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EDITORIAL

Neoadjuvant treatment for resectable pancreatic cancer: Time for phase Ⅲ testing?

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Abstract

This paper discusses the rationale for phase Ⅲ testing of neoadjuvant therapy in patients affected by resectable pancreatic adenocarcinoma. The therapeutic management of patients affected by resectable pancreatic cancer is particularly troublesome due to the aggressiveness of the disease and to the limited efficacy and sometimes unfavourable risk-benefit ratio of the available therapeutic tools. Conflicting data on the role of adjuvant chemoradiation have been reported, while adjuvant single-agent chemotherapy significantly improved overall survival (OS) when compared to surgery alone. However, the OS figures for adjuvant chemotherapy remain disappointing. In effect, pancreatic cancer exhibits a prominent tendency to recur after a brief median time interval from surgery and extra-pancreatic dissemination represents the predominant pattern of disease failure. Neoadjuvant treatment has a strong rationale in this disease but limited information on the efficacy of this approach is available from single arm trials with low levels of evidence. Thus, in spite of two decades of investigation there is currently no evidence to support the routine use of pre-surgical therapy in clinical practice. To foster knowledge on the optimal management of this disease, and to produce evidence-based treatment guidelines, there is no alternative to well designed randomized trials. Systemic chemotherapy is a candidate for testing because it is supported by a more robust rationale than chemoradiation. Combination chemotherapy regimens with elevated activity in advanced disease warrant investigation. Caution would suggest the running of an exploratory phase Ⅱ randomized trial before embarking on a large phase Ⅲ study.

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Key words: Pancreatic cancer; Neoadjuvant therapy; Phase Ⅲ trial; Chemotherapy

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INTRODUCTION

Pancreatic cancer represents the fourth most common cause of cancer death, bears the worst prognosis among solid tumors and has seen very limited progress over the last 30 years. Due to intrinsic chemo- and radio-resistance, surgical resection is considered the only therapy that may have an impact on the natural history of the disease and may increase chance for cure. However, 5-year overall survival (OS) rates of less than 20% can be expected even after a curative resection, which is related to a non-negligible risk of mortality and morbidity. Therefore, the therapeutic management of patients affected by resectable pancreatic cancer is particularly troublesome due to the aggressiveness of the disease and to the limited efficacy and, sometimes, unfavourable risk-benefit ratio of the available therapeutic tools.

STANDARD TREATMENT IN RESECTABLE PANCREATIC CANCER

Adjuvant fluorouracil-based chemoradiation followed[1,2] or not[3] by maintenance systemic chemotherapy with 5-fluorouracil has been tested against surgery alone in a few phase Ⅲ trials with conflicting results, ranging from a significant improvement[1] to a detrimental impact on OS[2]. Accordingly, the use of this strategy is a highly controversial topic in the management of patients with resected pancreatic adenocarcinoma.

More recently, randomized trials have suggested that both adjuvant 5-fluorouracil and gemcitabine may obtain an improvement in median survival of 2.6-4.5 mo and in 2-year OS of 6%-10% over pancreatic resection alone[2,4], with no significant difference between the two drugs[5,6].

It is noteworthy that the OS benefit achieved by adjuvant chemotherapy, in addition to being modest, does not apply to the whole population of patients submitted to pancreatic resection. In fact, up to 25% of patients have a complicated course after surgery and are unable to receive the planned treatment in due time[3,7]. Also, patients with evidence of persistent local disease or metastatic disease at the first post-operative radiological assessment are ineligible for prospective trials on adjuvant chemotherapy, whose results are, consequently, not fully generalizable.

PATTERN OF DISEASE RECURRENCE AFTER STANDARD TREATMENT

In the subset of patients receiving postoperative treatment, pancreatic cancer exhibits a prominent tendency to recur locally and to metastasize after a brief median time interval of about 13 mo from surgery[4]. Early relapse after curative surgery may be explained by the presence of micro-metastases or minimal residual disease not detectable at the time of surgery, or by the spread of cancer cells into the portal vein, lymphatic vessels, and the peritoneal cavity due to surgical manipulation of the tumor. In spite of adjuvant systemic chemotherapy, extra-pancreatic dissemination represents the predominant pattern of disease failure, affecting 63%-83% of patients, and occurs earlier than isolated local failure, which can be observed in 17%-37% of cases[2,4-6,8,9].

RATIONAL FOR NEOADJUVANT THERAPY

In this scenario, the administration of neoadjuvant systemic chemotherapy may offer several theoretical advantages. Firstly, micro-metastatic disease may be immediately treated, thus avoiding the harmful delay of at least 2 mo which occurs for patients submitted to upfront surgery. Second, a larger proportion of patients may receive an active systemic treatment compared with the adjuvant setting. Third, the treatment itself may be better tolerated, resulting in a higher rate of treatment compliance and improved dose-intensity. Fourth, neoadjuvant chemotherapy potentially reduces intraoperative tumor spillage. Fifth, the delivery of treatment before surgical manipulation may be favored by better tissue oxygenation, facilitating the distribution of chemotherapy agents into the tumor, and increasing normal tissue tolerance. Moreover, the administration of chemotherapy before surgery allows an *in vivo* assessment of tumor chemo-sensitivity. Finally, neoadjuvant chemotherapy may also lead to more definitive surgical resections by reducing the risk of tumoral infiltration of lymph nodes and of resection margins in the surgical specimen.

On the other hand, the neoadjuvant approach is subject to hypothetical risks such as (1) inaccurate staging and the consequent overtreatment of very early disease; (2) erroneous histology; (3) diagnostic inaccuracy due to difficulties in distinguishing between intra-pancreatic bile duct adenocarcinoma and pancreatic adenocarcinoma; (4) increase in operative morbidity and mortality; and (5) the possibility that the disease might metastasize or become unresectable during the course of induction therapy. The first topic appears to be of little relevance in pancreatic cancer since systemic treatment administration is warranted at virtually any stage of disease, aside from, perhaps, stage I , which is exceedingly rare. Similarly, the risk of yielding an inaccurate pathological diagnosis is limited as the widespread and systematic use of endoscopic ultrasound and fine needle aspiration considerably reduces the possibility of errors. As regards surgical complications, no increase in morbidity or mortality after neoadjuvant therapy has been reported in prior trials[10-12]. Conversely, the topic of disease progression during pre-surgical treatment is of considerable concern because, among patients whose disease was deemed resectable at the time of trial enrolment, only 45%-74% were actually submitted to surgical resection after induction chemoradiation[12-16] and 38%-70% after induction chemotherapy followed[10] or not[11] by chemoradiation. Proponents of neoadjuvant therapy consider these figures another advantage of this strategy, claiming that patients who experience disease progression during induction treatment suffer from an extremely aggressive tumor, which cannot be cured by extensive surgery. In fact, avoiding the risk of surgical mortality and morbidity in this subset of patients may be appealing. However, this is not necessarily true for patients who experience only local progression during neoadjuvant therapy, and in any case, no comparative information from randomized trials on the impact of the different management strategies is available in order to rule out a detrimental impact of delaying surgery. Furthermore, the proper aim for pre-surgical therapy should be that of downstaging disease and of improving both disease control and, ultimately, cure rate, rather than improving patient selection for surgery. Overall, the balance between the theoretical advantages and disadvantages of neoadjuvant therapy in pancreatic cancer appears uncertain.

PRIOR EXPERIENCE WITH NEOADJUVANT THERAPY

There have been no large randomised controlled studies

on the use of neoadjuvant therapy in resectable pancreatic cancer and the sample size of prospective series has usually been limited. In addition to the abovementioned disappointing resection rates, reported median OS and 2-year OS in this single arm selected series ranged from 8 to 23 mo and from 27% to 40%[10-17]. Altogether, these figures do not appear to represent a remarkable improvement when compared to those of patients submitted to surgery alone (median OS 11-17 mo; 2-year OS 15%-31%)[1-3], or to compare favorably with those of adjuvant therapy (median OS 14-25 mo; 2-year OS 29%-55%)[1-4,6,7]. It is noteworthy that prior experiences with adjuvant combination chemotherapy reported more promising results (median OS 27-44 mo; 2-year OS 53%-58%)[9,18,19]. However, inter-trial comparisons, which already have several limitations, are in this case subject to an additional bias due to the different enrolment timing. In fact, the typical population enrolled in a prospective adjuvant trial is better selected than the typical population enrolled in a neoadjuvant trial because it does not include patients with intraoperative or postoperative detection of metastases, patients who die due to surgical complications or those who experience severe morbidity and delayed surgical recovery.

Thus, in spite of two decades of investigation of neoadjuvant therapy in resectable pancreatic cancer, there is currently no evidence to support its routine use in clinical practice, and even a detrimental effect on outcome cannot be ruled out.

TRIAL DESIGN TO ASSESS THE ROLE OF NEOADJUVANT THERAPY

Single arm trials with historical or literature comparison, and divergent study designs and entry criteria have produced modest therapeutic progress and do not allow a proper assessment of the role of neoadjuvant therapy in resectable pancreatic cancer. To foster knowledge regarding the optimal management of this disease and to produce evidence-based treatment guidelines, there is no alternative to well designed randomized trials. Since timing and sequencing of treatments appears to be a crucial and as yet unanswered issue, patients in the ideal trial should be randomly allocated to receive exactly the same treatment for the same period of time before and after surgery. Otherwise, the attribution of any potential outcome improvement to treatment type, timing or duration will be irremediably confounded and trial interpretation inconclusive.

CANDIDATES FOR PROSPECTIVE ASSESSMENT

As mentioned above, pancreatic cancer has an elevated risk of both local and systemic failure after surgery. In this perspective, local therapy represents a poor chance of considerably improving cure rates while the concomitant administration of radiotherapy and systemic chemotherapy may simultaneously address both troubles. The main radiosensitizing antitumor agents available for pancreatic cancer are gemcitabine and 5-fluorouracil. Unfortunately, gemcitabine has to be administered at suboptimal doses which are unlikely to achieve any effect against systemic disease, due to the overlapping toxicity with radiotherapy and both drugs yield scarce activity. In fact, gemcitabine and 5-fluorouracil obtained objective response rates around 10% in advanced disease[20-26]. Any chemotherapy with a low rate of tumor shrinkage is clearly unable to provide any major advantage in terms of either micro-metastatic or local disease control for the majority of patients and may therefore be assumed to have a limited role in the neoadjuvant setting. More active combination chemotherapy regimens appear to be more promising candidates for testing but have feasibility limitations with concomitant irradiation. Furthermore, the value of radiotherapy in this disease is controversial and, at the moment, does not represent the most burning question, while the rational endorsement of the assessment of the role of combination chemotherapy as pre-surgical therapy is more convincing. Among several regimens with conventional or target agents that have been assessed for use against advanced pancreatic cancer, objective response rates over 20% have rarely been reported, while gemcitabine-cisplatin and gemcitabine-oxaliplatin doublets obtained a response rate of 26%[22] and 28%[23], respectively. Unfortunately, these figures were not reproduced in larger trials where partial plus complete response rate was in the range of 10% to 13% with gemcitabine-cisplatin[24,25] and 9% with gemcitabine-oxaliplatin[26]. Response rates with triplets including gemcitabine, a fluoropyrimidin and either a platinating agent (18%-33%)[27-29] or docetaxel (29%)[30], FOLFOXIRI (5-fluorouracil-oxaliplatin-irinotecan; 26%)[31] and G-FLIP (gemcitabine-5-fluorouracil-irinotecan-cisplatin; 26%)[32] regimens have shown promise in single phase Ⅱ series, but no phase Ⅲ or confirmatory trials are available. A PEFG (cisplatin, epirubicin, 5-fluorouracil, gemcitabine) regimen was proven, in a phase Ⅲ trial, to be both clinically and statistically more effective than single agent gemcitabine as upfront treatment in advanced pancreatic cancer[33]. It is noteworthy that this combination chemotherapy had manageable toxic effects and the significant survival improvement was not achieved at the cost of impaired quality of life[34]. Four consecutive trials with the PEFG regimen and its variants reproduced a radiological response rate in the range of 38.5%-51%[33,35-37]. The substitution of infusional 5-fluorouracil by oral capecitabine originated the PEXG regimen that further confirmed the activity figures[38] and rendered the schedule more suitable for clinical use. The reliability of the response rate was also endorsed by the biochemical response rate. In effect, a major biochemical response (i.e. CA19.9 reduction at nadir relative to baseline value reduction $\geqslant$ 90%) was observed in 30% of patients treated with quadruplets *vs* 7% with single agent gemcitabine[39]. The superiority of this four-drug combination over other regimens was also suggested by a recent survey on treatment trends and outcomes of 650 patients with stage Ⅲ pancreatic adenocarcinoma[40]. Based on these data and

considerations, the PEXG regimen appears to be the most deserving candidate for a prospective assessment in the neoadjuvant setting.

CONCLUSION

The topic of treatment sequencing for patients affected by resectable pancreatic adenocarcinoma is of paramount importance and warrants further investigation. Time is mature for the running of a randomized prospective study, which is the only approach capable of providing evidence-based answers. To date, on the basis of activity data from trials on advanced pancreatic cancer, the most robust candidate for testing is the PEXG regimen. However, the lack of a large randomized trial confirming survival improvement over single agent gemcitabine in advanced disease suggests caution before embarking on a phase Ⅲ study in the neoadjuvant setting. An exploratory phase Ⅱ randomized trial seems to embody the optimal approach to avoid the risk of wasting resources and time. Accordingly, a clinical trial involving more than 20 Italian institutions has been designed as a three-arm calibrated study[41] and is currently underway. Patients are randomly allocated to receive either an adjuvant treatment with gemcitabine for 6 mo (calibration arm) or an adjuvant treatment with PEXG for 6 mo or a perioperative treatment (3 mo before and 3 mo after surgery) with PEXG. After completion of recruitment for the phase Ⅱ part of the study, an analysis of the results will be performed to decide whether to continue to the subsequent phase Ⅲ part of the study. It is hoped that this trial will contribute to an expansion of knowledge on the optimal therapeutic management of resectable pancreatic cancer.

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EDITORIAL

Role of endoscopic ultrasound during hospitalization for acute pancreatitis

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Abstract

Endoscopic ultrasound (EUS) is often used to detect the cause of acute pancreatitis (AP) after the acute attack has subsided. The limited data on its role during hospitalization for AP are reviewed here. The ability of EUS to visualize the pancreas and bile duct, the sonographic appearance of the pancreas, correlation of such appearance to clinical outcomes and the impact on AP management are analyzed from studies. The most important indication for EUS appears to be for detection of suspected common bile duct and/or gall bladder stones and microlithiasis. Such an approach might avoid diagnostic endoscopic retrograde cholangio-pancreatography with its known complications. The use of EUS during hospitalization for AP still appears to be infrequent but may become more frequent in future.

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Key words: Acute pancreatitis; Endoscopic ultrasound; Acute biliary pancreatitis; Endoscopic retrograde cholangio-pancreatography; Idiopathic pancreatitis

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INTRODUCTION

In recent years endoscopic ultrasound (EUS) has emerged as a very useful diagnostic modality in the evaluation of patients with acute pancreatitis (AP). Studies have shown EUS to be highly accurate in the diagnosis of gallstone disease (including microlithiasis), chronic pancreatitis, pancreatic tumors and other causes of AP which have negative or inconclusive results as assessed by other imaging methods. However, most of these studies have been carried out after the acute attack has subsided[1-4].

There are very limited data regarding the role of EUS during AP. We have reviewed the literature as to the role of EUS during an episode of acute pancreatitis and attempted to find out its utility in terms of making a diagnosis, finding an etiology and predicting the severity and outcomes in AP.

We could find only 8 studies (7 published and 1 in abstract form) so far which have investigated EUS during an episode of AP. Of these, only one study[5] looked specifically at the diagnostic usefulness of EUS in AP and another[6] looked at its prognostic value. Most of the other studies looked at the role of EUS in evaluation of gallstone pancreatitis and compared it to other diagnostic modalities such as transabdominal US and endoscopic retrograde cholangio-pancreatography (ERCP). We reviewed these studies in an attempt to answer the following questions with regard to AP: (1) how good is EUS in visualizing the pancreas and bile duct? (2) what are the findings

on EUS? (3) whether these findings correlate with the clinical outcomes and (4) what is the role of EUS in suspected biliary pancreatitis?

VISUALIZATION OF THE PANCREAS AND THE BILE DUCT BY EUS DURING ACUTE PANCREATITIS

During acute pancreatitis, edema of the duodenal wall, pancreatic necrosis and inflammation or fluid collection around the pancreas can potentially hinder the visualization of the pancreas, gallbladder or bile duct by EUS. There are only two prospective studies (with 23 and 35 patients, respectively) which have commented about visualization of the pancreas during AP, and the entire pancreas could be visualized in all patients in both of the studies[5,7]. While there are four studies (with a total of 228 patients) which have shown complete visualization of the gallbladder and bile duct in all patients during AP[5,7-9], this was not possible in three studies[10-12]. In one study, the gallbladder could not be visualized in three out of 28 patients (10.7%)[10]. One out of the 3 patients had situs inversus, and in two the gall bladder was located abnormally. In the same study, visualization of the common bile duct (CBD) was complete in 32 patients (88.8%), partial in 3 (8.3%) and unsuccessful in 1 patient (2.7%). Prat *et al*[11] in their study of 123 patients, found that EUS imaging of the CBD was unsatisfactory in three out of 123 patients (2.4%), two with Billroth Ⅱ gastrectomy and one who underwent transcystic drainage. In another study, CBD could not be visualized in one out of 38 patients (2.6%) due to severe pancreatic necrosis[12].

These observations suggest that even though EUS can visualize the entire pancreas, gallbladder and bile duct in acute pancreatitis in most patients, there may be occasional difficulties encountered in patients with severe pancreatic necrosis, unusual location of gallbladder or altered gastroduodenal anatomy.

We would like to stress here that while the entire pancreas can be visualized in AP, changes of chronic pancreatitis and small tumors can be missed and hence EUS for these indications should be avoided during an episode of AP. Another question that needs to be addressed is, how well can the pancreatic duct be imaged by EUS during AP? This has been mentioned in only one study of 23 patients, out of which the main pancreatic duct was seen in 78% of patients[5]. There are no data concerning sensitivity and specificity of EUS in detecting pancreatic duct disruptions and strictures during the course of AP.

EUS FINDINGS OF THE PANCREAS IN ACUTE PANCREATITIS

There are 4 studies[5-7,10] that looked prospectively at the EUS findings in AP early in the disease course. In 3 of the studies[6,7,10], EUS was performed within 72 h of admission and in the other it was done within the 1st week[5]. In two prospective studies by Sugiyama *et al*[5,7], EUS showed a normal or diffusely enlarged pancreas with a normal or diffusely low internal echo pattern in all patients with edematous pancreatitis. The details of these findings were given in only one out of the 2 studies, involving 23 patients[5]. In this study, the pancreas was normal in size in 37.5% of patients with edematous pancreatitis. The echogenicity was normal in 25% and the remaining 75% had diffusely hypoechoic pancreas. However, in another study by Chak *et al*[10], the pancreas appeared normal in size in 63.8% of patients. Only eight out of 36 patients (22.2%) with edematous pancreatitis had a hypoechoic pattern on EUS. Four patients (11.1%) had hyperechoic pattern and the remaining 24 (66.6%) had a mixed pattern. Based on these findings, it appears that in edematous pancreatitis, EUS can show a normal-sized or enlarged pancreas, while hypoechoic pattern may be quite common. In our experience, in some patients the only findings on EUS suggestive of AP can be peripancreatic inflammation/fluid collection (unpublished data). Table 1 shows the salient EUS features of acute edematous pancreatitis.

In the studies carried out by Sugiyama *et al*[5,7], EUS also demonstrated extrapancreatic inflammatory spread as a hypoechoic area. When results of these two studies were combined, EUS correctly identified fluid in the lesser sac in all 18 patients (100%) while fluid in the retroperitoneum was identified in 20 out of 25 patients (80%).

Sugiyama *et al*[5,7] also found that all patients with necrotizing pancreatitis (n = 6) had focal hypoechoic areas with or without interspersed hyperechoic spots. The location and size of focal hypoechoic regions on EUS corresponded to those of avascular pancreatic necrosis on contrast-enhanced CT. Schoefer *et al*[6] in their abstract commented that EUS was able to detect pancreatic necrosis early in 3 patients. However, the number of patients with necrotizing pancreatitis in these studies was very small and therefore these results cannot be extrapolated to all patients with acute necrotizing pancreatitis.

CORRELATION OF EUS FINDINGS WITH CLINICAL OUTCOMES IN AP

Chak *et al*[10], in their study of 36 patients, found that in patients with peripancreatic fluid collection on EUS, there was a trend toward longer duration of hospital stay but it was not statistically significant (9.2 d *vs* 5.7 d, $P < 0.1$). They also found that patients whose pancreas had a coarse echotexture (n = 6) had a significantly shorter hospitalization (2.6 d) as compared to those with fine (n = 19) or grainy (n = 11) echotexture (6.6 and 8.2 d, respectively). However, size of the inflamed gland, parenchymal heterogeneity, parenchymal echogenicity and gastroduodenal wall edema did not correlate with the duration of hospital stay.

Schoefer *et al*[6], in a prospective study of 31 patients, developed an EUS score from 1-30 based on findings such as organ size, aspect of outer margin, echogenicity, location and degree of peripancreatic fluid. The score was correlated with clinical course and CT severity index. EUS score correlated significantly with duration of hospital stay ($P < 0.0001$), number of days with fever ($P < 0.001$),

Table 1 Endoscopic ultrasound features in acute edematous pancreatitis

Study	No. of patients	Normal-sized pancreas (%)	Normal echogenicity (%)	Hypoechoic (%)	Hyperechoic (%)	Mixed (%)
Sugiyama *et al*[5], 1995	16	37.5	25.0	75.0	None	None
Chak *et al*[10], 1999	36	63.8	None	22.2	11.1	66.6

Table 2 Sensitivity, specificity, positive and negative predictive value of endoscopic ultrasound to detect common bile duct stones in acute pancreatitis

Study	No. of patients	Sensitivity	Specificity	Positive predictive value	Negative predictive value
Sugiyama *et al*[7], 1998	35	100	100	-	-
Chak *et al*[10], 1999	36	91	100	100	95
Liu *et al*[8], 2001	100	97	98	-	-
Stabuc *et al*[12], 2008	38	96	85	92	92

days spent in ICU ($P < 0.0001$) and CT severity index ($P = 0.0004$). However, this study was published only in abstract form and therefore requires further validation.

ROLE OF EUS IN SUSPECTED ACUTE BILIARY PANCREATITIS

There are many studies which have looked at the utility of EUS in the diagnosis of CBD stones. However, only 4 studies have so far investigated this during the course of AP[7,8,10,12]. These studies showed that EUS had a sensitivity of 91% to 100% and specificity of 85% to 100% to detect CBD stones (Table 2). It is generally believed that in patients with acute pancreatitis and intermediate probability of CBD stones, EUS can be done safely and may avoid diagnostic ERCP, with its attendant complications. A recent meta-analysis based on 4 randomized controlled trials performed by Petrov *et al*[13] showed that use of EUS for the selection of patients who will need therapeutic ERCP results in significantly lower risk of complications in comparison with the use of ERCP for both diagnosis and treatment of choledocholithiasis. However, it should be noted that only one of these 4 RCTs was carried out in the setting of AP[9]. In this trial, 140 patients with first episode of suspected biliary AP were randomized to EUS or ERCP, within 24 h of admission. In the EUS group, therapeutic ERCP with endoscopic sphincterotomy (ES) and stone extraction was performed if EUS detected choledocholithiasis; otherwise the patients were managed conservatively. None of the patients with negative EUS for CBD stones had recurrent pancreatitis or symptoms suggestive of biliary stones during a median follow up of 26 mo. The only statistically significant difference between the two groups was that EUS could be performed in 100% of patients, while successful cannulation of the CBD in the ERCP group was possible in 86% ($P = 0.001$).

While there were no complications related to EUS, four patients in the ERCP arm had post-ES bleeding. However, there was no significant difference in morbidity or mortality between the 2 groups.

CAN EUS REPLACE CT OR TRANSABDOMINAL ULTRASOUND?

Sugiyama *et al*[5] pointed out that EUS did not have some of the drawbacks of CT such as radiation exposure and contrast load and therefore could be potentially used for both the diagnosis and detection of the cause of AP. However, in patients with edematous pancreatitis, EUS can show normal pancreas. Moreover, the findings on EUS are variable in edematous pancreatitis (hypoechoic, hyperechoic or mixed). Although there is a suggestion that EUS might be able to detect pancreatic necrosis, this is based on a small sample size and therefore larger studies are needed for its validation. Again, EUS cannot detect fluid collection in the retroperitoneal area very well. At the same time, patients with severe pancreatitis may require multiple CT scans to evaluate the dynamics of local complications. Whether EUS, an invasive test, will be feasible and cost effective under these circumstances is a question that needs to be addressed.

Chak *et al*[10] recommended that if the patient would undergo EUS regardless of the transabdominal US findings, it made sense to forgo the expense of transabdominal US. However, in the same study there were 3 patients in whom the gallbladder was not seen on EUS but was seen only on conventional US and all 3 had gallstones.

Therefore, based on current evidence, one cannot propose that EUS can replace CT or transabdominal US. However, in certain situations such as patients with renal failure, contrast allergy or early pregnancy when CT scan cannot be carried out, EUS might have a role to play.

CONCLUSION

The data regarding the role of EUS during AP are limited because its use in this situation is evolving. There is a wide variation in appearance of the pancreas on EUS during AP and it may even be normal in some patients. The finding of focal hypoechoic areas on EUS has been suggested to indicate pancreatic necrosis, but this finding needs to be validated with larger trials. Preliminary data

have shown that some findings on EUS might be able to predict the severity of AP, but further studies are needed for confirmation. One of the important indications for performing EUS in AP is suspected acute biliary pancreatitis when transabdominal US and CT do not show biliary calculi. Although the EUS-guided ERCP approach has been shown to be beneficial in patients with suspected choledocholithiasis, data supporting this in the setting of acute pancreatitis are limited. Identifying other causes such as tumors during the acute stage, while possible, is often done after the resolution of the attack. EUS definitely has a role in the drainage of pancreatic fluid collection and in helping to obtain access to perform necrosectomy. Thus, at the present time, the role of EUS during the acute stage of AP is still limited.

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EDITORIAL

Clostridium difficile infection and inflammatory bowel disease: Understanding the evolving relationship

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Abstract

Clostridium difficile (*C. difficile*) infection (CDI) is the leading identifiable cause of antibiotic-associated diarrhea. While there is an alarming trend of increasing incidence and severity of CDI in the United States and Europe, superimposed CDI in patients with inflammatory bowel disease (IBD) has drawn considerable attention in the gastrointestinal community. The majority of IBD patients appear to contract CDI as outpatients. *C. difficile* affects disease course of IBD in several ways, including triggering disease flares, sustaining activity, and in some cases, acting as an "innocent" bystander. Despite its wide spectrum of presentations, CDI has been reported to be associated with a longer duration of hospitalization and a higher mortality in IBD patients. IBD patients with restorative proctocolectomy or with diverting ileostomy are not immune to CDI of the small bowel or ileal pouch. Whether immunomodulator or corticosteroid therapy for IBD should be continued in patients with superimposed CDI is controversial. It appears that more adverse outcomes was observed among patients treated by a combination of immunomodulators and antibiotics than those treated by antibiotics alone. The use of biologic agents does not appear to increase the risk of acquisition of CDI. For CDI in the setting of underlying IBD, vancomycin appears to be more efficacious than metronidazole. Randomized controlled trials are required to clearly define the appropriate management for CDI in patients with IBD.

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Key words: *Clostridium difficile*; Inflammatory bowel disease; Antibiotics; Colectomy

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INTRODUCTION

Clostridium difficile (*C. difficile*) infection (CDI) is the leading identifiable etiology for antibiotic-associated diarrhea and is associated with substantial morbidity and mortality. Since the initial report of this bacterium as a cause of antibiotic-associated pseudomembranous colitis in 1978[1], the incidence of CDI has increased over the years. Although knowledge in epidemiology, pathogenesis, risk factors, diagnosis and management of CDI has tremendously increased, the frequency and severity of CDI continue to increase at an alarming rate[2-4]. A hypervirulent strain of *C. difficile*, BI/NAP1/027 was reported from North America

and Europe which was associated with a more severe and complicated disease and a higher mortality[3,4]. In addition to its effect on morbidity and mortality, CDI is also associated with increasing duration of hospitalization and costs. The expected health care costs due to CDI alone are estimated as being up to 3.2 billion dollars per year in the US[5]. Clearly the impact of CDI on the health care system continues to grow with emergence of community-acquired CDI[6,7].

Inflammatory bowel disease (IBD), consisting of Crohn's disease (CD) and ulcerative colitis (UC), are chronic relapsing inflammatory conditions. IBD patients frequently require corticosteroids, antibiotics (in CD), immumomodulators, and biological therapy. Some of these agents can increase the risk of acquisition of CDI. In a large population-based cohort study, the use of biologic agents does not appear to increase the risk for CDI[8]. Recently published single-center studies and national inpatient database studies reported rising rates of CDI among IBD patients and their contributions to an increased rate of hospitalizations and mortality[9-12]. The risk of CDI in IBD patients appears to persist even after colectomy. CDI can involve the small bowel[13]. CDI has also been reported in UC patients with restorative proctocolectomy with ileal pouch-anal anastomosis (IPAA)[14,15] .However, the exact pathogenic role of *C. difficile* in these clinical settings are unclear. *C. difficile* may cause an isolated infectious colitis superimposed on IBD, or in some patients, may precipitate an IBD flare leading to two separate but simultaneous inflammatory processes. The other possibility is that *C. difficile* may be just a colonizer and that IBD flare probably occurs independently. When patients with IBD develop worsening symptoms and *C. difficile* is isolated in their stool, there are no clear guidelines to suggest whether to withhold or continue IBD-related medications, including antibiotics, corticosteroids, immunosuppressants, or biologics, while instituting appropriate therapy for CDI. In a retrospective study, patients treated by combination therapy of antibiotics and immunomodulators had a trend towards increased mortality when compared with those treated by antibiotics alone[16]. Lastly, there is no consensus on which antibiotic regimen should be considered as the first-line agent for the treatment of CDI complicating IBD.

Given the uncertainty in the pathogenesis and controversy on management of patients with concurrent CDI and IBD, we embarked on this project to clarify some issues on evolving CDI in IBD. The main goals of this article are to provide information on the pathogenesis and impact of CDI on disease course of IBD, to discuss diagnosis and treatment modalities of CDI in IBD, and to compare the clinical, laboratory, macroscopic and microscopic features between isolated CDI and superimposed CDI on IBD.

REVIEW CRITERIA

In February 2010, we searched MEDLINE from 1970 to the present using the Medical Subject Headings terms "Clostridium difficile, inflammatory bowel disease, Ulcerative colitis, Crohn's disease, Clostridium difficile and inflammatory bowel disease, Clostridium difficile and diagnosis, Clostridium difficile and treatment". Full papers and abstracts without language restrictions were considered. Important developments in research, reports from centers of excellence, and our own research developments form the basis of this article.

EPIDEMIOLOGY

CDI occurs predominantly in hospitalized patients and the incidence is increasing across the US with 3 million new cases of CDI occurring each year and as many as 10% of patients being affected within 2 d of hospitalization[17]. The prevalence of carrier state of the bacterium ranges from 0% to 3% in healthy individuals to 20% in hospitalized patients[17]. Interestingly, only one third of all infected patients developed diarrhea, while the remaining two thirds were asymptomatic carriers. Antibiotic exposure is the major risk factor for CDI.

The Center for Disease Control (CDC) reports of community acquired *C. difficile* colitis in the US has made the picture more concerning[6,7]. The traditional risk factors, such as recent hospitalization, being elderly, or having an underlying health condition were often absent. Close to 25% of patients who developed community acquired *C. difficile* colitis were young, healthy patients with no recent hospitalization in the past year.

Recent papers have highlighted a hypervirulent form of *C. difficile* strain, BI/NAP1/027 that was shown to be associated with a more severe and complicated disease course and a higher mortality. This strain appears to spread across the US. In a recent CDC report with regard to the BI/NAP1/027 strain of *C. difficile*, 38 states were reported to have the hypervirulent strain of the bacterium in their population[6,7]. This particular strain of *C. difficile*, toxinotype Ⅲ, North American PFGE type 1, and PCR ribotype 027 (NAP1/027) carries the binary toxin gene cdtB (cytolethal distending toxin B gene) and an 18-base pair deletion in tcdC; it produces 16-23 times more toxin A and B than the routine strain[3,5]. In addition, this hypervirulent strain was reported to be associated with increased disease severity[18] and possibly transmissibility and to cause outbreaks in Europe and the US[3,4]. The increasing use of fluoroquinolones may be one of the reasons for selecting the hypervirulent BI/NAP1/027 *C. difficile* strain since it is resistant to this class of antibiotics and possibly less responsive to other antibiotics.

