OS, the efficacy of capecitabine in adjuvant treatment of stage III colon cancer was comparable to that of 5FU + leucovorin.

There has been much consideration of the need for adjuvant chemotherapy in patients who have already received preoperative chemoradiation. The EORTC 22921

study^[28,105] assessed the value of adjuvant chemotherapy in patients undergoing preoperative CRT followed by surgery. Patients were allocated into four arms: preoperative RT; preoperative CRT; preoperative RT + adjuvant chemotherapy; and preoperative CRT + adjuvant chemotherapy. No difference in OS was found between the groups receiving preoperative and postoperative chemotherapy ($P = 0.12$). The addition of chemotherapy after preoperative CRT did not impact rates of local recurrence or survival. Of concern, however, is the fact that only 42.9% of assigned patients adhered to their postoperative chemotherapy regimens. DFS and OS benefits were shown in the subgroup of patients with pathological T (ypT) 0-2 cancers^[106]. These results indicate that patients who do obtain downstaging after CRT may be more likely to derive a survival benefit from adjuvant chemotherapy as well.

Given that most of the patients in EORTC 22921 did not receive adequate doses of adjuvant chemotherapy, we cannot assume from this study that adjuvant chemotherapy is definitely not beneficial to patients receiving neoadjuvant chemoradiation. To date, preoperative chemoradiation followed by surgery and adjuvant chemotherapy remains the standard of practice in the United States for treating stage II and III rectal cancers^[6] regardless of final pathological results. Options for adjuvant chemotherapy include four months of FOLFOX, capecitabine, or 5FU + leucovorin.

CLINICAL COMPLETE RESPONSE: CAN SURGERY BE AVOIDED?

Habr-Gama *et al*^[107] have proposed a definition of clinical complete response (cCR), based on the findings of clinical and endoscopic examination, as follows: (1) whitening of the mucosa in an area of the rectal wall; (2) any associated telangiectasia; (3) scarring of the rectal wall (which manifests as a slight stiffness of the wall during insufflation); and (4) if tumor cannot be felt or seen. These parameters are subjective, however, and require more clinical substantiation. Moreover, cCR may not be equivalent to pCR. Surgery is still the standard of management following neoadjuvant chemoradiation^[6,86].

There have been studies of local excision as an option for patients with cCR after neoadjuvant chemoradiation^[108,109]. However, complete response of the primary tumor cannot predict response in regional lymph nodes, which are involved in 7%-17% of patients who have cCR of the primary tumor^[110-112]. For this reason local excision may not be adequate treatment of these patients. In the setting of low-lying rectal cancer, sphincter-preserving procedures (intersphincteric dissection with coloanal anastomosis) may be performed in patients who obtain a tremendous response from neoadjuvant chemoradiation^[20].

Habr-Gama *et al*^[113] have also reported very interesting long-term outcomes of observational management in patients with cCR following neoadjuvant chemoradiation. They selected the 122 of 361 patients who received

neoadjuvant chemoradiation and were considered to have cCR. These patients had very low rates of both local recurrence and distant metastasis (5% and 7%, respectively). Isolated local recurrences confined to the rectum developed in 5% of patients over a median follow-up period of 59.9 mo^[114]. Significantly more data from larger studies will be required to evaluate this approach. Nevertheless, observational management may be an alternative choice for some carefully selected patients.

CONCLUSION

Concurrent chemoradiotherapy is widely accepted as an effective way to achieve local control and survival in patients with locally advanced rectal cancer. The accuracy of preoperative staging is crucial in preventing under- or over-treatment. ERUS and MRI are suitable imaging tools for local preoperative staging, while CT and PET are helpful in evaluation of metastasis. Fluoropyrimidine-based chemotherapy (either 5FU or capecitabine) used concurrently with radiotherapy is the standard treatment. The addition of oxaliplatin increases toxicity without having any significant impact on tumor sterilization.

Optimal timing of surgery remains unclear. Previous recommendations to resect at 6-8 wk after neoadjuvant chemoradiation appear reasonable; but a longer interval may increase tumor downsizing. However, the effect of a longer waiting time on long-term outcome is yet to be defined.

The accuracy of studies attempting to determine tumor response remains problematic. Clinical complete response, as determined by existing methods, may not be equivalent to pCR. At this time, surgery remains the standard of care for rectal cancer patients following neoadjuvant chemoradiation. Observational management is still investigational but may be used in carefully selected high-risk patients, ideally in the setting of a clinical trial. Pathologic features such as ypT, ypN, tumor response, and CRM are accurate prognostic factors associated with long-term outcome.

Extrapolating from colon cancer trials, adjuvant chemotherapy (5FU/leucovorin, capecitabine or FOLFOX) remains the standard of care following rectal cancer resection, irrespective of pathologic response. Future directions will require a rethinking of management strategies, and may include different optimal drug combinations, treatment sequences, and approaches to neoadjuvant radiation, chemotherapy and/or targeted therapy. The goal is to achieve improvement in response and patient survival. The challenges include individualizing care to improve long-term oncologic outcome, while minimizing toxicity and maintaining quality of life.

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Experimental and clinical evidence of antioxidant therapy in acute pancreatitis

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Abstract

Oxidative stress has been shown to play an important role in the pathogenesis of acute pancreatitis (AP). Antioxidants, alone or in combination with conventional therapy, should improve oxidative-stress-induced organ damage and therefore accelerate the rate of recovery. In recent years, substantial amounts of data about the efficiency of antioxidants against oxidative damage have been obtained from experiments with rodents. Some of these antioxidants have been found beneficial in the treatment of AP in humans; however, at present there is insufficient clinical data to support the benefits of antioxidants, alone or in combination with conventional therapy, in the management of AP in humans. Conflicting results obtained from experimental animals and humans may represent distinct pathophysiological mechanisms mediating tissue injury in different species. Further detailed studies should be done to clarify the exact mechanisms of tissue injury in human AP. Herein I tried to review the existing experimental and clinical studies on AP in order to determine the efficiency of antioxidants. The use of antioxidant enriched nutrition is a potential direction of clinical research in AP given the lack of clues about the efficiency and safety of antioxidant usage in patients with AP.

INTRODUCTION

Acute pancreatitis (AP) can present as a wide clinical spectrum ranging from a mild, self-limiting localized disease to fatal widespread multi-organ failure with high mortality rates. Mild pancreatitis is the inflammation and edema of the pancreas, with additional features of necrosis and secondary injury to extrapancreatic organs in severe pancreatitis^[1-3]. AP is characterized by acute inflammation and necrosis of pancreatic parenchyma, necrosis of pancreatic fat, hemorrhage, and inflammatory infiltration^[4]. Since pancreatic cell death occurs according to either necrosis or apoptosis mechanisms, many necrotic and apoptotic cells can be seen within pancreas parenchyma. The study of Booth *et al*^[5] found that oxygen free radical (OFR) induction in acinar cells promoted apoptosis whereas inhibition of OFR generation led to an increase in necrosis accompanied by reduced ATP. These findings suggest that OFR generation within acinar cells may be a protective response during pancreatitis. The main reason for necrotic or apoptotic cell death is the early activation of pancreas zymogens, especially cathepsin and trypsinogen inside the pancreas. Scott *et al*^[6] showed that excessive OFR in

a pathologic state can cause tissue and cell damage. The OFR participates in the pancreas edema process in AP, and may participate in the pancreas necrosis process. Furthermore, OFR are involved in the generation of pain as another important clinical feature of patients suffering from AP^[7]. The OFR, as highly reactive species, directly attacks lipids and proteins in the biological membranes and thus disrupts their functions. The action of OFR includes oxidation of lipids in the pancreatic cell membrane and oxidatively modified proteins, depolarization of the mitochondrial membrane, and induction of DNA fragmentation^[8].

Aerobic organisms require ground state oxygen to live; however, the use of oxygen during normal metabolism produces OFR^[9,10]. OFR are required for the maintenance of tissue homeostasis. Physiologic levels of OFR can regulate transcription, serve as signal molecules and defend against pathogen infections^[11]. The mitochondria, endoplasmic reticulum, cytosol, and nuclear membranes have all been shown to be sources of OFR^[12]. The primary function of the electron transport chain located in the inner mitochondrial membrane is ATP synthesis via oxidative phosphorylation^[13]. The superoxide radical formed during cellular metabolism is mainly produced during electron transport in the mitochondria and is released to both the mitochondrial matrix and intermembrane space^[9,10,14,15]. Superoxide, depending on its location, causes potential oxidative damage to different proteins and lipids, as well as DNA^[16]. The end product of the respiratory chain is water generated in a four-electron reduction of molecular oxygen. However, a small portion of oxygen is involved in the generation of OFR, including superoxide anion radicals, hydrogen peroxide, hypochlorous acid, and the extremely reactive hydroxyl radical^[10,17]. These free radicals, each containing an unpaired electron, are energetically unstable and highly reactive. OFR can attack the double bonds of unsaturated phospholipids in cell membranes, which eventually degrade the structural integrity of cell membranes. Lipid oxidation can lead to the loss of the integrity of both plasma membrane and intracellular membranes, such as that of lysosomes and the endoplasmic reticulum, which then leads to an intracellular leak of proteases or an influx of Ca²⁺ resulting in necrosis^[18]. OFR also impairs the functions of enzymes by causing fragmentation of polypeptide chains or cross-linking sulfhydryl groups in proteins. In addition, they cause strand breaks or abnormal cross-linking in DNA^[19-21]. Damage to DNA may lead to a DNA-damage response, including activation of p53 and poly-ADP ribose polymerase (PARP) (a nuclear enzyme). While activation of p53 causes apoptosis and cell cycle arrest, hyperactivation of PARP leads to necrosis^[22]. The hydroxyl radical is the most reactive species and can attack and damage almost all molecules found in the living cell. Being so reactive, it attacks DNA, a free radical chain reaction cascades through the DNA and causes chemical alterations of the bases as well as strand breakage. The best characterized biological dam-

age caused by hydroxyl radicals may be their ability to stimulate the free radical chain reaction known as lipid peroxidation^[12,23]. One hydroxyl radical can result in conversion of many hundred fatty acid chains into lipid hydroperoxides. Accumulation of lipid hydroperoxides in a membrane disrupts its functioning. Some of the products of lipid peroxidation, malonaldehyde (MDA) and 4-hydroxynonenal, increase the permeability and deformability of the membranes in which they are found. Mitochondrial membranes are particularly susceptible to this sort of damage, perhaps because they combine a high risk of OFR-mediated peroxidation^[12]. We have shown mitochondrial degeneration within pancreatic acinar cells in experimental pancreatitis^[1,24].

OFR indirectly act on the arachidonic acid cascade by increasing the production of thromboxane, which lowers tissue circulation by its potent platelet-aggregating and vasoconstricting effects^[25]. Additionally, OFR enhance the production of leukotriene B₄ which promotes activation of leukocytes and discharge of lysosomal enzymes^[26]. As a secondary effect, polymorphonuclear leukocytes are responsible for the respiratory burst that leads to an enhanced production of radical species and activated enzymes and further cell damage^[27]. The results of Rau *et al*^[28] indicate that OFR play an important mediator function in early and later courses of AP. Their findings suggest that OFR species are important mediators but not necessarily triggers of tissue damage in AP. The degree of oxidant-antioxidant balance changes in the early phase of human AP, correlating with the clinical severity of pancreatitis^[29]. Park *et al*^[30] reported higher plasma levels of lipid peroxides and myeloperoxides and lower superoxide dismutase (SOD) activity in patients with severe AP than in those with mild AP. OFR may thus be closely associated with the inflammatory process and the severity of AP. Thareja *et al*^[31] reported that high oxidative stress was observed during the early phase of AP and that gradually improving antioxidant status was associated with a better clinical outcome in patients with AP. The concentration of plasma lipid peroxides is a particularly meaningful index for determining the severity of the disease in humans^[30,32].

During the later stages of AP, the major pathophysiological role seems to be the attraction and activation of leukocytes, which in turn contribute to enhanced radical generation and acinar cell damage^[33,34]. Oxidative stress in the neutrophils (activated during the inflammatory response to acinar injury) may be responsible for further propagation of local and systemic inflammation^[5]. Synergy between pro-inflammatory cytokines and oxidative stress occurs in the development of the inflammatory response in AP^[35]. Proinflammatory cytokines such as interleukin (IL)-1, 6 and tumor necrosis factor (TNF) and microvascular ischemia are also important factors in the pathophysiology of AP^[33,34]. Serum levels of pro-inflammatory cytokines, such as TNF-alpha and IL-1beta, increase during the course of AP and appear to be the driving force for the initiation and propagation of

the systemic response. Accordingly, pretreatment with either an antibody against TNF- α or a blockade of TNF- α production with pentoxifylline ameliorates experimental AP^[36]. A cross-talk between oxidative stress and proinflammatory cytokines, particularly TNF- α amplifies the inflammatory cascade through different mechanisms, such as the activation of mitogen activated protein kinases and nuclear factor-kappa B (NF- κ B) and/or the inactivation of protein phosphatases^[35,37-39].

In addition to increased levels of plasma and tissue lipid peroxides, decreased levels of plasma antioxidants such as vitamin A, vitamin C^[40], vitamin E^[41], selenium^[42], β carotene, whole-blood glutathione, and the activity of plasma glutathione peroxidase^[21] have been found in patients with AP. Moreover, lower levels of selenium in toenails of AP patients and lower selenium concentrations in red blood cells of patients with severe AP have been detected^[40].

Since experimental^[42,43] and clinical studies^[31,44] have provided some support for the concept that oxidative stress is the common pathway for the pathogenesis of AP, one reasonable idea is to use antioxidant regimens in the management of AP to complement its traditional therapy. In fact elucidation of all of the mechanisms above mentioned and their interactions is critical in developing a treatment based on the pathophysiology of AP. However, clinical evidence showed that antioxidant administration to patients with AP was not as beneficial as the antioxidant administration to experimental animals. Perhaps the key steps in the pathogenesis of AP are not yet fully understood. It is not possible to clearly determine whether oxidative stress is a cause or an effect of AP. This review highlights the experimental and clinical evidence of the benefit of various antioxidants in AP.

ANTIOXIDANT THERAPY IN AP

Antioxidants may function to prevent the formation of, or to detoxify, free radicals or to scavenge OFR. Halliwell^[45] has proposed this useful definition, "an antioxidant is any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate". In experimental pancreatitis, the beneficial effects of antioxidants may be associated with the inhibition of NF- κ B activity^[46]. Types of antioxidants include antioxidant vitamins (e.g., ascorbic acid, α tocopherol, β -carotene), inorganic antioxidants (e.g., selenium), synthetic antioxidants (e.g., butylated hydroxyanisole), and a range of plant-derived polyphenols^[47]. Organisms widely use glutathione peroxidase, glutathione transferase, SOD, catalase (CAT), and a variety of other antioxidants to protect themselves against generation of OFR^[48-50]. Some authors have studied the effects of various antioxidants in experimental animals and patients with AP. They used both natural and synthetic antioxidants in order to protect the pancreas from deleterious effects of OFR. The following sections summarize data obtained

from experimental and clinical studies about the efficacy of some oxidants in AP.

Alpha tocopherol

Tocopherols belong to a class of phenolic antioxidants which can inhibit lipid auto-oxidation by scavenging free radicals and by reacting with singlet oxygen. The vitamin E activity of alpha-tocopherol may be attributed to its efficient inhibition of *in vivo* lipid oxidation^[51]. Fat-soluble antioxidants act directly in the lipid bilayer of plasma and cell membranes by interacting with membrane lipophilic components. A natural antioxidant, alpha-tocopherol has been found to be beneficial in inhibiting intermolecular connections of lipid peroxides in liver of dogs with AP^[52]. Vitamin E, including tocopherols and tocotrienols, is a fat-soluble antioxidant. To my knowledge the effect of vitamin E on AP has not been studied. Since they accumulate within tissues, fat-soluble substances have high toxic risk thereby limiting their clinical application and widespread utilization. The results of combined therapies, including vitamin E, will be discussed below.

Ascorbic acid

Ascorbic acid functions in multiple, complex ways, acting as a hydrogen donor, a metal inactivator, and a peroxide destroyer^[31]. The study of Bonham *et al*^[53] demonstrated that plasma ascorbic acid concentration was significantly below normal in patients with early phase AP; however, Sajewicz *et al*^[52] reported that patients with AP had double the plasma ascorbic acid values than healthy volunteers. Few studies have investigated the therapeutic efficacy of ascorbic acid in experimental animals with AP whereas many have examined its effects singly or within an antioxidant mixture in patients with AP. Two decades ago, Nonaka *et al*^[54] reported that CV3611, a synthetic free radical scavenger prepared from ascorbic acid, had an important therapeutic effect on the development of AP in mice. However since then, another experimental or clinical study evaluating the benefit of this agent in AP has not been performed. Du *et al*^[55] have reported that high dose vitamin C has a therapeutic effect in humans with AP. Their results indicate that vitamin C decreases hospitalization and duration of disease, and increases the cure rate by blocking lipid peroxidation, diminishing proinflammatory cytokines, and improving cellular immune function. The results of combination therapies will be discussed below.

Beta-carotene

Beta-carotene protects lipids by interfering with photosensitized oxidation, and behaves as a reducing agent by trapping radicals. In addition to its singlet oxygen-quenching properties, beta-carotene has good radical-trapping properties at low partial pressures of oxygen, a condition which prevails in healthy tissues. In biological systems, alpha-tocopherol and beta-carotene exhibit synergism by mutually reinforcing their respective activities.

Synergism also takes place in a cascade where ascorbic acid can be regenerated at the expense of more oxidizable substrates^[51]. In patients with mild AP, the concentrations of beta-carotene at final review has been found significantly higher than those in patients with severe AP^[56]. The correlation between low antioxidant level and high severity of disease suggests the utility of antioxidant supplementation therapies. Lavy *et al*^[57] have reported some possible protective effects of treatment with beta carotene in regards to the severity of post-endoscopic retrograde cholangiopancreatography pancreatitis (ERCP). In a double-blind trial, 321 patients were given a single dose of natural beta carotene. The rate of severe pancreatitis was found to be lower in the beta carotene-treated group. Adverse events were not reported.

Caffeic acid phenethyl ester

Caffeic acid phenethyl ester (CAPE) is a phenolic compound and an active substrate of propolis. Several investigators have shown that CAPE acts as an anti-inflammatory by inhibiting the release of arachidonic acid from cell membranes, and suppressing cyclooxygenase (COX)-1 and COX-2 enzyme activities^[58], and reducing antioxidant activity by lipoxygenase inhibition^[59,60]. Buyukberber *et al*^[46] have found CAPE to be beneficial in improving the biochemical and histopathological findings in cerulein-induced AP in rats. Turkyilmaz *et al*^[61] have also reported the beneficial effect of CAPE on acute necrotizing pancreatitis in rats. To my knowledge, no clinical data has been reported about the effect of CAPE on AP in humans. The data obtained from experimental animals are promising. CAPE has been shown to inhibit the production of proinflammatory cytokines by inhibiting nuclear transcription factor activity^[62].

Carnitine

As an antioxidant, acetyl l-carnitine culminate most probably protects tissues from oxidative stress by stabilizing cell membranes, rendering them more resistant to free radicals, perhaps by facilitating the repair of the phospholipid bilayer damaged by oxidant stress, rather than acting as a direct scavenger of free radicals or decreasing their generation^[63]. Data so far obtained would suggest that prior administration of acetyl l-carnitine ahead of caerulein challenge has proven protective efficacy that could possibly be ascribed, in part, to its regulation of the oxidant/antioxidant balance and modulation of nitric oxide (NO) release and myeloperoxidase activity that may ultimately lead to regulation of the inflammatory events associated with AP^[64].

Green tea

Another naturally occurring antioxidant comes from the caffeine-free extract from leaves of green tea (*Camellia sinensis*), shown to reduce the degree of AP and attenuate the activation of the transcription factor NF- κ B, as well as the formation of proinflammatory cytokines^[65]. It also significantly decreases lipid peroxidation^[65,66] and

the formation of nitrogen-derived radicals in rodents^[65].

Melatonin

Melatonin, the hormone produced mainly by the pineal gland, has been studied widely. Both *in vitro* and *in vivo* studies have identified melatonin as a potent scavenger of highly toxic hydroxyl radical and other oxygen-based radicals. Potent anti-inflammatory effects of melatonin may be related to a reduction of the inflammatory mediators produced during the inflammatory process^[67]. Several experimental studies showed that melatonin is effective in reducing oxidative stress-mediated AP in rodents^[68,69]. Recently, we reported a potent therapeutic effect of melatonin on caerulein-induced AP and associated liver injury^[1]. We also demonstrated its potent effect on the protection of cell ultrastructure^[43]. Melatonin decreases tissue MDA levels and increases antioxidant enzyme levels or activities^[1,3,43]. Melatonin treatment was found to promote the spontaneous regeneration process of pancreatic tissue in rats^[69]. Jaworek *et al*^[70] have reported that pinealectomy aggravates AP in the rat. Interestingly, Belyaev *et al*^[71] presented the dynamic changes of endogenous melatonin in the early phase of human AP. Melatonin concentrations during the first 24 h after the onset of pain in younger patients (< 35 years) were significantly higher than levels in older patients (> 35 years). They concluded that high endogenous serum melatonin levels in the first 24 h after the onset of AP played a protective role and favoured a mild disease course in humans, especially in young patients. Another clinical study performed by Chen *et al*^[72] indicates that delayed neutrophil apoptosis is associated with mild and severe AP in humans. Neutrophil apoptosis plays a critical role in minimizing the autodestructive potential of neutrophils. The data from the study show that melatonin promotes neutrophil apoptosis in human AP. Melatonin administration is promising for the treatment of AP in humans. However, further studies are still needed.

N-acetyl-cysteine

N-acetyl-cysteine (NAC) is a thiol compound which, by providing sulphhydryl groups, can act both as a precursor of reduced glutathione and as a direct free scavenger, hence regulating the redox status in the cells. In this way, it can interfere with several signaling pathways that play a role in regulating apoptosis, angiogenesis, cell growth and arrest, and inflammatory response^[73]. NAC has been found to be hugely effective in reducing oxidative stress-induced pancreatic injury in rats^[74,75] by enhancing the ability of acinar cells to produce IL-10^[74], preventing the impairment of Ca^{2+} ^[75], and downregulating the expression of chemokines, monocyte chemotactic protein-1, and macrophage inflammatory protein-2^[76]. Moreover extrapancreatic complications (liver and lung injury) during AP induced by bile-pancreatic duct obstruction were palliated by NAC treatment^[77]. Onur *et al*^[78] have reported that NAC, especially combined with hyperbaric oxygen, decreases oxidative stress parameters, serum amylase, cal-

cium, and lactate dehydrogenase levels, as well as the histopathological score. To our knowledge, only one study shows the effects of NAC in patients with AP. Recently, Milewski *et al*^[79] investigated the effects of NAC (administered by intravenous or oral route) on post-ERCP. Regrettably, NAC failed to demonstrate any significant preventive effect on post-ERCP pancreatitis or on serum and urine amylase activity. The results of combination therapies, including NAC, will be discussed below.

Resveratrol

Alcoholic AP predominates in countries where other forms of alcoholic drinks rather than wine are preferred. Presumably, wine contains factors that protect the pancreas against alcohol-induced AP. One of these factors may be resveratrol. Resveratrol, a naturally occurring antioxidant, acts as a free radical scavenger^[80], but to date, no clinical study has been performed on antioxidant and free radical scavenger potentials of resveratrol on AP. Resveratrol has been found beneficial in AP in rats^[81-83]. Lawinski *et al*^[84] found stilbene derivatives (resveratrol and diethylstilbestrol) effective in preventing pancreatic cells from structural changes to OOH-induced AP in Wistar albino rats. Resveratrol and diethylstilbestrol protect the pancreas against prooxidative activity of hydroperoxide; stilbene derivatives significantly inhibit the free radical generating reactions. Leonard *et al*^[85] have demonstrated that resveratrol can clear hydroxyl, superoxide, and metal inductive radicals. Kimura *et al*^[86] have found that resveratrol inhibits lipoxygenase, an enzyme that is converted into powerful inflammatory and white cell stimulating agents known as leukotrienes, hepoxillins, and lipoxins through arachidonic acid. Resveratrol may exert its therapeutic effect on severe AP by lowering pancreatic OFR and reducing pancreatic tissue infiltration of neutrophils^[87]. Due to its strong effect of inhibiting activation of NF- κ B and reducing secondary activation of cytokines, resveratrol is regarded as a promising drug for blocking the initiation and progress of AP. Though no document as yet illustrates the function of resveratrol in AP in humans, primary trials have found that resveratrol could inhibit the production of TNF and IL-6 in pancreatitis. More research is required^[87]. An increasing amount of data has confirmed that resveratrol could relieve the pathologic injury of the pancreas and extra-pancreatic organs (intestines, brain, lungs, *etc.*) induced by severe AP^[88-90]. Further clinical studies are needed.

Quercetin

Quercetin is a naturally occurring plant flavonoid abundantly present in onions, fruits, and Chinese herbs. Several studies pointed out the beneficial biological activities of quercetin which include antioxidant, antiinflammatory, antiatherosclerotic, and anti-tumor properties^[91-93]. The study of Carvalho *et al*^[94] has demonstrated that the flavonoid quercetin attenuates the severity of cerulein-induced AP in mice. In particular, they reported that quercetin treatment reduces pancreatic inflammation and

associated tissue injury through suppression of neutrophil infiltration, TNF- α , IL-1 β and IL-6 cytokine production, and TNF- α expression, and increased IL-10 cytokine production. Since high oral and subcutaneous doses of quercetin did not manifest any clinical signs of toxicity, it may be safe and nontoxic in the treatment of AP; however, further experimental and clinical investigations are needed.

Selenium

Selenium, a trace element necessary for cellular function in many organisms, is a co-factor for the antioxidant enzyme glutathione peroxidase^[95]. Glutathione peroxidase catalyses the reduction of both hydrogen peroxide and lipid hydroperoxides^[96] and as such acts as an intracellular defence against free radical injury^[97]. Nowadays, the role of micronutrients, in particular selenium, is receiving increased attention. Lower levels of selenium in toenails of AP patients and lower selenium concentrations in red blood cells of patients with severe AP have been found^[40]. Selenium administration 24 h after induction of experimental AP has been demonstrated to be associated with amelioration of pancreatic injury and lung injury although it has not reversed the diminished serum selenium level^[98]. Kuklinski *et al*^[99] reported their clinical results of 4 years of selenium therapy. They concluded that an improvement in the prognosis of AP could be achieved if antioxidative selenium therapy with sodium selenite was introduced in time. In rare cases, total necroses and complications in organs only occurred in those patients who were admitted to this therapy too late. Wollschläger *et al*^[100] reported that selenium therapy caused a significant increase in selenium, a moderate increase in glutathione peroxidase activity, and a significant decrease in MDA activity, while SOD remained unchanged in patients with AP. Lindner *et al*^[101] did report, however, that substitution of sodium selenite had no beneficial effect of the clinical outcome of patients with AP (32 selenium administered, 35 placebo administered). On the other hand, Kocan *et al*^[102] found selenium to be beneficial in decreasing inflammation in one patient with AP. The results of combination therapies including selenium will be discussed below.

RESULTS OF COMBINED ANTIOXIDANT THERAPIES

Recently we reported the beneficial effect of an antioxidant mixture of NAC and ascorbic acid on experimental AP in rats. The antioxidant mixture reduced tissue MDA levels and increased tissue CAT and glutathione peroxidase activities^[1,42] in L-arginine-induced experimental AP. Hardman *et al*^[103] have demonstrated that early exogenous anti-oxidant intravenous supplementation using a combination of NAC, selenium, and vitamin C reduces pancreatic injury in rats. However, a case-control study demonstrated no benefit from intravenous administration of these multicomponent anti-oxidants in clinical

AP although critically, many patients in the clinical study received antioxidants relatively late in the course of the disease^[104]. A randomized trial by Siriwardena *et al*^[105] also failed to show any benefit to AP patients given selenium intravenously as part of a cocktail of antioxidants including NAC and vitamin C. On the other hand, Sateesh *et al*^[106] demonstrated the beneficial effects of vitamin C and NAC on decreasing oxidative stress and improving antioxidant status in 23 patients with AP. They concluded that antioxidant supplementation associated with standard medical treatment may decrease the length of hospital stay and rates of complications in patients with AP, but a larger clinical trial is needed to support this hypothesis. The study of Bansal *et al*^[107] showed that vitamin-based antioxidant therapy had no significant beneficial effect on organ dysfunction or on clinical outcomes in severe AP during the hospital stay. They administered vitamin A (intramuscularly), E (orally) and C (intravenously) together for a period of 14 d. Kuklinski *et al*^[108] showed the beneficial effect of an adjuvant antioxidant therapy with selenium and D-alpha-tocopherol in 90 patients with necrotizing or mild AP. They reported that the average lethality rate of 34% fell to 1.1%. This study represents the vital benefits of antioxidant therapy. Uden *et al*^[109] administered organic selenium, beta carotene, vitamin C, vitamin E and methionine to patients with recurrent pancreatitis (idiopathic chronic 8, alcoholic chronic 7, and idiopathic acute 5) and they reported the beneficial role of antioxidants in pain reduction. Recently, Zhao *et al*^[110] reported the significant beneficial effects of the combination of ebselen and ethylhydroxyethyl cellulose (EHEC) on severe AP in Sprague Dawley rats. The mixture prevented pancreatitis-induced multiple organ injury. Ebselen (2-phenyl-1,2-benzisoxselenazol-3(2H-one) is a non-toxic seleno-organic drug with anti-inflammatory, antiatherosclerotic, and cytoprotective properties that downregulates the production of OFRs^[111,112]. Ebselen is a mimic of glutathione peroxidase that also reacts with peroxynitrite and can inhibit enzymes such as lipoxygenases, NO synthases, nicotinamide adenine dinucleotide phosphate oxidase, protein kinase C and H(+)/K(+)-ATPase^[113,114]. The combination of ebselen and EHEC may be a new potential for treatment of severe AP^[110].

While the trial clearly showed that the given combination of antioxidants was not effective in patients with AP when administered intravenously, antioxidants administered through another route may be beneficial, given that the benefits of enteral versus parenteral nutrition in patients with AP are well proven^[115,116]. In particular, a growing body of clinical evidence from other disease settings suggests that supplementation of enteral nutrition with antioxidants can be beneficial^[117,118].

In conclusion, based on the reported experimental studies, using antioxidant regimens in the management of AP as a supplement and combined with traditional therapy is rational and reasonable. If this hypothesis is correct, antioxidant therapy should reduce the inflammatory process involved in pancreatitis and thereby

accelerate the recovery rate. Some of the antioxidants have been shown to be beneficial in the treatment of human AP. However, the present studies indicate that insufficient clinical data support using antioxidants alone or in combination with conventional therapy in the management of AP. Further double blind, randomized, placebo-controlled clinical trials with a larger sample size need to be conducted.

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Dietary copper triggers onset of fulminant hepatitis in the Long-Evans cinnamon rat model

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Abstract

AIM: To investigate the impact of dietary copper given at different time points on the onset of fulminant hepatitis.

METHODS: The Long-Evans cinnamon (LEC) rat model of Wilson's disease (WD) was used to study the impact of high dietary copper (hCu) on the induction of fulminant hepatitis at early or late time points of life. High Cu diet was started in rat pups or in adults (month 5) for three months. Animals that received reduced dietary copper (rCu) throughout their lifetime served as a control. Hepatitis-associated serum markers (alanine aminotransferase, aspartate transaminase, bilirubin) were analyzed in animal groups receiving hCu or rCu. Liver copper content and liver histology were revealed

at sacrifice. A set of 5 marker genes previously found to be affected in injured liver and which are related to angiogenesis (*Vegfa*), fat metabolism (*Srebf1*), extracellular matrix (*Timp1*), oxidative stress (*Hmox1*), and the cell cycle (*Cdkn1a*) were analyzed by real-time polymerase chain reaction.

RESULTS: Regardless of the time point when hCu was started, LEC rats (35/36) developed fulminant hepatitis and died. Animals receiving rCu (36/36) remained healthy, did not develop hepatitis, and survived long term without symptoms of overt disease, although liver copper accumulated in adult animals ($477 \pm 75 \mu\text{g/g}$). With regard to start of hCu, onset of fulminant hepatitis was significantly ($P < 0.001$) earlier in adults ($35 \pm 9 \text{ d}$) that showed pre-accumulation of liver copper as compared to the pup group ($77 \pm 15 \text{ d}$). Hepatitis-associated serum markers, liver copper and liver histology, as well as gene expression, were affected in LEC rats receiving hCu. However, except for early and rapid onset of hepatitis, biochemical and molecular markers were similar at the early and late time points of disease.

CONCLUSION: Rapid onset of fulminant hepatitis in asymptomatic LEC rats with elevated liver copper suggests that there is a critical threshold of liver copper which is important to trigger the course of WD.

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Key words: Wilson's disease; Fulminant hepatitis; Acute liver failure; Dietary copper; Long-Evans cinnamon rat; *ATP7B*

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INTRODUCTION

Wilson's disease (WD) is a genetic disorder transmitted by a recessive gene located on chromosome 13^[1,2]. Diagnosis of WD is mostly established on the basis of combined biochemical and clinical parameters, and lately by specific genetic analysis^[3-6]. Presentation of WD includes hepatic insufficiency, acute and chronic active hepatitis, fulminant hepatitis and/or extra hepatic manifestations. Although it is a rare disease, fulminant hepatitis is frequently observed in WD patients and is followed by high mortality that accounts for approximately 5% of acute liver failure (ALF) observed worldwide. The disease is linked to an imbalance of copper homeostasis that is due to mutation of the *ATP7B* copper transporter gene expressed primarily in the liver^[7,8]. More than 600 mutations have been reported in human *ATP7B*. Effective drug treatment of WD has been established which aims at chelation or reduction of copper^[1]. However, compliance to take lifelong treatment or to reduce dietary copper intake is low in patients^[9]. Liver transplantation remains the only treatment option of WD patients encountering ALF.

In healthy individuals copper is absorbed *via* the intestine and delivered to the liver, where it is either transferred by *ATP7B* into ceruloplasmin that is secreted into blood or exported *via* bile^[2]. Malfunction of the *ATP7B* gene in WD patients leads to large quantities of toxic copper in the liver and other organs. However, manifestation and onset of the disease induced by toxic copper can vary to a great extent between WD patients^[10-12]. Individual mutations of the *ATP7B* gene as well as mutations outside of this locus have been implicated to be important for the course of the disease as suggested, e.g., by rare studies of twins having the same mutation of the *ATP7B* gene but presenting different courses of WD^[13,14]. Besides genetic factors, environmental factors, such as the amount of dietary copper intake, may also have an impact on WD^[15,16]. However, studies of such factors are difficult in humans.

Much has been learned from animal models of WD. The Long-Evans cinnamon (LEC) rat has a large deletion of the *ATP7B* gene^[17] and has many characteristics that are also observed in patients, e.g., low ceruloplasmin, high liver copper levels, and sensitivity to anti-copper therapy. The LEC rat strain can be housed on a commercial diet containing standard copper concentrations (about 7-15 mg/kg). Using this standard chow, 40%-60% of LEC rats spontaneously encounter fulminant hepatitis at the age of 80-120 d^[18]. The LEC rat is therefore a highly attractive model to analyze pathogenesis of the liver, and is also used to study the effect of

novel therapeutic approaches for liver disease, e.g., hepatocyte transplantation or gene transfer^[19,20].

In the present study, the role of low and high dietary copper given at different time points was investigated in male and female LEC rats. We hypothesized that pre-loading of copper may affect the course and onset of disease. A high copper (hCu) diet was given to juvenile and adult rats that could pre-accumulate liver copper. The impact on hepatitis, liver copper, histology, gene expression, and survival was examined.

MATERIALS AND METHODS

Animals

LEC rats that lack functional *ATP7B*^[17] and Long-Evans agouti (LEA) rats expressing wild-type *ATP7B* were a kind gift of S. Gupta (Albert Einstein College of Medicine, New York, United States). Rats were genotyped for *ATP7B* by polymerase chain reaction (PCR) analysis using primers essentially as described previously^[21]. For the hCu regimen, animals received a solid diet that contains a standard concentration (13 mg/kg) of copper (1324, Altromin, Germany). Animals of the hCu groups additionally received tap water containing 20 mg copper/L [copper (II) chloride, Sigma-Aldrich]. For the reduced copper (rCu) regimen, rats received a specialized solid diet (C1041, Altromin) with a copper content of 0.3 mg/kg and distilled tap water. Female rats ($n = 12$) which were reported to have higher sensitivity to copper^[18,22] and male rats ($n = 6$) were analyzed for each group. LEA rats, heterozygotes and LEC rats housed on standard diet were used as controls. The incidence of severe jaundice with strong disturbance of common as well as spontaneous behaviour, and weight loss ($> 20\%$) which was accompanied by coma, led to sacrifice of the animals. The protocol for animal use was approved by local authorities.

Analysis of serum samples

Blood was taken under retrobulbar anesthesia with isoflurane (1-chloro-2,2,2-trifluoroethyl difluoromethyl ether, Abbott). Activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and total concentration of bilirubin in serum were analyzed photometrically using a Cobas Modular System (Roche Diagnostics, Germany). Ceruloplasmin oxidase activity was determined using the modified protocol of Schosinsky *et al*^[23] adapted to a 96-well plate.

Liver histology

For histological evaluation, liver specimens were fixed by immersion for at least 24 h in 4% formaldehyde solution, and were subsequently dehydrated and embedded in paraffin wax in order to cut serial sections at a thickness of 5 μ m. Morphologic parameters were determined after hematoxylin and eosin staining using standard protocols, and scored by a blinded observer, e.g., for polyploidy, steatosis, apoptosis, and proliferation.

Table 1 Genes analyzed by real-time polymerase chain reaction in liver

Gene symbol	Synonym	Accession number	Primer (forward/reverse)
<i>Vegfa</i>	Vascular endothelial growth factor A	NM031836	CAGTTCGAGGAAAGGGAAGGCAAATGCTTTCTCCGCTCTG
<i>Hmox1</i>	Heme oxygenase (decycling) 1	NM012580	AGAGGCTAAGACCGCCTTCC AGGCCTCTGGCGAAGAAAC
<i>Cdkn1a</i>	Cyclin-dependent kinase inhibitor 1A	U24174	CTTGCACTCTGGTGCTCACG ATCGGCGCTTGGAGTGATAG
<i>Timp1</i>	TIMP metalloproteinase inhibitor 1	BC099821	CCTGGTTCCTGGCATAATC TTGCAAGGGATGGCTGAAC
<i>Srebf1</i>	Sterol regulatory element binding protein-1	AF286470	AGAAGGCCAGTGGGTACCTG TCGGGGCCACAAGAAGTAG

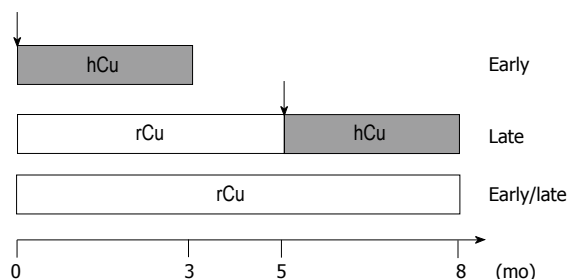


Figure 1 Schematic representation of study design. For the early copper regimen, Long-Evans cinnamon (LEC) rat pups received a high copper (hCu) diet up to month 3. For the late copper regimen, LEC pups were first housed on a reduced copper (rCu) diet until adult (month 5). Thereafter, rats received hCu for 3 mo up to the end of observation time (month 8). LEC rats received rCu diet throughout the observation period (early/late). *ATP7B* heterozygotes as well as Long-Evans agouti rats were also studied using an early hCu regimen. Arrow indicates start of hCu regimen.

Copper determination

For determination of copper content, liver samples were dried for 72 h at 70 °C. Dry weight was determined using the analytical balance ME235S (Sartorius, Germany). Liver was redissolved in 65% nitric acid (Merck, Germany) and copper concentration was determined by flame atomic absorption spectroscopy (Shimadzu AA6300, Japan).

Real-time PCR analysis

Randomly chosen liver samples were immediately frozen after resection. Total RNA was extracted after disruption of tissue using a homogenizer and RNeasy kit (Qiagen, Hilden, Germany). Reverse transcription-PCR was carried out as described^[24]. Briefly, RNA was reverse transcribed using SuperScript II according to the instructions of the manufacturer (Invitrogen). Primers for PCR (Table 1) were synthesized by MWG Biotech (Germany). For quantitative real-time PCR, a SYBR® Green kit (Eurogentec, Belgium) was used. PCR was performed on the ABI PRISM™ 7900HT sequence detector (PE Applied Biosystems, United States). Each sample was tested in three independent experiments. The ct value was normalized to the expression of the house-keeping gene *HPRT* (Δ ct method). Relative expression level of gene normalized to house-keeping gene was determined by the equation $2^{\Delta\text{ct}}$ and fold-change was calculated thereafter.

Statistical analysis

Statistical analysis was performed using SPSS 17.0 software. Data are given as mean \pm SE and median. Data were analyzed by the Student's two tailed *t* test using

Bonferroni correction for post-hoc pairwise comparisons or Kruskal-Wallis test. Kaplan-Meier survival test and logrank test were performed for time to event data.

RESULTS

Onset of fulminant hepatitis

The role of low and high dietary copper in the induction of severe liver disease was studied with respect to the start of a copper diet in LEC rats of different age (Figure 1). A hCu was started early in pups or late in adults at month 5. The latter animals received a rCu up to month 5. The time point of hCu in adults was chosen to encompass the phase of fulminant hepatitis that occurs in LEC rats at the age of 80-120 d when using commercial chow^[18]. LEC rats subjected to hCu displayed significantly elevated hepatitis-associated markers (Figure 2A), and developed severe jaundice (bilirubin > 2.0 mg/dL). With regard to start of hCu, levels of serum markers increased significantly earlier ($P < 0.001$) after the late regimen as compared with the early regimen (Figure 2B). Both genders displayed similar onset of significantly elevated serum markers (Table 2). LEC rats receiving a rCu diet or animals of the control groups did not develop hepatitis.

LEC rats (36/36) receiving rCu survived the observation period (8 mo), and no signs of overt morbidity were recorded in individual rats (18/18) as compared to controls, even when monitored beyond the observation period (up to month 20). In contrast, survival was significantly impaired ($P < 0.001$) in LEC rats (35/36) that received hCu (Figure 2C). LEC rats subjected to an early hCu regimen showed a mean survival of 77 ± 15 d that was only moderately shorter when compared to control LEC rats that were housed on a commonly used standard diet (84 ± 5 d). In contrast, LEC rats that received a late hCu regimen had a significantly reduced survival (35 ± 9 d) with regard to start of hCu. Survival of male and female LEC rats did not differ (Table 2).

Impact of dietary copper on liver histology

Nuclei of hepatocytes were found to be highly enlarged in LEC rats receiving early and late hCu regimens (Figure 3). Pronounced fatty acid changes, inflammation and, to a lesser extent, necrosis were observed in LEC rats receiving hCu. No marked changes in liver histology, including enlargements, fatty metamorphosis and inflammation, or cholangiofibrosis, that are routinely found in adults^[18] were observed in LEC rats that received a rCu diet. The liver histology in the control groups was found to be

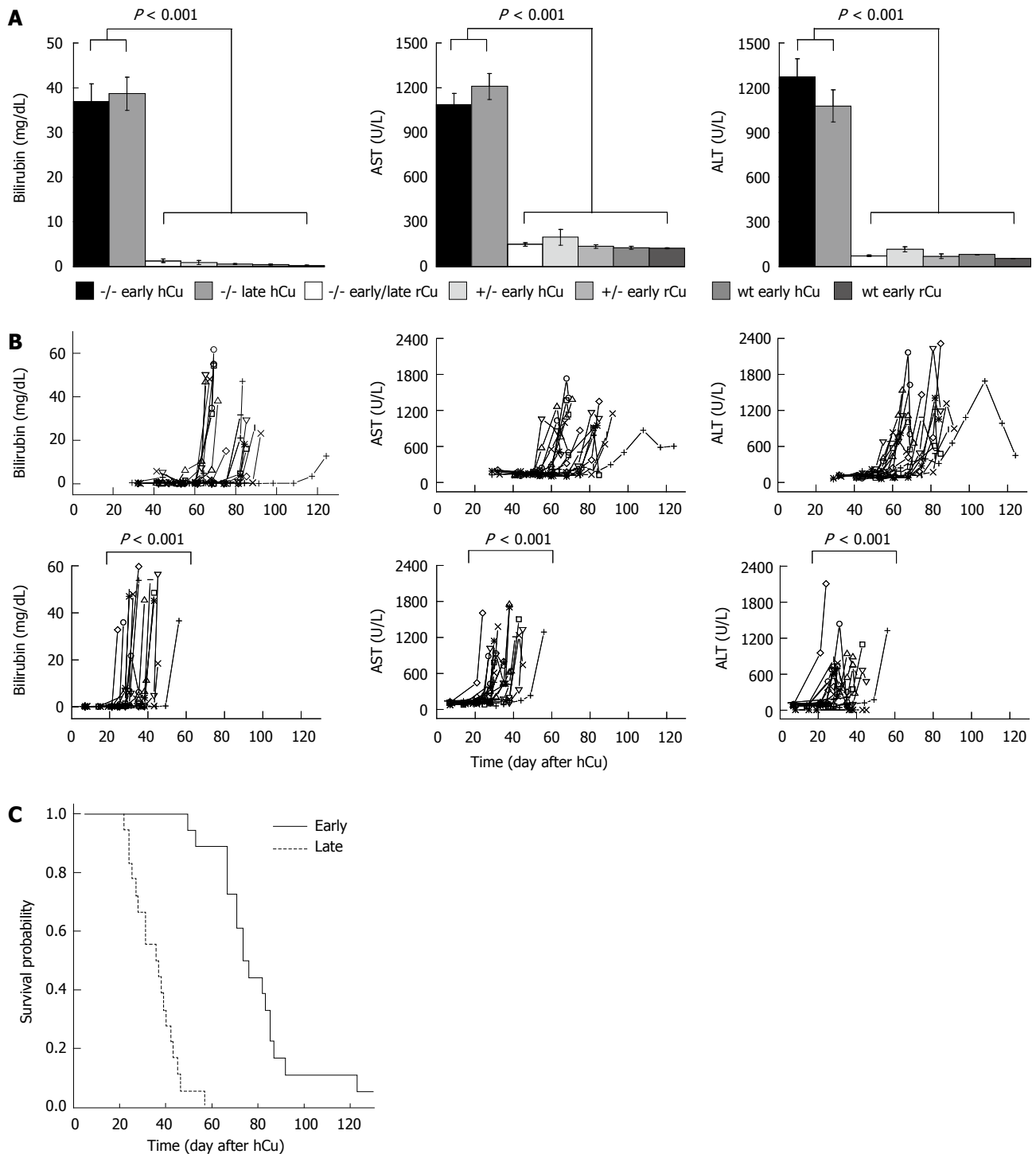


Figure 2 High copper regimen induces fulminant hepatitis. A: Bilirubin, aspartate transaminase (AST) and alanine aminotransferase (ALT) were determined in Long-Evans cinnamon (LEC) rats (-/-), heterozygotes (+/-) or Long-Evans agouti (LEA) rats (wt). Animals received a high copper (hCu) or reduced copper (rCu) diet as an early or late regimen. Median \pm SE of maximal values obtained from animals are shown; B: Time course of serum markers in LEC rats that received early (top) or late (bottom) hCu. X-axis represents day after start of hCu. Animals of other groups showed normal levels. Of note, the onset of increased values following late hCu was at day 189 ± 42 after birth. Each line represents one animal. Logrank test using threshold (mean \pm SE of LEA rats $\times 2$) was used; C: Kaplan-Meier survival curve of LEC rats receiving an early and late hCu regimen are shown. Survival is represented as day after start of hCu. Of note, age of animals at death in late group was 190 ± 9 d.

normal regardless of the copper regimen used.

Accumulation of liver copper

High concentrations of copper were determined in the livers of LEC rats (Figure 4) following hCu, resembling WD^[25]. LEC rats receiving rCu exhibited significant liver

copper after the early (422 ± 62 $\mu\text{g/g}$) and late regimen (477 ± 75 $\mu\text{g/g}$) as compared to heterozygotes or wild type rats. Male and female LEC rats did not significantly differ with respect to liver copper (Table 2). Ceruloplasmin activity, which is absent in the LEC rat strain^[20], was not affected by a copper diet (Figure 5).

Table 2 Male and female Long-Evans cinnamon rats after copper diet

		Early hCu		Late hCu		rCu		Long-Evans agouti
		Male	Female	Male	Female	Male	Female	Female
Bilirubin	> 0.4 mg/dL ¹ (d)	55 (67 ± 5)	63 (60 ± 1)	28 (30 ± 1)	28 (32 ± 1)	0.3 ² (0.3 ± 0)	0.2 ² (0.2 ± 0)	0.2 ² (0.2 ± 0)
AST	> 240 U/L ¹ (d)	74 (75 ± 3)	68 (68 ± 1)	28 (34 ± 3)	30 (33 ± 1)	115 ² (117 ± 4)	103 ² (103 ± 3)	114 ² (114 ± 5)
ALT	> 110 U/L ¹ (d)	44 (50 ± 2)	54 (54 ± 1)	28 (30 ± 2)	28 (31 ± 1)	69 ² (69 ± 6)	75 ² (75 ± 6)	54 ² (54 ± 1)
Liver Cu	(μg/g)	868 (880 ± 93)	1036 (1058 ± 59)	1494 (1452 ± 161)	1235 (1178 ± 59)	503 (495 ± 75)	309 (326 ± 29)	46 (44 ± 9)
Survival	(day after hCu)	85 (81 ± 4)	81 (75 ± 9)	37 (37 ± 6)	35 (35 ± 2)	NA	NA	NA

NA: Not available; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; hCu: High copper; rCu: Reduced copper. ¹Sera up to sacrifice were analyzed by logrank test using thresholds calculated from Long-Evans agouti rat (mean ± SE) × 2; ²Max values up to sacrifice are given. Data of male and females did not differ (logrank, *t* test, Kruskal-Wallis, Kaplan-Meier).

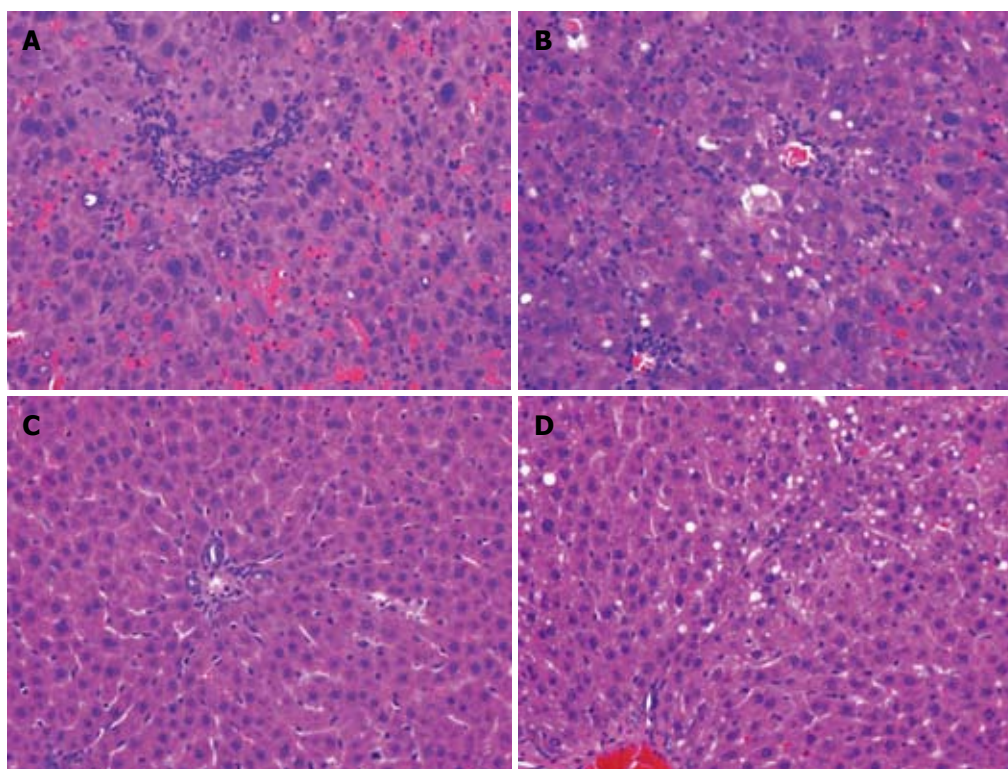


Figure 3 Liver of Long-Evans cinnamon rats is significantly affected by high copper regimen. Hematoxylin and eosin staining of liver. Livers were obtained from Long-Evans cinnamon rats that received high copper at an early or late time point (A, B) or after a reduced copper diet (C: late regimen). As a control, liver of Long-Evans agouti rat is depicted (D). Note highly irregular architecture of liver parenchyma, polyploidy, enlarged nuclei and inflammation (A, B) as compared to C and D. Liver histology of other groups was normal. One representative staining (40× magnification) of female rats is shown.

Gene expression in liver

In order to investigate whether the different copper diet regimens also have an impact on major biochemical pathways, gene expression in the liver was determined in animals at sacrifice. A small set of five marker genes was analyzed that relate to different areas of metabolism previously found to be affected in injured liver, including angiogenesis (*Vegfa*), fat metabolism (*Srebf1*), extracellular matrix (*Timp1*), oxidative stress (*Hmox1*), and the cell cycle (*Cdkn1a*). A highly different (fold-change > 8.5) expression of marker genes was observed when LEC rats receiving an early hCu regimen were compared to LEC rats receiving rCu (Figure 6). mRNA levels of *Vegfa* (8.5) and *Srebf1* (13.9) were downregulated at the time points of fulminant hepatitis in LEC rats receiving hCu, where-

as *Timp1* (9.8), *Cdkn1* (10.1) and *Hmox1* (10.6) were up-regulated at this time point. Marker gene expression was not affected in LEC rats receiving rCu, in LEA rats or in *ATP7B* heterozygotes (data not shown). Of note, none of the five genes displayed a significantly different (fold-change > 2) level of expression when LEC rats receiving early and late hCu regimen were compared.

DISCUSSION

The early hCu regimen used here for juvenile LEC rats closely resembles previous protocols. Onset of fulminant hepatitis was observed by us at about day 80, which is of a similar range as compared to others^[18,25,26]. The high dietary copper regimen was achieved by copper

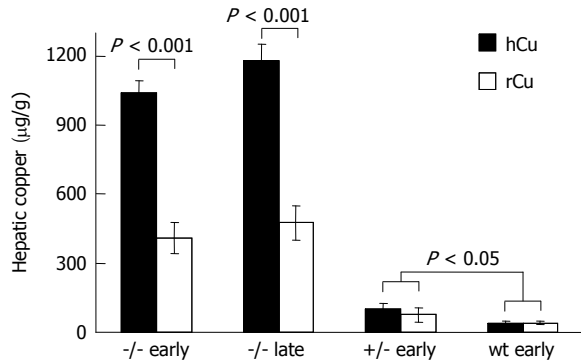


Figure 4 Increased liver copper is found in Long-Evans cinnamon rats irrespective of copper diet. Copper was determined in the liver of Long-Evans cinnamon rats (-/-; $n = 9$), heterozygotes (+/-; $n = 5$) and Long-Evans agouti rats (wt; $n = 3$) after receiving high copper (hCu) or reduced copper (rCu) diet according to an early or late regimen, respectively. Copper concentration is given as mean \pm SE of liver dry weight.

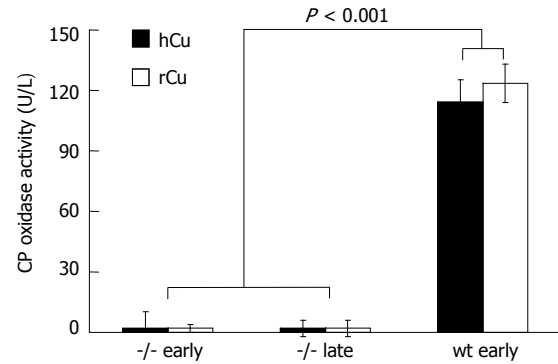


Figure 5 Ceruloplasmin oxidase is independent of high copper treatment. Ceruloplasmin (CP) oxidase was determined in Long-Evans cinnamon rats (-/-) that received high copper (hCu) or reduced copper (rCu). Sera ($n = 6$) were obtained from animals after an early or late hCu regimen. As control, values obtained from Long-Evans agouti rats (wt) ($n = 3$) are shown. Data are expressed as mean \pm SE.

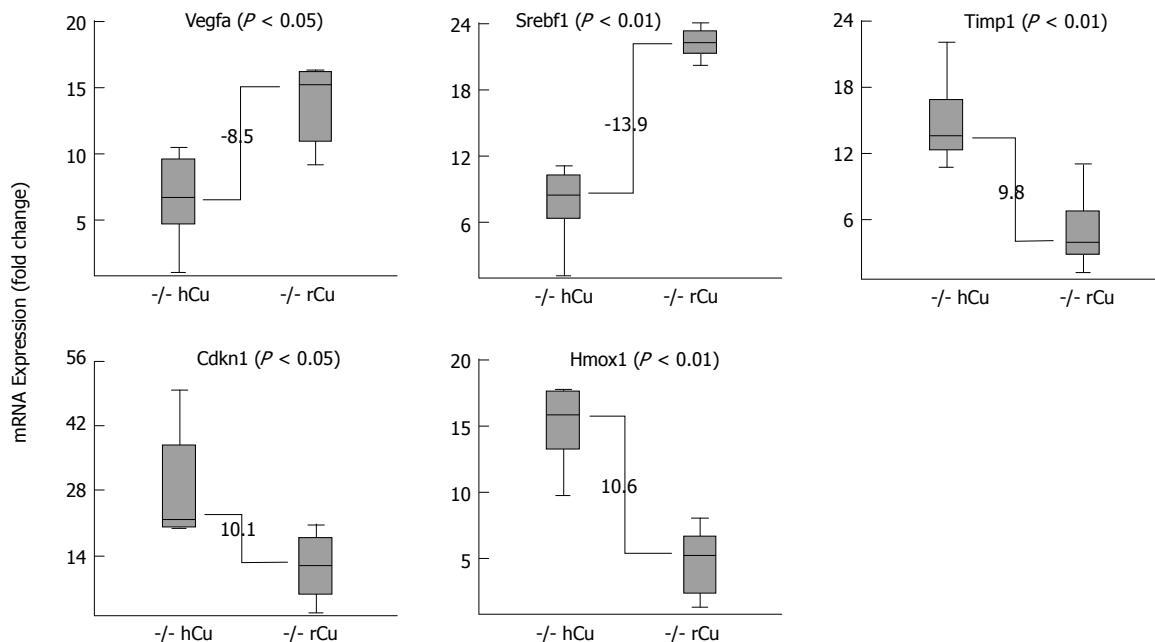


Figure 6 Liver gene expression of Long-Evans cinnamon rat is significantly altered after high copper regimen. Relative expression of individual mRNA in Long-Evans cinnamon (LEC) rats ($n = 7$) that were subjected to early high copper (hCu) regimen (left boxes) and in age-matched LEC rats ($n = 5$) receiving reduced copper (rCu) (right boxes). Animals were analyzed at fulminant hepatitis. Fold-change was calculated within groups by the $\Delta\Delta$ method using the *HPRT* gene for normalization. Data are shown as a box plot representation as calculated from three independent experiments. Numbers refer to difference of fold-change with regard to the median of each group.

enriched tap water (20 mg copper/L) and a standard solid diet (13 mg copper/kg). As compared to previous studies that employed a standard solid diet, only the overall dietary copper burden given to the LEC rats was moderately increased^[18,25,26]. Much higher dietary copper concentrations have been reported in LEC rats^[26-28], and intraperitoneal injection of 3 mg/kg copper for 3 d was shown to result in jaundice and fulminant hepatitis within 2 d after the last injection, suggesting that besides the absolute amount of copper the route of administration is also important^[29]. As one result of our high dietary copper regimen, all LEC rats (with the exception of one animal used for the early regimen) encountered

fulminant hepatitis and death. This is in contrast to previous studies using a standard solid diet only, where 40%-60% of LEC rats survived^[18,25,26]. Since the high dietary copper regimen can induce fulminant hepatitis in nearly all LEC rats, such protocols may now allow a more quantitative analysis of therapy, e.g., by cell-based approaches^[30].