PATHOGENESIS OF *C. DIFFICILE*-INDUCED DIARRHEA

Pathogenic strains of *C. difficile* produce two potent toxins, toxin A, an enterotoxin, and toxin B, a cytotoxin. The genes encoding toxin A and B are encoded in the *C. difficile* pathogenicity locus (*tcdA* and *tcdB*) which also encode two additional regulatory genes (*tcdC* and *tcdD*)[19]. The *tcdD* gene product up-regulates toxin transcription, while *tcdC* prob-

ably encodes a toxin gene repressor[19]. The fifth gene of the pathogenicity locus, *tcdE* is postulated to release both toxins A and B into the colonic lumen by lysing the cell walls[20]. Both toxins A and B have a 49% amino acid homology and possess a N-terminal domain that possesses cytotoxic activity, a transmembrane domain that facilitates toxin entry into the cytoplasm and a C-terminal domain that favors toxin binding to the epithelial cells[19]. Both toxins A and B are UDP-glucose hydrolases and glucosyltransferases and contribute to infectious and inflammatory diarrhea; however toxin B may be the major inflammatory toxin[21]. The toxins initially attach to non-proteinaceous disaccharide Gal beta 1-4GlcNac residues in the colon. Both toxins play a role in the initial binding to the colonic epithelial cells. After adhesion, the toxin enters the cell through receptor-mediated endocytosis and catalyzes the transfer of a glucose residue from UDP-glucose to guanosine triphosphate-binding rho proteins[19], the intracellular signaling molecules regulating cytoskeletal organization and gene expression. Glucosylation of rho proteins in turn leads to disruption of protein synthesis, and cell death. This leads to the inflammatory diarrhea seen in patients with CDI[22].

INFLAMMATORY BOWEL DISEASE AND CDI

Almost three decades before, LaMont *et al*[23] postulated that *C. difficile* toxin complicates chronic IBD and contribute to relapse in some patients. Since then, isolated case series of CDI contributing to symptomatic relapse in patients with IBD have been reported[24-27].

Incidence and prevalence

Paralleling the rising burden of CDI in the general population, recent years have witnessed a dramatic increase in CDI in patients with IBD. Recently, two single-center studies and two national inpatient database studies have reported a rising rate of CDI among IBD patients and their contributions of increased rates of hospitalization and mortality[9-12]. In a retrospective study of all confirmed CDI patients from a tertiary care center over a 7 years period, there was a doubling in the CDI rate in CD patients (9.5 to 22.3/1000 admissions) and tripling in UC patients (18.4 to 57.6/1000 admissions)[9]. A similar increase in the rate of CDI in IBD patients from 1.8% in 2004 to 4.6% in 2005 was observed in a subsequent study from a different tertiary-care center[10]. Furthermore, both studies identified that IBD patients, in particular those with UC, were at a disproportionately higher risk for acquiring CDI than non-IBD patients. In a large study utilizing the Healthcare Cost and Utilization Project Nationwide Inpatient Sample inpatient care database in the US, hospitalized patients with concurrent CDI and IBD had a 4 times greater mortality than those admitted to hospital for IBD or CDI alone[11]. In a subsequent study utilizing the same National Inpatient Sample database to study the temporal pattern of CDI, the prevalence of CDI among UC patients (37.3 per 1000) was higher than that among CD patients (10.9 per 1000), non-IBD gastrointestinal (GI) patients (4.8 per 1000), and general medicine patients (4.5 per 1000). In addition the incidence of CDI among UC patients almost doubled (26.6 per 1000 to 51.2 per 1000) over the 7-year period. CDI was independently associated with a greater mortality among patients with UC, but not CD[12].

Superimposed infections of pathogenic bacteria or viruses may contribute to exacerbation of IBD. Concurrent CDI is one of them. In a Scandinavian study in 1983, only 5% of patients admitted for a flare had CDI which would make routine screening not cost-effective[28]. However recent studies reported that approximately 5%-19% of newly-admitted patients for relapsing IBD tested positive for *C. difficile* toxins[29,30]. Similar to the adult population, pediatric IBD patients also seem to be susceptible to CDI as a recent Italian study identified *C. difficile* toxins in 24.7% of patients with diarrhea or abdominal pain[31]. *C. difficile* carriage status was studied with stool culture and molecular microbiological methods in IBD patients in clinical remission with no recent hospitalization or antibiotic exposure[32]. Toxigenic *C. difficile* was demonstrated more frequently in IBD patients (8.2%) than in healthy volunteers (1.0%). However, none of these patients developed CDI after a 6 mo follow-up and all the ribotypes identified were community-acquired, highlighting the acquisition of *C. difficile* in IBD patients even in remission from a wide variety of community sources[32]. The clinical relevance and significance of this community-acquired *C. difficile* carriage is interesting and its effect on the outcome of IBD has not been studied.

Risk factors

Environmental exposure continues to be the most common route of acquisition of CDI. Recent hospitalization increases the risk for nosocomial acquisition of CDI, the most common setting for the infection. Antibiotic-resistant *C. difficile* spores survive in hospital environment and can be isolated on toilets, bedrails, floors, telephones, call buttons, stethoscopes, and the hands of healthcare workers[19-21]. Sharing a room with an infected patient also increases the risk of infection[19].

Interestingly in a majority of IBD patients, CDI seems to often be contracted outside of the hospital. In a recent study, the median time to development of CDI in non-IBD patients was 4 d in contrast to less than a day with CDI in IBD patients[9]. In another study, 76%-79% of patients acquired CDI from the community[10]. Toxigenic *C. difficile* was demonstrated more frequently in IBD patients in complete remission with no recent hospitalization or antibiotic exposure (8.2%) than in healthy volunteers (1.0%) and the ribotypes identified were community-acquired, highlighting the acquisition of *C. difficile* in IBD patients[32]. In a case-control study from our institution, we also observed that 47.2% of patients had acquired the infection outside the hospital[33].

Almost any antibiotic has been associated with the development of CDI. The risk of CDI varies depending on the type of antibiotic, frequency, duration, route of antibiotic use, and the use of concurrent medications[34-37]. How-

ever, even a short-term use of prophylactic antibiotics can cause CDI[38]. The most common implicated antibiotics associated with CDI till recently were ampicillin, amoxicillin, cephalosporins, and clindamycin[34-37]. However, with widespread use, fluoroquinolones have become one of the common predisposing factors for CDI[38-41]. The exact mechanism of antibiotic-associated CDI in IBD is unclear. In addition, frequency of antibiotic exposure in relation to CDI risk in IBD has been highly variable. Antibiotic use prior to 3 mo before the development of CDI was seen in only 40% of IBD patients compared with 69% in the non-IBD population in a study[42]. A separate study showed that 61% of IBD patients with antibiotic exposure developed *C. difficile*[10]. In a cohort study, 57.2% of patients acquiring a CDI received antibiotics in the previous 6 mo[8]. In our recent study, antibiotic exposure within 30 d prior to *C. difficile* testing was found to be associated with an increased risk for CDI with odds ratio of 12.0 [95% confidence interval (95% CI): 1.2-124.2][33].

Immunosuppression is also proposed as another risk factor for the development of *C. difficile* infection. Cancer chemotherapy, particularly methotrexate[43] or patients with organ transplantation on immunosuppression appear to be at risk. The role of immunomodulators in the development of CDI is controversial. Previously studies have reported the association of immunomodulators with CDI in IBD[9,10]. However, in the studies from our institution and others suggest that immunosuppressive treatment was not associated with the risk of CDI[33,44,45].

The relationship of the use of corticosteroids to the risk of CDI has been studied in IBD patients. In a large cohort study of IBD patients, corticosteroid use with or without simultaneous use of other immunomodulating drugs was associated with a 3-fold increase in the risk of CDI (relative risk = 3.38, 95% CI: 1.88-6.10)[8]. Even in the absence of other immunomodulating drugs, the risk increased 2.5 fold in patients using corticosteroids[8]. Corticosteroids have also been shown to increase the risk of CDI relapse in solid organ transplant patients[44]; however corticosteroids and their risk of association to CDI have not been studied in IBD patients.

The use of biologics, specifically infliximab to the risk of CDI was studied in IBD patients and there appears to be no association of the use of biologics on the risk of development of CDI[8].

The normal bacterial flora in the bowel is an important natural defense and inhibits the growth of *C. difficile*[46]. In addition, the gastric acid barrier is a host mechanism to protect against ingested microorganisms[47]. The use of proton pump inhibitors and the risk for CDI is a subject of controversy, as published results of studies have been conflicting. Some initial studies demonstrated a higher risk of development of CDI with proton pump inhibitor therapy but this finding has not been consistently demonstrated[42,48].

Similar to the non-IBD populations, increasing age has been proposed as a risk factor for CDI in the IBD population[49-51]. IBD patients with CDI, however, were younger than the corresponding non-IBD population who developed CDI. In addition to immunosuppressive medication, host immunity, particularly the humoral arm, may play a role in determining susceptibility to CDI[52]. Thus serum and intestinal secretory antitoxin antibodies may afford protection and may be associated with mild colitis or carriage, while patients with deficient response develop severe or recurrent CDI[52]. Recurrent CDI is also suggested to be because of alterations in the fecal microbiota with markedly decreased diversity as demonstrated by phylogenetic analysis of 16S rRNA-encoding gene sequences[53].

IBD itself has been shown to be a specific risk factor for the development of CDI, particularly in those with colonic involvement[9,11,54]. Patients with UC appear to be at a higher risk for the development of CDI than CD and the presence of colonic disease conferred 3-fold greater risk (odds ratio = 3.12, 95% CI: 1.28-5.12) for CDI[11]. Similarly CD patients with colonic involvement seem to be at a greater risk for CDI than those with isolated small bowel disease[11]. The risk of CDI in relation to disease activity is unclear. A recent study suggested that patients with a greater disease activity may be at a higher risk for CDI[31]. However, in the population based study by Nguyen *et al*[12], the inverse association between CDI and colectomy rate led to the suggestion that IBD patients with CDI have lesser disease activity, although there was no information on the disease activity. Similarly, in our recent study we did not find any difference in the endoscopic disease activity in UC patients with and without CDI[33].

OUTCOME AFTER TREATMENT AND NATURAL HISTORY OF CDI

Short-term outcome

Patients with CDI are at risk for complications including toxic megacolon, colonic perforation, and peritonitis with sepsis. Patients with IBD are similarly at risk for these complications. Single-center and nationwide studies have studied the outcome of CDI in patients with IBD. The results have been highly variable with some studies reporting shorter stay in patients with CDI in IBD than those in non-IBD patients[42], and some studies showing similar lengths of stay[55], while other studies highlighting increased hospitalization duration and costs[10].

The colectomy rate in CDI is an important measurement of short-term outcome in CDI. Colectomy has been shown to be independently associated with a greater than 2-fold increase in inpatient mortality (incidence rate ratio = 2.4, 95% CI: 1.8-3.2)[56]. However studies have reported varying rates of colectomy for CDI in the setting of IBD. In a large study utilizing the Health Care inpatient care database, the development of CDI was inversely related to the risk of colectomy[11]. Similarly a subsequent study reported low rates for colectomy after CDI (1 of 15 patients)[42]. In our recent study with colectomy at 3 mo following CDI infection being the end point, we did not find CDI as a risk factor[33]. In a single-center case-control study, the rate of emergent colectomy in their CDI-UC population was 23% with the indication being toxic complications (4 of 11) or

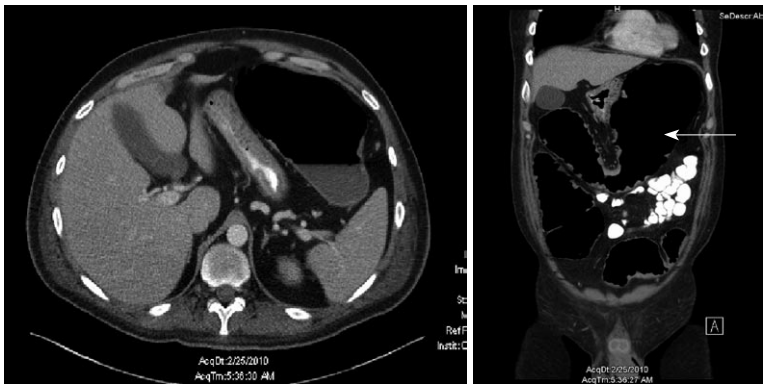

Figure 1 Toxic megacolon in a 27-year-old patient with *Clostridium difficile* infection who had underlying ulcerative colitis, resulting in emergent subtotal colectomy. Arrow indicate dilated colon.

medically refractory disease (7 of 11) compared to 13.4% in the *C. difficile*-negative IBD population[55]. However, it is interesting that 7/11 patients had medically refractory disease and may be *C. difficile* was a colonizer. Also there was no statistically significant difference in the short-term risk of colectomy at 1 mo[55]. In another study, underlying IBD was associated with 6 fold greater risk of bowel surgery compared with patients with CDI without underlying IBD[10]. We believe that the lower risk of colectomy with UC-CDI in most studies may be due to the fact that patients with UC exacerbation resulting from CDI are much more likely to improve with proper pathogen-directed medical therapy. Therefore, treating CDI in UC patients may actually prevent the need for colectomy in the short term (Figure 1).

Long-term outcome

There are limited studies available investigating the long-term outcome of CDI in patients with IBD. In a recent retrospective case control study, UC-CDI patients had worse clinical outcome than UC patients without CDI, with a follow-up of up to a year after CDI[55]. On the other hand, the study did not discriminate between recurrent CDI *vs* worse IBD disease activity because of the retrospective nature and study design. However, *C. difficile*-positive patients had significantly more UC-related hospitalizations (58 hospitalizations *vs* 27 hospitalizations) and emergency room visits in the year following initial admission (8 visits *vs* 1 visit). Also, up to a year following the index admission, patients with CDI had significantly higher rates of colectomy compared to *C. difficile*-negative patients (44.6% *vs* 25%). In a case-control study comparing the disease course for 1 year before and 1 year after the initial infection in 87 patients with IBD with *C. difficile,* colectomy occurred in only 10.3% of patients (9/87) following CDI[57]. While 8% had fewer hospitalizations in the year following infection, 41.3% of patients (36/87) followed for a year after CDI had no difference in the number of hospitalizations. However, 46% of patients (40/87) had more hospitalizations in the year following CDI (range 1-9 hospitalizations)[57]. Also 53% (46/87) of IBD patients with CDI required an escalation in their IBD medical therapy including initiation of biologic therapy (26%; 23/87), dose escalation of current biologic (8%; 7/87), escalation or initiation of azathioprine/6-MP (11.5%; 10/87) or methotrexate (7%; 6/87)[57]. Both these studies are limited by their retrospective nature and it is unclear whether underlying IBD severity was responsible for this outcome or whether *C. difficile* produces certain immunological changes that leads to a worse long term clinical outcome. Table 1 summarized both the short and long term outcome of CDI in IBD.

Table 1 Short and long-term outcomes with *Clostridium difficile* infection and inflammatory bowel disease

Short-term outcomes
Toxic megacolon
Colonic perforation
Peritonitis with sepsis
? Increased hospitalization duration and costs
Colectomy rates highly variable
Long-term outcomes
Increased UC related hospitalization and emergency room visits
? Escalation of medical treatment
Increased rate of colectomy

UC: Ulcerative colitis.

CLINICAL, RADIOGRAPHIC, ENDOSOPIC, AND HISTOLOGIC FEATURES

Patients with CDI can present with a wide variety of clinical manifestations ranging from an asymptomatic carrier state to fulminant colitis with megacolon. The most common clinical presentation of CDI is diarrhea and abdominal pain. The diarrhea is usually watery in patients with CDI; however in patients with underlying IBD, it may be bloody or mucous[49,58]. There are associated systemic symptoms and low-grade fever with a polymorphonuclear leukocytosis.

Although 0% and 3% of healthy adults may carry *C. difficile*, the frequency of asymptomatic carriage of *C. difficile* in patients with IBD is not exactly known. *C. difficile* carriage status in 122 IBD patients in clinical remission in the outpatient setting with no recent hospitalization or antibiotic exposure was studied with stool culture and molecular DNA-based microbiological methods. The strains were characterized by toxin typing, ribotyping, and pulsed-field gel electrophoresis. Toxigenic *C. difficile* was demonstrated more frequently in IBD patients (8.2%) than in healthy volunteers (1.0%). However, none of these patients developed CDI after a 6 mo follow-up and all the ribotypes identified were community acquired highlighting

the acquisition of *C. difficile* in IBD patients even in remission from a wide variety of community sources[32].

Some patients may present with severe disease causing paralytic ileus, which may evolve into toxic megacolon characterized by a dilated colon (> 7 cm in its greatest diameter), and signs and symptoms of severe toxicity (fever, chills, dehydration, high white count). There is associated dilatation of the small intestine in patients with megacolon mimicking an intestinal obstruction. Bowel perforation may also occur[59-61]. Diarrhea may be absent because of paralytic ileus, particularly in postoperative patients who receive narcotics for pain control. Patients may also have anasarca due to severe hypoalbuminemia[62]. Patients may present without diarrhea but only with abdominal pain, fever and leukocytosis (a leukemoid reaction with a white blood cell count up to 100 000 cells/cu.mm.)[62]. A high degree of suspicion is required to diagnose CDI in these settings.

Abdominal imaging

Plain radiography is usually normal in patients with CDI, unless they have complications like ileus or toxic megacolon or perforation. CT imaging is useful in the diagnosis of severe or fulminant CDI and the characteristic features include colonic-wall thickening, pericolonic stranding, the "accordion sign", and the "double-halo sign"[63]. The accordion sign is seen with oral contrast and shows the high attenuation in the colonic lumen alternating with a low attenuation inflamed mucosa, while the double-halo sign is seen with intravenous contrast[63]. The presence of these signs in the right clinical setting may suggest a diagnosis of CDI.

Endoscopy

Lower endoscopic visualization forms an important part in the evaluation of patients with CDI in IBD. Isolated CDI produces the classic endoscopic appearance of pseudomembrane formation which is described in 50% of patients[64,65]. However in patients with underlying IBD, classic endoscopic or histologic features of pseudomembranes are conspicuously absent, making it hard to diagnose CDI in patients with worsening diarrhea[10,42]. In fact, recently published studies from Milwaukee and Belgium did not identify pseudomembranes in any of the IBD patients with CDI who underwent endoscopic evaluation[10,42]. However endoscopy may be useful to assess disease activity of IBD and also to rule out other secondary causes of diarrhea including concurrent cytomegalovirus infection[66].

Histology

The classic histologic picture in CDI is the presence of pseudomembranes. Pseudomembrane formation is caused by sloughing and necrosis of the mucosa with ulceration secondary to inflammation. Pseudomembranes are actually characteristic "volcano" lesions with focal ulceration with inflammation composed of polymorphonuclear leukocytes, fibrin, chronic inflammatory cells, and epithelial debris[66]. In patients with IBD, pseudomembranes are not commonly present, CDI tends to produce a nonspecific mucopus; erythema and friability are commonly encountered endoscopic findings[10].

LABORATORY DIAGNOSIS

Although a variety of laboratory tests are used for the diagnosis of CDI, enzyme linked immunoassay (ELISA) is the most commonly used test to detect the toxin.

Enzyme linked immunoassay

These assays are based on the detection of toxins A and/or B using either a monoclonal antibody or a polyclonal antiserum that recognizes the specific toxin. The ELISA test is inexpensive and the results are available within 2-6 h. The most widely used ELISAs for detection of both toxins A and B in stool are somewhat less sensitive (70%-90%) than the cell cytotoxicity assay (see below). Up to 30% of tests may be falsely negative in comparison to the cell cytotoxicity assay or culture[67,68]. They do, however, demonstrate excellent specificity (99%)[68,69]. The lower sensitivity of these tests can be improved by performing ELISAs on 2 or 3 specimens rather than on 1 specimen, which increases the diagnostic yield by 5%-10%[70]. In IBD patients, the diagnostic yield of ELISA testing may be much lower. Four sequential stool samples were shown to increase the diagnostic yield to 92%[42].

Latex agglutination assay

Latex agglutination assay is based on the glutamate dehydrogenase (GDH) enzyme produced by *C. difficile*. The sensitivity of these tests approached almost 96%-100% in a recent study[71]. However, certain other organisms can also produce GDH and also the positivity indicates only the presence of the organism, rather than *in vivo* production of *C. difficile* toxins. It is not recommended for routine clinical use.

Cell cytotoxicity assay

Cell cytotoxicity assay is the gold standard test for diagnosis of CDI. It detects as little as 10 picograms of toxin and it is the most sensitive available test for detection of toxin B[72-75]. It is based on the principle that the toxins in the stool exert a cytopathic effect characterized by cell rounding which can be demonstrated in tissue culture. The high sensitivity (94%-100%) and specificity (99%) of the cytotoxicity assay is its major advantage. Disadvantages are its relatively high technical expertise and the 24-48 h needed to complete the assay[76].

C. difficile culture

Stool culture is seldom used for routine diagnosis because of labor intensiveness, long turnaround time (24-48 h) and a low specificity. The *in vivo* production of toxins can be seen in hospitalized patients who are asymptomatic carriers. It fails to differentiate toxin-producing from non-toxigenic strains. However, because culture permits molecular typing of the organisms, it is essential for monitoring molecular epidemiology and antibiotic susceptibility[72].

We do not recommend its routine use in the diagnosis of CDI in clinical practice.

Polymerase chain reaction for toxin gene detection

Polymerase chain reaction (PCR) based primers for the detection of genes for toxins A and is highly sensitive and specific for the diagnosis of CDI[77,78]. Culture of the organisms may be required for PCR, which makes the process more technically demanding and challenging. A study based on the nested PCR assay reported a 99% concordance with the cytotoxicity assay and a sensitivity of 96.3% and a specificity of 100%[78].

TREATMENT OF CDI

The Society for Healthcare Epidemiology of America recommends initiating empiric therapy for CDI immediately after stool procurement for patients with severe symptoms consistent with CDI[34]. Empiric treatment is warranted if the clinical suspicion is high without waiting for the results as early initiation of treatment is critical in improving the outcome. Agents that decrease intestinal motility, such as narcotics and loperamide, should be avoided because of the risk of decreasing toxin clearance and the risk for ileus and/or megacolon[79].

Specific antibiotic therapy should be initiated as soon as possible. Oral metronidazole in a dose of 250-500 mg four times a day for 10-14 d or oral vancomycin at 125-500 mg four times a day for 10-14 d is the treatment of choice in patients with CDI. Metronidazole can be administered intravenously (in doses of 500 mg four times daily) in patients who are unable to take oral agents[66]. Bacitracin, teicoplanin and fusidic acid have been used in the treatment of CDI, but their efficacy has not been proved superior to vancomycin/metronidazole in large systematic meta-analysis[80,81]. A large meta-analysis of 1157 patients from 12 randomized trials assessed the efficacy of eight antibiotics for the treatment of CDI. None of the antibiotics are superior to others for symptomatic cure and/or reduction in complications[82]. Thus metronidazole is the initial drug of choice because of similar efficacy, lower cost and lesser risk of selecting vancomycin resistant *enterococci* in mild to moderate disease. However in patients with severe disease, multiple studies have shown a failure rate of 22%-38% with metronidazole[83]. Studies have shown similar cure rates in patients with mild disease with either use of metronidazole or vancomycin, while in severe disease the eradication rate with metronidazole is 76%, as compared with vancomycin, which gives a cure rate of 97%[84]. These data support the use of vancomycin as the first line treatment for severe CDI, also in patients with mild to moderate CDI who do not improve within 72 h of initiation of treatment with metronidazole should be switched to vancomycin. Severe CDI requires aggressive treatment and doses up to 2 g/d of vancomycin may be required in patients with severe disease. A recent phase 3 trial compared the efficacy and safety of OPT-80, fidaxomicin that is bactericidal *via* inhibition of RNA polymerase and oral vancomycin in treating CDI. The clinical cure rates after OPT-80 (fidaxomicin) or vancomycin treatment were comparable[85]. However, OPT-80 was associated with a highly significant lower recurrence rate than vancomycin[85]. Further evidence of its efficacy needs to be studied. Anion-binding resins, such as cholestyramine and colestipol have also been used along with antibiotics[86]. These are proposed to bind to the *C. difficile* toxins and may have adjunctive benefit. However these agents have not been studied in IBD patients.

The efficacy of metronidazole or vancomycin specifically in the IBD population with CDI is unknown, but one study reported that just less than one quarter of the IBD patients with CDI required to be initiated on oral vancomycin because of lack of sufficient response with metronidazole[42]. Neither vancomycin nor rifaximin have been studied in randomized controlled trials for CDI in IBD patients.

There are no guidelines or evidence to suggest that one particular antibiotic regimen is better than the other in IBD patients who develop CDI. However colectomy rates in hospitalized patients with IBD was reportedly less from 45.5% in 2004, to 3.5% in 2006 in a single center study where vancomycin was adopted as the first line therapy in IBD patients with CDI after 2005[87,88].

Patients with fulminant colitis require initiation of treatment with oral vancomycin at a high dose of 500 mg every 6 h which may be administered with a nasogastric tube because of paralytic ileus. We also tend to use intravenous metronidazole along with vancomycin in these cases in our clinical practice. Emergent surgery is required for patients who do not respond to the above medical management and in patients with impending perforation and toxic megacolon. Patients usually undergo a subtotal colectomy and a temporary ileostomy and are associated with a high perioperative mortality rate approaching close to 40%[89].

There is no consensus on whether IBD-related medications, particularly immunomodulators and corticosteroids should be discontinued during the anti-CDI therapy. In a retrospective study of 155 patients from Europe with CDI complicating IBD, 104 (67%) were cotreated with antibiotics and immunomodulators (defined as the use of prednisone, azathioprine/6-mercaptopurine, methotrexate, biologics, cyclosporine, tacrolimus) for their *C. difficile*-associated IBD exacerbation, while the remaining 51 (33%) were treated with antibiotics alone[32]. The primary outcome of the study was colon perforation or toxic megacolon, shock, colectomy, and mortality. Patients treated by combination therapy had a trend towards a worse outcome when compared to those treated by antibiotics alone (likelihood ratio = 11.9; 95% CI: 0.9-157)[32]. Thus in most patients with CDI, it may be inappropriate to escalate immunosuppressive therapy during the acute CDI episode. However, the question of whether to add immunomodulator therapy in patients who are not on it before the CDI episode remains unanswered. In a recent survey of 169 North American gastroenterologists, there was significant disagreement on whether combination antibiotics and immunomodulators or antibiotics alone should be given

Table 2 Differentiating *Clostridium difficile* infection and inflammatory bowel disease

Features	Isolated CDI	CDI and IBD
Setting	Often hospital acquired	Often community-acquired
Risk factors	Antibiotic exposure prior to infection common Immunomodulator and corticosteroid use Increasing age	Many patients lacking of history of antibiotic exposure Immunomodulator and corticosteroid use playing even a greater role Increasing age Risk greater with ulcerative colitis than Crohn's disease, more with colonic involvement than small bowel disease
Clinical features	Usually watery diarrhea	May be bloody or mucous diarrhea
Outcome	Short term complications including toxic megacolon, colonic perforation, and peritonitis with sepsis	Short term complications including toxic megacolon, colonic perforation, and peritonitis with sepsis similar to patients without IBD Hospitalization costs and length of stay variable in studies Increased mortality in some studies Risk of colectomy unclear Long term outcome unclear, increased hospitalizations and escalation in medication use and colectomy rates reported with retrospective data
Diagnosis	ELISA testing for toxins	ELISA testing may be less sensitive
Endoscopy and histology	Pseudomembranes common	Pseudomembranes rare
Treatment	Metronidazole for mild to moderate severity Vancomycin for severe disease	? Vancomycin for any hospitalized IBD patient
Recurrence	20% after the first episode of CDI	Rates highly variable 10%-58%, may be higher
Extra-colonic gastrointestinal manifestations	Small bowel can be affected	Most cases of small bowel involvement in IBD patients Pouchitis can also be seen

IBD: Inflammatory bowel disease; ELISA: Enzyme linked immunoassay; CDI: *Clostridium difficile* infection.

to flaring IBD patients with CDI. Overall, 77/169 (46%) of the respondents elected to add on corticosteroids as a combined treatment with antibiotics, whereas 82/169 (54%) treated the flare with antibiotics alone. When maintenance azathioprine was regularly taken, only 11% of respondents withdrew it upon the diagnosis of CDI[90].

RECURRENT CDI

Recurrence of CDI is common, affecting approximately 20% of patients. Recurrence typically occurs 1 to 2 wk after stopping metronidazole or vancomycin, but it can be delayed for up to 12 wk[49,90]. Risk factors for recurrent CDI include a prior history of recurrence, increasing age, use of additional antimicrobials, and an inadequate protective immune response to *C. difficile* toxins[49,91].

There are limited data available on the risk of CDI recurrence in IBD patients. In a study published in abstract form from Milwaukee in 2005, recurrent CDI was reported in 27/46 (58%) of patients[92]. In a subsequent study from the same center, recurrent *C. difficile* occurred in (10/87) 11.5% of patients[57]. Thus the risk appears to be highly variable and prospective studies need to be undertaken to clearly clarify the risk of CDI recurrence in IBD patients.

Management of a first recurrence of CDI is identical to a primary episode. Long tapering courses of vancomycin or pulsed treatment reduce recurrence and are suggested for treating second episode of recurrence[93,94]. Because of the risk of often-irreversible neuropathy with long-term use of metronidazole, it is not used for treatment of second relapse. Recently, several small series reported the efficacy of rifaximin in treating recurrent CDI[95,96]. Similarly reconstitution of the fecal flora by administration of stool is effective in small series[97,98] as previous studies have shown loss of diversity of fecal flora[53]. Other treatments including the use of active and passive immunization by administration of immunoglobulins or oral administration of antibodies from colostrum of cows immunized against toxins are under investigation for future use[91].

The treatment of recurrent disease in IBD patients is unclear in the absence of evidence based studies. In a study from Milwaukee of 14 IBD patients, rifaximin at a dose of 200 mg three times a day for 2 wk, followed by 200 mg once daily for 2 wk and 200 mg every other day for the final 2 wk of the taper resulted in resolution of infection in all the patients[92]. In the absence of data, we recommend treating patients in a similar way to the non-IBD population as far as recurrence is concerned (Table 2).

CDI IN SPECIAL SITUATIONS

C. difficile enteritis

Small intestinal *C. difficile* has increasingly been reported. The spectrum of CDI has definitely expanded with small bowel involvement (Figure 2)[99]. They are more frequently reported in patients with IBD who have undergone total colectomy or some form of gastrointestinal surgery[13]. The most common presentation is increased ileostomy output with associated dehydration. In patients with small bowel CDI, the risk factors seem to be slightly different. Antibiotic use and IBD predispose to small bowel CDI similar to CDI of the colon. Prior surgeries of the colon/colectomy, and host factors including advanced age, immunocompromised state are proposed as additional risk factors for small bowel CDI[100]. More than 90% of patients reported in the literature had gastrointestinal surgery of the colon.

The reason for the predisposition of patients who undergo colonic surgery to small bowel CDI is not clear al-

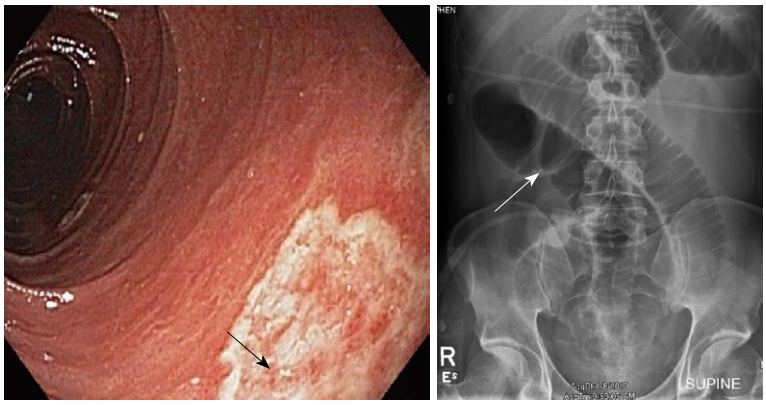

Figure 2 Recurrent *Clostridium difficile* enteritis in neoterminal ileum in a 36-year-old patient with diverting ileostomy for ileal pouch who had a preoperative diagnosis ulcerative colitis. Arrows: Enteritis due to *Clostridium difficile* infection and dilated loops of small bowel.

though multiple hypotheses are proposed. Firstly, changes occur in the small-bowel bacterial flora resembling colonic flora and after colectomy this may make it susceptible to overgrowth with *C. difficile,* particularly with concomitant antibiotic use[101]. This is based on the fact that the neoterminal ileum is colonized by colonic-type bacterial flora after ileocolonic resection[102]. Prolonged exposures to fecal stream may make the small bowel mucosa undergo metaplastic changes, as seen in patients with IPAA[103]. This notion was further supported by the fact that similar changes may occur in patients with end ileostomy and the long latent period between the surgery and the infection supports this. Changes occur in the ileostomy flora resembling the fecal flora[104]. In patients who develop infection in the immediate post operative period, a majority of patients had CDI of the colon prior to surgery which leads to the hypothesis that in those patients CDI of the small bowel may be secondary to migration of *C. difficile* into the small bowel after surgery. CDI is a toxin-mediated disease process. Although receptors for *C. difficile* toxins are typically on colonic epithelium, the receptors for toxin B is ubiquitous and may be present on small bowel epithelium which could mediate diarrhea in the immediate post operative period in the absence of colonic phenotype changes[105]. Secondly, colonization of the small bowel occurs because the protective mechanisms are compromised by colonic resection surgeries. The mechanical action of the ileocecal valve may be lost because of surgery[106]. In addition, continued peristalsis in the small bowel also inhibits colonization of the small bowel with *C. difficile*[106]. Therefore, surgeries involving only the left side of the colon with preservation of the ileocecal valve do not seem to increase the risk of CDI of the small bowel, highlighting the importance of the ileocecal valve in preventing colonization.

Initial studies highlighted that infection of the small bowel with *C. difficile* was associated with an increased mortality[13]. The increased permeability of the small intestinal mucosa was hypothesized to be due to result in profound sepsis[107]. However, recent studies showed a favorable prognosis. In fact, two large recent case series reported no mortality[100,108]. This may be probably secondary to increased awareness of the problem and early intervention.