Prevention of hepatitis and almost disease-free long term survival were observed in LEC rats that received a low copper diet throughout their lifetime. Hepatitis in LEC rats is closely associated with copper intake^[18,31], and it was therefore noted early that a copper deficient diet can reduce serum levels of AST and ALT in addition to

diminution of copper concentration in the liver^[27]. In the latter study of male LEC rats, animals at the age of 30 d were monitored for 35 d or 15 wk^[18,32]. A rCu diet that was enriched for L-proline could also improve survival (up to month 5) in male LEC rats^[26]. We could confirm previous results regarding the impact of reduced dietary copper intake in a long-term study (up to 20 mo). Since male LEC rats seem to be more resistant to copper^[33,34], we also investigated female LEC rats; however, a significant difference between genders was not observed for onset of hepatitis, liver copper, and survival.

Onset of hepatitis as determined by serum markers ALT, AST, and bilirubin was significant earlier (about 2 wk) in LEC rats that received a late hCu regimen. The finding of a significantly earlier onset of hepatitis and death in animals having elevated liver copper at start of hCu suggests that copper preloading of the liver above a critical threshold may accelerate the onset of disease. In contrast, LEC rats remained apparently asymptomatic showing liver copper levels that were about 10-fold higher as compared to LEA rats, suggesting that a high liver copper level can be tolerated for an extended time but induces a rapid onset of disease when dietary copper intake is increased. It should be stated that the effect of copper preloading could not be demonstrated by inspection of liver tissue since the stains obtained at early and late time points after hCu treatment did not significantly differ. Inflammation and necrosis were almost absent in stains of liver tissue at early and late time points when a rCu diet was used, suggesting that preloading with significant but subcritical levels of copper does not result in gross histological alterations of liver tissue and adverse immune responses.

The liver of LEC rats was characterized by determination of mRNA levels after a hCu regimen to assess the impact of dietary copper on gene expression. Notably, an almost identical gene expression was observed in juvenile and adult LEC rats after the hCu regimen, indicating that pre-accumulated copper levels do not affect gene expression at least at the time point of fulminant hepatitis. Marked differences were however observed between LEC rats at fulminant hepatitis and age-matched asymptomatic LEC rats that received a rCu diet. Inhibitory molecule Cdkn1a (p21) was found to be markedly upregulated suggesting that the cell cycle is blocked at G0 in the phase of fulminant hepatitis, confirming previous results obtained in LEC rats as well as in rats after hepatectomy^[35,36]. It is conceivable that arrest of the cell cycle within the liver is a common molecular determinant of fulminant hepatitis. Timp1, an important mediator of extracellular matrix remodeling during toxic liver injury^[37], was highly upregulated in LEC rats after hCu corroborating our observations of significant changes in liver architecture. The role (if any) of Timp-1 mRNA regulation by a copper diet with regard to the activity of metalloproteinase (MMP), e.g., MMP-9, which was shown to be highly induced in fulminant hepatic failure^[38], remains to be studied. Expression of *Srebfl* was down-regulated in

LEC rats after the hCu regimen, suggesting that copper overload may be followed by reduction of lipid biosynthesis. Of note, *Srebfl* activity was found to be increased in copper-deficient rats^[39]. *Hmox1*, which is related to oxidative stress induced by iron deposition that is also commonly observed in LEC rats, was found to be highly upregulated after the hCu regimen, as reported before^[36]. In contrast, *Vegfa*, a strong mediator of angiogenesis that was recently observed to be induced in hepatoma cells by hCu *via* a ceruloplasmin promotor, was down-regulated after hCu regimen. LEC rats which have a low ceruloplasmin expression may differ in this respect from wild type^[40]. Although our analysis of gene expression in the liver of LEC rats after hCu regimen showed a significant modulation of important markers of copper metabolism, the study is limited since only one time point was examined. Clearly, further studies are needed that are beyond the scope of this article to explore the molecular events following toxic copper exposure of the liver.

Accumulation of dietary copper in the liver over a lifetime may play a role in the course of WD even when the genetic constitution of patients is similar^[13-16]. While WD is frequently observed in the 2nd and 3rd decade of life, presentation of typical WD symptoms at the preschool age is relative rare and onset of disease above the age of 40 has been noted^[12]. The finding that LEC rats have moderate liver copper but no overt disease may possibly resemble the observation of rare asymptomatic WD patients^[41,42]. Conversion from a state of low to high dietary copper is followed by rapid development of severe disease in LEC rats, a situation that may also apply to WD patients after discontinuation of therapy^[9,43]. Our findings indicate the importance of a stringent maintenance of anti-copper therapy for management of WD patients in order to balance liver copper level below a critical threshold, and reinforce the lifetime risk of WD patients of developing liver failure when dietary copper intake is increased or anti-copper therapy has failed.

Taken together our results demonstrate that asymptomatic LEC rats having significantly elevated liver copper either rapidly develop fulminant hepatitis when dietary copper is increased or survive long term on a rCu diet, suggesting that there may exist a critical threshold of liver copper important to trigger onset of WD. A stringent diet of low copper throughout the lifetime is followed in LEC rats by escape from hepatitis, moderately low liver copper, and long term survival without overt disease. The data underline the importance of environmental factors, such as dietary copper, with respect to the phenotype and course of WD, and suggest that similar molecular and physiologic mechanisms are initiated when tolerable copper thresholds are exceeded during the lifetime.

ACKNOWLEDGMENTS

We thank Gerß J for statistical analysis and Cebulla K for technical support.

COMMENTS

Background

Fulminant hepatitis is a life-threatening disorder that is also observed in Wilson's disease (WD), an inherited disease that is caused by mutation of the *ATP7B* gene encoding a crucial copper transport protein in the liver. The dietary intake of copper during a lifetime can affect the course of WD. Analysis of dietary copper intake was assessed in a rat model of WD. The study is related to the understanding of copper toxicity and the molecular events of pathogenesis.

Research frontiers

High copper is toxic to cells. In humans, the liver has the role of excreting excessive copper and preventing toxicity. The molecular events following copper toxicity are not understood. The relationship between liver copper levels and onset of disease is important to understand for improving current therapy approaches.

Innovations and breakthroughs

Accumulation of liver copper in individuals carrying two allelic mutations of *ATP7B* is ultimately followed by development of WD; however, rate, onset, course of disease, including acute liver failure (ALF), varies greatly between patients. Studies of monozygotic twins having identical mutations of *ATP7B* are most informative to decipher the role of genetic and environmental factors for the course of WD. However, these studies are casual and rare. The Long-Evans cinnamon rat carrying a natural mutation of *ATP7B* has been established as a model to study the pathophysiology of WD. This study illustrates for the first time that a stringent copper-restricted diet can completely prevent development of fulminant hepatitis in the long-term (up to 20 mo). Reduced dietary copper intake is associated with no overt disease symptoms, although elevated levels of liver copper were observed. This state of disease rapidly shifts to severe disease, liver failure, and death if dietary copper intake is increased. The molecular events following induction of ALF seem to be identical in WD regardless of the time point of disease onset.

Applications

The observation of different degrees of disease progression with regard to liver copper levels underlines the importance of a stringent maintenance of drugs during anti-copper therapy of WD and provides a novel basis for efforts to achieve compliance. Counselling and monitoring of dietary aspects in WD could be reinforced for effective management of patients.

Terminology

Fulminant hepatitis is the appearance of severe, life-threatening complications that occur rapidly after the first signs of liver disease (such as jaundice). The damage to the liver is broad, leading to the death of most (80%-90%) of the liver cells.

Peer review

This article is about the correlation between dietary copper level and the onset of fulminant hepatitis in the rat model of WD, especially at different time points. The article presents an interesting topic of WD to simulate the liver injury and onset of fulminant hepatitis. Although the model has been previously described the current study provides additional new value to the field.

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MUC5AC/ β -catenin expression and *KRAS* gene alteration in laterally spreading colorectal tumors

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Abstract

AIM: To clarify differences in mucin phenotype, proliferative activity and oncogenetic alteration among subtypes of colorectal laterally spreading tumor (LST).

METHODS: LSTs, defined as superficial elevated lesions greater than 10 mm in diameter with a low vertical axis, were macroscopically classified into two subtypes: (1) a granular type (Gr-LST) composed of superficially spreading aggregates of nodules forming a flat-based lesion with a granulonodular and uneven surface; and (2) a non-granular type (NGr-LST) with a flat smooth surface and an absence of granulonodular formation. A total of 69 LSTs, comprising 36 Gr-LSTs and 33 NGr-LSTs, were immunohistochemically stained with MUC2, MUC5AC,

MUC6, CD10 (markers of gastrointestinal cell lineage), p53, β -catenin and Ki-67 antibodies, and examined for alteration in exon 1 of v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) and exon 15 of v-raf murine sarcoma viral oncogene homologue B1 (*BRAF*) by polymerase chain reaction followed by direct sequencing.

RESULTS: Histologically, 15 Gr-LST samples were adenomas with low-grade dysplasia (LGD), 12 were high-grade dysplasia (HGD) and 9 were adenocarcinomas invading the submucosa (INV), while 12 NGr-LSTs demonstrated LGD, 14 HGD and 7 INV. In the proximal colon, MUC5AC expression was significantly higher in the Gr-type than the NGr-type. MUC6 was expressed only in NGr-LST. MUC2 or CD10 did not differ. P53 expression demonstrated a significant stepwise increment in progression through LGD-HGD-INV with both types of LST. Nuclear β -catenin expression was significantly higher in the NGr-type. Ki-67 expression was significantly higher in the Gr-type in the lower one third zone of the tumor. In proximal, but not distal colon tumors, the incidence of *KRAS* provided mutation was significantly higher in the Gr-type harboring a specific mutational pattern (G12V). *BRAF* mutations (V600E) were detected only in two Gr-LSTs.

CONCLUSION: The two subtypes of LST, especially in the proximal colon, have differing phenotypes of gastrointestinal cell lineage, proliferation and activation of Wnt/ β -catenin or RAS/RAF/extracellular signal-regulated kinase signaling.

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Key words: Laterally spreading tumor; Mucin core protein; Colon; β -catenin; Immunohistochemistry; v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; Direct sequencing; Adenoma-carcinoma sequence

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INTRODUCTION

Colorectal cancer (CRC) is considered to arise from an adenoma precursor, and a model for genetic alterations in the adenoma-carcinoma sequence has been proposed^[1,2]. In this model, an adenomatous polyposis coli (*APC*) gene mutation occurs at the earliest stage, followed by a v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutation, as well as a change in p53. Along this sequence, the Wnt/*APC*/ β -catenin and RAS/RAF/extracellular signal-regulated kinase (ERK) signaling pathways also play important roles^[3-7]. Previous studies have primarily considered protruded adenomatous polyps as the most likely precursor of CRC^[1,2]. However, recent advances have led to changes in the diagnosis of early colorectal tumors, which can now be morphologically divided into three groups: classical protruded tumors, depressed tumors, and laterally spreading tumors (LSTs)^[8,9]. The latter two are possible candidates for alternative pathways of colorectal tumorigenesis. LSTs are considered to be less invasive, as neoplastic cells tend to spread along the surface of the lumen, and are usually categorized into two subtypes: granular type (Gr-LST) and flat- or non-granular type (NGr-LST); NGr-LSTs were more often associated with submucosal invasion compared to Gr-LSTs^[9,10]. An earlier report demonstrated unique cell kinetics in LSTs^[11]. Subsequent studies also evaluated alteration of *APC*^[12] or β -catenin^[12-15] for Wnt/*APC*/ β -catenin, mutation of *KRAS*^[11,12,14,16-19] or v-raf murine sarcoma viral oncogene homologue B1 (*BRAF*)^[12,14,19] for RAS/RAF/ERK and mutation of phosphoinositide-3-kinase (PI3K) catalytic- α polypeptide^[12,19] for the PI3K/AKT signaling pathway in LSTs. In addition, promoter methylation of CpG islands including its methylator phenotype (CIMP) in association with other genetic alterations has been shown to make important contributions to LST development^[14,18].

In an earlier study, *de novo* appearance of gastric mucin genes MUC5AC and MUC6, which are present in surface foveolar cells and mucous neck cells of the oxyntic mucosa and antral-type glands in the stomach^[20], was shown in colonic adenomas^[21]. Altered expression of MUC2 as an intestinal apomucin known to be expressed in goblet cells^[22], and CD10 expressed on the brush borders of intestinal epithelial cells^[23], occurred during progression of adenoma to early adenocarcinoma state^[24-26]. While considerable effort has been made to identify gene mutations and alteration of gastrointestinal phenotypes in conventional colorectal tumors, less attention has been paid to LSTs. Therefore, in the present study, we focused on differences in expression of MUC2, MU-

C5AC, MUC6 and CD10, p53 alteration, nuclear translocation of β -catenin, cell proliferation, and *KRAS*/*BRAF* mutations in morphologically different LST subtypes.

MATERIALS AND METHODS

Patients and materials

LSTs are defined as superficial elevated lesions greater than 10 mm in diameter with a low vertical axis^[8-10] and are macroscopically classified into two subtypes: (1) Gr-LSTs composed of superficially spreading aggregates of nodules forming flat based lesions with a granulonodular and uneven surface (Figure 1A and C); and (2) NGr-LSTs with flat smooth surfaces and an absence of granulonodular formation (Figure 1B and D).

The material for our study was a series of 69 LSTs (from 69 patients) resected endoscopically or surgically at Jundendo University Hospital (Tokyo, Japan) between May 2008 and July 2011. Patients with familial polyposis coli, hereditary non-polyposis colorectal carcinoma, multiple colorectal carcinomas, or inflammatory bowel disease were not included in the analysis. Proximal LSTs were classified as tumors proximal to the splenic flexure and the remaining tumors were defined as distal. Resected specimens were fixed in 15% formalin and processed for embedding in paraffin wax according to routine procedures and then sections were cut and stained with hematoxylin and eosin (H and E). This study was approved by the Institutional Review Board and the ethical committee of our hospital.

Pathological review

All slides stained with H and E were reviewed by two experienced gastrointestinal pathologists (Mitomi H, Yao T) independently. According to the World Health Organization classification^[27], adenoma was classified as tubular, tubulovillous or villous type with low grade dysplasia (LGD) or high grade dysplasia (HGD). Intramucosal carcinoma and carcinoma *in situ* were included in adenoma with HGD. Adenocarcinoma invading submucosa (INV) was classified as well, moderately or poorly differentiated. The subjects were classified according to the most advanced lesion identified. Interobserver variation was resolved by reevaluation and discussion to reach consensus.

Immunohistochemistry

Briefly, 4 μ m thick tissue serial sections were dewaxed in xylene and rehydrated, then endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxidase. For heat-induced antigen retrieval, sections were transferred to citrate buffer (10 mmol/L, pH 6.0) and heated at 100 °C in a water bath. The primary antibodies employed were MUC2 (Ccp58, 1:100 dilution; Leica Biosystems/Novocastra Laboratories, Newcastle Upon Tyne, UK), MUC5AC (CLH2, 1:100 dilution; Novocastra Laboratories), MUC6 (CLH5, 1:100 dilution; Novocastra Laboratories), CD10 (56C6, 1:100 dilution; Novocastra Laboratories), p53 (PAb 1801, 1:100; Leica Biosystems/Novocastra Laboratories), β -catenin (14/Beta-Catenin,

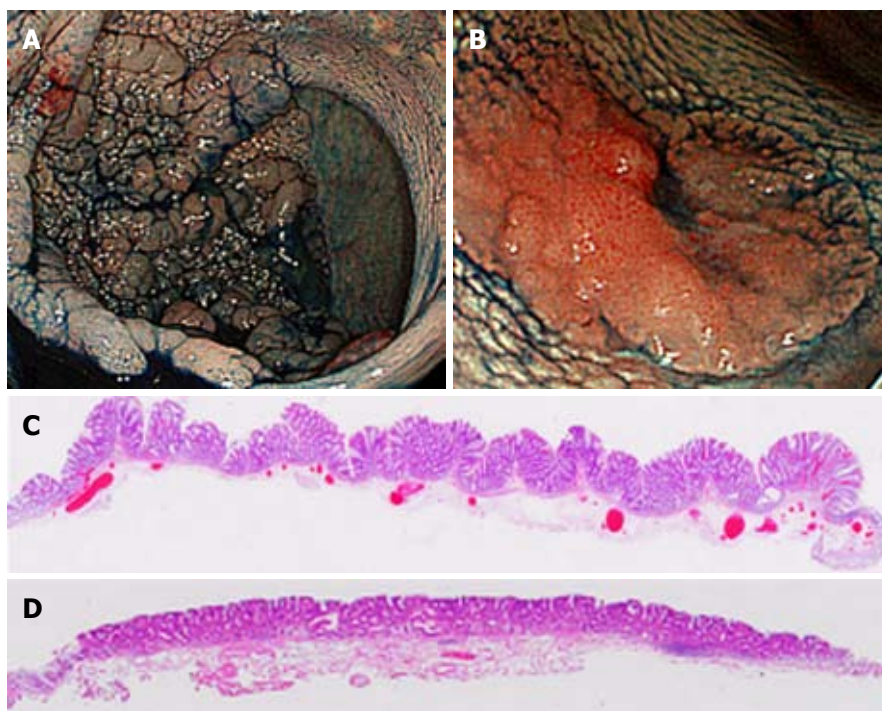


Figure 1 Macroscopic appearance (chromoscopic image) of laterally spreading tumors. (A) Granular type (Gr-LST) and (B) flat- or non-granular type (NGr-LST). Whole mount view of cut sections of LSTs stained with hematoxylin and eosin: (C) A Gr-LST showing tubulovillous structures with nodular surfaces and (D) an NGr-LST consisting of small tubular glands with flat surfaces. LSTs: Laterally spreading tumors.

dilution 1:100, BD Biosciences, San Diego, CA), and Ki-67 (MIB-1, 1:100; DAKO, Glostrup, Denmark), with incubation for 120 min at room temperature. Immunostaining was performed using the streptavidin-biotin-peroxidase complex method using a Histofine SAB-PO Kit (Nichirei Corp., Tokyo, Japan). The chromogen was 3,3'-diaminobenzidine and the sections were counterstained lightly with Mayer's hematoxylin to facilitate recognition of structures.

Assessment of immunostaining

Distinct cytoplasmic staining for MUC2, MUC5AC, MUC6 and apical staining for CD10 were considered positive, as was nuclear staining for p53 and Ki-67, regardless of the staining intensity. Expression of β -catenin, which generally showed an inverse relationship between membranous and nuclear (with cytoplasmic) reactivity, was evaluated only with respect to nuclear localization in this study. Immunostaining for all markers was evaluated by two of the authors (Mitomi H, Nakae K) independently, without prior knowledge of clinicopathological data. Discrepancies were resolved by re-evaluation to reach consensus.

Immunoreactive scores (IRSs) for MUC2, MUC5AC, MUC6 and CD10 were classified into five grades: 0 points, positive cells < 5% of tumor area; 1 point, 5%-24%; 2 points, 25%-49%; 3 points, 50%-74%; 4 points, \geq 75%. IRSs for p53 and nuclear β -catenin were also classified into five grades: 0 points, positive nuclei < 5% of tumor cells; 1 point, 5%-24%; 2 points, 25%-49%; 3 points, 50%-74%; 4 points, \geq 75%. For topological evaluation of the Ki-67 labeling index (LI; %), tumor glands in the lamina propria were separated into three equal zones (upper, middle and lower thirds), and the number of immunoreactive nuclei per approximately 300 tumor cells

were counted in each zone (for a total of approximately 1000 cells in whole glands).

Mutation analysis of KRAS and BRAF

Mutation analysis for KRAS and BRAF were performed using genomic DNA derived from formalin-fixed paraffin-embedded tissue of 29 Gr-LSTs (10 LGD, 10 HGD and 9 INV) and 27 NGr-LSTs (10 LGD, 10 HGD and 7 INV). Mutations were examined in exon 1 of KRAS and exon 15 of BRAF by polymerase chain reaction followed by direct sequencing. The primer sequences in this study were as previously described^[28].

Statistical analysis

All statistical analysis were carried out using StatView for Windows Version 5.0 (SAS Institute Inc., Cary, NC, United States). IRSs and LI are presented as mean \pm SD. Continuous data were compared with the Mann-Whitney *U* test. Categorical analysis of variables was performed using either the χ^2 test (with Yates' correction) or the Fisher's exact test, as appropriate. Correlations among expression levels of the encoded proteins were assessed with the Spearman's rank correlation coefficient. A *P* value < 0.05 was considered statistically significant, with classification into two grades: *P* < 0.05 and *P* < 0.01.

RESULTS

Clinicopathological characteristics of LST

The clinicopathological characteristics of the Gr-LSTs (15 LGD, 12 HGD, 9 INV) and NGr-LSTs (12 LGD, 14 HGD, 7 INV) are shown in Table 1. Gr-LSTs were located equally in proximal and distal colons, whereas NGr-LSTs were more frequently found in the distal colon, but without reaching statistical significance. Gr-LSTs were

Table 1 Clinicopathological characteristics of laterally spreading tumor of the colorectum

Variable	Gr-type (n = 36)	NGr-type (n = 33)
Age (yr)	68.5 \pm 10.9 (34-88)	69.0 \pm 10.3 (44-85)
Sex		
Male	21	23
Female	15	10
Location		
Proximal colon	18	12
Distal colon	18	21
Size of tumor (mm)	35.3 \pm 17.9 (12-80) ^b	23.0 \pm 9.1 (10-50) ^b
Histology		
LGD	15	12
Tubular type	11	11
Tubulovillous type	4	1
HGD	12	14
Tubular type ^d	0	13
Tubulovillous type ^d	12	1
INV	9	7
Well differentiated type	7	7
Others	2	0

Age and size of tumor are represented as mean \pm SD (range); Gr-type: Granular type of laterally spreading tumor; NGr-type: Flat- or non-granular type of laterally spreading tumor; LGD: Adenoma with low grade dysplasia; HGD: Adenoma with high grade dysplasia; INV: Adenocarcinoma invading submucosa; INV-others: Mixed well and moderately differentiated type (n = 1) and mixed well and poorly differentiated adenocarcinoma (n = 1); ^{b,d}P < 0.01, Gr-type *vs* NGr-type, tubular type *vs* tubulovillous type.

significantly larger than NGr-LSTs. Histologically, 12 out of 13 (92%) tubulovillous adenomas with HGD were Gr-LSTs, whereas all of the 13 tubular adenomas with HGD were NGr-LSTs. All NGr-INV and 7 out of 9 (78%) Gr-INV were well differentiated adenocarcinomas. The two other Gr-INV were an adenocarcinoma coexisting with well and moderately differentiated histology, and a mixed well and poorly differentiated type adenocarcinoma.

IRSs of MUC2, MUC5AC, MUC6, CD10, p53 and nuclear β -catenin in LSTs

For the MUC2 IRS (Figure 2A-C), significant stepwise decrement was evident in progression through LGD - HGD-INV in Gr- and NGr-LSTs. For the MUC5AC IRS (Figure 2D-F), the Gr-type value was significantly higher than that of the NGr-type, in the proximal colon. MUC6 was expressed only in NGr-LSTs (Figure 2G-I). A low extent of CD10 expression was detected in HGD and INV, but not LGD of both Gr- and NGr-LSTs (Figure 2J-L).

For the p53 IRS (Figure 3A), a stepwise increment in progression was noted through LGD to INV in Gr- and NGr-LSTs, which was statistically significant. For the nuclear β -catenin IRS (Figure 3B), the NGr-type value was significantly higher than the Gr-type. A similar trend for p53 and nuclear β -catenin IRSs was observed in proximal and distal LSTs (data not shown). For the Ki-67 LI (%) in the lower zone (Figure 3C), the Gr-type value was significantly higher than that of the NGr-type. A similar trend without statistical significance was found in the upper and middle zones (data not shown).

Representative H and E features and expression of

MUC2, MUC5AC, MUC6, CD10, p53 and β -catenin are illustrated in Figure 4.

Correlations among IRSs for the encoded proteins

Data for correlations among IRS are given in Table 2. Significant inverse relationships were found between p53 and MUC2 or MUC5AC. In addition, inverse associations were shown between nuclear β -catenin and MUC2 in NGr-LSTs, or MUC5AC in Gr-LSTs. Reciprocally, inverse associations were noted between Ki-67 and MUC2 in Gr-LSTs, or MUC5AC in NGr-LSTs.

KRAS and BRAF mutations

The results for KRAS and BRAF mutations analyzed are shown in Table 3. In proximal colon lesions, the incidence of KRAS mutations was significantly higher in Gr-LSTs (69%) than in NGr-LSTs (6%; *P* < 0.001). BRAF mutations were only detected in distal Gr-LSTs. In Gr-LSTs, 7 out of 29 (24%) tumors showed mutations from wild-type GGC (glycine) to GAC (aspartic acid) in codon 13 of the *KRAS* gene, and 5 (17%) tumors harbored mutations from GGT (glycine) to GTT (valine) in codon 12. In NGr-LSTs, 2 out of 27 (7%) tumors had mutations from GGC (glycine) to GAC (aspartic acid) in codon 13 of the *KRAS* gene. BRAF mutations were GTG (valine) to GAG (glutamic acid) in codon 600. A schematic representation of KRAS and BRAF mutational patterns is shown in Figure 5.

Association of KRAS or BRAF mutations with clinicopathological parameters and IRSs of the encoded proteins

Since there were only two cases of NGr-LST harbored mutations of KRAS, and neither had BRAF mutations, we analyzed only Gr-LSTs with respect to the relationship of KRAS or BRAF mutations with clinicopathological parameters (age, sex, location, size of tumor, and dysplastic grade) and IRSs of the encoded proteins. No significant link was observed with clinicopathological parameters. The MUC5AC IRS was significantly higher in mutated Gr-LSTs compared to non-mutated tumors (1.2 \pm 1.3 *vs* 0.2 \pm 0.6, *P* < 0.05). No significant differences were found in other IRSs (data not shown).

DISCUSSION

Only a few reports are available on the phenotypic expression of gastrointestinal cell lineage with immunohistochemistry of MUC2, MUC5AC, MUC6 and CD10 in relation to colorectal adenoma-carcinoma sequence^[24-26]. In proximal colon lesions, MUC5AC expression was significantly higher in Gr-LSTs than NGr-LSTs. Expression of MUC5AC or HGM was increased in adenoma with villous histology or polypoid growth^[21,24,25]. Enhanced MUC6 immunoreactivity was reported to be exhibited in large adenomas^[21]. In the current study, MUC6 was expressed only in NGr-LSTs, suggesting it as a marker of a morphologically distinct tumor. The molecular mechanisms responsible for the aberrant expression of gastric mucin,

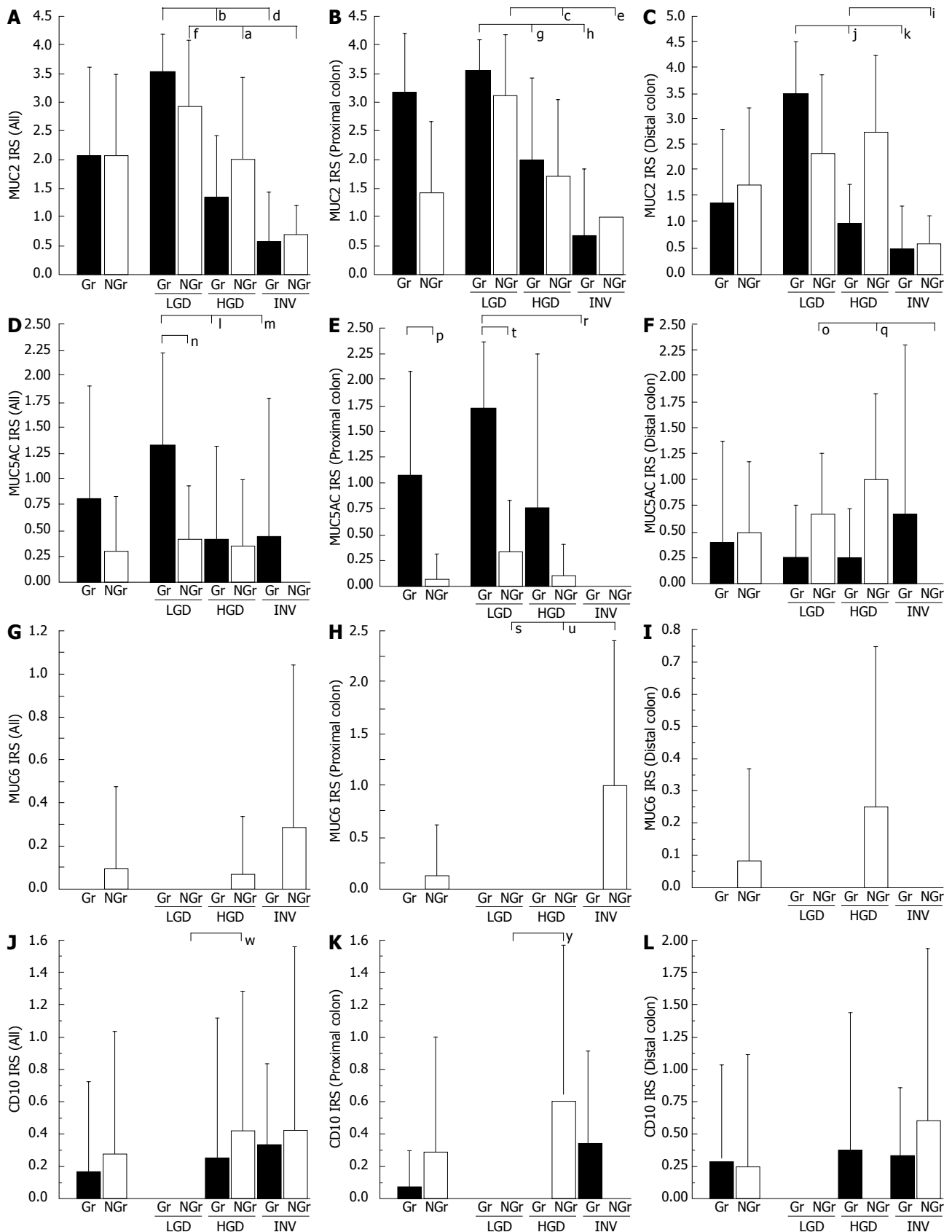


Figure 2 Immunoreactive scores for MUC2, MUC5AC, MUC6 and CD10 in all (A, D, G, J), proximal (B, E, H, K) and distal (C, F, I, L) laterally spreading tumors. Gr, Gr-LST (black bar); NGr, NGr-LST (white bar). IRS: Immunoreactive score; LGD: Adenoma with low grade dysplasia; HGD: Adenoma with high grade dysplasia; INV: Adenocarcinoma invading into submucosa; LSTs: laterally spreading tumors; Gr: Granular; NGr: Non-granular. Data are mean \pm SD; ^{a, c, e, g, i, k, m, o, q, s, u, w, y} $P < 0.05$, MUC2: all NGr-HGD vs NGr-INV; proximal colon NGr-LGD vs NGr-HGD or NGr-INV; Gr-LGD vs Gr-HGD; distal colon Gr-LGD vs Gr-INV; NGr-HGD vs NGr-INV; MUC5AC: all Gr-LGD vs Gr-INV; distal colon NGr-INV vs NGr-HGD or NGr-LGD; MUC6: proximal colon NGr-INV vs NGr-LGD or NGr-HGD; CD10: all NGr-LGD vs NGr-HGD; proximal colon NGr-LGD vs NGr-HGD; ^{b, d, f, h, j, l, n, p, r, t} $P < 0.01$, MUC2: all Gr-LGD vs Gr-HGD or Gr-INV; NGr-LGD vs NGr-INV; proximal colon Gr-LGD vs Gr-INV; distal colon Gr-LGD vs Gr-HGD; MUC5AC: all Gr-LGD vs NGr-LGD; Gr-LGD vs Gr-HGD; proximal colon Gr-type vs NGr-type; Gr-LGD vs NGr-LGD; Gr-LGD vs Gr-INV.

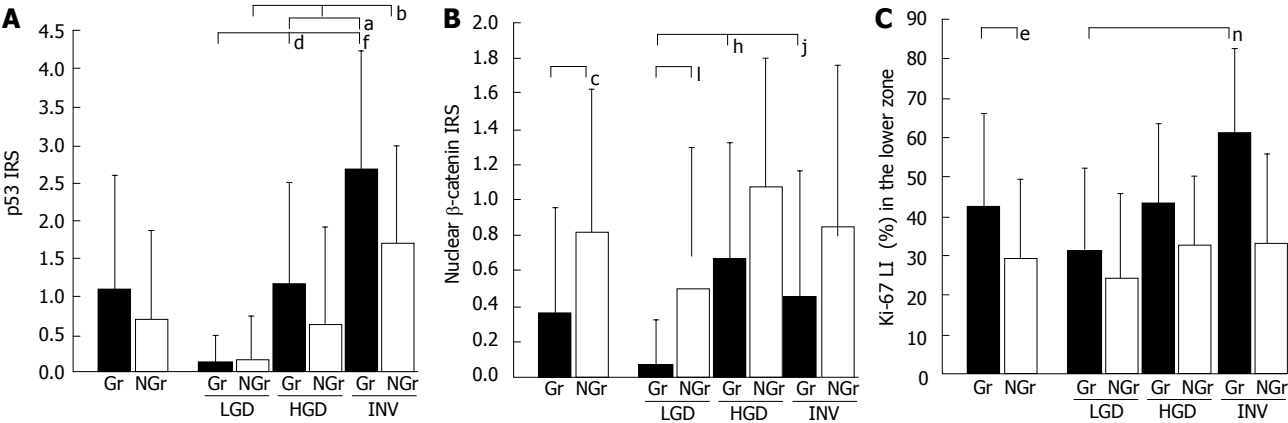


Figure 3 Immunoreactive scores for p53 (A) and nuclear β -catenin (B), and labeling indices for Ki-67 in the lower zone (C) in all laterally spreading tumors. Gr, Gr-LST (black bar); NGr, NGr-LST (white bar); LSTs: laterally spreading tumors. Data are mean \pm SD; ^{a, c, e} $P < 0.05$, p53: Gr-HGD vs Gr-INV; nuclear β -catenin: Gr-type vs NGr-type; Ki-67, Gr-type vs NGr-type; ^{b, d, f, h, j, l, n} $P < 0.01$, p53: NGr-LGD vs NGr-INV; Gr-LGD vs Gr-HGD or Gr-INV; nuclear β -catenin: Gr-LGD vs Gr-HGD or Gr-INV; Gr-LGD vs NGr-LGD; Ki-67, Gr-LGD vs NGr-INV. IRSs: Immunoreactive scores; LGD: Adenoma with low grade dysplasia; HGD: Adenoma with high grade dysplasia; INV: Adenocarcinoma invading into submucosa; Gr: Granular; NGr: Non-granular; LI: Labeling indice.

Table 2 Correlations among expression levels of the encoded proteins											
	MUC5AC		MUC6		CD10		p53		Nuclear β -catenin		Ki-67
	Gr-type	NGr-type	Gr-type	NGr-type	Gr-type	NGr-type	Gr-type	NGr-type	Gr-type	NGr-type	Gr-type
MUC2	0.550 ^b	0.486 ^b	NS	NS	-0.417 ^a	NS	-0.549 ^a	-0.450 ^a	NS	0.413 ^a	-0.397 ^a
MUC5AC		Blank	NS	NS	NS	NS	-0.430 ^a	-0.368 ^a	-0.334 ^a	NS	NS
MUC6		Blank		Blank	NS	NS	NS	NS	NS	NS	NS
CD10		Blank		Blank		Blank	NS	NS	NS	NS	NS
p53		Blank		Blank		Blank		Blank	NS	NS	0.514 ^b
Nuclear β -catenin		Blank		Blank		Blank		Blank		Blank	NS

Gr-type: Granular type of laterally spreading tumor; NGr-type: Flat- or non-granular type of laterally spreading tumor; Data indicates rho value examined by Spearman's correlation coefficient; NS: Not significant. ^a $P < 0.05$; ^b $P < 0.01$.

Table 3 Frequency of KRAS and BRAF mutations in laterally spreading tumor of the colorectum					
Type of tumors	Number (%) of mutated samples				
	<i>n</i>	All	Proximal colon		
		KRAS	BRAF	<i>n</i>	KRAS
Gr-type	29	15 (52) ^a	2 (7)	13	9 (69) ^c
LGD	10	6 (60)	0 (0)	7	6 (86) ^{d,e}
HGD	10	6 (60) ^b	1 (10)	3	2 (67)
INV	9	3 (33)	1 (11)	3	1 (33)
NGr-type	27	2 (7) ^a	0 (0)	18	1 (6) ^c
LGD	10	1 (10)	0 (0)	8	1 (13) ^d
HGD	10	0 (0) ^b	0 (0)	8	0 (0)
INV	7	1 (14)	0 (0)	2	0 (0)
Total	56	17 (30)	2 (4)	30	10 (33)

Gr-type: Granular type of laterally spreading tumor; NGr-type: Flat- or non-granular type of laterally spreading tumor; LGD: Adenoma with low grade dysplasia; HGD: Adenoma with high grade dysplasia; INV: Adenocarcinoma invading submucosa; KRAS: v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; BRAF: v-raf murine sarcoma viral oncogene homologue B1. ^{a, c, e} $P < 0.05$, All and proximal colon: Gr-type vs NGr-type; Gr-LGD: proximal vs distal colon; ^{b, d} $P < 0.01$, all and proximal colon: Gr-HGD vs NGr-HGD; proximal colon: Gr-type vs NGr-type.

MUC5AC and MUC6 in colorectal tumors are still unclear and may be due to changes in transcriptional regulation. It was previously reported that none of the four NGr-

LSTs harbored p53 mutations, whereas 7 out of 24 (29%) Gr-LSTs were positive^[16]. We have shown that no significant differences in p53 expression, along with an inverse relation of p53 to MUC2 or MUC5AC expression in two types of LSTs. An experimental study identified MUC2 expression as increased along with induction of wild-type p53 in carcinoma cell lines *in vitro*, and potential p53-binding sites in the MUC2 promoter, which contributes to stimulation of promoter activity^[29]. P53 overexpression as an indirect sign of loss of functional p53 by its mutation is possibly related to the down-regulation of MUC2 expression in colorectal LST. A detailed study on the relation of p53 to the altered mucin expression appears warranted.

We have documented high nuclear β -catenin expression in NGr-LSTs in line with a previous study^[15]. In an analysis of LSTs, no mutation of β -catenin exon 3 was found, while LOH at 5q (APC locus) was more frequently detected in NGr-LSTs than in Gr-LSTs^[12]. To date, there is only one case report of NGr-LST harboring interstitial deletion of β -catenin exon 3^[13]. Nuclear accumulation of β -catenin has been observed in tumors with mutations in the β -catenin or APC gene^[3]. Taken together, the available data suggest that activation of Wnt/APC/ β -catenin signaling in NGr-LSTs is due primarily to alterations in the APC gene. Furthermore, in-

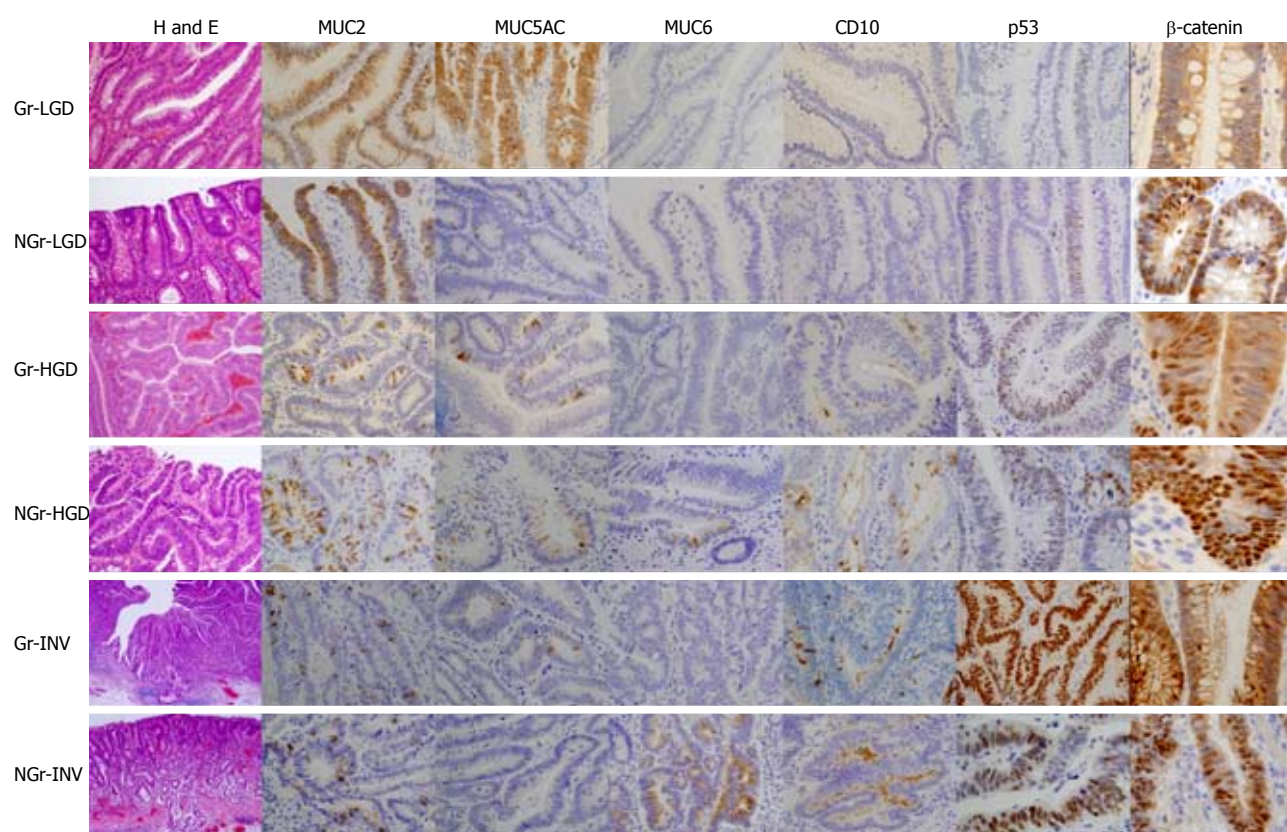


Figure 4 Histology and immunohistochemistry of laterally spreading tumors. Immunoreactive scores (IRSs) for Gr-LGD: MUC2, 4 points; MUC5AC, 2 points; MUC6, 0 points; CD10, 0 points; p53, 0 points; nuclear β -catenin, 0 points. IRSs for NGr-LGD: MUC2, 3 points; MUC5AC, 1 points; MUC6, 0 points; CD10, 0 points; p53, 0 points; nuclear β -catenin, 1 points. IRSs for Gr-HGD: MUC2, 1 points; MUC5AC, 1 points; MUC6, 0 points; CD10, 1 points; p53, 1 points; nuclear β -catenin, 1 points. IRSs for NGr-HGD: MUC2, 2 points; MUC5AC, 1 points; MUC6, 1 points; CD10, 1 points; p53, 1 points; nuclear β -catenin, 2 points. IRSs for Gr-INV: MUC2, 1 points; MUC5AC, 1 points; MUC6, 0 points; CD10, 1 points; p53, 4 points; nuclear β -catenin, 1 points. IRSs for NGr-INV: MUC2, 1 points; MUC5AC, 0 points; MUC6, 2 points; CD10, 2 points; p53, 2 points; nuclear β -catenin, 2 points (H and E, $\times 80$; immunoperoxidase: MUC2, MUC5AC and MUC6, $\times 100$; CD10 and p53, $\times 120$; β -catenin, $\times 300$); LGD: Adenoma with low grade dysplasia; HGD: Adenoma with high grade dysplasia; INV: Adenocarcinoma invading into submucosa; Gr: Granular; NGr: Non-granular.

verse associations were shown between nuclear β -catenin and MUC2 or MUC5AC in LSTs. Experimental studies have demonstrated that abrogation of MUC2 in tumors of the rat colon is related to nuclear β -catenin localization and its mutation^[30,31]. Interaction of mucin core protein with Wnt/APC/ β -catenin signaling may have some role in the progression of LSTs.

Ki-67, considered to be a reliable indicator for accurately assessing growth fraction, is increased with a shift in the proliferative zone toward the upper compartment in LSTs^[11]. In the current study of Ki-67, a similar upward shift was detected in Gr- and NGr-LSTs. Furthermore, higher proliferation in the lower compartment was more apparent in Gr-LSTs, which may explain the morphologic variation in LSTs. We also found an inverse association between tumor cell proliferation (Ki-67) and MUC2 in LST. This is in line with the fact that decreased *in vivo* expression of MUC2 is related to colon carcinogenesis, accompanied by increased cell proliferation^[32].

We have shown that, in proximal colon, the incidence of KRAS mutation was significantly higher in Gr-LST (69%) than NGr-LST (6%), with a relatively frequent and specific pattern in Gr-type for G12V, as it was for G12C in another report^[16]. Previously reported

incidences of KRAS mutation were 21%-83% in Gr-LST and 17%-26% in NGr-LST^[12,16,17-19]. In the current study, BRAF mutations (V600E) were only detected in two Gr-LSTs. Gr-LSTs, particularly those located in the proximal colon, exhibited frequent KRAS mutations and high CIMP^[18]. BRAF mutations are often characteristic of CIMP-high/microsatellite instability-high colorectal cancer^[6], and are infrequent in LST^[12,14,19]. These facts suggest that proximal Gr-LST is a possible candidate for early lesions of CIMP-high/microsatellite stable cancer. Furthermore, MUC5AC expression was significantly higher in KRAS mutated Gr-LSTs than in non-mutated tumors. Aberrant MUC5AC expression is thought to be related to KRAS mutations in experimental colon carcinogenesis^[31]. *In vitro*, upregulation of MUC5AC may occur through concomitant activation of the EGFR/RAS/RAF/ERK signaling pathway and Sp1 binding to the gene promoter^[33]. We therefore hypothesize that ERK signal activation induced by mutated RAS in relation to aberrant gastric mucin expression may play a role in the development and progression of Gr-LSTs in the proximal colon.

In conclusion, as summarized schematically in Figure 6, the two subtypes of LST have differing mucin pheno-

	KRAS				BRAF V600E
	G12D	G12S	G12V	G13D	
Gr-LGD					
Gr-LGD					
Gr-LGD					
Gr-LGD					
Gr-LGD					
Gr-LGD					
Gr-LGD					
Gr-LGD					
Gr-LGD					
Gr-LGD					
NGr-LGD					
NGr-LGD					
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NGr-INV					
NGr-INV					

Figure 5 v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog and v-raf murine sarcoma viral oncogene homologue B1 mutational patterns in laterally spreading tumors. D: Aspartic acid; E: Glutamic acid; G: Glycine; S: Serine; V: Valine; Black box: Mutated case; White box: Non-mutated case; LGD: Adenoma with low grade dysplasia; HGD: Adenoma with high grade dysplasia; INV: Adenocarcinoma invading into submucosa; Gr: Granular; NGr: Non-granular; KRAS: v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; BRAF: v-raf murine sarcoma viral oncogene homologue B1.

typic expression, proliferative activity, and activation of Wnt/ β -catenin or RAS/RAF/ERK signaling in progression from adenomas through to invasive carcinomas.

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COMMENTS

Background

Laterally spreading tumors (LSTs) in the colorectum are usually categorized

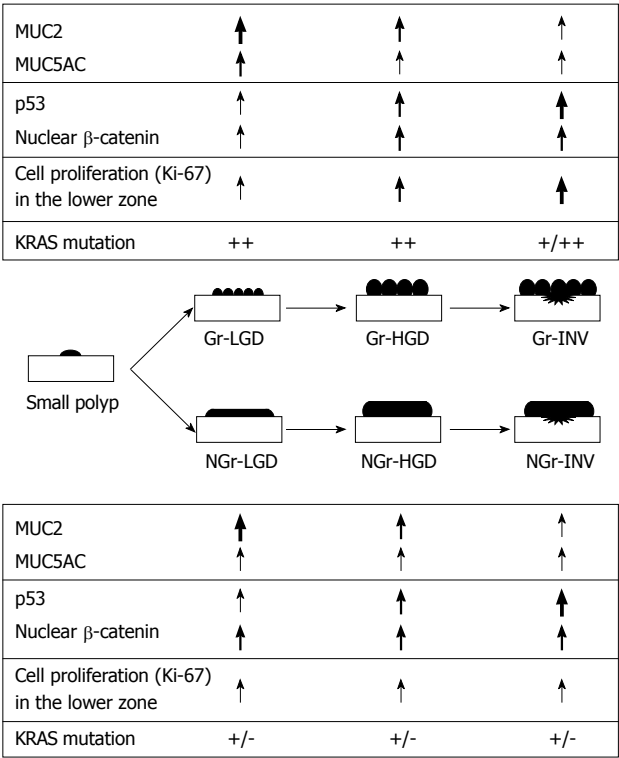


Figure 6 Alterations of expression of mucin core protein, p53 and β -catenin, cell proliferation and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog mutations in malignant transformation of laterally spreading tumors. Large arrow: Marked upregulation; Medium arrow: Moderate upregulation; Small arrow: Mild upregulation; ++: Frequently mutated; +: Infrequently mutated; -: Not mutated; LGD: Adenoma with low grade dysplasia; HGD: Adenoma with high grade dysplasia; INV: Adenocarcinoma invading into submucosa; KRAS: v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; Gr: Granular; NGr: Non-granular.

into two subtypes: granular (Gr-LST) and flat- or non-granular types (NGr-LST). While considerable effort has been made to identify gene mutations and alteration of gastrointestinal phenotypes in conventional colorectal tumors, less attention has been paid to LSTs.

Research frontiers

The authors focused on differences in expression of MUC2, MUC5AC, MUC6 and CD10, p53 alteration, nuclear translocation of β -catenin, cell proliferation, and v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS)/v-raf murine sarcoma viral oncogene homologue B1 mutations in morphologically different LST subtypes.

Innovations and breakthroughs

The authors showed that the two types of LSTs have different phenotypes, particularly with respect to MUC5AC (expression greater in Gr- vs NGr- types) and MUC6 (only expressed in NGr-type). They showed a higher nuclear β -catenin expression in NGr-type, and Ki-67 was much more prevalent in the Gr-type. Finally, the incidence of KRAS mutations was much more frequent in Gr-LST.

Applications

The subtypes of LSTs may be different candidates for alternative pathways of colorectal tumorigenesis. The results of the study represent a further impact on research in colorectal carcinogenesis.

Peer review

This is a good descriptive study in which the authors clarify differences in mucin phenotype, proliferative activity and oncogenetic alteration among subtypes of LST. The results are interesting and suggest that they are different candidates for alternative pathways of colorectal carcinogenesis.

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Software for automated classification of probe-based confocal laser endomicroscopy videos of colorectal polyps

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Abstract

AIM: To support probe-based confocal laser endomicroscopy (pCLE) diagnosis by designing software for the automated classification of colonic polyps.

METHODS: Intravenous fluorescein pCLE imaging of colorectal lesions was performed on patients undergoing screening and surveillance colonoscopies, followed by polypectomies. All resected specimens were reviewed by a reference gastrointestinal pathologist

blinded to pCLE information. Histopathology was used as the criterion standard for the differentiation between neoplastic and non-neoplastic lesions. The pCLE video sequences, recorded for each polyp, were analyzed off-line by 2 expert endoscopists who were blinded to the endoscopic characteristics and histopathology. These pCLE videos, along with their histopathology diagnosis, were used to train the automated classification software which is a content-based image retrieval technique followed by *k*-nearest neighbor classification. The performance of the off-line diagnosis of pCLE videos established by the 2 expert endoscopists was compared with that of automated pCLE software classification. All evaluations were performed using leave-one-patient-out cross-validation to avoid bias.

RESULTS: Colorectal lesions (135) were imaged in 71 patients. Based on histopathology, 93 of these 135 lesions were neoplastic and 42 were non-neoplastic. The study found no statistical significance for the difference between the performance of automated pCLE software classification (accuracy 89.6%, sensitivity 92.5%, specificity 83.3%, using leave-one-patient-out cross-validation) and the performance of the off-line diagnosis of pCLE videos established by the 2 expert endoscopists (accuracy 89.6%, sensitivity 91.4%, specificity 85.7%). There was very low power (< 6%) to detect the observed differences. The 95% confidence intervals for equivalence testing were: -0.073 to 0.073 for accuracy, -0.068 to 0.089 for sensitivity and -0.18 to 0.13 for specificity. The classification software proposed in this study is not a "black box" but an informative tool based on the query by example model that produces, as intermediate results, visually similar annotated videos that are directly interpretable by the endoscopist.

CONCLUSION: The proposed software for automated classification of pCLE videos of colonic polyps achieves high performance, comparable to that of off-line diagnosis of pCLE videos established by expert endoscopists.

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Key words: Colorectal neoplasia; Computer-aided diagnosis; Content-based image retrieval; Nearest neighbor classification software; Probe-based confocal laser endomicroscopy

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INTRODUCTION

Colorectal cancer is the second leading cause of cancer-related death in the United States^[1]. Its development includes several morphological stages, from benign to adenomatous polyps with low grade dysplasia to adenocarcinoma. Suspicious lesions are usually detected with standard colonoscopy by endoscopists who either perform confirmatory biopsy, or if high certainty exists, perform immediate therapy such as resection or ablation of diseased tissue. Because standard endoscopic imaging can only diagnose disease states with moderate levels of certainty^[2,3], histopathology remains the criterion standard for final diagnosis^[4]. However, the requirement for *ex vivo* histology implies a large proportion of unnecessary polypectomies and often requires a separate endoscopic procedure to be performed for treatment. It also increases the cost of colorectal cancer screening.

Probe-based confocal laser endomicroscopy (pCLE, Mauna Kea Technologies, France) enables the endoscopist to image the epithelial tissue *in vivo*, at the microscopic level with a confocal miniprobe, and in real-time during ongoing endoscopy. Preliminary findings by Meining *et al*^[5] demonstrated the applicability of pCLE in diagnosing colorectal neoplasia *in vivo* with high sensitivity and specificity (93% and 92%, respectively) in 13 patients with colorectal lesions. Venkatesh *et al*^[6] and De Palma^[7] pointed out that confocal endomicroscopy offers the ability to target biopsies much more precisely and thus to reduce the number of random biopsies. In a recent study including a large pool of 75 patients, Buchner *et al*^[8] compared off-line diagnosis of pCLE videos to virtual chromoendoscopy (Narrow-Band Imaging and Fujinon Intelligent Color Enhancement) and showed that off-line diagnosis of pCLE videos had higher sensitivity (91% *vs* 77%) with similar specificity (76%). As noted by Wallace *et al*^[9], endoscopists now have the challenging task of performing “optical biopsies” and diagnosing pCLE video sequences *in vivo*.

In order to provide an objective support for pCLE diagnosis, we aimed to design a computer-based system

for the automated classification of colonic polyps into neoplastic and non-neoplastic lesions. As physicians typically rely on similarity-based reasoning to establish a diagnosis from image queries, we propose a content-based image retrieval (CBIR) approach to automatically estimate the pathology of a new pCLE video. Indeed, contrary to “black box” classification systems, a CBIR-based classification system extracts, from a training database, annotated pCLE videos that are visually similar to the video of interest and directly interpretable by the endoscopist. The pathology of the video query is then estimated from the histopathological votes of these already diagnosed videos. Another advantage of CBIR-based classification is that the extracted similar videos can be presented to the endoscopist in a second reader paradigm to better support pCLE diagnosis.

The main goal of this study was to compare, using the same database of colonic polyps, the clinical performance of our automated pCLE classification software with that of off-line diagnosis of pCLE videos established by endoscopists expert in pCLE, with histopathology remaining the criterion standard reference.

MATERIALS AND METHODS

Patients

The patients included in the study were enrolled between November 2007 and March 2009 for previous studies approved by the Mayo Clinic Institutional Review Board, and from which we collected all available data to ensure as large a sample size as possible. These patients were enrolled into the study of Buchner *et al*^[8] and for further studies by the same Mayo Clinic group. Only the patients with complete diagnostic data were considered in our study. All study participants gave full written consent. Patients were enrolled if they were due for surveillance or screening colonoscopies, evaluation of known or suspected polyps on other imaging modalities, and endoscopic mucosal resection of larger flat colorectal neoplasia. Exclusion criteria were patients with non-corrected coagulopathy, women who were pregnant or breast feeding, those with documented allergy to fluorescein, and patients with no colorectal lesions found during a study colonoscopy. Twenty-four hours before the procedure, patients were prepped with 2-4 L polyethylene glycol solution. Conscious sedation was performed with intravenous administration of midazolam and meperidine.

Endoscopy equipment and procedure

All procedures were performed by the authors (either Wallace MB or Buchner AM) using a high-definition colonoscope (Fujinon EC450HL5 or 490 ZW, Fujinon, Ft Wayne, NJ, United States; Olympus CFH180, Olympus, Center Valley, NY, United States). The system was equipped with the EPX 4400 processor (Fujinon Inc.) or CV 180 Exera (Olympus, Co.). The primary screening method was white-light high-definition colonoscopy. Then, either Fujinon Intelligent Color Enhancement

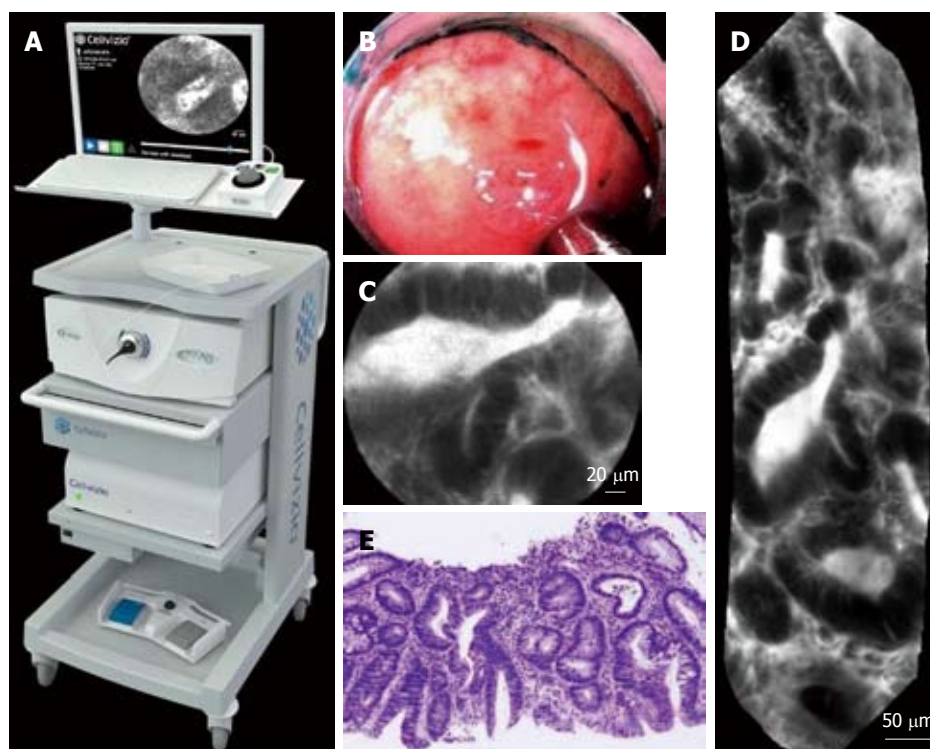


Figure 1 Imaging modalities of colonic polyps. A: Setup of probe-based confocal laser endomicroscopy (pCLE) imaging system (Cellvizio, Mauna Kea Technologies); B: Endoscopic image of tubular adenoma, and the pCLE mini-probe; C: An image of the pCLE video sequence; D: A pCLE mosaic image built with the video mosaicing tool; E: Histopathology image.

mode 4 with a Fujinon colonoscope or Narrow-Band Imaging with an Olympus 180 series scope was used to characterize lesions in all patients.

The surface pit pattern of the lesion was classified according to Kudo criteria. Anatomical site and morphological class of lesions were recorded in accordance with the Paris classification^[10]. Fluorescein sodium 2.5-5.0 mL 10% (AK Fluor, Akorn Pharmaceutical, Lake Forest, IL, United States) solution was administered intravenously after the first polyp was identified. Immediately after fluorescein injection, pCLE video sequences of the lesions were acquired and recorded. According to the visual examination of both endoscopic and pCLE images, real biopsies were targeted to the most suspicious parts of the polyp. Appropriate treatment procedures, ranging from simple polypectomies to complex endoscopic mucosal resection of lesions, were then performed.

Probe-based confocal laser endomicroscopy acquisition protocol

During a pCLE acquisition protocol, the endoscopist typically inserts, through the working channel of a standard endoscope, a confocal miniprobe (Coloflex UHD, Cellvizio GI) of external diameter 2.5 mm, which is made of 30 000 optical fibers bundled together. The pCLE imaging setup, shown in Figure 1, allows the acquisition of pCLE images of field-of-view 240 µm at a rate of 9 to 12 frames per second. In stable pCLE video sequences, the probe is in constant contact with the tissue. Representative endoscopic, pCLE, and histopathology images of tubular adenoma are shown in Figure 1.

Prior to pCLE evaluation of the study polyps, the 2 expert endoscopists (Wallace MB, Buchner AM) viewed extensive published material on pCLE and performed a

self-calibration on training pCLE videos of 20 polyps of known pathology (10 neoplastic and 10 non-neoplastic). These “training” polyps were evaluated by a gastrointestinal pathologist (Krishna M) and came from 9 patients not included in the study. Once acquired, the pCLE videos of the study lesions were evaluated off-line and in random order by the 2 expert endoscopists, who were blinded to histology diagnosis and endoscopic appearance of the lesion. The off-line diagnosis of pCLE videos was made based on the established modified Mainz criteria^[11] for diagnosis of colorectal neoplasia, and according to pit pattern and overall crypt and vessel architecture. Of the whole pCLE video imaging of a polyp, the sequence of the video containing the most malignant pCLE features was considered to represent the polyp.

Histopathology as criterion standard diagnosis

All resected specimens were reviewed by a reference gastrointestinal pathologist (Krishna M) blinded to the pCLE information. Only the size and anatomic location were provided, which is the routine clinical practice at the Mayo Clinic institution. Intraepithelial neoplasia were defined using modified Vienna criteria^[12,13]: benign polyps and hyperplastic polyps were classified as non-neoplastic lesions, while tubular adenoma, villous adenoma, tubulovillous adenoma and adenocarcinoma were classified as neoplastic lesions.

Standard bag-of-visual-words technique for content-based image retrieval

As endoscopists use perceptual similarities between pCLE videos of known diagnosis to establish a diagnosis on a new pCLE video, we propose a content-based retrieval approach to design the automated pCLE

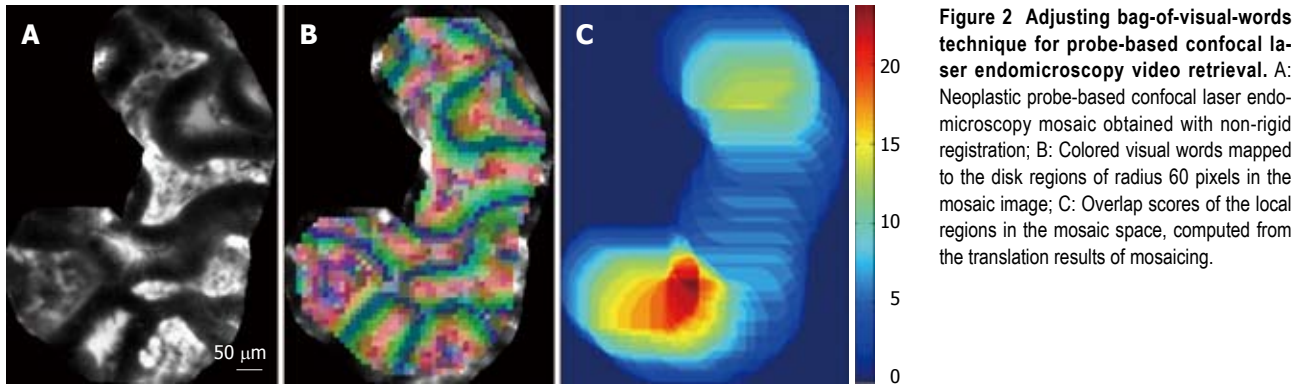


Figure 2 Adjusting bag-of-visual-words technique for probe-based confocal laser endomicroscopy video retrieval. A: Neoplastic probe-based confocal laser endomicroscopy mosaic obtained with non-rigid registration; B: Colored visual words mapped to the disk regions of radius 60 pixels in the mosaic image; C: Overlap scores of the local regions in the mosaic space, computed from the translation results of mosaicing.

video classification software. We revisited the standard bag-of-visual-words (BoW) technique which has been successfully used in many content-based image retrieval applications in computer vision^[14]. A thorough technical presentation of our methodology has been disclosed previously^[15], but without detailed clinical evaluation.

The standard BoW technique for image retrieval can be divided into four steps: region detection on the image, description of the regions, discretization of the feature space and similarity measuring between images. The detection step extracts salient regions in the image using sparse detectors. During the description step, a descriptor computes for each salient region its description vector. Then, the discretization step uses the result of a clustering method that builds K clusters, i.e., K visual words, from the union of the description vector sets gathered across all the images of the training database. Each description vector counts for one visual word, so an image can be represented by a signature of size K which is the histogram of its visual words. By construction, image signatures are invariant by viewpoint changes (image translation, rotation and scaling) and affine illumination changes. Finally, the similarity measuring step defines the similarity distance between two images as an adequate distance between their signatures: the most similar training images to the image of interest are defined as being the closest ones in terms of this distance.

Adjusting bag-of-visual-words technique for probe-based confocal laser endomicroscopy video retrieval

First, we observed that discriminative information is densely distributed in pCLE images. Second, we noticed that several pCLE image patterns have the same shape but represent different objects characterized by their different size (e.g., mesoscopic crypts and microscopic goblet cells both have a rounded shape). Therefore, pCLE image description must not be invariant by scaling. To avoid scale invariance and to extract all the image information, we decided to apply, instead of standard sparse detectors, a dense detector that was made of overlapping disks having a fixed radius and localized on a dense regular grid. We maintained the invariance by in-plane translation and rotation, because the pCLE miniprobe translates and rotates along the tissue surface. Besides, as the diffusion rate of fluorescein administered before

imaging procedure decreases through time, invariance by affine illumination changes is also preserved.

Expert endoscopists pointed out that the field-of-view of single still images may not be large enough to make a robust diagnosis. Thus, we decided to retrieve not single images but complete videos, using the video mosaicing technique^[16,17] (available in the Cellvizio software) to include spatial overlap between time-related images. Examples of mosaics built with the video mosaicing tool are shown in Figure 1. To ensure on-line retrieval, we use the translation results of the real-time version of the video-mosaicing technique to weight the contribution of each local image region to its visual word, as illustrated in Figure 2. Then, we computed the video signatures with a histogram summation technique. The whole pipeline of our retrieval-based software classification framework can be run on-line during ongoing colonoscopy (Figure 3).

Classification of probe-based confocal laser endomicroscopy videos using similarity distance

Once the visual signature of the video query is computed, the k -Nearest Neighbor search step identifies the k closest training videos to the video query, by relying on the similarity distance between the video signatures. We then used the known histopathology diagnosis of these training videos to classify the query video, either as neoplastic or as non-neoplastic. Each of the k most similar training videos delivers a “histopathological” vote which is weighted by the inverse of its similarity distance to the video query.

Due to the relatively small size of our pCLE database, we needed to learn from as much data as possible. To avoid any bias while having a large training set, we employed cross-validation. As several videos were acquired for the same patient, we performed a leave-one-patient-out cross-validation^[18]: all videos from a given patient were excluded from the training set before being tested as queries of our retrieval and classification software. Cross-validation also allowed us to find the optimal number of nearest neighbors, $k = 9$, which is the one that maximizes the accuracy of the retrieval-based software classification results.

Statistical analysis

All the reported results of the automated software classi-

Table 1 Study population characteristics

Study population	Summary (n = 71)
Age (yr), median (min, 25th, 75th, max)	75 (46, 68, 79, 93)
Gender, %	
Male	49
Female	51
History of colon cancer, %	9
Family history of colon cancer, %	10

Table 2 Colorectal lesion characteristics

Colorectal lesions	Summary (n = 135)
Polyp size (mm), median (min, 25th, 75th, max)	8 (1, 5, 20, 60)
Polyp location, %	
Cecum	24
Rectum	20
Ascending	18
Sigmoid	14.5
Transverse	15
Descending	5.5
Splenic flex	3
Histopathology diagnosis, %	
Hyperplastic	31
Tubular adenoma	52
Tubulovillous adenoma	11.5
Hyperplastic and adenomatous features	2.5
Adenocarcinoma	3
Neoplastic lesion, simplified histopathology, %	69
Paris classification, %	
1p	1
1s	57
2a	32
2b	5
2c	1
2a/c	4

fication were obtained using leave-one-patient-out cross-validation. Statistical analysis was performed by André B.

To test for statistical difference between the two methods of interest, namely automated software classification and off-line classification by expert endoscopists, we used McNemar's tests^[19] and showed the corresponding power calculations with a type I error $\alpha = 0.05$. Two-sided P values < 0.05 were assumed to indicate statistical significance.

In order to assess statistical equivalence between the two methods, we used the two-sided Z-test between proportions^[19,20] and computed 95% CI. Because the 135 pCLE videos constituted a small sample size, we used a correction for continuity for McNemar's test.

The statistics on overall accuracy are dependent on the relative fraction of non-neoplastic and neoplastic lesions examined, which in this study were 31.1% and 68.9%, respectively. Even though observations were made for more than one polyp in some patients, for the purposes of statistical analysis, individual polyps (and their corresponding videos) were assumed to constitute independent observations. It is recognized that there was multiple testing of outcome data arising from individual polyps. Since the statistical tests were meant to highlight

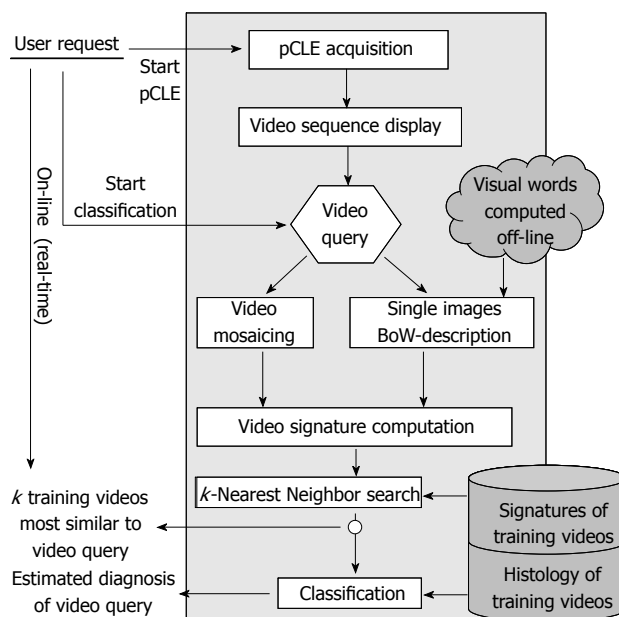


Figure 3 Pipeline of the probe-based confocal laser endomicroscopy retrieval-based software classification framework. From the acquisition of the probe-based confocal laser endomicroscopy (pCLE) video query by the Cellvizio system to the on-line automated diagnosis estimation.

differences, and since the correction by Bonferroni's method did not affect statistical significance in any of the comparisons, all P values are presented uncorrected for multiple testing.

RESULTS

Study population and colorectal lesion characteristics

Table 1 summarizes the demographic and general characteristics of the study population. None of the 71 patients experienced any endoscopic complications or adverse reactions to sodium fluorescein, with the exception of transient yellow discoloration of the skin and urine, which resolved by the time of discharge from the recovery room (skin) or within 24 h (urine). Histopathology and morphological classification of the 135 analyzed colorectal lesions are shown in Table 2.

Qualitative results of visual similarities between probe-based confocal laser endomicroscopy videos

The pCLE database contained 135 pCLE videos representing each of the 135 polyps. The pCLE appearance of neoplastic lesions, compared to that of non-neoplastic lesions, included dilated irregular vessels, fluorescein leakage, cellular features of epithelial mucin depletion, and histological features of villiform crypts with increased optical density along the epithelial border.

As the automated pCLE classification software is a similarity-based system that classified pCLE videos based on the votes of visually similar videos, its clinical relevance can be qualitatively evaluated by examining the intermediate results of video retrieval. Examples of mosaics built with the video mosaicing tool are shown in

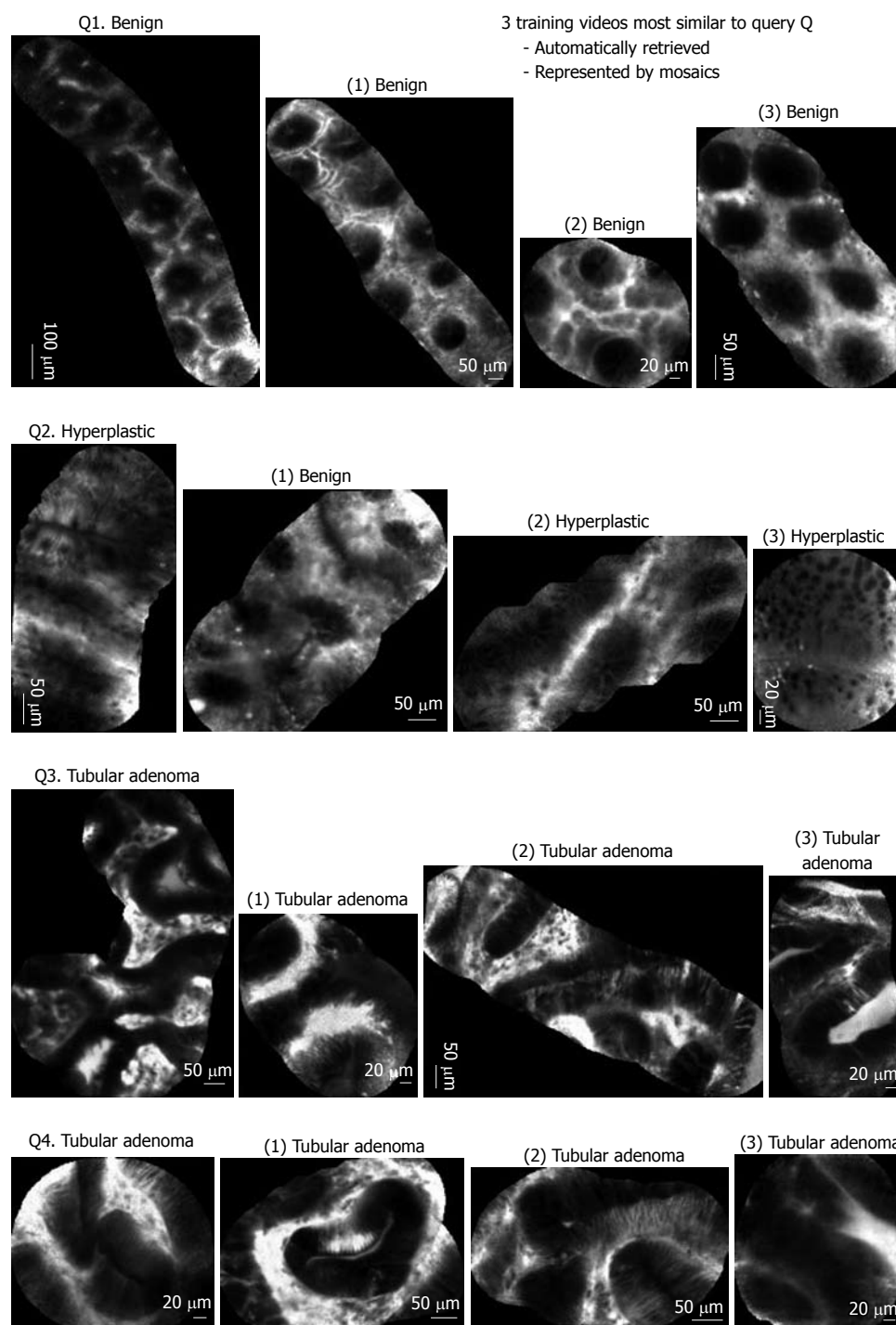


Figure 4 Typical results of automated probe-based confocal laser endoscopy video retrieval.

The probe-based confocal laser endoscopy (pCLE) videos are represented by mosaic images; they are annotated with their histopathology diagnosis. Video queries are highlighted in gray and followed by their 3 most similar videos. Automated software classification (hyperplastic vs neoplastic) of query videos is based on the votes of the similar videos. With respect to histopathology, both the automated software classification and the pCLE diagnosis established by expert endoscopists are correct for these queries.

Figures 4 and 5. Figure 4 shows 5 typical results of the automated pCLE retrieval software. We observed that, despite the high variability in appearance of a given histopathological class (neoplastic or non-neoplastic), the automatically retrieved videos called “neighbors” looked quite similar to the video queries (Q1, Q2, Q3 and Q4, respectively). In addition, we noticed that the closer the neighbor was to the query, the more similar it was to it.

In terms of classification, the pathological class was estimated by the weighted votes of the 3 retrieved neighbors. In Figure 4, video queries Q1, Q2, Q3 and Q4 have been correctly classified with respect to histopathology, both by automated software classification and by expert

endoscopists.

Figure 5 shows 3 other results that revealed some limitations of the automated pCLE retrieval software. Video query Q5 corresponds to a rare variety of hyperplastic polyp correctly classified as non-neoplastic by the expert endoscopists, but misclassified by the automated software classification because it was not represented in the training database for retrieval. Video query Q6 corresponds to the ambiguous serrated adenoma case, correctly classified as non-neoplastic by the automated software classification, but misclassified by the expert endoscopists who considered serrated adenomas as malignant. Video query Q7 corresponds to a tubulovillous

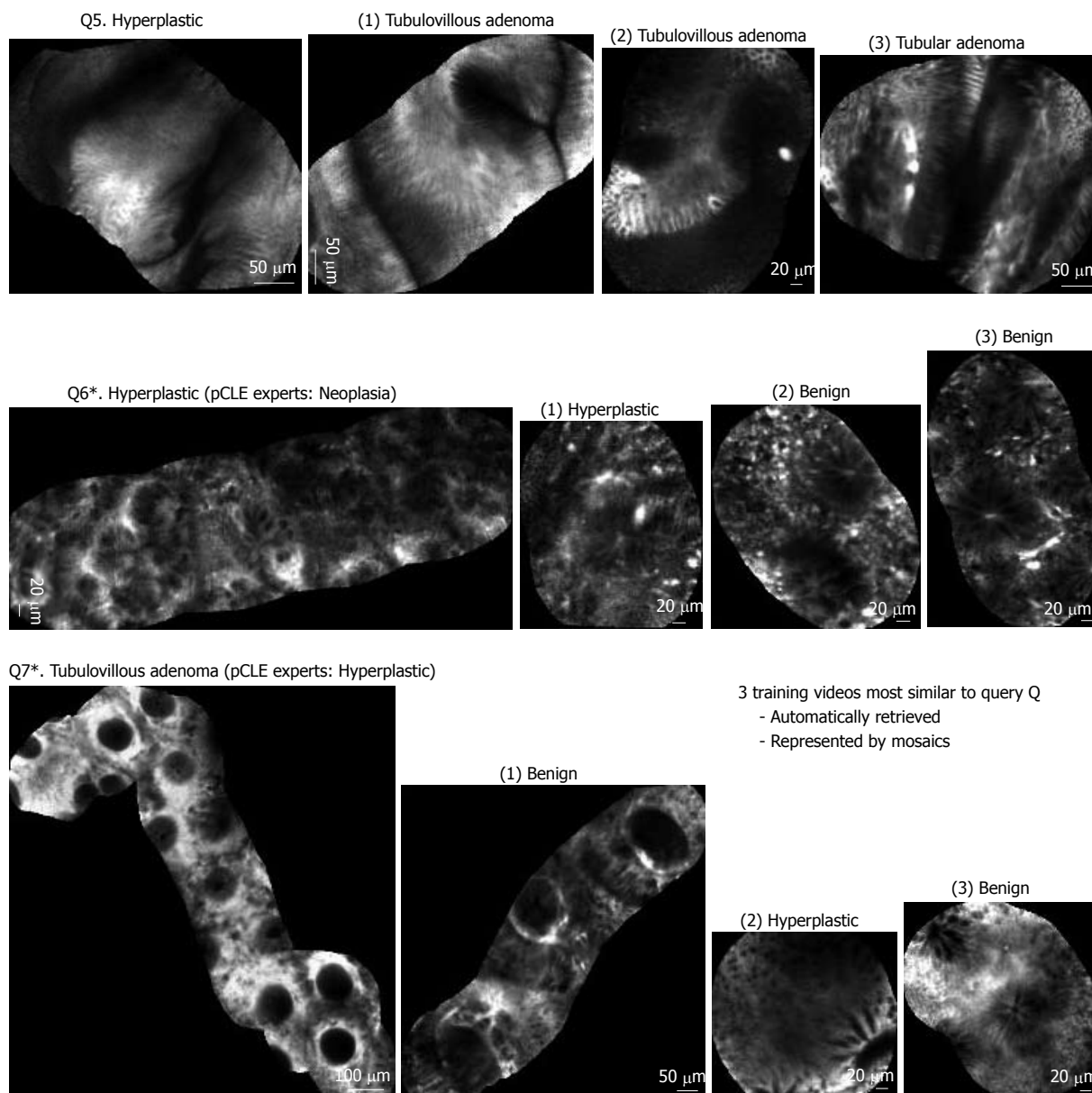


Figure 5 Results of automated probe-based confocal laser endomicroscopy video retrieval represented as mosaics. With respect to histology: the automated software classification is correct for video query Q6 but incorrect for video queries Q5 and Q7, whereas the off-line diagnosis of probe-based confocal laser endomicroscopy videos established by the expert endoscopists is correct for video queries Q5 but incorrect for video queries Q6 and Q7 (for which this disagreement is marked by *).

adenoma misclassified as non-neoplastic both by the expert endoscopists and by the automated software classification (this may be explained if a sampling error occurred and the corresponding biopsy was not performed exactly on the imaging spot).

Quantitative results of automated pCLE classification compared to off-line diagnosis of pCLE videos established by experts

Classification accuracy, sensitivity and specificity of the two methods, automated pCLE software classification (first method) and off-line diagnosis of pCLE videos established by the 2 expert endoscopists (second method), are listed in Table 3. Automated software classification reached a sensitivity of 92.5%, a specificity of 83.3% for

a resulting accuracy of 89.6%. Expert review reached a sensitivity of 91.4%, a specificity of 85.7% and the same accuracy of 89.6%.

When testing for statistical difference, the *P* values provided by McNemar's tests showed that the differences between the 2 methods were not statistically significant and that there was very low power (< 6%) to detect the observed differences.

When testing for statistical equivalence, the 95% confidence intervals provided by two-sided Z-tests between proportions were: -0.073 to 0.073 for accuracy, -0.068 to 0.089 for sensitivity and -0.18 to 0.13 for specificity. These intervals included zero and were sufficiently small to suggest that the methods were equivalent. In particular, the -0.18 lower bound for specificity was acceptable

Table 3 Performance comparison between automated probe-based confocal laser endomicroscopy classification and off-line expert diagnosis of probe-based confocal laser endomicroscopy, for the differentiation between neoplastic and non-neoplastic colonic polyps

	Automated pCLE classification	Off-line expert diagnosis of pCLE
Accuracy		
%	89.6	89.6
Fraction	121/135	121/135
Sensitivity		
%	92.5	91.4
Fraction	86/93	85/93
Specificity		
%	83.3	85.7
Fraction	35/42	36/42
Statistical significance between (1) and (2)		
McNemar's test, alpha = 0.05		
Accuracy: (<i>P</i> , power)	(Not significant, 2.5%)	
Sensitivity: (<i>P</i> , power)	(Not significant, 6.5%)	
Specificity: (<i>P</i> , power)	(Not significant, 5.2%)	
Statistical equivalence between (1) and (2)		
Two-sided Z test		
95% CI for accuracy	-0.073-0.073	
95% CI for sensitivity	-0.068-0.089	
95% CI for specificity	-0.18-0.13	

pCLE: Probe-based confocal laser endomicroscopy.

if the automated pCLE classification software was only taken as a second-reader tool to support pCLE diagnosis.

DISCUSSION

The present study demonstrates that, using a fairly representative database of colonic polyps, our automated software for the pCLE video classification has overall high accuracy, sensitivity and specificity, that are comparable to those of the off-line diagnosis of pCLE videos established by two endoscopists expert in pCLE. As the automated classification software can be run on-line during ongoing colonoscopy, it could be used as a second-reader tool to support and improve not only off-line but also on-line diagnosis of pCLE established by endoscopists with various levels of expertise. In the majority of cases, the second reader would agree with a moderately experienced endoscopist, who would thus be comforted in his/her diagnosis. For cases when they disagree, the endoscopist would have the opportunity to rethink his/her diagnosis and have more accurate *in vivo* interpretation. Besides, especially for small polyps, this second-reader tool could assist the endoscopist in adopting the "Diagnose, Resect and Discard Strategy" that dispenses with histopathological examination.

Gomez *et al.*^[21] analyzed *in vivo* pCLE interpretation in distinguishing between neoplastic and non-neoplastic lesions among 3 expert endoscopists and estimated an average accuracy of 75% (sensitivity 76%, specificity 72%) with good to moderate interobserver agreement. Buchner *et al.*^[22] demonstrated that accurate interpretation of pCLE images by 11 endoscopists, considered

as non-expert in pCLE, can be learned rapidly with a short 2-h training session. The learning curve pattern of pCLE in predicting neoplastic lesions was demonstrated with improved accuracies in time from 63% to 86% as observers' experience increased. Thus, prospectively, the automated classification software could be valuable not only for *in vivo* diagnosis support, but also for training support to improve the learning curve of new endoscopists. Indeed, we have shown in a preliminary study^[23] how interpretation difficulty can be automatically estimated by the software, in order to develop a self-training simulator for pCLE diagnosis with adjustable level of difficulty. For surgical skills, evidence of the learning effect from the use of training tools have been provided in the thesis by Brydges^[24], however, further investigation is needed for the extension of learning effect analysis to diagnostic skills.

One of the advantages of our classification software is that it is not a "black box" but an informative tool based on the query by example model: it produces, as intermediate results, visually similar annotated videos that are directly interpretable by the endoscopist. From the qualitative observations of visual similarities between pCLE videos, we suggest that the visually convincing results of the intermediate video retrieval step account for the relevance of the whole pCLE classification software. As few similar videos (less than 10) are necessary to classify a video query with high accuracy, this visual information should be clinically useful for the endoscopist.

Further limitations of the classification software may include three main issues. First, a large training database is needed to be sufficiently representative of non-typical pCLE cases. This is even more challenging since the practice of pCLE is evolving and that new cases with atypical pCLE features may still be encountered. Second, the definition of "criterion standard" for colorectal cancer screening is debatable because expert endoscopists and pathologists do not always agree. This can be illustrated by many examples of hyperplastic polyps redefined later as sessile serrated lesions by gastrointestinal pathologists, as in the study by Khalid *et al.*^[25]. The third limitation is that an obtained biopsy may be acquired unintentionally from the area that does not correspond with the obtained pCLE imaging.

The task of the automated pCLE classification software is not to replace the endoscopist or the pathologist but to assist the endoscopist in making an informed decision. Before using the computer-based classification tool during an ongoing endoscopy procedure, more work is needed to improve its accuracy and to develop underlying tools that are both ergonomic and complementary. In particular, the on-line display of the retrieval outputs, for instance of the 3 most similar videos to the video query, together with their histopathology and possible multimodal clinical data, may be a precious underlying indicator for diagnosis decision. Such a sophisticated "Smart Atlas" for pCLE would allow endoscopists in different centers to share and enrich their pCLE knowl-

edge during ongoing endoscopy. Further studies are warranted to evaluate the impact of using automated pCLE retrieval and classification software on the pCLE learning curve and on the diagnostic performance of the endoscopists.

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COMMENTS

Background

Histopathology is the criterion standard for the diagnosis of colorectal cancers, but it implies a large proportion of unnecessary polypectomies and an inherent delay in diagnosis. Probe-based confocal laser endomicroscopy (pCLE) is a recent technology that enables, during ongoing endoscopy, *in vivo* imaging of the epithelium at the microscopic level.

Research frontiers

Several studies have already demonstrated the applicability of pCLE in diagnosing colorectal neoplasia *in vivo* with high sensitivity and specificity. Because pCLE is a relatively recent imaging technology, the interpretation of pCLE videos of colonic polyps for diagnostic purposes is still challenging for many non-expert endoscopists.

Innovations and breakthroughs

This is believed to be the first study to propose, with the aim of supporting *in vivo* diagnosis of colorectal cancers, content-based image retrieval-based classification software that automatically extracts visually similar annotated videos directly interpretable by the endoscopist. The extracted annotated videos can be presented to the endoscopist in a second reader paradigm to better support pCLE diagnosis. Furthermore, this study demonstrates that this novel software achieves a high diagnostic performance, which is statistically comparable to that of off-line diagnosis of pCLE videos established by expert endoscopists.

Applications

The classification software proposed in this study is an objective tool which has the potential to support the interpretation of pCLE videos of colonic polyps for diagnostic purposes. Further studies are warranted to evaluate the impact of using the automated classification software on the pCLE learning curve and on the diagnostic performance of endoscopists.

Terminology

pCLE: An imaging system that allows the endoscopist to visualize the epithelium *in vivo*, at the microscopic level and in real-time during ongoing endoscopy; Content-based image retrieval: A computer vision technique that automatically extracts, given a query image, several training images with the most similar appearance to the query.

Peer review

This is a good descriptive study in which authors support probe-based confocal laser endomicroscopy diagnosis by designing a software for automated classification of colonic polyps. The results are interesting and suggest that the proposed software for automated classification of pCLE videos of colonic polyps achieves high performance, comparable to that of off-line diagnosis of pCLE videos established by expert endoscopists.

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Quantitation of HBsAg predicts response to entecavir therapy in HBV genotype C patients

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Abstract

AIM: To analysis the factors that predict the response to entecavir therapy in chronic hepatitis patients with hepatitis B virus (HBV) genotype C.

METHODS: Fifty patients [hepatitis B e antigen (HBeAg)-negative:HBeAg-positive = 26:24] with HBV genotype C, who received naïve entecavir therapy for > 2 years, were analyzed. Patients who showed HBV DNA levels \geq 3.0 log viral copies/mL after 2 years of entecavir therapy were designated as slow-responders, while those that showed < 3.0 log copies/mL were termed rapid-responders. Quantitative hepatitis B surface antigen (HBsAg) levels (qHBsAg) were determined by the Architect HBsAg QT immunoassay. Hepatitis B core-related antigen was detected by enzyme immunoassay. Pre-C and Core promoter mutations were determined using by polymerase chain reaction (PCR). Drug-resistance mutations were detected by the PCR-Invader method.

RESULTS: At year 2, HBV DNA levels in all patients in

the HBeAg-negative group were < 3.0 log copies/mL. In contrast, in the HBeAg-positive group, 41.7% were slow-responders, while 58.3% were rapid-responders. No entecavir-resistant mutants were detected in the slow-responders. When the pretreatment factors were compared between the slow- and rapid-responders; the median qHBsAg in the slow-responders was 4.57 log IU/mL, compared with 3.63 log IU/mL in the rapid-responders ($P < 0.01$). When the pretreatment factors predictive of HBV DNA-negative status at year 2 in all 50 patients were analyzed, HBeAg-negative status, low HBV DNA levels, and low qHBsAg levels were significant ($P < 0.01$). Multivariate analysis revealed that the low qHBsAg level was the most significant predictive factor ($P = 0.03$).

CONCLUSION: Quantitation of HBsAg could be a useful indicator to predict response to entecavir therapy.

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Key words: Chronic hepatitis B; Quantitation of hepatitis B surface antigen; Entecavir; Hepatitis B virus genotype C; Slow-responders; Hepatitis B core-related antigen; Core promoter mutation; Pre-C mutation

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INTRODUCTION

Hepatitis B virus (HBV) is a major causative agent of chronic liver diseases^[1]. Various strains of HBV have been isolated all over the world, and have been classified as HBV genotypes from A to J^[2]. In Japan, about 85% of patients have HBV genotype C, and about 12% have HBV genotype B^[3]. Worldwide, HBV genotypes show specific geographical distributions. HBV genotypes A and D are prevalent in the United States and Europe, while HBV genotypes B and C are prevalent in Asia^[4]. Disease progression and prevalence of hepatitis B e antigen (HBeAg)-positive status are often associated with HBV genotypes^[5]. Therefore, we analyzed the clinical and virological features of patients with HBV genotype C and homogenous backgrounds, because HBV genotype C is the predominant type in Japan.

Entecavir is widely used as a first-choice nucleot(s)ide analog (NA) for chronic hepatitis B patients, because less than 1% of entecavir-naïve patients developed resistant mutants after 5 years of therapy^[6-8]. However, in HBeAg-positive patients, the response rate to entecavir therapy is less favorable compared with HBeAg-negative patients^[6,7]. In addition, some patients show a slow-response, which indicates that serum HBV DNA levels remain high after long-term entecavir therapy. However, it is unclear which patients become slow-responders. Therefore, the aim of this study is to clarify the virological and clinical characteristics of the slow-responders before and during long-term entecavir therapy for HBV genotype C.

MATERIALS AND METHODS

Patient population

From July 2007, 102 consecutive hepatitis B surface antigen (HBsAg)-positive patients with chronic liver disease were enrolled in a naïve entecavir therapy in our hospital. Ten patients dropped out, 15 patients discontinued therapy, 10 patients received immunosuppressive therapy during entecavir therapy, and 10 patients received entecavir for less than 2 years. Thus, 57 patients were analyzed in this prospective, single center study. The institutional review board of the hospital approved the study. Serum samples were drawn from the patients after obtaining written informed consent.

All the patients received 0.5 mg of entecavir daily. Patients with poor adherence were excluded from the study.

All patients were positive for HBsAg for more than 6 mo, had serum HBV DNA of ≥ 3 log viral copies/mL, were negative for anti-HCV, and were negative for anti-human immunodeficiency virus before entecavir therapy. Patients with decompensated cirrhosis, acute hepatitis, or acute exacerbation were excluded. Liver biopsy was not performed in some patients; therefore, the liver disease status was diagnosed by the clinical, laboratory, and imaging tests.

HBeAg-positive patients whose serum HBV DNA levels remained ≥ 3.0 log copies/mL after 2 years of entecavir therapy were considered to be slow-responders,

Table 1 Baseline characteristics of the patients with hepatitis B virus genotype C

	HBeAg-negative group	HBeAg-positive group	P value
No.	26	24	NS
Age (yr)	57.2 (35-80)	44.2 (35-71)	< 0.01
Gender, M:F	15:16	16:11	NS
Diseases, CH:LC/HCC	21:5	23:1	NS
ALT (IU/mL)	38 (13-950)	102 (812-602)	< 0.01
Platelet counts ($\times 10^4$ /mL)	18.6 (3.4-4.9)	18.0 (8.4-26.8)	NS
Albumin (mg/dL)	4.3 (3.4-4.9)	4.2 (2.3-5.0)	NS
Serum HBV DNA level (log copies/mL)	5.1 (3.9-8.8)	7.6 (5.6-8.8)	< 0.01

All data are shown by median (range). HBeAg: Hepatitis B e antigen; HBV: Hepatitis B virus; ALT: Alanine aminotransferase; CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; NS: Not significant.

while patients with < 3.0 log copies/mL were designated as rapid-responders.

Laboratory tests

Quantitation of HBsAg (qHBsAg) was performed using the Architect HBsAg QT immunoassay (Abbott Japan, Tokyo, Japan), in accordance with the manufacturer's instructions^[9]. The detection range was 0.05 to 250 IU/mL. If HBsAg levels were found to be higher than 250 IU/mL, samples were diluted to 1:500 to 1:20 000. In this study, the results of the quantitative HBsAg levels are shown as the logarithmic value. HBV genotypes were detected by enzyme immunoassay (EIA) (Institute of Immunology, Tokyo, Japan)^[10]. The HBV core-related antigen (HBcrAg) was detected by a chemiluminescent EIA method (Fujirebio Inc., Tokyo, Japan)^[11]. Pre-C mutation and Core promoter mutations were detected by polymerase chain reaction (PCR) (HBV DNA precore/core promoter mutation decision kit; Roche Diagnostics Japan, Tokyo, Japan). Drug-resistant mutations in HBV against nucleotide analogs (NAs; lamivudine, adefovir and entecavir) were detected by the PCR-Invader method (BML Inc., Tokyo, Japan)^[12].

RESULTS

Of the 57 patients, 50 patients were genotype C, three patients were HBV genotype A, one was genotype D, and three were of indeterminate genotype on EIA. Thus, the 50 patients with HBV genotype C were analyzed. Baseline characteristics of the 50 patients are shown in Table 1. The median age of the HBeAg-negative group age was significantly higher, the median alanine aminotransferase (ALT) level was significantly lower, and the median HBV DNA level was significantly lower than those in the HBeAg-positive group.

After 2 years of entecavir therapy, the rates of normalization (< 40 IU/L) of ALT levels were 87.0% in the HBeAg-negative group and 92.5% in the HBeAg-positive group ($P =$ Not significant).

In contrast, at year 2, the rates of reduction in HBV

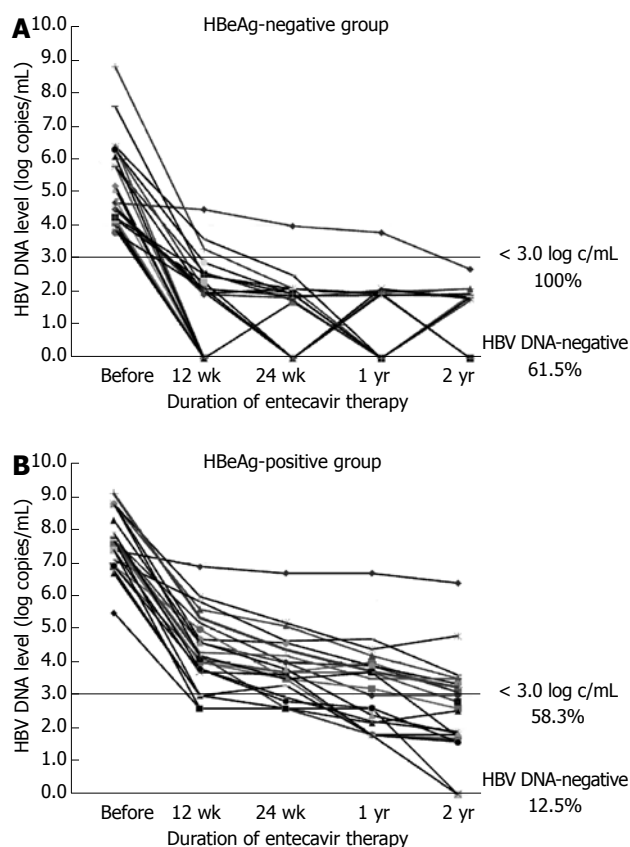


Figure 1 Hepatitis B virus DNA levels before and during entecavir therapy. A: In the hepatitis B e antigen (HBeAg)-negative group, hepatitis B virus (HBV) DNA levels in all patients decreased to < 3.0 log copies/mL at year 2. Of these patients, 61.5% shown to be negative for HBV DNA by the real-time polymerase chain reaction method; B: In the HBeAg-positive group, 58.3% of patients (rapid-responders) showed < 3.0 log copies/mL at year 2, while 41.7% of patients (slow-responders) showed ≥ 3.0 log copies/mL. In addition, at year 2, only 12.5% of the patients were negative for HBV DNA.

DNA to < 3.0 log copies/mL were 100% in the HBeAg-negative group and 58.3% in the HBeAg-positive group ($P < 0.01$). Thus, in the HBeAg-positive group, 58.3% of patients were designated as rapid-responders, and 41.7% were designated as slow-responders (HBV DNA levels ≥ 3.0 log copies/mL at year 2) (Figure 1). In addition, in the HBeAg-negative group, real-time PCR indicated that 61.5% of the patients were negative for HBV DNA, compared to 12.5% of the HBeAg-positive patients ($P < 0.01$).

Baseline data

When pre-treatment factors were compared between the rapid- and slow-responders (Table 2), age, gender, disease, platelet counts, and albumin were not significantly different. The median ALT level in the rapid-responders group was 131 IU/L compared with 31 IU/L in the slow-responders ($P = 0.02$). The pre-treatment median HBV DNA levels were 7.4 log copies/mL in the rapid-responders, and 8.3 in the slow-responders ($P = 0.06$). There was no difference in the rate of Pre-C and Core promoter mutations between the responder groups. In contrast, the rate of Pre-C mutations in the HBeAg-negative group was 83.3%, compared with 0% in the

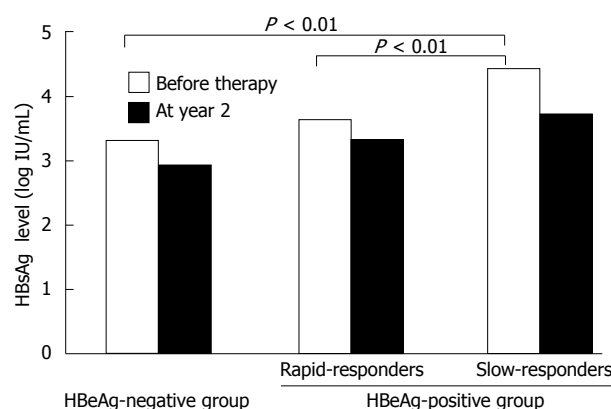


Figure 2 Median quantitative hepatitis B surface antigen levels among patients receiving entecavir therapy. Quantitative hepatitis B surface antigen (HBsAg) levels (qHBsAg) levels in slow-responders in the HBeAg-positive group were significantly higher than those in rapid-responders and the hepatitis B e antigen (HBeAg)-negative group. The median qHBsAg level at year 2 in the slow-responders remained higher than in other groups.

HBeAg-positive group. Pre-treatment HBsAg levels did not differ among the three groups. In contrast, the pre-treatment median qHBsAg level was 3.63 log IU/mL in the rapid-responders, compared with 4.57 log IU/mL in the slow-responders ($P < 0.01$).

Data at year 2

At year 2 of therapy, the median qHBsAg level in the rapid-responders was 3.25 log IU/mL, compared with 4.12 log IU/mL in the slow-responders ($P = 0.01$). The median HBsAg level in the rapid-responders was 5.85 log U/mL, compared with > 6.8 (the upper limit of the detection range) in the slow-responders ($P < 0.01$). In Figure 2, qHBsAg levels before treatment and at year 2 are shown for the HBeAg-negative group, and the rapid-responder and slow-responders in the HBeAg-positive group.

Among all the slow-responders, no entecavir-resistant mutations were found, although three patients showed M204I lamivudine-resistant mutations (Table 3).

Comparison between HBV DNA-negative and -positive patients at year 2 during entecavir therapy in all the patients

At year 2 of entecavir therapy, among 50 patients, real-time PCR showed that 19 (38.0%) were negative for HBV DNA, compared with 31 (62.0%) who were still positive for HBV DNA (Table 4). The pretreatment clinical and virological characteristics between the HBV DNA-negative and -positive groups were compared by univariate analysis. In the HBV DNA-negative group, the median ALT level was significantly lower, the rate of HBeAg-negative status was significantly higher, the median HBV DNA level was lower, and the median qHBsAg level was lower, than those in the HBV DNA-positive group.

However, when multivariate analysis using logistic regression analysis was performed, the median qHBsAg level was the only significant factor that predicted the negative HBV DNA status at year 2 of entecavir therapy

Table 2 Clinical and virological results among the hepatitis B e antigen-negative group, the rapid-responders, and the slow-responders in the hepatitis B e antigen-positive group during 2 years of entecavir therapy

Characteristics	HBeAg-negative group	HBeAg-positive group		P value
	(n = 26)	RR (n = 14)	SR (n = 10)	RR vs SR
<Baseline data>				
Age	58 (35-80)	45 (34-68)	43 (31-71)	NS
Gender (male:female)	13:13	9:5	6:4	NS
Disease (CH:LC/HCC)	21:5	13:1	6:4	NS
ALT (IU/L)	38 (13-950)	131 (12-602)	31 (13-108)	0.02
Platelet count (× 10 ⁴ /mL)	18.6 (3.4-35.1)	17.1 (8.4-22.4)	20.0 (11.0-26.8)	NS
Albumin (mg/dL)	4.3 (3.4-4.9)	4.0 (2.3-5.0)	4.4 (3.7-4.6)	NS
HBV genotype C	100%	100%	100%	NS
HBV DNA (log copies/mL)	5.1 (3.9-8.8)	7.4 (5.6-8.8)	8.3 (7.1-8.8)	NS
qHBsAg (log IU/mL)	3.17 (0.70-4.58)	3.63 (1.68-4.34)	4.57 (4.35-4.76)	< 0.01
HBcrAg (log U/mL)	3.6 (3.0-> 6.8)	> 6.8 (6-> 6.8)	> 6.8 (> 6.8-> 6.8)	NS
Pre-C mutation (%)	83.3	0	0	NS
Core promoter mutation (%)	58.3	57.1	50.0	NS
<At year 2 during therapy>				
HBV DNA (log copies/mL)	0.0 (0.0-2.7)	2.1 (0.0-2.1)	3.5 (3.1-6.9)	-
ALT (IU/L)	18 (9-75)	17.5 (10-31)	23 (13-37)	NS
HBeAg seroconversion	-	23.50%	0%	NS
HBsAg seroclearance	0%	0%	0%	NS
qHBsAg (log IU/mL)	2.91 (0.62-3.9)	3.25 (1.70-3.92)	4.12 (3.23-4.47)	0.01
HBcrAg (log U/mL)	3.0 (3.0-5.4)	5.9 (4.0-> 6.8)	> 6.8 (5.2-> 6.8)	< 0.01
Resistant mutations against entecavir	UDL	UDL	0%	-

HBeAg: Hepatitis B e antigen; ALT: Alanine aminotransferase; CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; UDL: Under the detection limit; HBV: Hepatitis B virus; HBsAg: Hepatitis B surface antigen; qHBsAg: Quantitation of HBsAg level; HBcrAg: HBV core-related antigen; NS: Not significant; RR: Rapid-responder; SR: Slow-responder.

Table 3 Drug resistant mutations in the slow-responders at year 2

Patient	Age (yr)	Gender	Previous therapy	HBV genotype	Drug resistant mutations against						
					Lam L180	Lam M204	Lam/Ade A181	Ade N236	Ent T184	Ent S202	Ent M205
1	52	Male	No	C	Wild	Wild	Wild	Wild	Wild	Wild	Wild
2	35	Male	No	C	Wild	Wild	Wild	Wild	Wild	Wild	Wild
3	68	Male	No	C	Wild	Wild	Wild	Wild	Wild	Wild	Wild
4	56	Female	No	C	Wild	M204I	Wild	Wild	Wild	Wild	Wild
5	36	Female	No	C	Wild	M204I	Wild	Wild	Wild	Wild	Wild
6	45	Male	No	C	Wild	M204I	Wild	Wild	Wild	Wild	Wild
7	35	Male	No	C	Wild	Wild	Wild	Wild	Wild	Wild	Wild
8	67	Female	No	C	Wild	Wild	Wild	Wild	Wild	Wild	Wild
9	39	Male	No	C	Wild	Wild	Wild	Wild	Wild	Wild	Wild
10	44	Female	No	C	Wild	Wild	Wild	Wild	Wild	Wild	Wild

Lam: Lamivudine; Ade: Adefovir; Ent: Entecavir; HBV: Hepatitis B virus.

(odds ratio 8.16, 95% CI: 1.28-52.18, *P* = 0.03).

DISCUSSION

In this study, the clinical and virological features of patients with HBV genotype C who received naïve-entecavir therapy were analyzed. After 2 years of entecavir therapy, about 42% of the HBeAg-positive patients showed HBV DNA levels ≥ 3 log copies/mL, while all of the HBeAg-negative patients showed < 3 log copies/mL. Therefore, the factors associated with the slow response to entecavir therapy among the HBeAg-positive group were studied initially. In addition, among the 50 patients, 38% showed HBV DNA-negative status at year 2. Thus, the pretreatment factors that predict the loss of HBV DNA

were analyzed in all 50 patients. According to the multivariate analysis, qHBsAg levels are the most important factor for predicting the response to entecavir therapy in patients with HBV genotype C.

In Japan, HBV genotype C is the most prevalent^[3]. The response rates to interferon or NA therapy in patients with HBV genotype C, as well as D, are poor when compared to those with HBV genotype B or A^[13]. Thus, in this study, only subjects with HBV genotype C were studied.

Recently, a decline in HBsAg levels during PEG-interferon therapy was reported to be significant in the evaluation of the response to therapy^[14-17]. In these reports, HBsAg levels were found to be one of the best viral markers for predicting response to anti-viral therapy and viral

Table 4 Pretreatment clinical and virological characteristics between hepatitis B virus DNA-negative and -positive group at year 2 during entecavir therapy

Characteristics	HBV DNA-negative group (<i>n</i> = 19)	HBV DNA-positive group (<i>n</i> = 31)	<i>P</i> value	
			Univariate analysis	Multivariate analysis
Age	51 (31-73)	52 (32-80)	NS	
Gender (male:female)	12:7	20:11	NS	
Disease (CH:LC/HCC)	17:2	27:4	NS	
ALT (IU/L)	36 (12-366)	108 (13-602)	0.03	NS
Platelet counts ($\times 10^4$ /mL)	19.0 (8.8-35.1)	17.8 (3.4-26.8)	NS	
Albumin (mg/dL)	4.35 (3.84-4.85)	4.14 (2.28-4.72)	NS	
HBV genotype (B:C:others)	0:19:0	0:31:0	NS	
HBeAg status (positive:negative)	3:16	21:10	< 0.01	NS
HBV DNA (log copies/mL)	5.1 (3.1-7.4)	7.6 (3.7-8.8)	< 0.01	NS
qHBsAg level (log IU/mL)	3.31 (1.90-4.08)	4.20 (3.06-4.87)	< 0.01	0.03
HBcrAg level (log U/mL)	3.45 (3.0-> 6.8)	> 6.8 (3.0-> 6.8)	NS	
Pre-C mutation (%)	75.0	43.3	NS	
Core promoter mutation (%)	37.5	60.0	NS	

CH: Chronic hepatitis; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma; ALT: Alanine aminotransferase; HBV: Hepatitis B virus; HBeAg: Hepatitis B e antigen; qHBsAg: Quantitation of hepatitis B surface antigen level; HBcrAg: HBV core-related antigen; NS: Not significant.

activity levels in hepatocytes, correlating with cccDNA levels^[14,18]. Although qHBsAg is a predictor of response to entecavir therapy^[19], there have been no reports in a homogeneous HBV genotype setting. In this study, qHBsAg was demonstrated to be the most significant predictor of entecavir therapy in patients with HBV genotype C.

HBV DNA levels are also considered an important factor associated with response to anti-viral therapy^[17]. In this study, there was a tendency for higher HBV DNA levels in the slow-responders as compared to rapid-responders. The association between HBV DNA levels and response to therapy may be clarified further in a larger number of patients.

HBcrAg levels indicate the serum HB core antigen levels plus HBeAg levels^[11]. HBcrAg levels are reported to be associated with cccDNA levels in hepatocytes^[20]. However, this study showed no association between HBcrAg levels and slow response. This may be explained by the narrow quantitation range of HBcrAg levels, because HBcrAg levels in most patients in the HBeAg-positive group were greater than the upper level of the detection range of the assay.

The association between Core promoter mutation and response rate to NAs is also interesting, because the replication level of HBV is thought to be high in patients with Core promoter mutations. We reported previously that, in patients with HBV genotype C, the rates of HBeAg-positive status and Core promoter mutations are higher than those in patients with HBV genotype B^[5]. In this study, a higher rate of Core promoter mutations was observed in the HBeAg-positive patients with HBV genotype C. In addition, a higher rate of Pre-C mutations was observed in the HBeAg-negative group. However, no association between the mutation rate and response rate to therapy was demonstrated in this study.

Higher ALT levels are considered an important factor in predicting good response to PEG-interferon therapy^[21]. However, low ALT levels were observed in the

HBV DNA-negative group at year 2 of therapy, because a high proportion of the HBeAg-negative patients had low ALT levels, compared to the HBeAg-positive patients. Thus, we consider that, during entecavir therapy, ALT levels are not associated with treatment response.

In this study, no resistant mutations against entecavir were found during 2 years therapy. As reported previously, resistant mutations against entecavir are rarely developed during 5 years of entecavir therapy^[8]. Therefore, slow response was not caused by entecavir-resistant mutants.

In conclusion, we suggest that qHBsAg is a significant and convenient indicator for predicting response to entecavir therapy.

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COMMENTS

Background

Entecavir is a nucleot(s)ide analog that is widely used for the treatment of chronic hepatitis B patients. The efficacy of entecavir is very good for hepatitis B e antigen (HBeAg)-negative patients, but not so good for HBeAg-positive patients. The prognosis and response to anti-viral therapies depend on hepatitis B virus (HBV) genotype. The factors that affect the efficacy of entecavir therapy are still unclear, especially in patients with HBV genotype C.

Research frontiers

As quantitation assay of serum hepatitis B surface antigen (HBsAg) has been recently developed, allowing the serum level of HBsAg to be determined over a very wide range. The upper range of HBsAg levels could be detected to 6.7 log IU/mL by the Architect HBsAg QT immunoassay when samples were diluted to 1:20 000. Thus, the authors could analyze the relationship between the efficacy of entecavir therapy and various HBV markers.

Innovations and breakthroughs

This study showed that the quantitative HBsAg level is a significant factor for predicting the efficacy of entecavir therapy in patients with HBV genotype C. Patients with low levels of HBsAg before entecavir therapy often show HBV DNA levels < 3.0 log copies/mL or are negative for HBV DNA at year 2 during therapy.

Applications

Using the quantitation of HBsAg, the efficacy of various anti-viral therapies can be predicted before treatment. The quantitation of HBsAg could be a useful tool for determining the treatment schedule for chronic hepatitis B patients.

Peer review

Although small in patient numbers, the subject matter is interesting and original. This study adds to the emerging data suggesting HBsAg decline is a predictor of response.

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Inhibition of gastric perception of mild distention by omeprazole in volunteers

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Abstract

AIM: To evaluate the effects of omeprazole on gastric mechanosensitivity in humans.

METHODS: A double lumen polyvinyl tube with a plastic bag was introduced into the stomach of healthy volunteers under fluorography and connected to a barostat device. Subjects were then positioned so they were sitting comfortably, and the minimal distending pressure (MDP) was determined after a 30-min adaptation period. Isobaric distensions were performed in stepwise increments of 2 mmHg (2 min each) starting from the MDP. Subjects were instructed to score feel-

ings at the end of every step using a graphic rating scale: 0, no perception; 1, weak/vague; 2, weak but significant; 3, moderate/vague; 4, moderate but significant; 5, severe discomfort; and 6, unbearable pain. After this first test, subjects received omeprazole (20 mg, after dinner) once daily for 1 wk. A second test was performed on the last day of treatment.

RESULTS: No adverse effects were observed. Mean MDP before and after treatment was 6.3 ± 0.3 mmHg and 6.2 ± 0.5 mmHg, respectively. One subject before and 2 after treatment did not reach a score of 6 at the maximum bag volume of 750 mL. After omeprazole, there was a significant increase in the distension pressure required to reach scores of 1 ($P = 0.019$) and 2 ($P = 0.017$) as compared to baseline. There were no changes in pressure required to reach the other scores after treatment. Two subjects before and one after omeprazole rated their abdominal feeling < 1 at MDP, and mean (\pm SE) abdominal discomfort scores at MDP were 0.13 ± 0.09 and 0.04 ± 0.04 , respectively. Mean scores induced by each MDP + 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 (mmHg) were 1.1 ± 0.3 , 2.0 ± 0.4 , 2.9 ± 0.5 , 3.3 ± 0.4 , 4.6 ± 0.3 , 5.2 ± 0.3 , 5.5 ± 0.2 , 5.5 ± 0.3 , 5.7 ± 0.3 , and 5.4, respectively. After omeprazole, abdominal feeling scores for the same incremental pressures over MDP were 0.3 ± 0.1 , 0.8 ± 0.1 , 2.0 ± 0.4 , 2.8 ± 0.4 , 3.8 ± 0.4 , 4.6 ± 0.4 , 4.9 ± 0.3 , 5.4 ± 0.4 , 5.2 ± 0.6 , and 5.0 ± 1.0 , respectively. A significant decrease in feeling score was observed at intra-bag pressures of MDP + 2 mmHg ($P = 0.028$) and + 4 mmHg ($P = 0.013$), respectively, after omeprazole. No significant score changes were observed at pressures \geq MDP + 6 mmHg.

CONCLUSION: Although the precise mechanisms are undetermined, the present study demonstrated that omeprazole decreases mechanosensitivity to mild gastric distension.

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Key words: Functional dyspepsia; Acid exposure; Omeprazole; Barostat test; Mechanosensitivity

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Iida A, Kaneko H, Konagaya T, Funaki Y, Tokudome K, Izawa S, Tamura Y, Mizuno M, Ogasawara N, Sasaki M, Kasugai K. Inhibition of gastric perception of mild distention by omeprazole in volunteers. *World J Gastroenterol* 2012; 18(39): 5576-5580 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i39/5576.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i39.5576>

INTRODUCTION

According to Rome III, the classification for functional gastrointestinal disorders published in 2006, functional dyspepsia (FD) is defined as the presence of one or more dyspepsia symptoms (postprandial fullness, early satiation, epigastric pain, or epigastric burning) that originate from the gastroduodenal region in the absence of any organic, systemic, or metabolic disease that is likely to explain the symptoms^[1]. Alteration of gastric motility, visceral hypersensitivity, impaired accommodation of meals, gastritis induced by *Helicobacter pylori* (*H. pylori*) infection, and dysfunction of the central nervous system have all been implicated in the pathophysiology of FD^[2-4]. Duodenal acidification increases proximal gastric mechanosensitivity, induces proximal gastric relaxation, and seems to inhibit the proximal gastric accommodation to a meal^[5]. Hypersensitivity not only to acid in the duodenum but also gastric distension has been proposed among the possible mechanisms of FD. Thus, the mechanism by which decreased duodenal acid exposure affects symptoms of dyspepsia warrants investigation.