The treatment of small bowel CDI is controversial and stratification of the disease severity as CDI of the colon could be used to initiate appropriate management plan. In a series of 11 patients, more than 50% responded to metronidazole alone[108]. In another series, all six patients were treated with a combination of metronidazole and vancomycin[100]. Thus similar to colonic CDI, oral vancomycin may be a first-line agent for severe CDI, while in mild to moderate disease, metronidazole may be used. However in patients who do not improve within 72 h of initiation of treatment with metronidazole, vancomycin needs to substituted instead of metronidazole.

C. difficile pouchitis

CDI has been reported in patients with IPAA[14,15,109,110]. CDI in IPAA can either present with asymptomatic colonization or with chronic antibiotic-refractory pouchitis or occasionally with fatal outcome. As the majority of patients have a history of short- or long- term exposure to antibiotics, CDI should be excluded in pouch patients with persistent symptoms with or without endoscopic findings of pouchitis or other pouch disorders.

In patients with IPAA, the epithelium of pelvic pouches undergoes morphologic changes facilitating fecal flora establishment[109]. These histologic adaptive changes include villus atrophy, Paneth cell hyperplasia, and a partial transition to colonic mucin phenotype without complete metaplasia[103]. In a recent study of 115 patients with IPAA, 21 (18.3%) were tested positive for *C. difficile* toxin A or B[14]. Three of those patients had chronic antibiotic-refractory pouchitis and all 3 patients had clinical remission and disappearance of *C. difficile* toxin from the stool with anti-*C. difficile* treatment with rifaximin or tinidazole. Three additional patients with other pouch-associated disorders also symptomatically improved with treatment of CDI. We also recently reported a patient who developed CDI of the pouch and neoterminal ileum immediately after ileostomy closure with a fatal outcome[111]. Fulminant outcomes of CDI of the pouch have also been described recently in a case report[112]. Similar to IBD patients with CDI who do not have the classic endoscopic or histologic features of pseudomembranes[10,42], superimposed CDI in pouch patients hardly have endoscopic or histologic features of pseudomembranes which makes the diagnosis challenging.

The treatment of CDI in IPAA is empiric at this point. There are no published prospective trials. The traditional

drugs used in the management of CDI are metronidazole and vancomycin. Previous studies suggest that metronidazole may be not completely protective against CDI of the pouch, as the bacterial infection can develop while the patients had been still on metronidazole[109,110]. Therefore, in patients with *C. difficile*-associated pouchitis, metronidazole may not be considered as the first-line agent. Based on our own experience and limited published literature, rifaximin, tinidazole, or vancomycin have been used with satisfactory results[14,110].

C. difficile infection in diverted bowel

Diversion colitis is common in segments of the colorectum after surgical diversion of the fecal stream, which may persist indefinitely unless the excluded segment is reanastomosed[113]. Patients with diverted bowel appear not immune to the development of CDI in the excluded downstream bowel segment. There has been a case report in which, following subtotal colectomy and end-ileostomy for medically refractory disease, a UC patient subsequently developed severe CDI in the rectal remnant (Hartmann pouch) and the patient responded to metronidazole suppositories[110].

RECOMMENDATIONS

In patients with IBD who present with worsening symptoms, CDI needs to be thought off and ruled out. In patients with a suspected diagnosis of CDI in IBD, stool studies for CDI are sent and empiric treatment is started. ELISA is the most commonly used method of diagnosis of CDI. We do not usually wait for the stool studies to return back to start treatment. We start all our IBD patients with suspected CDI on vancomycin 125 mg orally every 6 h and continue their previous immunosuppressive therapy. We do not add any new immunomodulators or escalate immunosuppressive medications in patients with suspected CDI in IBD unless CDI is ruled out with serial stool studies (at least 3-4). The duration of antibiotic use is 14 d. Routine endoscopy is not performed in these patients as the yield of pseudomembranes is very low unless an alternative diagnosis such as cytomegalovirus infection is being entertained. We also follow these patients serially to study the impact of CDI on the short term and long term outcome of IBD.

FUTURE DIRECTIONS

The pathogenesis and natural history of CDI in IBD patients is not entirely clear. The role of CDI in IBD exacerbation needs to be further investigated. It is unclear how to distinguish whether CDI is precipitating an IBD flare or whether it is an innocent bystander, as medical treatment targeted CDI does not necessarily induce IBD into remission. There is need for research to study the role of asymptomatic carriage of *C. difficile* and its impact on the longer-term outcomes of CDI in IBD. Although some retrospective studies have suggested worse long-term outcome of CDI in IBD patients, it needs to be prospectively studied. Management of these patients can be challenging. Future studies to ascertain the appropriate management of CDI in IBD is required in particular, as there is little consensus on whether antibiotics and immunomodulators or antibiotics alone should be administered to these patients. Randomized controlled trials comparing metronidazole and vancomycin are also required to clearly understand the best management of *C. difficile* flares in IBD patients. A multidisciplinary approach involving gastroenterologists and colorectal surgeons, together with a team of GI pathologists and GI radiologists is necessary to successfully manage and treat patients with these disorders. Development of animal models with concurrent CDI and IBD would help us to understand the pathogenesis and manage these patients better.

CONCLUSION

CDI has continuously evolved over the years rising from a relative "benign" disease entity due to antibiotic exposure to a significant public health problem. CDI poses substantial challenge to epidemiologists, infection control practitioners, infectious disease specialists, gastroenterologists, gastrointestinal surgeons and hospital administration. The rising incidence, with increasing hospitalization rate, length of hospital stay, morbidity and mortality is of great concern. There has been a tremendous increase in the burden of CDI over the past few years with higher rates of surgery and mortality in the IBD population compared with the non-IBD cohort. The increase in the risk of community-acquired CDI in IBD population highlights that a high index of suspicion should be maintained even in the absence of conventional risk factors, such as antibiotic use or health care exposure. Patients with IBD even after colectomy are not immune to CDI. Pseudomembranes on endoscopy and histology appear to be uncommon in CDI superimposed on IBD. Randomized controlled trials are required to define the appropriate strategy for risk stratification and management for CDI in patients with IBD. In addition, preventive measures are the key and require concerted effort from all quarters from epidemiologists to hospital administration and clinicians.

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TOPIC HIGHLIGHT

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Alcoholic hepatitis 2010: A clinician's guide to diagnosis and therapy

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Abstract

Alcoholic hepatitis (AH) remains a common and life threatening cause of liver failure, especially when it is severe. Although the adjective "acute" is frequently used to describe this form of liver injury, it is usually subacute and has been developing for weeks to months before it becomes clinically apparent. Patients with this form of alcoholic liver disease usually have a history of drinking heavily for many years. While certain aspects of therapy, mainly nutritional support and abstinence are well established, significant debate has surrounded the pharmacologic treatment of AH, and many institutions practice widely varying treatment protocols. In recent years a significant amount of literature has helped focus on the details of treatment, and more data have accumulated regarding risks and benefits of pharmacologic treatment. In particular, the efficacy of pentoxifylline has become increasingly apparent, and when compared with the risks associated with prednisolone, has brought this drug to the forefront of therapy for severe AH. This review will focus on the clinical and laboratory diagnosis and pharmacologic therapies that should be applied during hospitalization and continued into outpatient management. We conclude that the routine use of glucocorticoids for severe AH poses significant risk with equivocal benefit, and that pentoxifylline is a better, safer and cheaper alternative. While the full details of nutritional support lie beyond the scope of this article, nutrition is a cornerstone of therapy and must be addressed in every patient diagnosed with AH. Finally, while traditional psychosocial techniques play a major role in post-hospitalization care of alcoholics, we hope to make the medical clinician realize his or her role in reducing recidivism rates with early and frequent outpatient visits and with the use of baclofen to reduce alcohol craving.

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Key words: Alcoholic hepatitis; Alcoholic liver disease; Pentoxifylline; Baclofen

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INTRODUCTION

The term alcoholic hepatitis (AH) was first used by Beckett *et al*[1] in 1961, but reports of clinical jaundice after excessive ethanol consumption were not unusual in the early medical literature and they likely represented instances of AH[1,2]. Despite this longstanding observational relationship between alcohol consumption and liver disease, significant work has been required to determine how much alcohol must be consumed to cause liver disease, and which cohort of patients are at highest risk of developing significant liver injury.

VOLUME OF ALCOHOL REQUIRED

Observational studies have shown an increased risk of cirrhosis with ingestion of greater than 10-20 g of alcohol per day in women and more than 20-40 g/d in men[3]. In addition to total duration of alcohol intake, a variety of genetic, environmental and gender-related factors appear to independently influence the development of alcoholic liver disease. Age, female gender, and excess body weight [body mass index (BMI) > 27 kg/m^2 in men, BMI > 25 kg/m^2 in women] have been identified as independent risk factors for development of liver disease including AH[3-6]. In addition to a smaller volume of distribution, women are at higher risk due to a relative deficiency of gastric alcohol dehydrogenase compared to men[7]. An oral dose of alcohol in a woman is more like an intravenous dose. We have recently seen several women who developed AH after gastric bypass, in which the amount of gastric mucosa available to metabolize alcohol was reduced. More severe forms of AH are associated with consumption of large amounts of alcohol or binge drinking, and concomitant malnutrition[8]. Not surprisingly, the presence of coexisting hepatitis C has also been linked to a poorer prognosis[9].

DIAGNOSIS

Diagnosing AH can be challenging as the disease has widely varying presentations and in severe cases can mimic a bacterial infection and/or biliary obstruction. A detailed and thorough history remains the cornerstone of diagnosis[10]. Obtaining such a history can be rather difficult if patients feel ashamed about their drinking habits. Often, lengthy discussions are required to reveal the full extent of alcohol intake.

Medical history

Questions that are relevant to ask are detailed in Table 1. Patients regularly tell us that they "quit drinking" when in reality they simply reduced the amount or switched from hard liquor or fortified wine to beer. It is important to obtain the exact time sequence and volume and type of alcohol consumption. Many patients have the mistaken impression that beer is not alcohol. It is actually difficult to drink enough beer on a daily basis to develop AH-perhaps 48 beers per day. To develop AH, patients usually have to supplement beer with wine, fortified wine, and/or hard liquor.

Alcoholism and alcohol-related health problems are common in patients seen in county or university healthcare systems. Detailed histories regarding alcohol are therefore common in these settings. In contrast, patients admitted or seen in private systems may not be questioned at all about alcohol or may be asked only a few superficial questions that the patient finds easy to respond negatively to.

In general, patients with AH have been drinking heavily for years and then report a dramatic increase in the amount of alcohol intake, usually relating to a major life stressor, such as death of a parent, loss of a job, divorce, *etc*. Also, patients have often stopped drinking alcohol days to weeks prior to presentation due to malaise, poor appetite, and/or the realization that their drinking finally "caught up" with them. Most commonly patients present with nonspecific complaints such as anorexia, nausea and vomiting, abdominal pain, and weight loss (Table 2)[10,11].

Table 1 Questions to ask patients with suspected alcoholic hepatitis

When did you first start to drink alcohol?
How many days per week do you usually drink?
How many years have you been drinking on a regular or daily basis?
How many times have you been arrested for driving under the influence of alcohol?
How many times have you been arrested for public intoxication?
What type of alcohol do you usually drink? Beer? Wine? Hard liquor?
How many drinks of each type of alcohol do you drink on an average day?
Do you usually drink at home? Bars?
Have you been through an alcohol rehabilitation program? What type-inpatient or outpatient? How many times?
Have there been prolonged times when you drank no alcohol?
When was your last drink?

Table 2 Symptoms and signs of alcoholic hepatitis

	%
Common Presenting Symptoms of Alcoholic Hepatitis[10-13]	
Anorexia	27-77
Nausea and vomiting	34-55
Abdominal pain	27-46
Weight loss	29-43
Physical Examination Findings	
Hepatomegaly	71-81
Ascites	35
Encephalopathy (from asterixis to coma)	18-23
Gastrointestinal bleeding requiring transfusion	23
Jaundice	37-100
Malnutrition	56-90
Hepatic bruit	59

Physical examination

In addition to the patient's history, noteworthy physical findings that may help the clinician focus on AH as the diagnosis include hepatomegaly, ascites, encephalopathy (ranging from asterixis only to coma) and gastrointestinal bleeding requiring transfusion, especially if the cause is esophageal varices. Nonspecific findings of jaundice and malnutrition are also commonly seen[10-12]. With severe AH, jaundice is present in essentially 100% of patients. Notably, fever ranging from 100.4º to 104º, due to AH and not attributable to infection can be seen in over half of patients diagnosed with severe AH[11]. Fever is a common cause of confusion among physicians and can lead to extensive and relatively useless evaluations for fever of unknown origin, when AH should be the obvious explanation. The presence of a hepatic bruit is also very helpful in providing strong evidence for AH, if there is no malignant mass that could also cause a bruit. In one large series, 59% of patients with severe AH had a bruit[13].

Laboratory data

Laboratory findings in AH are often nonspecific, but can on occasion provide clues to the diagnosis. These include mild to moderately elevated transaminases, usually with an aspartate aminotransferase (AST) to alanine aminotransferase (ALT) ratio above 1.5 with AST greater than 45 U/L but usually < 300 U/L[10,14]. However, an unusual variant of AH, known as alcoholic foamy degeneration, can lead to an AST as high as 730 U/L[15]. A serum bilirubin > 2 mg/dL is often required to make a diagnosis, but clinical jaundice is usually present and the bilirubin is regularly greater than 10 mg/dL. Other nonspecific but established markers of alcohol intake include gamma-glutamyltransferase activity (GGT) and erythrocyte mean corpuscular volume[14]. As the severity of alcohol-related liver injury increases, the bilirubin can increase with a concomitant decrease in GGT[16]. Also, it has been our experience that total blood cholesterol levels < 100 mg/dL can predict poor outcome; the lower the cholesterol, the worse the prognosis. Finally, a mild to very elevated leukocytosis (up to 40 000/mm^3) is very characteristic of AH. While less common, reports of severe leukemoid reactions are readily found with documented peripheral white blood cell counts > 130 000/mm^3[17-20]. In general, a severe leukemoid reaction in AH portents a very poor prognosis[17,19].

Clinicians can be led astray by the presence of both fever and prominent leukocytosis, leading to concern for sepsis and overshadowing the possibility of AH, particularly if the patient's history is unclear or unattainable due to altered mental status. As these patients may be profoundly nutrient deficient and commonly have comorbidities such as cirrhosis, they are generally at increased risk of infection and therefore an evaluation for bacterial infection should be performed[11]. This evaluation should, at a minimum, include a chest X-ray, blood cultures, abdominal paracentesis (ascites is frequently present), and urinalysis with urine culture. More specific testing should be pursued for localizing clinical signs of infection such as excessive sputum production or diarrhea. However, the clinician must also remember that profound leukocytosis can be seen without a concomitant infection, and extensive evaluation and prolonged use of broad spectrum antibiotics can lead to loss of time and resources, but more importantly, can carry specific risks (superinfection with resistant bacteria or fungi) and delay appropriate therapy[17-19].

Liver scanning

In rare instances in which diagnosis is still unclear despite a thorough history, physical examination and laboratory evaluation, a technetium sulfur colloid liver spleen scan can confirm the diagnosis of AH noninvasively[21]. This is perhaps the last remaining indication for this very old imaging modality. However the single photon updated version, otherwise known as the perfused hepatic mass, may have utility in assessing prognosis in parenchymal liver disease.

The dramatic "colloid shift" to the bone marrow and spleen seen in the older version of this scan is characteristic of severe AH and is unusual in other diagnoses. The liver may be nearly invisible on a liver spleen scan and the bone marrow may be so visible that one can count ribs easily.

Liver biopsy

Liver biopsy is rarely needed outside of research protocols or perhaps when the patient and family fabricate a story of total alcohol abstinence when AH is clinically obvious. This conspiracy is common when the patient is pursuing liver transplantation. A histologic diagnosis of AH rules out the possibility of liver transplantation, at least in the Unites States. Because of the high risk of recidivism, organs can not be allocated to patients with a diagnosis of AH. Only patients who survive AH and continue to have liver failure after 6 mo of observed and documented abstinence can be listed for transplant. Patients usually improve so dramatically with several months of abstinence that a transplant is not needed.

The rare biopsy is usually performed transjugularly because of ascites and/or coagulopathy. The most common characteristic finding on pathology is macrovesicular steatosis which can also be seen in nonalcoholic fatty liver disease[22]. Intrahepatic cholestasis can also be seen and requires the clinician to rule out mechanical obstruction of the bile ducts, and evaluate the patient for other causes of cholestasis such as drug toxicity or viral hepatitis. Mallory bodies can be seen in up to 65% of patients with AH but can also be found in other causes of hepatocyte injury and has been described as indicative but not pathognomonic of AH[2]. Giant mitochondria, and in particular Type I megamitochondria, can also provide a diagnostic clue for AH and correlate with the presence and amount of daily alcohol consumption[23,24]. Ultimately however, biopsy must be correlated with the patient's history and will rarely provide all the diagnostic information needed in the absence of other details.

Some inexperienced clinicians may assume that such a biopsy could represent nonalcoholic steatohepatitis (NASH). However, patients with NASH do not present with deep jaundice, ascites, coagulopathy, *etc*. NASH is a much less inflammatory condition. In fact, patients with NASH do not develop jaundice until their advanced cirrhosis is near terminal.

CLINICAL ASSESSMENT AND TREATMENT

Once a diagnosis is made, treatment should be initiated that addresses all aspects of the disease, including alcohol cessation, correction of nutritional deficiencies and initiation of pharmacologic therapy when needed. In fact, a 3-pronged approach can help clinicians formulate a plan to guide therapy from the time of presentation through hospitalization and following into the outpatient setting to help reduce recidivism and prevent recurrence.

Nutrition

The first consideration for the hospitalized patient, after

evaluation and treatment for any signs of alcohol withdrawal, should be nutrition and electrolyte repletion, because AH induces a profound catabolic state. In part because of malnutrition, AH carries a considerably high mortality rate, therefore nutrition remains a key aspect of therapy. Nutrition should be provided orally if the patient is able to eat or via nasojejunal feeding if nausea, vomiting or poor appetite prevent adequate intake of calories. Calorie counting is essential to assure adequate intake as patients require a higher than average caloric intake (approximately 1.2-1.4 times the normal resting intake)[25]. Furthermore, nighttime supplementation of nutrition (approximately 700 kcal/d) may prevent muscle wasting and improve lean muscle mass and should be considered in hospital and beyond if the patient has any evidence of cirrhosis[26]. Furthermore, patients with longstanding alcohol abuse usually require liberal multivitamin, folic acid and thiamine supplementation. Many of these patients are profoundly depleted of potassium due to high aldosterone levels and lack of intake of solid food for weeks to months. Many have been living on a total liquid alcohol diet. Serum potassium levels do not accurately reflect intracellular levels. It may take many days of potassium repletion to finally achieve normokalemia. As these nutritional considerations are being addressed, the next step for the clinician is deciding upon whether pharmacologic therapy and anticipating potential complications of AH.

Ascites

Patients with pure severe AH in the absence of cirrhosis have relatively little problem with ascites. They eat so little that they do not take in enough sodium to retain much fluid. Maintenance intravenous fluids should be avoided to minimize fluid retention. When cirrhosis is also present, they may have more problematic fluid retention. In this case, if blood urea nitrogen and creatinine are normal, spironolactone can be given. This drug will increase urinary excretion of sodium and water, increase serum potassium, and decrease the need for potassium supplementation. Once serum potassium is normal without supplementation, oral furosemide can be added, if needed. If azotemia occurs, diuretics should be stopped and the patient should be evaluated for hepatorenal syndrome. The first step is to give 1 g of 25% albumin/kg body weight (100 g maximum) intravenously daily for 2 d and to monitor creatinine. If creatinine improves with albumin, the azotemia is probably diuretic-induced. If creatinine continues to rise, hepatorenal syndrome is probably present, as this commonly occurs in severe AH (see below).

Variceal hemorrhage

Similar to the situation with ascites, the authors have observed that patients with pure severe AH in the absence of cirrhosis have relatively little problem with upper gut hemorrhage. The relatively short duration of AH usually does not lead to formation of varices that are large enough to bleed. However, patients with underlying cirrhosis can bleed from esophageal varices. Urgent endoscopy with banding of varices is warranted when this occurs. Patients with severe AH are very intolerant of hypotension and seldom survive shock superimposed on AH.

Pharmacologic therapy

Deciding upon appropriate pharmacologic management of patients with AH relies heavily upon assessment of the severity of disease. Mild forms of AH may improve with abstinence and conservative management, while more severe disease is associated with significant mortality and should be treated more aggressively[27]. The Maddrey discriminant function (DF) [4.6 × (prothrombin time (PT) in seconds - control PT) + serum bilirubin (mg/dL)] was first introduced in 1978 in order to aid in the assessment of disease and guide therapy, which at that time relied mostly upon corticosteroid use[28]. A cutoff value of 32 was used to identify patients with a mortality rate above 50% without pharmacologic therapy. The shortcomings of the DF were outlined by Dunn *et al*[10], the most notable of which are the lack of standardized PT measurement techniques and values across different laboratories, and more importantly a relatively high risk of mortality (up to 17%) with DF values under 32. The model for end-stage liver disease (MELD) has been evaluated and compared with DF in predicting mortality and has helped guide initiation of pharmacologic therapy[10,29,30]. A MELD score of 11 has equal sensitivity but higher specificity then DF in predicting 30-d mortality[29]. A MELD score of 20 or higher at the time of admission has the highest sensitivity and specificity for predicting in-hospital mortality and outperformed both DF and Child-Pugh-Turcotte (CPT)[30]. We propose using a MELD score ⩾ 20 or a DF of 32 or higher to prompt initiation of pharmacologic therapy (Figure 1).

There may be a component of malabsorption of vitamin K due to jaundice in addition to poor synthesis of coagulation components by the diseased liver. The International Normalized Ratio regularly decreases after 3 daily doses of 10 mg of vitamin K intravenously or subcutaneously. Oral dosing of vitamin K is not appropriate because of poor absorption in the setting of deep jaundice.

After diagnosing and assessing the severity of AH, the decision of which pharmacologic therapy to initiate has become a major point of debate among experts, but recent publications may help narrow the choices down considerably. A few small trials had suggested that glucocorticoids can improve short-term survival in patients with the severe AH (DF ⩾ 32)[31]. Since then, however, significant work has challenged the efficacy of steroids and raised concerns regarding side effects. The first of these was a meta-analysis by Christensen *et al*[32] in 1995 which did not support the routine use of glucocorticosteroids in these patients. In addition, a 2008 Cochrane review of 15 randomized controlled trials with a total of 721 patients concluded that glucocorticosteroids did not statistically reduce mortality compared with placebo or no intervention. Only when a subset of patients with DF ⩾ 32 or with encephalopathy were evaluated was a mortality benefit seen[33]. In addition to the limited efficacy found by these analyses,

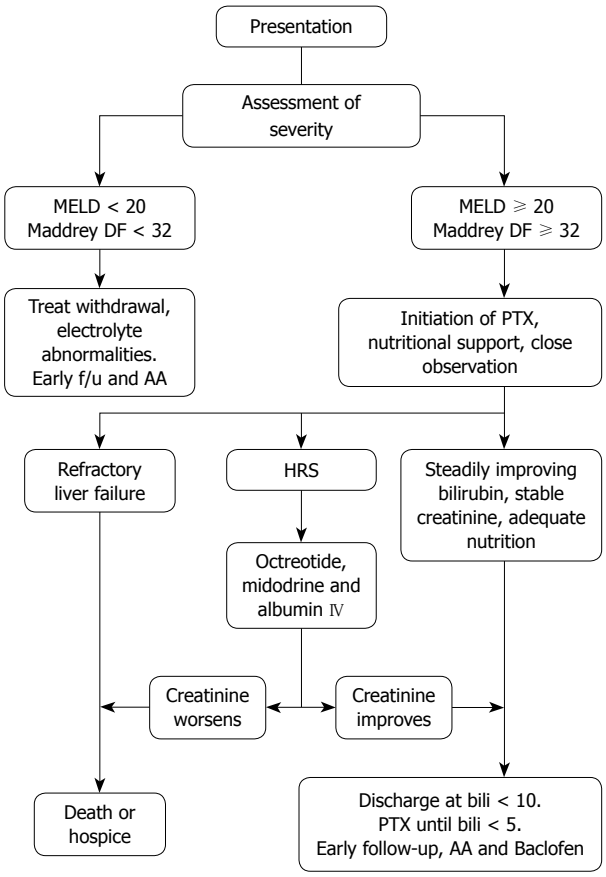

Figure 1 Treatment algorithm for hospitalized patients with alcoholic hepatitis. MELD: Model for end stage liver disease; DF: Discriminant function [4.6 × prothrombin time (PT) in seconds - control PT]; PTX: Pentoxifylline; AA: Alcoholics anonymous; HRS: Hepatorenal syndrome; Bili: Bilirubin (in mg/dL).

there is also a propensity for side effects with even relatively short-term use of steroids. The clinician must take these side effects into consideration. Approximately 16% of patients experience adverse effects, primarily in the form hyperglycemia or Cushing's syndrome, but also with increased risk of infection as compared with only a 4% adverse event rate in control patients [relative risk (RR), 3.63; 95% confidence interval (CI): 1.95-6.76][33].

As there is a lack of strong evidence of a survival benefit and a propensity for adverse events, routine use of glucocorticosteroids is discouraged. In the ongoing obesity epidemic, many patients with severe AH now have frank diabetes or insulin resistance. Corticosteroids can make diabetes overt or convert non-ketosis-prone patients to a ketosis-prone state.

Another serious practical issue regarding corticosteroid treatment is discontinuation of this highly problematic drug. In some of the trials, the drug was stopped abruptly. In others the dose was tapered. Many physicians are reluctant to stop steroid treatment abruptly and the patients may remain on it too long. Many patients with severe AH are in county hospitals or other settings in which they see a different physician in the clinic than in the hospital. The patient may be homeless and not return to the clinic at all.

In the USA these patients are frequently taken to or transferred to an academic hospital but then discharged to a county hospital because of a lack of insurance that is required to return to the academic system. The new clinic physician may not have access to the patient's medical information and may not even know why the patient is on steroids. It has been the authors' experience that too often the drug is continued for more than 30 d because the clinic physician does not know what to do with the steroid dose. Too often it is just continued at the high dose, putting the patient into what the authors call "steroid auto-pilot". The drug may be continued for months in this setting with the dose being adjusted up or down, for no apparent reason, unless someone finds out that it is supposed to be stopped. Then the issue of tapering the dose comes up.

Yet another issue is prednisone *vs* prednisolone for treatment. When steroids are avoided, such a debate is unlikely.

Unless a physician makes a commitment to see the same patient in the clinic and stop the drug when appropriate, it is the authors' opinion that the physician should not start steroid treatment for this condition. The hospitalist movement in the USA has led to different physicians caring for the patient in the hospital *vs* in the clinic and has made continuity and follow-through on a plan of care very difficult.

More recently, trials and reviews of pentoxifylline (PTX) have shown a better risk benefit profile than that of steroids, and point to PTX as a better first-line agent in treatment of severe AH than glucocorticosteroids. The efficacy of PTX in severe AH was demonstrated by Akriviadis *et al*[13] in 2000 with a randomized, placebo-controlled trial showing significant benefit in both short-term survival and in preventing development of hepatorenal syndrome (HRS), a key cause of mortality in AH.

Since then, there has been debate regarding the magnitude of the effect PTX has on mortality, and recent publications have examined the results of the Akriviadis trial along with other available data to clarify this issue. A Cochrane analysis of PTX, published in 2009, performed a detailed analysis of all the combined randomized, controlled trials available at that time. Routine meta-analysis showed reduced mortality (RR, 0.64; 95% CI: 0.46-0.89) and reduced hepatic-related mortality due to HRS (RR, 0.40; 95% CI: 0.22-0.71), and trial sequential analysis found strong support for PTX in lowering serum creatinine, a surrogate marker of HRS. However, the group concluded that there was not a significant effect on mortality based primarily upon trial sequential analysis which included, among other data, an abstract by Lebrec *et al*[34] which was published fully in 2010. This abstract did not demonstrate the same mortality benefit as that seen in the Akriviadis trial and therefore led to doubt regarding overall efficacy. Since that time, however, the full manuscript by Lebrec has been published and reveals that a likely reason for the discrepancy is based upon the significantly different populations used in each study[13,34]. The recent article included only CPT class C patients with cirrhosis while the older study excluded such patients completely. This may explain the lack of a clear cut mortality benefit, as any advanced cirrhosis patient diagnosed with AH

stands to have a predictably poorer outcome than his counterpart without cirrhosis. More important than the differences between the 2 trials, however, is the common finding that PTX therapy correlated significantly with a lower rate of liver-related complications (including HRS and hepatic encephalopathy).

A recent randomized trial comparing PTX to steroids in the setting of severe AH (DF ⩾ 32) also showed significantly lower mortality with PTX (35.29% *vs* 14.71%, $P = 0.04$) and no incidence of HRS[35]. In terms of adverse events with the use of PTX, gastrointestinal upset including diarrhea, vomiting and or epigastric pain, are the primary complaints. Publications vary widely on the reported rate of adverse events, with gastrointestinal complaints ranging from 9.9% to 26.5%, and overall events including headache, skin rash, spontaneous bacterial peritonitis and urinary tract infection reported as 67.3% for PTX *vs* 28.2% in the control group. In our experience of treating many hundreds of patients with PTX, side effects rarely lead to discontinuation of this life-saving drug. These patients have so many digestive symptoms routinely that they do not notice the upset stomach that healthy patients may experience with PTX. No life threatening or severe reactions were reported with PTX and therefore a trial should be attempted. We have treated hundreds of patients with PTX with results similar to the Akriviadis trial.

We therefore recommend PTX as the routine first line treatment of severe AH at a dose of 400 mg orally 3 times daily for a period of at least 4 wk. We usually continue this safe, inexpensive drug until the bilirubin is < 5 mg/dL. Patients are usually discharged from hospital when the bilirubin is approximate 10 mg/dL (see below). The drug is usually stopped in the outpatient setting. This strategy may require up to several months of treatment with the latter component taking place in the clinic.

Another advantage of PTX over steroids is that it can be safely given for months, whereas steroids can not.

Hepatorenal syndrome

Due to the prevalence of HRS in AH patients, the clinician must also be prepared to diagnose and treat this disorder. If the serum creatinine is abnormal or the patient has only minimal fluid overload on admission, it is prudent to withhold diuretics. If diuretics are initiated, it can be confusing as to whether subsequent azotemia is diuretic-induced or HRS. A retrospective review of patients with advanced liver disease and renal failure found that misdiagnosis of HRS occurred in approximately 40% of cases[36]; therefore care must be taken to make an appropriate diagnosis before initiating therapy, and a table paraphrasing the diagnostic criteria set by the International Ascites Club is provided[37] (Table 3). Diagnosis should begin with a careful review of medications, cessation of diuretics and any potentially nephrotoxic drugs, urinalysis and urine electrolytes to give a rapid assessment of underlying nephropathy and acute tubular necrosis. This should be followed by a 24 h urine collection to rule out proteinuria, and Doppler ultrasound of the kidneys to rule out parenchymal disease and obstructive uropathy.

Table 3 International ascites club criteria for hepatorenal syndrome[37]

Cirrhosis with ascites
Serum creatinine > 1.5 mg/dL (> 133 μmol/L)
No improvement in serum creatinine (< 1.5 mg/dL) after at least 2 d with diuretic withdrawal, and volume expansion with intravenous albumin. The recommended dose is 1 g/kg of body weight per day up to a maximum of 100 g/d
Absence of shock
No current or recent treatment with nephrotoxic drugs
Absence of parenchymal kidney disease as indicated by proteinuria > 500 mg/d, microhematuria (> 50 red blood cells per high power field) and/or abnormal renal ultrasonography

After the appropriate evaluation is performed and HRS is diagnosed one should initiate therapy with intravenous albumin infusion of 1 g/kg per day (100 g maximum) for a total of 2 d and pharmacotherapy. A study of octreotide and midodrine used in combination showed significant reduction in mortality (43% *vs* 71%, $P < 0.05$) and sustained reduction in serum creatinine (40% *vs* 10%, $P < 0.05$)[38]. We recommend initiation of octreotide 50 μg/h continuous infusion and midodrine 5 mg orally given every 8 h followed by gradual increases in the dose of midodrine by 2.5 mg increments with each dosing. There is no reason to wait 24 h between dose increases. Time is of the essence in treating this life-threatening complication of severe AH. The goal is to achieve an increase in mean arterial pressure of 15 mmHg or until a systolic blood pressure of 140 mmHg is reached.

Finally, consideration of the use of an anabolic steroid, in particular oxandrolone, can be made. In a study comparing oxandrolone to prednisolone and placebo, oxandrolone was found to improve conditional mortality rates beyond 30 d. This effect was especially pronounced in patients with moderate severity AH[39]. A recent review of oxandrolone use in AH, as well as other catabolic diseases associated with muscle wasting, showed evidence of clinical efficacy and few side effects[40]. Improvements in body composition, muscle strength and function, recovery from acute catabolic injury and nutritional status have been shown in various trials[40].

It has been the authors' policy to add oxandrolone at a dose of 40 mg orally daily for 30 d maximum in the following circumstances: (1) Maddrey score ⩾ 80 on admission, or (2) lack of improvement in Maddrey score or MELD after 10-14 d of PTX. Androgenic steroids could theoretically increase the risk of hepatocellular or prostate carcinoma. Because of this potential risk, physicians who have used oxandrolone extensively for severe AH do not prescribe it for more than 30 d. Oxandrolone seems to improve survival in patients with ultra-severe or refractory AH (Table 4).