Omeprazole, a proton pump inhibitor that strongly decreases acid secretion, has been prescribed in the treatment of FD worldwide. This is the best first-line treatment when compared with ranitidine, cisapride, and placebo for primary care of *H. pylori*-negative dyspepsia patients^[6,7]. However, changes in patients' perceptions of gastric distention after omeprazole administration have yet to be clarified. The gastric barostat test has been reported to be the gold standard method for evaluating gastric perception^[8-11]. In the present study, we examined the effects of omeprazole on mechanosensitivity in humans using the barostat test.

MATERIALS AND METHODS

Study subjects

Ten healthy volunteers were recruited (8 men, 2 women; mean age, 31.6 ± 2.1 years; range, 23 to 41 years). None of the subjects had any history of gastrointestinal dis-

ease, nor were they taking any medications. Per individual interviews, no subjects had any dyspepsia symptoms (postprandial fullness, early satiation, epigastric pain, or epigastric burning). A check for *H. pylori* infection was not requested. Written informed consent was obtained from each participant. The ethics committee of Aichi Medical University approved all the study protocols (No. 369-3).

Gastric barostat

The barostat system was set up as reported previously^[12]. After the patient had fasted for 12 h, a double lumen polyvinyl tube with an adherent finely folded plastic bag (Mui Scientific, Mississauga, Ontario, Canada) was introduced through the mouth into the stomach under fluorography. The polyvinyl tube was then connected to a barostat device (Distender series II TM; G and J Electronics, North York, Ontario, Canada). The bag was unfolded by inflation with 300 mL of air and was positioned in the proximal stomach with the subject in a recumbent position, after which the bag was deflated. Subjects were then positioned so they were sitting comfortably with their knees bent (80°) and trunk upright.

Gastric sensitivity to intragastric bag distension

After a 30-min adaptation period, minimal distending pressure (MDP) was determined by increasing the intrabag pressure by 1 mmHg every 3 min until a volume of 30 mL was reached. Gastric perception of distention by a barostat device was examined^[12]. Isobaric distensions were performed in stepwise increments of 2 mmHg (2 min each) starting from the MDP while the corresponding intragastric volume was recorded continuously. Subjects were instructed to score feelings in the upper abdomen at the end of every distending step using a graphic rating scale that included verbal descriptors, as follows: 0, no perception; 1, weak/vague; 2, weak but significant; 3, moderate/vague; 4, moderate but significant; 5, severe discomfort; and 6, unbearable pain. The endpoint of each sequence of distensions was established at an intrabag volume of 750 mL or when the subject reported unbearable pain (score 6).

Effects of omeprazole on the barostat test

This study was investigated the effects of omeprazole (Omepral[®]; AstraZenca, Osaka, Japan) on the barostat test. After the first barostat test, 10 volunteers received omeprazole (20 mg, once after dinner) as the medication for 1 wk. The second barostat study was performed on the morning after the last medication.

Statistical analysis

Data are expressed as mean ± SE. Statistical evaluation was performed using one way-analysis of variance. Values of *P* < 0.05 were considered statistically significant. Analyses were performed using a personal computer with Office Excel 2003 software (Microsoft, Redmond, WA, United States), and Analyse-it Software (Analyse-it Software, Ltd., Road Leeds, United Kingdom).

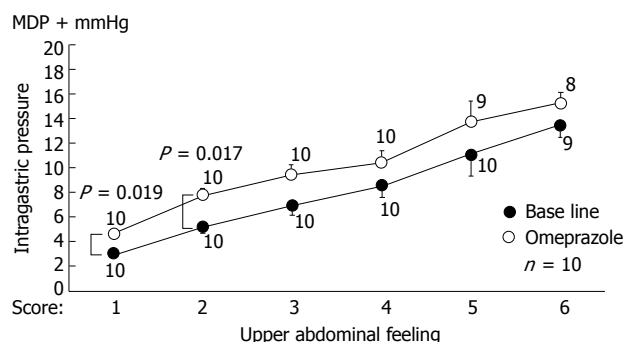


Figure 1 Intra-gastric pressure by a barostat device at each score of upper abdominal feeling. The distention pressure required to induce a weak/vague feeling (score 1) in the upper abdomen increased from 3.0 ± 0.5 mmHg over the minimal distending pressure (MDP) at baseline to 4.6 ± 0.4 mmHg over the MDP after omeprazole, $P = 0.019$. The distention pressure to induce a weak but significant feeling (score 2) increased from 5.2 ± 0.7 mmHg to 7.8 ± 0.6 mmHg, $P = 0.017$. The n sizes for each data point are indicated on the graph.

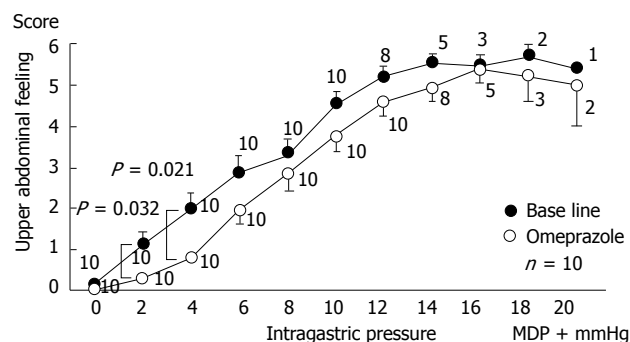


Figure 2 Score of upper abdominal feeling during gastric distension by a barostat device. The mean scores at the intrabag pressures of minimal distending pressure (MDP) + 2 mmHg and + 4 mmHg were decreased from baseline after omeprazole: 1.1 ± 0.3 vs 0.3 ± 0.1 , $P = 0.028$; and 2.0 ± 0.4 vs 0.8 ± 0.1 , $P = 0.0132$, respectively. No significant changes were observed at pressures > MDP + 4 mmHg. The n sizes for each data point are indicated on the graph.

RESULTS

No adverse effects were observed with omeprazole. Oral intubation with subsequent positioning of the barostat bag was well-tolerated by all subjects. The mean MDP before and after omeprazole was 6.3 ± 0.3 mmHg and 6.2 ± 0.5 mmHg, respectively. One subject at baseline and 2 after omeprazole did not reach discomfort scores of 6 at the maximum bag volume of 750 mL.

The distention pressures (mmHg) required to induce scores of weak/vague (score 1, $n = 10$), weak but significant (score 2, $n = 10$), moderate/vague (score 3, $n = 10$), moderate but significant (score 4, $n = 10$), severe discomfort (score 5, $n = 10$), and unbearable pain (score 6, $n = 9$) were 3.0 ± 0.5 , 5.2 ± 0.7 , 7.0 ± 1.0 , 8.6 ± 0.9 , 11.1 ± 0.8 , and 13.5 ± 0.9 , respectively, at baseline. After 1 week of daily omeprazole, the distention pressure required to induce the same scores ($n = 10$ for scores 1 to 4; $n = 9$, score 5; $n = 8$, score 6) were 4.6 ± 0.4 , 7.8 ± 0.6 , 9.4 ± 0.9 , 10.4 ± 1.0 , 13.8 ± 1.8 , and 15.3 ± 1.0 , respectively. The distended pressure needed to reach scores of 1 ($P = 0.019$) and 2 ($P = 0.017$) (Figure 1) increased significantly after omeprazole. No changes in pressure were demonstrated for other scores after treatment.

Eight subjects scored 0 for the MDP at baseline and 9 scored 0 for the MDP after omeprazole. The mean discomfort scores (\pm SE) at MDP before and after omeprazole were 0.13 ± 0.09 and 0.04 ± 0.04 , respectively.

The mean scores induced by each MDP + 2, 4, 6, 8, 10, 12, 14, 16, 18 and 20 mmHg were 1.1 ± 0.3 ($n = 10$), 2.0 ± 0.4 ($n = 10$), 2.9 ± 0.5 ($n = 10$), 3.3 ± 0.4 ($n = 10$), 4.6 ± 0.3 ($n = 10$), 5.2 ± 0.3 ($n = 8$), 5.5 ± 0.2 ($n = 5$), 5.5 ± 0.3 ($n = 3$), 5.7 ± 0.3 ($n = 2$), and 5.4 ($n = 1$), respectively. After omeprazole, the abdominal feeling scores for the same incremental pressures over MDP were 0.3 ± 0.1 ($n = 10$), 0.8 ± 0.1 ($n = 10$), 2.0 ± 0.4 ($n = 10$), 2.8 ± 0.4 ($n = 10$), 3.8 ± 0.4 ($n = 10$), 4.6 ± 0.4 ($n = 10$), 4.9 ± 0.3 ($n = 8$), 5.4 ± 0.4 ($n = 5$), 5.2 ± 0.6 ($n = 3$), and 5.0 ± 1.0 ($n = 2$), respectively. A significant decrease in the feeling score was observed at intrabag pressures of MDP + 2 mmHg (P

$= 0.028$) and + 4 mmHg ($P = 0.013$), respectively, after omeprazole (Figure 2). No significant score changes were seen at pressures \geq MDP + 6 mmHg.

DISCUSSION

In this study, we have shown for the first time the inhibitory effect of 1 wk of treatment with omeprazole on perception of mechanical distension by a barostat device. Namely, a significant increase was observed in the pressure required to induce a weak/vague feeling (score 1) and a weak but significant feeling (score 2). In other words, the perceived intensity of MDP + 2 and + 4 mmHg were significantly decreased after omeprazole treatment.

We recruited normal volunteers who were paid for their participation, and not all participants scored a weak/vague abdominal feeling (score 1) at intrabag pressure of MDP, indicating that the results obtained in the present study are limited to dyspepsia-free normal subjects.

The mechanisms by which omeprazole decreases perceptions of discomfort associated with gastric distension remain unknown. Omeprazole is a proton pump inhibitor that strongly decreases acid secretion. In a previous report of 5-d treatment with omeprazole at a similar dose (20 mg) to the present study, the number of hours with intragastric pH greater than 4 during a 24-h period was found to be $10.5 \text{ h}^{[13]}$. Omeprazole has been reported to be most effective in patients who rated epigastric pain or heartburn as their most bothersome symptom^[6,7]. Visceral hypersensitivity as well as gastric dysmotility, impaired accommodation to the meals, *H. pylori*-induced gastritis, and dysfunction of the central nervous system have all been implicated in the pathophysiology of FD^[2-4]. Hypersensitivity to gastric distension and acid has especially been demonstrated in patients with FD. After the first report that gastric distension-induced perception was significantly increased in subjects with FD compared with controls in 1991^[14], it has been reported that the hypersensitivity against distension might present in 34% to 66% of patients with FD^[15]. In addition, duodenal acidification-

induced dyspeptic symptoms occur more significantly in patients with FD than in healthy volunteers^[16].

A growing set of evidence has clarified the role of acid on dyspeptic symptoms and mechanosensitivity. First, acid infusion into the stomach induced dysmotility-like predominant dyspeptic symptoms in healthy Japanese control subjects^[17]. Second, intraluminal infusion of hydrochloric acid affected sensitization of stomach mucosal mechanoreceptors^[18]. Next, distal esophageal infusion also increased gastric sensitivity to distension^[19]. Finally, intraduodenal acid infusion increased fundic compliance, decreased the tone and phasic contractile activities of the fundus, and increased proximal gastric mechanosensitivity^[5,20,21]. These data suggest that the presence of acid in the upper gut might enhance mechanosensitivity. This putative mechanism has been supported by animal studies. Protons evoke multiple currents in primary afferent neurons that are carried by several acid-sensitive ion channels. Among these, acid-sensing ion channels (ASICs) and transient receptor potential vanilloid-1 (TRPV1) ion channels have been most thoroughly studied^[22-24]. Taken together, it is speculated that an omeprazole-induced decrease in acid secretion followed by the downregulation of chemoreceptors might suppress distension-induced mechanosensitivity, resulting in the reduced perception of gastric distension demonstrated in the present study.

In this study, the inhibitory effect of omeprazole on perception induced by intragastric bag distension was demonstrated only with mild distension stimulus and weak feeling in the abdomen in normal volunteers. The precise reasons for these limited effects are unclear. Jones *et al.*^[22] demonstrated that both TRPV1 and ASIC3 knock-out mice were significantly less sensitive to colon distension, with an average response magnitude only 58% and 50% of controls, respectively. The data may suggest that afferent nociceptive signal transfer occurs mainly *via* a mechanoreceptor, rather than a chemoreceptor at moderate and severe distension.

It is well-known that gut infection is one of the risk factors for functional gastrointestinal disorders such as irritable bowel syndrome and FD^[25]. Visceral hypersensitivity after infection has been reported in both animals^[26] and humans^[27]. Proton pump inhibitors have been reported to have potential anti-inflammatory effects apart from acid suppression^[28]. Therefore, it is possible that the omeprazole-induced decreased perception of gastric distension might be mediated by the anti-inflammatory action of omeprazole.

In conclusion, although the precise mechanisms are undetermined, the present study demonstrated that omeprazole decreases mechanosensitivity to mild gastric distension, suggesting that the drug might be effective in the treatment of FD.

COMMENTS

Background

Hypersensitivity to gastric distension has been proposed among the possible

mechanisms of functional dyspepsia (FD). Omeprazole has been prescribed in the treatment of FD. They examined the effects of omeprazole on mechanosensitivity in humans.

Research frontiers

The gastric barostat test is the gold standard method for evaluating gastric perception. They examined the effects of omeprazole on mechanosensitivity in humans using the barostat test.

Innovations and breakthroughs

Omeprazole is a proton pump inhibitor that strongly decreases acid secretion. The data suggest that suppression of acid secretion may decrease mechanosensitivity of the proximal stomach as well as dyspeptic symptoms.

Applications

Although the precise mechanisms are undetermined, the present study demonstrated that omeprazole decreases mechanosensitivity to mild gastric distension.

Terminology

These studies used the gastric barostat test in which a plastic bag with a double lumen polyvinyl tube was introduced into the stomach under fluorography. The tube was connected to a barostat device, which is a computer-controlled pump. Isobaric distensions were performed in stepwise increments. This method is the gold standard for evaluating gastric perception.

Peer review

This is a good descriptive study in which authors evaluate the effects of omeprazole on gastric mechanosensitivity in humans. The results are interesting and suggest that omeprazole decreases mechanosensitivity to mild gastric distension.

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Thioredoxin and thioredoxin-interacting protein as prognostic markers for gastric cancer recurrence

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Abstract

AIM: To evaluate the potential of thioredoxin (TXN) and thioredoxin-interacting protein (TXNIP) expression as biomarkers for predicting gastric cancer recurrence.

METHODS: TXN and TXNIP expression levels were acquired from gene expression microarray data for 65 human gastric cancer tissues. We determined whether each gene expression level was associated with cancer recurrence and investigated the relationship between the two genes. For validation, the expression levels of TXN and TXNIP were measured by quantitative real-time reverse transcription polymerase chain reaction in

68 independent stage III gastric cancer patients. The correlation between gene expression and cancer prognosis was evaluated. Immunohistochemical staining was performed to investigate the protein expression levels of TXN and TXNIP and to characterize the expression patterns of each protein.

RESULTS: TXN was a prognosis-related gene ($P = 0.009$), whereas TXNIP, a TXN inhibitor, demonstrated a negative correlation with TXN in the gene expression microarray data. In the 68 stage III patients, the expression levels of both TXN and TXNIP had a statistically significant effect on recurrence-free survival (RFS, $P = 0.008$ and $P = 0.036$, respectively). The low TXN and high TXNIP expression group exhibited a better prognosis than the other groups, and the high TXN and low TXNIP expression group exhibited a poorer prognosis ($P < 0.001$ for RFS and $P = 0.001$ for overall survival). More than half of the patients in the simultaneously high TXN and low TXNIP expression group experienced a recurrence within 1 year after curative surgery, and the 5-year survival rate of the patients in this group was 29%, compared with 89% in the low TXN and high TXNIP expression group. The TXN protein was overexpressed in 65% of the gastric cancer tissues, whereas the TXNIP protein was underexpressed in 85% of the cancer cells. In a correlation analysis, TXN and TXNIP were highly correlated with many oncogenes and tumor suppressors as well as with genes related to energy, protein synthesis and autophagy.

CONCLUSION: TXN and TXNIP are promising prognostic markers for gastric cancer, and performing personalized adjuvant treatment based on TXN and TXNIP expression levels would be an effective practice in the treatment of gastric cancer.

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Key words: Gastric cancer; Thioredoxin; Thioredoxin-interacting protein; Biomarker; Prognosis

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INTRODUCTION

Gastric cancer is the second leading cause of global cancer mortality, and it has the highest mortality rates in East Asia, including South Korea, Japan and China^[1]. The standard treatment for gastric cancer is surgery. However, relapses occur in many patients who undergo curative resection, even after adjuvant therapy^[2,3]. Even among individuals with the same stage of cancer, gastric cancer patients present with diverse clinical manifestations and prognoses. The clinical diversity of gastric cancer arises from its molecular biological diversity, which is caused by changes in different genes. Molecular markers are important in predicting patient outcome and in personalizing treatments according to the individual biology of the patient. Importantly, in the adjuvant treatment setting, uniform adjuvant treatment after curative resection of gastric cancer has been performed regardless of the individual molecular prognostic markers of the cancer. However, cancer researchers have identified biomarkers that can predict recurrence and survival^[4-8], and doctors should consider using biomarker-driven personalized adjuvant treatment.

Thioredoxin (TXN) is a low-molecular-weight redox protein and a putative oncoprotein that provides growth and survival advantages to tumor cells through the activation of redox-sensitive transcription factors, such as nuclear factor kappa B (NF- κ B), p53, and activator protein-1 (AP-1)^[9,10]. TXN inhibits apoptosis via apoptosis signaling kinase-1 (ASK-1) and phosphatase and tensin homolog (PTEN)^[11]. TXN also induces hypoxia-inducible factor-1 α (HIF-1 α), which increases the production of vascular endothelial growth factor and leads to tumor angiogenesis and drug resistance^[12]. The overexpression of TXN has been found in several cancers, including lung^[13], pancreatic^[14], cervical^[15], and colorectal cancers^[16] as well as hepatomas^[17]. The increased expression of TXN in tumors has been associated with decreased patient survival in several cancers and with resistance to anticancer drugs^[18,19]. The TXN-interacting protein (TXNIP), also known as vitamin D3 up-regulated protein-1 (VDUP1) and TXN-binding protein-2 (TBP-2), inhibits the interaction between TXN and other factors. TXNIP overexpression inhibits TXN activity, which in turn inhibits tumor cell proliferation and cell cycle progression^[20,21]. Recently,

it was reported that knockout of the *TXNIP* gene in a mouse model induced *Helicobacter pylori* (*H. pylori*)-related gastric cancer^[22]. However, there is little information regarding the prognostic value of the expression level of TXN and TXNIP in gastric cancer.

In this study, we evaluated the use of TXN expression combined with TXNIP expression as prognostic markers to individualize the postoperative treatment strategy following gastric cancer removal.

MATERIALS AND METHODS

Gene expression microarray data analysis

The previously generated gene expression data from gastric cancer patients are available in the NCBI's GEO public database (microarray data accession number, GSE13861)^[23]. Sixty-five gastric cancer patients underwent curative surgery as a primary treatment, with clinical data obtained from the Yonsei University Severance Hospital (Table 1). Sixty-five surgically removed frozen gastric adenocarcinoma tissues and 19 normal surrounding tissue samples were used for the microarray experiments. The total RNA was extracted from the fresh-frozen tissues using a mirVana RNA Isolation Labeling Kit (Ambion, Austin, TX, United States). For the labeling and hybridization, 500 ng of total RNA was used, according to the manufacturer's protocols (Human-HT12 v.3 Expression BeadChip, Illumina, San Diego, CA, United States). The microarray data were normalized using the quantile normalization method in the Linear Models for Microarray Data package in the R language environment. The expression level of each gene was transformed into a log2 base prior to further analysis. The random variance *t* test was applied to identify the differentially expressed genes between the two tissue types. The gene expression differences were considered significant if the *P* value was less than 0.001. Cluster analysis was performed with Cluster 3.0 and TreeView^[24]. Univariate analysis was performed by dividing the patients into two groups based on the median value of each gene expression level to search for prognostic genes.

Quantitative reverse transcription-polymerase chain reaction and analysis

Paraffin-embedded cancer tissues were collected from gastric adenocarcinoma patients who underwent curative surgery between 1999 and 2007 as a primary treatment at Gangnam Severance Hospital. The clinical data of the patients were reviewed to obtain age, sex, tumor location, tumor differentiation, and stage based upon the American Joint Committee on Cancer 2002 criteria. The patients were followed up for more than 36 mo after surgery or until recurrence or death within 36 mo after surgery.

Sixty-eight stage III gastric cancer tissues were chosen to validate the microarray data (Table 1). The total RNA was extracted according to the manufacturer's instructions (RecoverAll™ Total Nucleic Acid Isolation; Applied Biosystems, Foster City, CA, United States). The

Table 1 Clinicopathological factors of the gastric cancer patients

Characteristics	Microarray (<i>n</i> = 65)	qRT-PCR (<i>n</i> = 68)	TMA (<i>n</i> = 328)
Age			
mean (range), yr	63 (32-83)	56 (26-82)	57 (25-82)
Sex, <i>n</i> (%)			
Male/female	46 (71)/19 (29)	36 (53)/32 (47)	204 (62)/124 (38)
Follow up duration			
Mean (95% CI), mo	41.7 (41-42)	89.5 (79-100)	99.8 (97.5-102)
Histological type, <i>n</i> (%)			
Intestinal	23 (35)	14 (21)	100 (30)
Diffuse	42 (65)	54 (79)	228 (70)
TNM stage, <i>n</i> (%)			
I	12 (18)	0	101 (31)
II	11 (17)	0	79 (24)
III	26 (40)	68 (100)	110 (33)
IV	16 (25)	0	38 (12)
Location, <i>n</i> (%)			
Cardia	5 (8)	8 (12)	25 (8)
Non cardia	60 (92)	60 (88)	303 (92)
Adjuvant chemotherapy, <i>n</i> (%)			
Yes	49 (75)	59 (87)	230 (70)
No	16 (25)	9 (13)	98 (30)

qRT-PCR: Quantitative reverse transcription-polymerase chain reaction;
TMA: Tissue microarray; TNM: Tumor node metastasis.

TXN and *TXNIP* genes were assayed using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) with TaqMan gene-specific primers (Applied Biosystems, Foster City, CA, United States). Real-time RT-PCR amplification was performed using the 7900HT Fast Real-Time PCR System with a 384-well block module (Applied Biosystems, Foster City, CA, United States). The cycling conditions were as follows: 48 °C for 30 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and at 60 °C for 60 s. The relative amounts of mRNA were calculated from the threshold cycle (C_t) number using the expression of β -2 microglobulin as an endogenous control. All of the experiments were performed in triplicate, and the values were averaged.

Tissue microarray construction and immunohistochemical staining

Paraffin-embedded tissue microarray blocks of gastric cancer tissue specimens were created from tissues from 328 patients. Each block had 3-mm cores of gastric cancer tissue. The 4- μ m thick sections were deparaffinized and processed to block endogenous peroxidase activity. Next, an antigen retrieval step was performed. Subsequently, primary anti-*TXN* (Polyclonal, 1:500, Abcam, Cambridge, MA, United States) and anti-*TXNIP* antibodies (Polyclonal, 1:100 Sigma, St. Louis, MO, United States) were applied to the sections. The sections were then incubated with a secondary antibody (HRP-rabbit/mouse), and the stains were developed using a Nova-RED substrate kit (VECTOR Laboratory, Burlingame, CA, United States). The samples were then counterstained with Harris hematoxylin.

The *TXN* and *TXNIP* protein expression levels were evaluated by two pathologists. Over-expression was

defined as staining higher in more than 50% of cancer cells compared to the matching normal cells, regardless of cytoplasmic or nuclear location. Underexpression was defined as no staining or staining positivity lower than that of the matching normal tissue, and normal expression was defined as a level of staining positivity similar to that of the matching normal tissue. For slides that were heterogeneously stained within a tumor, we graded the highest intensity within the tumor.

Statistical analysis

The BRB-Array Tools system was used for the analysis of the microarray data. Statistical analysis were primarily performed with PASW Statistics 17.0 (SPSS Inc., Chicago, IL, United States). A χ^2 test was used to compare the difference between the groups. Kaplan-Meier plots and a log-rank test were used to estimate patient survival. Associations between the expression levels of the two targets and in-trans correlation were analyzed using the Pearson correlation coefficient. A *P* value of less than 0.05 was considered statistically significant, and all tests were two tailed.

RESULTS

Expression of *TXN* mRNA in microarray data for gastric cancer

First, a hierarchical clustering analysis was applied to the gene expression data from the 65 human primary tumor tissue samples. Unsupervised clustering revealed 2 distinctive subtypes with clear differences in overall gene expression patterns^[23]. Recurrence-free survival (RFS) was found to differ significantly between the 2 clusters (*P* = 0.001 by the log rank test), indicating that the molecular features of these tumors reflected in gene expression patterns might be strong independent predictors of clinical outcomes.

We next sought to identify genes whose expression was unique to the poor prognostic subgroup by cross-comparing gene lists. Between the two groups, 1258 genes were differentially expressed (*P* < 0.001) and presented more than a 1.5-fold difference between the two groups. Next, a univariate analysis identified 84 prognostic genes (*P* < 0.01). Of the 84 genes, *TXN* was a prognosis-related gene (*P* = 0.009 by univariate analysis) and was up-regulated in the poor prognostic group. From the correlation analysis between the expression levels of *TXN* and associated genes, we determined the relative correlations. *TXNIP*, a *TXN* inhibitor, demonstrated a negative correlation with *TXN* (*r* = -0.295, *P* = 0.024). The survival analysis based on the hierarchical clustering of *TXN*- or *TXNIP*-related genes demonstrated that each cluster influenced patient survival (*P* = 0.025 and *P* = 0.036, respectively) (Figure 1).

qRT-PCR validation of *TXN* and *TXNIP* expression

To determine if *TXN* and *TXNIP* gene expression was associated with prognosis among patients with the same

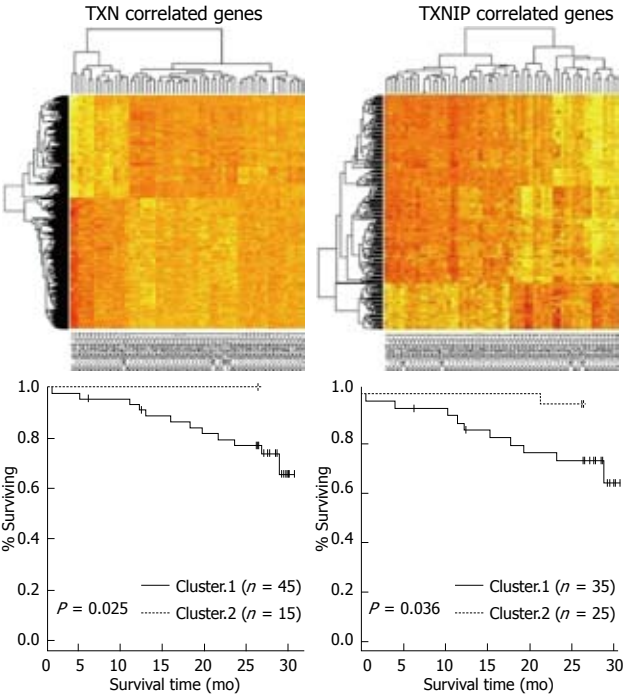


Figure 1 Survival analysis based on hierarchical clustering of thioredoxin or thioredoxin-interacting protein correlated genes.

stage of cancer, 68 stage III patients were randomly selected for qRT-PCR analysis (Table 1). The median follow-up duration after curative resection was 89.5 mo. By the last follow-up visit, 44 patients had experienced a recurrence, and 16 patients had died of gastric cancer. The patients were divided into high or low expression groups according to the TXN and TXNIP expression levels. RFS and overall survival (OS) were analyzed between the two groups (Figure 2A-D). Both TXN and TXNIP expression influenced RFS, and these associations were significant ($P = 0.008$ and $P = 0.036$, respectively). The patients in the high TXN expression or low TXNIP expression groups presented a poor prognosis. TXN expression also influenced OS ($P = 0.015$), but TXNIP expression did not have a significant relationship with OS ($P = 0.053$).

Stage III patients were classified into the following three combination groups based on the TXN and TXNIP expression levels: Group 1, simultaneously low TXN and high TXNIP levels ($n = 20$); Group 2, neither Group 1 nor Group 3 ($n = 35$); and Group 3, simultaneously high TXN and low TXNIP levels ($n = 13$). As expected, Group 1 exhibited a better prognosis than the other groups, and Group 3 presented with a poor prognosis (Figure 2E and F; $P < 0.001$ for RFS and $P = 0.001$ for OS). More than half of the Group 3 patients experienced a recurrence within 1 year after curative surgery, and their 5-year survival rate was one-third (29%) that of Group 1 (89%) (Table 2). There was no relationship between the TXN/TXNIP expression levels and either tumor stage or histological cell type.

Immunohistochemical assay of TXN and TXNIP expression

The gastric glands were relatively well stained with the anti-TXN antibody regardless of the presence of cancer-

Table 2 Prognoses of stage III gastric cancer patients according to the thioredoxin and thioredoxin-interacting protein gene expression levels

Group	Mean RFS, (95% CI), mo	3-yr recurrence-free rate (%)	5-yr survival rate (%)
1: Low TXN and high TXNIP	Non applicable	70	89
2: Neither group 1 nor group 3	36.7 (15.5-57.9)	51	73
3: High TXN and low TXNIP	11.9 (9.5-14.2)	7	29
P value by log rank test		$P < 0.001$	$P = 0.001$

RFS: Recurrence-free survival; TXN: Thioredoxin; TXNIP: Thioredoxin-interacting protein.

Table 3 Protein expression of thioredoxin and thioredoxin-interacting protein in gastric cancers

Characteristic (n)	TXN overexpression n (%)	TXNIP underexpression n (%)
Total (328)	213 (65)	278 (85)
Histologic type		
Diffuse type (228)	149 (65)	201 (88)
Intestinal type (100)	64 (64)	77 (77)
	$P = 0.9$	$P = 0.012$
Stage		
Stage I / II (180)	116 (64)	142 (79)
Stage III / IV (148)	97 (66)	136 (92)
	$P = 0.767$	$P = 0.009$

TXN: Thioredoxin; TXNIP: Thioredoxin-interacting protein.

ous and non-cancerous lesions based on the immunohistochemical assay results. Thioredoxin was overexpressed in approximately 65% of cancer tissues regardless of the histological type and TNM stage (Table 3). Thioredoxin staining was observed in the cytoplasm or nucleus or in both areas of the cancer cells. The histological location of thioredoxin in the cytoplasm or the nucleus in cancer tissues varied among patients, and there was heterogeneity in the staining intensity within the individual samples. In contrast to the staining with the anti-thioredoxin antibody, the staining of the gastric cancer tissue with the anti-TXNIP antibody was weak. TXNIP was underexpressed in almost 85% of the cancer cells and was found in the cytoplasm or nucleus of the cancer cells. There was a tendency for TXNIP to be underexpressed in two poor prognosis groups: diffuse histology type ($P = 0.009$) and high-stage gastric cancer ($P = 0.011$) (Table 3). Representative immunohistochemical staining results are shown in Figure 3.

Bioinformatic in-trans correlation analysis of TXN and TXNIP expression

Approximately 7000 genes were significantly correlated with TXN according to the bioinformatic in-trans correlation analysis of TXN expression with whole mRNA-expressing genes ($P < 0.001$). These findings suggest that TXN plays an important role in gastric cancer. Glutaredoxin 2 (GLRX2) and peroxiredoxin 4 (PRDX4) were highly correlated with TXN ($r = 0.76$, $P < 0.001$ and $r = 0.72$, $P < 0.001$, respectively; Table 4), and this correlation explains the function of TXN as a reactive

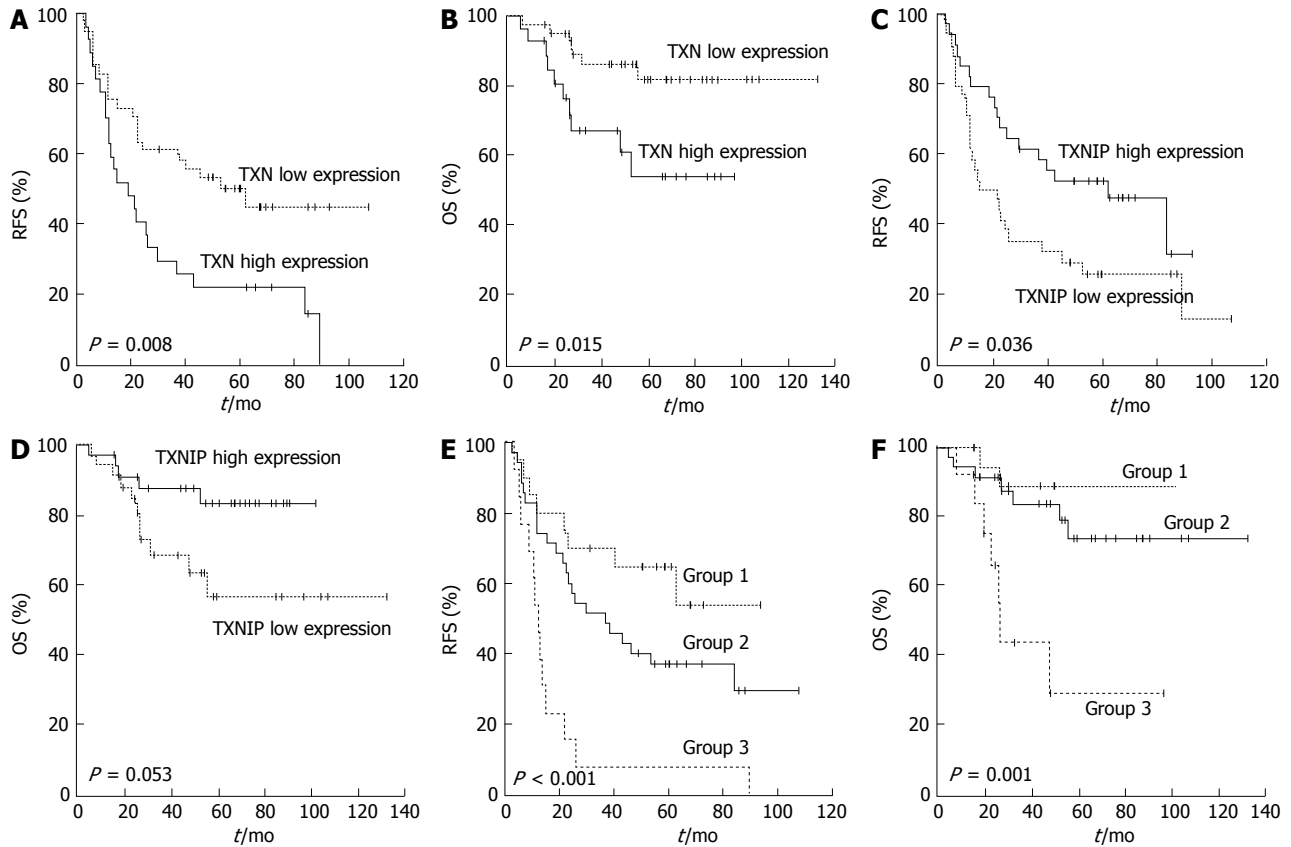


Figure 2 Kaplan-Meier plot of recurrence-free survival and overall survival according to gene expression. Gene expression was measured using quantitative reverse transcription-polymerase chain reaction. Stage III gastric cancer patients were divided into two or three groups according to their gene expression levels. A, B: Survival curves according to thioredoxin (TXN) expression level; C, D: Survival curves according to thioredoxin-interacting protein (TXNIP) expression levels; E, F: Survival curves according to the combination of the TXN and TXNIP expression levels (Group 1: Simultaneous low TXN and high TXNIP expression; Group 2: Neither group 1 nor group 3; Group 3: Simultaneous high TXN and low TXNIP expression). The prognosis was compared across the groups using the log-rank test.

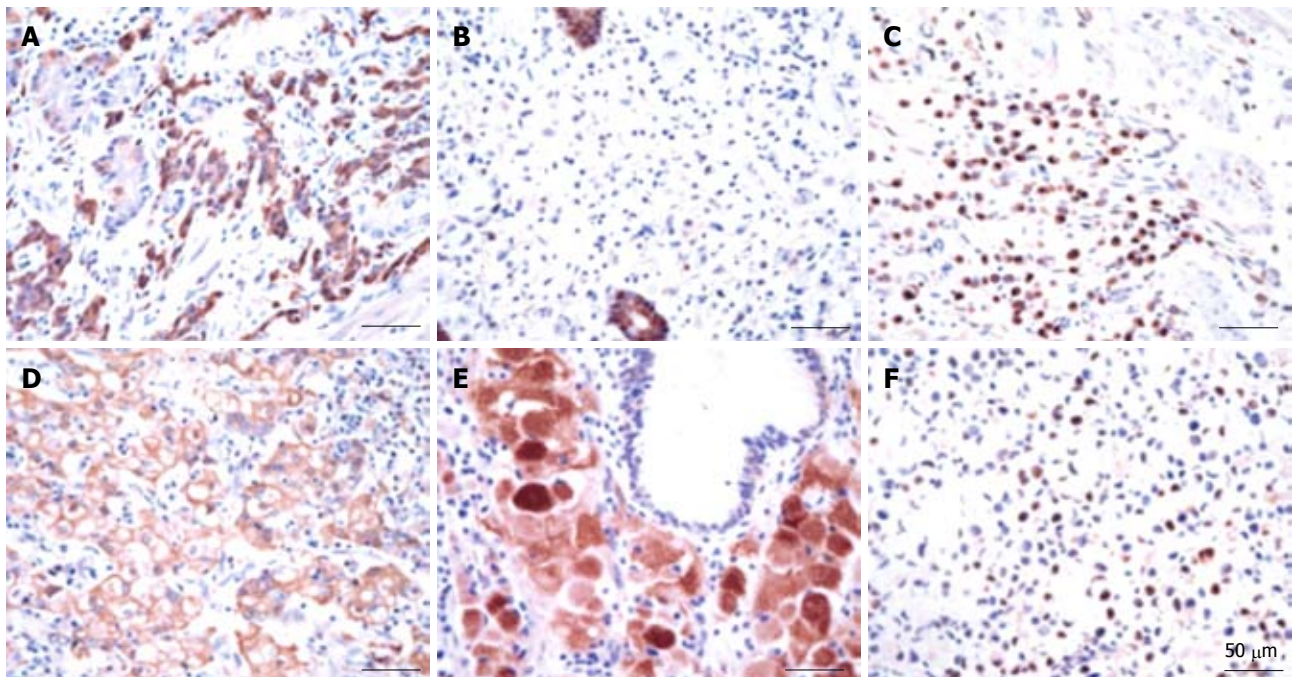


Figure 3 Representative images of immunohistochemical assay (×400). A: The cytoplasm of gastric cancer cells was strongly stained with the anti-thioredoxin (TXN) Ab; B: The cytoplasm of gastric cancer cells was not stained with the anti-TXN Ab; C: The nuclei of gastric cancer cells were strongly stained with the anti-TXN Ab; D: The nuclei of gastric cancer cells were not stained with the anti-TXN Ab; E: The cytoplasm of gastric cancer cells was strongly stained with the anti-thioredoxin-interacting protein (TXNIP) Ab, but the nuclei were not stained; F: The nuclei of gastric cancer cells were strongly stained with the anti-TXNIP Ab, but the cytoplasm was not stained.

Table 4 In-trans correlation analysis of thioredoxin and thioredoxin-interacting protein with whole mRNA expressing genes

Symbol	Gene name	Correlation with TXN		Correlation with TXNIP	
		<i>r</i>	<i>P</i> value	<i>r</i>	<i>P</i> value
TXN	Thioredoxin	1.000	0.000	-0.295	0.022
RPL6	Ribosomal protein L6	0.779	< 0.001	-0.335	0.009
GLRX2	Glutaredoxin 2	0.764	< 0.001	-0.314	0.015
RPL29	Ribosomal protein L29	0.757	< 0.001	-0.360	0.005
MRPL22	Mitochondrial ribosomal protein L22	0.751	< 0.001	-0.225	0.085
MRPL42	Mitochondrial ribosomal protein L42	0.734	< 0.001	-0.181	0.166
MRPS17	Mitochondrial ribosomal protein S17	0.732	< 0.001	-0.387	0.002
ATP5S	ATP synthase-coupling factor B	0.728	< 0.001	-0.182	0.165
PRDX4	Peroxioredoxin 4	0.722	< 0.001	-0.321	0.012
AURKA	Aurora kinase A	0.611	< 0.001	-0.486	< 0.001
ERO1L	ERO1-like	0.586	< 0.001	-0.200	0.125
HIG2	Chromosome 7 open reading frame 68	0.574	< 0.001	-0.089	0.498
CCNB1	Cyclin B1	0.563	< 0.001	-0.503	< 0.001
ERBB3	HER3	0.004	0.975	-0.536	< 0.001
TXNIP	Thioredoxin interacting protein	-0.295	0.022	1.000	0.000
ATG12	ATG12 autophagy related 12 homolog	-0.482	< 0.001	0.072	0.582
ATG16L2	ATG16 autophagy related 16-like 2	-0.722	< 0.001	0.216	0.098
ATG10	Autophagy related 10 homolog	-0.756	< 0.001	0.266	0.040

TXN: Thioredoxin; TXNIP: Thioredoxin-interacting protein.

oxygen species (ROS) scavenger with these known redox molecules. Ribosomal proteins and mitochondrial ribosomal proteins (i.e., RPL6, RPL29, MRPL22, MRPL42, MRPS17 and ATP5S) were highly correlated with TXN ($P < 0.001$; Table 4), which suggests the active involvement of TXN in protein synthesis and mitochondrial ATP synthesis for energy production. TXNIP was negatively correlated with TXN and known poor prognostic markers, such as AURKA, ERBB3, CCNB1, and many genes that are significantly correlated with TXN (Table 4). These results indirectly support the functional role of the TXNIP in the inhibition of TXN.

DISCUSSION

We demonstrated that TXN and TXNIP are poor and good prognostic gastric cancer markers, respectively. In stage III patients who underwent curative gastrectomy, the 3-year recurrence-free rate ($P < 0.001$) and the 5-year survival rate ($P = 0.001$) were significantly different between patients with different TXN and TXNIP expression levels (Table 2). High TXN and low TXNIP patients had a definitively poor prognosis. Most of these patients experienced a recurrence within 2 years and died within 4 years. Thus, it is necessary to perform intensive treatment and plan post-adjuvant treatment for these patients.

The gene expression microarray data imply that TXN plays an important role in gastric cancer. TXN functions as a ROS scavenger with known redox molecules, such as GLRX2 and PRDX4, and TXN is actively involved in protein synthesis and mitochondrial ATP synthesis for energy production. Hypoxia-induced genes [i.e., ERO1L and hypoxia inducible gene 2 (*HIG2*)] were significantly correlated with TXN. Tumors with high TXN expression also exhibited elevated *ERO1L* and *HIG2* levels; therefore, these tumors are in a relatively high hypoxic state

compared with tumors with low TXN expression. Additionally, autophagy-related genes (i.e., *ATG10*, *ATG16L2*, and *ATG12*) were negatively correlated with TXN expression, which indirectly suggests that TXN is involved in autophagic inhibition in a hypoxic cancer state. Autophagy is associated with cancer pathogenesis and chemotherapy resistance; therefore, the function of TXN in autophagy should be elucidated through additional studies.

Grogan *et al.*^[25] demonstrated that TXN was localized to tumor cells and was overexpressed in gastric cancer tissues compared with the levels in normal gastric mucosa. TXN overexpression was typically found in both the nucleus and the cytoplasm of neoplastic cells. These findings are consistent with our immunohistochemical staining results. Furthermore, high TXN expression was observed regardless of the gastric cancer stage or cell type.

Our study confirmed that TXNIP was significantly underexpressed in gastric cancer tissues compared with normal tissues; furthermore, it was expressed at the lowest levels in cancer patients with poor prognoses. TXNIP is a known potent tumor suppressor whose expression is markedly decreased in various human cancers, including gastric cancer. Knockout of the TXNIP gene in a mouse model was associated with *H. pylori*-related gastric cancer^[22]. In an *in vitro* experiment, TXNIP overexpression in pancreatic cells resulted in a higher basal level of apoptosis and an increased sensitivity to cisplatin and oxaliplatin^[26]. In microarray data analysis, TXNIP expression was negatively correlated with the expression of TXN expression, known as poor prognostic cancer biomarkers^[27-29], and many genes that are significantly correlated with TXN. These results indirectly confirm the functional role of the TXNIP in TXN inhibition. Additionally, autophagy-related genes were correlated with TXNIP, and these data support that TXNIP induces cancer cell autophagy.

In this study, we analyzed the gene expression profile

of human gastric cancer to identify potential biomarkers that could be used to classify patients according to prognosis after curative resection. We found that TXN and TXNIP were significantly associated with prognosis. TXN up-regulation and the simultaneous down-regulation of TXNIP were associated with a poor prognosis in gastric cancer patients. Bioinformatic analysis revealed that TXN and TXNIP were highly correlated with many oncogenes and tumor suppressor genes and demonstrated that TXN and TXNIP were associated with genes related to energy, protein synthesis and the modulation of autophagy under hypoxic or other stressful conditions. One of the limitations of this study is that we did not elucidate how TXN and TXNIP affect the recurrence of gastric cancer after curative resection. Further investigation of TXN and TXNIP in gastric cancer would likely identify unknown pathogenic mechanisms. In addition, because our results were derived from retrospective assessment, it is necessary to utilize *TXN* and *TXNIP* gene expression-based prediction in a prospective randomized trial(s) to validate the true clinical relevance of TXN and TXNIP. It will be beneficial to identify effective anti-tumor treatments other than the current standard adjuvant chemotherapy for gastric cancer patients with potent recurrence factor, high TXN and low TXNIP expression. Furthermore, TXN-targeted agents or those that up-modulate TXNIP could be used in targeted therapy in the treatment of gastric cancer patients who are selected based on biomarker gene signatures.

In conclusion, TXN and TXNIP are promising prognostic markers for gastric cancer, and performing personalized adjuvant treatment based on TXN and TXNIP expression levels would be an effective practice in the treatment of gastric cancer.

COMMENTS

Background

The standard of treatment for gastric cancer is surgery. After curative resection, even among individuals with the same stage of cancer, diverse recurrence patterns are present. However, uniform adjuvant treatment after curative resection has been performed. Authors evaluated the use of thioredoxin (TXN) expression combined with thioredoxin-interacting protein (TXNIP) expression as prognostic markers to individualize the postoperative treatment strategy following gastric cancer removal.

Research frontiers

TXN is putative oncoprotein that provides growth and survival advantages to tumor cells through the activation of redox-sensitive transcription factors. TXNIP is a potent tumor suppressor and inhibits the interaction between TXN and other factors. They have been recognized as cancer-related markers in diverse malignancies.

Innovations and breakthroughs

High TXN and low TXNIP expression in human gastric cancer tissue definitively related with poor prognosis of gastric cancer patients who had undergone curative resection.

Applications

The establishment of adjuvant treatment strategy based on our discovery would be useful to accomplish better outcomes after surgical treatment. The patients with high TXN and low TXNIP expressions in cancer tissue require novel treatment and should be more carefully monitored after surgery.

Terminology

Redox (reduction-oxidation) reactions: Many important biological processes involve redox reactions. Free radicals are a part of redox molecules and can be-

come harmful to the human body if they do not reattach to the redox molecule or an antioxidant. Unsatisfied free radicals can trigger the mutation of cells they encounter and are thus cancer causing.

Peer review

This study investigated the potential of TXN and TXNIP genes as a prognostic marker after curative resection of gastric cancer. Patients with high TXN and low TXNIP expression in cancer tissue manifested significantly more recurrence and shorter survival. However, it is necessary to validate through the prospective trial and to elucidate the functional mechanism of TXN and TXNIP.

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Photodynamic therapy prolongs metal stent patency in patients with unresectable hilar cholangiocarcinoma

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Abstract

AIM: To evaluate the effect of photodynamic therapy (PDT) on metal stent patency in patients with unresectable hilar cholangiocarcinoma (CC).

METHODS: This was a retrospective analysis of patients with hilar CC referred to our institution from December, 1999 to January, 2011. Out of 232 patients, thirty-three patients with unresectable hilar CC were treated. Eighteen patients in the PDT group were treated with uncovered metal stents after one session of PDT. Fifteen patients in the control group were treated with metal stents alone. Porfimer sodium (2 mg/kg) was administered intravenously to PDT patients. Forty-eight hours later, PDT was administered using a diffusing fiber that was advanced across the tumor by either endoscopic retrograde cholangiopancreatography or percutaneous cholangiography. After performance of PDT, uncovered metal stents were inserted to ensure

adequate decompression and bile drainage. Patient survival rates and cumulative stent patency were calculated using Kaplan-Meier analysis with the log-rank test.

RESULTS: The PDT and control patients were comparable with respect to age, gender, health status, pre-treatment bilirubin, and hilar CC stage. When compared to control, the PDT group was associated with significantly prolonged stent patency (median 244 ± 66 and 177 ± 45 d, respectively, $P = 0.002$) and longer patient survival (median 356 ± 213 and 230 ± 73 d, respectively, $P = 0.006$). Early complication rates were similar between the groups (PDT group 17%, control group 13%) and all patients were treated conservatively. Stent malfunctions occurred in 14 PDT patients (78%) and 12 control patients (80%). Of these 26 patients, twenty-two were treated endoscopically and four were treated with external drainage.

CONCLUSION: Metal stenting after one session of PDT may be safe with acceptable complication rates. The PDT group was associated with a significantly longer stent patency than the control group in patients with unresectable hilar CC.

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Key words: Bile duct cancer; Palliative endoscopic stenting; Photodynamic therapy; Outcome

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INTRODUCTION

Hilar cholangiocarcinoma (CC) is a rare tumor that is asymptomatic early on, making it difficult to diagnose, and is associated with a high mortality^[1]. Surgical resection with negative histologic margins is the most robust predictor of long-term survival. However, due to the lack of characteristic early symptoms, a definitive diagnosis is often not reached until the tumor is at an advanced stage. As a result, a large proportion of patients are beyond the scope of curative treatment by the time of diagnosis, and only palliative management is possible^[2]. Palliative therapies for unresectable CC such as stent, radiotherapy and chemotherapy, have generally been disappointing in prolonging life^[3]. Endoscopic or percutaneous biliary drainage alleviates jaundice, but there is no evidence that it prolongs survival.

Although endoscopic biliary metal stenting is the mainstay of palliative treatment in patients with unresectable hilar CC^[4], tumor ingrowth or overgrowth is a significant problem in uncovered stents. In particular, the duration of metal stent patency for hilar CCs was shorter than that for distal bile duct cancer because the malignant hilar stricture provides an acute angle that hinders full expansion of the metal stent and promotes biliary sludge formation within. Several clinical trials have reported the therapeutic effect of photodynamic therapy (PDT) for unresectable hilar CC^[5,6]. The ability of PDT to destroy cancer and neovascular cells may prolong biliary stent patency. However, the effect of PDT on stent patency has not yet been determined. Therefore, the primary aim of this study was to explore the effect of PDT on stent patency in unresectable hilar CC. We also evaluated overall survival and procedure-related complications after PDT compared with endoscopic biliary drainage alone for unresectable hilar CCs.

MATERIALS AND METHODS

Patients

This was a non-randomized, retrospective study. From December, 1999 to January, 2011, 232 patients with hilar cholangiocarcinoma were referred to our institution. This study included patients with unresectable hilar CC without chemoradiation who were palliated with only metal stents (control group) or with one session of PDT followed by metal stents (PDT group). Patients were deemed inoperable if they met the following criteria: the presence of type Bismuth-Corlette IV lesions, advanced type III lesions containing T3 tumors, or other types II-III tumors if surgery was contraindicated due to lymph nodes or liver metastases; and age > 80 years or the presence of other co-morbid conditions. Among these patients, metal stents were placed when they met the following conditions: the patient did not want an additional PDT due to the high cost, the patient was referred from a distant location; and there was a change to poor performance status during follow-up. We included patients with lymph node and liver metastasis but excluded those

with extrahepatic distant metastasis whose prognoses are expected to be extremely poor.

Using these criteria, 199 patients were excluded because of surgery ($n = 38$), a plastic stent ($n = 72$), palliative chemotherapy or radiotherapy ($n = 30$), more than two sessions of PDT ($n = 21$), a Karnofsky performance status of less than 60 ($n = 10$), percutaneous biliary drainage only ($n = 11$), unavailability for follow-up ($n = 4$), refusal of endoscopic treatment ($n = 12$) and sudden death during diagnostic workup ($n = 1$). After these exclusions, 33 patients with unresectable hilar CC were included in this study. Eighteen patients were treated using uncovered metal stents after one session of PDT (PDT group) and 15 patients were treated by endoscopic metal stenting alone (control group). All patients underwent a standard pretreatment evaluation that included thin-section, contrast-enhanced, multiphase spiral computed tomography and/or magnetic resonance imaging of the abdomen. Tissue diagnosis was obtained by endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous transhepatic cholangioscopy (PTCS) or direct peroral cholangioscopy with biopsy and/or cytology. Patients with no histological confirmation were diagnosed as having malignant disease on the basis of clinical outcome during follow-up for at least 12 mo. All tumors were staged using the American Joint Committee (7th edition) on Cancer Staging criteria for perihilar bile duct cancer^[7]. Clinical, laboratory, radiological, endoscopic, and histopathologic data were collected prospectively and analyzed retrospectively. The local institutional review board approved this study.

Photodynamic therapy

PDT was administered by methods described previously^[8,9]. Briefly, patients received porfimer sodium (Photofrin II, Axcan Pharma, Quebec, Canada) intravenously at a dose of 2 mg/kg 48-h before ERCP or PTCS. For light distribution, we used flexible cylindrical diffuser probes (BioLitec, Stirling, Scotland) mounted on a 400- μ m quartz fiber with an active distal tip length of 2 cm. The light source was a diode laser system (Ceralas PDT 633; CeramOptec, Bonn, Germany) with a maximum power output of 2 W and a wavelength of 633 ± 3 nm. The power emitted by the diffuser tip was calibrated to 400 mW/cm before PDT was conducted using an integrating sphere power meter. The mean irradiation time was 452 s (range: 400-600 s), using a power density of 300-400 mW/cm and energy dose of 180-200 J/cm (of diffuser length). The PDT with PTCS was usually applicable for more advanced Bismuth type-III lesions. To perform the percutaneous PDT procedure, a guide wire was first inserted through the stricture into the common bile duct. A 6-F guiding catheter was then inserted along the guide wire, after which the guide wire was removed. A diffuser fiber was then inserted into the guiding catheter and the stricture site was irradiated from the distal to the proximal region under cholangioscopy and fluoroscopy.

Peroral PDT with ERCP was performed in patients who had coagulopathy or refused percutaneous mo-

dality or bismuth II and some of III. To perform the peroral PDT, the preloaded catheter (catheter and PDT fiber) was advanced across the bile duct tumor using a 0.035-inch guide wire. The tip of the catheter was cut just below its metal marker, allowing the fiber to pass. Tumor segments were treated sequentially proximal to distal. In both percutaneous or peroral PDT, PDT was performed in both sides of the intrahepatic duct in Bismuth III and IV strictures. In the case of multiple strictures, as many second branches as possible were treated. After PDT was performed, uncovered metal stents (MI tech, Seoul, South Korea) with diameters of 10 mm and stent lengths of 6 and 8 cm were inserted to ensure adequate decompression and bile drainage. In patients with percutaneous PDT, metal stent was inserted using the Rendezvous technique.

Definition of events and follow-up

The clinical outcome was evaluated according to the following parameters: (1) functional success, as measured by a decrease in the bilirubin level to less than 50% of the pretreatment value within 7 d; (2) early complications (occurring within 30 d); (3) late complications including stent malfunction; and (4) revision methods such as endoscopic retrograde biliary drainage or percutaneous transhepatic biliary drainage (PTBD). Stent patency was calculated as the interval between stent insertion and stent occlusion. Stent occlusion was defined as signs of jaundice or cholangitis (e.g., fever, tenderness in the right upper quadrant, and/or > 2-fold elevation of the total serum bilirubin above baseline after stent insertion).

Follow-up of symptoms and biochemical parameters was done at 1 mo intervals. Tumor markers for carbohydrate antigen (CA) 19-9 and abdominal computed tomography scans were done every 3 mo. ERCP was performed to confirm obstruction and to perform biliary decompression if a new episode of cholangitis occurred. Enrolled patients were followed until death. If the patient died with a patent stent, the time interval was recorded as censored data.

Statistical analysis

Statistical analysis were performed using SPSS software (ver. 18.0; SPSS Inc., Chicago, IL). Numerical data are presented as the median with range. Intergroup comparisons were performed using the Mann-Whitney *U* test. Estimates of probabilities of survival for the follow-up study and cumulative stent patency were calculated using the Kaplan-Meier method with the log-rank test. Data are presented as medians and 95% CI. For survival rates, all deaths related to CC and procedures were included, but deaths unrelated to CC were treated as censored patients. All of the data were analyzed using an intention-to-treat analysis. Intention-to-treat survival was calculated from the day of treatment until death or the last follow-up. *P* values ≤ 0.05 were deemed to indicate statistical significance.

Table 1 Patient characteristics *n* (%)

	PDT group (PDT with metal stenting) (<i>n</i> = 18)	Control group (metal stent only) (<i>n</i> = 15)
Age, yr (range)	65.6 (44-89)	67 (53-82)
Male sex	12 (66)	12 (80)
AJCC stage ¹		
II	3 (17)	0 (0)
III A/III B	0 (0)/6 (33)	3 (20)/5 (33)
IV A/IV B	7 (39)/2 (11)	1 (7)/6 (40)
Bismuth type		
II	3 (17)	4 (27)
III	5 (28)	7 (47)
IV	10 (55)	4 (27)
Lymph node metastasis	13 (72)	10 (67)
Liver metastasis	2 (11)	2 (17)
CA 19-9 (U/mL)	229 (0.9-4800)	480 (1.3-4800)
Pre-albumin (g/dL)	3.5 (2.1-4.5)	3.2 (2.0-4.3)
Bilirubin (mg/dL)		
Pre-treatment	8.5 (0.6-31.9)	12.3 (0.9-26.0)
Post-treatment	1.3 (0.5-14.3)	2.9 (0.3-26.0)
Successful drainage ²	15 (83)	12 (86)
Histologic confirmation	14 (78)	10 (67)
Unilateral/bilateral stenting	10 (55)/8 (45)	11 (73)/4 (27)
Early complications of procedure	3 (17)	2 (13)
Stent malfunctions	14 (78)	12 (80)
Follow-up period (median, d)	437	230

¹American Joint Committee Cancer (AJCC) staging 7th edition; ²Decrease of bilirubin to less than 50% of the pretreatment value within 7 d. Data are expressed as medians (range). PDT: Photodynamic therapy; CA: Carbohydrate antigen.