ABSTINENCE AND POST-HOSPITALIZATION CARE

The final consideration, for those patients who survive

Table 4 Use of oxandrolone for alcoholic hepatitis

Dose	Oxandrolone 40 mg orally daily
Duration of therapy	30 d maximum
Circumstances for use	Maddrey score ≥ 80 on admission No improvement in Maddrey score or MELD after 10-14 d of pentoxifylline

MELD: Model for end-stage liver disease.

the initial bout of AH, involves establishing a plan for increasing the likelihood of abstinence from alcohol. This usually involves a multidisciplinary approach involving self-help or 12-step programs, some level of psychiatric or behavioral therapy and also pharmacotherapy. The efficacy of self-help programs is well established and usually accessible if not always funded, and we recommend routine referral and strong encouragement of attendance[41].

The role of the clinician in this recovery program has been relatively undefined, primarily as most pharmacologic therapies have been less than efficacious. Furthermore, clinicians may feel that closely monitoring a patient's abstinence may be difficult to confirm without measurable markers.

With regard to pharmacotherapy, baclofen has recently been evaluated in terms of safety and efficacy in the setting of alcoholic cirrhosis. Baclofen significantly reduced alcohol cravings and significantly lengthened time to relapse with no significant adverse effects noted after 12 wk of continuous use in a well run randomized, controlled trial[42]. Notably, patients with CPT class C cirrhosis had the most significant effect from treatment, and may reflect the importance of the patient's commitment to the treatment program. Discharge is considered as the bilirubin level approaches 10 mg/dL, and the clinician can use this to help plan initiation of baclofen.

Many of the early randomized trials of treatment for AH were conducted on a dedicated approximate 100 bed Liver Unit at the University of Southern California. This unit was mostly populated by patients with AH. The founders of this unit, Drs Reynolds and Redeker, determined through 5 decades of experience in treating these patients, that a bilirubin approximate 10 mg/dL was a good marker of stability for discharge. When patients were sent out with higher bilirubin levels, they were regularly rapidly readmitted with further deterioration.

Currently, we are initiating baclofen in the final days of hospitalization, starting at 5 mg orally 3 times daily then increasing to 10 mg on day 3. The authors have had great success in eliminating alcohol craving and eliminating alcohol consumption with baclofen. No side effects have been recognized in this patient population. We are continuing it indefinitely. Some patients who have stopped it have then redeveloped an alcohol craving and requested that it be continued.

With respect to monitoring abstinence, close follow-up with interview may not be adequate and usually requires a committed family member or friend to corroborate abstinence and honestly report recidivism. In addition to, or in place of such an informant, the clinician may find the use of serologic markers useful for monitoring or diagnosing alcoholic recidivism. Carbohydrate-deficient transferrin (CDT) has been approved by the US Food and Drug Administration for identification of heavy alcohol use and is found in high prevalence in alcoholics. Furthermore, CDT is not detectable after approximately 2 wk of abstinence and may help confirm patient reports of abstinence[14]. A second helpful test is measurement of ethyl glucuronide (EtG), a non-volatile, water-soluble, direct metabolite of ethanol which tests positive shortly after consumption and remains positive for up to 80 h after complete alcohol excretion[43]. EtG may play a role in monitoring for recidivism or in drug and alcohol treatment programs that monitor patients more closely.

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REVIEW

Diagnosis and management of angioedema with abdominal involvement: A gastroenterology perspective

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Abstract

Abdominal involvement in angioedema is often a challenge to diagnose. Acute onset abdominal pain is its most common presenting symptom, and misdiagnosis may lead to unnecessary surgical intervention. Familiarity with the types and presentations of angioedema can be invaluable to clinicians as they consider the differential diagnoses of a patient presenting with abdominal pain. Detailed personal and family histories, careful physical examination of the patient, combined with knowledge of angioedema types, can help clinicians perform their diagnostic evaluation. An accurate diagnosis is essential in order to provide appropriate treatment to patients with angioedema. Depending upon the diagnosis, treatment may be the avoidance of provoking factors (such as allergens or medications), inhibiting histamine-provoked reactions, or treating C1 esterase inhibitor deficiency.

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Key words: Acquired angioedema; Angiotensin-converting enzyme-induced angioedema; Gastrointestinal; Hereditary angioedema; C1 esterase inhibitor deficiency

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INTRODUCTION

About 10% to 20% of people worldwide will develop an episode of angioedema or urticaria at some point in their lifetime, women being more prone than men[1]. Angioedema is characterized by localized temporary swelling, which can affect all layers of the skin or of the walls of hollow viscera, such as the oropharynx, respiratory system, or the gastrointestinal (GI) tract. Peripheral non-pitting edema is typical of cutaneous manifestations. However, the effects of visceral angioedema are variable, ranging from life-threatening episodes when the respiratory system (larynx) is involved, to pain of varied severity associated with nausea or vomiting, when abdominal viscera such as the intestines are involved[2].

Abdominal pain associated with angioedema may manifest as severe acute onset abdominal pain, or as chronic recurrent abdominal pain of moderate severity. The abdominal pain is described as cramping or colicky and is rated as severe to excruciating in 87% of patients[3]. Vomiting and diarrhea occur in 78% and 65%, respectively, of patients with abdominal symptoms[3].

Patients with these symptoms typically present to one of four medical specialists, namely emergency room physicians, primary care physicians (internists, pediatricians, family medicine), general surgeons, and gastroenterologists. Physicians in each of these specialties should be familiar with the signs and symptoms of cutaneous and visceral angioedema, and be able to order appropriate tests to investigate this group of differential diagnoses. This brief review provides a perspective of angioedema diagnoses and management, including new therapeutic options, for practicing gastroenterologists.

Table 1 Molecular mechanisms of angioedema[2,4,5]

Type of AE	Mediator	Mechanism
Allergic AE	Histamine (mast cells)	Allergens react with IgE antibodies on the surface of mast cells, causing degranulation and release of histamine
ACE-I-induced	Bradykinin	ACE-Is prevent the conversion of bradykinin to inactive metabolites, leading to bradykinin accumulation
NSAID-induced AE	Leukotrienes (mast cells)	Inhibition of COX-1 leads to overproduction of vasoactive substances by shunting arachidonic acid metabolism through the lipoxygenase pathway, creating leukotrienes. Vasoactive leukotrienes act on cell-surface receptors to increase vascular permeability and promote inflammation
HAE type 1	Bradykinin	Genetic mutations in the *C1 INH* gene result in low levels of C1 INH. Major roles of C1 INH include inactivating coagulation factors XIIa, XIIf and XIa; blocking C1 complement autoactivation; and inhibiting activated kallikrein. Removal of these inhibitory actions results in complement activation and elevated bradykinin levels
HAE type 2	Bradykinin	Genetic mutations in the *C1 INH* gene result in normal levels of C1 INH, but the C1 INH is dysfunctional. Plasma cascades are unregulated in the presence of dysfunctional C1 INH, leading to bradykinin accumulation as in HAE type 1
Inherited AE with normal C1 INH	Bradykinin	Missense mutation in factor XII gene confers a significant increase in the protease activity of each activated factor XII molecule, which increases bradykinin generation. Decreased activity of enzymes such as ACE and aminopeptidase P have also been noted
Acquired AE	Bradykinin	Type 1: Immune complex formation associated with rheumatologic, lymphoproliferative, and neoplastic disorders continuously activate C1, causing C1 INH depletion and bradykinin accumulation Type 2: Autoantibodies inactivate C1 INH, leading to bradykinin accumulation
Idiopathic recurrent AE	Unknown	Unknown

AE: Adverse events; ACE-I: Angiotensin converting enzyme inhibitor; HAE: Hereditary angioedema; COX-1: Cyclooxygenase-1; NSAID: Non-steroidal anti-inflammatory drug; C1 INH: C1 esterase inhibitor.

TYPES OF ANGIOEDEMA AND THEIR CHARACTERISTICS

Classification of angioedema types is based on their etiology or pathophysiology. Broadly, these include allergic angioedema, angiotensin converting enzyme inhibitor (ACE-I)-mediated angioedema, non-steroidal anti-inflammatory drug (NSAID)-mediated angioedema, hereditary angioedema (HAE), inherited angioedema with normal C1 esterase inhibitor (formerly called HAE type 3), and acquired C1 esterase inhibitor deficiency angioedema [acquired angioedema (AA)]. Angioedema can result from mast cell degranulation with massive histamine release or from increased accumulation of bradykinin either *via* increased production or decreased inactivation (Table 1).

Allergic angioedema is caused by reaction to foods (such as shellfish, nuts, some fruits), medications, insect bites, latex, or other environmental allergens, and results from IgE-mediated mast cell degranulation, with resultant histamine release that causes local tissue swelling[2]. Sensitization, through prior exposure to the allergen, is usual. Swelling in allergic angioedema can occur throughout the body and is typically associated with urticaria and pruritus. Ingested allergens may cause angioedema symptoms that include abdominal pain and vomiting. Most episodes of allergic angioedema resolve 1 to 3 d after ceasing contact with the allergen[2].

A variety of medications can induce a non-IgE-mediated form of angioedema, including ACE-Is, NSAIDs, and rarely, angiotensin-2-receptor blockers (ARBs).

ACE-I-induced angioedema occurs in 0.1% to 2.2% of patients receiving these drugs[6,7]. It manifests within the first month of treatment in one quarter of patients taking ACE-Is, but delay of onset as long as 10 years has been reported[8]. Bradykinin is converted by ACE into inactive metabolites. Thus, ACE-Is inhibit the degradation of bradykinin, causing it to accumulate[9]. This accumulation causes angioedema *via* bradykinin-induced vasodilation, increased capillary permeability, and plasma extravasation[8,10]. ACE-I-induced angioedema primarily affects the head and neck (especially the lips and tongue), and is more common in women and people of African descent[8]. However, there have been case reports of abdominal visceral involvement with ACE-I-induced angioedema presenting with abdominal pain as the only symptom[11]. ACE-Is should always be considered in the differential diagnosis of unexplained abdominal pain. Although switching patients to ARBs is safe in most patients with ACE-I-induced angioedema, continued bouts of angioedema have been reported in some patients after switching to ARBs[12]. Observational data suggest that the combined use of ACE-I and ARBs may be more likely to result in angioedema[13].

NSAID-induced angioedema is present in 0.1% to 0.3% of patients receiving NSAIDs[14,15]. This is a class-specific reaction mediated by inhibition of cyclooxygenase (COX)-1, which results in the over-production of a variety of vasoactive substances, including cysteinyl leukotrienes. It is often characterized by periorbital swelling and occurs in combination with respiratory symptoms in one third of patients[14]. Observational data suggest that the combined use of ACE-Is and NSAIDs may also be more likely to result in angioedema adverse effects[13].

HAE occurs in 1:10 000 to 1:50 000 people and results from mutations in the C1 esterase inhibitor (*C1 INH)* gene[16]. Type 1 HAE is caused by a deficiency in the amount of functional C1 INH produced, while type 2 HAE is characterized by dysfunctional C1 INH. Although primarily inherited in an autosomal dominant manner, HAE appears *de novo* in one quarter of patients

due to new mutations. C1 INH plays an important role in complement, contact, and fibrinolytic pathways, which have been described in other literature[17]. The end result of quantitative or functional C1 INH deficiency is massive bradykinin release, which is thought to mediate many symptoms of HAE and AA[17]. Bradykinin causes edema, ascites, and swelling *via* increasing vascular permeability; congestion, hypotension, and erythema due to vasodilation; and cramps, spasms, and pain due to contraction of nonvascular smooth muscle[4].

HAE can manifest anywhere in the body, including the head and neck, extremities, GI tract, genitals, trunk, and larynx, and shows wide variability in presentation within patients and families[18,19]. Up to 80% of patients with HAE have recurrent abdominal pain, while half will have a potentially life-threatening laryngeal attack[19-22]. Such abdominal pain symptoms may occur for many years without concomitant cutaneous or respiratory symptoms[23]. HAE-mediated abdominal pain can be mistaken for other causes of abdominal pain, such as acute appendicitis[16]. Attacks are frequently accompanied by a prodromal phase. In the case of GI manifestations, nonspecific complaints of irritability, aggressiveness, fatigue, or hunger may precede an attack[3]. Intestinal wall swelling (thickening on imaging studies), ascites, and rarely, hypovolemic shock, occur due to massive fluid accumulation in the intestinal wall and lumen, and in the peritoneal cavity[23]. Attacks typically resolve over 2 to 5 d, and may be triggered by trauma, stress, medical procedures (e.g. instrumentation or surgery), estrogens, and certain medications (e.g. ACE-Is)[4].

Inherited angioedema with normal C1 INH is a rare disorder caused by a mutation in the coagulation factor Ⅻ gene, which in turn leads to increased gene expression, and consequently increased levels of bradykinin[24]. It is clinically indistinguishable from HAE, is thought to be autosomal dominant, and although occurring predominantly in women, has also been reported in men[25]. Similar to HAE, estrogen-containing birth control pills, estrogen-replacement therapy, and pregnancy may precipitate or worsen symptoms[25,26].

AA is a rare syndrome that occurs as a result of increased catabolism of C1 INH and overactivation of the classical complement pathway[27]. Although the clinical picture is identical to HAE, the underlying immunologic disturbance is not hereditary in nature, and precise mechanisms remain unclear. AA has been associated with lymphoproliferative/neoplastic disorders (type 1 AA) or autoimmunity (type 2 AA). Symptoms often localize to the abdomen and upper respiratory tract, as well as skin. Abdominal symptoms of angioedema have been described previously. Symptoms typically resolve over 2 d.

Idiopathic recurrent angioedema refers to swelling episodes that occur at least three times within a 6 to 12 mo period, have no identifiable cause, and are typically recalcitrant to treatment[1]. The mechanism is unknown, but autoimmune processes have been implicated. In most cases, swelling is accompanied by urticaria. When urticaria is present, it is accompanied by pruritus. Swelling can last from hours to days. Abdominal symptoms include pain, nausea, and vomiting.

MOLECULAR MECHANISMS AND PATHOPHYSIOLOGY OF ANGIOEDEMA TYPES

Different cells, and different cell components, play roles in the prevention or causation of various types of angioedema.

Mast cells, with cell surface IgE receptors, mediate allergic angioedema when an allergen interacts with the Fab fragment of the IgE molecule (Figure 1). Such allergen-Fab interaction results in intracellular signaling, which causes degranulation of the mast cells with release of histamine and leukotrienes, thereby resulting in angioedema symptoms.

HAE and AA occur due to the loss of inhibitory control of the contact/fibrinolytic pathway and the classical complement pathway caused by low levels or subnormal activity of C1 INH (Figure 2). Unlike allergic angioedema, these events occur in the extracellular milieu. C1 esterase inhibitor is synthesized mainly by hepatocytes and, to a lesser extent, by circulating blood monocytes. In AA, C1 INH is catabolized at a rate which is faster than the rate of synthesis, thus resulting in attacks of angioedema. HAE is caused by genetic mutations which result either in significantly low production of normal C1 INH (type 1), or production of normal or elevated quantities of a dysfunctional C1 INH which is unable to bind the usual substrates and thus is unable to inhibit activation of the contact/fibrinolytic and classical pathways (type 2)[4].

Angiotensin converting enzyme (ACE) is also known as kininase Ⅱ, because one of its main roles is to metabolize bradykinin into inactive metabolites. When ACE activity is inhibited, another enzyme - aminopeptidase P - metabolizes bradykinin, thus preventing excess accumulation. ACE-I-induced angioedema results from accumulation of bradykinin after inhibition of ACE by ACE-I drugs, and appears to occur more often among individuals who have subnormal activity of aminopeptidase P. Such subnormal aminopeptidase P activity may be genetic (caused by a mutation), or acquired (caused by another drug the patient is taking)[28].

Inherited angioedema with normal C1 INH results from missense mutations in the factor Ⅻ gene, which confer a significant increase in the protease activity of transcribed factor Ⅻ, resulting in increased bradykinin production[29]. Some of these patients also appear to have concomitant mutations in the ACE gene, which result in production of ACE with reduced ability to degrade bradykinin[30]. Angioedema attacks are therefore more common in patients with these mutations due to increased production, and decreased metabolism, of bradykinin.

NSAID-induced angioedema results from the inhibition of COX-1, which results in the channeling of larger quantities of arachidonic acid into the lipoxygenase pathway, resulting in the increased production of vasoactive leukotrienes (Figure 3).

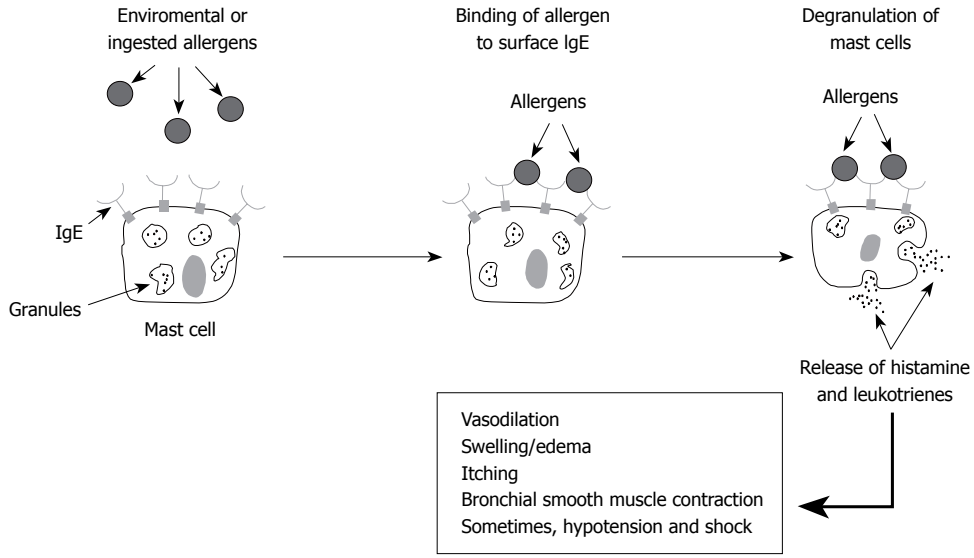

Figure 1 Allergic angioedema pathway.

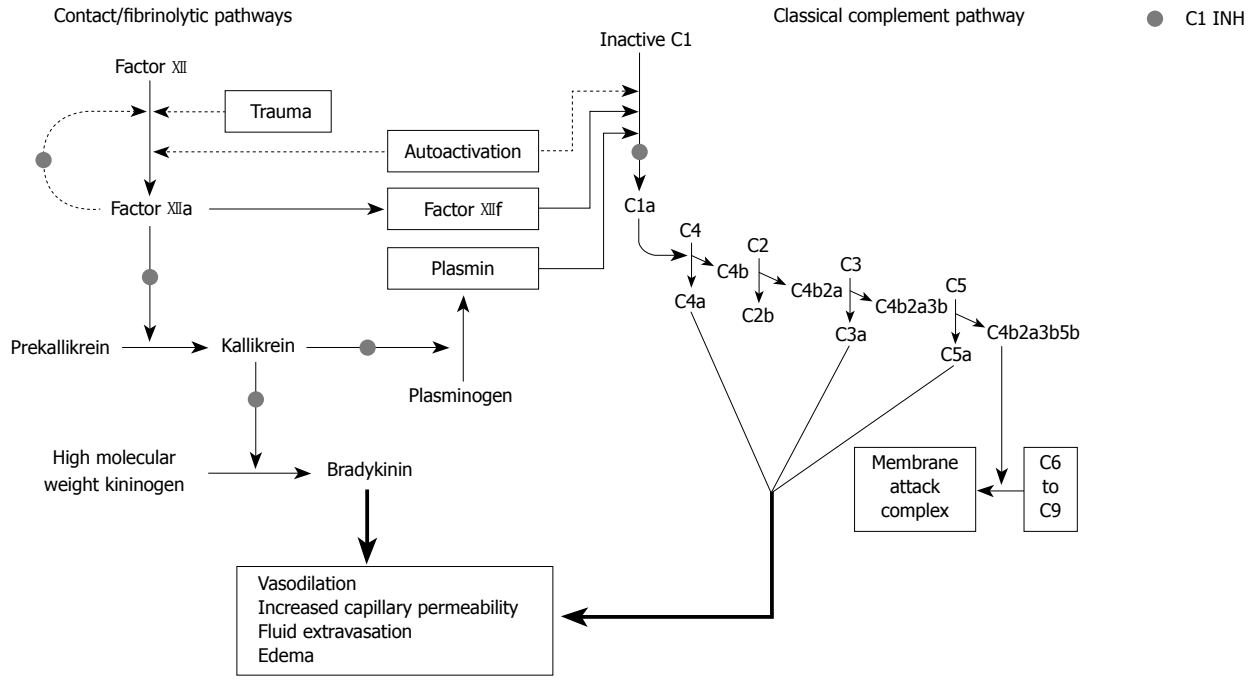

Figure 2 Pathways Involved in hereditary angioedema and acquired angioedema. C1 INH: C1 esterase inhibitor.

DIAGNOSIS

For the practicing gastroenterologist, several patients with abdominal involvement by angioedema will present as referrals for evaluation at a time when the acute episode has subsided, or during a subacute episode of abdominal pain. In such cases, identification of angioedema triggers (e.g. medications, allergens, trauma, or disease) and careful consideration of patient and family history may provide clues. The absence of a family or prior personal history of angioedema should not preclude consideration of this diagnosis.

A thorough history of patient illness, family history, and review of current and recent medications will raise suspicion of the diagnosis in appropriate circumstances. Physical examination, cross-sectional imaging studies of the abdomen, and relevant laboratory tests will often confirm the diagnosis during the acute episode. The physical examination may reveal characteristics of cutaneous angioedema (urticaria, pruritis, cutaneous swelling). For episodes with abdominal involvement, palpation may reveal diffuse abdominal tenderness, with or without rebound[23]. Bowel sounds may be hypoactive or hyperactive[20]. Shifting dullness may be present.

Contrast-enhanced abdominal computed tomography (CT) scan may show intestinal wall and mucosal thickening consistent with edema, fluid accumulation in dilated small or large bowel loops, and ascites[23]. Plain

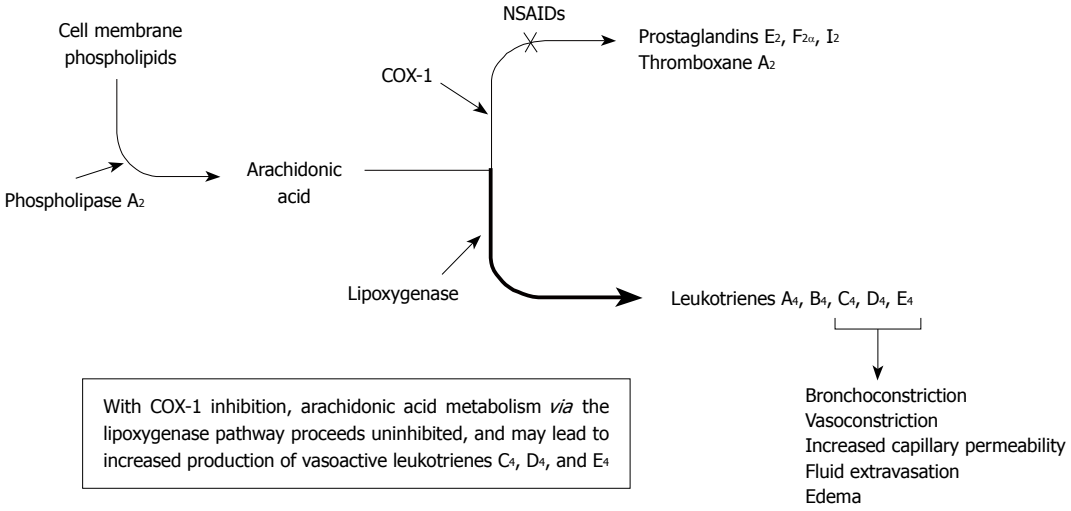

Figure 3 Non-steroidal anti-inflammatory drug-induced angioedema pathway. NSAIDs: Non-steroidal anti-inflammatory drugs; COX-1: Cyclooxygenase-1.

Table 2 Distinguishing features of angioedemas[8,27,28,33]

	HAE	Inherited	AA	Idiopathic	ACE-I	NSAID	Allergic
Location	Anywhere	Anywhere	Anywhere	Especially lips and face	Especially lips, tongue, intestines	Especially face	Anywhere
Urticaria	No	No	No	Usually	Rare	Usually	Usually
Family history	Yes	Yes	No	No	No	No	No
Age of onset	6-20 yr	6-20 yr	> 40 yr	Any age	Any age	Any age	Any age
Trauma as trigger	Yes	Yes	Yes	No	No	No	No
C1q	Normal	Normal	Low (type 1) Low/normal (type 2)	Normal	Normal	Normal	Normal
C1 INH levels	Low (type 1) Normal (type 2)	Normal	Low (type 1) Low/normal (type 2)	Normal	Normal	Normal	Normal
C1 INH function	Low (type 1) Low (type 2)	Normal	Low	Normal	Normal	Normal	Normal
C4	Low	Normal	Low	Normal	Normal	Normal	Normal
C3	Normal	Normal	Low/normal	Normal	Normal	Normal	Normal

HAE: Hereditary angioedema; AA: acquired angioedema; ACE-I: Angiotensin converting enzyme inhibitor; NSAID: Non-steroidal anti-inflammatory drug; C1 INH: C1 esterase inhibitor.

abdominal X-ray may show various degrees of obstruction with or without air-fluid levels, thumb printing, and dilated intestinal loops. Abdominal ultrasonography may detect ascites and edematous viscera. These findings are visible only during acute angioedema attacks and are fully reversible.

Endoscopy of the GI tract or oropharynx is not recommended for patients with suspected HAE due to the risk of inducing a potentially life-threatening laryngeal attack. In cases where endoscopy must be used for additional clinical reasons, prophylactic measures to protect against the possibility of laryngeal swelling should be initiated. Diffuse mucosal edema and erythema, and bulging masses of gastric mucosa (resembling a submucosal tumor), have been reported upon upper GI endoscopy in patients with HAE and abdominal attacks[31].

Laboratory testing should be conducted for patients suspected of angioedema to aid in differential diagnosis. Elevated serum tryptase and urine histamine levels can detect IgE-mediated angioedema, and allergy testing can be used to help identify the source of the offending allergen[32]. Assessment of complement markers provides useful information for helping delineate between acquired and hereditary forms of angioedema. Patient characteristics and test results can help differentiate between types of angioedema, as shown in Table 2.

MANAGEMENT

The various types of angioedema have symptoms that overlap with several other acute and subacute conditions, and as noted above, can occasionally present atypically. As such, the diagnosis may be obscured. Isolated abdominal pain from angioedema, without associated skin or respiratory system symptoms, may lead to wrong diagnoses or unnecessary interventions. In one study, misdiagnosis of abdominal symptoms led to unnecessary appendectomy, laparotomy, or both, in 35% of patients[20]. Such inappropriate treatment may be attributed to the multiple interpretations that can be made of GI findings. For example,

Table 3 Clinical studies of agents used in the treatment of hereditary angioedema

	Trial design	Primary efficacy outcome result	AE	Other safety notes
Routine prophylaxis				
CINRYZE[34] C1 inhibitor (human) (1000 units every 3-4 d for 12 wk) Ⅳ	Randomized, double-blind, placebo-controlled, cross-over study (n = 24)	Decreased the number of attacks (mean 12.7 for placebo *vs* 6.1, P < 0.0001)	Sinusitis, rash, headache, upper respiratory tract infection	Precautions: hypersensitivity reactions, thrombotic events, and risk of transmission of infectious agents
DANOCRINE[35,36] Danazol (range, 40-1000 mg, mean: 171.2 mg/d) oral route	Retrospective (n = 118)	Decreased the number of attacks (33.3 per year when untreated *vs* 9.7 per year when treated)	Clinical: weight gain, menstrual irregularities, virilization in women, headache Laboratory: elevated liver enzymes, elevated cholesterol	8 patients with long-term therapy had serious adverse events (i.e. myocardial infarction, stroke, deep vein thrombosis, acute pancreatitis, hepatocellular adenoma) Warnings: use in pregnancy is contraindicated. Thrombotic events, peliosis hepatis, benign hepatic adenoma, and intracranial hypertension have been reported
Acute attacks				
BERINERT[37] C1 esterase inhibitor (human) (10 or 20 units/kg body weight) Ⅳ	Randomized, double-blind, placebo-controlled study (n = 124)	20 mg/kg dose: reduction in time to onset of symptom relief (> 4 h for placebo *vs* 50 min, P = 0.0016)	Headache, nausea, abdominal pain, dysgeusia, vomiting	Treatment-emergent AEs: laryngeal edema, facial attack with laryngeal edema, swelling (shoulder and chest) exacerbation of HAE, and laryngospasm Precautions: hypersensitivity reactions, thrombotic events, and risk of transmission of infectious agents
FIRAZYR[38,39] Icatibant (30 mg) SQ	Randomized, double-blind, comparator-group study [n = 56 (placebo comparator study)] [n = 74 (tranexamic acid comparator study)]	Decreased time to onset of symptom relief (4.6 h placebo *vs* 2.5 h, P = 0.142; 12 h tranexamic acid *vs* 2 h, P < 0.001)	Injection site reactions; common events include recurrent attacks, nausea, abdominal pain, headache, asthenia, rash	Precautions: caution should be used in patients with acute ischemic heart disease or unstable angina pectoris, and in patients in the weeks following a stroke
KALBITOR[40] Ecallantide (30 mg) SQ	Randomized, double-blind, placebo-controlled trial (n = 96)	Decreased symptom severity measured by Mean Symptom Complex Severity scores (-0.4 placebo *vs* -0.8, P = 0.010)	Headache, nausea, diarrhea, pyrexia, injection site reactions, nasopharyngitis	Warnings: hypersensitivity reactions, including anaphylaxis
KALBITOR[40] Ecallantide (30 mg) SQ	Randomized, double-blind, placebo-controlled trial (n = 72)	Improved symptom response to treatment measured by Treatment Outcome Scores (36 placebo *vs* 63, P = 0.045)		

AE: Adverse events; HAE: Hereditary angioedema; SQ: By subcutaneous route.

abdominal pain with intestinal wall edema on CT scan can be misinterpreted as ischemia and result in unnecessary laparotomy. Abdominal pain with intestinal obstruction in cases of severe edema may be interpreted as an "acute abdomen", resulting in unnecessary laparotomy. Abdominal pain with gallbladder distension may be interpreted as biliary colic, and result in cholecystectomy. Abdominal pain with transient ascites on CT scan or ultrasonography may misdirect the focus to the liver as the cause of abdominal symptoms. Surgical exploration of abdominal symptoms should be avoided in the absence of signs of acute abdomen (i.e. absence of fever, leukocytosis, or peritoneal signs; presence of bowel sounds)[23]. Alternate diagnoses for recurrent abdominal symptoms should be considered to avoid unnecessary medical procedures.

Without intervention, angioedema attacks typically last from 1 to 5 d, depending on the underlying cause. However, acute treatment is often necessary to prevent a fatal outcome when the respiratory system is involved, or to alleviate pain when the abdominal viscera are involved.

Acute treatment of angioedema varies by type. Airway integrity is the first priority. Establishing intravenous access early and initiating appropriate intravenous hydration is also essential. For acute allergic angioedema, adrenaline by the intramuscular or subcutaneous route, and intravenous diphenhydramine, will help reduce edema. Hydrocortisone or methylprednisolone can reduce the risk of relapse[32]. Allergen avoidance is the best prophylaxis for allergic angioedema[32]. The same principles are true for acute treatment of medication-induced angioedema. Clinicians need to consider the short-term risk of relapse despite the discontinuation of the culprit medication. Hospital admission may be necessary to ensure close observation following a medication-induced attack.

Table 3 reviews clinical trial data of agents for the treatment of patients with HAE. For HAE, C1 INH concentrate has shown efficacy in aborting acute attacks of angioedema and has been used in this capacity in Europe for over 20 years. In the emergency room or critical care setting, administration of C1 INH may be a necessary ur-

gent intervention in a patient with severe oropharyngeal, respiratory or abdominal symptoms who has a known diagnosis of HAE. Antihistamines, glucocorticoids or epinephrine typically do not improve acute HAE exacerbations[5]. Fresh frozen plasma (FFP) has also shown efficacy in aborting acute attacks of HAE, with only mild and transient adverse events[41]. However, because FFP contains contact-system proteins that could contribute to increased bradykinin production, there is a small risk of exacerbating an attack[18]. Although long available in Europe, the plasma-derived C1 INH, BERINERT® P [C1 esterase inhibitor (human); CSL Behring GmbH, Marburg, Germany] was approved by the United States Food and Drug Administration (FDA) in October 2009 [under the name BERINERT®, C1 esterase inhibitor (human); CSL Behring LLC, Kankakee, IL] for the treatment of acute abdominal and facial attacks of HAE in adolescents and adults in the United States[37]. Patients who received the recommended dose experienced a significantly shorter time to symptom relief compared with patients given placebo. Common potential side effects of BERINERT include subsequent HAE attack, headache, abdominal pain, nausea, muscle spasms, pain, diarrhea, and vomiting[37]. Rarely, a paradoxical increase in severity of pain associated with HAE may occur with BERINERT treatment[37].

Two agents that work using different mechanisms have recently become available for treating acute attacks of HAE. Icatibant (FIRAZYR®; Jerini AG, Berlin, Germany) is a bradykinin-2 receptor antagonist that was approved by the European Commission in July 2008 for the treatment of acute attacks of HAE in adults[38]. In December 2009, the kallikrein inhibitor, ecallantide (KALBITOR®; Dyax Corporation, Cambridge, MA), was approved by the FDA for the treatment of acute attacks of HAE in patients aged 16 and older[40]. A black box warning notes that ecallantide should only be administered by a healthcare professional with appropriate medical support due to a risk of anaphylaxis after administration of ecallantide.

In Europe, the C1 esterase inhibitor, CETOR® (Sanquin; Amsterdam, Netherlands) is approved for the treatment of acute attacks of AA[42]. No treatments are approved for acute attacks of AA in the United States. Acute treatment has typically paralleled treatment of acute attacks of HAE, with the use of FFP or C1 INH concentrate. Although patients with AA generally require higher doses of C1 INH, treatment of the underlying malignancy or lymphoproliferative disorder will best prevent recurrent symptoms and laboratory abnormalities. Additionally, plasmapheresis is sometimes necessary to decrease autoantibody levels in AA type 2.

ATTACK PROPHYLAXIS

Although patient safety and symptom resolution are the primary goals of acute angioedema treatment, routine and pre-procedure prophylaxis focus on preventing acute events and are particularly appropriate in HAE, AA, and idiopathic recurrent angioedema. For patients with idiopathic recurrent angioedema, routine prophylaxis includes avoidance of provoking factors and low sedation antihistamines, supplemented as necessary with sedating antihistamines at night[1].

In HAE and AA, pre-procedure prophylaxis increases the quantity of circulating C1 INH prior to invasive medical or surgical procedures, so as to reduce the risk of developing life-threatening acute angioedema during the peri-operative period. Thus, an accurate diagnosis of angioedema type is central to developing an appropriate long-term management strategy. Once a gastroenterologist has reached a diagnosis of HAE or AA, a physician specializing in allergy and immunology should be consulted to guide further management.

Attenuated androgens (e.g. danazol and stanozolol), C1 INH, and the antifibrinolytic, tranexamic acid, are used in Europe for prophylaxis[43]. In the United States, the nanofiltered C1 INH concentrate, CINRYZE™ [C1 esterase inhibitor (human); ViroPharma Incorporated, Exton, PA; approved by the FDA in October 2008 for intravenous administration] and oral attenuated androgens (e.g. danazol), are the only FDA-approved treatments for routine prophylaxis of HAE attacks[34,35]. Antifibrinolytics (e.g. tranexamic acid) have also been shown to be effective. However, the nanofiltered C1 INH concentrate has become the preferred therapy for attack prophylaxis due to the significant risk for adverse events associated with attenuated androgens and antifibrinolytics[5]. Biweekly administration of CINRYZE (every 3-4 d) has been shown to reduce angioedema attack rate, attack severity, and time to symptom resolution in patients with HAE[34]. Common potential side effects include upper respiratory infections, sinusitis, skin rash, and headache[34]. CINRYZE is approved for patient self-administration.

Prior to any surgical or dental procedure, or any invasive procedure associated with tissue trauma, patients with HAE should receive prophylaxis with either C1 INH concentrate 1 h prior to surgery with subsequent doses available, or oral attenuated androgens starting 5 d before and 2 d after the procedure, or FFP at least 1 h before surgery[16,28]. These measures will help prevent an acute HAE attack during the peri-operative period.

Since C1 INH is purified from human plasma, there exists a theoretical risk of transmission of viral infections or Creutzfeldt-Jakob agent[34,37]. To reduce the risk of viral transmission, both C1 INH products referenced in this paper start with plasma donor screening for human immunodeficiency virus, hepatitis B, and hepatitis C viruses. Further steps within the manufacturing process are designed to reduce the risk of viral transmission, with both products ultimately using different processes to this end. For CINRYZE, no transmission of disease has been reported[34]. For BERINERT, a few suspected cases of viral transmission have been reported[37]. C1 INH has been associated with a risk of thrombosis if used off-label at high doses[34,37]. Severe hypersensitivity reactions may rarely occur, and since such reactions are clinically indistinguishable from an acute HAE attack, epinephrine injection should

Table 4 Food and Drug Administration approved drugs for prophylaxis and treatment of hereditary angioedema attacks[18,34,35,37,38,40]

Drug	FDA approved indication	Usual adult dose	Range
Cinryze	Prophylaxis	1000 units IV	Every 3rd or 4th day
Danazol	Prophylaxis	200 mg/d	100 mg every 3rd day to 600 mg/d
Berinert	Acute attacks	20 units/kg body weight IV	Per attack
Icatibant	Acute attacks	30 mg SQ	Per attack
Ecallantide	Acute attacks	30 mg SQ	Per attack

FDA: Food and Drug Administration.

be available at all times during administration of C1 INH, for use if necessary[34,37]. Table 4 summarizes the FDA approved drugs for prophylaxis and treatment of HAE.

CONCLUSION

Patients experiencing angioedema with abdominal involvement often present to the gastroenterologist with a history of recurrent episodes of abdominal pain. A careful history and physical examination, imaging studies, laboratory assessments, and awareness of angioedema types can help the clinician order appropriate tests to explore the differential diagnosis. Suspicion may be raised from the patient or family history, but the physical examination, imaging studies, and laboratory tests may confirm a diagnosis. Several reports have shown that abdominal pain can sometimes be the only manifestation of HAE or drug-induced angioedema. Therefore, these differential diagnoses need to be considered even in the absence of a history of prior cutaneous, oropharyngeal or respiratory symptoms, because recurrent abdominal symptoms may predate these other presentations by several years. Once a correct diagnosis has been made, appropriate treatments can be considered.

It is this author's opinion that patients receiving ACE-Is who present with unexplained recurrent or chronic abdominal pain should be tested for HAE. However, since these drugs can have abdominal pain adverse effects independent of a diagnosis of HAE, *via* a pathway leading to excessive bradykinin concentration, negative tests for HAE should not completely remove these drugs from consideration as a cause of the patient's symptoms. Furthermore, the absence of classical intestinal thickening on imaging studies such as CT scan should not automatically absolve this drug class of blame in cases of unexplained abdominal pain, without a brief trial of drug withdrawal to see if symptoms improve.

If a diagnosis of HAE is made, appropriate immediate and short-term treatment with one of the approved agents should be undertaken. In addition, a plan for long-term management should be discussed with the patient and their family. Appropriate testing of family members, to identify those at risk, should be offered.

A multi-disciplinary approach to management of angioedema is necessary. A specialist in allergy and immunology should be engaged to guide additional evaluation and to provide the patient with long-term management.

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ORIGINAL ARTICLE

Anti-inflammatory effects of *Mangifera indica* L. extract in a model of colitis

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Abstract

AIM: To investigate the effect of aqueous extract from *Mangifera indica* L. (MIE) on dextran sulfate sodium (DSS)-induced colitis in rats.

METHODS: MIE (150 mg/kg) was administered in two different protocols: (1) rectally, over 7 d at the same time as DSS administration; and (2) once daily over 14 d (by oral gavage, 7 d before starting DSS, and rectally for 7 d during DSS administration). General observations of clinical signs were performed. Anti-inflammatory activity of MIE was assessed by myeloperoxidase (MPO) activity. Colonic lipid peroxidation was determined by measuring the levels of thiobarbituric acid reactive substances (TBARS). Reduced glutathione (GSH) levels, expression of inflammatory related mediators [inducible isoforms of nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2, respectively] and cytokines [tumor necrosis factor (TNF)-α and TNF receptors 1 and 2] in colonic tissue were also assessed. Interleukin (IL)-6 and TNF-α serum levels were also measured.

RESULTS: The results demonstrated that MIE has anti-inflammatory properties by improvement of clinical signs, reduction of ulceration and reduced MPO activity when administered before DSS. In addition, administration of MIE for 14 d resulted in an increase in GSH and reduction of TBARS levels and iNOS, COX-2, TNF-α and TNF R-2 expression in colonic tissue, and a decrease in IL-6 and TNF-α serum levels.

CONCLUSION: MIE has anti-inflammatory activity in a DSS-induced rat colitis model and preventive administration (prior to DSS) seems to be a more effective protocol.

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Key words: Oxidative stress; Ulcerative colitis; Inflammation; Polyphenols; *Mangifera indica*; Antioxidants

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INTRODUCTION

Ulcerative colitis (UC) is a chronic, idiopathic inflammatory bowel disease that is characterized by bloody diarrhea, colonic mucosal ulceration and, in severe cases, systemic symptoms. An abnormal immune response against antigens of the colonic microbiota in genetically predisposed individuals is suggested to be involved in the etiology of UC[1]. Several authors have proposed that such intestinal conditions are mediated by the activation of lymphocytes and non-lymphoid cells such as macrophages and neutrophils. Once a large number of neutrophils and macrophages are activated, these cells enter the injured mucosa of the large intestine, which leads to over-production of oxygen free radicals that can cause injury to target cells in inflamed tissue[2]. Many animal models have been designed to study pathogenic events during colitis development. The symptoms and colonic histopathology of the rodent colitis model induced by dextran sulfate sodium (DSS) salt resemble more human UC than other chemically induced colitis, and has become a research model for the pathogenesis of UC and for the development of new drugs[3].

In spite of several pharmacological treatments for UC, new therapies must be developed to increase the number and duration of remissions. In this regard, traditional medicine worldwide is nowadays being re-evaluated by extensive research on different plants and their therapeutic principles. Many plants produce antioxidant compounds to control the oxidative stress caused by sunbeams and oxygen, and represent a source of new compounds with antioxidant activity[4]. An aqueous stem bark extract from *Mangifera indica* (*M. indica*) L. (Anacardiaceae family) has been traditionally used as a nutritional supplement. The composition is a defined mixture of polyphenols, flavonoids, triterpenoids, steroids, phytosterols, fatty acids and microelements (mainly zinc, copper and selenium)[5]. The extract has been described as an antioxidant with anti-inflammatory and immunomodulatory activities in several experimental settings[6-8]. In addition, some experimental models have demonstrated that *M. indica* extract (MIE) improves its effects when it is given on various days before the induction of damage[9]. For example, when administered orally 1 h before lipopolysaccharide (LPS), MIE inhibited LPS-induced tumor necrosis factor (TNF)-α production in mice dose-dependently with ED_{50} 64.5 mg/kg. However, the extract inhibited the TNF serum levels but with ED_{50} 37.4 mg/kg when it was administered orally during 7 d before LPS challenge. The increasing evidence related to the positive effects of natural compounds with antioxidant and anti-inflammatory properties on UC prompted us to investigate whether MIE could protect colonic mucosa of rats from damage induced by oral administration of DSS, using two different treatment protocols, and to elucidate the possible mechanism(s) involved.

MATERIALS AND METHODS

Materials

Twenty-eight male outbred Wistar Hannover rats (HsdRccHan:Wist, from Harlan Spain), initially weighing 190-200 g, were housed five per cage and maintained in an animal holding room controlled at a constant temperature of 24 ± 2℃, with a relative humidity of 70% ± 5% and a 12-h light/dark cycle. Animals were fed a standard pellet chow with free access to fresh tap water. All experimental protocols followed the guidelines of the Animal Welfare Committee of the Universidad Complutense according to European legislation (2003/65/EC). Chemicals were from Sigma (Spain) or as indicated.

Extract preparation

M. indica L. was collected from a cultivated field located in the region of Pinar del Rio, Cuba. Voucher specimens of the plant (Code: 41722) were deposited at Herbarium of the Academy of Sciences, Institute of Ecology and Systematics, Ministry of Science, Technology and Environment, La Habana, Cuba. Stem bark extract was concentrated by evaporation and spray-dried to obtain a fine brown powder, which is used as the standardized active ingredient of MIE formulations. It melts at 210-215℃ with decomposition. The chemical composition of MIE has been characterized by chromatographic (planar, liquid and gas) methods, mass spectrometry, nuclear magnetic resonance (NMR), and UV-V spectrophotometry (fully described in[5]). The elemental inorganic composition has been determined by inductively coupled plasma spectrometry[6]. Extracts were prepared by suspending powder in 0.5% carboxymethylcellulose for oral administration and in melted suppository vehicle (Witepsol H15; Sasol, Witten, Germany) for rectal administration.

Colitis model

The experiment lasted for 21 d. The rats were randomly divided into four groups (Table 1). Control, A and B groups received vehicle orally during 2 wk. Group C received MIE (150 mg/kg) orally once daily. At day 15, oral administration was stopped and colitis was induced by 4% DSS (MP Biomedicals) in drinking water during 7 d for groups A, B and C. The control group received water. At the same time, groups B and C were co-treated rectally with extract at an equal dose while the controls and group A received vehicle rectally.

Macroscopic assessments, including weight changes, visible fecal blood and stool consistency were determined. The severity of diarrhea was evaluated according to the following score: no diarrhea = 0; mild diarrhea = 2; severe watery diarrhea = 3; and severe watery diarrhea with blood = 4[10]. Seven days after DSS (or 21 d from the onset of the study), animals were sacrificed after terminal anesthesia with sodium pentobarbital, and the entire colon

Table 1 Experimental design of dextran sulfate sodium-induced colitis model

Group	Treatment regimens			*n*
	Week 1	Week 2	Week 3	
Control	Vehicle *po*	Vehicle *po*	DSS no + vehicle	4
A	Vehicle *po*	Vehicle *po*	DSS yes + vehicle	8
B	Vehicle *po*	Vehicle *po*	DSS yes + MIE 150 mg/kg rectal	8
C	MIE 150 mg/kg *po*	MIE 150 mg/kg *po*	DSS yes + MIE 150 mg/kg rectal	8

MIE: *Mangifera indica* L.; DSS: Dextran sulfate sodium.

was removed. The colon length was measured and colon samples were collected for biochemical determinations and histological assessment.

Histological assessment

Each removed colon was washed in saline solution and cut longitudinally. Distal fractions were immediately embedded in Tissue-Teck OCT (Sakura), frozen and cut in transverse sections (7 μm) in a microtome cryostat. Samples were mounted on glass slides, cleaned and stained with hematoxylin and eosin for histological evaluation. Each slide was coded and analyzed in a blinded fashion by two investigators who assigned to each sample a histological score based on mucosal injury, with particular attention paid to alterations of the colonic crypts and the presence of inflammation in the colon. Colonic epithelial damage was assessed as: grade 0, normal; grade 1, slight damage and a few inflammatory cells infiltrated in a small area of mucosa; grade 2, moderate damage in two or more areas of the mucosa, with slight bleeding of the submucosa and mild inflammatory infiltrate; and grade 3, severe damage of the mucosa that extended into the muscular mucosa, with loss of the epithelium, and a large inflammatory infiltrate[1].

Myeloperoxidase activity

Immediately after removal, colon samples were minced on ice and homogenized (glass/glass) in 0.5% hexadecyltrimethylammonium bromide, 0.5% Nonidet P40 (Boehringer, Mannheim, Germany) in 20 mmol/L phosphate buffer, pH 6.0. The homogenates were then centrifuged for 20 min at 12000 *g*. Tissue levels of myeloperoxidase (MPO) were determined in supernatants using hydrogen peroxide as a substrate for the enzyme. A unit of MPO activity was defined as that which converted 1 μmol hydrogen peroxide to water in 1 min at 40℃[11].

Lipid peroxidation

Lipid peroxidation was measured by the thiobarbituric acid test for malondialdehyde (MDA) following a previously described method[12] with some modifications. Colonic samples were homogenized (glass/glass) in 10 vol 50 mmol/L phosphate buffer and deproteinized with 40 % trichloroacetic acid and 5 mol/L HCl, followed by addition of 2 % (w/v) thiobarbituric acid in 0.5 mol/L NaOH. The reaction mixture was heated in a water bath at 90℃ for 15 min and centrifuged at 12000 *g* for 20 min. The pink chromogen was measured at 532 nm in a Beckman DU-7500 spectrophotometer. The results were expressed as nmol/mg protein.

Glutathione determination

Reduced glutathione (GSH) levels were determined in accordance with a procedure described by Kamencic *et al*[13]. Frozen colonic samples were homogenized (glass/glass) in 20 vol cold 50 mmol/L Tris buffer, pH 7.4. Homogenates were centrifuged at 12000 *g* for 20 min and the supernatants were collected. The samples were then treated with monochlorobimane (mCB) 100 μmol/L and glutathione-s-transferase 1 U/mL, and were incubated at room temperature for 30 min. The GSH-mCB adducts were measured in a Labsystems Fluoroskan reader with excitation at 380 nm and emission measured at 470 nm. Concentration of GSH in samples were calculated by standard curve of GSH and expressed as μg/mg protein.

Western blotting analysis

To determine the levels of inducible nitric oxide synthase (iNOS), inducible cyclooxygenase (COX)-2, TNF-α and its receptors TNF-R1, and TNF-R2, tissues were homogenized at 4℃ in 5 vol buffer that contained 320 mmol/L sucrose, 1 mmol/L, DL-dithiothreitol, 10 μg/mL leupeptin, 10 μg/mL soybean trypsin inhibitor, 2 μg/mL aprotinin and 50 nmol/L Tris brought to pH 7.0, and supernatants after centrifugation at 12000 *g* for 20 min were used. The supernatants were diluted (Laemmli) and heated at 90℃ for 10 min. After loading (20 μg protein), proteins were sized-separated in 10% or 14% (for TNF-α analysis) SDS-PAGE (90 mV). The gels were processed against the antigens and after blotting onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA), they were incubated with specific goat polyclonal anti-rat COX-2 (1:1000), rabbit polyclonal anti-rat iNOS (1:1000), polyclonal rabbit anti-rat TNF-α (1:1000), polyclonal rabbit anti-rat TNF-R1 (1:500) and polyclonal rabbit anti-rat TNF-R2 (1:500) antibodies (all from Santa Cruz Biotechonology, Santa Cruz, CA, USA, except anti-rat TNF-α that was purchased from PeproTech EC). The correspondent peroxidase secondary antibody was used and proteins recognized by the antibody were visualized on X-ray film by chemiluminescence following the manufacturer's instructions (Amersham Ibérica, Madrid, Spain). Autoradiographs were quantified by densitometry (Software Total Lab Dynamics Ltd, Phoretix, Newcastle, UK), and several time expositions were analyzed to ensure the linearity of the band intensities.

Detection of serum TNF-α and interleukin-6

ABC-ELISAs of double antibodies sandwich were adopted for determination of the two cytokines (kits were obtained from R&D Corporation).

Statistical analysis

All results are presented as mean ± SE. Data were ana-

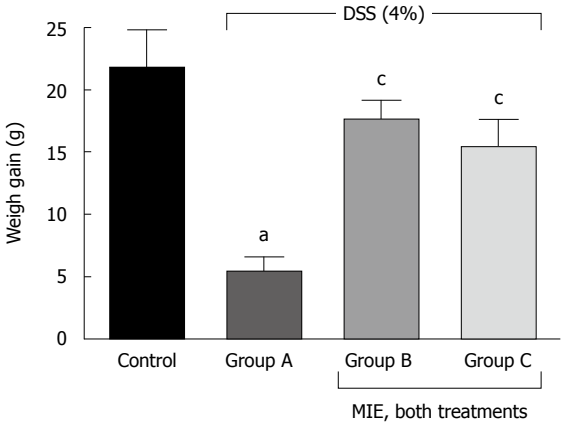

Figure 1 Effect of *Mangifera indica* L. on weight gain in dextran sulfate sodium-treated rat ulcerative colitis. Experimental colitis was induced by 4% dextran sulfate sodium (DSS) dissolved in drinking water for 7 d. Control group received common water; Group A was exposed to 4% DSS and vehicle; Group B was co-treated with rectal *Mangifera indica* L. (MIE) for 7 d, at the same time as DSS administration; Group C was treated with MIE (150 mg/kg) orally during 14 d prior to DSS administration, and co-administered rectally during DSS exposure. Each bar represents the difference between the weight at the beginning and ending of the experiment, and it was expressed as the mean ± SE of each group. [a]Significant differences *vs* control group; [c]Significant differences *vs* group A; $P < 0.05$.

lyzed using the Graph Pad Prism 4 statistical software. One-way analysis of variance followed by Newman-Keuls test were used for statistical evaluation of the parametric data. Non-parametric data were analyzed by Kruskal-Wallis one-way analysis followed by Dunn's test. $P < 0.05$ was considered as statistically significant.

RESULTS

General observations

None of the animals in the four experimental groups died throughout the experiment. The intake of drinking water in the three groups administered with DSS (A-C) decreased significantly from the beginning compared with that in the control group (data not shown). The weight gain of rats in the DSS group (A) was significantly lower than in the control group. Administration of MIE in the both pre/co-treated (C) and co-treated only (B) groups prevented this effect (Figure 1). On the other hand, all groups with DSS exhibited an increase in diarrhea and rectal bleeding from day 4 post-DSS until the end of experiment. However, in the case of group C (pre/co-treated group), diarrhea score was found to be less severe than in the DSS group (A) at days 4 and 5 post-DSS. Group B did not show any significant differences compared to the group that received DSS alone (Figure 2A). Colon length is a useful assessment of colitis and it is considered as a marker of inflammation. As shown in Figure 2B, 7 d after DSS administration, there was a significant shortening of the colon length in the group given DSS only (group A: 14.1 ± 0.1 cm) compared with the control group (17.4 ± 0.2 cm). In both pre-and co-treated groups (C and B), MIE significantly improved this inflammatory marker.

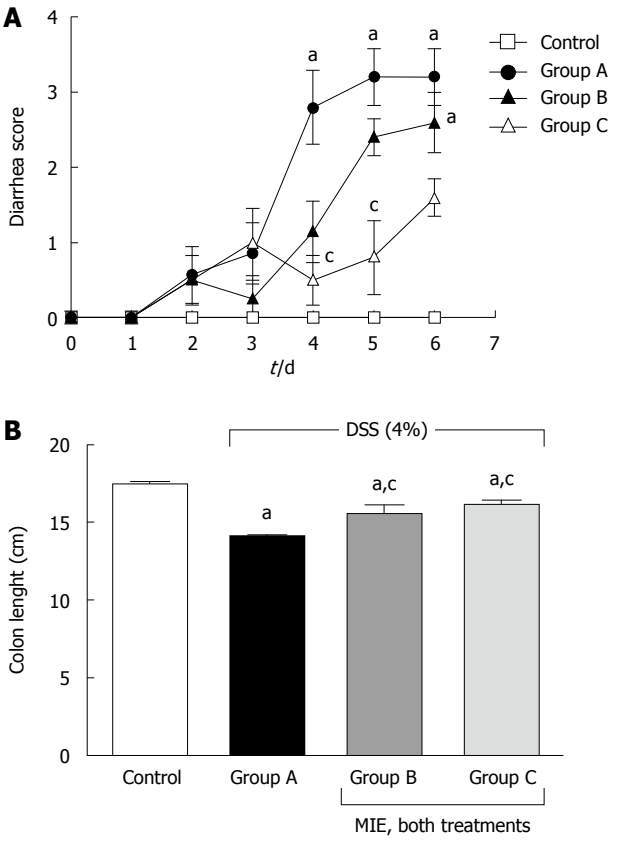

Figure 2 Effect of *Mangifera indica* L. on diarrhea and colon length in dextran sulfate sodium-treated rat ulcerative colitis. Induction of experimental colitis, the control group, and groups A, B and C were as described for Figure 1. Changes in diarrhea score (A) and colon length (B) after 4% dextran sulfate sodium treatment in the presence or absence of *Mangifera indica* L. (MIE) are presented. [a]Significant differences *vs* control group; [c]Significant differences *vs* group A; $P < 0.05$. DSS: Dextran sulfate sodium.

Histological findings

The occurrence of UC was corroborated on the basis of histological damage and inflammatory infiltrate as shown in Figure 3. Figure 3D summarizes the microscopical damage scores from DSS rats and DSS rats treated with MIE. The control group exhibited normal mucosal morphology. Rats that received DSS and vehicle (group A) showed extensive mucosal damage with a large number of inflammatory cells, obtaining as a result, the highest score in the microscopic analysis. MIE in both treatment protocols (groups B and C) decreased the grade and number of ulcerations and diminished the inflammatory infiltrate.

Effect of MIE on MPO activity

DSS colitis was also characterized by increased MPO activity in colonic tissue, an indicator of polymorphonuclear leukocyte accumulation. The DSS group (A) showed a significant elevation of MPO levels in colonic tissue (21.1 ± 2.7 mU/mg), $P < 0.05$ *vs* the control group. The increase observed in the DSS group was clearly diminished by both treatments with MIE as shown in Figure 4A.

Effect of MIE on lipid peroxidation

The effect of MIE on lipid peroxidation - an indicator of

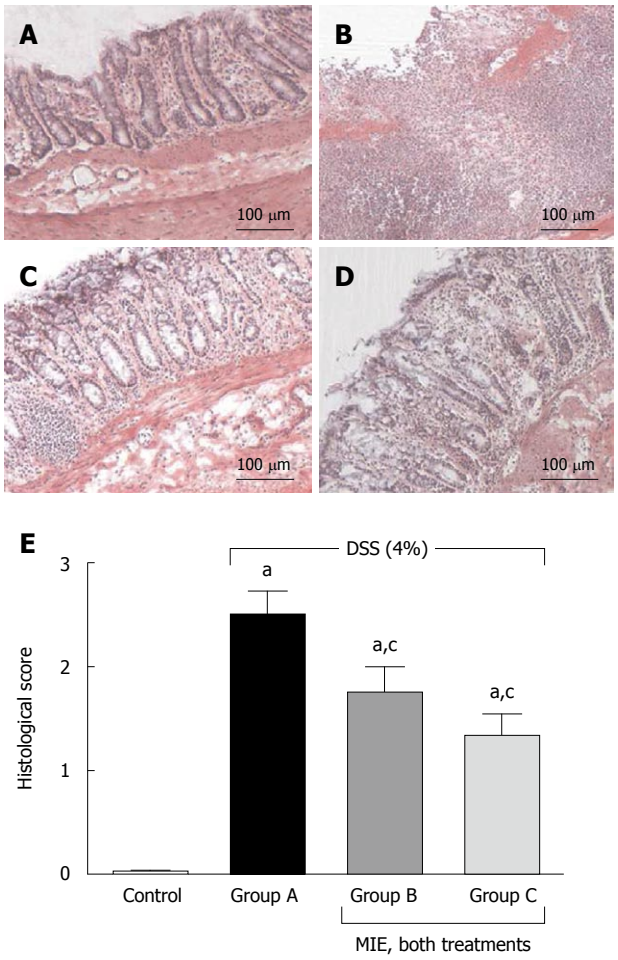

Figure 3 Hematoxylin and eosin staining of colons obtained from rats untreated or treated with dextran sulfate sodium (4%) and *Mangifera indica* L.. A: Control group: Received common water (100 ×); B: Exposed to 4% dextran sulfate sodium (DSS) and vehicle (100 ×); C: Co-treated with *Mangifera indica* L. (MIE) rectally (150 mg/kg) for 7 d, at the same time as DSS administration (100 ×); D: Treated with MIE (150 mg/kg) orally during 14 d prior to DSS administration, and co-administered rectally during DSS exposure (100 ×); E: Changes in histological score were assigned according to criteria defined in the Material and Methods. Each bar represents the mean ± SE of the different groups. [a]Significant differences *vs* control group; [c]Significant differences *vs* group A; $P < 0.05$.

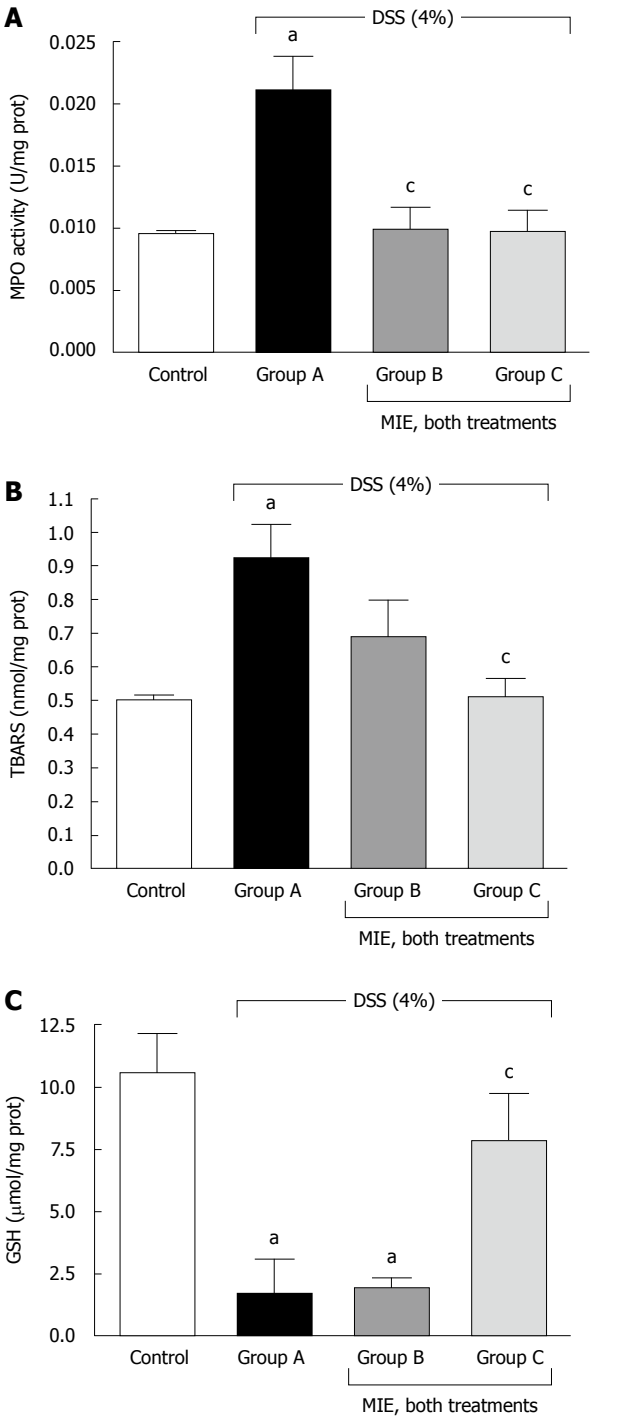

Figure 4 Effect of *Mangifera indica* L. on myeloperoxidase activity, lipid peroxidation and glutathione levels in dextran sulfate sodium-treated rat colon tissue. Induction of experimental colitis, the control group, and groups A, B and C were as described for Figure 1. Each bar represents the mean ± SE of each group. [a]Significant differences *vs* control group; [c]Significant differences *vs* group A; $P < 0.05$. A: Myeloperoxidase (MPO) levels were determined; B: Lipid peroxidation was estimated according to the presence of thiobarbituric acid reactive substances (TBARS); C: Glutathione (GSH) levels were determined. MIE: *Mangifera indica* L.; DSS: Dextran sulfate sodium.

cell membrane damage as a result of oxidative toxicity - in rats treated with 4% DSS is shown in Figure 4B. In the DSS-induced colitis rats, the level of TBARS was significantly increased (0.71 ± 0.07 nmol/mg) when compared with the control group (0.41 ± 0.05 nmol/mg). Although previous administration of MIE resulted in a reduction in TBARS level (group C, 0.4 ± 0.04 nmol/mg), co-treatment with MIE (group B) did not decrease TBARS level (0.57 ± 0.16 nmol/mg) compared with that in the DSS-treated rats.

Effect of MIE on GSH levels

GSH is one of the most important endogenous antioxidants. Figure 4C shows a significant decrease of GSH in group A (1.68 ± 1.4 μg/mg) compared to the control group (10.55 ± 1.6 μg/mg). In this case, there were no significant differences between the DSS group and group B (co-treated but not pre-treated with 1.91 ± 0.4 μg/mg MIE). However, the administration of MIE prior to 4% DSS resulted in an increase in GSH level (7.80 ± 1.91 μg/mg) compared with that in the 4% DSS treatment group.

Effects of MIE on expression of iNOS and COX-2

When rats were treated with 4% DSS, the levels of inflam-

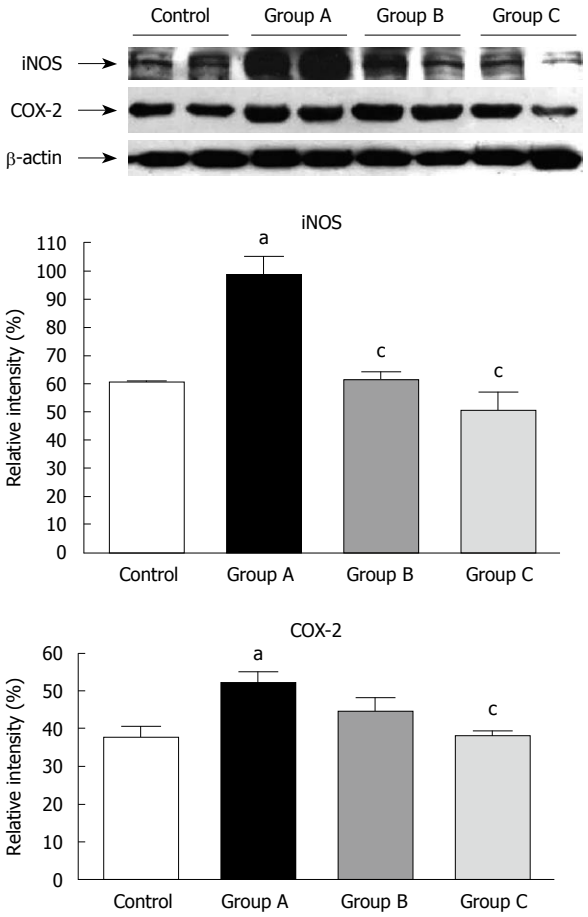

Figure 5 Effect of *Mangifera indica* L. on isoforms of nitric oxide synthase and cyclooxygenase-2 production in dextran sulfate sodium-treated rat colon tissue. Induction of experimental colitis, the control group, and groups A, B and C were as described for Figure 1. The protein extracts were obtained as described in the Materials and Methods. β-actin was used as an internal control. Expression of isoforms of nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 proteins were analyzed by Western blotting using iNOS and COX-2 polyclonal antibodies. The relative intensity was calculated using imaging software. Each bar represents the mean ± SE of each group. [a]Significant differences *vs* control group; [c]Significant differences *vs* group A; $P < 0.05$.

mation-related proteins (iNOS and COX-2) in colonic tissue were significantly increased (Figure 5). In the case of iNOS, both treatments with MIE resulted in a decrease of expression. However, for COX-2 expression, attenuation of band intensities was observed only in group C (pretreated group).