RESULTS

Thirty-three patients (24 male), mean age 67.9 years (range: 44-89 years) were treated. A total of 18 (55%) patients received PDT (percutaneous PDT = 11, peroral PDT = 7) and metal stenting, whereas 15 (45%) underwent metal stenting alone. Baseline clinical and demographic profiles in the two treatment groups were similar, except for bismuth type IV (Table 1). There were more patients with bismuth type IV in the PDT group than in the control group (10/18 *vs* 4/15; *P* = 0.025). There were no significant differences between the groups with regard to age, gender, pre-procedure bilirubin, CA 19-9, stent length, or tumor stage. The diagnosis was confirmed histologically in 14 PDT patients (78%) and 10 control patients (67%). Histological confirmation of malignancy in PDT group was obtained by brush cytology in three ERCP patients and by intraductal biopsy examination in 11 PTCS patients. In the control group, histological confirmation was obtained by brush cytology in nine ERCP patients, and by intraductal biopsy in one direct cholangioscopy (using the mother-baby scope technique). Successful drainage was achieved in 83% and 86% of patients in PDT and control patients, respectively. No significant difference was observed in the degree of decrease of total bilirubin between the two groups (*P* = 0.2).

Stent patency and survival

The metal stent patency duration was longer in the PDT

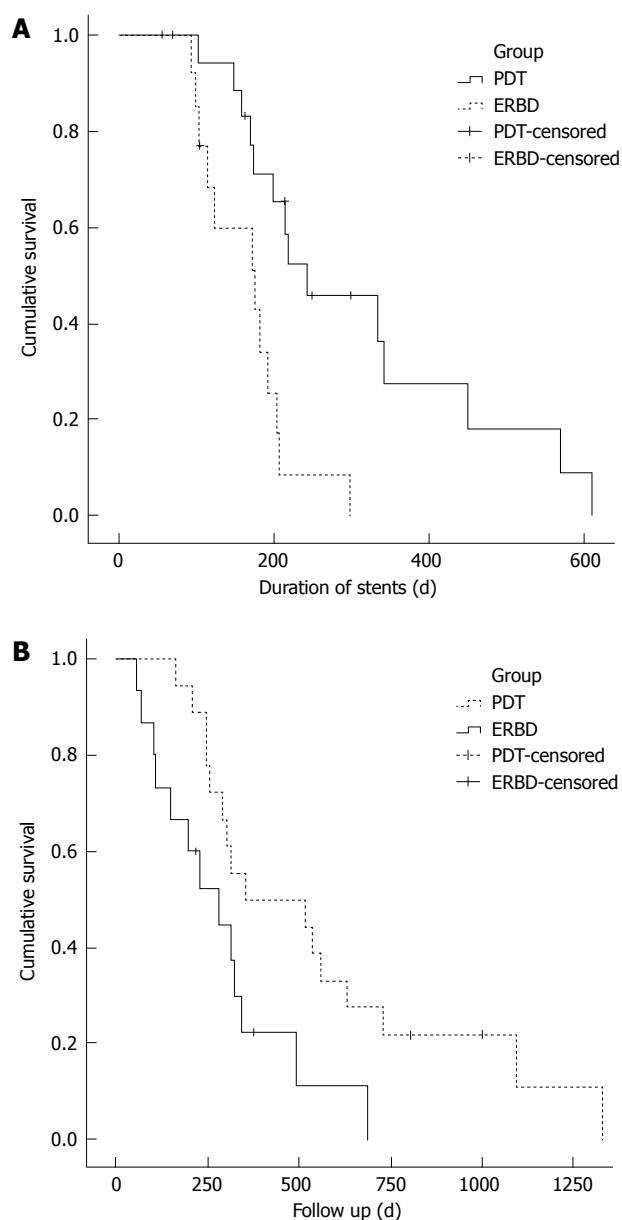


Figure 1 Kaplan-Meier analysis of metal stent patency rates and overall survival in the photodynamic therapy and control groups. A: Kaplan-Meier analysis of metal stent patency rates. The median stent patency was longer in the photodynamic therapy (PDT) group than in the control group (244 d vs 177 d; $P = 0.002$); B: Kaplan-Meier analysis of overall survival. The median survival in the PDT group was 356 d, vs 230 d in the stent-only group ($P = 0.006$). ERBD: Endoscopic retrograde biliary drainage.

group than in controls (Figure 1A). The median stent patency (range) was 244 ± 66 d (72-570 d) in the PDT group and 177 ± 45 d (70-309 d) in the controls ($P = 0.002$). Table 2 shows the results of univariate analysis of all variables associated with stent patency in all patients. PDT treatment ($P = 0.002$) was a significant predictive factor of longer stent patency on univariate analysis (Table 2). Factors including age, gender, bismuth type, pretreatment CA 19-9 and total bilirubin, T stage, number of endoprostheses (unilateral vs bilateral), and PDT method (PTCS vs ERCP) did not significantly affect stent patency according to univariate analysis.

Table 2 Univariate analysis of all prognostic factors associated with stent patency and patient survival in all patients

Variables	Univariate (Kaplan-Meier and log-rank test)				
	Cases	Median stent patency (d)	P value	Median patient survival (d)	P value
Age (yr)					
< 65 vs ≥ 65	12:21	174:206	0.524	539:290	0.103
Gender					
Male vs female	24:9	193:298	0.278	322:303	0.766
CA 19-9 (U/mL)					
< 100 vs ≥ 100	7:26	215:177	0.308	250:317	0.145
Pre-PDT bilirubin (mg/dL)					
< 3.0 vs ≥ 3.0	15:18	244:181	0.112	493:250	0.026
T stage ¹					
T1,2 vs T3,4	11:22	174:207	0.388	317:283	0.527
Number of endoprostheses					
Unilateral vs bilateral	21:12	206:193	0.454	317:322	0.758
Bismuth type					
II, III vs IV	19:14	206:193	0.644	283:356	0.551
PDT method					
PTCS vs ERCP	11:22	200:334	0.075	322:214	0.621
Treatment group					
PDT vs ERBD	18:15	244:177	0.002	356:283	0.023

¹American Joint Committee Cancer (AJCC) staging, 7th edition. PDT: Photodynamic therapy; PTCS: Percutaneous cholangioscopy; ERBD: Endoscopic retrograde biliary drainage; CA: Carbohydrate antigen; ERCP: Endoscopic retrograde cholangiopancreatography.

Compared with the controls, the PDT group was associated with longer patient survival (Figure 1B). The median survival time (range) was 356 ± 213 d (163-1330 d) in the PDT group and 230 ± 73 d (56-687 d) in the controls ($P = 0.006$). Table 2 shows the results of the univariate analysis of all variables considered. Statistically significant predictors of longer survival were PDT treatment and a lower total bilirubin level before the procedure.

Complications

Early complications were experienced by two control patients (13%) and three (17%) in the PDT group. In the control group, cholangitis occurred in 2 patients (13%), while in the PDT group, cholangitis and cholecystitis were each observed in one patient (6%). PDT-specific adverse events occurred in one patient in the PDT group, who experienced dermal phototoxicity. No stent migration occurred in either group.

Stent malfunctions occurred in 14 (78%) and 12 (80%) of the control and PDT patients, respectively. Among these 26 patients, 22 (85%) with tumor ingrowth were treated endoscopically with insertion of one or two plastic stents (10F) into unilateral or bilateral ducts through metal stents. In four patients with tumor recurrence, tumor ingrowth and overgrowth occurred in the hilar portion and more proximal intrahepatic duct, resulting in revisionary treatment with PTBD.

DISCUSSION

Hilar CC has an extremely poor prognosis, with an average five-year survival rate of 5%-10%. Surgery provides

the only possibility for a cure, but due to its anatomical location and natural history, the disease is locally advanced in most patients at the time of diagnosis. Therefore, effective palliation to alleviate symptoms associated with jaundice and the prevention of biliary sepsis are the fundamental goals for most patients with hilar CC^[5]. Although relief of biliary obstruction by endoscopic placement of metal stents is regarded as an optimal palliative measure in hilar CC, the clinical course after even successful stent insertion is one of disease progression and death from liver failure or cholangitis within 4-9 mo^[10]. This clinical course^[1] is related to the ability to decompress affected proximal segments^[2] and recurrent stent occlusion, because these stents are unable to remodel malignant tissues^[11].

PDT is an evolving therapy for treatment of cancers that are resistant to standard oncologic treatment. PDT involves the injection of an intravenous photosensitizing drug followed by endoscopic application of light to the tumor bed. The interaction between light and the photo-agent causes death of cancer cells and tumor thrombosis by generating oxygen free radicals. PDT is currently being used for cases of hilar cholangiocarcinoma^[12,13]. Even in patients with advanced hilar CC, PDT has been shown to improve survival, quality of life, and to have a performance superior to that of biliary stenting in uncontrolled and randomized controlled trials^[6,14-16]. In our study, Kaplan-Meier analysis demonstrated improved survival in the PDT group compared with the stent-only group (356 d *vs* 230 d, $P = 0.006$), in accordance with previous reports^[11,14,17].

Effective palliation is essential, because biliary drainage and prevention of cholestasis are crucial for prevention of pruritus, cholangitis, and death in patients with hilar CC. The approach to palliative decompression has evolved from surgery and percutaneous to endoscopic management in order to prevent cholestasis and improve mortality. Endoscopy of hilar CCs is generally challenging and complex due to the involvement of multiple bile ducts requiring two or more stents; indeed, patency rates of endobiliary stents are lower than those of distal tumors^[16,18,19]. Moreover, the efficacy of endoscopic stenting in a hilar CC is often limited by stent patency, which is related to proximal tumor obstruction, because the stent does not affect tissue remodeling, unlike benign conditions^[11,20,21]. To address this issue, multiple studies have investigated the positive effects of the combination of bile duct stenting with PDT on patient survival^[6,14,15]. However, a paucity of information exists regarding the effect of PDT on stent patency.

In our study, metal stent patency was longer in the PDT group than in the stent-only group. The median stent patency was 244 d in the PDT group and 177 d in the control group ($P = 0.002$). The main causes of obstruction of metal stents in bile ducts is tumor ingrowth or overgrowth^[22]. PDT offers the possibility of tumor "remodeling", which can enhance or prolong the decompression effect^[23]. Accepting this hypothesis, the

ability of PDT to destroy cancer cells and lessen cholestasis may prolong stent patency. In this context, this study is meaningful because the longer stent patency that is achieved by PDT may diminish the need for further procedures, such as stent revision or percutaneous biliary drainage, improving the quality of life of hilar CC patients whose prognosis is poor.

This study was limited because of its retrospective nature and small sample size. Thus it may be not possible to reach statistical significance in terms of differences in overall survival between the groups. Because hilar CC is a rare malignancy and PDT is offered at few tertiary centers in South Korea, inclusion of sufficient patients to complete a well-designed palliative study is problematic. However, the study population was derived from a larger cohort of patients with CC, who were followed until death. Median survival of this cohort was comparable to that of other cohorts reported in the literature, which may decrease the possibility of significant selection bias.

In summary, metal stenting after one session of PDT may be safe with acceptable complication rates. The PDT group was associated with a significantly longer stent patency period and patient survival compared with the control group in patients with unresectable hilar CC. A prospective randomized multicenter study is required to confirm these data.

COMMENTS

Background

Although endoscopic biliary metal stenting is the mainstay of palliative treatment in patients with unresectable hilar cholangiocarcinoma (CC), tumor ingrowth or overgrowth in uncovered stents is a significant problem. The duration of metal stent patency for hilar CCs was shorter than that for the distal bile duct. Photodynamic therapy (PDT) is an evolving therapy for treatment of hilar CCs that are resistant to standard oncologic treatment.

Research frontiers

The authors observed that the one session of PDT with metal stenting was associated with a significantly longer stent patency period and patient survival compared with the metal stent only group in patients with unresectable hilar CC.

Innovations and breakthroughs

Metal stent patency was longer in the PDT group than in the stent-only group. The median stent patency was 244 d in the PDT group and 177 d in the control group ($P = 0.002$). The ability of PDT to destroy cancer cells and lessen cholestasis may prolong stent patency.

Applications

Just one session of PDT with endoscopic retrograde cholangiopancreatography (ERCP) or percutaneous transhepatic cholangioscopy (PTCS) before metal stent placement is promising because it prolongs stent patency in unresectable hilar CC. A prospective randomized multicenter study is required to assess this technique.

Terminology

PDT is an emerging palliative strategy for unresectable hilar CC based on the intravenous administration of photosensitizing agents that preferentially accumulate in malignant cells. PDT is performed to the targeted area of bile ducts using ERCP or PTCS.

Peer review

PDT in patients with CC has been reported as effective palliative treatment providing improvement in cholestasis and quality of life, and prolonging survival. The authors conclude that metallic stenting after PDT may be a safe, acceptable modality associated with longer stent patency in patients with unresectable hilar CC.

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Surgical outcome of pancreatic cancer using radical antegrade modular pancreatectomy procedure

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Abstract

AIM: To evaluate the surgical outcomes following radical antegrade modular pancreatectomy (RAMPS) for pancreatic cancer.

METHODS: Twenty-four patients underwent RAMPS with curative intent between January 2005 and June 2009 at the National Cancer Center, South Korea. Clinicopathologic data, including age, sex, operative findings, pathologic results, adjuvant therapy, postoperative clinical course and follow-up data were retrospectively collected and analyzed for this study.

RESULTS: Twenty-one patients (87.5%) underwent distal pancreatectomy and 3 patients (12.5%) underwent total pancreatectomy using RAMPS. Nine patients (37.5%) underwent combined vessel resection, including 8 superior mesenteric-portal vein resections and 1 celiac axis resection. Two patients (8.3%) underwent

combined resection of other organs, including the colon, stomach or duodenum. Negative tangential margins were achieved in 22 patients (91.7%). The mean tumor diameter for all patients was 4.09 ± 2.15 cm. The 2 patients with positive margins had a mean diameter of 7.25 cm. The mean number of retrieved lymph nodes was 20.92 ± 11.24 and the node positivity rate was 70.8%. The median survival of the 24 patients was 18.23 ± 6.02 mo. Patients with negative margins had a median survival of 21.80 ± 5.30 mo and those with positive margins had a median survival of 6.47 mo ($P = 0.021$). Nine patients (37.5%) had postoperative complications, but there were no postoperative mortalities. Pancreatic fistula occurred in 4 patients (16.7%): 2 patients had a grade A fistula and 2 had a grade B fistula. On univariate analysis, histologic grade, positive tangential margin, pancreatic fistula and adjuvant therapy were significant prognostic factors for survival.

CONCLUSION: RAMPS is a feasible procedure for achieving negative tangential margins in patients with carcinoma of the body and tail of the pancreas.

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Key words: Carcinoma; Pancreas; Surgical resection; Survival; Radical antegrade modular pancreatectomy

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INTRODUCTION

Distal pancreatectomy, originally described at the Mayo Clinic in 1913, has been the standard procedure for carcinoma of the body or tail of the pancreas^[1]. This traditional approach is, however, associated with a high tangential margin positive rate and is not based on the physiologic lymphatic drainage of the pancreas^[2]. So far, there has been little progress in overcoming these shortcomings. Radical antegrade modular pancreatosplenectomy (RAMPS), which was first introduced by Strasberg *et al*^[3] in 2003 as a surgical treatment for carcinomas of the body and tail of the pancreas, is known as a feasible procedure for achieving a higher rate of negative tangential margins than the traditional approach. RAMPS is performed in a right-to-left fashion after early ligation of blood vessels, whereas pancreatosplenectomy is performed in a left-to-right fashion. In fact, RAMPS improves the tangential margin negative rate and provides better surgical exposure, especially in obese patients^[3].

Strasberg *et al*^[2] reported that negative tangential margins were obtained in 91% of all patients and that the 5-year overall survival rate was 26%. However, there have been few studies on the clinical outcomes following RAMPS^[4]. The purpose of this study is to evaluate the surgical outcomes following RAMPS for carcinoma of the body and tail of the pancreas.

MATERIALS AND METHODS

Patients

Twenty-four patients underwent RAMPS with curative intent between January 2005 and June 2009 at the National Cancer Center in South Korea. History-taking, physical examination, liver function tests, tumor marker (carbohydrate antigen 19-9) levels, and abdominal computed tomography (CT) scans were used for diagnostic and staging workup. A whole-body positron emission tomography scan was added when necessary. Clinico-pathologic data, including age, sex, operative findings, pathologic results, adjuvant therapy, postoperative clinical course and follow-up data were collected and analyzed retrospectively. A pancreatic fistula after surgery was defined based on the International Study Group of Pancreatic Fistula definition^[5]: a drain output of any measurable volume on or after postoperative day 3 with an amylase concentration greater than 3 times the serum amylase concentration. Postoperative complications were reviewed and graded using the Clavien-Dindo classification^[6].

Operative procedures

The operation was performed according to the procedure introduced by Strasberg *et al*^[2]. After dissection of the gastro-colic ligament, we entered the lesser omentum and dissected the celiac axis, hepatic artery, and trunk of the splenic artery to divide the splenic artery. The neck of the pancreas was divided using electrocautery, and the pan-

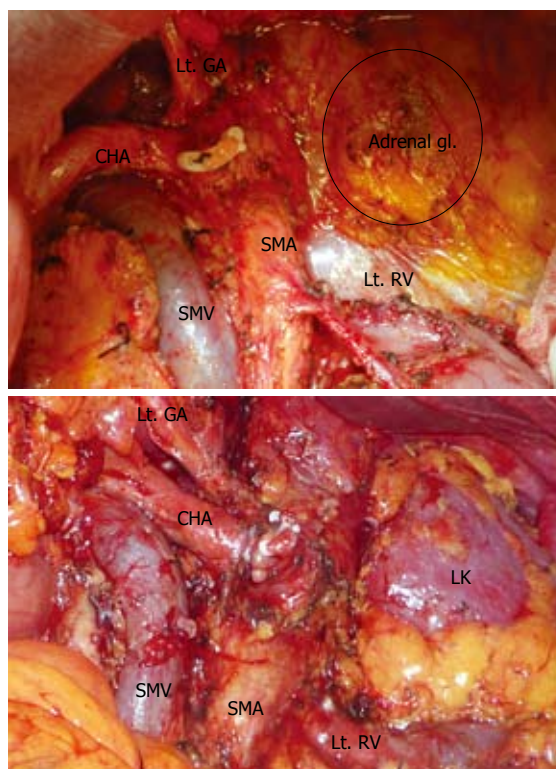


Figure 1 Photographs of operative field. A: Anterior radical antegrade modular pancreatosplenectomy (RAMPS); B: Posterior RAMPS. Lt. GA: Left gastric artery; CHA: Common hepatic artery; SMA: Superior mesenteric artery; SMV: Superior mesenteric vein; Lt. RV: Left renal vein; LK: Left kidney; gl: Gland.

creatic duct stump was ligated. After we divided the neck of the pancreas, the vertical plane of dissection reached the level of the aorta where the left renal vein was exposed. The left adrenal vein was ligated and divided when a posterior RAMPS was performed. During lymph node dissection, we removed regional nodes along the common hepatic and celiac artery. The soft tissue to the left of the hepatoduodenal ligament and superior mesenteric artery was also dissected. Lymph nodes along the splenic artery and splenic hilum were removed en-bloc with the specimen. Removal of the nerve plexus and lymph nodes was performed in the same manner (Figure 1).

Adjuvant therapy

We performed adjuvant concurrent chemoradiation therapy (CCRT) on all patients without severe medical comorbidities or poor physical status. Several studies have demonstrated the positive effects of CCRT^[7-17]. Twenty patients (83.3%) underwent adjuvant CCRT using multiple-field techniques. The initial irradiated field, which was defined as the tumor bed plus regional nodes, received 45 Gy in 25 fractions using a 4-field technique (anteroposterior, posteroanterior and paired laterals) with 15-MV X-rays. The tumor bed boost field received an additional 5.4-10.8 Gy in 3 to 6 fractions of 1.8 Gy. Concomitant 5-fluorouracil-based chemotherapy during radiotherapy was given to all patients. Patients were followed every 3 mo with CT scans and tumor marker levels to detect recurrent disease.

Table 1 Pathological results

Parameters	n (%)
Mean tumor diameter	4.09 ± 2.15 cm
Tumor diameter in negative margin (n = 22)	3.80 ± 1.73 cm
Tumor diameter in positive margin (n = 2)	7.25 cm
Histologic grade	
Well-differentiated	2 (8.3)
Moderately-differentiated	17 (70.8)
Poorly-differentiated	5 (20.8)
T stage	
T2	3 (12.5)
T3	21 (87.5)
Lymph node metastasis	
(+)	17 (70.8)
(-)	7 (29.2)
Vascular invasion	
(+)	12 (50)
(-)	12 (50)
Lymphatic invasion	
(+)	15 (62.5)
(-)	9 (37.5)
Perineural invasion	
(+)	21 (87.5)
(-)	3 (12.5)
Tangential margin	
(+)	2 (8.3)
(-)	22 (91.7)

Table 2 Postoperative complications

Complications	n (%)	Grade (number)
Pancreatic fistula	4 (16.7)	A (2), B (2) ¹
Wound dehiscence	2 (8.3)	IIIa (2) ²
Intraabdominal fluid collection	1 (4.2)	IIIa (1) ²
Pleural effusion	1 (4.2)	IIIa (1) ²
PV-SMV thrombosis	1 (4.2)	IIIa (1) ²

¹Graded by the International Study Group on Pancreatic Fistula classification; ²Graded by Clavien-Dindo classification. PV: Portal vein; SMV: Superior mesenteric vein.

Statistical analysis

The χ^2 test, the independent *t* test, the Kaplan-Meier method with the log-rank test and a Cox regression model were used for statistical analysis. A *P* value of less than 0.05 was considered significant. SPSS[®] version 19.0 (Chicago, IL, United States) was used for all statistical analyses. This study was approved by the Institutional Review Board of the National Cancer Center in South Korea.

RESULTS

Operative findings and histopathologic results

The mean age of the total 24 patients was 60.00 ± 7.79 years with a male:female ratio of 1.18:1. The mean operative time was 305.42 ± 155.68 min. One patient required a blood transfusion during surgery. Three patients (12.5%) who had cancer in the body of the pancreas with diffuse infiltration into the head and tail were converted to a total pancreatectomy. Nine patients (37.5%) underwent combined vessel resection, including 8 superior

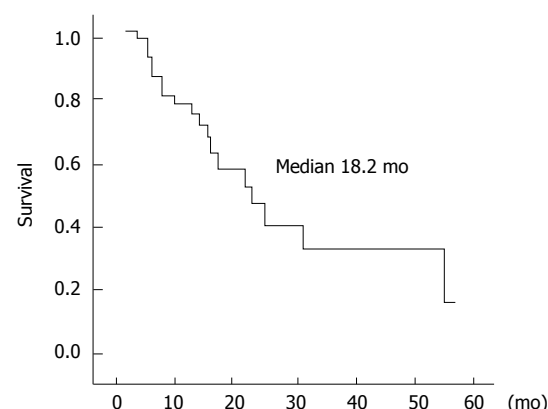


Figure 2 Overall survival curve of the 26 patients.

mesenteric-portal vein (SMV-PV) resections and 1 celiac axis resection. Two patients (8.3%) underwent combined resection of other organs, including the colon, stomach or jejunum. Posterior RAMPS, including resection of the adrenal gland, was performed in 5 patients (20.8%).

Histopathologic examination showed that 21 cases (87.5%) were T3. As for histologic grade, moderately differentiated adenocarcinoma was found in 17 patients (70.8%). The mean number of retrieved lymph nodes was 20.92 ± 11.24 and node positivity was observed in 17 patients (70.8%). The pancreatic parenchymal resection margin was negative for all patients with a mean distance of 16.52 ± 13.8 mm. Negative tangential margins were obtained in 22 patients (91.7%). The mean tumor diameter of all patients was 4.09 ± 2.15 cm. Two patients with positive tangential margins had a mean tumor diameter of 7.3 cm (Table 1). Among 9 patients with combined vessel resection, 1 patient had no evidence of vascular invasion on pathology.

Nine patients (37.5%) had postoperative complications (Table 2). Pancreatic fistula occurred in 4 patients (16.7%): 2 patients had a grade A fistula and 2 had a grade B fistula. Intra-abdominal fluid collections and grade B pancreatic fistulae were treated with percutaneous drainage and intravenous antibiotics. Patients with PV-SMV thrombosis were treated with percutaneous thrombectomy and stenting. There were no postoperative mortalities.

Survival and prognostic factors

In this study, the median survival was 18.23 ± 6.02 mo with a median follow-up period of 20.06 ± 14.46 mo (Figure 2). Twenty-one patients (87.5%) had recurrence at follow-up. Two patients (8.3%) had local recurrence, 14 patients (58.3%) had distant metastasis, and 5 patients (20.8%) had both.

Celiac axis resection (Appleby operation) was performed in 1 patient who died of recurrent disease 12.5 mo after surgery. Three patients who underwent total pancreatectomy had a median survival of 21.23 ± 4.25 mo which was similar to the median survival of all patients in this study. One patient was free of cancer at his last

Table 3 Prognostic factors by univariate analysis

Factors		n	Median survival (mo)	P value
Age	≤ 60 yr	11	21.84 ± 12.90	0.249
	> 60 yr	13	16.93 ± 8.54	
Gender	Male	13	21.80 ± 6.84	0.995
	Female	11	18.23 ± 4.77	
Transfusion	(+)	1	26.1	0.345
	(-)	23	19.79 ± 14.73	
Operative time	≤ 300 min	18	21.80 ± 7.42	0.534
	> 300 min	6	12.53 ± 6.96	
Type of RAMPS	Anterior	19	21.80 ± 4.34	0.421
	Posterior	5	12.53 ± 6.65	
Combined vascular resection	(+)	9	16.93 ± 5.48	0.333
	(-)	15	26.27 ± 10.81	
Tumor size	≤ 4 cm	15	26.27 ± 9.44	0.862
	> 4 cm	9	18.23 ± 4.91	
Histologic grade	WD	2	16.93	0.001
	MD	17	26.27 ± 5.87	
	PD	5	6.46 ± 1.06	
T-stage	2	3	21.17 ± 10.94	0.448
	3	21	18.23 ± 5.79	
N-stage	0	7	21.80 ± 16.32	0.485
	1	17	18.23 ± 4.70	
Tangential margin	(+)	2	6.46	0.031
	(-)	22	21.80 ± 5.30	
Complication	(+)	9	12.53 ± 4.77	0.385
	(-)	15	21.80 ± 4.05	
Pancreatic fistula	(+)	4	6.46 ± 1.52	0.003
	(-)	20	26.27 ± 6.49	
Adjuvant therapy	(+)	20	26.27 ± 6.49	< 0.001
	(-)	4	6.30 ± 0.48	

RAMPS: radical antegrade modular pancreateosplenectomy; WD: Well differentiated; MD: Moderately differentiated; PD: Poorly differentiated.

visit, while the other 2 patients had local or systemic recurrence.

Twenty patients (83.3%) underwent adjuvant CCRT. CCRT was also performed in patients with pancreatic fistula with the exception of 1 patient with grade A pancreatic fistula who was not a candidate because of age.

On univariate analysis, histologic grade, positive tangential margin, pancreatic fistula and adjuvant therapy were significant prognostic factors for survival (Table 3). Unfortunately, this study was not powered to show significant factors on multivariate analysis.

DISCUSSION

Because carcinomas of the body and tail of the pancreas are often found in a larger size than those of the head, unresectable cases are more common and the recurrence rate after resection is also higher^[18-22]. The goals of pancreatic cancer surgery are to obtain tumor-free margins and perform a sufficient regional lymphadenectomy. However, conventional distal pancreatectomy, which is performed in the left-to-right direction along the anterior border of Gerota's fascia, is inappropriate for achieving this goal because the tumor easily infiltrates the retroperitoneum and spreads to lymph nodes at an early stage. There have been few surgical methods to overcome these limitations. Yang *et al.*^[23] introduced retro-

grade distal pancreatectomy, which cuts the neck of the pancreas first and proceeds with dissection in the right-to-left direction. This procedure is a useful method for exposing the portal-superior mesenteric vein junction, which helps to avoid operative injuries. RAMPS was introduced with the theoretical advantages of obtaining a higher rate of negative tangential margins and a higher lymph node count. Therefore, we applied RAMPS to all distal pancreatectomy cases.

The margin-negative rate of RAMPS reported by Strasberg *et al.*^[2] and our institute was 91% and 91.7%, respectively, which is higher than the margin-negative rate of conventional distal pancreatectomy (70% to 80%)^[5,7,8]. In our study, only 2 patients showed positive resection margins. These 2 patients had a lower median survival than patients with negative margins (6.47 mo *vs* 21.80 ± 5.30 mo, *P* = 0.03). The first patient with a positive tangential margin underwent posterior RAMPS and was stage T3N1 with a tumor size of 10.5 cm. He received adjuvant CCRT after recovering from grade B pancreatic fistula, but had multiple liver metastases at follow-up and survived 6.5 mo. The second patient underwent anterior RAMPS with combined jejunal resection due to gross invasion. He had stage T3N0 disease with a tumor size of 4.0 cm. He had both local recurrence and distant metastasis after adjuvant CCRT and survived 19.0 mo.

RAMPS has also been applied to laparoscopic distal pancreatectomy because it provides good surgical exposure for performing a right-to-left distal pancreatectomy. Kang *et al.*^[24] and Choi *et al.*^[25] reported outcomes of laparoscopic distal pancreatectomy performed on well-selected patients with left-sided pancreatic ductal adenocarcinomas.

In a previous study performed by Strasberg *et al.*^[2], the mean number of retrieved lymph nodes and lymph node positivity rate were 14.3 and 48%, respectively. In our study, the mean number of retrieved lymph nodes was 20.92 ± 11.24 and the lymph node positivity rate was 70.8%. Lymph node status is known to be a very important prognostic factor in pancreatic cancer. Some previous reports emphasized the lymph node ratio (the number of positive lymph nodes/the total number of retrieved lymph nodes) in pancreatic cancer^[26]. However, few studies have mentioned the number of retrieved lymph nodes, which makes it difficult to compare the surgical efficacy of RAMPS with conventional distal pancreatectomy.

Strasberg *et al.*^[2] reported that the median survival time and the 5-year survival rate after RAMPS were 21 mo and 26%, respectively, which were better than those of previous reports^[18]. It is known that patients with left-sided pancreatic cancer have poorer survival rates than patients with right-sided pancreatic cancer. However, surgical outcomes for patients with left-sided cancer were better than those with right-sided pancreatic cancer. Our patients had a median survival of 18.23 ± 6.02 mo, which was a little better than the survival following distal pancreatectomy reported by previous studies of conven-

tional distal pancreatectomy. However, our patients were mostly T3 or higher (87.5%), and lymph node positivity was 70.8%, which means that most of our patients were advanced stage. When viewed in this light, our results are better than those reported by previous studies and are comparable with those of right-sided pancreatic cancer.

We had 19 patients (79.2%) with systemic recurrence. Therefore, even though RAMPS has surgical advantages in terms of local control, there are limitations in preventing cancer progression, especially through systemic recurrence.

We performed distal pancreatectomy for carcinomas of the body and tail of the pancreas using RAMPS, with a negative tangential margin of 91.7%, no mortality and acceptable morbidity. It is suggested that RAMPS is a safe and feasible procedure for carcinomas of the body and tail of the pancreas. Further studies with larger sample sizes are needed to confirm our results.

COMMENTS

Background

Radical antegrade modular pancreatosplenectomy (RAMPS) is known as a feasible procedure for achieving a higher rate of negative tangential margins than the traditional approach. Although RAMPS is widely performed, there have been few studies on its clinical outcomes.

Applications

This study provides reference data for future larger studies.

Terminology

RAMPS was first introduced in 2003 as a surgical treatment for carcinomas of the body and tail of the pancreas.

Peer review

RAMPS seems to be a feasible procedure for achieving negative tangential margins for patients with carcinoma of the body and tail of the pancreas.

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Biochemical characteristics of neonatal cholestasis induced by citrin deficiency

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Abstract

AIM: To explore differences in biochemical indices between neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) and that with other etiologies.

METHODS: Patients under 6 mo of age who were referred for investigation of conjugated hyperbilirubinaemia from June 2003 to December 2010 were

eligible for this study. After excluding diseases affecting the extrahepatic biliary system, all patients were screened for the two most common *SLC25A13* mutations; the coding exons of the entire *SLC25A13* gene was sequenced and Western blotting of citrin protein performed in selected cases. Patients in whom homozygous or compound heterozygous *SLC25A13* mutation and/or absence of normal citrin protein was detected were defined as having NICCD. Cases in which no specific etiological factor could be ascertained after a comprehensive conjugated hyperbilirubinaemia work-up were defined as idiopathic neonatal cholestasis (INC). Thirty-two NICCD patients, 250 INC patients, and 39 infants with cholangiography-confirmed biliary atresia (BA) were enrolled. Laboratory values at their first visit were abstracted from medical files and compared.

RESULTS: Compared with BA and INC patients, the NICCD patients had significantly higher levels of total bile acid (TBA) [all measures are expressed as median (inter-quartile range): 178.0 (111.2-236.4) $\mu\text{mol/L}$ in NICCD vs 112.0 (84.9-153.9) $\mu\text{mol/L}$ in BA and 103.0 (70.9-135.3) $\mu\text{mol/L}$ in INC, $P = 0.0001$]. The NICCD patients had significantly lower direct bilirubin [D-Bil 59.6 (43.1-90.9) $\mu\text{mol/L}$ in NICCD vs 134.0 (115.9-151.2) $\mu\text{mol/L}$ in BA and 87.3 (63.0-123.6) $\mu\text{mol/L}$ in INC, $P = 0.0001$]; alanine aminotransferase [ALT 34.0 (23.0-55.0) U/L in NICCD vs 108.0 (62.0-199.0) U/L in BA and 84.5 (46.0-166.0) U/L in INC, $P = 0.0001$]; aspartate aminotransferase [AST 74.0 (53.5-150.0) U/L in NICCD vs 153.0 (115.0-239.0) U/L in BA and 130.5 (81.0-223.0) U/L in INC, $P = 0.0006$]; albumin [34.9 (30.7-38.2) g/L in NICCD vs 38.4 (36.3-42.2) g/L in BA and 39.9 (37.0-42.3) g/L in INC, $P = 0.0001$]; glucose [3.2 (2.0-4.4) mmol/L in NICCD vs 4.1 (3.4-5.1) mmol/L in BA and 4.0 (3.4-4.6) mmol/L in INC, $P = 0.0014$] and total cholesterol [TCH 3.33 (2.97-4.00) mmol/L in NICCD vs 4.57 (3.81-5.26) mmol/L in BA and 4.00 (3.24-4.74) mmol/L in INC, $P = 0.0155$] levels. The D-Bil to total bilirubin (T-Bil) ratio was significantly lower in NICCD patients [all measures

are expressed as median (inter-quartile range): 0.54 (0.40-0.74)] than that in BA patients [0.77 (0.72-0.81), $P = 0.001$] and that in INC patients [0.74 (0.59-0.80), $P = 0.0045$]. A much higher AST/ALT ratio was found in NICCD patients [2.46 (1.95-3.63)] compared to BA patients [1.38 (0.94-1.97), $P = 0.0001$] and INC patients [1.48 (1.10-2.26), $P = 0.0001$]. NICCD patients had significantly higher TBA/D-Bil ratio [3.36 (1.98-4.43) *vs* 0.85 (0.72-1.09) in BA patients and 1.04 (0.92-1.14) in INC patients, $P = 0.0001$], and TBA/TCH ratio [60.7 (32.4-70.9) *vs* 24.7 (19.8-30.2) in BA patients and 24.2 (21.4-26.9) in INC patients, $P = 0.0001$] compared to the BA and INC groups.

CONCLUSION: NICCD has significantly different biochemical indices from BA or INC. TBA excretion in NICCD appeared to be more severely disturbed than that of bilirubin and cholesterol.

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Key words: Cholestasis; Biliary atresia; Infants; Idiopathic neonatal cholestasis; *SLC25A13*

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INTRODUCTION

Citrin deficiency, caused by mutations in the *SLC25A13* gene on chromosome 7q21.3, is an autosomal recessive disease that was first discovered in Japan and thereafter identified worldwide^[1-8]. At least two main phenotypes of citrin deficiency have been established: neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD, OMIM #605814)^[9-11] and adult-onset type II citrullinemia (CTLN2, OMIM #603471)^[11,12]. The clinical features and diagnostic criteria of CTLN2 have been well established, but those of NICCD have not yet been established.

Children with NICCD usually have transient intrahepatic cholestasis that disappears by the age of 1 year with appropriate management^[13]. However, some patients need liver transplantation or may die from the disease during infancy^[14-17]. Others may develop severe CTLN2 symptoms unexpectedly one to several decades later^[13]. Prompt detection and specific lactose-free and/or medium-chain triglyceride formula may contribute to the avoidance of a complicated course in the NICCD phase. However, the prompt diagnosis of NICCD is still a challenge because the clinical features of cholestasis induced by citrin deficiency are presently not fully understood^[18].

Some biochemical indices, including total bilirubin

(T-Bil), direct bilirubin (D-Bil), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), γ -glutamyltranspeptidase (GGT), and α -fetoprotein (AFP) have been analyzed or compared between patients with NICCD, biliary atresia (BA) or idiopathic neonatal cholestasis (INC), with significant differences found between them^[4]. However, comparisons of serum total bile acid (TBA) and total cholesterol (TCH) levels between different causes of neonatal cholestasis has rarely been reported before^[19].

Bilirubin, bile acids and cholesterol are all mainly physiologically excreted by the hepatobiliary system. Usually, the blood levels of all these compounds increase in the event of blockage of the extrahepatic biliary system. However, they may be affected to different extents in intrahepatic cholestasis with different etiology. For instance, blood TCH and TBA levels are usually elevated significantly in Alagille syndrome, even in cases in which the bilirubin level is only mildly elevated; however, the blood TCH level is usually normal in cases of progressive familial intrahepatic cholestasis type 1 or 2 despite significant elevation of blood TBA and bilirubin levels. Therefore, comparison of these biochemical indices in neonatal cholestasis cases with different etiologies will enable better characterization of the biochemical changes of the disease and may help elucidate the mechanism of cholestasis caused by citrin deficiency.

Therefore, the aims of this study were to explore the differences in biochemical indices, including D-Bil, TBA, TCH and their ratios between cholestasis with different etiologies, and to explore the mechanism of cholestasis caused by citrin deficiency.

MATERIALS AND METHODS

Subjects

Patients under 6 mo of age who were referred to the Children's Hospital of Fudan University, a tertiary referral hospital and primary specialized paediatric hospital in Eastern China, from June 2003 to December 2010, for investigation of conjugated hyperbilirubinaemia were eligible for this study. Conjugated hyperbilirubinemia was defined as serum T-Bil levels $> 75 \mu\text{mol/L}$, with a conjugated fraction accounting for $> 20\%$ of the total, or having conjugated bilirubin levels $> 17 \mu\text{mol/L}$ with serum T-Bil levels $< 75 \mu\text{mol/L}$ ^[20]. Patients who had obvious extrahepatic abnormalities or prolonged prothrombin time that could not be corrected by parenteral administration of vitamin K1 were excluded. Subjects who satisfied the above criteria as well as the following specific criteria for each of the three groups were included.

BA group: Patients with neonatal cholestasis in whom no isotope excretion was demonstrated by hepatobiliary iminodiacetic acid (HIDA) scintigraphy, and in whom diagnosis of BA was confirmed by laparoscopic or laparotomic cholangiography were eligible for this group.

INC group: Intrahepatic cholestasis was defined as con-

Table 1 Differential diagnosis have been excluded

Affecting bile duct	Infectious	Metabolic	Others
Biliary atresia	Herpes viruses	α -1 antitrypsin deficiency	Endocrinological
Choledochal cyst	Rubella virus	Neonatal iron storage disease	Hypothyroidism
Cholelithiasis	Enteroviruses	Amino acid disorders	Panhypopituitarism/septo-optic dysplasia
Inspissated bile	Hepatitis viruses	Tyrosinemia	Genetic
Tumor	Human immunodeficiency virus	Hypermethioninemia	ATP8B1 deficiency
Hemangioma	Syphilis	Mevalonate kinase deficiency	ABCB11 deficiency
Bile duct stenosis/stricture/perforation	Toxoplasmosis	Glucogen storage diseases	ABCB4 deficiency
Neonatal sclerosing cholangitis	Bacterial sepsis	Gaucher disease	Bile acid synthetic defects
Caroli disease	Urinary tract infections	Niemann-Pick disease	Neonatal Dubin-Johnson syndrome
Alagille syndrome		Wolman disease	Various trisomies
		Zellweger syndrome	Argyrogryposis
		Infantile Refsum disease	Hematological
		Mitochondrial disorders	Hemophagocytic lymphohistiocytosis
		Urea cycle disorders	Langerhans cell histiocytosis
			Miscellaneous drug effects
			Total parenteral nutrition

jugated hyperbilirubinemia following the exclusion of diseases affecting the extrahepatic biliary system (Table 1) by imaging of the hepatobiliary system. The imaging procedures included an ultrasound scan and HIDA scintigraphy in each case and laparotomic/laparoscopic cholangiography in selected cases. Idiopathic neonatal cholestasis (INC) was defined when no specific etiological factor (Table 1) could be ascertained after a comprehensive conjugated hyperbilirubinemia test^[21]. Patients with at least single-allele mutation of *SLC25A13* gene were excluded from this group as well.

NICCD group: The strategy of testing for *SLC25A13* gene mutations in intrahepatic cholestatic infants had been reported previously^[22,23]. All intrahepatic cholestasis infants with unknown causes were screened for the two most common mutations of the *SLC25A13* gene in Chinese, 851del4 and 1638ins23. For patients with various aminoacidemia or patients with only single-allele mutation who were found by the above screening method, the entire 18 coding exons together with the flanking sequence of the *SLC25A13* gene were amplified by polymerase chain reaction and directly sequenced. Western blotting analysis of citrin protein was performed on patients with biopsied liver specimens available. Only patients in whom homozygous or compound heterozygous *SLC25A13* gene mutation and/or absence of normal citrin protein were demonstrated, for whom a definite diagnosis of citrin deficiency could be made, were regarded as NICCD patients in this study. Patients with a probable diagnosis of citrin deficiency, that is, in whom there was only a heterozygous *SLC25A13* gene mutation and in whom absence of normal citrin protein could not be demonstrated by Western blotting were excluded.

Retrospective analyses of biochemical indices

The medical files of the patients who satisfied the above inclusion and exclusion criteria were reviewed following the approval of the Institute's Ethics Review Committee. Sex, birth weight, gestation age or term/preterm, age at

which conjugated jaundice was first noticed, and the biochemical indices at presentation, were abstracted. Liver function tests and other routine laboratory data were obtained using standard methods.

Statistical analysis

Statistical analysis was performed using Stata/SE 10.0 for Windows (StataCorp LP, College Station, TX, United States of America). The descriptive data of the quantitative variables were reported in box-whisker plots and compared using Kruskal-Wallis rank tests among the three groups. For results with overall statistical significance, a Mann-Whitney test with a Bonferroni correction was further performed to test the medians between a series of pairwise groups. All *P* values were two-sided. Results were considered statistically significant at the 0.05 level.

RESULTS

Basic information

In total, 32 patients (19 male and 13 female) with a definite diagnosis of citrin deficiency were included in the NICCD group. Thirty-nine patients (24 male and 15 female) were included in the BA group. Two hundred and fifty patients (174 male and 76 female) were included in the INC group. The birth weight and the days at which conjugated jaundice was first noticed in the three groups are illustrated in Figure 1 or Table 2. The median birth weight was lowest in the NICCD group, but the difference did not reach statistical significance (Table 2). Conjugated jaundice was noticed earlier in the BA group compared with the INC group (*P* < 0.05, Figure 1A).

Comparison of biochemical indices among three groups

The biochemical data of the three groups were compared (Figure 1B-E, Table 2). The NICCD group had significantly lower ALT, AST, total protein, albumin, and glucose levels compared with the BA and INC groups, suggesting that the synthetic function and glucose metabolism were more severely damaged in the NICCD

Table 2 Comparison of birth weight, biochemical indices and their ratios among the three groups

	Reference range and unit	BA (<i>n</i> = 39)		INC (<i>n</i> = 39)		NICCD (<i>n</i> = 32)	
		Median	Inter-quartile	Median	Inter-quartile	Median	Inter-quartile
Birth weight	2.5-4.0 kg	3.2	2.9-3.8	3.1	2.8-3.5	2.9	2.4-3.4
Biochemical indices							
T-Bil ^{ac}	2-20 mmol/L	159.5	140.2-201.4	133.8	90.0-190.4	112.7	64.4-165.4
D-Bil ^{ac}	0-6 mmol/L	134	115.9-151.2	87.3	63.0-123.6	59.6	43.1-90.9
ALT ^{ce}	< 40 IU/L	108	62.0-199.0	84.5	46.0-166.0	34	23.0-55.0
AST ^{ce}	< 40 IU/L	153	115.0-239.0	130.5	81.0-223.0	74	53.5-150.0
GGT ^{ac}	< 50 IU/L	558	300.0-1086.0	155	91.0-294.0	187.5	136.0-253.0
TBA ^{ce}	< 40 mmol/L	112	84.9-153.9	103	70.9-135.3	177.9	111.2-236.4
Total protein ^{ce}	55-78 g/L	57.4	55.3-63.1	57.2	52.5-62.8	48.5	44.5-53.9
Albumin ^{ce}	35-55 g/L	38.4	36.3-42.2	39.9	37.0-42.3	34.9	30.7-38.2
Glucose ^{ce}	3.9-5.9 mmol/L	4.1	3.4-5.1	4	3.4-4.6	3.2	2.0-4.4
TCH ^{ce}	3.12-5.20 mmol/L	4.57	3.81-5.26	4	3.24-4.74	3.33	2.97-4.00
Ratios							
D-Bil/T-Bil ^{ac}		0.77	0.72-0.81	0.74	0.59-0.80	0.54	0.40-0.74
AST/ALT ^{ce}		1.38	0.94-1.97	1.48	1.10-2.66	2.46	1.95-3.63
TBA/D-Bil ^{ac}		0.85	0.72-1.09	1.04	0.92-1.14	3.36	1.98-4.43
D-Bil/TCH ^{ac}		30.2	22.8-34.0	21.5	16.7-31.2	18.7	13.9-26.6
TBA/TCH ^{ce}		24.7	19.8-30.2	24.2	21.4-26.9	60.7	32.4-70.9

^a*P* < 0.05 between BA and INC; ^c*P* < 0.05 between BA and NICCD; ^e*P* < 0.05 between INC and NICCD. NICCD: Neonatal intrahepatic cholestasis caused by citrin deficiency; BA: Biliary atresia; INC: Idiopathic neonatal cholestasis; TBA: Total bile acid; T-Bil: Total bilirubin; D-Bil: Direct bilirubin; AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; GGT: γ -glutamyltranspeptidase; TCH: Total cholesterol.

group than in the other two groups. The NICCD group also had significantly higher TBA and lower D-Bil and cholesterol levels compared with the BA and INC groups, indicating that the excretion of bile acids, D-Bil and cholesterol might be affected differently in NICCD patients. Significantly lower T-Bil and GGT levels were noticed in the NICCD group only when compared with the BA group.

Comparison of ratios of biochemical indices

To compare further the different biochemical indices, a series of ratios was calculated. The highest ratio of D-Bil to T-Bil was found in the BA group and the lowest in the NICCD group (Table 2, Figure 1F). A much higher AST/ALT ratio was found in the NICCD group compared to the INC and BA groups (Table 2).

The ratios between D-Bil, bile acids and cholesterol were also compared (Table 2). The ratio of serum TBA to D-Bil was significantly higher in the NICCD group than the ratios in the BA and INC groups (Figure 1G, *P* < 0.05). Significant differences were also found for the ratio of TBA to TCH between the NICCD and BA groups and between the NICCD and INC groups (Figure 1H, *P* < 0.05).

DISCUSSION

Citrin deficiency is one of the most common classical inborn errors of metabolism of amino acids, organic acids and fatty acid oxidation in Eastern Asia, including China^[24]. The biochemical characteristics and mechanism of cholestasis caused by citrin deficiency are still not fully understood. Although differences in some indices among patients with BA, INC and NICCD have been reported previously, the very small sample sizes of

the studies precluded a definite conclusion^[4,19,23]. In the present study, the cohorts of NICCD, INC and BA had numbers large enough to test previous findings. Additionally, by comparing the elevation of D-Bil, TBA and cholesterol levels and the ratios of these compounds, it was found that the excretion of bile acids appeared to be more severely affected in NICCD than in BA and INC.

A previous study with a small number of subjects demonstrated that patients with cholestasis caused by citrin deficiency had lower ALT and AST levels and higher AST to ALT ratios compared to those with BA or idiopathic neonatal hepatitis^[25]. Low albumin and glucose levels were also associated with NICCD in a previous case series^[26,27]. In the present study, those findings were confirmed. Previous studies also showed that patients with NICCD had lower birth weight compared to normal controls or to the national standard. In our study, although a lower median birth weight in the NICCD group was noticed, the differences did not reach statistical significance compared to patients with BA or INC. This could be explained by the different control groups (normal control or national standard used in previous studies *vs* patients with cholestasis of other causes) and the large difference observed within the NICCD group in this study.

The serum TBA level in NICCD has previously been compared with that in BA and INC in a study that had very few subjects^[19]. In the present study, the serum level of TBA as well as the ratio of serum TBA to D-Bil and cholesterol levels was compared. In BA, we can suppose that excretion of D-Bil, bile acids and cholesterol is affected to the same extent in consideration of complete blockage of the biliary system. If the ratio of TBA to D-Bil in the BA group was taken as the reference value, the median for INC was found to be 1.22 (1.04/0.85) times higher

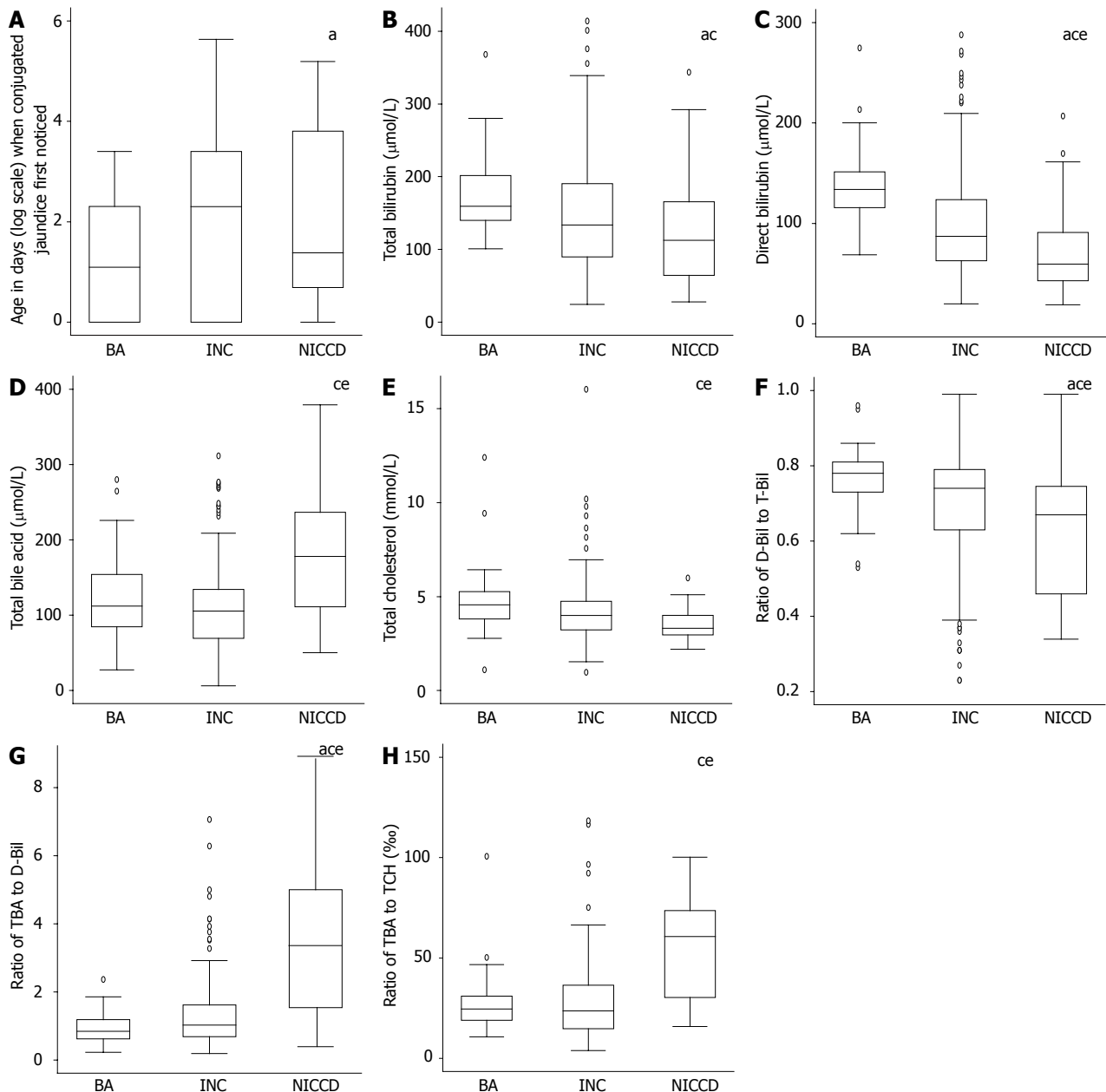


Figure 1 Comparison of the age when conjugated jaundice was first noticed (A), biochemistry indices (B-E), and ratios of some biochemical indices (F-H) in patients with neonatal intrahepatic cholestasis caused by citrin deficiency, idiopathic neonatal cholestasis and biliary atresia. ^a $P < 0.05$ between BA and INC; ^c $P < 0.05$ between BA and NICCD; ^e $P < 0.05$ between INC and NICCD. Normal range: T-Bil (2-20 $\mu\text{mol/L}$), D-Bil (0-6 $\mu\text{mol/L}$), total bile acids ($< 40 \mu\text{mol/L}$), and TCH (3.12-5.20 mmol/L). NICCD: Neonatal intrahepatic cholestasis caused by citrin deficiency; BA: Biliary atresia; INC: Idiopathic neonatal cholestasis; TBA: Total bile acid; T-Bil: Total bilirubin; D-Bil: Direct bilirubin; TCH: Total cholesterol.

and that of NICCD 3.95 (3.36/0.85) times higher. For the ratio of TBA to cholesterol, if the median value of the BA group was taken as a standard, the median in the INC group was nearly the same as the standard but that in the NICCD group was 2.46 (60.7/24.7) times higher. These results indicate that the excretion of bile acids is much more severely affected than the excretion of bilirubin and cholesterol in NICCD patients. As a consequence, we may speculate that the failure to excrete bile acids from hepatocytes to the canalicula is the main mechanism of cholestasis caused by citrin deficiency.

The main limitation of this study was its retrospec-

tive nature. It could be argued that some biochemical indices were affected by the drugs that patients were taking. However, prior to the determination of a clear diagnosis, the management of patients had been similar in the three groups; therefore, the patients in the different groups would have been affected by these variables in the same way. Another measure that was used to avoid sample bias was using the first available laboratory data obtained when patients were referred to us. Although significant differences in TBA and TBA ratios were found between the NICCD and other two groups, no cut-off levels can be presented at this time.

COMMENTS

Background

Citrin deficiency is one of the most common metabolic disorders in Eastern Asia. It has at least two main phenotypes: neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD) and adult-onset type II citrullinemia. The clinical features of and the mechanism of cholestasis in NICCD have yet to be established.

Research frontiers

Some biochemical indices of patients with NICCD have been compared to those of patients with biliary atresia (BA) and of patients with idiopathic neonatal cholestasis (INC). Comparison of these biochemical indices in neonatal cholestasis cases with different etiologies will better characterize the biochemical changes of the disease and may further the understanding of the mechanism of cholestasis caused by citrin deficiency.

Innovations and breakthroughs

Apart from confirming previous findings that NICCD patients had significantly lower alanine aminotransferase (ALT) level, lower direct bilirubin (D-Bil) to total bilirubin ratio, and significantly higher aspartate aminotransferase to ALT ratio compared to the BA and INC patients, this study specifically compared the serum level of total bile acid (TBA) and its ratio to D-Bil and cholesterol, and found that NICCD patients had significantly higher TBA levels as well as higher TBA to D-Bil and TBA to cholesterol ratios than patients with BA and INC.

Applications

The excretion of TBA appears to be much more severely disturbed than that of D-Bil and cholesterol in cholestasis caused by citrin deficiency. Further study of this condition will help elucidate the mechanism of cholestasis in NICCD, and the ratios could be further developed as indices for the differential diagnosis of neonatal cholestasis.

Peer review

The authors present an interesting retrospective study comparing liver specific biochemical parameters in different groups of infants with cholestasis.

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Colonic stenting vs emergent surgery for acute left-sided malignant colonic obstruction: A systematic review and meta-analysis

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Abstract

AIM: To investigate the effects of emergent preoperative self-expandable metallic stent (SEMS) vs emergent surgery for acute left-sided malignant colonic obstruction.

METHODS: Two investigators independently searched the MEDLINE, EMBASE and Cochrane Central Register of Controlled Trials, as well as references of included studies to identify randomized controlled trials (RCTs) that compared two or more surgical approaches for acute colonic obstruction. Summary risk ratios (RR) and 95% CI for colonic stenting and emergent surgery were calculated.

RESULTS: Eight studies met the selection criteria, involving 444 patients, of whom 219 underwent SEMS

and 225 underwent emergent surgery. Seven studies reported difference of the one-stage stoma rates between the two groups (RR, 0.60; 95% CI: 0.48-0.76; $P < 0.0001$). Only three RCTs described the follow-up stoma rates, which showed no significant difference between the two groups (RR, 0.80; 95% CI: 0.59-1.08; $P = 0.14$). Difference was not significant in the mortality between the two groups (RR, 0.91; 95% CI: 0.50-1.66; $P = 0.77$), but there was significant difference (RR, 0.57; 95% CI: 0.44-0.74; $P < 0.0001$) in the overall morbidity. There were no significant differences between the two groups in the anastomotic leak rate (RR, 0.60; 95% CI: 0.28-1.28; $P = 0.19$), occurrence of abscesses, including peristomal abscess, intraperitoneal abscess and parietal abscess (RR, 0.83; 95% CI: 0.36-1.95; $P = 0.68$), and other abdominal complications (RR: 0.67; 95% CI: 0.40-1.12; $P = 0.13$).

CONCLUSION: SEMS is not obviously more advantageous than emergent surgery for patients with acute left-sided malignant colonic obstruction.

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Key words: Acute obstruction; Colonic cancer; Self-expandable metallic stent; Stoma placement; Meta-analysis; Systematic review

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INTRODUCTION

Colorectal cancer is the fourth most common malignancy worldwide, with an estimated number of 1 023 000 new cases and 529 000 deaths each year^[1]. The incidence of colorectal cancer has been increasing rapidly in Asia over the past few decades^[2]. About 7%-29% of the patients with colorectal cancer present with bowel obstruction^[3,4]. And benefit of surgical management of malignant large bowel obstruction remains controversial, especially for left-sided colonic obstruction. Emergent colorectal surgery for acute obstruction is associated with a mortality rate of 15%-20% and a morbidity rate of 15%-20%, both significantly higher than that in the elective situation^[5-7]. Emergent surgery is an independent factor of mortality and morbidity, and about two-thirds of patients end up with a permanent stoma^[3,6,8,9].

In 1991, colonic stenting was introduced to restore luminal patency in patients with malignant obstruction of the left side colon^[10]. Tejero *et al*^[11] used self-expandable metallic stent (SEMS) as a bridge to surgery in two patients with colonic obstruction in 1994. Stent placement before elective surgery, also known as a bridge to surgery, improved the clinical condition of the patient and seemed to decrease the mortality, morbidity, and number of colostomies in uncontrolled studies^[12-15]. Although preoperative SEMS insertion has such advantages, it may result in the related complications such as perforation, stent migration, and reobstruction. As shown in the recent randomized controlled trials (RCTs), whether preoperative SEMS can reduce mortality, complication rate and stoma rate is still a big controversy^[16-18]. Thus, this meta-analysis was performed to evaluate the effects of preoperative SEMS *vs* emergent surgery for acute left-sided malignant colonic obstruction.

MATERIALS AND METHODS

Search strategy

SEMS was first used in 1991. We therefore, searched the databases, including the Cochrane Central Register of Controlled Trials (1991-April 2011), MEDLINE (1991-September 2011), EMBASE (1991-September 2008), Elsevier ScienceDirect (1998-September 2008), SpringLink (up to September 2011), Ovid LWW (1991-September 2011) and BMJ Journals Online (up to September 2008). The following keywords were used: "intestinal obstruction", "colon", "rectum", "left-sided colon", "surgery", "resection", "stents", "randomized" and "controlled study". The detailed search strategy is available from the authors. All included studies also had access to the PubMed "related articles" function and the Science Citation Index. In addition, the reference lists of included studies were scrutinized. No language restrictions were applied.

Data extraction

Data were independently abstracted from each study by two researchers, and disagreement was resolved by consensus. Data were extracted from each study using a pre-

designed review form. Data to be extracted were as follows: (1) treatment details: primary anastomosis rate, and the incidence of stoma creation; (2) short-term adverse events: mortality and morbidity such as anastomotic leak rate, abscess and extra abdominal complications; and (3) long-term outcomes: follow-up stoma rate.

Inclusion and exclusion criteria

Studies fulfilling the following criteria were included in the meta-analysis: (1) RCTs or other comparative studies comparing SEMS as a bridge to surgery and emergent surgery; (2) reports on at least one of the outcome measures mentioned below; and (3) studies reporting patients with malignant acute left-sided colonic obstruction.

Quality of methodology

The quality of nonrandomized studies was assessed using the Newcastle-Ottawa Scale with some modifications to meet the needs for this meta-analysis^[19], and the quality of randomized studies was evaluated by means of the modified Jadad score^[20]. The quality of the studies was evaluated based on three items: patient selection, comparability of study groups, and assessment of outcome. Studies achieving five or more stars were considered high quality. The quality of randomized studies was evaluated by means of the modified Jadad score including the following four areas: (1) randomization method; (2) hidden subgroups; (3) blinding; and (4) the description of the loss to follow-up and drop-out and the intention-to-treat. The total score of 1 to 3 points were ascribed to low-quality studies, whereas a total score of 4 to 7 points to high-quality researches.

Statistical analysis

Using the Cochrane Collaboration's RevMan 5.1 software provided by meta-analysis, the results of included measurement of indicators were all count data, and 95% CI was used for the efficacy analysis. The heterogeneity between studies was tested. When there was homogeneity among studies ($P > 0.1$, $I^2 < 50\%$), a fixed effects model was used for meta-analysis; if there is significant heterogeneity among studies ($P < 0.1$, $I^2 > 50\%$), the random effects model was used. We also analyzed the different quality of the possible causes, and conducted subgroup analysis. If the heterogeneity among the studies was too large, descriptive analysis was performed.

RESULTS

Selection of trials

The initial search strategy retrieved 88 articles after screening all titles, abstracts and full texts. Twenty-two articles were excluded due to lack of comparison with other surgical strategies in most of the cohort studies, 45 articles were excluded because of comparison stenting *vs* surgery without a bridge to the surgery, and 13 studies were excluded because there was no control. Finally, 8 trials with 444 patients were included, of whom 219

Table 1 Randomized controlled trials involved in the meta-analysis

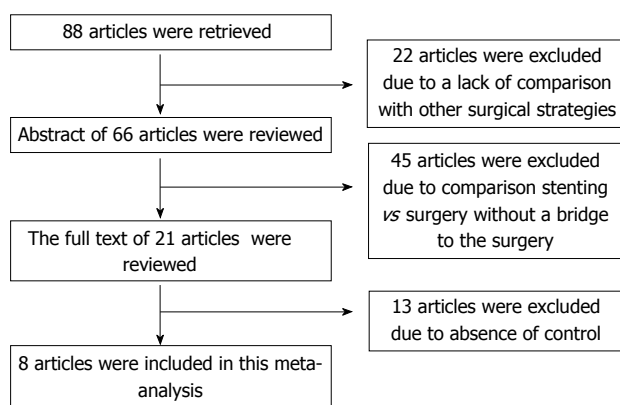
Year	Author	Region	Total	SEMS	Surgery	Concealment of allocation	Jaded score	Quality
2009	Cheung	Hongkong	60	30	30	Appropriate	5	High
2010	Pirlet	France	48	24	24	Appropriate	5	High
2011	Van Hooft	Holland	98	47	51	Appropriate	5	High

SEMS: Self-expandable metallic stent.

Table 2 Basic characteristics of included nonrandomized controlled studies in the meta-analysis

Year	Author	Design	Total	SEMS	Surgery	Match	Study quality (rate, max 11)
2008	Dastur	R	43	19	23	1,2,3,4,5,6	8
2002	Martin	R	52	26	26	1,2,3,4,5,8	7
2006	Ng	R	60	20	40	1,2,3,5,6,8	8
2007	Pessione	R	16	9	7	1,2,3,4,5,8	7

Matching: 1 = age, 2 = sex, 3 = diagnosis, 4 = tumor site, 5 = tumor stage, 6 = American Society of Anesthesiologists score, 7 = body mass index, 8 = comorbidity. SEMS: Self-expandable metallic stent. "R" represents that the study was a randomized clinical trial.

**Figure 1** Flow chart of selection of studies and reasons for exclusion from the meta-analysis.

(49.3%) successfully underwent stent insertion and 225 (50.7%) underwent emergent surgery. There were 17 (7.8%) deaths in the SEMS as a bridge to surgery group and 21 (9.3%) deaths in the emergent surgery group. There were only three RCTs^[16-18] and five nonrandomized controlled studies (NRCTs)^[12-14,21,22]. The flow chart of selection of studies and reasons for exclusion is presented in Figure 1. Characteristics of studies included in the meta-analysis are presented in Tables 1 and 2.

Quality of studies

Three RCTs were of moderate to good methodological quality evaluated by the modified Jadad score^[20]. Because of the special strategies under assessment, no blind method was used in all the RCTs. All five NRCTs contained groups matched for age, sex, and diagnosis; five articles contained information on tumor site, tumor stage, American Society of Anesthesiologists score, or body mass index, respectively. All studies were scored more than five stars using the modified Newcastle-Ottawa scale^[19].

Meta-analysis of treatment details and long-term outcomes

Our meta-analysis showed statistically significant difference between the SEMS group (175 patients) and the emergent surgery group (201 patients) in seven studies with regard to the one-stage stoma rate [risk ratios (RR): 0.60, 95% CI: 0.48-0.76; $P < 0.0001$]. There was no significant heterogeneity between the studies ($P = 0.14$, $I^2 = 37\%$) (Figure 2A). Only three RCTs compared the two groups (101 patients in the SEMS group and 105 in the emergent surgery group) and described the follow-up stoma rates. There was neither significant difference in follow-up stoma rates (RR: 0.80; 95% CI: 0.59-1.08; $P = 0.14$), nor heterogeneity ($P = 0.17$, $I^2 = 44\%$) between the two groups (Figure 2B).

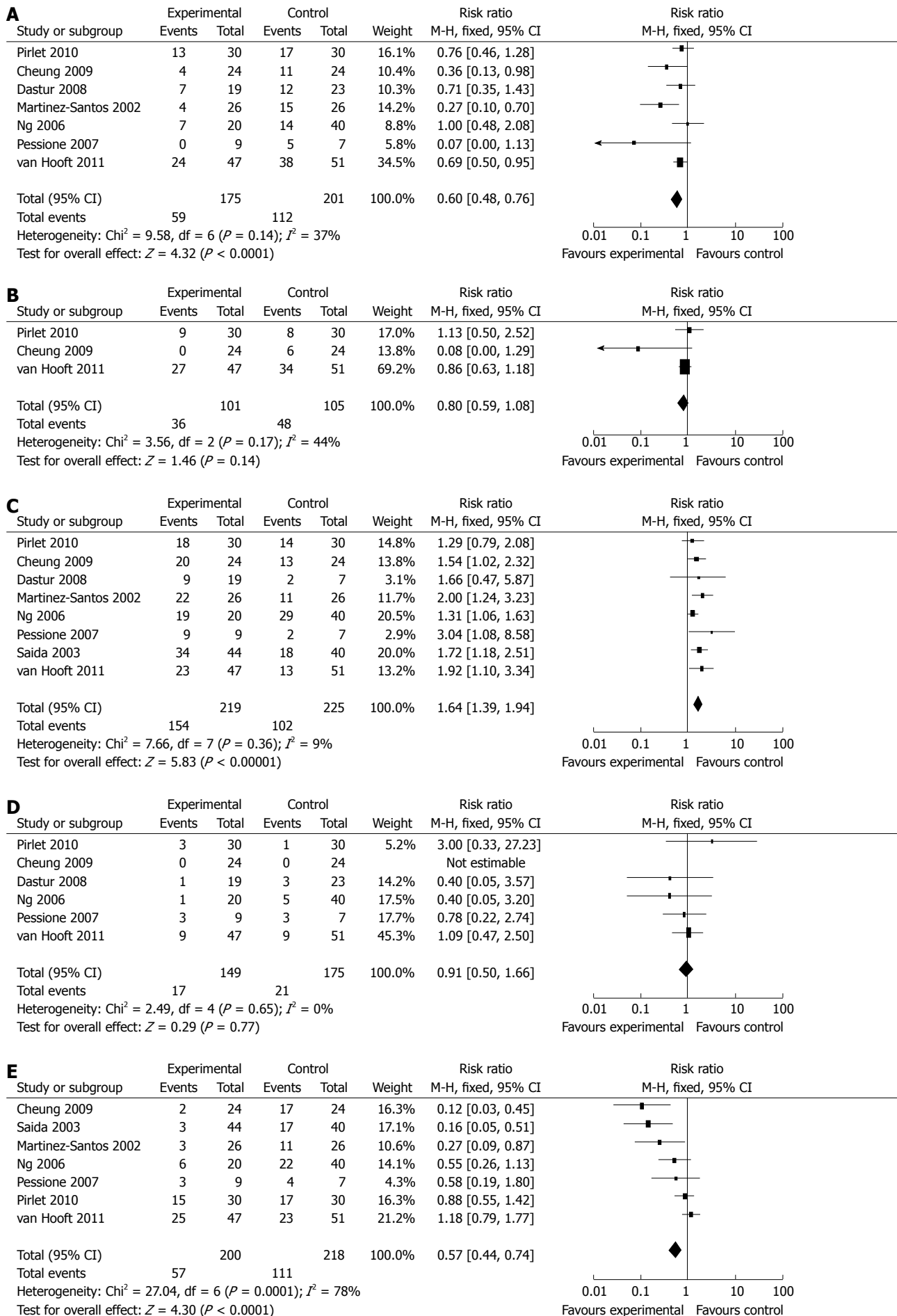
Anastomosis rates were reported by all eight trials involving 219 patients in the SEMS group and 225 in the emergent surgery group. There was a significant difference between the two groups, with a pooled RR of 1.64 (95% CI: 1.39-1.94; $P < 0.00001$) (Figure 2C).

Meta-analysis of short-term adverse events: Mortality and morbidity such as anastomotic leak rate, abscess and extra abdominal complications

Six trials reported mortality in 149 patients in the SEMS group and 175 patients in the emergent surgery group. There was no significant difference (RR: 0.91; 95% CI: 0.50-1.66; $P = 0.77$) and heterogeneity ($P = 0.65$, $I^2 = 0\%$) between the two groups (Figure 2D).

Seven trials with 200 patients in the SEMS group and 218 patients in the emergent surgery group reported the overall morbidity. There was no significant difference between the two groups with a pooled RR of 0.57 (95% CI: 0.44-0.74; $P < 0.0001$). However, significant heterogeneity was observed ($P = 0.0001$, $I^2 = 78\%$) (Figure 2E).

Further complication analysis was also performed, such as anastomotic leak, abscess and extra abdominal complications. The incidence of anastomotic leakage in



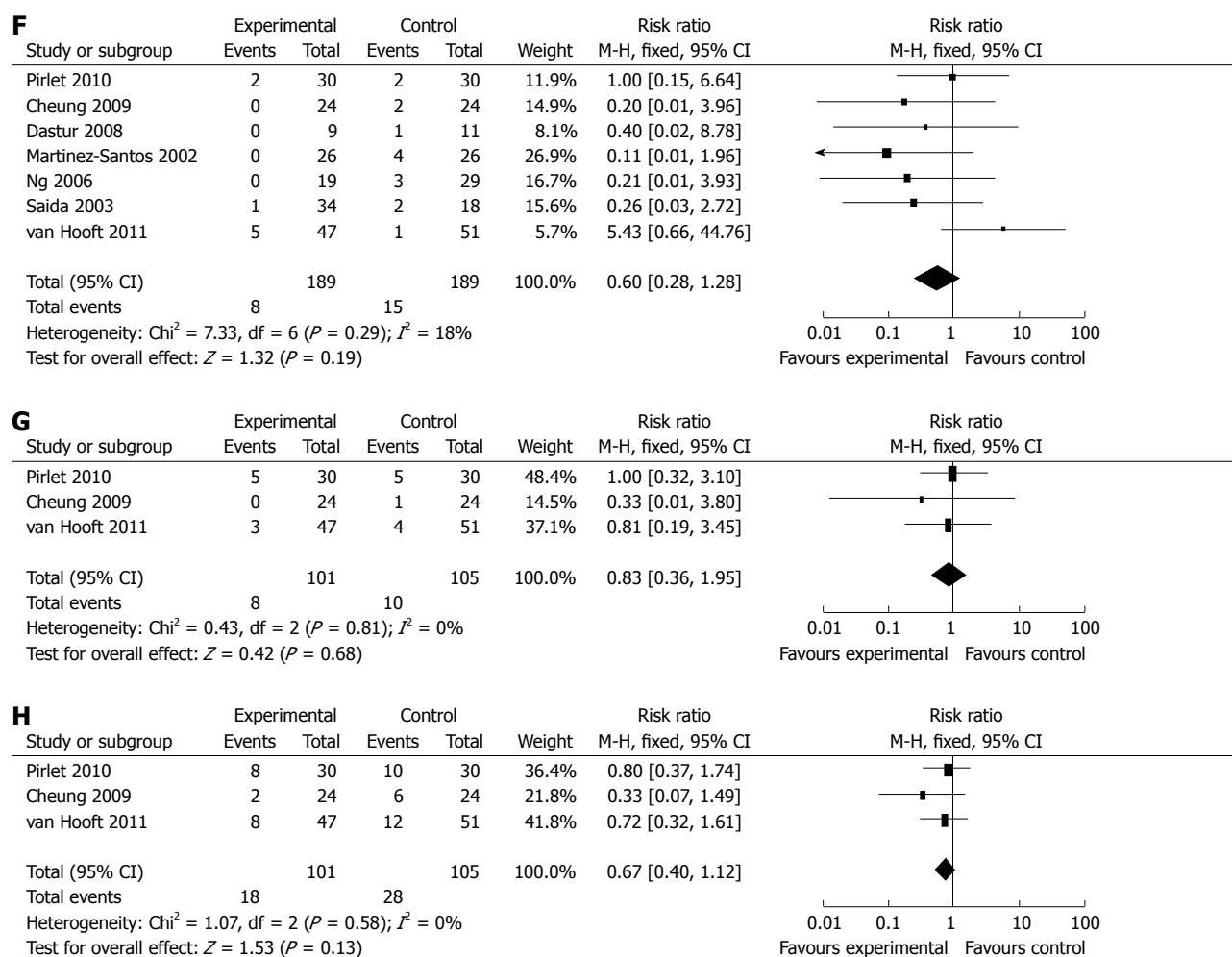


Figure 2 Colonic stenting *vs* emergent surgery. A: One-stage stoma rates; B: Follow-up stoma rates; C: Anastomosis rates; D: Mortality; E: Morbidity; F: Anastomotic leak; G: Abscesses; H: Extra abdominal complication. M-H: Mantel Haenszel.

the two groups was reported in seven studies. SEMS did not reduce the incidence of anastomotic leakage in patients treated with emergent surgery (RR: 0.60; 95% CI: 0.28-1.28; $P = 0.19$). No significant heterogeneity was observed between the two groups ($P = 0.29$, $I^2 = 18\%$) (Figure 2F).

Three studies compared the rates of abscesses, including peristomal abscess, intraperitoneal abscess and parietal abscess and extra abdominal complication. The analyses showed no significant difference in occurrence of abscess (RR: 0.83, 95% CI: 0.36-1.95; $P = 0.68$) and extra abdominal complications (RR: 0.67, 95% CI: 0.40-1.12; $P = 0.13$). No significant heterogeneity was found in abscess ($P = 0.81$, $I^2 = 0\%$) (Figure 2G) and extra abdominal complications ($P = 0.58$, $I^2 = 0\%$) (Figure 2H).

RCTs *vs* NRCTs

We also compared three RCTs with other NRCTs. There were significant differences in the morbidity (RCT group, RR 1.18; 95% CI: 0.79-1.77; $P = 0.08$ *vs* NRCTs group, RR 0.34; 95% CI: 0.21-0.56; $P < 0.0001$), anastomotic leak rate (RCT group, RR 5.43; 95% CI: 0.66-44.76; $P = 0.53$, *vs* NRCT group, RR 0.21; 95% CI: 0.05-0.82;

$P = 0.02$), and anastomosis rates (RCT group, RR 1.57; 95% CI: 1.10-2.08; $P = 0.002$, *vs* NRCT group, RR 1.69; 95% CI: 1.38-2.08; $P < 0.00001$). There were no differences in the stoma rates (RCT group, RR 0.65; 95% CI: 0.50-0.85; $P = 0.002$, *vs* NRCT group, RR 0.52; 95% CI: 0.34-0.80; $P = 0.003$) and mortality (RCT group, RR 1.09; 95% CI: 0.47-2.50; $P = 0.53$, *vs* NRCT group, RR 0.54; 95% CI: 0.2-1.45; $P = 0.22$).

DISCUSSION

Meta-analysis can be used to evaluate the existing literature both qualitatively and quantitatively by comparing and integrating the results of different studies and taking into account the variations in characteristics that could influence the overall estimate of the outcome of interest^[23]. Although meta-analysis is traditionally applied and best confined to RCTs, meta-analytical techniques using NRCTs might be a good method in some clinical settings in which either the number or the sample size of RCTs was insufficient^[24].

The concept of colonic stenting as a bridge to elective surgery in patients with acute left-sided malignant

colonic obstruction has been established to reduce the morbidity, mortality and number of colostomies. The nonrandomized or retrospective studies showed a significant reduction of morbidity and mortality, and need for stoma placement when SEMS was inserted before surgery with palliative intent. In contrast to these studies, two RCTs failed to confirm the findings.

Our meta-analysis illustrated that SEMS placement significantly decreased the one-stage stoma rates (RR: 0.60; 95% CI: 0.48-0.76; $P < 0.0001$) and increased anastomosis rates (RR: 1.64; 95% CI: 1.39-1.94; $P < 0.00001$), but no difference was found by the end of follow-up. The results of 206 patients in three RCTs which reported follow-up stoma rates demonstrated no difference. The difference of the stoma rate in the follow-up was partly caused by the high leakage rate of primary anastomosis in one stent group^[16], probably because bowel decompression and improvement of the patients' clinical condition were insignificant at the time of elective operation. Another reason might be that more patients with complete obstruction had been selected in a Holland study^[16]. In a retrospective study from a renowned tertiary referral centre, complete obstruction has been identified as a risk factor for complications^[25]. Additionally, the elective nature of the operation and the surgeons' faith in the idea of bridge to surgery might have made the surgeons less conservative than the emergent surgery group.

The perioperative mortality is frequently used to evaluate the outcome of SEMS. Our study failed to reveal the difference between the two groups. Six studies including 324 patients came to a conclusion that there was no difference (RR: 0.91; 95% CI: 0.50-1.66; $P = 0.77$) between SEMS group and emergent surgery group. These outcomes might imply the potential benefits for preoperative SEMS as a bridge to surgery. It is hard to evaluate this outcome as the patients picked up did not match with the clinical stages. Clinical stage is considered as one of independent factors for prognosis. When SEMS is used as a bridge to surgery, there is concern about the oncologic outcome of those patients whose disease is potentially curable, because theoretically SEMS placement could induce tumor dissemination and worsen long-term survival^[26]. More subgroup analyses should be performed to obtain a more accurate assessment.

In acute colonic obstruction, doctors and patients both want to remove the tumor with primary anastomosis with a shorter hospital stay. But the major concern is how to avoid complications. In right-sided bowel obstruction, a resection with primary anastomosis has been generally accepted by surgeons, but it is controversial in left-sided bowel obstruction. It is believed that the left colon obstruction has a high risk of radical resection and anastomosis with a high incidence of complications. Even with modern enema techniques and nutritional support, the rates of postoperative complications are still as high as 40%-50% (both significantly higher than in the elective situation with a $< 14.0\%$ anastomotic leakage

rate and 10.0% operative mortality)^[5,6,15,17,21-23,27,28] because of the long procedure time, peritoneal contamination, thin proximal wall of obstruction bowel, inflammatory edema, and poor blood supply. Although preoperative SEMS can potentially ameliorate bowel edema, there was no improvement in the overall situation. There was a significant difference between the two groups in the overall complications in seven studies with 418 patients, (RR: 0.57; 95% CI: 0.44-0.74; $P < 0.0001$). We found that SEMS, as a bridge to elective surgery, could decrease the incidence of anastomotic leakage. Leakage occurred in eight patients in the stenting group as compared with 15 patients in the control group. Anastomotic leakage could increase local recurrence and postoperative mortality. SEMS as a bridge to surgery can provide abundant bowel preparation to decrease tissue edema. But, there was no difference in the operation-related complications as shown in three studies involving 206 patients with abscess (RR: 0.83; 95% CI: 0.36-1.95; $P = 0.68$) and extra abdominal complications (RR: 0.67; 95% CI: 0.4-1.12; $P = 0.13$). Abscess was found as the main complication and pneumonia as the most common adverse event.

The major adverse events occurring in the SEMS group was bowel perforation during the stent placement procedure. Procedure- and stent-related complications were found in 5%-23.1% of patients, with an average rate of stent-related perforations of 5%^[29,30]. The oncological consequences of potential tumor dissemination caused by perforations are unclear^[15]. The data from NRCTs are inconsistent, ranging from no difference between colonic stenting and emergent surgery to a significantly reduced 5-year survival rate for patients treated with colonic stenting before elective surgery^[27]. But the possibility of dissemination should be taken into account and the silent perforations should not be disregarded.

A cost-effectiveness analysis, including cost per quality-adjusted life-year as an outcome measure, was also performed in the meta-analysis. As a result, mortality, morbidity, quality-of-life dimensions, and stoma rates between treatment groups suggest that the probability of colonic stenting which could become more effective than emergent surgery is negligible. As only two authors have evaluated the cost-effectiveness, the results may have limitations, and a large sample would be gathered and assessed.

As this meta-analysis has a few limitations, there might be bias in the results. First, only three studies were RCTs, and confounding factors such as age and gender inevitably existed. Second, there was difference in the selection criteria, such as different protocols, method of procedures and so on. Finally, publication bias might exist when the meta-analysis was based on published studies, because positive results are more likely to be published than negative results.

In summary, the current meta-analysis demonstrated that SEMS as a bridge to surgery for obstructed left-sided colon cancer decreased the incidence of primary stoma rates and anastomotic leakage. But the consequence

failed to show the effect on mortality and complications related to surgery. Therefore, preoperative SEMS can be used as an alternative approach for emergent surgery, but should be used with caution, mainly because of concerns of overt and silent perforations. Future studies are needed to further investigate the oncological outcomes and establish whether specific groups of patients could benefit more from either colonic stenting or emergent surgery.

COMMENTS

Background

Colorectal cancer is one of the most commonly diagnosed malignancies worldwide. And many patients with colorectal cancer present with an acute left-sided colonic obstruction. The benefit of surgical management of malignant large bowel obstruction remains controversial. Stent placement before elective surgery as a bridge to surgery is an alternative for emergent surgery in patients with acute left-sided malignant colonic obstruction. Its benefits are uncertain.

Research frontiers

The authors performed a systematic review of the literature and meta-analysis of the one-stage stoma rates, follow-up stoma rates, anastomotic leakage rates, abscess, extra abdominal complications, morbidity and mortality of SEMS compared with emergent surgery.

Innovations and breakthroughs

The current meta-analysis demonstrated the advantage of SEMS as a bridge to surgery for obstructed left-sided colon cancer in term of decreasing the incidence of primary stoma rates and anastomotic leakage. But the consequence failed to show the effect on mortality and complications related to surgery.

Applications

The analysis has shown that preoperative SEMS can be used as an alternative approach for emergent surgery, but should be used with caution, mainly because of the concerns of overt and silent perforations. Future studies are needed to further investigate oncological outcomes and establish whether specific groups of patients could benefit more from either colonic stenting or emergent surgery.

Terminology

SEMS: SEMS is a metallic tube or stent, used to hold a structure in the gastrointestinal tract in order to allow the passage of food, stool, or other secretions required for digestion; Systematic review: A literature review focused on a research question that tries to identify, appraise, select and synthesize all high quality research evidence relevant to that question; Meta-analysis: A combination of the results of several studies that address a set of related research hypotheses.

Peer review

Overall, this is a nice review with good metrics and appropriate analysis.

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Comparison between different reconstruction routes in esophageal squamous cell carcinoma

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Abstract

AIM: To compare postoperative complications and prognosis of esophageal squamous cell carcinoma patients treated with different routes of reconstruction.

METHODS: After obtaining approval from the Medical Ethics Committee of the Sun Yat-Sen University Cancer Center, we retrospectively reviewed data from 306 consecutive patients with histologically diagnosed esophageal squamous cell carcinoma who were treated between 2001 and 2011. All patients underwent radical McKeown-type esophagectomy with at least two-field lymphadenectomy. Regular follow-up was performed in our outpatient department. Postoperative complica-

tions and long-term survival were analyzed by treatment modality, baseline patient characteristics, and operative procedure. Data from patients treated *via* the retrosternal and posterior mediastinal routes were compared.

RESULTS: The posterior mediastinal and retrosternal reconstruction routes were employed in 120 and 186 patients, respectively. Pulmonary complications were the most common complications experienced during the postoperative period (46.1% of all patients; 141/306). Compared to the retrosternal route, the posterior mediastinal reconstruction route was associated with a lower incidence of anastomotic stricture (15.8% *vs* 27.4%, $P = 0.018$) and less surgical bleeding (242.8 ± 114.2 mL *vs* 308.2 ± 168.4 mL, $P < 0.001$). The median survival time was 26.8 mo (range: 1.6-116.1 mo). Upon uni/multivariate analysis, a lower preoperative albumin level ($P = 0.009$) and a more advanced pathological stage (pT; $P = 0.006$; pN; $P < 0.001$) were identified as independent factors predicting poor prognosis. The reconstruction route did not influence prognosis ($P = 0.477$).

CONCLUSION: The posterior mediastinal route of reconstruction reduces incidence of postoperative complications but does not affect survival. This route is recommended for resectable esophageal squamous cell carcinoma.

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Key words: Esophageal carcinoma; Route of reconstruction; Posterior mediastinal; Retrosternal; Comparison

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INTRODUCTION

The therapeutic treatment of esophageal cancer has undergone important changes over the past several decades. For patients with localized esophageal cancer, subtotal esophagectomy with a thoracic-abdominal-cervical incision (McKeown-type esophagectomy), combined with extensive lymphadenectomy, is now generally recognized as the optimal treatment in terms of long-term survival^[1-5].

After subtotal esophagectomy, a gastric tube formed by resection of the lesser curvature is generally considered to be the most suitable esophageal substitute available^[6-10]. Reconstruction under such circumstances commonly uses either the posterior mediastinal (PM) or retrosternal (RS) route^[6,10,11]. However, the optimal route remains controversial, principally because most previous studies focused exclusively on postoperative complications and quality of life, rather than prognosis^[6-9,12].

Thus, for the first time, we conducted a retrospective study to compare not only the incidence of postoperative complications but also prognosis in patients with esophageal squamous cell carcinoma (ESCC) treated *via* the PM and RS routes.

MATERIALS AND METHODS

Patient selection

After approval was obtained from the Medical Ethics Committee of Sun Yat-Sen University Cancer Center, 306 patients diagnosed with ESCC were consecutively recruited between January 2001 and March 2011. Careful preoperative evaluations were conducted to ensure that there were no contraindications to surgical treatment. All patients included in the present evaluation underwent radical esophagectomy. Exclusion criteria included any history of malignant disease, the presence of a second primary tumor, prior non-curative resection (R1/R2), prior use of an esophageal substitute that was not a gastric tube, and any prior neoadjuvant treatment. Disease was staged based on the recommendations of the American Joint Committee on Cancer (AJCC 2010). The baseline characteristics of the 306 patients enrolled in the present study are shown in Table 1.

Surgery and complications

All patients underwent McKeown-type esophagectomy with at least two-field lymphadenectomy, as described in previous studies^[1,4,5]. Three-field lymphadenectomy was performed only if the cervical lymph nodes were thought to be abnormal upon preoperative evaluation. No definitive criteria have been established, therefore,

Table 1 Baseline patient characteristics

Characteristic	Data	Route of reconstruction (%)	
		Posterior mediastinal	Retrosternal
Age (yr)			
Median	58.3	58.6	58.2
Range	32-79	32-79	34-78
≤ 65 ¹	233	90 (75.0)	143 (76.9)
> 65	73	30 (25.0)	43 (23.1)
Sex			
Male	237	95 (79.2)	142 (76.3)
Female	69	25 (20.8)	44 (23.7)
BMI (kg/m ²)			
Median	22.3	22.2	22.3
Range	15.2-35.9	15.2-33.8	15.2-35.9
Smoking index			
Median	444.2	483.6	418.7
Range	0-3330	0-3330	0-2000
≤ 400 ²	174	67 (55.8)	107 (57.5)
> 400	132	53 (44.2)	79 (42.5)
Tumor location			
Upper thorax	46	8 (6.7)	38 (20.4)
Middle thorax	153	54 (45.0)	99 (53.2)
Lower thorax	107	58 (48.3)	49 (26.3)
pT status (UICC 7th)			
pT1	30	11 (9.2)	19 (10.2)
pT2	55	26 (21.7)	29 (15.6)
pT3	221	83 (69.2)	138 (74.2)
pN status (UICC 7th)			
pN0	139	58 (48.3)	81 (43.5)
pN1	93	36 (30.0)	57 (30.6)
pN2	53	16 (13.3)	37 (19.9)
pN3	21	10 (8.3)	11 (5.9)
Tumor grade (UICC 7th)			
G1	90	41 (34.2)	49 (26.3)
G2	168	59 (49.2)	109 (58.6)
G3	48	20 (16.7)	28 (15.1)

¹This signals the commencement of "older age" as defined by the World Health Organization (WHO); ²Risk stratification for lung cancer as defined by the WHO. BMI: Body mass index; G: Grade; pN status: Pathological node status; pT status: Pathological tumor status; UICC: International Union Against Cancer.

each reconstructive option was determined by the individual surgeon. Pyloroplasty was performed if a patient showed abnormal gastric motility preoperatively. Neither a manubrium nor a partial clavicle was reconstructed.

Complications that occurred during hospital stay and during long-term follow-up were recorded. These included anastomotic leakage, chylothorax, pulmonary complications, cardiac complications, and recurrent laryngeal nerve (RLN) palsy. Drainage, conservative management, symptomatic and function-supportive treatment, and observation, respectively, were used as initial treatments. The treatment of choice for anastomotic stricture (the options included bougienage and stent placement) depended on the extent and history of the stricture. Surgical details, and complications, are summarized in Table 2.

Follow-up

After completion of primary treatment, patients were followed up in our outpatient department every 4-6 mo for the first 3 years and every 12 mo thereafter. Radio-

Table 2 Complications arising when either route of reconstruction was used

Variable	Route of reconstruction (%)		P value
	PM	RS	
No. of patients	120	186	
Lymphadenectomy			
Three-field	29 (24.2)	32 (17.2)	0.137
Two-field	91 (75.8)	154 (82.8)	
Pyloroplasty	5 (4.2)	9 (4.8)	0.784
Anastomosis			
Left neck	110 (91.7)	176 (94.6)	0.360
Right neck	8 (6.7)	6 (3.2)	
Intrathoracic	2 (1.7)	4 (2.2)	
Thoracic duct			
Reserved	27 (22.5)	80 (43.0)	< 0.001
Ligation	80 (66.7)	104 (55.9)	
Resected	13 (10.8)	2 (1.1)	
Surgical bleeding (mL)	242.8 ± 114.2	308.2 ± 168.4	< 0.001
Complications (short-term)			
Anastomotic leakage	15 (12.5)	23 (12.4)	0.573
Cervical	13 (10.8)	19 (10.2)	
Intrathoracic	1 (0.8)	2 (1.1)	
Mediastinal	0 (0)	2 (1.1)	0.008
Esophagotracheal	1 (0.8)	0 (0)	
Chylothorax	2 (1.7)	17 (9.1)	0.214
Pulmonary	50 (41.7)	91 (48.9)	0.494
Cardiac	16 (13.3)	20 (10.8)	0.707
RLN palsy	22 (18.3)	31 (16.7)	
Complications (long-term)			
Anastomotic stricture	19 (15.8)	51 (27.4)	0.018

PM: Posterior mediastinal; RS: Retrosternal.

therapy and/or chemotherapy and/or surgical resection were adopted if and when local recurrence and/or metastasis occurred. The chosen treatment modality was determined by consideration of symptoms, the physical condition of the patient, and the clinical stage of disease. Patient-specific therapeutic schedules used the best available remedies at any time. Survival status was explored *via* direct telecommunication with patients or family members in October 2011.

Statistical analysis

We used SPSS 19.0 (SPSS, Inc., Chicago, IL) for statistical analysis. Overall survival (OS) was defined as the interval from the date of surgery to the date of death or final clinical follow-up. Correlations between the reconstructive route and clinicopathological characteristics and postoperative complications were assessed using the *t* and χ^2 tests. To detect factors associated with an increased risk of chylothorax, crude and adjusted analyses were performed using both univariate and multivariate logistic regression. Survival was analyzed *via* the Kaplan-Meier method and the differences between curves were assessed with the aid of the log-rank test. Multivariate Cox's regression analysis was performed with inclusion of parameters that prior univariate analysis had identified as significant. $P < 0.05$ was considered to be significant.

RESULTS

Of the 306 patients, 120 and 186 were treated *via* PM

Table 3 Association between recurrent laryngeal nerve palsy, lymphadenectomy, and development of an anastomotic fistula

Variable	Recurrent laryngeal nerve palsy (%)	P value
Lymphadenectomy		
Three-field	15 (24.6)	0.094
Two-field	38 (12.4)	
Anastomotic fistula		
No	42 (15.6)	0.043
Yes	11 (28.9)	

and RS reconstruction, respectively. Baseline patient characteristics are summarized in Table 1.

Pulmonary complications were the most common during the perioperative period (46.1% of all patients; 141/306). Patients in the RS group were more likely to preserve the thoracic duct ($P < 0.001$). The mean blood loss was 282.6 mL for the entire cohort and was 65.4 mL greater in the RS group than in the PM group ($P < 0.001$). A positive association between development of chylothorax and use of the RS route was observed ($P = 0.008$). Anastomotic stricture, the combined incidence of which was 12.4% (38/306), was more common in the RS than in the PM group ($P = 0.018$). Details of the operations and complications are listed in Table 2. Upon subgroup analysis, RLN palsy was found to be highly associated with three-field lymphadenectomy ($P = 0.094$) and anastomotic fistula ($P = 0.043$) (Table 3).

The median follow-up interval was 32.1 mo for surviving patients. A total of 201 patients were alive at last follow-up. The predicted 1-, 3- and 5-year overall survival rates after primary surgery were 75%, 60% and 50% respectively. The median survival time was 26.8 mo (range: 1.6-116.1 mo).

Preoperative albumin level ($P = 0.009$), pT status ($P = 0.006$), and pN status ($P < 0.001$) were independent factors prognostic for survival upon uni/multivariate analysis (Table 4). However, reconstruction route was not a significant prognostic factor ($P = 0.477$; Figure 1).

DISCUSSION

In the present study of patients with histologically diagnosed esophageal squamous cell carcinoma who underwent radical McKeown-type esophagectomy with at least two-field lymphadenectomy, we compared survival and complications in patients according to whether the RS or PM route was used. Our work had the advantages that it was performed in patients with the same disease etiology treated with a uniform therapeutic modality, and who underwent long-term follow-up focusing not only on postoperative complications but also on prognosis.

We confirmed that the reconstruction route was not associated with any significant variance in the extent of cardiac ($P = 0.494$) or pulmonary ($P = 0.214$) complications, as has been shown in previous studies^[6,7,10].

The incidence of RLN palsy was 17.3% in our entire cohort, and did not differ between the PM and RS groups ($P = 0.707$). In esophagectomy patients, the

Table 4 Univariate and multivariate Cox's regression analysis predicting overall survival

Factor	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P value ¹	HR (95% CI)	P value ¹
Age (yr) ²	1.642 (1.097-2.460)	0.016	1.013 (0.991-1.035)	0.245
Sex ³	0.690 (0.431-1.107)	0.124	0.690 (0.428-1.112)	0.127
BMI (kg/m ²)	0.974 (0.917-1.035)	0.400		
Smoking index ⁴	1.499 (1.019-2.204)	0.040	1.000 (1.000-1.001)	0.596
Preoperative albumin level (g/L)	0.952 (0.917-0.988)	0.009	0.949 (0.912-0.987)	0.009
Tumor location ⁵	0.872 (0.663-1.148)	0.330		
pT ⁶	1.768 (1.209-2.585)	0.003	1.708 (1.163-2.508)	0.006
pN ⁷	1.903 (1.571-2.306)	< 0.001	1.848 (1.525-2.239)	< 0.001
Tumor grade ⁸	0.984 (0.731-1.324)	0.915		
Surgical bleeding	1.001 (0.999-1.002)	0.285		
Lymphadenectomy	0.655 (0.384-1.115)	0.119	0.779 (0.454-1.339)	0.367
Reconstruction ⁹	1.157 (0.775-1.727)	0.477		

¹Cox's proportional hazards model; ²≤ 65 years of age vs > 65 years of age; ³Male vs female; ⁴≤ 400 vs > 400; ⁵Upper thoracic vs middle thoracic vs lower thoracic; ⁶pT1 vs pT2 vs pT3; ⁷pN0 vs pN1 vs pN2 vs pN3; ⁸G1 vs G2 vs G3; ⁹Posterior mediastinal reconstruction vs retrosternal reconstruction. HR: Hazard ratio; pN: Pathological node; pT: Pathological tumor.

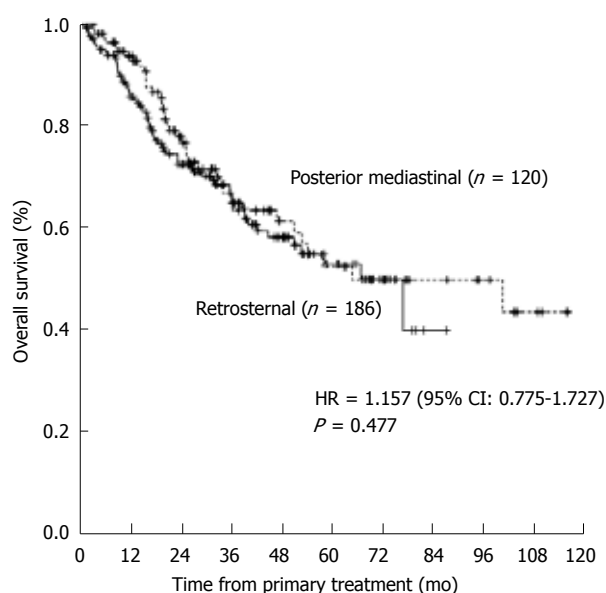


Figure 1 Kaplan-Meier curve showing overall patient survival stratified by route of reconstruction. HR: Hazard ratio.

prime etiology of RLN palsy is direct mechanical injury inflicted on the RLN during dissection^[5,13]. Fang *et al*^[14] reported that development of RLN palsy was closely linked to cervical dissection (22.9% vs 9.6%, $P = 0.089$) and anastomotic leakage (53.8% vs 13.5%, $P = 0.001$). These data lend support to our conclusion that surgical trauma and fistula-induced secondary corrosion play important roles in the development of RLN palsy (Table 3). Thus, RLN injury would be reduced if trauma during cervical lymphadenectomy were minimized and new anastomotic fistulae were managed in a timely manner.

The conduit is longer when the posterior route of reconstruction is used, therefore, a higher frequency of anastomotic leakage would be expected because a segment of stomach that is more remote from the blood supply is used (compared to the RS route)^[8,9]. However, a recent meta-analysis by Urschel *et al*^[10] showed that the

reconstruction route chosen did not affect the frequency of anastomotic fistula (95% CI: 0.35-2.94; $P = 0.98$), in agreement with our data. Additionally, we found a significant association between anastomotic stricture and use of the RS route ($P = 0.018$). After review of the literature, we suggest that patients undergoing RS reconstruction are more at risk of anatomic stricture because of the narrow entrance to the thoracic inlet and the severe foregut angulation that are created when the RS route is used^[7,11,15]. In this context, some authors recommend removal of the manubrium and the sternoclavicular joints^[16,17]. We did not take these options; rather we prioritized thoracic stability and better patient appearance. Also, application of cervical anastomosis, which permits better intraoperative exposure at the cost of more severe postoperative pressure, has been reported to decrease fistula but increases stricture development^[18]. This was not observed in our present work (anastomotic fistula, $P = 0.182$; anastomotic stricture, $P = 0.110$) (data not shown).

Chylothorax, the overall incidence of which was 6.2%, was significantly associated with use of the RS route ($P = 0.008$). However, prophylactic thoracic duct ligation or resection, which is known to mitigate against chylothorax^[19,20], was more likely to be performed in patients of the PM group ($P < 0.001$). Additionally, each reconstructive option was determined by the preference of the individual surgeon. Therefore, surgeon characteristics and decisions regarding whether to perform thoracic duct ligation or resection seemed to be the major factors contributing to the higher incidence of chylothorax observed in the RS group. The route of reconstruction may not be important, in agreement with the data of previous studies^[11,12].

Turning to surgical bleeding, Turnbull *et al*^[21] considered that such bleeding was reduced when the PM technique was used because the extent of tunneling associated with this approach is less than that required when RS reconstruction is used. This is in line with the findings of the present study ($P < 0.001$). Although bleeding

often requires blood transfusion, the clinical significance of a 64.5-mL loss of blood is marginal.

In the present study, the 5-year OS rate was 50% (median: 26.8 mo), which was better than in previous studies^[1,3]. This can be attributed to the strict enrolment criteria. Specifically, to facilitate objective comparisons, we excluded patients with pT4-stage disease, those with preoperative metastasis, and those who underwent non-radical (R1/R2) resections.

To date, the most commonly cited argument as to why the RS route of reconstruction should be favored is that it affords a radiotherapeutic advantage if local recurrence occurs^[6,7], thus contributing to a favorable prognosis. However, to the best of our knowledge, no prior study has evaluated long-term survival in patients treated *via* the RS and PM reconstructive routes. Only the biological behavior of a tumor and the chosen treatment modality could influence survival. Thus, the objective of the present study was not to verify the independently prognostic significance of a given reconstructive route but to ascertain whether the route of reconstruction influenced the efficacy of a uniform treatment modality, thereby inducing a difference in survival. To explore this question, we performed prognostic analysis but failed to demonstrate any significant survival difference according to whether the RS or PM route was used (median OS, 25.4 mo *vs* 27.4 mo; $P = 0.477$) (Figure 1). Several possible explanations may be advanced. First, improvements in patient selection and surgical techniques, especially in the context of McKeown-type esophagectomy combined with extended lymphadenectomy, have decreased the rates of local recurrence^[4,7,22]. Second, various treatment modalities (chemotherapy and/or radiotherapy and/or surgical resection) have been shown to be satisfactory in terms of efficacy and to allow acceptable levels of post-recurrence survival^[23-26]. Thus, recurrence is no longer an intractable problem for which no effective treatment is available.

Several prognostic factors were identified upon uni/multivariate survival analysis; these included preoperative albumin level and pathological stage (pT and pN status). Lower preoperative albumin levels have long been regarded as indicative of poor nutritional status, abnormal liver function, and a metabolic response to acute phase disease^[27,28]. A lower albumin level was significantly associated with a shorter survival time ($P = 0.009$). A similar result was reported by Lien *et al*^[29] in patients with adenocarcinoma of the gastric cardia. The importance of pathological stage, the best-established prognostic factor for malignant disease, was emphasized once again in our present work.

The present study has both strengths and weaknesses. The work was retrospective in nature. All work was performed in a single institution, with patients who had disease of uniform etiology and who were identically treated. However, the data may be biased to some extent, because we could not control for surgical experience or guarantee that all documentation was completely accu-

rate. However, we attempted to minimize the latter possible source of error by using consistent definitions when performing our review of records, and all data were independently checked.

In conclusion, this is believed to be the first retrospective study to investigate systematically the influence of reconstructive route on both clinical complications and prognosis. Use of the PM route of reconstruction reduces the incidence of postoperative complications, without compromising survival, and must be recommended for use in patients with resectable ESCC.

COMMENTS

Background

For patients with localized esophageal cancer, subtotal esophagectomy with a thoracic-abdominal-cervical incision (McKeown-type esophagectomy), combined with extensive lymphadenectomy, is now generally recognized as the optimal treatment in terms of long-term survival. Reconstruction under such circumstances commonly uses either the posterior mediastinal (PM) or retrosternal (RS) route. Despite studied and debated for decades, there remains no consensus as to the optimal route of reconstruction after subtotal esophagectomy.

Research frontiers

Postoperative complications and quality of life are hot topics and have been analyzed in previous studies. A recent meta-analysis showed that PM and anterior mediastinal routes of reconstruction are associated with similar outcomes.

Innovations and breakthroughs

To date, the most commonly cited argument as to why the RS route of reconstruction should be favored is that it affords a radiotherapeutic advantage if local recurrence occurs, thus contributing to a favorable prognosis. However, no prior study has evaluated long-term survival and, their results may be biased based on their small sample size. The authors' work had the advantages that it was performed in a large cohort with the same disease etiology treated with a uniform therapeutic modality, and who underwent long-term follow-up focusing not only on postoperative complications but also on prognosis.

Applications

Use of the PM route of reconstruction reduces the incidence of postoperative complications but does not affect survival. This route is recommended for treatment of patients with resectable esophageal squamous cell carcinoma.

Peer review

This paper is well written. The authors retrospectively reviewed 306 consecutive patients. They described that choice of reconstruction method was determined by surgeon preference, because there are still no criteria. Therefore, the surgeon factor should be included in all analyses. Especially in the analysis of risk for postesophagectomy chylothorax, the major factor seemed to be the surgeon and not route of reconstruction.

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Carbon dioxide insufflation for endoscopic retrograde cholangiopancreatography: A meta-analysis and systematic review

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Abstract

AIM: To assess the safety and efficacy of carbon dioxide (CO₂) insufflation during endoscopic retrograde cholangiopancreatography (ERCP).

METHODS: The Cochrane Library, Medical Literature Analysis and Retrieval System Online, Excerpta Medica Database, Science Citation Index Expanded, Chinese Biomedical Literature Database, and references in relevant publications were searched up to December 2011 to identify randomized controlled trials (RCTs) comparing CO₂ insufflation with air insufflation during ERCP. The trials were included in the review irrespective of sample size, publication status, or language. Study selection and data extraction were performed by two independent authors. The meta-analysis was performed using Review Manager 5.1.6. A random-effects model was used to analyze various outcomes.

Sensitivity and subgroup analyses were performed if necessary.

RESULTS: Seven double-blind RCTs involving a total of 818 patients were identified that compared CO₂ insufflation ($n = 404$) with air insufflation ($n = 401$) during ERCP. There were a total of 13 post-randomization dropouts in four RCTs. Six RCTs had a high risk of bias and one had a low risk of bias. None of the RCTs reported any severe gas-related adverse events in either group. A meta-analysis of 5 RCTs ($n = 459$) indicated that patients in the CO₂ insufflation group had less post-ERCP abdominal pain and distension for at least 1 h compared with patients in the air insufflation group. There were no significant differences in mild cardiopulmonary complications [risk ratio (RR) = 0.43, 95% CI: 0.07-2.66, $P = 0.36$], cardiopulmonary (e.g., blood CO₂ level) changes [standardized mean difference (SMD) = -0.97, 95% CI: -2.58-0.63, $P = 0.23$], cost analysis (mean difference = 3.14, 95% CI: -14.57-20.85, $P = 0.73$), and total procedure time (SMD = -0.05, 95% CI: -0.26-0.17, $P = 0.67$) between the two groups.

CONCLUSION: CO₂ insufflation during ERCP appears to be safe and reduces post-ERCP abdominal pain and discomfort.

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Key words: Systematic review; Meta-analysis; Carbon dioxide insufflation; Endoscopic retrograde cholangiopancreatography; Abdominal pain

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INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) refers to radiographic visualization of the pancreatobiliary system by retrograde injection of contrast media through the ampulla of Vater^[1]. ERCP, which was first introduced in the late 1960s^[2], is now widely performed by endoscopists to diagnose various pancreatic and biliary diseases^[1,3]. Although the exact number of patients undergoing ERCP worldwide each year is unknown, it has been reported that approximately 54 000 and 500 000 patients undergo ERCP annually in the United Kingdom and United States, respectively^[4-6]. ERCP has become an invaluable tool in the diagnosis of numerous pancreatic and biliary diseases and is considered to be the gold standard study for the pancreatobiliary system^[1,3-5].

Gas is deliberately insufflated into the bowel lumen during ERCP to provide operating space to ensure adequate visualization by the camera and manipulation of instruments in the duodenum^[1-4]. Currently, air is the most commonly used gas for insufflation during ERCP worldwide^[6]. However, air has some potential disadvantages. Air is not absorbed by the bowel and must be passed from the gastrointestinal tract in the form of flatus, which may lead to post-ERCP abdominal pain and discomfort (e.g., abdominal distension) because of gas retention in the gastrointestinal tract^[7]. Recently, carbon dioxide (CO₂) has been introduced as an alternative to air for insufflation during ERCP^[8-14].

CO₂ is rapidly absorbed from the bowel and is delivered directly to the lungs by the circulation^[15,16]. Ultimately, it is excreted by the lungs during respiratory exchange^[15,16]. Theoretically, CO₂ has the potential to reduce post-ERCP abdominal pain and discomfort due to lower gas retention^[15,16]. However, the absorption of CO₂ may cause hypercapnia and acidosis, which must be prevented through hyperventilation^[17]. The absorption of CO₂ may be associated with various cardiopulmonary side effects such as tachycardia, cardiac arrhythmias, hypoxemia, and pulmonary edema^[17]. Elderly patients with cardiopulmonary diseases are more likely to suffer from these adverse events^[9,17].

The use of CO₂ insufflation during ERCP is controversial. Some authors suggest that the application of CO₂ insufflation reduces post-ERCP abdominal pain and discomfort^[8,11,12,14], thus they recommend the use of CO₂ in ERCP, whereas others do not think so^[9,10,13]. To date, we have been unable to identify any meta-analysis that assesses the role of carbon dioxide insufflation during ERCP. We conducted a meta-analysis and systematic review to assess the safety

and efficacy of CO₂ insufflation during ERCP for reduction of post-ERCP abdominal pain and discomfort.

MATERIALS AND METHODS

Identification of trials and data extraction

We searched the following databases up to December 2011 to identify randomized controlled trials (RCTs): The Cochrane Library, Medical Literature Analysis and Retrieval System Online (MEDLINE), Excerpta Medica Database (EMBASE), Science Citation Index Expanded, and Chinese Biomedical Literature Database (CBM). Search strategies for these databases are shown in Table 1. We also hand searched the references in relevant publications to explore additional relevant clinical trials. After completing all searches, we merged the search results using the software package Endnote X4 (reference management software) and removed duplicated records. Two independent authors (Lin YX and Xiong XZ) scanned the title and abstract of every record identified by the searches for inclusion. If compliance with inclusion criteria was not clear from the abstract, we retrieved full texts for further assessment. Only RCTs comparing CO₂ insufflation with air insufflation during ERCP were considered for the review, irrespective of sample size, publication status, or language. Two independent authors (Wu SJ and Lu J) independently extracted and confirmed the data and entered them into an electronic data collection form. We resolved all differences between authors by discussion.

Outcomes

Data for the following outcomes were extracted: abdominal pain (pain scores *via* visual analogue scale and number of pain-free patients at various time points after ERCP), abdominal distention, gas-related complications (severe gas-related adverse events and mild cardiopulmonary complications), cardiopulmonary changes (heart rate, blood pressure, blood pH, *etc.*), cost analysis, and total procedure time.

Assessment of methodological quality

Two authors (Cheng Y and Cheng NS) independently assessed the methodological quality of the included trials using the quality checklist recommended by the Cochrane Handbook^[18]. We assessed the risk of bias of the trials based on the following domains: random sequence generation, allocation concealment, blinding of participants and personnel, blinding of outcome assessment, incomplete outcome data, selective reporting and other sources of bias^[18]. Following the evaluation of the above domains, an included trial was judged as a trial with a low risk of bias if it was evaluated as "low" in all of the above domains. If the risk of bias was judged as "unclear" or "high", then the trial was listed under the group of trials with "high risk of bias". We resolved all disagreements by discussion and referral to a third author (Wu TX) for adjudication.

Statistical analysis

We performed the meta-analysis using the software pack-

Table 1 Search strategies

Databases	Period of search	Search strategies
The Cochrane library	Until 1st December 2011	1. MeSH descriptor Cholangiopancreatography, Endoscopic Retrograde explode all trees 2. (endoscopic retrograde cholangiopancreatograph*): ti, ab, kw OR (ERCP): ti, ab, kw 3. MeSH descriptor Carbon Dioxide explode all trees 4. (carbon dioxide): ti, ab, kw OR (CO ₂): ti, ab, kw 5. 1 OR 2 6. 3 OR 4 7. 5 AND 6
MEDLINE <i>via</i> PubMed	Until 1st December 2011	1. "Cholangiopancreatography, Endoscopic Retrograde" [MeSH] OR endoscopic retrograde cholangiopancreatograph* [tiab] OR ERCP [tiab] 2. "Carbon Dioxide" [Mesh] OR carbon dioxide [tiab] OR CO ₂ [tiab] 3. 1 AND 2
EMBASE <i>via</i> embase.com	Until 1st December 2011	1. 'endoscopic retrograde cholangiopancreatography'/exp OR 'endoscopic retrograde cholangiopancreatography' 2. 'ercp'/exp OR ercp 3. 'carbon dioxide'/exp OR 'carbon dioxide' 4. 'CO ₂ '/exp OR CO ₂ 5. 1 OR 2 6. 3 OR 4 7. 5 AND 6
Science citation index expanded	Until 1st December 2011	1. TS = ('endoscopic retrograde cholangiopancreatograph*' OR ERCP) 2. TS = ('carbon dioxide' OR CO ₂) 3. 1 AND 2
CBM	Until 1st December 2011	Search strategy in Chinese. Includes search terms similar to the terms used in MEDLINE

MEDLINE: Medical Literature Analysis and Retrieval System Online; EMBASE: Excerpta Medica Database; CBM: Chinese Biomedical Literature Database; MeSH: Medical Subject Heading; ERCP: Endoscopic retrograde cholangiopancreatography.

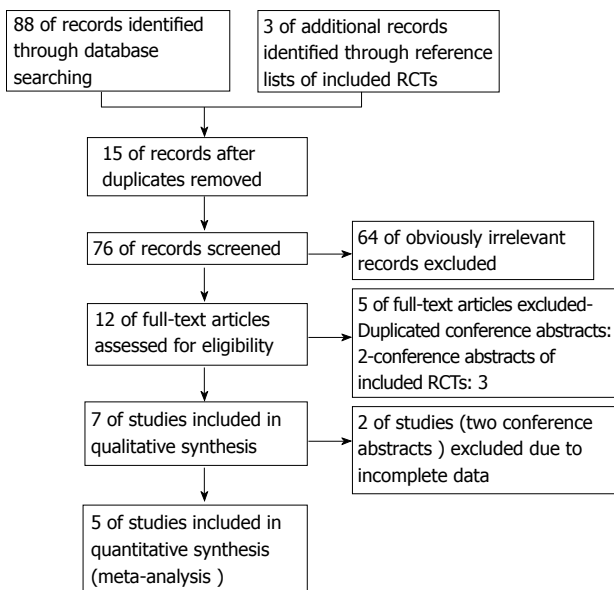


Figure 1 Flow diagram showing study selection process. RCT: Randomized controlled trial.

age Review Manager 5.1.6. Two authors (Wu SJ, Lu J) confirmed and entered all data into Review Manager independently. For dichotomous outcomes, we calculated the risk ratio (RR) with 95% CI^[19]. For continuous outcomes, we calculated the mean difference (MD) with 95% CI^[19]. For continuous outcomes with different measurement scales in different RCTs, we calculated the standardized mean difference (SMD) with 95% CI^[19]. We described the heterogeneity with the Chi-squared test^[19]. A *P* value less than 0.10 was considered to be sig-

nificant heterogeneity^[19]. We also used the *I*² statistic to measure the quantity of heterogeneity^[19]. For all analysis, we employed the random-effects model. We intended to perform funnel plots and assessed their visual asymmetry to determine reporting biases^[20]. In case of missing data, we contacted the original investigators to request further information. If there was no reply, we performed the analysis on an "intention-to-treat" (ITT) principle, if applicable^[21]. Otherwise, we adopted the available-case analysis (also known as per-protocol analysis and PP analysis). We conducted the meta-analysis and systematic review according to the Cochrane Handbook for Systematic Reviews of Interventions^[22] and Preferred Reporting Items for Systematic reviews and Meta-Analysis (PRISMA)^[23].

RESULTS

Search results

We identified a total of 88 records through electronic searches of The Cochrane Library (*n* = 4), MEDLINE (*n* = 16), EMBASE (*n* = 47), Science Citation Index Expanded (*n* = 20), Chinese Biomedical Literature Database (*n* = 1), and hand search of the references of the included RCTs (*n* = 3). We excluded 15 duplicates and 64 clearly irrelevant records by reading titles and abstracts. Twelve full-text articles were retrieved for further assessment. We excluded five articles for the reasons listed in Figure 1.

Description of included trials

Seven RCTs (5 articles^[8-12] and 2 conference abstracts^[13,14]), which were published between 2007 and 2011, were identified that fulfilled the inclusion criteria. A total of

Table 2 Study characteristics

Author	Year	Country	Study design	Participants	Participants (CO ₂ /Air)	Mean age (CO ₂ /Air)
Bretthauer <i>et al</i> ^[8]	2007	Norway	Multi-centers	Low risk	118 (58/58)	57/54
Maple <i>et al</i> ^[12]	2009	United States	Single-center	Low risk	105 (50/50)	57/51.7
Dellon <i>et al</i> ^[9]	2010	United States	Single-center	High risk and low risk	78 (36/38)	60.1/59.7
Kuwatani <i>et al</i> ^[10]	2011	Japan	Multi-centers	Low risk	80 (40/40)	66.1/68.7
Luigiano <i>et al</i> ^[11]	2011	Italy	Single-center	Low risk	78 (39/37)	66.1/67.1
Mei <i>et al</i> ^[13]	2011	Australia	Single-center	Not mentioned	61 (34/27)	Not mentioned
Arjunan <i>et al</i> ^[14]	2011	India	Single-center	Low risk	298 (147/151)	Not mentioned

Low risk refers to patients without chronic obstructive pulmonary diseases; high risk refers to patients with chronic obstructive pulmonary diseases.