Effects of MIE on expression of TNF-α and TNF receptors

Administration of 4% DSS induced a significant increase in TNF-α (Figure 6, lanes 3-5) and TNF R-2 in inflamed tissue (Figure 6, lanes 3-6). Treatment with MIE 15 d before 4% DSS resulted in a gradual weakness of band intensities for TNF-α and TNF R-2. Relative band intensities of increased TNF-α and TNF R-2 expression caused by DSS were reduced by prior treatment with MIE (group C) in 17.8 and 22.8% respectively *vs* DSS. There were no significant differences between relative intensities in group B compared with the group treated with DSS alone. Expression of TNF R-1 was not affected by DSS supplementation.

Table 2 Serum levels of tumor necrosis factor-α and interleukin-6 in experimental groups

Group	TNF-α	IL-6
A	146.17 ± 13.1	118.15 ± 16.7
C	101.92 ± 9.3[a]	61.36 ± 18.8[a]

Results are presented as % of control group, mean ± SE of each group. [a]$P < 0.05$ ($n = 8$ in both groups). TNF-α: Tumor necrosis factor-α; IL-6: Interleukin-6.

Effect of MIE on TNF-α and interleukin-6 serum levels

Based on the effects of MIE given before colitis induction (group C) on tissue cytokine expression, we tested the systemic levels of cytokines. Administration of 4% DSS produced an increase in TNF-α serum levels (46.2%), whereas interleukin (IL)-6 serum levels showed a tendency to elevation in group A (treated with DSS alone) but this was not statistically significant (18.1%). Treatment with MIE, before DSS intake, clearly decreased TNF-α levels by 44.3% and reduced IL-6 serum levels (down to control serum levels) by 58.8% (Table 2).

DISCUSSION

UC is a chronic, relapsing disease that causes inflammation and ulcerations of the colonic mucosa with a variable extent and severity. The etiology of UC remains essentially unknown but the results from many studies in humans and animal models suggest that it is related to an abnormal immune response in the gastrointestinal tract, possibly associated with genetic and environmental - mainly microbial - factors[14]. Aminosalicylates, glucocorticoids and immunosuppressive drugs have been mainly used for the treatment and maintenance of remission of UC, but the side effects or toxicity of these drugs represents a major clinical problem[15]. For these reasons, natural medicine has become an alternative therapy in addition to the conventional therapies that are used to treat UC[16].

In the present study, we demonstrated that MIE has an anti-inflammatory effect on colonic injury provoked by oral supplementation with DSS in rats, mainly when it is administered before the induction of damage. DSS-induced colitis is a well-established model that is phenotypically similar to UC in humans[17]. Oral administration of DSS for several days, leads to colonic epithelial lesions and acute inflammation characterized by the presence of neutrophils and macrophages within damaged segments. The reason for the deleterious effects of DSS is not well understood, however, epithelial cell permeability and macrophage activation have been proposed as potential mechanisms. We administered *M. indica* extract in two different protocols to evaluate the role of pretreatment with this product. The decrease in colitis induced by MIE was accompanied by a lower weight loss of rats and a partial

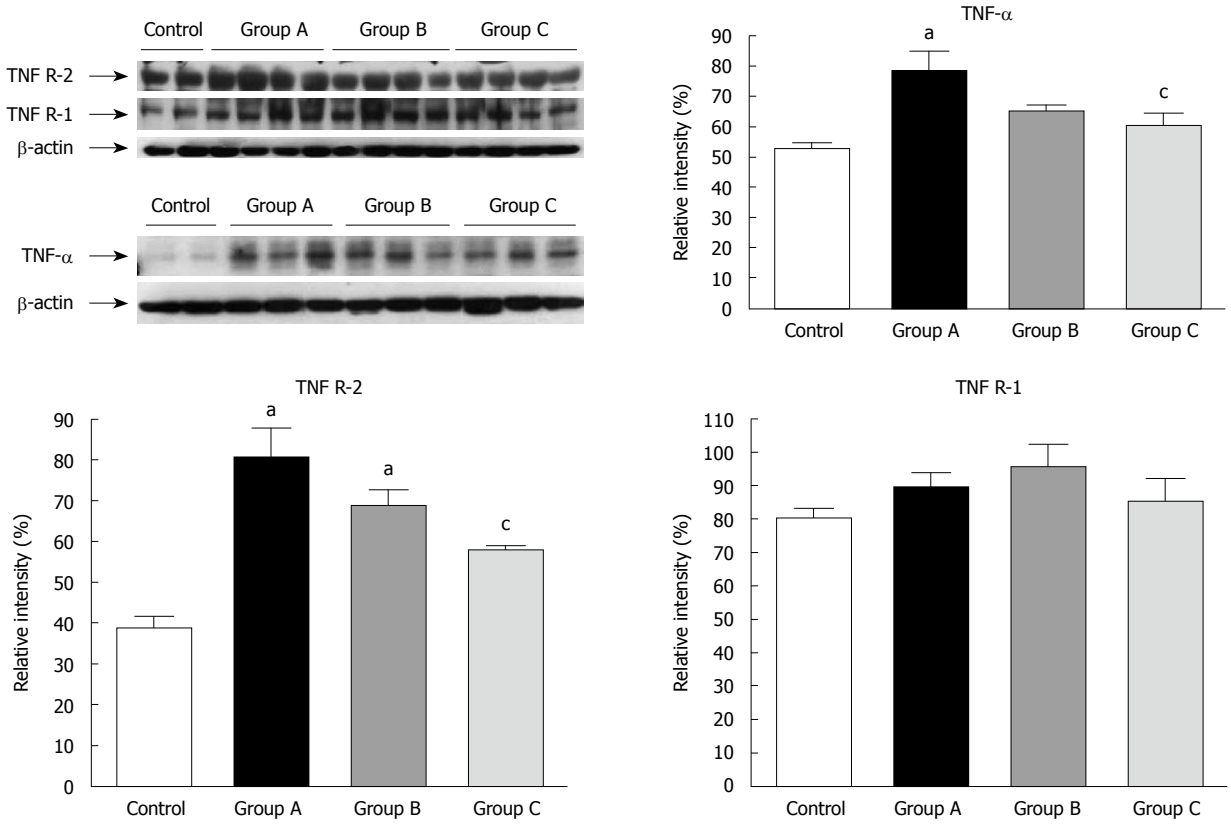

Figure 6 Effect of *Mangifera indica* L. on tumor necrosis factor-α and tumor necrosis factor receptor production in dextran sulfate sodium-treated rat colon tissue. Induction of experimental colitis, the control group, and groups A, B and C were as described for Figure 1. The protein extracts were obtained as described in the Materials and Methods. β-actin was used as an internal control. Expression of tumor necrosis factor (TNF)-α and TNF receptor proteins was analyzed by western blotting using TNF-α, TNF R-1 and TNF R-2 polyclonal antibodies. The relative intensity was calculated using imaging software. Each bar represents the mean ± SE of each group. [a]Significant differences *vs* control group; [c]Significant differences *vs* group A; $P < 0.05$.

restoration of colon length, which is an indirect assessment of colon inflammation. However, a decrease in the occurrence of diarrhea was only observed when MIE was administered before DSS. Confirming clinical results, microscopic analysis established a protective action of MIE, which was measured as a decrease in ulceration, conservation of epithelial crypts, and a reduction in infiltrated cells. These effects were more evident in the pretreated group.

The infiltration of leukocytes into the mucosa contributes significantly to the tissue necrosis and mucosal dysfunction, as they represent a major source of reactive oxygen species (ROS)[18]. MPO is an enzyme that is found predominantly in neutrophils, and a good marker of inflammation and tissue injury. Therefore, the decrease of MPO activity can be explained through the reduction of neutrophil accumulation in inflamed tissue[19]. In addition, oxygen radicals and NO can interact and exert a cytotoxic effect by causing lipid peroxidation, which results in the formation of MDA[20]. Our results showed that MIE in both treatment protocols inhibited MPO activity, whereas the decrease in MDA production was only observed when animals received MIE before DSS administration. A decrease of MPO activity with MIE treatment in different experimental models of inflammation (ear and paw edema) has been described[21]. In addition, several studies have established the high antioxidant capacity of the extract by blocking oxygen radical formation[22,23]. The mechanism involved is associated with the antioxidant activity reported for mangiferin, which has a low redox potential that proves its ROS scavenger ability[24]. Therefore, the antioxidant capacity of the extract, administered prior to colitis development, probably leads to a decrease of lipid peroxidation and MPO activity. However, the results presented here indicated that co-treatment with MIE was not sufficient to reduce MDA levels. This could be related to the necessary oxidative pre-conditioning that has been described for many antioxidants[25]. We hypothesize that MIE could be useful in the prevention of relapse in patients with quiescent UC.

Furthermore, the increased generation of highly toxic ROS in UC exceeds the limited intestinal antioxidant defense system, thereby contributing to intestinal oxidative injury. Glutathione, as the most abundant cellular antioxidant system in animal cells, plays an essential role in modulating cell responses to redox changes[26]. GSH deficiency predisposes animals to organ failure and death after an otherwise nonlethal period of hypotension[27,28]. GSH deficiency is associated with severe injury such as inflammation and sepsis, therefore, treatment strategies that maintain GSH stores might decrease the incidence of organ failure. Our findings demonstrated that MIE administered before colitis induction produced a significant increase in GSH levels, which were probably associated with the radical scavenger capacity of the extract

and the protection of thiol groups described by numerous polyphenols[29]. Polyphenols are the main constituent of MIE (around 50%)[5].

Moreover, pathological invasion of inflammatory cells into the mucosa produces increased concentrations of inflammatory cytokines such as interleukins, TNF-α and interferon-γ[30]. Pro-inflammatory cytokines induce the expression of genes associated with inflammation, such as iNOS, and stimulate iNOS activity, which increases the production of the free radical NO[31]. Studies in knockout mice have demonstrated that iNOS plays an important role in the pathogenesis of colitis[32], and the role of iNOS in the pathogenesis of human UC has been previously suggested[33]. In the present study, MIE inhibited iNOS expression, as described in other inflammatory experimental settings[34].

In addition to iNOS, DSS-induced expression of COX-2 was also inhibited by prior administration of MIE. Previous studies in endotoxin-stimulated macrophages also have demonstrated that MIE inhibits COX-2 protein and mRNA levels, but at doses higher than those required for iNOS inhibition, which suggests that longer treatments or higher doses of MIE than those needed for inhibition of COX-2[34] are necessary. This might explain the lack of effect when the extract was administered only in the co-treatment regimen. The synthesis and activity of iNOS and COX-2 are induced by almost the same pro-inflammatory stimuli and are associated with inflammatory conditions. Therefore, it is possible that inhibition of iNOS and COX-2 induced by prior treatment with MIE could provide the most potent anti-inflammatory effect.

On the other hand, TNF-α has been described as a key molecule in UC pathogenesis, and a monoclonal antibody against this molecule, such as infliximab, has proven to be effective in the treatment of moderate to severe UC[35]. This cytokine, by interaction with its receptors I and Ⅱ, recruits leukocytes to inflammatory sites, stimulates monocytes and vascular endothelial cells to express cytokines, induces the cascade effects for other cytokines, and finally results in inflammatory lesions in tissues[36,37]. Our results demonstrated that prior administration of MIE inhibits DSS-induced increased TNF-α and TNF R-Ⅱ expression. TNF R-Ⅰ is expressed constitutively, whereas TNF R Ⅱ is induced by diverse stimuli and plays a key role in the local inflammatory response[38]. Previous *in vivo* and *in vitro* studies have appointed MIE as a potent TNF-α inhibitor[9] and some polyphenols structurally related to those present in MIE inhibit lymphocyte proliferation and cytokine production[39,40]. Moreover, the reduction of TNF R-Ⅱ receptor expression seems to enhance the inhibitory action of the extract on the TNF-α signaling system.

The reduction of inflammatory enzymes iNOS and COX-2, TNF-α and TNF R-Ⅱ expression induced by MIE can be correlated with its antioxidant properties. The effects of antioxidant agents have been ascribed by some authors to inhibition of activation of the nuclear transcription factor nuclear factor (NF)-κB, which is activated by ROS with the subsequent induction and expression of various cytokines (such as TNF-α) and enzymes (i.e. iNOS and COX-2)[41,42] that are involved in the induction and development of UC. Although *in vitro* studies have demonstrated that MIE inhibits NF-κB in macrophages[43], further research is necessary to demonstrate that MIE exerts an inhibitory effect on NF-κB signaling pathways.

In addition, TNF-α and IL-6 serum levels were determined in our study. Administration of DSS produced an increase in systemic TNF-α levels, which was reversed by prior administration of MIE. This fact is probably associated with the molecular changes found in the local inflammatory focus. Although several studies have establish an increase in IL-6 serum levels after DSS supplementation[3,44], our results demonstrated a non-significant tendency to increase IL-6 levels in serum. Nevertheless, prior administration of MIE produced a significant decrease in this cytokine. A previous study has demonstrated the ability of MIE to modulate macrophage function through inhibition of chemotaxis and phagocytosis[43]. Macrophages are one of the main sources of cytokines (i.e IL-6 and TNF-α), therefore, a possible modulation of macrophage activity by MIE could influence the decrease in cytokine production. This result suggests an important role for MIE as a modulator of the immune system and should be taken into account for future investigations.

In conclusion, the results showed that MIE administered in co-treatment regimens is able to prevent body weight loss and colon shortness, as well as modulate MPO activity and reduce iNOS expression levels. However, when MIE is administered before DSS damage, its protective effects are broader and enhanced, as demonstrated by a decrease in diarrhea and lipid peroxidation; an increase in GSH levels; a decrease in iNOS, COX-2, TNF-α and TNF R-Ⅱ expression levels, as well as a reduction in TNF-α and IL-6 serum levels.

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COMMENTS

Background

Ulcerative colitis (UC) is a chronic inflammatory bowel disease (IBD) that is characterized by bloody diarrhea, colonic mucosal ulceration and, in severe cases, systemic symptoms. An exaggerated immune response against antigens of the colonic microbiota in genetically predisposed individuals is suggested to be involved in the etiology of UC. Current available treatment includes anti-inflammatory and immunosuppressive agents, all of which have many adverse reactions after long-term treatment to prevent remissions of the disease.

Research frontiers

New therapies must be developed to increase the number and duration of remissions. In this vein, traditional medicine around the world is now being re-evaluated by extensive research on different plants and their therapeutic principles. Many plants produce antioxidant compounds to control oxidative stress. An aqueous stem bark extract from *Mangifera indic*a L. (MIE, Anacardiaceae family), has been traditionally used as a nutritional supplement. The composition is a defined mixture of polyphenols, flavonoids, triterpenoids, steroids, phytosterols, fatty acids

and microelements (mainly zinc, copper and selenium). The extract has been described as an antioxidant with anti-inflammatory and immunomodulatory activities in several experimental settings.

Innovations and breakthroughs

MIE has an anti-inflammatory effect on colonic injury in a rat model of UC, mainly when it is administered before the induction of damage. The decrease in colitis induced by MIE was accompanied by a lower weight loss of rats and a partial restoration of colon length, which is an indirect assessment of colon inflammation. Furthermore, a decrease was also observed in occurrence of diarrhea, which is the main clinical finding. By confirming the clinical results, microscopic analysis established protective activity of MIE, as measured by a decrease in ulceration, conservation of epithelial cells, and a reduction in infiltrating cells. Finally, MIE modulated most of the inflammatory mediators in colitis: inducible nitric oxide synthase (NOS), inducible cyclooxygenase (COX), and consequent lipid peroxidation. MIE inhibited two of the main inflammatory cytokines, tumor necrosis factor (TNF)-α and interleukin (IL)-6.

Applications

MIE is able to prevent body weight loss and colon shortness, as well as decrease some of the intra- and intercellular mechanisms of inflammatory damage in the colon, in an animal model of UC. In this way, this study might represent a future strategy for therapeutic intervention in the preventive management of patients with UC.

Terminology

NOS and COX are two enzymatic sources of inflammatory mediators, and their activation leads to an increase in reactive oxygen species, which can damage cells. Peroxidation of lipid components of the cell membranes is the result of this damage. Cytokines are a family of pleiotropic intercellular proteins, mainly in immunological cells, and most of them are pro-inflammatory, such as TNF-α and IL-6.

Peer review

This is a novel and interesting study that demonstrates the anti-inflammatory effects of MIE on colonic mucosa in a DSS colitis model in rats. The results are important and potentially relevant for designing therapy in IBD. The results have are well presented and support the authors' conclusions. However, the addition of another colitis model would improve the paper.

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ORIGINAL ARTICLE

HBx-induced reactive oxygen species activates hepatocellular carcinogenesis *via* dysregulation of PTEN/Akt pathway

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Abstract

AIM: To investigate the role of hepatitis B virus X-protein (HBx)-induced reactive oxygen species (ROS) on liver carcinogenesis in HBx transgenic mice and HepG2-HBx cells.

METHODS: Cell growth rate was analyzed, and through western blotting, mitogenic signaling was observed. Endogenous ROS from wild and HBx transgenic mice and HepG2-Mock and HBx cells were assayed by FACScalibur. Identification of oxidized and reduced phosphatase and tensin homolog (PTEN) was analyzed through N-ethylmaleimide alkylation, nonreducing electrophoresis.

RESULTS: We observed that the cell-proliferation-related phosphoinositide 3-kinase/Akt pathway is activated by HBx *in vivo* and *in vitro*. Increased ROS were detected by HBx. Tumor suppressor PTEN, *via* dephosphorylation of Akt, was oxidized and inactivated by increased ROS. Increased oxidized PTEN activated the mitogenic pathway through over-activated Akt. However, treatment with ROS scavenger N-acetyl cysteine can reverse PTEN to a reduced form. Endogenously produced ROS also stimulated HBx expression.

CONCLUSION: HBx induced ROS promoted Akt pathways *via* oxidized inactive PTEN. HBx and ROS maintained a positive regulatory loop, which aggravated carcinogenesis.

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Key words: Hepatitis B virus X protein; Hepatocellular carcinoma; Akt; Reactive oxygen species; Phosphatase and tensin homolog

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of cancer mortality. Among other risk factors (including alcohol abuse, cirrhosis, and aflatoxin B1), chronic hepatitis B virus (HBV) infection plays a central role in the etiology of

HCC[1]. About 53% of HCC cases are related to HBV, and the risk of HCC in chronic HBV carriers is approximately 100 times greater than in uninfected individuals[2]. Among the four proteins encoded by the HBV genome, X protein (HBx) is a multifunctional regulatory protein that is closely linked to HCC, but its role in tumor growth has not been fully clarified. Prior work from this laboratory has shown that HBx induces liver cancer in transgenic mice[3]. HBx does not bind directly to DNA, but affects transcriptional activation through interaction with nuclear transcription factors and by cytoplasmic modulation of signal transduction pathways[4]. HBx also mediates the activation of the Ras/Raf/extracellular signal-regulated kinase and mitogen-activated protein kinase kinase kinase-1/c-Jun NH_2-terminal kinase cascades, which leads to the induction of activator protein-1 and nuclear factor κB[5,6]. One of the most well-known pathways activated by HBx is phosphoinositide 3-kinase (PI3K)/Akt, which is associated with anti-apoptotic activity and cell proliferation[7-9]. Therefore, HBx is thought to be associated with the development of human HCC, but the precise function of HBx in the tumorigenic transformation of liver cells remains unclear.

Previous studies have indicated that HBx protein directly interacts with the membrane proteins of mitochondria, the major site of reactive oxygen species (ROS) production, and alters the mitochondrial membrane potential in a hepatoma cell line. HBx also increases the level of mitochondrial ROS and lipid peroxide production[10]. The results of many previous studies have shown that normal cells exposed to low levels of H_2O_2 can increase their proliferation[11]. In this context, many types of cancer cells manifest increased production of H_2O_2[12].

Protein tyrosine phosphatases (PTPs) are a group of enzymes that remove phosphate groups from phosphorylated tyrosine residues on proteins. Together with tyrosine kinases, PTPs regulate the phosphorylation state of many important signaling molecules. They have been suggested to be direct targets of H_2O_2[13,14]. In general, PTPs exert an inhibitory effect on cancer signaling by opposing the tyrosine phosphorylation initiated by activated receptor kinases. Cell stimulation induces the transient activation of class-I PI3K, and the subsequent production of PI 3,4,5-trisphosphate (PIP_3) which is important for the activation of a variety of downstream signaling molecules, including the protein kinase Akt, that mediate promotion of cell proliferation and survival[15]. The reaction catalyzed by PI3K is reversed by phosphatase and tensin homolog (PTEN), which functions as a PIP_3 3-phosphatase. Indeed, by negatively modulating the PI3K signaling pathway, PTEN acts as a tumor suppressor. PTEN is also a member of the PTP family. It has been previously demonstrated that Cys-124 in the catalytic site of human PTEN is readily oxidized by exogenous H_2O_2 to form a disulfide with Cys-71[16].

In the present study, we attempted to determine the effect of HBx on the activated Akt pathways. We showed that HBx-produced H_2O_2 induces reversible inactivation of PTEN and activation of Akt. We suggest that scavenging H_2O_2 could be a therapeutic target for abnormal cell signaling to reactivate PTEN.

MATERIALS AND METHODS

Transgenic mice

The production of HBx transgenic mice used in this study has been reported previously[3]. HBx homozygous (+/+) transgenic mice were produced by mating HBx heterozygous transgenic mice with each other. To generate HBx homozygous transgenic mice on a mixed background of C57BL/6 and CBA strains, HBx homozygous mice with C57BL/6 backgrounds were crossed with CBA wild-type mice. The heterozygous transgenic offspring with a mixed background of C57BL/6 and CBA strains were cross mated. Among their offspring, HBx homozygous transgenic mice were selected by genotyping the next generation. Selected mice were then crossed up to F12, which is applicable for the study as an inbred strain with a mixed genetic background (C57BL/6 and CBA). In the current study, these F12 mice were used for *in vivo* analyses. HBx (+/+) transgenic mice were verified by polymerase chain reaction (PCR) analysis. The PCR primers used were as follows: one set was sense primer 5'-TTCTCATCTGCCGGTCCGTG-3' and antisense primer 5'-GGGTCAATGTCCATGCCCCA-3', and another set was sense primer 5'-GAAAACACACTCACTGTTCAGAG-3' and antisense primer 5'-GTAAGCCGCTTTCTCTTATGCAG-3'. The wild-type mice were derived from littermates between HBx heterozygous transgenic male and female mice, with a mixed genetic background (C57BL/6 and CBA). Mice were housed in a specific pathogen-free environment. Mice were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee at the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea).

Cell lines and cell culture conditions

HepG2-HBx cells derived from HepG2 cells were stably transfected and expressed HBx. HepG2 cells were grown in an atmosphere that contained 5% CO_2 at 37℃ in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 U/mL streptomycin.

Proliferation assay

Cell proliferation was determined by the crystal violet staining method, as described previously[17].

Western blotting analysis

Proteins (20 μg/sample) were separated on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). The membranes were blotted at 4℃ overnight with primary antibodies. The membranes were washed five times with 10 mmol/L Tris-HCl (pH 7.5) plus 150 mmol/L NaCl (Tris-buffered saline; TBS) that contained 0.2% Tween-20, and incubated with horseradish peroxidase (HRP)-conjugated IgG. After the removal of excess antibodies by washing with TBS, specific binding was detected using a chemiluminescence detection system (Amersham, Berks, UK) according to the manufacturer's in-

structions. Mouse monoclonal antibody to PTEN was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antibodies to phospho-Akt (Ser-473), Akt, and monoclonal antibodies to cyclin D1, were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit polyclonal antibodies to GAPDH were from Lab Frontier (Seoul, Korea), and HRP-conjugated goat antibodies to mouse or rabbit IgG were from Amersham and Sigma.

RNA isolation and quantitative-PCR analysis

Total RNA was isolated from the HepG2-HBx cells, or liver tissues from HBx transgenic mice, using TRIzol reagent (Invitrogen, Seoul, Korea) according to the manufacturer's specifications. The concentration of total RNA in the final elutes was determined by nano-drop. Total RNA was converted into single-strand cDNA using a cDNA synthesis kit (Fermentas, Glen Burnie, MD, USA). Amplification of the target genes by real-time reverse transcriptase (RT)-PCR was conducted using SYBR Green (Takara, Otsu, Shiga, Japan) followed by analysis using the ExicyclerTM 96 Real-Time Quantitative Thermal block (Bioneer, Daejeon, Korea). Relative gene expression was calculated using the comparative Ct (2-ΔΔCt) method.

Identification of reduced and oxidized PTEN by immunoblot analysis

Cells were harvested, washed once with PBS, and resuspended in 0.2 mL 100 mmol/L Tris-HCl (pH 6.8) that contained 2% SDS and 40 mmol/L N-ethylmaleimide (Sigma). Protein (20 μg/sample) was loaded and subjected to SDS-PAGE under nonreducing conditions. The separated proteins were then transferred to nitrocellulose membranes and immunoblotted with a mouse anti-PTEN antibody. Binding was detected by an HRP-conjugated anti-mouse Ig (1:10 000, Sigma) and enhanced chemiluminescence reagents (Pierce, Rockford, IL, USA).

Isolation of primary hepatocytes

Hepatocytes were isolated using the same methods as previously reported[18].

ROS detection

Cells treated with 500 μmol/L H_2O_2 and 10 mmol/L N-acetylcysteine (NAC) were stained for 15 min with 5 μmol/L H_2O_2-sensitive fluorescent dye dichlorofluorescein diacetate (DCFDA, FL-1; Molecular Probes, Eugene, OR, USA) at 37℃ in the dark, washed three times with PBS, and subsequently assayed by FACSCalibur (BD Biosciences, San Jose, CA, USA).

Statistical analysis

Comparisons were analyzed for statistical significance by unpaired or paired Student's *t* test using Microsoft Excel software. $P < 0.001$ was considered as significant. All data are reported as mean ± SD.

RESULTS

HBx promotes tumor formation

The HBx protein is considered to be closely associated with the development of HCC. HBx transgenic mice, previously developed in this laboratory[3], developed dysplasia around 4 wk of age, and hepatic tumors developed from 6 mo of age[19]. Several studies have shown that HBx stimulates cell proliferation and growth through the activation of signal transduction pathways such as Akt. To study the role of the HBx protein in cancer generation at the cellular level, HepG2-HBx cells were obtained by stably transfecting HepG2 cells with an HBx expression plasmid. The growth rate of the HepG2-HBx cells was approximately double that of the HepG2 control cells (Figure 1A and B). There were differences not only in cell growth, but also in morphology. HepG2-HBx cells showed aberrant actin bundling. Taken together, these results show that HBx has a role in the development of the liver tumor by activating proliferation and changing cell characteristics.

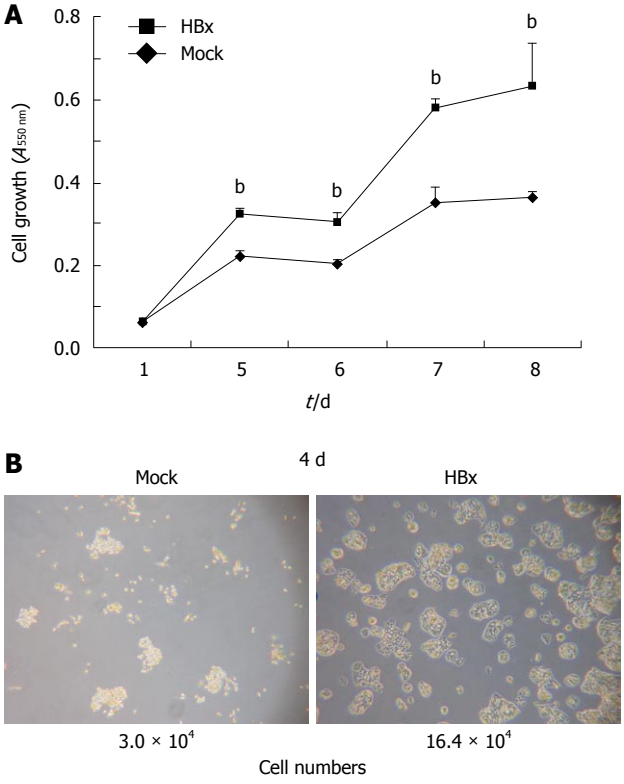

Figure 1 Effect of hepatitis B virus X-protein on induction of aberrant cell growth. A: Cell growth analysis by crystal violet staining and $A_{550\ nm}$ detection. 10^4 cells were seeded, and Stained at 1, 5, 6, 7 and 8 d after seeding. Values represent mean ± SD ($n = 3$). $^bP \leq 0.001$ compared with mock transfectants; B: Morphology of the cells was observed by optical microscopy. HBx: Hepatitis B virus X-protein.

Tumorigenesis in HBx transgenic mice and HepG2-HBx cells through activation of the Akt pathway

The PI3K/Akt signaling pathway is crucial to many aspects of cell growth and survival. To determine whether HBx-associated HCC is also accompanied by activation of the Akt pathway, lysates from the mouse liver tissue and cells transfected with HBx or an empty vector were used. As expected, the livers of HBx transgenic mice and HepG2-HBx cells displayed an activated Akt pathway. Accumulated β-catenin, phosphorylated Akt, and increased cyclin D1 were detected (Figure 2A). Even though cancer

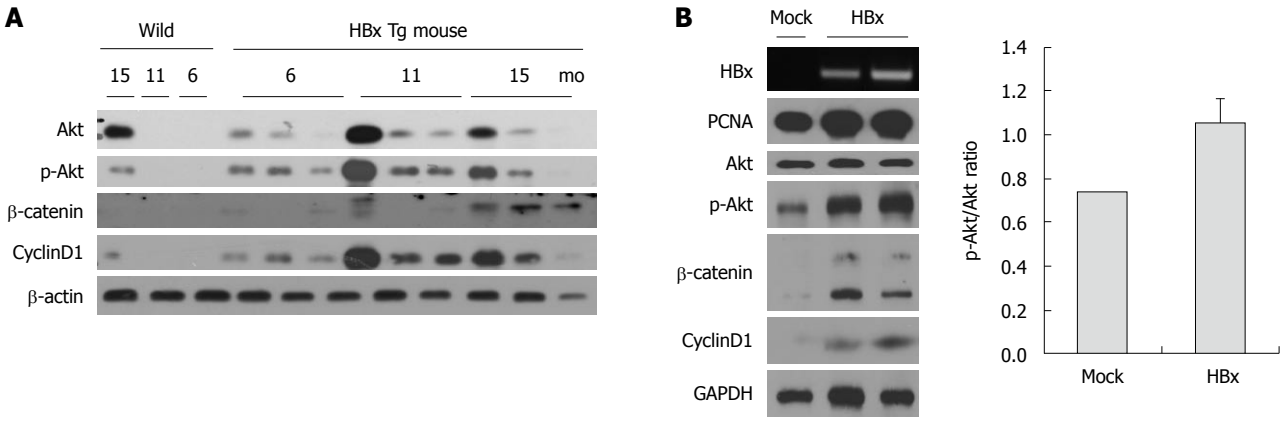

Figure 2 Effect of hepatitis B virus X-protein on activation of the Akt pathway. A: Activation of Akt pathway was examined by Western blotting with liver tissue extracts from 6-, 11- and 13-mo-old hepatitis B virus X-protein (HBx) transgenic and wild-type mice; B: Western blotting was also performed on extracts from stable HepG2-Mock and HBx cell lines.