Table 3 Risk of bias in included trials

Studies	Sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting
Bretthauer <i>et al</i> ^[8]	Low risk	Low risk	Low risk	Low risk	High risk	High risk
Maple <i>et al</i> ^[12]	Low risk	Low risk	Low risk	Low risk	High risk	High risk
Dellon <i>et al</i> ^[9]	Low risk	Low risk	Low risk	Low risk	Low risk	Low risk
Kuwatani <i>et al</i> ^[10]	Low risk	Unclear risk	Low risk	Low risk	Low risk	High risk
Luigiano <i>et al</i> ^[11]	Low risk	Low risk	Low risk	Low risk	High risk	High risk
Mei <i>et al</i> ^[13]	Unclear risk	Unclear risk	Low risk	Low risk	Unclear risk	Unclear risk
Arjunan <i>et al</i> ^[14]	Unclear risk	Unclear risk	Low risk	Low risk	Unclear risk	Unclear risk

818 patients were included: 404 were assigned to the CO₂ group and 401 were allocated to the air group (13 post-randomization dropouts). Details on the included studies are shown in Table 2.

Risk of bias of included studies

The risk of bias is summarized in Table 3. Six RCTs^[8,10-14] had a high risk of bias and one RCT^[9] had a low risk of bias. All trials were double-blind RCTs with a parallel group study design. There were a total of 13 post-randomization dropouts in four RCTs^[8,9,11,12] which were not included in the analysis. Only one RCT^[9] reported costs.

Effects of interventions

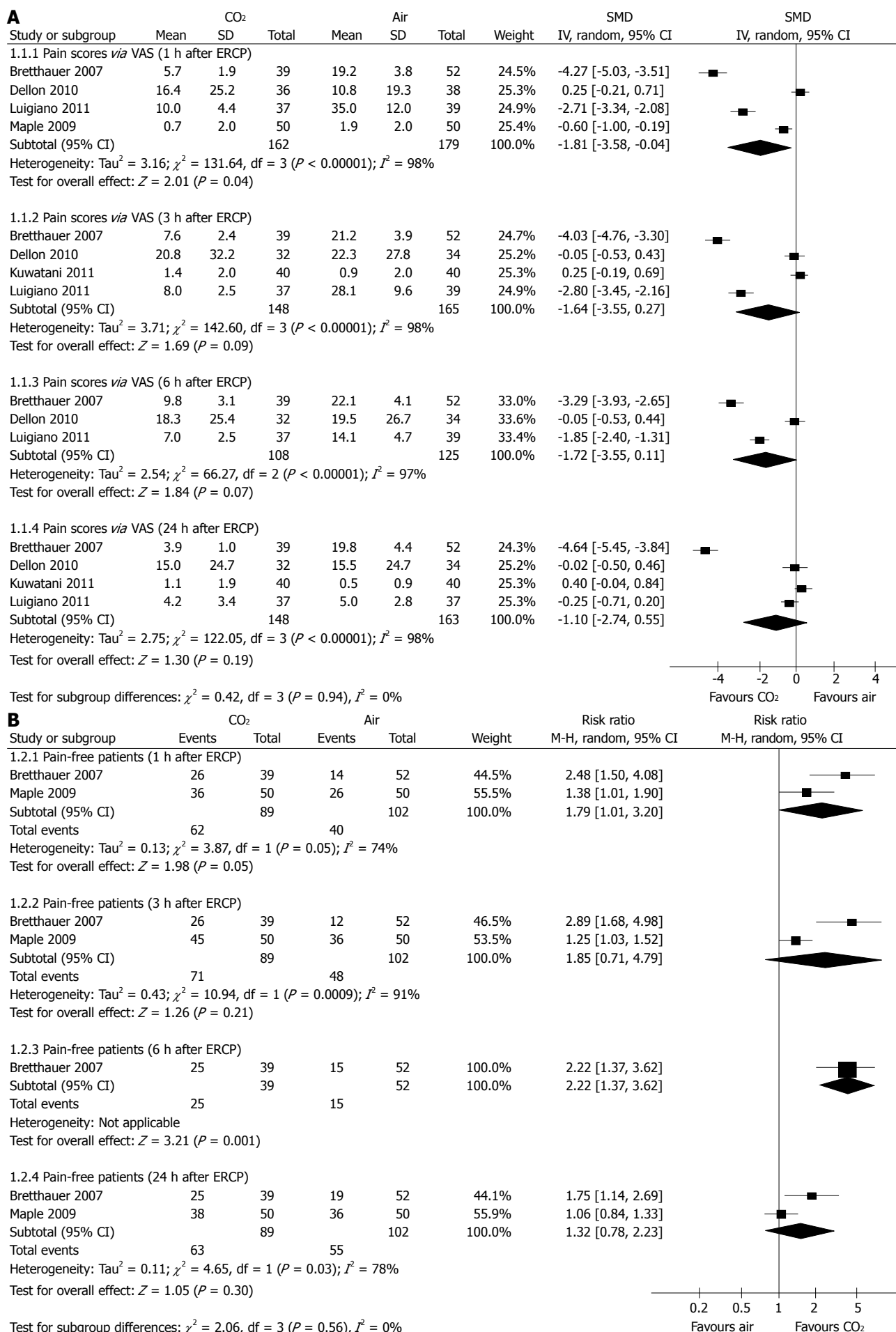
Abdominal pain scores (intensity of pain): Seven RCTs reported the abdominal pain scores. Pain was measured with a 10-point visual analogue scale (VAS)^[10,12-14] or a 100-mm VAS^[8-9,11] at various time points, including before the procedure, during the procedure, 30 min, 1 h, 90 min, 3 h, 6 h and 24 h after ERCP. Four RCTs^[8,11,12,14] showed decreased post-ERCP abdominal pain scores in the CO₂ group compared with the air group, while the other three RCTs^[9,10,13] did not. The meta-analysis of 5 RCTs^[8-12] showed that the abdominal pain scores 1 h after ERCP was significantly lower in the CO₂ group than the air group ($I^2 = 98\%$; SMD = -1.81, 95% CI: -3.58--0.04, $P = 0.04$). The pain scores 3 h, 6 h and 24 h after ERCP were also lower in the CO₂ group than in the air group, but the differences were not significant (Figure 2A).

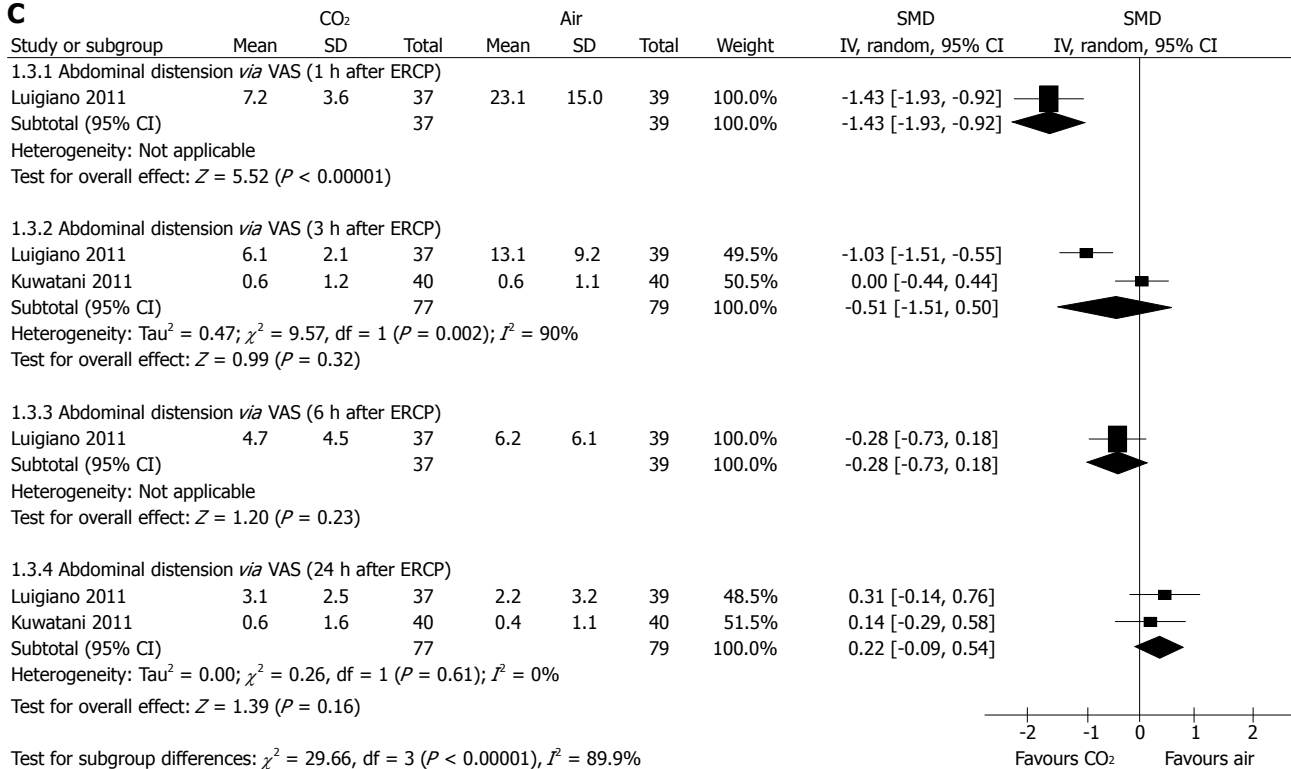
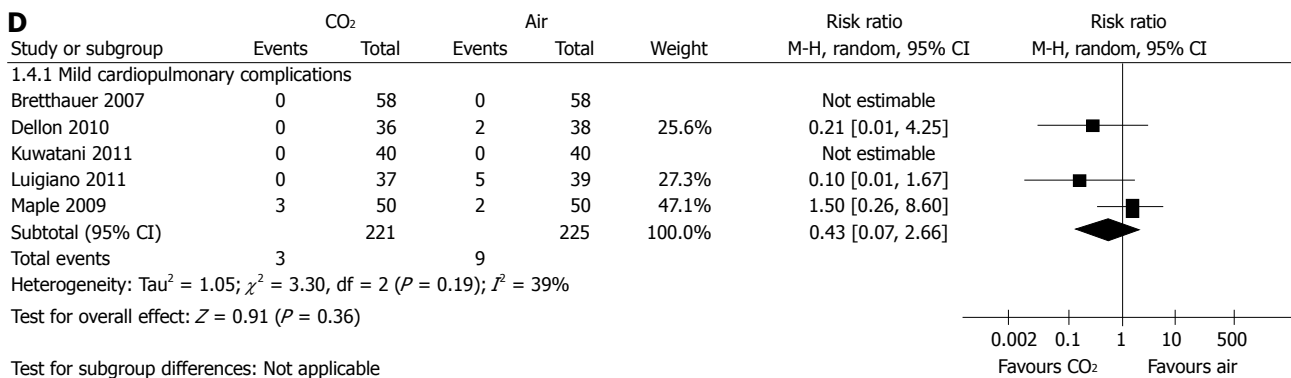
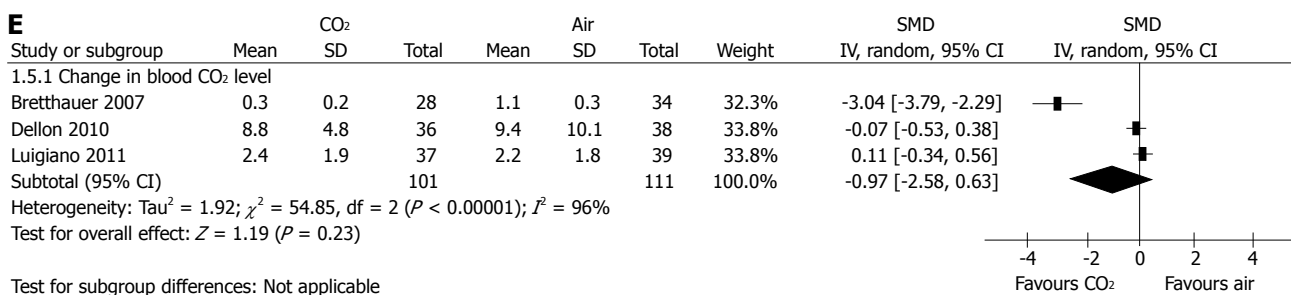
Pain-free patients (incidence of pain): Only two RCTs^[8,12] reported the number of pain-free patients at various time points, including 1, 3, 6 and 24 h after ERCP. The meta-analysis showed that the rate of pain-free patients 1 h and

6 h after ERCP was significantly higher in the CO₂ group than in the air group: ($I^2 = 74\%$; RR = 1.79, 95% CI: 1.01-3.20, $P = 0.05$) and (RR = 2.22, 95% CI: 1.37-3.62, $P = 0.001$), respectively. The rate of pain-free patients 3 h and 24 h after ERCP were also higher in the CO₂ group than in the air group, but the differences were not significant (Figure 2B).

Abdominal distention: Abdominal distention was measured with a 100-mm VAS in two RCTs^[10,11]. The meta-analysis showed that abdominal distention 1 h after ERCP was significantly lower in the CO₂ group than in the air group (SMD = -1.43, 95% CI: -1.93--0.92, $P < 0.00001$). There was no significant difference in abdominal distention between the two groups at 3 h, 6 h and 24 h after ERCP (Figure 2C). Three RCTs^[9,12,14] reported the increase in abdominal girth. Maple *et al*^[12] and Arjunan *et al*^[14] stated that CO₂ insufflation was associated with less increase in abdominal girth than air insufflation. However, Dellon *et al*^[9] indicated that there was no significant difference between groups with regard to increase in abdominal girth. One RCT^[8] reported the number of patients with bowel distention as assessed by X-ray photographs. There were fewer patients with bowel distention in the CO₂ group than in the air group (RR = 0.76, 95% CI: 0.60-0.98, $P = 0.03$).

Complications: None of the RCTs reported any severe gas-related adverse events in either group (e.g., death, embolism, cardiac arrhythmias, and significant respiratory events). Mild cardiopulmonary complications (e.g., respiratory depression, hypotension, and bradycardia) were reported in five RCTs^[8-12]. There was no significant difference in the number of patients with any mild car-



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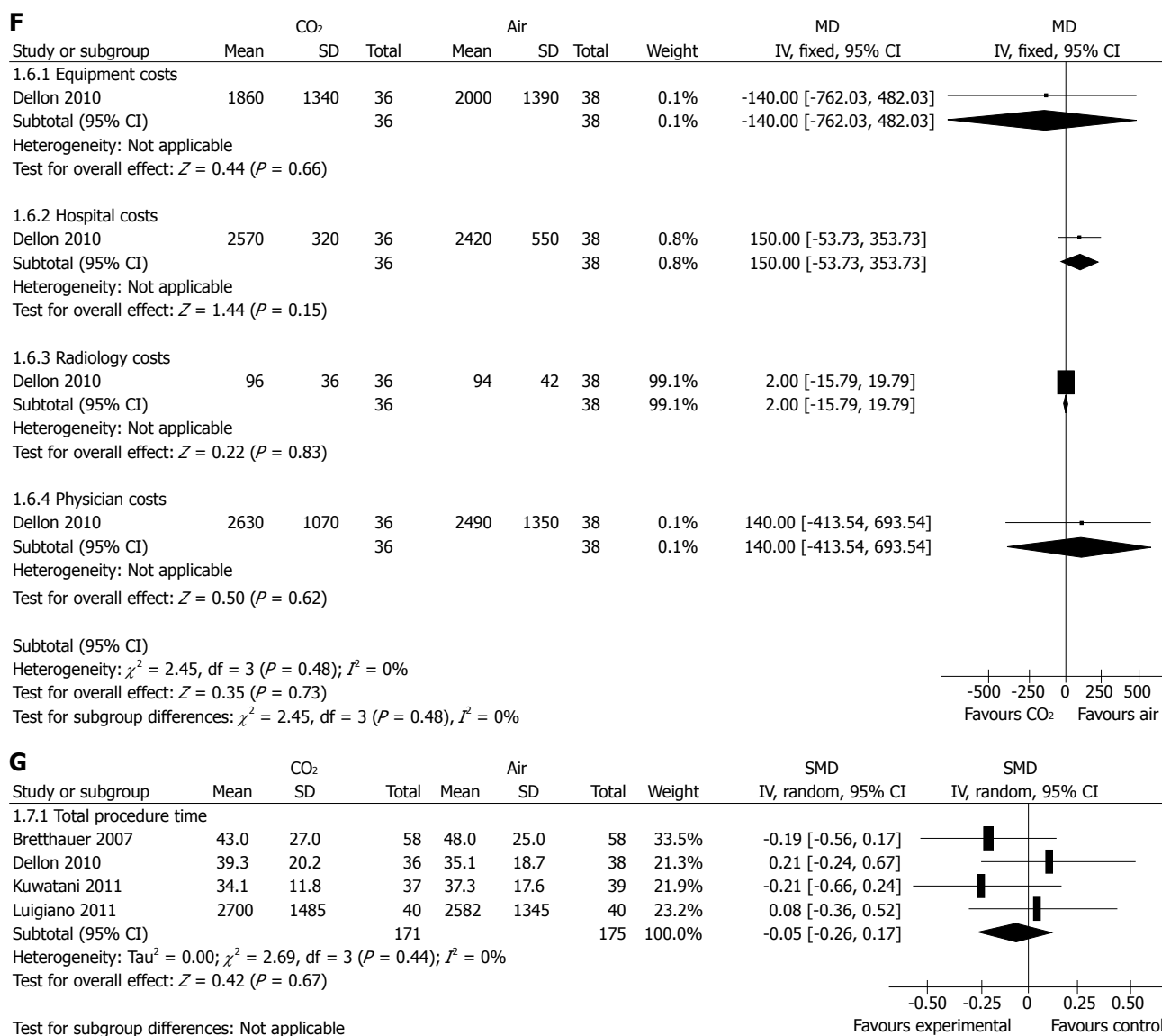


Figure 2 Forest plots of meta-analysis. A: Carbon dioxide vs air in abdominal pain scores; B: Carbon dioxide vs air in pain-free patients; C: Carbon dioxide vs air in abdominal distension; D: Carbon dioxide vs air in mild cardiopulmonary complications; E: Carbon dioxide vs air in change in blood carbon dioxide level; F: Carbon dioxide vs air in total costs; G: Carbon dioxide vs air in total procedure time. IV: Inverse-variance; M-H: Mantel Haenszel; ERCP: Endoscopic retrograde cholangiopancreatography; VAS: Visual analogue scale; MD: Mean difference; SMD: Standardized mean difference.

diopulmonary complications between groups ($I^2 = 39\%$; $RR = 0.43$, 95% CI: 0.07-2.66, $P = 0.36$) (Figure 2D).

Cardiopulmonary changes: Changes in blood CO₂ levels were reported in three RCTs^[8,9,11]. There was no significant difference in the change in blood CO₂ level between the two groups ($I^2 = 96\%$; $SMD = -0.97$, 95% CI: -2.58-0.63, $P = 0.23$). Only one RCT^[10] reported blood oxygen saturation (SpO₂); Kuwatani *et al*^[10] stated that there was no significant difference between the two groups at any time point (Figure 2E).

Cost analysis: Only one RCT^[9] reported the cost analysis. There were no significant differences in the total costs for ERCP ($I^2 = 0\%$; $MD = 3.14$, 95% CI: -14.57-20.85, $P = 0.73$), including equipment costs, hospital costs, radiology costs, and physician costs (Figure 2F).

Total procedure time: Four RCTs were included in the meta-analysis^[8-11]. There was no significant difference in the total procedure time between the two groups ($I^2 = 0\%$; $SMD = -0.05$, 95% CI: -0.26-0.17, $P = 0.67$). Maple *et al*^[12] stated that there was no difference in total procedure time between the two groups (Figure 2G).

DISCUSSION

This systematic review with meta-analysis of RCTs assessed the safety and efficacy of CO₂ insufflation *vs* air insufflation during ERCP for reduction of abdominal pain and discomfort. The meta-analysis of 5 RCTs indicated that CO₂ insufflation during ERCP was associated with less post-ERCP abdominal pain and distension for at least 1 h. There were no significant differences in mild cardiopulmonary complications, cardiopulmonary

changes, cost analysis, and total procedure time between the two groups.

Post-ERCP abdominal pain and discomfort are common in clinical practice^[24]. Abdominal pain related to insufflation is nonspecific and may mimic the symptoms of severe post-ERCP complications, including pancreatitis and perforation, which can be a source of stress to both patients and endoscopists^[9]. Some patients need hospitalization for further evaluation and observation of post-ERCP abdominal pain and discomfort^[7,15]. Post-ERCP abdominal pain and discomfort may result from air insufflation, as air is difficult to dissolve in blood and stays in the bowel for several hours after ERCP^[7,16]. CO₂ has unique characteristics in that it is cheap, colorless, nonflammable, non-explosive, easily excreted, and non-toxic to patients^[16]. CO₂ insufflation during ERCP was first introduced by Bretthauer *et al*^[8] in 2007. Bretthauer *et al*^[8] found that CO₂ insufflation effectively reduced post-ERCP abdominal pain and discomfort and recommended its routine use in ERCP. Then the benefits of CO₂ insufflation were further demonstrated by three later RCTs^[11,12,14]. On the contrary, three additional RCTs^[9,10,13] showed no differences in post-ERCP abdominal pain or discomfort between CO₂ insufflation and air insufflation. Although this meta-analysis suggested that CO₂ insufflation was associated with less abdominal pain and discomfort for at least 1 h, it included only 5 RCTs and all had small sample sizes. Consequently, the data from three ongoing RCTs performed by Mei *et al*^[13], Arjunan *et al*^[14] and Janssens *et al*^[15] (Trial number: UMIN000005755) are anticipated to resolve the controversy.

The safety of CO₂ insufflation during ERCP is another major concern for patients and endoscopists. Air insufflation is associated with rare but severe adverse events such as combustion when using electrocautery and gas embolisms^[16,25]. CO₂ is non-flammable and more soluble than air. In theory, CO₂ insufflation is safer than air insufflation with regard to combustion and embolisms. CO₂ is the most commonly used gas for insufflation in laparoscopy. The safety of CO₂ insufflation for endoscopy has been well established in colonoscopy^[16]. None of the included RCTs reported any severe CO₂-related adverse events (e.g., death, embolism, cardiac arrhythmias, or significant respiratory events). With regard to other adverse effects from CO₂ insufflation, the meta-analysis showed there were no differences in mild cardiopulmonary complications and cardiopulmonary changes between the two groups. However, all RCTs except one^[9] excluded patients with chronic obstructive pulmonary diseases (COPD). The safety of CO₂ insufflation during ERCP for high-risk patients (e.g., patients with cardiopulmonary diseases or American Society of Anesthesiologists (ASA) Physical Status classification III or IV) needs further evaluation.

There are other potential benefits of CO₂ insufflation for ERCP, such as the possibility of immediate computed tomography cholangiopancreatography after ERCP, which has recently been introduced to obtain clear images, and the

possibility of intraoperative ERCP during laparoscopy^[16,26].

The feasibility of CO₂ insufflation during ERCP is as follows. First, to date there have been three types of commercial CO₂ insufflators available: Olympus KeyMed ECR, Olympus UCR, and CO₂-EFFICIENT^[15]. In addition, the cost of a CO₂ insufflator is not very high, at approximately 7000 euros^[15]. CO₂ gas is inexpensive and convenient to obtain^[15]. Dellon *et al*^[9] found that the total costs for ERCP (including equipment costs, hospital costs, radiology costs, and physician costs), is similar between groups (\$7170 in the CO₂ group *vs* \$7000 in the air group). Moreover, the safety of CO₂ insufflation during ERCP has been well documented in the RCTs (see above). Thus, it appears that widespread implementation of CO₂ insufflation during ERCP is anticipated.

This review included a total of seven RCTs. Most excluded patients with COPD, whereas only one study^[9] included patients with COPD. Thus, the results of this review may only be relevant to low-risk patients without cardiopulmonary diseases.

The overall quality of the current best evidence was low. Only one RCT had a low risk of bias. There were a total of 13 post-randomization dropouts in four RCTs. All four RCTs adopted the available-case analysis (also known as per-protocol analysis and PP analysis) without performing an intention-to-treat (ITT) analysis. Only one RCT reported the cost analysis. Further RCTs are anticipated to perform both PP analysis and ITT analysis in case of post-randomization dropouts (missing data) and report the costs of ERCP.

Dellon *et al*^[7] conducted a systematic review published in 2009 which assessed the role of CO₂ insufflation for flexible sigmoidoscopy, colonoscopy, ERCP, and double-balloon enteroscopy. This review included nine RCTs, but only one used CO₂ insufflation for ERCP. In addition, the authors did not perform a meta-analysis because of obvious heterogeneity. Our findings are similar to the previous systematic review in terms of the safety of CO₂ insufflation. In contrast to our study, the previous systematic review showed a reduction in abdominal pain for at least 6 h in the CO₂ insufflation group when compared with the air group.

The limitations of our review are as follows. The first concerns the small number of included RCTs and the small sample size of each RCT. We included only seven RCTs with 404 patients undergoing ERCP with CO₂ insufflation; thus, there is a lack of available data on this issue to date. In addition, we did not perform funnel plots to assess the publication bias due to the small number of included RCTs.

This review currently provides the best available evidence for comparison of CO₂ insufflation versus air insufflation during ERCP. On the basis of this evidence, CO₂ insufflation during ERCP appears to be safe for the majority of patients and results in less post-ERCP abdominal pain and discomfort than air insufflation. Further RCTs with a low risk of bias and a greater number of patients are necessary to assess the role of CO₂

insufflation in high-risk patients. Future RCTs need to be conducted and reported according to the Consolidated Standards of Reporting Trials (CONSORT) statement^[27].

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COMMENTS

Background

Endoscopic retrograde cholangiopancreatography (ERCP) is now widely performed by endoscopists to diagnose various pancreatic and biliary diseases. Currently, air is the most commonly used gas for insufflation during ERCP worldwide. However, air has some potential disadvantages.

Research frontiers

Recently, carbon dioxide (CO₂) has been introduced as an alternative to air for insufflation during ERCP. Many studies, including randomized controlled trials (RCTs), have been conducted to assess the safety and efficacy of CO₂ insufflation during ERCP for reduction of post-ERCP abdominal pain and discomfort. However, the use of CO₂ insufflation during ERCP is controversial.

Innovations and breakthroughs

The authors identified all RCTs comparing CO₂ insufflation with air insufflation during ERCP. They conducted a meta-analysis and systematic review according to the Cochrane Handbook for Systematic Reviews of Interventions and Preferred Reporting Items for Systematic reviews and Meta-Analysis. They found that CO₂ insufflation during ERCP appears to be safe and reduces post-ERCP abdominal pain and discomfort.

Applications

Due to reduced post-procedure abdominal pain and discomfort, CO₂ insufflation may be preferable to air insufflation during ERCP and should be recommended in clinical practice.

Terminology

ERCP refers to radiographic visualization of the bile duct and pancreatic duct by retrograde injection of contrast media into the pancreatobiliary system; RCTs refer to trials in which people are allocated at random to receive one of several clinical interventions.

Peer review

This is the first well-designed meta-analysis on the role of CO₂ insufflation in ERCP. The results are interesting and suggest that CO₂ insufflation may be preferable to air insufflation during ERCP. However, this article has several limitations, such as a small number of RCTs.

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Clostridium perfringens bacteremia caused by choledocholithiasis in the absence of gallbladder stones

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INTRODUCTION

Clostridium species are the second most common causes of anaerobic bacteremia, with *Clostridium perfringens* the most frequently isolated. Malignancies, gastrointestinal disorders and other chronic illnesses have been associated with *Clostridium perfringens* bacteremia. *Clostridium perfringens* can cause food poisoning, gas gangrene, necrotizing enterocolitis, tuboovarian abscess, emphysematous cholecystitis, discitis, and liver abscess. Choledocholithiasis has rarely been reported as a source of *Clostridium perfringens* bacteremia. We describe a case of *Clostridium perfringens* bacteremia caused by choledocholithiasis in the absence of gallbladder stones and with normal common bile duct (CBD) diameter and discuss management and review of the literature of this interesting but rare entity.

CASE REPORT

A 67-year-old male with medical history of coronary artery disease, diabetes mellitus, hypertension, hypothyroidism, chronic obstructive pulmonary disease (COPD) and diverticulosis presented with abdominal pain and fever. Fever with chills started four days prior to admission. Abdominal pain was sharp, periumbilical, non-radiating, lasted for 4 h, unrelated to food and resolved on its own. He denied nausea, vomiting, blood in stools but had chronic constipation. His last colonoscopy was

Abstract

A 67-years-old male presented with periumbilical abdominal pain, fever and jaundice. His anaerobic blood culture was positive for *Clostridium perfringens*. Computed tomogram scan of the abdomen and abdominal ultrasound showed normal gallbladder and common bile duct (CBD). Subsequently magnetic resonance cholangiopancreatogram showed choledocholithiasis. Endoscopic retrograde cholangiopancreatogram with sphincterotomy and CBD stone extraction was performed. The patient progressively improved with antibiotic therapy. Choledocholithiasis should be considered as a source of *Clostridium perfringens* bacteremia especially in the setting of elevated liver enzymes with cholestatic pattern.

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Key words: Choledocholithiasis; Clostridium perfringens; Bacteremia

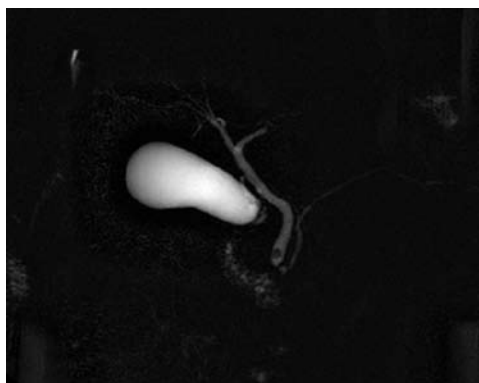


Figure 1 Magnetic resonance cholangiopancreatography shows filling defects within the distal common bile duct.

two years prior and showed sigmoid diverticulosis and colon polyps. On physical examination his heart rate was 103 and he had scleral icterus. The remainder of physical examination including abdominal examination was normal. Initial laboratory data showed white cell count of 8.2 (4.8-10.5), total bilirubin: 3 (0.2-1.0 mg/dL), direct bilirubin: 0.4 (0.0-0.2 mg/dL), aspartate transaminase: 131 (15-46 U/L), alanine transaminase: 86 (7-56 U/L) and alkaline phosphatase: 254 (38-126 U/L). His liver enzymes were normal one year earlier. Computed tomography (CT) scan of the abdomen and pelvis showed normal gallbladder, normal CBD diameter and colonic diverticulosis without diverticulitis. Ultrasound (US) of the abdomen revealed no stone in the gallbladder and the CBD diameter was 4.5 mm. Anaerobic blood culture was positive for *Clostridium perfringens*. Patient was treated with vancomycin, aztreonam and metronidazole. He was allergic to penicillin. Urine culture was negative. Magnetic resonance cholangiopancreatography (MRCP) showed revealed a 6 mm ovoid filling defect and additional smaller filling defects within the distal common bile duct (Figure 1). Subsequently, endoscopic retrograde cholangiopancreatography confirmed the diagnosis of choledocholithiasis for which sphincterotomy and stone extraction was performed (Figure 2). Following the procedure, liver enzymes improved. He was discharged home on ertapenem and he had colonoscopy on outpatient basis that showed sigmoid diverticulosis.

DISCUSSION

Clostridium bacteremia is a rare occurrence that can lead to a devastating outcome. Early recognition and treatment of *Clostridium* bacteremia can be life saving. The aim of this report is to present a rare case of *Clostridium perfringens* bacteremia caused by choledocholithiasis. There are few epidemiological studies documenting the incidence of *Clostridium* bacteremia. A retrospective population-based surveillance for clostridial bacteremia among all residents of the Calgary Health Region (population 1.2 million) during 2000-2006 showed that the incidence of *Clostridium* bacteremia was 1.8/100 000

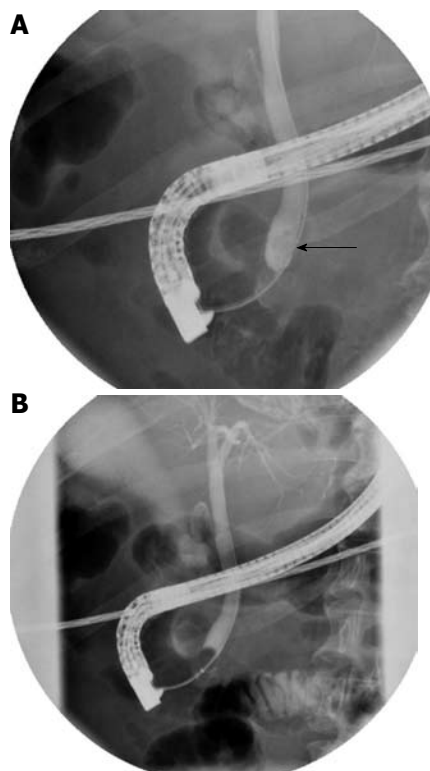


Figure 2 Endoscopic retrograde cholangiopancreatography. A: Filling defects within the distal common bile duct (arrow); B: Filling defects after sphincterotomy and balloon sweep.

per year. *Clostridium perfringens* was the most common isolate accounting for 42% of cases followed by *Clostridium Septicum*, *Clostridium ramosum*, *Clostridium clostridioforme*, and *Clostridium difficile*^[1]. Another review of blood cultures drawn in a rural hospital in Wisconsin, United States from 1990-1997 yielded clostridium infection in 0.12% of drawn cultures with *Clostridium perfringens* being the most common (21.7%)^[2]. A review of blood cultures in a Japanese tertiary center during 2001-2009 showed that only 18 patients had *Clostridium perfringens* bacteremia^[3]. *Clostridium perfringens* is an anaerobic gram positive rod that can produce a wide spectrum of diseases related to toxin production. *Clostridium perfringens* produce four principal toxins including alpha, beta, epsilon, and iota toxins^[4]. Alpha toxin can produce gas gangrene^[5] while beta toxin can produce necrotic enteritis^[6]. *Clostridium perfringens* has also been reported to cause tuboovarian abscess^[7], necrotizing enterocolitis^[8], emphysematous cholecystitis^[9], discitis^[10], and liver abscess^[11]. *Clostridium perfringens* bacteremia has been reported to occur following colonoscopy and gynecological procedures^[12,13]. Advanced age and co-morbidities such as hemodialysis, cancer, heart disease, diabetes, Crohn's disease, COPD, stroke and asthma increase the risk for clostridial infections. Advanced age increases the risk independent of co-morbidities which could be explained by age-related increase of clostridial species in the normal intestinal flora^[1]. *Clostridium perfringens* bacteremia has been associated with intravascular hemolysis and death. Alpha toxin

produced by *Clostridium perfringens* can cause hemolysis, platelet destruction and widespread capillary damage. Intravascular hemolysis can be fatal unless treatment is instituted early. Sudden severe hemolytic anaemia, very low MCV, hemolyzed blood samples and negative Coombs test in a patient with fever should prompt the clinician to consider *Clostridium perfringens* infection. The morphological findings seen on blood cell examination are spherocytes, microspherocytes, and neutrophils with vacuoles or Dohle bodies^[14-16]. Fortunately, our patient did not manifest these abnormalities. In our patient it is difficult to determine if the predominance of indirect hyperbilirubinemia is caused by intravascular hemolysis that was limited by early antibiotics use or by choledocholithiasis. Therefore we would encourage clinicians to obtain peripheral blood smear in patients with *Clostridium perfringens* bacteremia with any evidence suggestive of intravascular hemolysis.

When the clinician encounters *Clostridium perfringens* bacteremia, discovery of the source is very important. Sources of *Clostridium perfringens* bacteremia include colon, biliary tree, lungs, tuboovarian, endometrium and decubitus ulcer. Unlikely sources include urinary tract, pancreas, small bowel, esophagus and brain abscess or unknown source^[2]. Choledocholithiasis has been rarely reported as a cause of *Clostridium perfringens* bacteremia^[17]. We report an unusual case of *Clostridium perfringens* bacteremia caused by choledocholithiasis in the absence of gallbladder stones and with normal CBD diameter. This case demonstrates the importance of pursuing an extensive differential diagnosis because the identification of the underlying source may be necessary to prevent a fatal outcome. Our patient presented with *Clostridium perfringens* bacteremia along with newly elevated liver function tests leading to consideration of biliary source for his bacteremia. This led to further imaging for evaluation of the biliary tract despite normal gallbladder and common bile duct seen on abdominal US and CT scan of the abdomen.

Clostridium perfringens bacteremia is a rare occurrence. We would like to emphasize the importance of discovery of the source of bacteremia and the early administration of antibiotics. It is important to recognize the occurrence of intravascular hemolysis with *Clostridium perfringens* bacteremia. Imaging of the biliary system by MRCP or endoscopic US is needed despite normal ultrasound and CT scan of the abdomen when biliary source of *Clostridium perfringens* bacteremia is suspected.

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S- Editor Cheng JX L- Editor A E- Editor Li JY

A *de novo* germline *MLH1* mutation in a Lynch syndrome patient with discordant immunohistochemical and molecular biology test results

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Airaud F, Küry S, Valo I, Maury I, Bonneau D, Ingster O, Bezieau S. A *de novo* germline *MLH1* mutation in a Lynch syndrome patient with discordant immunohistochemical and molecular biology test results. *World J Gastroenterol* 2012; 18(39): 5635-5639 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i39/5635.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i39.5635>

Abstract

We describe a patient with a *Homo sapiens* mutL homolog 1 (*MLH1*)-associated Lynch syndrome with previous diagnoses of two distinct primary cancers: a sigmoid colon cancer at the age of 39 years, and a right colon cancer at the age of 50 years. The mutation identified in his blood and buccal cells, c.1771delG, p.Asp591Ilefs*25, appears to be a *de novo* event, as it was not transmitted by either of his parents. This type of *de novo* event is rare in *MLH1* as only three cases have been reported in the literature so far. Furthermore, the discordant results observed between replication error phenotyping and immunohistochemistry highlight the importance of the systematic use of both pre-screening tests in the molecular diagnosis of Lynch syndrome.

INTRODUCTION

Lynch syndrome or hereditary non-polyposis colorectal cancer syndrome (MIM#120435)^[1] is a familial form of cancer mainly involving the colon, rectum or endometrium and, more rarely, the small bowel, urinary tract, ovaries, stomach, brain or skin. The pathology is transmitted in an autosomal dominant mode of inheritance with an incomplete penetrance. It is linked to mutations in the genes of the DNA mismatch repair system (MMR), including *Homo sapiens* mutL homolog 1 (*MLH1*), *Homo sapiens* mutS homolog 2 (*MSH2*), and to a lesser extent,

Homo sapiens mutS homolog 6 (*MSH6*) and PostMeiotic Segregation increased 2 (*PMS2*). International scientific community defined the clinical and familial criteria which had to be fulfilled to consider a likely diagnosis of Lynch syndrome, and thereby the value of a mutation screening of *MMR* genes in a patient. The initial recommendations following the Amsterdam (1991)^[2] and Amsterdam II (1999) criteria^[3] were too restrictive. New criteria were therefore proposed at the Bethesda conference in 2004^[4], in order to increase the sensitivity of mutation carrier detection. These recommendations include an age at diagnosis younger than 50 years, or younger than 60 years when the tumor has a microsatellite instability-high phenotype (MSI-high), which is the hallmark of tumors linked to a defective *MMR* gene system^[5,6].

Here we report on the unique case of a *de novo* mutation encountered by our Oncogenetic Laboratory through its diagnostic activity in Lynch syndrome. In our molecular diagnostic strategy, replication error (RER) phenotyping is performed systematically before sequencing of the *MMR* genes whenever tumor tissue is available, and this is done concomitantly with immunohistochemistry in order to direct the sequencing as far as possible. Sequencing of either *MLH1*, *MLH2* or both genes is initiated each time a tumor is found to be MSI-high or -low (low microsatellite instability: one system with replication error), even when the immunohistochemistry results are ambiguous or show no extinction of a *MMR* protein. The sequencing step is also initiated under three specific circumstances: (1) the finding of an microsatellite stable (MSS) phenotype with validated Amsterdam criteria; (2) when no tumor material is available; and (3) in the case of a discrepancy between the immunohistochemistry results and the RER phenotype.

Whenever necessary, DNA samples are sent to another laboratory within the French *MMR* network (Groupe Génétique et Cancer *MMR*) for further testing of *MSH6*.

CASE REPORT

The patient is a 54 year-old man who had previously presented two distinct primary cancers, a sigmoid colon cancer at the age of 39 years, and a right colon cancer at the age of 50 years. He is the sixth of a family of 10 siblings, three of whom have had polyps removed: two brothers and one sister, at the ages of 38, 42 and 38 years respectively. A fourth sibling, another of his sisters, was diagnosed with cervical cancer at 45 years of age, but it is not in the tumor spectrum for Lynch syndrome. Among the older generations of the patient's family, the maternal grandfather died of colon cancer at the age of 84 years and the mother's medical records report an ovarian cyst and ovariectomy (Figure 1).

The right colon tumor had been removed by a colectomy covering 30 cm of the right colon and 5 cm of the ileum. The tumor measured 7 cm × 5 cm × 1.5 cm and was located in the caecum, invading the terminal ileum. The pathology examination revealed a grade 3 stage

for the poorly differentiated, burgeoning and stenosing adenocarcinoma, and cancer staging was classified as pT-3N0Mx. Immunohistochemistry and molecular biology analyses were performed on formalin-fixed paraffin-embedded material from this tumor.

The immunohistochemistry analysis for the *MMR* proteins *MLH1*, *MSH2* and *MSH6*^[7] was performed but did not demonstrate a loss of expression, albeit a very weak expression was detected for *MLH1* and *MSH2*.

RER phenotyping was then performed and showed an instable phenotype for four of the five markers routinely tested in our laboratory (*BAT25*, *BAT26*, *NR21*, *NR22* and *NR24*)^[8,9], with stability noted only for the *NR24* system. The tumor was thus classified MSI-high, and the patient was considered for mutational analysis of *MMR* genes, according to the Bethesda criteria.

In keeping with routine practice at our laboratory, we started with the search for large rearrangements using a commercial Multiplex Ligation-dependent Probe Amplification (MLPA[®]) kit for *MLH1* and *MSH2* (P003-B1 kit, MRC-HOLLAND, Amsterdam, The Netherlands)^[10]. This revealed the isolated loss of *MLH1* exon 16. In such cases of single exon deletion or duplication, we systematically verify the hybridizing sequence of the MLPA probes to exclude a false result caused by a single nucleotide polymorphism (SNP). However, in the present case, the sequence analysis showed a single nucleotide deletion of a G in position 1771 (NM_000249.3: c.1771delG, p.Asp591Ilefs*25). This mutation had previously been described in a Taiwanese Lynch family^[11]. It had been classified as deleterious because of the occurrence of a premature stop codon at position 615, which disrupts the interacting protein domains to *Pms2* and exonuclease 1^[12].

From this point on, we set out to investigate the status of the mutation within the tumor. Finding the mutation in a homozygous or hemizygous state would have meant that the second hit in the carcinogenetic process was a loss of heterozygosity, and that the mutation was the first hit. Unfortunately, the poor quality of the DNA extracted from the Formaldehyde-fixed Paraffin-embedded material did not enable us to get readable sequences. We confirmed the presence of mutation c.1771delG (p.Asp591Ilefs*25) in a second independent sample (DNA extracted from a buccal swab spotted onto a FTA paper). Once the mutation had been confirmed, a pre-symptomatic test was offered to the patient's family. In this context, we tested both parents but did not identify any parental origin for the mutation as both parents tested negative for the mutation, in both blood and buccal cell samples. We then performed the complete sequencing of *MLH1* to exclude any other mutational event that could have been the true cause of the patient's personal and familial history of cancer, but we did not identify anything else.

These results led us to question the patient's paternity, given that a meta-analysis performed in 2005 reported false paternity in a median of 3.7% of births^[13]. Nevertheless, a genetic fingerprinting kit (AmpF[®]STR[®] SGM

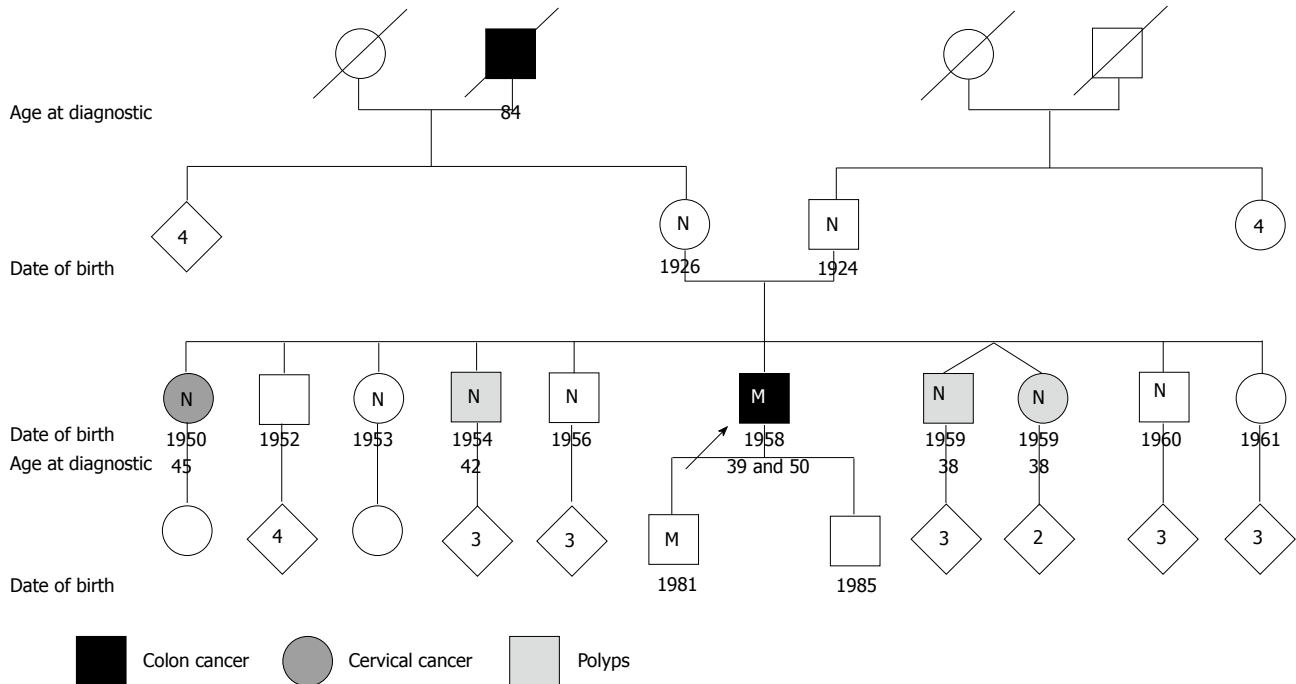


Figure 1 Pedigree. Family tree shows the segregation and clinical manifestations in the family with *de novo* *Homo sapiens* mutL homolog 1 mutation. N: Normal carrier status; M: Mutated carrier status.

Plus®; Applied) confirmed the paternal and maternal status of the supposed parents without ambiguity.

In order to further investigate the possibility of a mosaicism, we performed a haplotyping study using two frequent SNP within the *MLH1* gene (rs1800734 and rs2241031) chosen for their high heterozygosity frequencies and their belonging to different ancestral haplotypes. However, the lack of proband informativity meant that this analysis did not enable us to identify the maternal or paternal origin of the chromosome harboring the mutation. However, because we had tested two different types of tissues of different origins, i.e., mesodermic for blood cells and ectodermic for buccal cells, we were able to exclude mosaicism with quite a high level of confidence. We were finally able to conclude that the Lynch syndrome-causing mutation observed in our patient was a *de novo* event.

Up to now, we have been able to test seven of the patient's nine siblings and have not identified any of them as a carrier of the mutation, even those who have had polyps or cervical cancer. This could be considered as a further argument for a *de novo* mutation, since it does not follow the Mendelian transmission ratio of 1:2 and it is not concordant with polyp antecedents.

Finally, a presymptomatic test in our patient's 30 year-old son revealed the presence of mutation c.1771delG (p.Asp591Ilefs*25) in a heterozygous state, thereby highlighting the transmission (and conservation) of this *de novo* mutation by the proband.

DISCUSSION

Here we report on a French Lynch family in whom we

have identified a frame-shift mutation that induces a premature stop codon in a crucial part of the *MLH1* gene. This mutation has never been reported in the open access MMR mutation database (INSIGHT, Newfoundland *etc.*), or in the French MMR network database (unpublished data). Moreover, we have never found this mutation in 592 chromosomes of patients of similar geographic origin who have been tested in our laboratory as part of routine Lynch syndrome screening.

In contrast with certain other genes, such as *NF1*, which exhibit a *de novo* mutation rate of about 50%, this event in *MLH1* is relatively rare (1% to 5%) according to the study recently published by Win *et al*^[14]. To our knowledge, a *de novo* point mutation in *MLH1* has only been described three times until now. The first occurrence was a c.2101C>T (p.Gln701X) mutation in exon 18, which was detected in a 35 year-old man^[15]. The second was a c.666dupA (p.Asn222Lysfs*4) mutation in exon 8 found in a 31 year-old man^[16]. The third mutation was a nonsense mutation in exon 13, c.1459C>T (p.Arg487X) identified in a 36 year-old patient^[14]. In all three cases the patients had no family history of colorectal cancer and seemed to develop cancer younger than inherited mutation carriers. In addition to these three single nucleotide mutations, two large deletions have already been published, one of the entire *MLH1* gene and one of exon 15, once again in a young man without any family history of cancer^[14,17]. *De novo* mutations seem to be more frequent in *MSH2*, for which four different mutations have already been described^[14,18,19], including the recurrent mutation c.942+3A>T. The latter can even be considered as a kind of mutation hotspot, as it has been proved to occur *de novo* with a relatively high frequen-

cy^[20]. The nucleotide implicated in this mutation is part of the BAT26 homopolymer containing 26 adenines. This particular context is hypothesized to be responsible for misalignment during replication or recombination. Even though the *de novo* *MLH1* mutation described here arose in two families of different ethnic origin, a similar explanation cannot be considered.

This case report confirms the relevance of preceding *MMR* gene sequencing by the combination of the two prescreening tests (RER phenotyping and immunohistochemistry) in the molecular diagnostic strategy in Lynch syndrome, especially for young patients without familial antecedents. Indeed, it is worth noting that most of these patients would not have been considered as candidates for mutation analysis according to the Amsterdam I and II criteria; this might be the reason why *de novo* events in *MLH1* were not described prior to the implementation of the Bethesda criteria. It is also interesting to point out the discordance between immunohistochemistry and RER phenotyping results for our patient's tumor, which confirms the benefit of a dual approach for the screening of Lynch syndrome patients. Indeed, MSI-high phenotypes with conservation of protein expression have already been described and can easily be explained when they concern a missense mutation that does not occur in the epitope of the antibody used. Inversely, extinction of a protein associated with an MSS or MSI-low phenotype can also be encountered, especially when the *MSH6* gene is affected^[21]. In glioblastoma, in the context of Turcot syndrome, changes in microsatellite profiles have also been described as more subtle than those in colorectal tumors^[22] and thus have to be considered very carefully.

In conclusion, the frequency of *de novo* mutations in *MMR* genes may be higher than actually observed in diagnostic laboratories because once a mutation is identified, the parents of the proband are not systematically analyzed in routine practice. This may be because they are deceased, as the average age at molecular diagnosis of our index cases is 53 years, or because they do not wish to be tested.

Moreover, we show here that the combined use of molecular biology and immunohistochemistry should be recommended when screening patients with suspected Lynch syndrome. This combined strategy should help to avoid missing a tumor linked to a deficiency in a *MMR* gene, and also to orientate the subsequent sequencing to one of these genes in a more precise and therefore cost-effective manner.

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Ischemic colitis and large bowel infarction: A case report

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In these cases, aggressive investigation and therapeutic decisions must be taken whenever possible. Despite an absence of standardized protocols, angiographic evaluation and revascularization procedures have beneficial outcomes. Current advances in endovascular therapy, such as percutaneous transluminal angioplasty with stenting, should be increasingly used in patients with chronic mesenteric ischemia. Such therapy can avoid the risks that are associated with open repair. However, technical difficulties, especially in severe stenotic lesions, frequently occur.

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Key words: Ischemic colitis; Intestinal infarction; Mesenteric thrombosis; Acute mesenteric ischemia; Intestinal angina; Mesenteric atherosclerosis

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Abstract

Ischemic bowel disease results from an acute or chronic drop in the blood supply to the bowel and may have various clinical presentations, such as intestinal angina, ischemic colitis or intestinal infarction. Elderly patients with systemic atherosclerosis who are symptomatic for the disease in two or more vascular beds have multiple comorbidities and are particularly at risk. The clinical evolution and outcome of this disease are difficult to predict because of its pleomorphic aspects and the general lack of statistical data. In this paper, we present the case of a patient who was monitored in our unit for six years. For this patient, we encountered iterative changes in the clinical pattern, beginning with chronic "intestinal angina" and finishing with signs of acute mesenteric ischemia after an episode of ischemic colitis. This evolution is particularly rare in clinical practice, and the case is instructive because it raises discussions about the natural history of the condition and the therapeutic decisions that should be made at every stage of the disease. An important lesson is that ischemic bowel disease should always be considered in patients who have multiple risk factors for atherosclerosis and have experienced recurrent "indistinct" abdominal symptoms.

INTRODUCTION

Ischemic colitis (IC) was first described by Boley *et al*^[1]. The condition is a common form of ischemic injury to the gastrointestinal tract and represents approximately one half of all cases involving gastrointestinal ischemia^[2]. On average, this disease is found in association with approximately 1-3/1000 acute hospital admissions, but occasionally a mild and transient clinical course may lead to misdiagnoses. Elderly patients are typically affected, and the most common admission signs are hematochezia, abdominal pain and diarrhea^[3]. The rapid onset of abdominal pain, tenderness over the affected

bowel area (typically the left side of the colon) and mild to moderate hematochezia are the classic signs, although confusion can appear in cases that are considered to be inflammatory bowel disease or various forms of common bacterial colitis^[4]. There is a lack of data regarding the natural history and outcomes of IC. A recent study^[5] found that IC is clinically presumed in only 24.2% of cases, and unfavorable outcomes (as defined by mortality and/or the need for surgery) occur at a rate of 12.9%, including an overall mortality rate of 7.7%.

The taxonomy of the disease is somewhat unclear, especially when “puzzling” terms, such as ischemic colitis and colonic ischemia, are employed. According to the American Gastroenterological Association (AGA) technical review on intestinal ischemia^[6], ischemic bowel disease can be either acute or chronic. Whereas the acute condition includes various forms of acute mesenteric ischemia (AMI) up to intestinal infarction, the milder chronic variant includes chronic mesenteric ischemia (CMI) (also known as “intestinal angina”) and colonic ischemia (CI). CI can appear under various clinical-endoscopic aspects, including (1) reversible colopathy (submucosal or intramural hemorrhage); (2) transient colitis; (3) chronic colitis; (4) stricture; (5) gangrene; and (6) fulminant universal colitis. Most of these aspects have a mixed, ischemic-inflammatory pattern in which ischemic colitis results from an inadequate perfusion that leads to potentially life-threatening colonic inflammation^[7]. In its chronic forms, healing is associated with a degree of fibrosis that determines colonic stenosis.

Beyond this almost scholastic classification, it is important to bear in mind that in current practice, these three major categories (AMI, CMI and CI) may be quite intricate. Moving from one to another occurs in a mix of clinical presentations that can appear in the same patient at various moments, depending on factors such as localization, extent of vascular disease, severity of ischemia, or bowel distension, among others. Our case reflects such a patient, in whom the clinical pattern changed several times in a couple of years. The case began as chronic “intestinal angina” and finished with signs of acute mesenteric ischemia after an episode of ischemic colitis.

CASE REPORT

We present the case of a male Caucasian patient who was monitored in our unit between 2006 and 2012. The hallmark of his long medical background was a severe vascular pathology that was facilitated by the combination of several risk factors: heavy smoking since the age of 18 (more than 20 cigarettes/d), type 2 diabetes that was poorly controlled by diet and medication for more than 15 years, obesity, dyslipidemia and moderate to severe hypertension since 1993. An episode of acute thrombosis of the left femoral artery occurred in 1999, which resulted in the amputation of his left thigh. The patient continued to smoke and neglect the recommended therapy, and four years later, he suffered another acute thrombotic ep-

isode, this time of the right lower limb with the occlusion of the popliteal artery. His right foot was amputated just below the knee. A complete hemostasis investigation was performed at that time, and there was no sign of coagulation abnormality (including less common genetic defects, such as deficiencies of protein C/S, antithrombin III, or factor V Leiden mutation). Aspirin and oral anticoagulation by coumadin were strongly recommended, but the patient appeared to disregard the advice by taking his treatment erratically. Finally, in 2005, a coronarography was performed to investigate an episode of unstable angina. The procedure showed a narrow stenosis (75%) of the circumflex artery; a stent (3.5 × 30 mm) was placed after the dilatation with a balloon catheter.

The patient was first hospitalized in the gastroenterology unit in September 2006, when he was 61 years old. The patient complained of postprandial pain that occurred especially after taking his medication, which consisted of beta blockers, thiazides, angiotensin enzyme inhibitors, aspirin, statins and coumadin. Gastrointestinal, biliary, pancreatic and colonic pathologies were eliminated by upper and lower digestive endoscopy, echography and computed tomography (CT) scans. The initial diagnosis was irritable bowel disease, but a lack of response to specific medication, the suggestive medical history and an improvement in the abdominal pain with sublingual nitroglycerin led to the suspicion of intestinal angina. A mesenteric arteriography was performed in January 2007, which showed a severe (> 75%) incomplete thrombotic occlusion of the upper mesenteric artery and a weak perfusion of the affected bowel loop (Figure 1). The case was concluded to be chronic mesenteric ischemia, but endovascular mesenteric revascularization or surgical bypass were not performed because of the patient's lack of compliance. The patient was discharged with a prescription of long-acting nitrates, and pentoxifyllin was added to his antihypertensive, antiagregant and coumadin treatments.

The evolution was more or less stable until March 2011, when the patient was re-admitted to the unit with episodes of persistent diarrhea and hematochezia, which were spaced by sub-occlusive events of colicky left-lower-quadrant abdominal pain and distension, vomiting and vasovagal symptoms. The examination showed mild pyrexia, tachycardia and a distended abdomen, and there was pain and tenderness in the left lower quadrant upon palpation. A digital rectal examination confirmed hematochezia but did not detect any other lesions. Laboratory investigations showed mild anemia (hemoglobin = 11.1 g/dL), neutrophil leukocytosis (14.800 leukocytes/mm³ with 78% neutrophils), increased values of ESR (130 mm/h), hyperfibrinogenemia (780 mg/dL) and elevated C-reactive protein titers (27 mg/L). Other biochemical tests were within normal ranges. A plain abdominal radiography did not detect pneumoperitoneum or air-fluid levels. The recent use of antibiotics was denied by the patient, and stool cultures for aerobic/anaerobic pathogens (*Clostridium difficile* included) were negative. Amoebiasis serology,



Figure 1 Selective upper mesenteric artery angiogram showing severe incomplete thrombosis with more than a 75% reduction of the arterial lumen and poor perfusion of the bowel loop.

a parasitological investigation and tuberculin intradermo-reaction were also negative.

A contrast-enhanced CT scan (Figure 2) revealed segmental colitis involving the splenic flexure and the descending colon. The wall of the colon was markedly thickened with homogeneous enhancement and sharp definition, and it had a “dry” appearance. Concentric layers of low and high attenuation of the colonic wall (“double halo” sign) could be observed on sagittal sections, which suggested colonic edema. A long and narrow area of axial stenosis was present just below the splenic flexure. The stenosis extended to the distal third of the descending colon. Cecal and right colic distension were also observed, but there were no pericolic streakiness or fluid collections.

A colonoscopy found segmental edematous and hemorrhagic areas of the colonic mucosa surrounding large and deep ulcerations that were covered by pseudomembranes. When the pseudomembrane was washed off, the ulcerations revealed an erythematous and congestive granulation tissue. “Geographic”-like areas of mucosal denudation and “cobble stoning” were also observed (Figure 3A). The rectum was spared by an abrupt transition between normal and affected mucosa, and the ulcerative lesions extended to the sigmoid and descending colon. A narrow, long axial inflammatory stenosis was observed just below the splenic flexure. Biopsies showed edema, submucosal hemorrhage and necrotic areas, and an inflammatory infiltration with intravascular thrombi. The crypts had an atrophic appearance, but no cryptitis/cryptic abscesses or granulomas were observed (Figure 3B). Hemosiderin-laden macrophages were present in the mucosa and submucosa, and hyalinization and hemorrhages were observed in the lamina propria, which histologically confirmed ischemic colitis.

The therapeutic decision was to pursue a conservative treatment. After one week of bowel rest, fluid replacement, broad spectrum antibiotics and parenteral nutrition, the patient recovered well and was discharged. Six months later, in November 2011, the patient returned to the emergency room with acute abdominal pain, sudden evacuation of his bowel contents, abdominal distention,

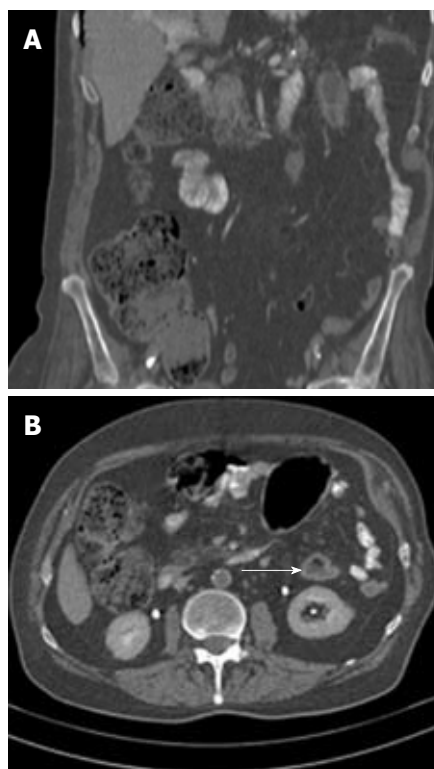


Figure 2 Contrast-enhanced abdominal computed tomography scans suggesting segmental colitis involving the splenic flexure and the descending colon. A: Coronal sections with zones of mural thickening of the colon just below the splenic flexure and a long and narrow axial stenosis of the descending colon; B: Sagittal sections showing (arrow) the “double halo” sign, a tomographic equivalent of the classic “thumbprinting” that is observed in barium enemas.

fever, vomiting and gross hematochezia. Abdominal tenderness, rebound and guarding occurred rapidly with tachycardia, polypnea and hypotension. A CT scan showed a huge thrombus in the abdominal aorta, which extended below the first lumbar vertebra and occluded more than 95% of the aortic lumen (Figure 4). The thrombosis spared the emergence of the celiac trunk and the upper mesenteric artery and appeared to completely occlude the origin of the inferior mesenteric; in the clinical context, this outcome suggested an intestinal infarction. An emergency laparotomy was performed in the following hours. The laparotomy showed a segmental infarction of the descending and sigmoid colon with a transmural hemorrhage and necrosis. Similar lesions were identified at the hepatic flexure. The attached mesentery was also hemorrhagic, and marked colic distention was observed. A total colectomy with ileostomy was successfully performed *per primam*, but unfortunately, the patient was lost in the following days after sepsis and cardiovascular complications.

DISCUSSION

This instructive case presented several clinical aspects of atherothrombotic disease of the mesenteric territory. Most surveys do not include mesenteric ischemia among the major clinical consequences of atherosclerosis, especially considering that coronary and cerebrovascular

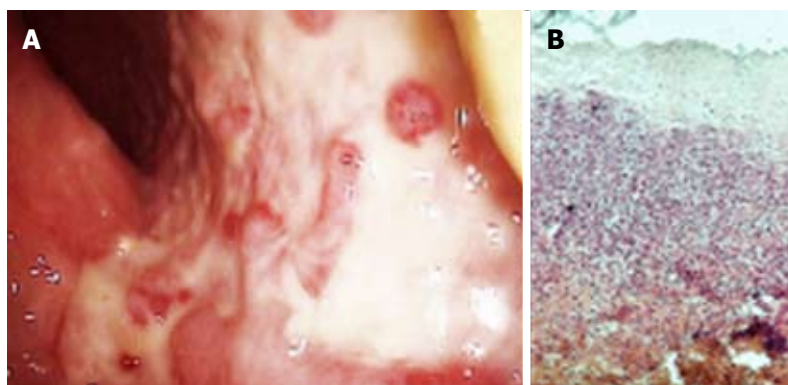


Figure 3 Morphologic changes confirming ischemic colitis by lower digestive endoscopy and histology. A: Areas of mucosal ulceration, pseudomembranes and subsequent granulation observed at colonoscopy; B: Biopsy specimens from affected areas showing submucosal necrosis, inflammatory infiltration, cryptal atrophy and hyalinization. Hematoxylin-eosin staining, $\times 100$. Courtesy Dr. Simionescu.

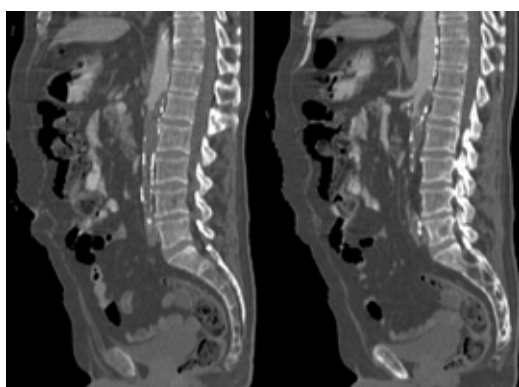


Figure 4 Calcification of the abdominal aorta with extensive thrombosis inside, which completely obstructed the lumen below the 2nd lumbar vertebra. Note that the celiac trunk and upper mesenteric artery are spared, whereas the lower mesenteric is completely occluded.

events with peripheral arterial disease may be credited with 60%-75% of the clinical manifestations of the disease^[8-10]. However, even if mesenteric ischemia is not listed in those studies, there is a significant degree of overlap, as 41% of patients present symptoms of the disease in two or more vascular beds^[11]. Moreover, recent research^[12] found a higher incidence than was preliminarily reported^[13,14], increasing the estimate of hospitalizations to 16.4/100 000. Multi-vascular disease is encountered in 24% of cases, and comorbidities, such as hypertension (72%), diabetes (21%) and coronary artery disease (21%), frequently occur^[12]. This observation is consistent with our case. In addition to mesenteric arterial thrombosis and embolization, other medical conditions can cause bowel ischemia, such as mesenteric venous thrombosis, trauma, small vessel disease (i.e., diabetes mellitus, vasculitis, amyloidosis, rheumatoid arthritis, radiation), hematologic disorders (i.e., protein C/S or antithrombin III deficiency, sickle cell disease), shock, medications (i.e., digitalis, diuretics, nonsteroidal anti-inflammatory drugs (NSAIDs), catecholamines, estrogens, danazol, neuroleptics), colonic obstruction, cocaine abuse or long-distance running^[15]. In our case, iterative mesentero-aortic thrombosis was the primary cause of the clinical manifestations of the ischemic bowel disease that was encountered in our patient; however, other secondary factors, such as diabetic vasculopathy and even

chronic obstructive pulmonary disease^[16], should not be overlooked.

Clinical patterns of mesenteric ischemia may be either acute ("gangrenous") or chronic ("nongangrenous") forms^[15,17]. Nongangrenous forms (80%-85%) are typically transient and reversible but can progress to chronic and irreversible strictures (10%-15%) or chronic segmental colitis (20%-25%). Acute forms^[18] have four major causes: sudden complete arterial occlusion by emboli (50%), thrombosis of atherosclerotic stenosis (20%), small vessel occlusion (20%) and venous thrombosis (10%). Whereas embolic occlusion of the mesenteric arteries is typically brutal and dramatic, often presenting the hallmark "pain out of proportion with physical findings", acute thrombosis of a stenotic atherosclerotic lesion may have a more insidious onset, and there may be a history of intestinal angina, which was the case in our patient. It is important not to miss the clinical signs of chronic mesenteric ischemia in a vascular patient, and it is also important to use angiography to aggressively evaluate the arterial damage in the mesenteric territory. Following the individual patient's anatomic and comorbidity considerations, early revascularization with percutaneous angioplasty/stenting or open repair may prevent a worse outcome, although protocols are not yet fully standardized, and symptomatic recurrences that require reinterventions occur as frequently as 20% of the time^[19].

Chronic nongangrenous colonic ischemia, which is frequently expressed by ischemic colitis, indicates a higher degree of impairment of the intestinal blood flow and, as a consequence, more severe vascular damage. The colon is particularly susceptible to hypoperfusion, and low-flow states often precipitate to preexisting mesenteric microvascular atherosclerosis. "Watershed areas", such as the splenic flexure (Griffith's point), ileocecal junction and rectosigmoid, are specific localizations of ischemic lesions, but diffuse and extended ischemic injury of the entire bowel can also be encountered^[20]. Ischemia is segmental and superficial, and it only extends to the mucosa and submucosa ("partial mural ischemia")^[20,21]. The subsequent inflammation and fibrosis that follow this decrease in the blood supply lead to a "true" colitis that includes edema, neutrophil influx and hyalinization. Ghost cells and hemosiderin-laden macrophages are highly specific findings that can facilitate

diagnosis; these findings should be differentiated from other entities, such as infectious colitis (i.e., *Salmonella*, *Shigella*, *Campylobacter*, *E. coli* O157:H7, *Clostridium difficile*), inflammatory bowel disease, medication-induced colitis (i.e., NSAIDs, hormones, anticoagulants, diuretics, antibiotics), malignancy, fecal impaction with stercoral ulcer, systemic disorders and amyloidosis^[22]. Although therapy classically relies on conservative treatment and anticoagulation, the recurrence of disease, especially in high-risk vascular patients, can impose angiographic evaluation and revascularization procedures that may have beneficial outcomes despite an absence of standardized protocols^[22,23].

In conclusion, ischemic bowel disease may be an expression of systemic atherosclerosis, especially in patients who have cumulative risk factors, such as dyslipidemia, diabetes or smoking, and a multi-vascular atherothrombotic pathology. These patients occasionally present all of the clinical forms of mesenteric ischemia, including intestinal angina, ischemic colitis and intestinal infarction. The symptoms may develop over several years and may be intricate, so their recognition is important. Simple anticoagulation does not always prevent major incidents, and revascularization procedures, such as percutaneous angioplasty or stenting, may ensure a better prognosis. In patients with ischemic bowel disease, the important lesson is to always consider aggressive investigations and therapeutic decisions while taking into account the associated benefits and risks. Angiographic evaluation and revascularization procedures are associated with beneficial outcomes despite an absence of standardized protocols. Current advances in endovascular therapy, such as percutaneous transluminal angioplasty with stenting, should be increasingly used in patients with chronic mesenteric ischemia. These procedures will limit the risks that are associated with open repair. However, technical difficulties, such as undistensible stenotic lesions, frequently occur.

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Gastrointestinal stromal tumor presenting with prominent calcification

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that the tumor was growing from the upper gastric body, with calcification in the peripheral ring area. A laparoscopic partial gastrectomy was performed, and the resected specimen revealed a well-circumscribed tumor with exophytic growth from the gastric muscularis propria. Microscopic examination revealed spindle-shaped tumor cells with calcification and hemorrhage. Additionally, positive immunoreactivity of the tumor to KIT and CD34 and a low mitotic index resulted in the diagnosis of very low risk GIST. There are a few case reports of heavily calcified GIST, although solitary or punctate calcification of primary GIST has been reported in several case series. Dystrophic calcification of necrotic or degenerative tissue is the supposed cause of primary calcified GISTs. In contrast, appearance of calcification after administration of imatinib mesylate, which may be one indicator of disease response, is possibly caused by a different mechanism.

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Key words: Gastrointestinal stromal tumor; Calcification; Stomach; Computed tomography; Imatinib mesylate

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Abstract

We present a rare case of a gastrointestinal stromal tumor (GIST) in the stomach with prominent calcification at presentation. A 61-year-old woman visited our hospital because of epigastric discomfort. A spherical calcified lesion with a diameter of about 30 mm was incidentally shown in the left upper quadrant on an abdominal X-ray. Computed tomography demonstrated

Izawa N, Sawada T, Abiko R, Kumon D, Hirakawa M, Kobayashi M, Obinata N, Nomoto M, Maehata T, Yamauchi S, Kouro T, Tsuda T, Kitajima S, Yasuda H, Tanaka K, Tanaka I, Hoshikawa M, Takagi M, Itoh F. Gastrointestinal stromal tumor presenting with prominent calcification. *World J Gastroenterol* 2012; 18(39): 5645-5648 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i39/5645.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i39.5645>

INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are the most common primary mesenchymal neoplasms of the gastrointestinal tract^[1,2]. In the past decade, histological and clinical features of GISTs have become increasingly clear^[1,2], however, calcified tumor mass is an unusual feature of GISTs at initial presentation, and the mechanism is not clear. We describe here an uncommon case of gastric GIST with prominent calcification.

CASE REPORT

A 61-year-old woman without a significant medical history visited our hospital due to epigastric discomfort. There were no positive findings on physical examination. Initial laboratory tests demonstrated low hemoglobin level (9.6 g/dL), which was probably due to iron deficiency anemia.

An abdominal X-ray showed a spherical calcified lesion measuring approximately 30 mm in the left upper quadrant (Figure 1A). An upper gastrointestinal barium study revealed an exogastric mass (Figure 1B). A plain computed tomography (CT) demonstrated calcification in the peripheral ring area of the tumor growing from the upper gastric body. With an abdominal enhanced CT, the tumor was enhanced heterogeneously, except for the calcified section (Figure 2A and B). There was no evidence of liver metastasis or diffuse peritoneal spread. Magnetic resonance imaging of the central portion of the tumor showed low signal intensity on T1-weighted images (Figure 2C) and high signal intensity on T2-weighted images (Figure 2D). Indicative of calcification, peripheral portions showed low intensity on both T1 and T2-weighted images. Endoscopic examination revealed a round submucosal tumor with the upper part depressed (Figure 3A). Endoscopic ultrasonography (EUS) indicated the tumor originated from the fourth layer of the gastric wall (Figure 3B). Although EUS-guided fine needle aspiration was considered for the diagnosis of the submucosal lesion, it was impossible to perform due to calcification of the tumor wall with a sonic shadow (Figure 3B). After informed consent was obtained, laparoscopic partial gastric resection was performed.

The resected specimen revealed a well-circumscribed tumor measuring 32 mm × 23 mm × 23 mm originating from the gastric muscularis propria. The sliced surface of the tumor showed a firm, solid, whitish-gray parenchyma with circular calcification and internal bleeding (Figure 4A). Microscopic investigation revealed spindle-shaped tumor cells (Figure 4B) with calcification and hemorrhage. The cells exhibited low mitotic activity of fewer than 5 mitoses per 50 high power field (HPF) and no prominent signs of nuclear atypia. Immunohistochemical staining demonstrated positive reactivity to KIT (Figure 4C) and CD34 (Figure 4D). On the other hand, there was negative reactivity to S-100 protein, smooth muscle actin, and desmin (not shown). Since the mitotic index of this tumor was less than 5/50 HPF, its

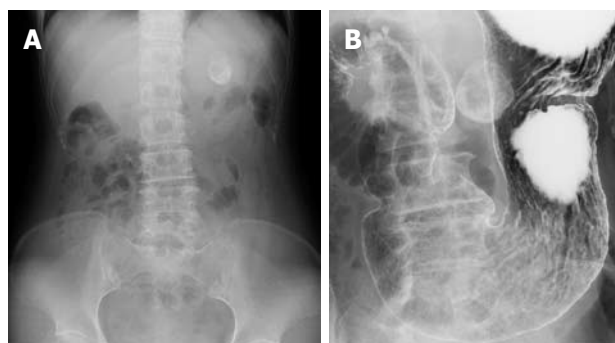


Figure 1 Radiological findings. A: Abdominal X-ray indicated a spherical calcified lesion in the left upper quadrant; B: Barium study showed a round calcification bordering the gastric wall.

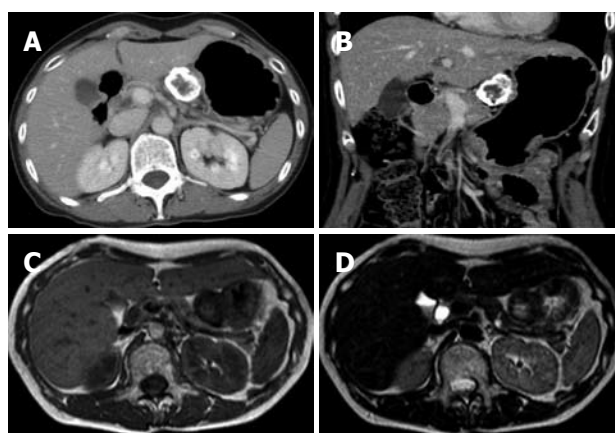


Figure 2 Computed tomography and magnetic resonance imaging findings. Contrast-enhanced axial (A) and coronal (B) computed tomography examination demonstrated that the marginal zone of the tumor was calcified, and that the internal portion of the tumor was enhanced heterogeneously. Magnetic resonance imaging T1-weighted image (C) and T2-weighted image (D) revealed a low intensity marginal zone of tumor reflecting calcification.

diagnosis was established as very low risk GIST according to risk stratification guidelines^[1]. The patient had an uneventful postoperative course and was discharged on the 7th postoperative day. The patient has been followed up for 10 mo without any signs of recurrence.

DISCUSSION

GISTs are defined as mesenchymal tumors in the gastrointestinal tract that express KIT, a tyrosine kinase growth factor receptor^[1]. Clinical, histological and molecular features of GISTs have become increasingly clear in the past decade^[1,2], and radiologic appearances of these tumors have also been well described^[2-7]. Small GISTs (< 5 cm) are often seen as well-defined with a homogeneous appearance on contrast-enhanced scans, whereas larger lesions (> 10 cm) are more likely to have irregular and lobulated margins with heterogeneous enhancement^[2-4].

Although several cases of primary GIST with calcification have been reported^[3-6], solitary or punctate calcification was shown in most cases^[4,5]. In contrast, there are only six case reports describing heavily calcified

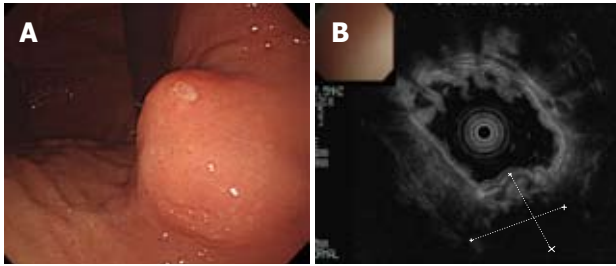


Figure 3 Endoscopy and endoscopic ultrasound of stomach. A: Endoscopic examination revealed a round submucosal tumor; B: The tumor originated from the fourth layer of the gastric wall as indicated by endoscopic ultrasound. The deeper section could not be visualized because of calcification.

GISTs^[8-13]; three cases located in the stomach, one in the colon, and two in the rectum. Although Ghanem *et al*^[4] referred to a solitary calcification as an aggressive finding, the evidence was not clearly shown. Regarding primary lesion, Tateishi *et al*^[5] suggested that the frequency of calcification was not a reliable finding for the distinction of low- and high-grade GISTs. Particularly, with respect to malignant GIST of the stomach, no CT feature (including calcification) other than size was found to have predictive value^[6].

The mechanism of calcification at presentation is unclear. Yoshida *et al*^[8] suggested that the calcification of GIST may be developed mostly from necrotic tissue. In the earlier literature, a true GIST would be included in the imaging studies of leiomyoma, leiomyoblastoma, or leiomyosarcoma arising in the gastrointestinal tract. Prior to the appearance of the definition of GIST, the major mechanism of calcified gastric submucosal tumors was also supposed to be dystrophic calcification of necrotic or degenerative tissue^[14].

In addition, several cases have demonstrated increasing calcification in mesenteric metastasis after therapy with imatinib mesylate^[7,15]. Histological examination of liver metastasis after therapy showed myxomatous degeneration, leaving small pyknotic nuclei in an eosinophilic myxoid background, with no signs of an inflammatory reaction or necrosis^[16,17]. Imatinib mesylate alters the balance from cellular proliferation to apoptosis by inhibiting the tyrosine kinase activity of KIT^[16]. Because cellular death of GISTs following imatinib therapy is by apoptosis and not by necrosis, which is the principal mechanism of cell death following conventional cytotoxic chemotherapy^[7], the mechanism of calcification following administration of imatinib is probably different from that of primary calcification.

[¹⁸F]-fluorodeoxyglucose-positron emission tomography has been reported as useful for the evaluation of early response to imatinib mesylate treatment^[16,18,19]. Furthermore, a decrease in tumor size or a tumor density on post-treatment CT has been reported as a good predictor for tumor response^[19,20]. Sandrasegaran *et al*^[7] also reported that four of twenty-two mesenteric masses showed calcification as well as cystic changes following at least 6 mo of therapy. It is suggested that calcification,

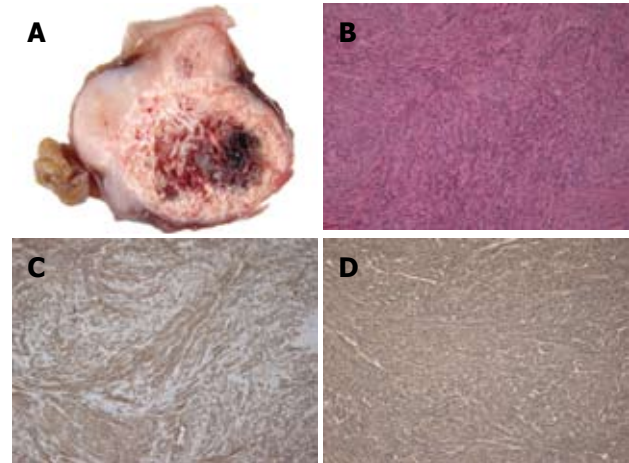


Figure 4 Pathological findings. A: Sliced sections of the resected mass demonstrated a firm, solid, whitish-gray parenchyma with circular calcification and internal bleeding; B: Microscopically, the tumor was characterized by spindle-shaped tumor cells (hematoxylin and eosin, original magnification $\times 100$); Immunohistochemically, the tumor cells were positive for KIT (C) and CD34 (D).

in addition to a reduction in tumor size and extensive cystic changes, may indicate disease response^[15].

In conclusion, we present here a rare case of gastric GIST with prominent calcification. To assess the mechanism and clinical relevance of calcification of GIST at presentation and following administration of imatinib, further accumulation of patients with pathological investigation would be required.

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Hepatothorax due to a right diaphragmatic rupture related to duodenal ulcer perforation

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Baek SJ, Kim J, Lee SH. Hepatothorax due to a right diaphragmatic rupture related to duodenal ulcer perforation. *World J Gastroenterol* 2012; 18(39): 5649-5652 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i39/5649.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i39.5649>

Abstract

Here, we present the case of a 53-year-old man with a hepatothorax due to a right diaphragmatic rupture related to duodenal ulcer perforation. On admission, the patient complained of severe acute abdominal pain, with physical examination findings suspicious for a perforated peptic ulcer. Of note, the patient had no history of other medical conditions or recent trauma, and the initial chest radiography and laboratory findings were not specific. A subsequent abdominal computed tomography revealed intrathoracic displacement of the liver, gallbladder, transverse colon and omentum through a right diaphragmatic defect. The patient then underwent an explorative laparotomy that confirmed duodenal ulcer perforation. A primary repair of the duodenal perforation was performed, and the diaphragmatic defect was repaired using a polytetrafluoroethylene patch after the organs were reduced and the cavity irrigated. This particular case proves interesting as right-sided spontaneous diaphragmatic ruptures are very rare and difficult to diagnose. Additionally, the best treatment for such large diaphragmatic defects is still controversial, especially in cases of intrathoracic or intra-abdominal contamination.

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INTRODUCTION

Non-traumatic, spontaneous diaphragmatic ruptures are extremely rare, accounting for approximately 1% of all diaphragmatic ruptures. Here, we present the case of an acute right diaphragmatic rupture related to duodenal ulcer perforation which resulted in displacement of the liver, gallbladder, transverse colon and omentum into the intrathoracic cavity.

CASE REPORT

A 53-year-old male patient presented to the emergency room complaining of acute abdominal pain, which reportedly started 30 min previously. Of note, he denied any relevant past medical history, any recent trauma, and any medication use. At the time of admission, the patient continued to complain of severe epigastric pain, and a physical exam revealed a rigid abdomen, with both tenderness and rebound tenderness observed throughout the abdomen. His vital signs were relatively stable, with a blood pressure of 120/80 mmHg, a heart rate of 95 beats

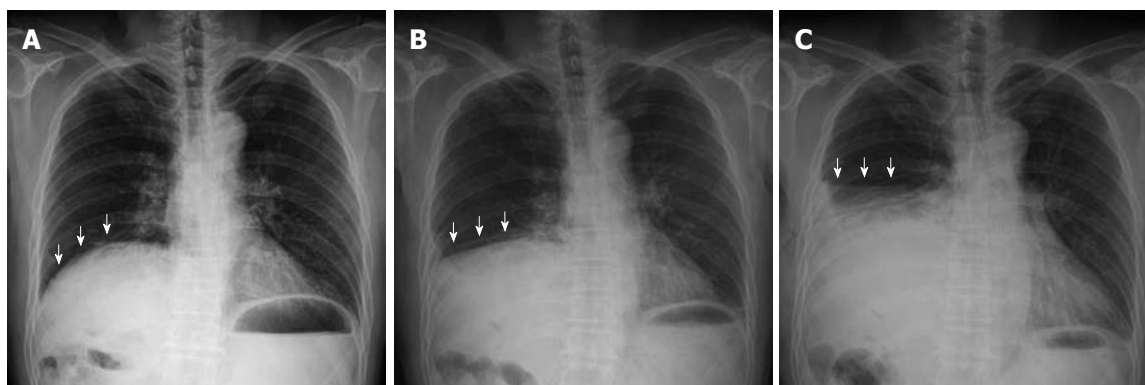


Figure 1 Gradual elevation of the right diaphragmatic border can be seen (arrows). A: Chest radiography on admission; B: Two hours after admission; C: After thoracostomy.



Figure 2 Chest computed tomography coronal reformat images revealing intrathoracic displacement of the liver, bowel, and omentum through the defect (arrowheads) of the diaphragm (arrows).

per minute, oxygen saturation of 98%, and a body temperature of 37 °C. The initial chest radiograph revealed a slight elevation of the right diaphragmatic border, though at the time this was not deemed to be significant (Figure 1A). Further laboratory findings showed no other abnormalities, such as anemia or leukocytosis (Figure 1). Due to the generalized peritonitis on the physical exam, a perforated peptic ulcer was suspected, and thus an abdominal computed tomography (CT) was performed. Unexpectedly, no free air or fluid collections were observed in the abdominal cavity. Instead, the CT showed significant displacement of the right lobe of the liver, the bowel and the omentum into the right hemithorax, with the reformat images providing better views of

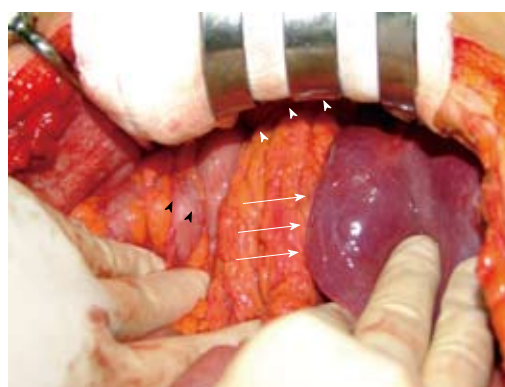


Figure 3 Herniation of the right hepatic lobe, gallbladder, transverse colon, and omentum through the diaphragmatic defect (white arrowheads), showing only the left hepatic lobe (white arrows) and residual transverse colon (black arrowheads) in the right upper abdomen.

the right diaphragmatic border and showing a loss of a part of the diaphragm (Figure 2). Notably, pneumothorax and hydrothorax were also observed, and an injury to the lung parenchyma could not be ruled out.

Initially, a tube thoracostomy was performed, with significant care taken to avoid any injury to the liver and/or bowel. As foul-smelling fluid and bowel contents were noted in the drainage from the chest tube, bowel perforation was also suspected along with diaphragmatic rupture. Accordingly, an exploratory upper midline laparotomy was performed 4 h after admission. At first no evidence of bowel perforation - such as fluid collection or fecal material - was observed in the abdomen. Instead, only the left lobe of the liver was found to have protruded into the right upper abdomen, with the right lobe rotated and herniated into the right hemithorax through the right diaphragmatic defect, along with the gallbladder, transverse colon, and omentum (Figure 3). Exploration of the entire abdomen revealed the site of perforation (approximately 0.5 cm in size) at the first portion of the duodenum immediately distal to the pylorus. Before reducing the herniated organs, a primary repair of the duodenal perforation was performed.

The medial portion of the right side of the diaphragm



Figure 4 Chest radiograph 12 d after surgery showing normal positioning of the right diaphragmatic border compared to the preoperative chest radiograph.

was nearly lost, with only the lateral portion remaining. As this lateral segment blocked reduction, the herniated organs were returned to their respective appropriate locations after the lateral diaphragm was incised, leaving a 12 cm × 10 cm defect. Because of the large size of this defect, primary repair was not possible. Accordingly, another chest tube was inserted just above the diaphragm after the thoracic cavity was thoroughly irrigated. Repair of the diaphragmatic rupture was then performed using a polytetrafluoroethylene patch (GoreTex; W.L. Gore, Flagstaff, AZ, United States) with 1-0 prolene sutures. No other evidence of perforation was noted in the herniated bowel. The abdominal cavity was then irrigated, and an omental patch was applied to the site of the duodenal perforation repair. After surgery, the patient was immediately sent to the intensive care unit for mechanical ventilation, whereby the thoracostomy tubes and abdominal drains were removed in sequence. A post-operative chest radiograph revealed that the right diaphragmatic border had returned to a normal position (Figure 4). After marked improvement in duodenal edema, the patient was discharged on post-operative day 20 with no further complications. Although the patient took oral antibiotics for only one week after discharge, no postoperative complications, including infections, occurred during the following year.

DISCUSSION

Diaphragmatic rupture is a rare complication of abdominal or thoracic trauma, reported in 1%-7% of major blunt trauma patients and 10%-15% of penetrating trauma patients^[1,2]. However, approximately 1% of all diaphragmatic ruptures occur spontaneously, often resulting from a sudden increase in abdominal pressure secondary to heavy physical effort, sudden twisting movements, childbirth, and/or severe coughing^[3-5]. Other cases have been reported to be caused by static sport activities (e.g., Pilates) or associated with endometriosis^[6]. As the patient described here had no recent history of trauma, the exact reason for his diaphragmatic rupture remains unclear. However, given the clinical context, we conjecture that

leaked digestive juice may have corroded the diaphragm, as occurs in cases of empyema. Another possibility is that portions of the diaphragm, such as the septum, may have been weakened or altered by previously unrecognized trauma. If this occurred, the pain resulting from the subsequent duodenal ulcer perforation and the associated abdominal muscle tension may have increased the intra-abdominal pressure, thus prompting the weakened diaphragm to rupture. We cannot determine the exact order of these incidents with any certainty. However, for whatever reason, the perforation of the duodenal ulcer seems to have acted as the precipitating event for the diaphragmatic rupture. Roughly 90% of diaphragmatic ruptures occur on the left side^[1,2]. Right-sided diaphragmatic ruptures are comparatively rare and difficult to diagnose, as chest radiography often does not reveal any specific signs other than elevation of the right diaphragmatic border. Accordingly, delayed diagnosis is common in right-sided ruptures, often resulting in severe complications, such as strangulation ileus and intrathoracic herniation of the hollow organs (stomach, colon, and small bowel)^[2,3].

The initial diagnostic tool is conventional chest radiography, though this modality has limited sensitivity and specificity (17%-40%). Currently, CT is most helpful for emergency diagnosis, as the coronal and sagittal reformatted images offer superior diagnostic value for right diaphragmatic rupture, with a specificity of nearly 100% and a sensitivity of 50%^[7,8].

Cases of right diaphragmatic rupture with hepatothorax may result in severe atelectasis of the right lung or tension mediastinum, thereby severely impeding respiration and circulation. As such, if concern exists for right diaphragmatic rupture, an abdominal CT should be performed quickly, and surgical repair via a trans-thoracic or trans-abdominal approach should be considered immediately following radiographic confirmation. Because the size of the defect is often too large for a primary repair to be performed, prosthetic mesh may prove necessary^[9,10]. Mesh infection was a significant concern in the case described here, given the intra-abdominal and intra-thoracic contamination from the bowel perforation. Fortunately, such complications did not occur and were likely prevented by the extensive irrigation and intensive antibiotic therapy.

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Tracheobronchial nodules and pulmonary infiltrates in a patient with Crohn's disease

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Abstract

Crohn's disease is a granulomatous systemic disorder of unknown etiology. Obvious pulmonary involvement is exceptional. Tracheal involvement in Crohn's disease is even more unusual, only a few cases have been reported to date. We herein report a rare case of tracheobronchial nodules and pulmonary infiltrates in both lungs as a complication of Crohn's disease. A 42-year-old man underwent pancolectomy for multiple broken colon caused by Crohn's disease. Forty days later pulmonary symptoms and radiologic abnormalities were noted. A search for bacterial (including mycobacteria) and fungal in the repeated sputum proved negative. The treatment consisted of intravenous antimicrobials for one month, but there was no improvement in pyrexia or cough and radiologic abnormalities. Fiberoptic bronchoscopy (FOB) was performed and revealed nodes in the trachea and the right upper lobe opening. Histopathology of tracheobronchial nodules and bronchial mucosa biopsy specimen both showed granulomatous inflammation with proliferation of capillaries and inflammatory cells. Oral steroid and salicylazosulfapyridine were commenced and

led to marked improvement in symptoms and an almost complete resolution of his chest radiograph. Repeated FOB showed that nodes in the trachea disappeared and the ones in the right upper lobe opening diminished obviously. Crohn's disease can be associated with several respiratory manifestations. The form of tracheal and bronchopulmonary involvement in Crohn's disease is rare and responded well to steroids.

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Key words: Crohn's disease; Inflammatory bowel disease; Lung; Extracolonic involvement

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INTRODUCTION

Crohn's disease has long been recognized to cause both intestinal and extraintestinal complications. Extra-intestinal manifestations occur in at least 25% of Crohn's disease patients^[1]. Although some papers have reported respiratory tract involvement in Crohn's disease, such as tracheobronchitis^[2], tracheobronchial stenosis^[3], upper bronchial pseudotumors and stenosis^[4], granulomatous bronchiolitis^[5], reports of involvement in both respiratory tract and lung parenchyma in Crohn's disease are very rare. We describe a patient with Crohn's disease who presented with tracheobronchial nodules and pulmonary infiltrates.

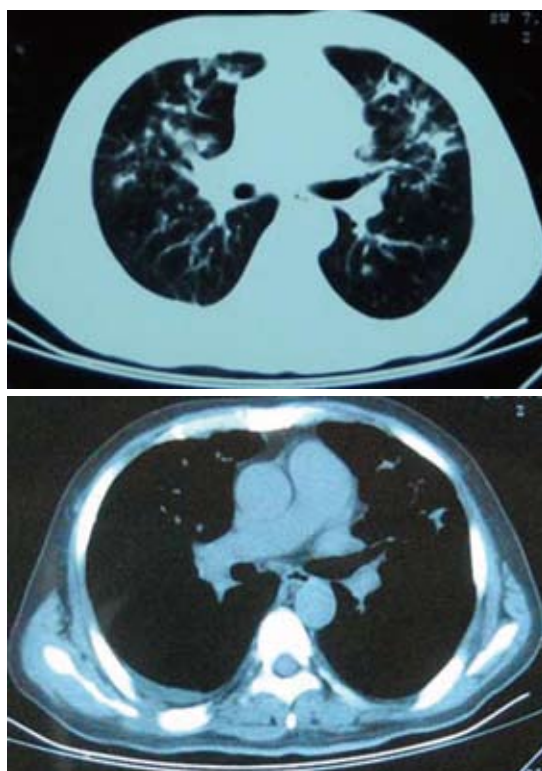


Figure 1 Computed tomography scan of the thorax (January 19, 2009), showing multiple patchy shadows in both lung fields with a little right pleural effusion.



Figure 2 Fiberoptic bronchoscopy (March 9, 2009), showing nodes in the right upper lobe opening.

CASE REPORT

A 42-year-old man was admitted to our department because of a slow onset of cough and pyrexia in January 19, 2009. Forty days prior to this presentation, severe abdominal pain and diarrhea led to his referral to abdominal computed tomography (CT) that revealed free gas under the diaphragm. The patient underwent celiotomy. During operation, multiple broken colon was identified and pancolectomy was performed. The pathology of resected specimen revealed Crohn's disease of colon. His symptoms were resolved after the operation. On admission, he was expectorating about 20 mL of grossly frothy sputum

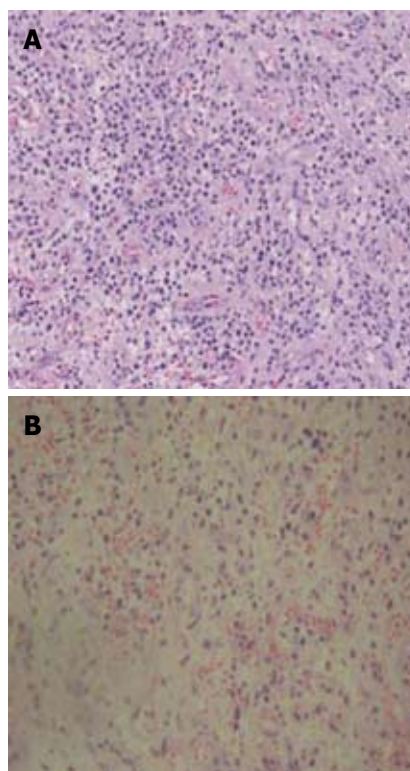


Figure 3 Transfiberoptic bronchoscopy mucosal biopsy, showing polypoid hyperplasia of granulation tissue and hyperplasia of capillary with inflammatory cells (hematoxylin and eosin stain, $\times 200$). A: Tracheobronchial nodules; B: Left lingular lobe.

per day and he had a pyrexia of 37.7 °C. Laboratory data on admission were: white cell counts $8.5 \times 10^9/L$, 63% segmented neutrophils, a moderate increase of erythrocyte sedimentation rate (30 mm in the first hour). A CT scan of the chest revealed bilateral lung patchy shadows (Figure 1). Initial treatment consisted of intravenous azithromycin (0.5 g, once a day) with aztreonam (2.0 g, twice a day) for 2 wk. A search for bacterial (including mycobacteria) and fungal in the repeated sputum proved negative. The purified protein derivative skin test and cytoplasmic-antineutrophil cytoplasmic antibodies (c-ANCA) were also negative. Intravenous cefoperazone/sulbactam (3.0 g, twice a day) was substituted for 2 wk, but there was no improvement in pyrexia or cough, and his chest CT revealed no dissipation. On day 36 after admission, the patient underwent fiberoptic bronchoscopy (FOB) which showed nodes in the trachea and the right upper lobe opening (Figure 2). Transbronchial lung biopsy was performed in tracheobronchial nodules and left lingular lobe. Histopathology of tracheobronchial nodules and bronchial mucosa biopsy specimen both showed granulomatous inflammation with proliferation of capillaries and inflammatory cells (Figure 3). Fibercoloscopy was performed in the following day, which showed cobblestones in the residual sigmoid colon. On day 55, antibiotic treatment was discontinued and the patient was started on prednisone 50 mg daily and salicylazosulfapyridine (SASP) (1.0 g, four times a day) for a presumptive

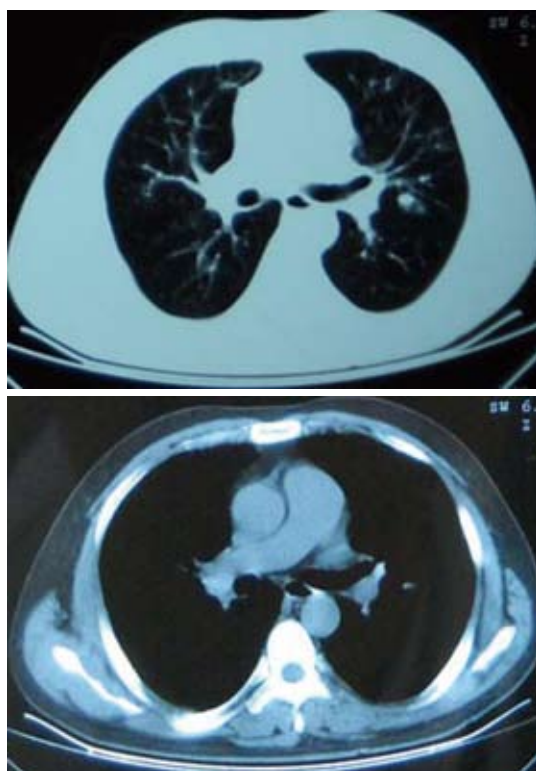


Figure 4 Computed tomography scan of the thorax (April 5, 2009), showing multiple patchy shadows in both lung fields almost disappeared.

diagnosis of Crohn's disease related tracheobronchial nodules and tracheobronchitis associated with pulmonary infiltrates. Within 48 h, the patient's temperature turned normal. There was improvement in cough and the volume of sputum being expectorated a week later. A follow-up CT 3 wk later showed disappearance of the bilateral patchy shadows (Figure 4). He underwent repeated FOB in the following day which showed that nodes in the trachea disappeared and the ones in the right upper lobe opening diminished obviously (Figure 5).

DISCUSSION

Crohn's disease is a granulomatous inflammation of unknown etiology that mainly affects the small bowel, although extracolonic manifestations are common. Extraintestinal manifestations occur in at least 25% of Crohn's disease patients, most commonly in the skin and genitourinary system^[6]. It has been rarely reported in the lung. The overall prevalence of concomitant bronchopulmonary manifestations is as low as 0.4%^[7]. Several forms of involvement of the lung parenchyma are recognized^[8-11]. Diffuse, nongranulomatous interstitial inflammatory lymphoid infiltration has also been described in Crohn's disease^[12].

The clinical features of extraintestinal involvement in our patient seem to be interesting for several reasons. Both tracheal-bronchus and lung parenchyma are involved. Transfiberoptic bronchoscopy biopsy of the tracheobronchial nodules and bronchial mucosa in the



Figure 5 Fibreoptic bronchoscopy (April 6, 2009), showing nodes in the right upper lobe opening diminished obviously.

left lingular lobe revealed a benign, nongranulomatous inflammation with proliferation of capillaries and inflammatory cells. Although tracheobronchial involvement in Crohn's disease has been described in literature, the extent of involvement seen in the current patient with nodules in trachea and right upper lobe bronchus, seems quite unusual. There are few previous reports of tracheobronchial nodules. Crohn's disease can involve the lung parenchyma, the tracheobronchial tree, and the pleura^[13]. The patient underwent colectomy prior to admission. The pulmonary manifestations of Crohn's disease in some patients may only become clinically remarkable after surgery^[14]. Colectomy may aggravate respiratory symptoms^[15]. However, the pathogenesis of Crohn's disease-related airway disease remains unknown. It may involve an inflammatory component because a large proportion of these patients are responsive to steroid therapy. The lung and the gastrointestinal tract have a similar embryological origin (a potential source of common antigenicity), and some authors considered a breakdown in the intestinal barrier and entry of dietary antigens as the cause of extracolonic involvement in Crohn's disease^[16]. One proposed mechanism is that many of the extraintestinal manifestations of Crohn's disease, including lung disease, are secondary to circulating inflammatory mediators released by the inflamed bowel mucosa^[17]. These inflammatory mediators may survive in the pulmonary system for a long time, causing smoldering injuries. This explains the development of pulmonary disease after colectomy in some of the patients with Crohn's disease.

Clinically, when assessing the relationship between respiratory disease and Crohn's disease, one should always keep in mind that the two conditions may have occurred together simply by chance, and therefore other differential diagnoses should be made, such as sarcoidosis, a systemic vasculitis (Wegener's granulomatosis or Churg-Strauss disease), or a prior pulmonary process. In the current patient, the diagnosis of Crohn's disease was initially clearly established on histological grounds of the resected bowel specimen. Although sarcoidosis and Crohn's disease may coexist in the same patient^[18], the absence of typical sarcoid granulomata in tracheobron-

chial biopsies, and the lack of evidence for extrapulmonary sarcoid involvement led the authors to rule out sarcoidosis. In view of the absence of histological findings suggestive of Wegener's granulomatosis, the lack of c-ANCA and no extrapulmonary involvement, the diagnosis of Wegener's granulomatosis was unlikely. Post-obstructive pneumonia results from airway obstruction, commonly due to lung cancer. A mismatch in scope of bilateral and diffuse lung infiltrates with tracheobronchial nodules excluded a diagnosis of post-obstructive pneumonia.

The initial lesion of tracheobronchial Crohn's disease seems to be mucosal inflammation, with symptoms of cough and pyrexia^[19]. Mucosal inflammation may be accompanied by bronchial suppuration. Biopsy shows either severe nonspecific chronic inflammation or non-caseating tuberculoid granulomas. These appearances have been associated with those in the bowel, and it is possible that the gut and the lung are both affected because they share common antigens. The lung and gastrointestinal tract contain submucosal lymphoid tissues and play crucial roles in host mucosal defense. The similarity in the mucosal immune system causes the same pathogenetic changes. The clinical courses between upper airway and gastrointestinal tract disease are not entirely parallel^[20]. It was thought that the tracheobronchial nodules and pulmonary infiltrates were both caused by Crohn's disease, because the pathological finding resembled that of the colon. In addition, they almost vanished as good response to steroids while multiple antibiotic regimens failed to produce clinical improvement.

Drug-induced disease must be kept in mind in patients taking sulfasalazine, mesalamine, methotrexate, and anti-tumor necrosis factor- α . Sulfasalazine and 5-aminosalicylic acid derivatives have been reported to cause interstitial pneumonitis, eosinophilic pneumonia, or bronchiolitis obliterans organizing pneumonia in rare cases^[21,22]. Other commonly used medications in inflammatory bowel disease patients include 6-mercaptopurine, azathioprine, and methotrexate. These drugs may also cause pneumonia *via* opportunistic infections, or acute pneumonitis or pulmonary fibrosis^[23]. Lung abnormalities observed in our patient were unrelated to pharmacological treatment.

Patients with pulmonary involvement of Crohn's disease can be cured with glucocorticoid and SASP, for they are strikingly steroid-responsive frequently. Pulmonary manifestations almost vanished after receiving oral prednisone and SASP in the present case. In all patients with airway disease, marked and long-lasting responses were seen following systemic or inhaled administration of steroids. These findings were not commonly observed for patients with chronic bronchitis without Crohn's disease. Intravenous steroids were required in the initial management of life-threatening complications such as asphyxiating subglottic stenosis or extensive interstitial lung disease. Bronchial lavages with methylprednisolone

were effective in some patients with severe airway inflammation. Bronchoscopic dilatation or stent placement should be considered in cases of tracheal stenosis due to Crohn's disease refractory to steroid treatment^[24]. Other interventional pulmonology techniques, such as bronchoscopic resection or ablation, could not be generally indicated for treatment modalities potentially contraindicated because of the risk of stenosis or fistula.

In summary, this case illustrates a rare form of tracheal and bronchopulmonary involvement in Crohn's disease with nodes and multiple patchy shadows in both lung fields. As is the case with other forms of pulmonary involvement in Crohn's disease, this manifestation responded well to steroids. It is believed that early investigation and vigorous treatment of bronchopulmonary involvement of Crohn's disease is essential.

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Severe acute cholangitis after endoscopic sphincterotomy induced by barium examination: A case report

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Zhang ZH, Wu YG, Qin CK, Su ZX, Xu J, Xian GZ, Wu SD. Severe acute cholangitis after endoscopic sphincterotomy induced by barium examination: A case report. *World J Gastroenterol* 2012; 18(39): 5658-5660 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v18/i39/5658.htm> DOI: <http://dx.doi.org/10.3748/wjg.v18.i39.5658>

Abstract

Endoscopic sphincterotomy (EST) is considered as a possible etiological factor for severe cholangitis. We herein report a case of severe cholangitis after endoscopic sphincterotomy induced by barium examination. An adult male patient presented with epigastric pain was diagnosed as having choledocholithiasis by ultrasonography. EST was performed and the stone was completely cleaned. Barium examination was done 3 d after EST and severe cholangitis appeared 4 h later. The patient was recovered after treated with tienam for 4 d. Barium examination may induce severe cholangitis in patients after EST, although rare, barium examination should be chosen cautiously. Cautions should be also used when EST is performed in patients younger than 50 years to avoid the damage to the sphincter of Oddi.

INTRODUCTION

Endoscopic retrograde cholangiopancreatography (ERCP) is an important diagnostic technique for biliary and pancreatic diseases. Endoscopic sphincterotomy has become a well established modality for common bile duct (CBD) stones^[1,2]. However, endoscopic sphincterotomy is considered as a possible etiological factor for recurrent ascending cholangitis^[3,4]. Here we report a case of severe cholangitis after endoscopic sphincterotomy induced by barium examination, and the patient was recovered after treatment with tienam.

CASE REPORT

A 40-year-old man presented with dull aching epigastric pain for 2 wk. He had a history of CBD stones for 2 years. The patient was averagely built and nourished. No jaundice was present. Biochemical parameters were all

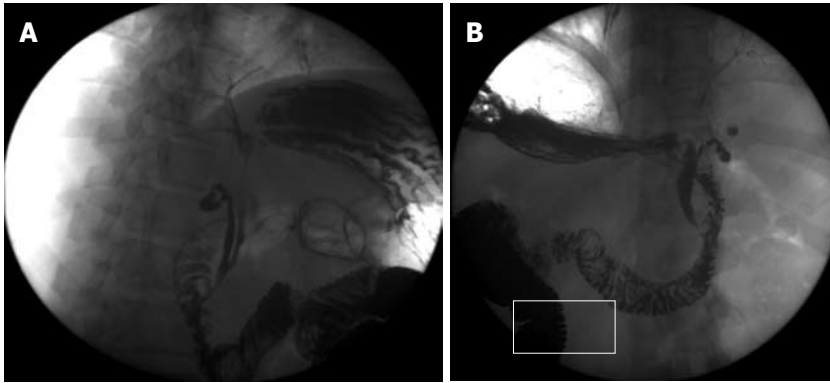


Figure 1 Barium refluxed into the biliary tree of the patient after endoscopic sphincterotomy.

within normal limits. Total white blood cell (WBC) count and hemoglobin measurement were normal. Liver function tests showed that serum bilirubin was 13.5 $\mu\text{mol/L}$, alkaline phosphatase 55 U/L, aspartate aminotransferase (AST) 40 U/L and alanine aminotransferase (ALT) 35 U/L. Ultrasonography revealed CBD stones in the ampulla of Vater and a dilated CBD of 12 mm in diameter. ERCP displayed a dilated CBD. Endoscopic sphincterotomy (EST) was performed in the Second Affiliated Hospital of China Medical University and stones were removed from the biliary tract. The general condition of the patient was good except for slight dull epigastric pain.

To rule out the disease of stomach, barium examination was made 3 d after EST. In the process of barium examination, barium could be seen refluxed into the biliary tree (Figure 1). Four hours later, the patient had a high fever and chills, and the body temperature was climaxed to 39.8 °C. On investigation, total WBC count was increased to $22.45 \times 10^9/\text{L}$, and hemoglobin was normal. Liver function tests showed serum bilirubin 33.5 $\mu\text{mol/L}$, alkaline phosphatase 155 U/L, AST 143 U/L and ALT 235 U/L, urine amylase 85 U/L and blood amylase 110 U/L. Ultrasonography showed a dilated CBD and no specific manifestation of the pancreas. Severe cholangitis after endoscopic sphincterotomy induced by barium examination was then diagnosed. The patient was recovered after treated with tienam for 4 d. He is still alive 5 years after the treatment, but suffered from 1-3 recurrent episodes of reflux cholangitis each year.

DISCUSSION

Endoscopic papillotomy with stone extraction continues to be a therapeutic choice and the reference standard in the treatment of symptomatic choledocholithiasis, especially for the solitary bile duct stones up to 12 mm in diameter. The overall success rate with EST was 95.7% in the present report. The most frequent complication encountered was bleeding, followed by acute pancreatitis, acute cholangitis, and perforation^[5,6]. It has been found recently that the incidence of acute biliary pancreatitis may be lowered by pancreatic duct stenting^[7].

After endoscopic sphincterotomy, the biliary sphincter is rendered permanently insufficient. The loss of this physiologic barrier between duodenum and biliary tract

results in duodenocholedochol reflux and bacterial colonization of the biliary tract. Our previous study showed that about 35.9% of the patients with a T-tube after cholecystectomy and choledochotomy had duodenal-biliary reflux. Most of them had hypomotility of the sphincter of Oddi^[8]. After EST, biliary reflux of duodenal chyme occurs in most patients, aerobilia is seen in about half, and bacterobilia in all the patients^[9,10]. Most patients with bacterobilia did not inevitably develop symptomatic recurrent cholangitis, but 20% patients had upper abdominal pain during the follow-up of about 36 mo^[11]. Another study showed that 65% patients after EST had duodenobiliary reflux, detected by barium studies, although no clinical symptoms were observed^[12]. The presence of bacteria in the biliary system, which is sterile under physiologic conditions, might lead to complications after EST. Misra *et al.*^[13] studied the incidence of duodenobiliary reflux and acute cholangitis after placement of self-expanding metal stent across the main duodenal papilla, and found that severe reflux of barium was evident in all the patients. However, none of them developed acute cholangitis because of reflux.

To avoid the damage to the biliary sphincter, endoscopic papillary balloon dilation (EPBD) was introduced as a less traumatic alternative to EST in the management of biliary tract stones^[14]. However, a high incidence rate of procedure-induced pancreatitis was reported^[15]. But, more recently, May *et al.*^[16] and Mathuna *et al.*^[17] found that the complication rates of pancreatitis by EPBD were similar to those by EST. Toda *et al.*^[18] studied the early results of EST and EPBD and found no difference in the early complications between EST and EPBD. The incidence rates of cholangitis by EST and EPBD were 4.0% and 4.2%, respectively. Yasuda *et al.*^[19] showed that preservation of papillary function after EPBD was not complete, but remained somewhat reduced. Preservation was more successful with EPBD than with EST. The incidence of pneumobilia was significantly higher in post-EST than in post-EPBD patients. Yasuda *et al.*^[20] also found that during long-term follow-up, patients who underwent endoscopic sphincterotomy (ES) had significantly more biliary complications than those who underwent EPBD. The biliary sphincter dysfunction after ES results in additional late complications.

Reflux of duodenal contents into the biliary tract af-

ter EST was the consequence of reduction or abolition of sphincter activity, as documented by manometry even 15 years following the sphincterotomy^[21]. One study found that sphincterotomy was associated with a 5-fold higher incidence of recurrent brown CBD stones compared with choledocholithotomy^[22]. One study reported the potentials of endoscopic papillary large balloon dilatation (EPLBD) with minor EST for the complete removal of CBD stones and found that the recurrence of CBD stones was especially low in cases of periampullary diverticulum treated with EPLBD by minor EST^[23].

In our patient after EST, severe cholangitis occurred 4 h after gastrointestinal barium X-ray examination was done. In the process of barium examination, barium refluxed to the CBD, gallbladder and intrabiliary bile duct. Barium examination may induce severe cholangitis in patients after EST. Although the incidence is low, we should avoid early barium examination in patients after EST. The patient was younger than 50 years, if choledochotomy was performed and the sphincter of Oddi was preserved, reflux cholangitis might not occur.

In conclusion, EST, as a standard treatment of choledocholithiasis, destroyed the integrity of sphincter of Oddi which may induce reflux cholangitis. As a motivation factor, barium examination may induce severe cholangitis in patients after EST, although rare, it should be performed cautiously. Cautions should also be used when EST was performed in patients younger than 50 years to avoid the damage to the sphincter of Oddi.

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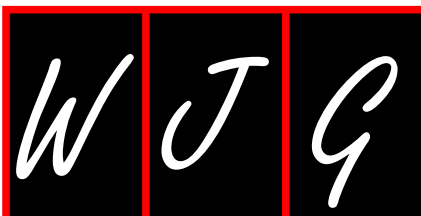
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Meeting 2012
Kuala Lumpur, Malaysia

January 19-21, 2012
American Society of Clinical
Oncology 2012 Gastrointestinal
Cancers Symposium
San Francisco, CA 3000,
United States

January 19-21, 2012
2012 Gastrointestinal Cancers
Symposium
San Francisco, CA 94103,
United States

January 20-21, 2012
American Gastroenterological
Association Clinical Congress of
Gastroenterology and Hepatology
Miami Beach, FL 33141,
United States

February 3, 2012
The Future of Obesity Treatment
London, United Kingdom

February 16-17, 2012
4th United Kingdom Swallowing
Research Group Conference
London, United Kingdom

February 23, 2012
Management of Barretts
Oesophagus: Everything you need
to know
Cambridge, United Kingdom

February 24-27, 2012
Canadian Digestive Diseases Week
2012
Montreal, Canada

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Nutrition and Growth 2012
Paris, France

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Meeting
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Omaha, NE 68197, United States

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Mayo Clinic Gastroenterology and
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Orlando, FL 32808, United States

March 26-27, 2012
26th Annual New Treatments in
Chronic Liver Disease
San Diego, CA 92121, United States

March 30-April 2, 2012
Mayo Clinic Gastroenterology and
Hepatology
San Antonio, TX 78249,
United States

March 31-April 1, 2012
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Chronic Liver Disease
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9th International Symposium on
Functional GI Disorders
Milwaukee, WI 53202, United States

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Singapore, Singapore

April 15-17, 2012
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Prague, Czech

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Amman, Jordan

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San Diego, CA 92101, United States

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMCID:2516377 DOI:10.1161/01.HYP.0000035706.28494.09]

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No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRS-A Careaction* 2002; 1-6 [PMID: 12154804]

Books*Personal author(s)*

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/eid/index.htm>

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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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