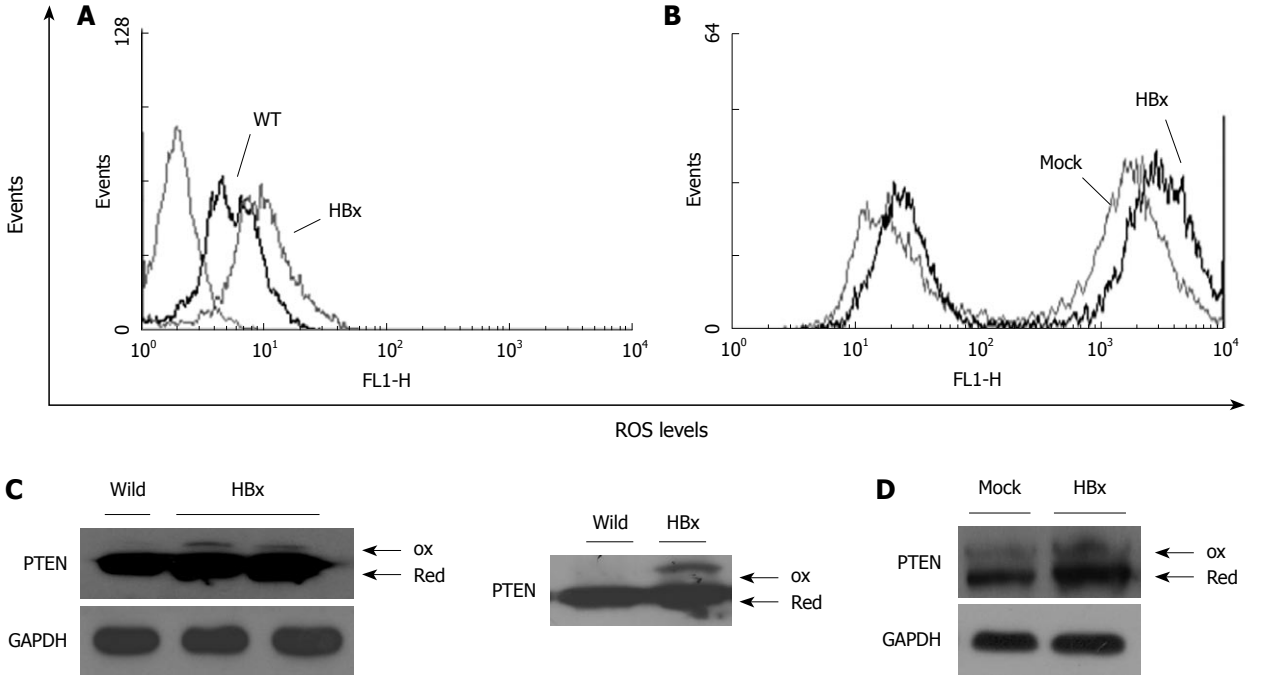

Figure 3 Effect of hepatitis B virus X-protein-induced endogenous reactive oxygen species and phosphatase and tensin homolog oxidation. Endogenous reactive oxygen species (ROS) level was examined by flow cytometry. A, B: Increased production of ROS in hepatitis B virus X-protein (HBx) primary hepatocytes compared to the wild-type hepatocytes and HepG2-HBx compared to the Mock cells. Oxidized phosphatase and tensin homolog (PTEN) was detected by N-ethylmaleimide alkylation, and non-reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis; C: In the upper panel, 20 μg protein was loaded, and for the lower panel, 50 μg was loaded; D: Parallel experiments were performed with extracts from HepG2-Mock and HBx cell lines.

cell lines might have activated Akt, total Akt per p-Akt of HepG2 HBx cells was increased 1.4-fold compared with the HepG2 control cells (Figure 2B).

HBx-induced endogenous ROS cause PTEN inactivation via cysteine oxidation

Peroxides are known to modify PTPs by oxidation. PTEN is also known to be inactivated through H_2O_2-mediated oxidation[20]. FACS analysis was used to verify HBx-induced ROS in mice and HepG2 cells. Primary hepatocytes were isolated from HBx transgenic and wild-type mice at the same age. ROS levels were significantly increased in HBx transgenic hepatocytes and HepG2-HBx cells compared to controls (Figure 3A and B). HBx expression was also associated with decreased mitochondrial membrane potential (data not shown). To examine the effect of HBx-induced ROS on PTEN inactivation, a PTEN oxidation assay was performed. HBx-expressing cells had higher ROS levels, and showed higher levels of oxidized PTEN when evaluated in primary hepatocytes and in HepG2 cells. HBx-induced ROS inactivated PTEN by promoting oxidation of cysteine residues within PTEN, thereby inactivating PTEN and promoting the function of Akt.

Inactivated PTEN correlates with upregulation of the PI3 kinase/ Akt pathway

To investigate the activation of Akt in the presence of ROS-inactivated PTEN, we examined the Akt pathway

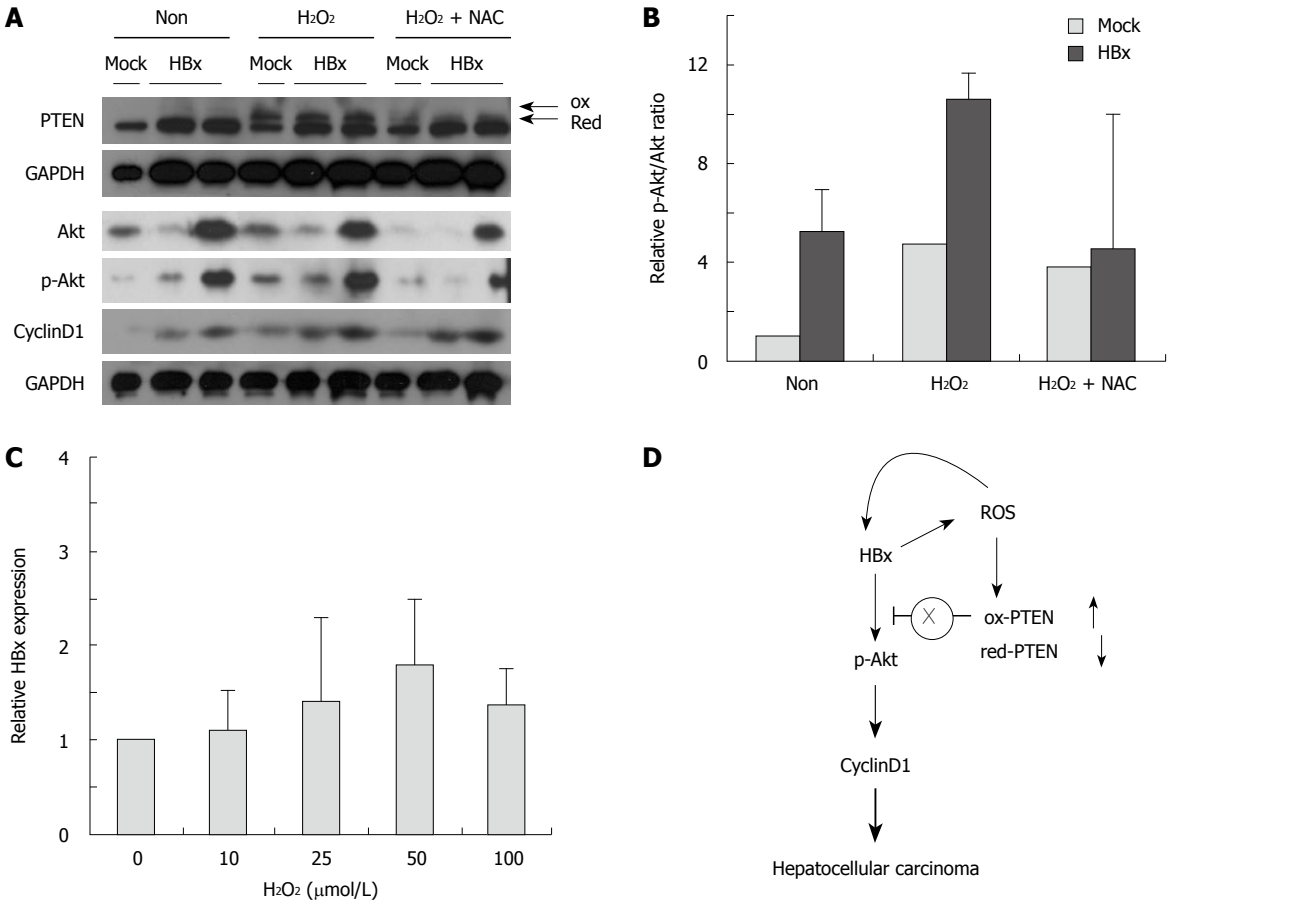

Figure 4 Effect of reactive oxygen species on phosphatase and tensin homolog oxidation, Akt pathway and hepatitis B virus X-protein expression. A, B: H_2O_2 treatment induced phosphatase and tensin homolog (PTEN) oxidation and activation of Akt pathway (increased relative p-Akt/total Akt ratio, cyclin D1 expression). Reactive oxygen species (ROS) scavenging through N-acetylcysteine (NAC) treatment reduced PTEN oxidation and Akt pathway; C: ROS effect on hepatitis B virus X-protein (HBx) expression, by quantitative reverse transcriptase polymerase chain reaction; D: Proposed scheme for ROS effect for activating Akt pathway *via* PTEN oxidation in HBx-induced hepatocarcinogenesis.

activity, which was detected in 0 and 500 μmol/L H_2O_2. Increases in oxidized PTEN were associated with a higher p-Akt/total Akt ratio and increased cyclin D1 expression. To investigate further whether induced ROS is required for activation of the Akt pathway, HepG2 cells were treated with H_2O_2 in the presence or absence of NAC, a ROS quencher. Scavenging ROS through NAC were able to block the Akt pathway (Figure 4A and B). These observations are consistent with the hypothesis that HBx-mediated generation of ROS inactivates PTEN, thereby activating the Akt pathway in carcinogenesis. In addition, elevated ROS was also associated with elevated levels of HBx (Figure 4C).

DISCUSSION

One of the HBV-encoded proteins, HBx, is considered to be a major risk factor for HCC. It is well known that HBx activates cell signal transduction pathways, such as PI3K. Mutations or inactivation of the tumor suppressor, PTEN, regulates Akt activation[21]. This is considered one of the reasons for activation of Akt signaling in cancer. For example, endogenously produced H_2O_2 has been shown to inactivate PTEN in a macrophage cell line and cancer cell lines[16,22]. In this study, HBx-triggered ROS were associated with the oxidation and functional inactivation of PTEN. Although quantification of the extent of PTEN oxidation in the cells was not possible, the level of oxidized, inactivated PTEN was associated with several factors, such as Akt activation and accelerated HepG2 cell growth, and thus might be associated with hepatocarcinogenesis in HBx transgenic mice. Both cell growth and abnormal actin filaments were observed in HepG2-HBx cells. It has been reported that reorganization of actin filaments can cause loss of focal adhesions and cell-cell contact, which leads to an epithelial-mesenchymal transition that consequently disrupts monolayer integrity[23]. The HBx-induced ROS appear to stimulate HBx expression further, which suggests the existence of a positive feedback loop. Such feedback would be expected to cause a rapid increase in the abundance of H_2O_2. This localized H_2O_2 accumulation would be expected to result in the oxidation of only those PTEN molecules located nearby, possibly explaining the small proportion of PTEN molecules that undergo oxidative inactivation in HepG2-HBx cells and mouse livers.

The scheme presented in Figure 4D represents the HBx-induced generation of H_2O_2. H_2O_2 participates in

intracellular signaling by targeting PTEN, and regulation of HBx gene expression, depending on the concentration. The results of the present study suggest that the HBx-mediated activation of Akt is regulated, at least in part, by the effects of HBx-induced ROS upon PTEN.

In summary, these studies further strengthen the case for a close relationship between oxidative stress and tumorigenesis. The studies reported herein have shown that HBx-induced generation of ROS can promote cellular transformation signaling by altering the function of PTEN. H_2O_2-oxidized PTEN leads to the activation of Akt. This is significant from a mechanistic as well as therapeutic point of view. Hence, drugs that scavenge endogenous ROS might slow down progression to HBx-induced liver cancer.

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COMMENTS

Background

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world. HCC is closely associated with hepatitis B virus (HBV) infection, especially in Asia. Among the HBV-encoding proteins, X protein (HBx) is a potential candidate for involvement in HBV-related HCC. One of the best-known pathways activated by HBx is phosphoinositide 3-kinase (PI3K)/Akt, which is associated with anti-apoptotic activity and cell proliferation. The reaction catalyzed by PI3K is reversed by phosphatase and tensin homolog (PTEN), which functions as a PI 3,4,5-trisphosphate 3-phosphatase. Indeed, by negatively modulating the PI3K signaling pathway, PTEN acts as a tumor suppressor.

Research frontiers

HCC is one of the cancers with poor prognosis. HBV carriers are approximately 100 times greater than in uninfected individuals. Finding a diagnostic marker and preventing severe liver damage are important areas in liver cancer research.

Innovations and breakthroughs

There have been several studies about HBx-induced reactive oxygen species (ROS). However, most of the studies have used *in vitro* models. This is believed to be the first study of HBx-induced ROS in mice and HepG2 cells, and the increased ROS promoted Akt pathways *via* oxidized inactive PTEN.

Applications

The suggestions in this study are significant not only from a mechanistic point of view - HBx-induced ROS activate the Akt pathway - but also from a therapeutic point of view - prevention of overactivation of the Akt pathway by scavenging ROS.

Peer review

In this experimental study, the molecular pathway of HBx-associated HCC tumorigenesis *via* PI3K/Akt was addressed. The authors demonstrated an important role for ROS as HBx-dependent tumorigenesis mediators. This paper is well written and concise.

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BRIEF ARTICLE

Peri-nuclear antibodies correlate with survival in Greek primary biliary cirrhosis patients

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Abstract

AIM: To investigate possible associations of anti-nuclear envelope antibody (ANEA) with disease severity and survival in Greek primary biliary cirrhosis (PBC) patients.

METHODS: Serum samples were collected at diagnosis from 147 PBC patients (85% female), who were followed-up for a median 89.5 mo (range 1-240). ANEA were detected with indirect immunofluorescence on 1% formaldehyde fixed Hep2 cells, and anti-gp210 antibodies were detected using an enzyme linked immunosorbent assay. Findings were correlated with clinical data, histology, and survival.

RESULTS: ANEA were detected in 69/147 (46.9%) patients and 31/147 (21%) were also anti-gp210 positive. The ANEA positive patients were at a more advanced histological stage (Ⅰ-Ⅱ/Ⅲ-Ⅳ 56.5%/43.5% *vs* 74.4%/25.6%, $P = 0.005$) compared to the ANEA negative ones. They had a higher antimitochondrial antibodies (AMA) titer (≤ 1:160/> 1:160 50.7%/49.3% *vs* 71.8%/28.2%, $P = 0.001$) and a lower survival time (91.7 ± 50.7 mo *vs* 101.8 ± 55 mo, $P = 0.043$). Moreover, they had more advanced fibrosis, portal inflammation, interface hepatitis, and proliferation of bile ductules ($P = 0.008$, $P = 0.008$, $P = 0.019$, and $P = 0.027$, respectively). They also died more frequently of hepatic failure and/or hepatocellular carcinoma ($P = 0.016$). ANEA positive, anti-gp210 positive patients had a difference in stage (Ⅰ-Ⅱ/Ⅲ-Ⅳ 54.8%/45.2% *vs* 74.4%/25.6%, $P = 0.006$), AMA titer (≤ 1:160/> 1:160 51.6%/48.4% *vs* 71.8%/28.2%, $P = 0.009$), survival (91.1 ± 52.9 mo *vs* 101.8 ± 55 mo, $P = 0.009$), and Mayo risk score (5.5 ± 1.9 *vs* 5.04 ± 1.3, $P = 0.04$) compared to the ANEA negative patients. ANEA positive, anti-gp210 negative patients had a difference in AMA titer (≤ 1:160/> 1:160 50%/50% *vs* 71.8%/28.2%, $P = 0.002$), stage (Ⅰ-Ⅱ/Ⅲ-Ⅳ 57.9%/42.1% *vs* 74.4%/25.6%, $P = 0.033$), fibrosis ($P = 0.009$), portal inflammation ($P = 0.018$), interface hepatitis ($P = 0.032$), and proliferation of bile ductules ($P = 0.031$). Anti-gp210 positive patients had a worse Mayo risk score (5.5 ± 1.9 *vs* 4.9 ± 1.7, $P = 0.038$) than the anti-gp210 negative ones.

CONCLUSION: The presence of ANEA and anti-gp210 identifies a subgroup of PBC patients with advanced disease severity and poor prognosis.

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Key words: Primary biliary cirrhosis; Antimitochondrial antibodies; Antinuclear antibodies; Antibodies against nuclear envelope antigens; Anti-gp210 antibodies

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INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic, progressive, cholestatic liver disease of probable autoimmune etiology, characterized by destruction of small intrahepatic bile ducts, portal inflammation, and, eventually, development of liver cirrhosis and hepatic failure. Elevated cholestatic enzymes, compatible liver histology, and detectable antimitochondrial antibodies (AMA), at titers higher than 1:40, are the three diagnostic criteria used for the diagnosis of PBC. Two of the three criteria suffice for making a definite diagnosis of PBC[1].

In fact, AMA are the specific marker of PBC, detected in 90%-95% of patients[2]. Antinuclear antibodies (ANA) are also present in 30% to 50% of patients[3-6], and are detected by indirect immunofluorescence (IIF) with various patterns. The peri-nuclear pattern is the most common IIF pattern of ANA, which detects nucleoporin 62 (p62)[7,8] and gp210[4] (nuclear pore complexes, NPC), as well as the lamin B receptor and lamin A/C[9,10], all antigens of the nuclear envelope. The multiple nuclear dot IIF pattern of ANA, is due to nuclear antigens such as sp100, SUMO, and PML. Another ANA IIF pattern found in PBC patients is the anti-centromere (ACA) one[11,12].

Although AMA are not associated with disease progression[1], ACA and anti-nuclear envelope antibody (ANEA) (ANA against the nuclear envelope antigens) seem to be associated with disease severity[4,13]. In fact, anti-NPC presence has been associated with more active and severe disease[7,13]; anti-gp210 and ACA positive patients have been reported to be associated with either hepatic failure or portal hypertension respectively[4,13,14]. However a study on Spanish and Greek patients did not confirmed these findings[15].

The purpose of the present study is to examine associations between the presence of serum ANEA and the severity and survival in a homogeneous cohort of Greek patients with AMA positive and negative PBC, in a single referral centre.

MATERIALS AND METHODS

Patients

From January 1989 to June 2009, 232 PBC patients were diagnosed by standard criteria and followed up at the Department of Gastroenterology of the University Hospital of Heraklion, Crete. Patients that had frozen (-80℃) serum collected at diagnosis, had negative hepatitis viral markers, were regularly followed up and gave their consent, were included in the study. These criteria were fulfilled by 147 patients. They were followed-up for 1-240 mo (mean 96.1 ± 55.8 mo, median 89.5 mo) after the initial diagnosis of PBC. Nineteen (12.9%) patients were AMA negative and 128 (87.1%) were AMA positive, with a titer > 1/40, M2 positive. Twenty-two patients (15%) were males and 125 (85%) were females. The mean age at diagnosis was 59.2 ± 10.9 years (median 60, range 31-80). According to the Ludwig classification, 97 patients (66%) were at an early stage (Ⅰ-Ⅱ), 50 (34%) were at a late stage (Ⅲ-Ⅳ), of whom 32/50 were at stage Ⅳ. The mean Mayo risk score at the time of diagnosis was 5.1 ± 1.6. All patients were treated with ursodeoxycholic acid (UDCA) at a dose of 13-15 mg/kg, starting after the time of serum collection. Other coexisting autoimmune diseases were: Sjogren syndrome in 12 patients, Raynaud phenomenon in two, psoriasis in one, sarcoidosis in one, discoid lupus erythematosus in one, autoimmune atrophic gastritis in two, and vitiligo in one.

During the follow-up period 14 patients developed variceal bleeding, 15 developed ascites, two developed hepatic encephalopathy, and six developed hepatocellular carcinoma. Four patients underwent orthotopic liver transplantation. Thirty patients died during follow-up (five from liver unrelated causes). The remaining 117 patients are alive and are still being followed up at our Department at the end of the study. The study was approved by the Ethics Committee of the Hospital. All patients have given a written, informed consent.

Methods

The Autoantibodies studied were correlated with clinical data, histology at diagnosis, the major events occurring during the follow-up period, and survival.

Ninety-eight biopsy specimens with more than three portal tracts were reexamined and graded by a single pathologist. The histological variables analyzed included: fibrosis (1-3) (1 = mild, 2 = moderate, 3 = severe), interface hepatitis (0-3) (0 = absent, 1 = mild, 2 = moderate, 3 = severe), portal inflammation (1-3) (1 = mild, 2 = moderate, 3 = severe), intralobular inflammation (0-2) (0 = absent, 1 = mild, 2 = moderate), epithelioid granulomas (0-1) (0 = absent, 1 = present), and proliferation of bile ductules (0-1) (0 = absent, 1 = present).

Cell culture

Human Hep2 cells (larynx and cervical carcinoma) were used. The cells were cultured in Minimum Essential Medium supplied with 10% Fetal Bovine Serum 100 U/mL penicillin/streptomycin and 1% non-essential amino acids.

They were maintained in humidified atmosphere at 37℃ and 5% CO_2. All the culture media were from Gibco (Invitrogen, UK)

IIF for serum autoantibody analysis

An IIF assay of Nova Lite™ (IFA) ANA plus Mouse Kidney & Stomach (Inova Diagnostics, San Diego CA, Inc) was used for screening and semi-quantitative determination of AMA IgG antibodies, according to the manufacture's instructions.

IIF was performed for detection of ANEA, as previously described[16]. Briefly, Hep2 cells were grown overnight on coverslips and washed with PBS (Gibco, Invitrogen, UK).

The cells were fixed with 1% and 4% formaldehyde (Sigma-Aldrich, Germany) for 10 min. The cells were washed with PBS. To quench auto-fluorescence and enhance antigenicity, the fixed cells were incubated with 20 mmol/L glycine (Sigma-Aldrich, Germany) in PBS for 5 min. After blocking with PBS containing 0.2% TritonX-100, 2 mmol/L $MgCl_2$, and 1% gelatin from cold water fish skin (Sigma-Aldrich, Germany) for 10 min, cells were incubated with serum (dilution 1:80) in blocking buffer for 45 min. Subsequently, the coverslips were washed with blocking buffer for 10 min and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG (dilution 1:500, H+L secondary antibody, Chemicon, Millipore, Germany) for 45 nin. Finally, cells were rinsed in PBS and mounted with mounting medium containing Dapi (Santa Cruz Biotechnology, Inc, Germany). All steps were performed at room temperature. Fluorescence was observed under a Leica SP confocal microscope.

Enzyme linked immunosorbent assay for gp210

For the assessment of anti-gp210 antibodies, a Quanta lite™ enzyme linked immunosorbent assay (ELISA) (Inova Diagnostics, San Diego CA, Inc) kit was used according to the manufacture's instructions.

Statistical analysis

Data are presented as percentages (%) or as mean ± SD, unless otherwise stated. Differences between autoantibody positive and negative patients for various clinical, histological, and serological measurements were compared using multivariate regression analysis. The survival time was estimated by the Kaplan-Meier method, and compared by the Breslow test. Fisher's exact test was used to compare causes of death between ANEA positive and negative and gp-210 positive and negative patients. A P value < 0.05 was considered significant. Statistical analyses were performed using SPSS v.15.0 and Excel 2003 software.

RESULTS

Fixation was important in visualization of ANEA by immunofluorescence, 1% fixation allowed for much better discrimination of antinuclear antibodies (Figure 1).

Parameters used in multivariate analysis are shown in Tables 1-3. The ANEA were detected by IIF on Hep2 cells giving a typical peri-nuclear staining pattern (Figure 1). ANEA were detected in 69 (46.9%) of 147 patients. Comparisons between ANEA positive and negative patients are shown in Tables 1 and 2. Although there was no significant difference in the number of alive/dead between positive and negative ANEA patients [51 (77.3%)/15 (22.7%) *vs* 66 (86.8%)/10 (13.2%), NS], there was a statistical significance in survival period between the two groups (91.7 ± 50.7 mo *vs* 101.8 ± 55 mo, P = 0.043) (Table 1 and Figure 2). Moreover, causes of death were significantly different between ANEA positive and negative patients (Figure 3).

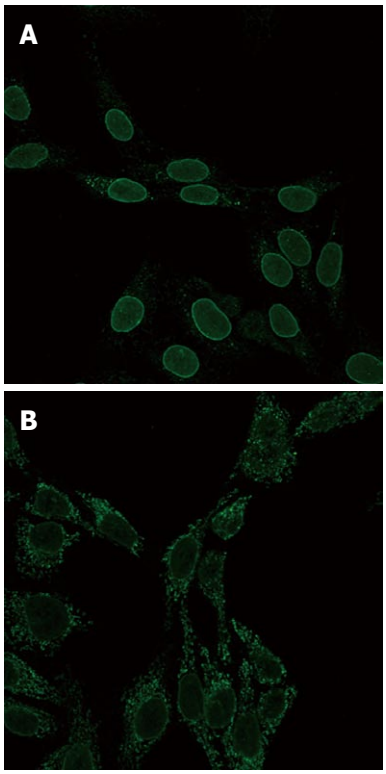

Figure 1 Typical peri-nuclear staining showing anti-nuclear envelope antibody positive sera in indirect immunofluorescence. A: Cells fixed with 1% formaldehyde; B: Cells fixed with 4% formaldehyde.

Table 1 Comparison of clinical parameters between anti-nuclear envelope antibody positive and anti-nuclear envelope antibody negative patients (mean ± SD) *n* (%)

	ANEA		*P* value
	Positive	Negative	
Patients	69 (46.9)	78 (53.1)	
Age (yr)	59.3 ± 11.8	59.1 ± 10.1	0.330
AMA titer ≤ 1:160/ > 1:160[1]	35 (50.7)/34 (49.3)	56 (71.8)/22 (28.2)	0.001
Stage Ⅰ-Ⅱ/ stage Ⅲ-Ⅳ	39 (56.5)/30 (43.5)	58 (74.4)/20 (25.6)	0.005
Alive/dead	51 (77.3)/15 (22.7)	66 (86.8)/10 (13.2)	0.162
Survival	91.7 ± 50.7	101.8 ± 55	0.043
Mayo risk score	5.19 ± 1.8	5.04 ± 1.3	0.239

[1]1/160 is the median antimitochondrial antibodies titer. ANEA: Anti-nuclear envelope antibody.

Table 2 Comparison of histological parameters between anti-nuclear envelope antibody positive and anti-nuclear envelope antibody negative patients *n* (%)

	Histological parameters		*P* value
	ANEA positive (*n* = 69)	ANEA negative (*n* = 78)	
Fibrosis			
1	10 (22.7)	22 (40.7)	0.008
2	17 (38.6)	22 (40.7)	
3	17 (38.6)	10 (18.6)	
Portal inflammation			
1	8 (18.2)	22 (40.7)	0.008
2 + 3	36 (81.8)	32 (59.3)	
Interface hepatitis			
0 + 1	16 (36.4)	31 (57.4)	0.019
2 + 3	28 (63.6)	23 (42.6)	
Intralobular inflammation			
0	11 (25)	9 (16.7)	0.359
1	19 (43.2)	35 (64.8)	
2	14 (31.8)	10 (18.5)	
Proliferation of bile ductules			
0	12 (27.3)	25 (46.3)	0.027
1	32 (72.7)	29 (53.7)	
Epithelioid granuloma			
0	31 (70.5)	39 (72.2)	0.425
1	13 (29.5)	15 (27.8)	

ANEA: Anti-nuclear envelope antibody.

Table 3 Comparison of histological parameters between anti-nuclear envelope antibody positive, gp210 negative, and anti-nuclear envelope antibody negative patients *n* (%)

	Histological parameters		*P* value
	Gp210 negative (*n* = 38)	ANEA negative (*n* = 78)	
Fibrosis			
1	7 (25.0)	22 (40.7)	0.009
2	8 (28.6)	22 (40.7)	
3	13 (48.4)	10 (18.6)	
Portal inflammation			
1	5 (17.9)	22 (40.7)	0.018
2 + 3	23 (82.1)	32 (59.3)	
Interface hepatitis			
0 + 1	10 (35.7)	31 (57.4)	0.032
2 + 3	18 (64.3)	23 (42.6)	
Intralobular inflammation			
0	8 (28.6)	9 (16.7)	0.274
1	14 (50.0)	35 (64.8)	
2	6 (21.4)	10 (18.5)	
Proliferation of bile ductules			
0	7 (25.0)	25 (46.3)	0.031
1	21 (75.0)	29 (53.7)	
Epithelioid granuloma			
0	19 (67.9)	39 (72.2)	0.342
1	9 (32.1)	15 (27.8)	

ANEA: Anti-nuclear envelope antibody.

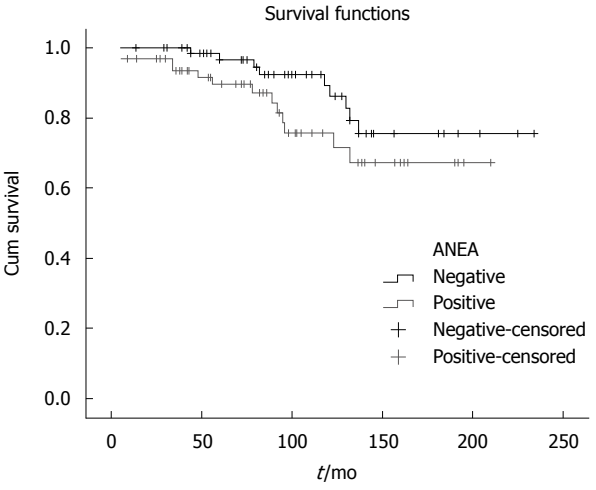

Figure 2 Kaplan-Meier curve of survival between anti-nuclear envelope antibody negative and anti-nuclear envelope antibody positive patients (*P* = 0.043 by Breslow test). ANEA: Anti-nuclear envelope antibody.

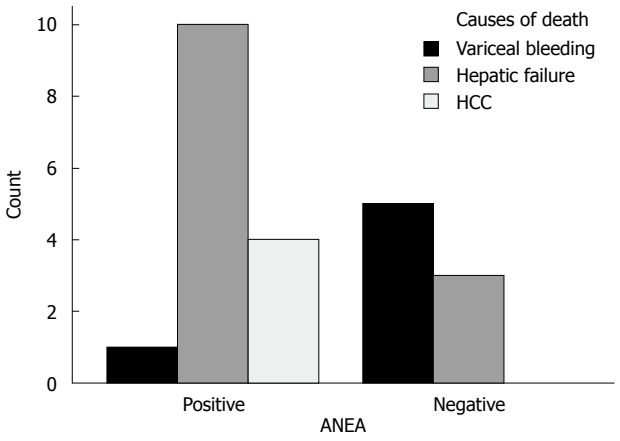

Figure 3 Causes of death between anti-nuclear envelope antibody positive and anti-nuclear envelope antibody negative patients (*P* = 0.016). Anti-nuclear envelope antibody (ANEA) positive patients died more frequently of hepatic failure and/or hepatocellular carcinoma (HCC), while ANEA negative patients died more frequently as a result of variceal bleeding.

AMA titers

AMA titers were not associated with disease severity. Kaplan-Meier analysis showed $P > 0.7$ when AMA titers were examined in relation to patient survival.

Anti-Gp210

We tested all 69 ANEA positive patients (18 dead, five from liver unrelated death) for the anti-gp210 antibodies by ELISA and found 38 (55.1%) negative and 31 (44.9%) positive, representing 21% of all studied patients.

Comparing the anti-gp210 positive patients ($n = 31$) with the ANEA negative patients ($n = 78$) we found significantly higher AMA titer (≤ 1:160/> 1:160 51.6%/48.4% *vs* 71.8%/28.2%, $P = 0.009$), more late stages (Ⅰ-Ⅱ/Ⅲ-Ⅳ 54.8%/45.2% *vs* 74.4%/25.6%, $P = 0.006$), higher Mayo risk score (5.5 ± 1.9 *vs* 5.04 ± 1.3, $P = 0.04$) and shorter survival period (91.1 ± 52.9 mo *vs* 101.8 ± 55 mo, $P = 0.009$) (Figure 4).

Comparing the 38 ANEA positive-gp210 negative patients with the 78 ANEA negative patients, we found that the ANEA negative ones had lower AMA titers (≤ 1:160/> 1:160 50%/50% *vs* 71.8%/28.2%, $P = 0.002$), earlier stage (Ⅰ-Ⅱ/Ⅲ-Ⅳ 57.9%/42.1% *vs* 74.4%/25.6%, $P = 0.033$), less severe fibrosis, portal inflammation, in-

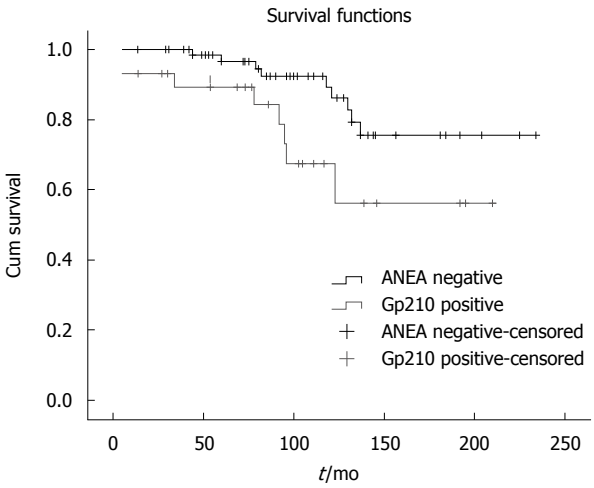

Figure 4 Kaplan-Meier curve of survival between anti-gp210 positive and anti-nuclear envelope antibody negative patients (P = 0.009 by Breslow test). ANEA: Anti-nuclear envelope antibody.

terface hepatitis, and proliferation of bile ductules (P = 0.009, P = 0.018, P = 0.032 and P = 0.031, respectively) (Table 3).

Between anti-gp210 positive (n = 31) and ANEA positive, anti-gp210 negative (n = 38) patients the only parameter that differed was Mayo risk score (5.5 ± 1.9 *vs* 4.9 ± 1.7, P = 0.038). No difference between the two groups was found for any of the other clinical, demographic, histological parameters or for survival (mean 91.1 ± 52.9 and 92 ± 49.6 mo respectively).

Causes of death were no different between gp-210 positive and negative patients.

DISCUSSION

In recent years, significant steps have been made in the clarification of the pathogenesis of PBC, although definitive data have not yet been provided[17,18]. Moreover, the mechanisms controlling disease severity and, therefore, prognosis in this clinically heterogeneous disease are still not well established. In recent years, more patients have been diagnosed in at the presymptomatic or asymptomatic stage than before. Some of these patients undergo a benign course and others a more progressive one, with early appearance of symptoms and rapid deterioration, leading to liver transplantation or death[1,19,20].

Prognostic scores based on clinical parameters (Mayo risk score and bilirubin) have been developed in patients with advanced disease, but they have not been validated in presymptomatic or asymptomatic patients.

Earlier studies failed to demonstrate an association of disease severity and progression, with ANEA[21,22]. However in 2001, Invernizzi *et al*[7] reported an association of antibodies to NPC with disease activity and severity, which was confirmed, in particular for anti-gp210, in Italian PBC patients two years later[23]. In American and Canadian patients, ANEA were associated with increased risk of liver failure[24].

Nakamura *et al*[4] in 276 Japanese PBC patients reported a prevalence of 26% for anti-gp210. In that study, anti-gp210 presence correlated with survival and a hepatic failure pattern of disease progression. By contrast, in a cohort of 170 Spanish and 162 Greek patients, only 10.4% of patients were anti-gp210 positive. In that study, there was no correlation with survival or histological severity, although correlations with Mayo risk score, ALP, and bilirubin were reported[15]. The authors stated that the low prevalence could be an ethnic or geographic variation and that, although anti-gp210 represents a disease severity marker, it is not a prognostic one. However, our results of anti-gp210 prevalence, using the same ELISA kits, in a homogeneous Greek population from the island of Crete, are similar to the Japanese report.

Similarly to the Japanese report, our ANEA positive patients died more frequently of hepatic failure and/or hepatocellular carcinoma, while ANEA negative patients died more frequently as a result of variceal bleeding.

Indeed, we found that 46.9% of the patients were ANEA positive and 21% anti-gp210 positive, a similar percentage to the Japanese patients (26%). The ANEA positive patients at the time of diagnosis were at later histological stages, with more severe fibrosis, portal inflammation, bile ductular proliferation, and interface hepatitis. They had higher AMA titers and, as expected, shorter survival periods than the ANEA negative patients. The anti-gp210 positive patients differed from the rest of the ANEA positive patients only in their higher Mayo risk scores. It should be noted that, with the usual 4% formaldehyde fixation used with Hep2 cells, there might be a difficulty in discrimination between peri-nuclear and cytoplasmic fluorescence caused by the presence of high titers of antimitochondrial antibodies. By contrast, our slight modification using 1% fixation instead allowed for much better visualization of peri-nuclear staining; therefore, we strongly recommend this fixation for further use.

In conclusion, our data confirm that, in Greek PBC patients, there is a correlation between the presence of ANEA antibodies and disease severity and shorter survival. The presence of anti-gp210 seems to be an additional factor, reducing survival. Therefore, we suggest that presence of ANEA and anti-gp210 should be routinely checked, because their presence identifies a subgroup of PBC patients with poor prognosis. The mechanism underlying the association of ANEA with prognosis requires further elucidation.

COMMENTS

Background

Primary biliary cirrhosis (PBC) is an autoimmune disease of unknown etiology, where genetic and environmental factors have roles in disease pathogenesis. The clinical course of the disease is variable, and no specific serological markers can predict disease progression. Peri-nuclear antibodies have not been adequately evaluated as predictive markers and the results so far are contradictory.

Research frontiers

Peri-nuclear antibodies were evaluated using a modification of the immunofluorescent identification technique, which allows for better visualization of these antibodies and avoids a possible confusion with antimitochondrial antibodies. The presence of anti-nuclear envelope antibody (ANEA), was associated with decreased patient survival and causes of death.

Innovations and breakthroughs

The modified technique might explain the reported differences with other European patient cohorts. Moreover, the patient population in this study is racially homogeneous, thus excluding possible racial differences as a factor of genetic influence in the results.

Applications

The findings of the present study suggest that identification of the presence of ANEA should be included in the routine work up of patients with PBC, because they identify a subgroup with worse prognosis, for whom a more intense follow up scheme should be applied.

Peer review

In this manuscript, the authors describe the apparent association between antinuclear antibodies, especially ANEA, and the severity and survival of 147 patients with PBC in a single-center cohort in Greek. They examined ANEA and anti-gp210 antibodies in sera at diagnosis and found that ANEA positivity, as well as anti-gp210 positivity, could identify a subgroup of PBC patients with poor prognosis. These results are coincident with previous results from Japan and look very interesting.

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BRIEF ARTICLE

Pancreatic function, quality of life and costs at long-term follow-up after acute pancreatitis

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Abstract

AIM: To evaluate long-term endocrine and exocrine pancreatic function, quality of life and health care costs after mild acute pancreatitis and severe acute pancreatitis (SAP).

METHODS: Patients prospectively included in 2001-2005 were followed-up after 42 (36-53) mo. Pancreatic function was evaluated with laboratory tests, the oral glucose tolerance test (OGTT), fecal elastase-1 and a questionnaire. Short Form (SF)-36, was completed.

RESULTS: Fourteen patients with a history of SAP and 26 with mild acute pancreatitis were included. Plasma glucose after OGTT was higher after SAP (9.2 mmol/L *vs* 7.0 mmol/L, $P = 0.044$). Diabetes mellitus or impaired glucose tolerance in fasting plasma glucose and/or 120 min plasma glucose were more common in SAP patients (11/14 *vs* 11/25, $P = 0.037$). Sick leave, time until the patients could take up recreational activities and time until they had recovered were all longer after SAP ($P < 0.001$). No significant differences in SF-36 were seen between the groups, or when comparing with age and gender matched reference groups. Total hospital costs, including primary care, follow-up and treatment of complications, were higher after SAP (median €16 572 *vs* €5000, $P < 0.001$).

CONCLUSION: Endocrine pancreatic function was affected, especially after severe disease. SAP requires greater resource use with long recovery, but most patients regained a good quality of life.

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Key words: Acute pancreatitis; Endocrine function; Exocrine function; Quality of life; Cost

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INTRODUCTION

Acute pancreatitis is associated with a mild and uneventful course in 80% of cases. However, severe acute pancreatitis (SAP) is associated with the risk of complications both in and around the pancreas, and eventually has a fatal outcome in up to 15%-20% of cases[1]. Exocrine and endocrine pancreatic function has mainly been studied after SAP, with divergent results[2-9]. In mild, edematous-interstitial cases of acute pancreatitis, the pancreas can recover completely. After SAP, however, morphological changes may often remain and functional recovery is not always complete[3,4,9]. Impairment of exocrine pancreatic function with steatorrhea has been reported to diminish over time[8,10]. Damaged pancreatic acinar cells may recover with improvement in pancreatic function[4]. However,

endocrine dysfunction with diabetes mellitus (DM) seems to be more common over time[9,11].

Until recently, results of treatment strategies were determined only in terms of cure, impairment of pancreatic functions, disability or death. However, there is a need for critical assessment of outcome measures that also include quality of life and health economical aspects. In some studies, SAP patients have been reported to regain a satisfactory quality of life[12-15], while others report a clear impairment[16,17].

Severe disease requires prolonged hospitalization, frequently including a stay in the intensive care unit (ICU). Sometimes there is also a need for multiple radiological and surgical interventions. Long rehabilitation of survivors can be expected, resulting in not only a great challenge for the individual but also a substantial need for resources together with high associated costs for society. There have only been a few previous cost analyses performed for acute pancreatitis[11,15,18-21].

The aim of the present study was to evaluate both pancreatic endocrine and exocrine function, as well as general recovery, quality of life and costs in patients who had recovered from mild acute pancreatitis, as well as from SAP, and to compare these groups at long-term follow-up.

MATERIALS AND METHODS

Study population

Case records from patients with mild acute pancreatitis and SAP, previously evaluated for participation in 2 nutritional studies[22,23], were examined. Severity definition was performed according to the Atlanta classification[24]. Exclusion criteria were dementia, malignancy, an additional history of SAP and chronic pancreatitis. Two out of the 20 SAP patients had died, one had dementia and 2 had developed chronic pancreatitis. In total, 15 patients with SAP, and a group of 30 gender- and age-matched patients from the group with mild disease were asked to participate in this follow-up survey. Invitation was performed by letter followed by a telephone call, offering an appointment at the outpatient clinic. Five patients, one of whom was from the SAP group, declined to participate. Finally, 14 patients with a history of SAP and 26 with a history of mild disease were included. Thus, 40 patients participated in this long-term evaluation for a median of 42 (36-53) mo after the episode of acute pancreatitis. APACHE Ⅱ, scored in all patients at initial admission to hospital, was a median of 7 (6-10) in patients who later developed severe disease and 6 (5-7) in the group of patients with mild disease ($P = 0.012$). Five out of 14 patients in the SAP group had an APACHE Ⅱ < 8 and 3/26 in the group with mild acute pancreatitis had a score ≥ 8.

All patients were seen by the same surgeon at the outpatient clinic. A thorough physical and physiological investigation was performed. Blood samples were taken after fasting and during an oral glucose tolerance test (OGTT), and a fecal sample was collected. All patients completed a questionnaire examining current pancreatic function, medication, abdominal surgical interventions, eating and drinking habits, readmissions for pancreatitis, ability to return to normal daily activity and time until they had recovered from the acute pancreatitis episode. Actual working capacity (including retirement/early retirement/sick leave/still working) was evaluated. Body weight and height was measured. Quality of life forms were completed. Several aspects of the patients' current condition were evaluated, using a visual analogue scale (VAS: 0-100).

Exocrine pancreatic function

Fecal elastase-1 concentration, a specific human protease synthesized by the acinar cells, was measured in stool samples using a commercial enzyme-linked immunosorbent assay, (ScheBo Biotech, Giessen, Germany). It is non-invasive, stable and correlates well with exocrine pancreatic function tests[25,26]. A value > 200 μg elastase/g stool is considered normal. Subjective pancreatic function was evaluated *via* a questionnaire, including questions about the incidence of abdominal discomfort, bowel habit including frequency of defecation, presence of diarrhea and steatorrhea, intolerance to fat and other food, unintentional weight loss and use of pancreatic enzyme supplementation.

Endocrine pancreatic function

Fasting plasma (FP) glucose, C-peptide and insulin were measured in all patients. In non-diabetic patients ($n = 39$), a 75 g, 2 h OGTT was performed to detect impaired glucose tolerance (IGT) and DM. Glucose and C-peptide were measured in venous plasma at 0, 15, 30, 60 and 120 min. Insulin was determined at 0 and 120 min. The guidelines and definitions established by the World Health Organisation were followed[27]. FP glucose $\geq$ 7.0 mmol/L met the criteria for DM and 6.1-6.9 mmol/L for IGT. OGTT plasma glucose values $\geq$ 11.1 mmol/L at 2 h were defined as DM and values $\geq$ 7.8 and $<$ 11.1 mmol/L as IGT. Measurements of baseline and stimulated insulin and C-peptide values allowed the differentiation of DM induced by insulin resistance or beta cell failure. The homeostasis model assessment (HOMA) for evaluating insulin resistance [HOMA IR = fasting insulin (mIE/mL) × FP glucose (mmol/L)/22.5] was calculated[28]. Fasting glycosylated hemoglobin A1c (HBA1c) was measured for assessing long-term glucose homeostasis.

Quality of life

The Swedish version of Standard Short Form 36 (SF-36), a widely used general quality-of-life questionnaire that has been validated in a variety of medical settings, was used[29]. The SF-36 examines 8 areas consisting of social and physical function, physical and emotional well-being, bodily pain, vitality, mental health and overall general health perception. Swedish normative data of age-matched controls were used for comparison.

Costs

Costs were calculated as total hospital costs per patient at the primary hospital stay, including expenses on the ward,

ICU stay, anesthesia and operating costs, radiological and clinical physiology expenses, and costs for laboratory analysis and blood products. Subsequent costs, both for in-hospital stay and outpatient care, directly related to the primary acute pancreatitis episode, were also calculated. Sick leave days were retrieved from the patient's medical records and from the patients at follow-up. All costs are given in 2008 price levels, inflated using the Swedish consumer price index. The costs have been converted from Swedish krona (SEK) to Euros (€) using the yearly average exchange rate for 2008 (9.6055 SEK to €1).

Statistical analysis

Continuous variables are presented as medians with 25th and 75th percentiles. Categorical variables are given as frequencies and percentages. Univariate analysis for continuous variables was conducted with the Wilcoxon test. Categorical variables were analyzed by the χ^2-test, except when expected frequencies were less than 5, in which case Fisher's exact test was used. The Kaplan-Meier estimate was used to calculate time to event. The log-rank test was used to compare the difference between the groups. Data were analyzed using Hmisc, Survival and Design packages of the R software (R Foundation for Statistical Computing, Vienna, Austria), version 2.8.1. The level of significance was set at $P < 0.05$. The study was approved by the Human Ethics Committee, Lund University.

RESULTS

Of the 40 patients finally included in the study, 16 (40%) were men. The median age was 61 (48-68) years at follow-up. Body mass index was 28 (26-31) kg/m^2, with no difference between patients with severe or mild acute pancreatitis. Patient data from the episode with acute pancreatitis are presented in Table 1. There was no difference in follow-up time between the groups. Most routine laboratory parameters, such as hemoglobin, creatinine, calcium, bilirubin and lipase showed no difference at follow-up, though pancreas-specific amylase was lower in patients with a history of SAP, and alanine aminotransferase was higher (Table 2). Sick leave, time until the patients could regain recreational activities and time until recovery were all significantly longer after SAP (Table 2). A total of 7 patients from both groups did not feel that they had fully recovered at the time of follow-up, a median 39 (33-49) mo after the episode of acute pancreatitis (Figure 1). There was no difference between the groups when using the subjective VAS for expressing abdominal pain, fatigue, nausea, anxiety, working capacity and energy for recreational activities. The question whether actual difficulties were thought to be late effects of the acute pancreatitis episode also showed no difference comparing SAP and mild acute pancreatitis, 3 (2-43) *vs* 3 (0-13), $P = 0.33$. Additional hospital visits, including both scheduled follow-ups and emergency visits due to abdominal pain, excluding any additional acute pancreatitis episodes, were more common after severe disease. In SAP, 12 (86%) patients had one or more visits and in the mild group, 7 (27%) had only one and none any additional visits, $P < 0.001$. At follow-up, no patient in either the SAP or the mild group used medication regularly for abdominal pain.

Table 1 Patient characteristics and parameters during the acute care for pancreatitis, divided into mild and severe acute pancreatitis cases

Parameter	Mild acute pancreatitis	Severe acute pancreatitis	All patients	Difference between groups (*P*)
Gender, male	10	6	16	0.79
Etiology				
Gallstone	16 (62)	4 (29)	20 (50)	0.096
Alcohol	5 (19)	5 (36)	10 (25)	0.278
Post ERCP	0	3 (21)	3 (8)	0.037
Unknown	5 (19)	2 (14)	7 (18)	1.0
Other diseases	13 (50)	5 (36)	18 (45)	0.39
ASA class[1]	1.5 (1-2)	1 (1-1.75)	1 (1-2)	0.14
APACHE II[1]	6 (5-7)	7 (6-10)	6 (5-7)	0.012
Weight loss	13 (50)	11 (79)	24 (60)	0.079
Weight loss in kg[1]	5 (1.5-6)	9 (7-10)	6.4 ± 4.7 6 (3-9)	0.003
Pancreatic surgery	0	4 (29)	4 (10)	0.004
Pancreatic pseudocysts	0	7 (50)	7 (18)	< 0.001
Hospital stay in days[1]	7.5 (4-9)	18 (16-24)	10 (6-16)	< 0.001
ICU stay	0	8 (57)	8 (20)	< 0.001

Values are median and in parentheses are percentages or [1]interquartile range. ERCP: Endoscopic retrograde cholangiopancreatography; ASA: American Society of Anesthesiologists; APACHE Ⅱ: Acute Physiology and Chronic Health Evaluation Ⅱ; ICU: Intensive care unit.

Table 2 Patient characteristics and parameters at follow-up after mild and severe acute pancreatitis

Parameter	Mild acute pancreatitis	Severe acute pancreatitis	All patients	Difference between groups (*P*)
Time to follow-up (mo)	41 (35-50)	47 (37-63)	42 (36-53)	0.14
Age (yr)	61 (51-70)	58 (45-67)	61 (48-68)	0.72
BMI (kg/m^2)	27 (25-32)	29 (26-31)	28 (26-31)	0.82
ASA	2 (1-2)	1 (1-2)	1.5 (1-2)	0.68
Serum pancreas amylase (μkat/L)	0.45 (0.37-0.53)	0.27 (0.18-0.43)	0.043 (0.27-0.52)	0.007
P-ALAT (μkat/L)	0.30 (0.23-0.47)	0.39 (0.33-0.54)	0.34 (0.29-0.50)	0.035
Sick leave days	14 (7-30)	120 (70-165)	30 (14-97)	< 0.001
Time to activity (d)	10.5 (0-21)	90 (60-365)	21 (2-60)	< 0.001
Time to recovery (d)	21 (14-60)	270 (180-[1])	60 (14-365)	0.005

[1]Less than 75% of the patients had recovered at time of follow-up. Values are given as median (interquartile range). BMI: Body mass index; ASA: American Society of Anesthesiologists; P-ALAT: Plasma alanine aminotransferase.

Exocrine pancreatic function

There were no statistically significant differences between the severe and mild groups concerning incidence of steatorrhea (1/14 *vs* 2/26), change in bowel habits (4/14 *vs* 10/25) or the need for pancreatic enzyme supplementa-

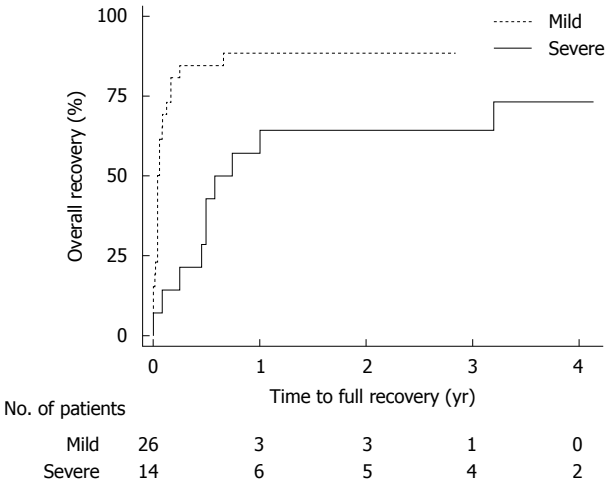

Figure 1 Log-rank test for time to recovery after acute pancreatitis in mild and severe acute pancreatitis patients.

tion (2/14 *vs* 1/26). Most patients had weight loss during the acute disease. At the time of follow-up, 4/14 *vs* 5/26 still had a decreased weight compared to body weight before the disease, with no difference between the groups. A change in diet was more common after SAP (9/14 *vs* 6/26, $P = 0.01$). Fecal elastase-1 was found to be decreased only in one of the patients (with SAP). Plasma albumin did not differ between the groups.

Endocrine pancreatic function

One patient with a history of mild acute pancreatitis was excluded from this part of the follow-up because of type 2 DM, already treated with insulin before the acute pancreatitis episode. Another patient had a history of insulin treatment starting immediately after the SAP episode, but had no treatment for DM for the 9 mo prior to the acute episode. FP glucose was 5.2 mmol/L and the patient was included in the OGTT. FP glucose had a tendency to be higher and plasma glucose was higher after the OGTT in patients with a history of SAP as compared to those with mild acute pancreatitis ($P = 0.055$ and $P = 0.044$, respectively; Figure 2). A higher level was also registered for HBA1C ($P = 0.041$). Patients with a history of SAP more frequently fulfilled the criteria for DM and/or IGT in either FP glucose or 120 min plasma glucose, or both, (11/14 *vs* 11/25, $P = 0.037$). There was no significant difference in the incidence of DM when comparing different etiologies of acute pancreatitis; 4/10 with alcohol as the etiological factor and 3/19 with underlying gallstone disease had DM ($P = 0.193$). The 4 patients subjected to pancreatic surgery all fulfilled the criteria for DM or IGT. Plasma C-peptide was higher in patients fulfilling the criteria for DM, both fasting and after the OGTT, and a significant difference was also seen for serum insulin (Table 3). Fasting C-peptide as well as HOMA-IR had a tendency, although not significant, to be lower in patients with DM and/or IGT after severe as compared to mild disease.

Quality of life

An exact gender- and age-matched reference group (n = 84) was randomly selected for the SAP group from the Swedish SF-36 norm database (n = 8930). Six control subjects were used for each patient (quota = 6:1). The numbers of reference subjects were decided from the lowest number representing one study patient (female, 83 years old). The corresponding figures for mild acute pancreatitis had a reference group of 182 persons, and a quota = 7:1 decided from the lowest number representing one study patient (male, 79 years old). No significant differences were

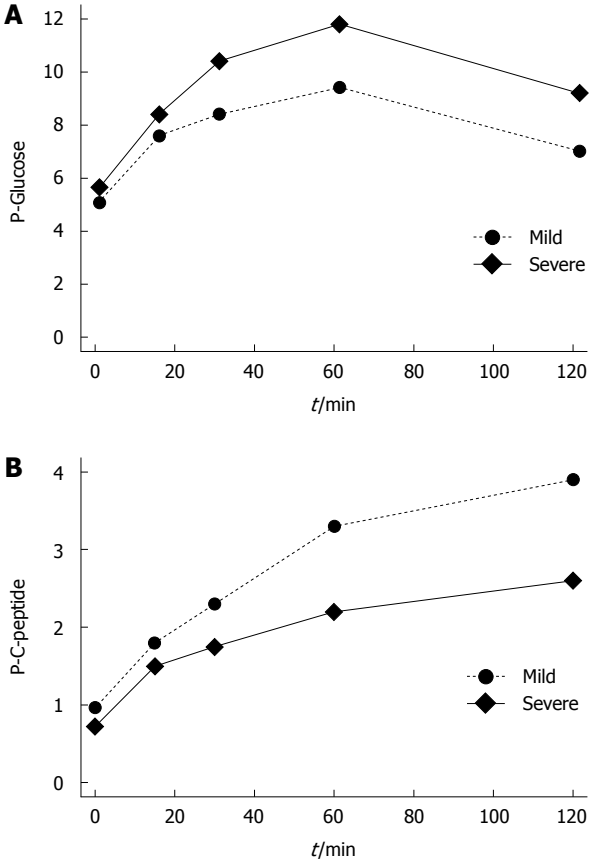

Figure 2 Relationship between mild and severe acute pancreatitis patients in the oral glucose tolerance test evaluating plasma glucose (mmol/L, A) and C-peptide values (nmol/L, B).

Table 3 Endocrine parameters in patients classified as having diabetes according to the World Health Organization definition and patients not fulfilling the criteria

Parameter	Diabetic patients (n = 9)	Non-diabetic patients (n = 30)	Difference between groups (P)
Fasting plasma glucose, 0 min[1]	6.9 (6.0-7.3)	5.1 (4.6-5.5)	< 0.001
Plasma glucose 120 min[1]	13 (12-14)	6.8 (5.5-8.2)	< 0.001
Plasma C-peptide 0 min[2]	1.7 (1.3-2)	0.72 (0.6-1.0)	< 0.001
Plasma C-peptide 120 min[2]	4.8 (4.1-5.9)	2.9 (2.2-4.4)	0.024
Serum insulin 0 min[3]	16 (13-17)	6 (4-9)	0.001
Serum insulin 120 min[3]	103 (79-126)	42 (28-60)	0.001
HOMA-insulin resistance	4.2 (3.7-5.4)	1.3 (0.9-2.2)	< 0.001
HBA1c	5.3 (5.0- 5.6)	4.6 (4.5-4.8)	< 0.001

[1]mmol/L; [2]nmol/L; [3]mIE/L; Values are given in median (interquartile range). HOMA: Homeostasis model assessment; HBA1c: Fasting glycosylated hemoglobin.

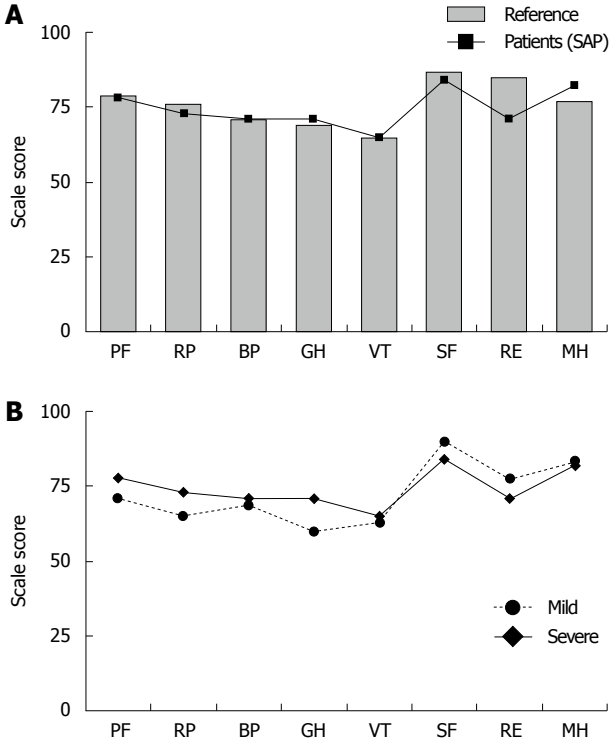

Figure 3 The graphs shows quality of life, measured by Short Form-36, in mild and severe acute pancreatitis patients, comparing age- and gender-matched control group. A: Mean values for patients after mild (black squares) acute pancreatitis and severe (black circles) acute pancreatitis in the 8 Short Form-36 domains; B: Mean value for patients after severe (black squares) acute pancreatitis compared with an age- and gender-matched control group (grey bars) in the 8 SF-36 domains. SAP: Severe acute pancreatitis; PF: Physical function; RP: Role physical; BP: Bodily pain; GH: General health; VT: Vitality; SF: Social functioning; RE: Role emotional; MH: Mental health.

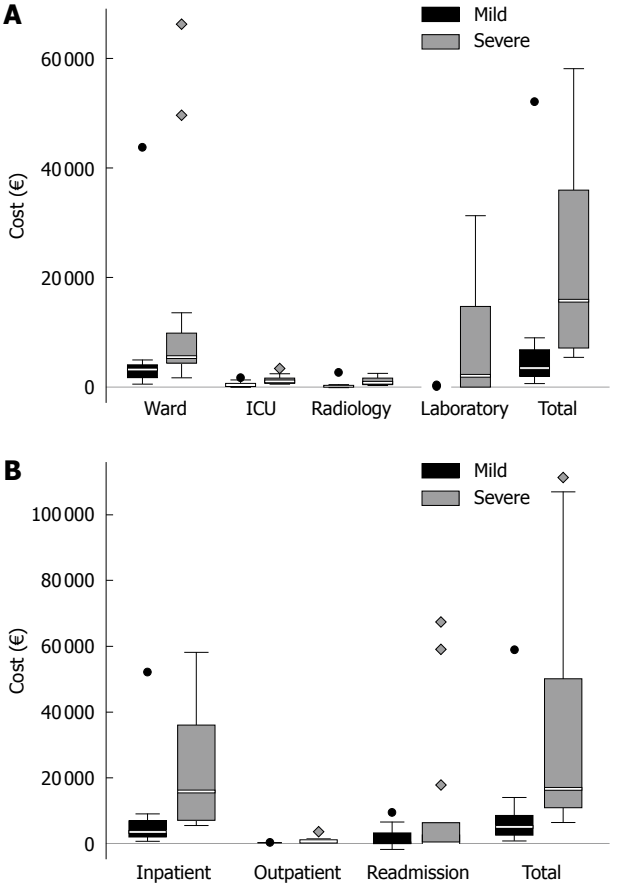

Figure 4 Box-plots showing the specified hospital costs in acute pancreatitis patients. A: In-hospital costs during primary care for acute pancreatitis, in mild and severe acute pancreatitis patients; B: Costs at follow-up, including cost for primary care and all subsequent inpatient and outpatient costs, in mild and severe acute pancreatitis patients. ICU: Intensive care unit.

seen between the groups with a history of severe and mild disease in the 8 SF-36 domains (Figure 3A). When comparing patients with SAP with their respective reference group, no significant differences could be found (Figure 3B).

Costs

There was a pronounced difference between severe and mild acute pancreatitis when comparing costs for the primary hospital stay, a median of €15 774 (€7455-€35 960) in SAP *vs* €3480 (€2049-€6662) in mild acute pancreatitis and a mean of €23 592 ± €18 821 *vs* €5908 ± €9740, ($P < 0.001$, Figure 4A). When including total hospital costs, adding costs for follow-up, both including in-hospital stay and outpatient care, the difference between severe and mild disease was a median of €16 572 (€11 017-€45 619) *vs* €5000 (€2562-€8384) and a mean of €35 427 ± €36 790 *vs* €7536 ± €11 228 ($P < 0.001$, Figure 4B). This means that the severe cases were about 3.3 times more expensive regarding the hospital costs. For the subgroup of SAP patients requiring stay in the ICU ($n = 8$), the total hospital costs, including care for late complications was a median of €30 026 (€17 636-€84 323) and a mean of €49 894 ± €41 905.

DISCUSSION

At the Marseilles symposium on pancreatitis in 1963 it was stated that after recovery complete restitution of the pancreas was the norm[30]. Since then several studies have been performed, mostly on SAP patients, with different follow-up times and measured parameters, with somewhat divergent results. In 1983 Mitchell and coworkers published a study including patients with both mild acute pancreatitis and SAP. All had initial exocrine insufficiency and 80% recovered after 12 mo[8]. The 1984 Marseilles Symposium acknowledged that both exocrine and endocrine function of the pancreas can be disrupted to varying degrees and duration following an acute attack of pancreatitis[31]. There is thus a dysfunction during the initial stage after acute pancreatitis, but functional recovery of the gland is controversial. Full recovery has been described[6], but some dysfunction is the usual scenario, especially after severe disease with necrosis[2,4,9,15].

After surgical treatment, a persistent exocrine insufficiency has been noted in up to 80%-85% of patients[3,4]. Acute pancreatitis with alcohol as an etiological factor may more likely be associated with exocrine impairment[32], though this has not been confirmed by others[9]. Exocrine insufficiency may not necessarily be clinically relevant and evaluation is difficult, particularly when non-invasive methods are used. Invasive tests also have their drawbacks. Furthermore, pancreatic insufficiency is often not

obvious until 90% of the gland has been destroyed. In the present study, only one patient had an objective exocrine insufficiency, as measured by fecal elastase-1. The evaluation of this test has been proven as a highly sensitive and specific pancreatic function tests, even comparable with oral pancreatic function tests, such as the pancreaolauryl test[26,33]. When analyzing the patient's answers regarding change in stool habits, including frequency, mild impairment was common, with a fluctuation over time, and constant steatorrhea at follow-up was uncommon. Medication with pancreatic enzyme supplements was used by a very limited number of patients. Change in diet was also a common answer, which can be due to a number of factors. Thus, there is a grey area concerning when and to what extent an exocrine dysfunction is present and of clinical relevance.

Endocrine dysfunction with glucosuria and elevated blood sugar levels is common during the acute phase of pancreatitis, but is usually self-limiting and resolves. Endocrine dysfunction with DM and IGT is, however, more common over time[9]. DM overall is also known to occur more often after operative treatment[11]. The 4 patients subjected to pancreatic surgery in the present survey all fulfilled the criteria for DM or IGT. We further found DM or IGT in 79% after SAP and in 42% after mild acute pancreatitis. Loss of β-cell function is expected after severe necrotizing disease, and the patients in the present study also had lower C-peptide and insulin levels after SAP as compared with mild disease. Fasting C-peptide and insulin levels were, however, higher in the patients with DM, as compared with the other patients, indicating that insulin resistance is an additional and important explanation for the development of DM. The insulin resistance was obvious, not only with hyperinsulinemia, but also with an elevated HOMA-IR in both groups, with a tendency to be more pronounced after mild disease. The present rate of DM and IGT found at follow-up in acute pancreatitis patients is much higher than expected in the population. Insulin resistance is furthermore associated with different conditions such as obesity, liver failure and inflammatory conditions, though patients in the present survey did not fulfill any of these criteria. Insulin resistance is a prominent feature in patients after pancreatic resection[34], implying the coexistence of pancreatic damage and hyperinsulinemia[5,35]. The pathophysiological mechanisms involved are, however, not clarified. The result in the present study indicates that the insufficiency after acute pancreatitis may be an underestimated problem. When taking the risk for untreated DM patients with poor metabolic control into consideration, a follow-up of these patients may be more important than hitherto proposed.

In follow-up studies, the focus has been on pancreatic dysfunction and less attention has been paid to other aspects of recovery and long-term detriment. With an increasing number of patients surviving SAP, more attention has been directed towards quality of life and long-term outcomes. Until recently, results of treatment strategies were determined only in terms of death, disability and cure. Quality of life as an outcome measure has only sporadically been reported, with a tendency for, or a statistically significant, reduced quality of life after acute pancreatitis[12,15-17]. In the present study, SF-36 was used, which in 36 questions, divided into 8 scales, estimates both function and wellbeing, and can be summarized as health-related quality of life. In a Finnish study, a difference was seen in the SF-36 general health domain, but the conclusion was that there were no clinically significant differences in quality of life compared with that of the normal population[14]. This is in accordance with our results, where no difference was noted between groups, nor between these and the normal reference population. After debridement for pancreatic necrosis, quality of life has been shown to far exceed that noted in patients with other severe medical diseases, such as congestive heart failure and severe hypertension[12]. It may be that experiencing critical illness, such as SAP, might change the opinion about what is really important in life, contributing to explaining why some patients feel well, despite possible persisting restrictions in daily life activities and not being entirely recovered.

Only a few reports on cost analyses in acute pancreatitis have been made, mainly focusing on severe cases[15,20]. One study has estimated hospital costs for acute pancreatitis patients, regardless of severity grade, with figures obtained from a large national hospital database, reporting a cost of $9870 per hospitalization and a good long-term outcome[19]. In the present study, we calculated not only costs for the primary admission but also additional hospital costs directly related to the pancreatitis episode. Data on total hospital costs has not previously been presented. Expressed as percentages, the increase was highest in the mild group due to subsequent treatment of gallstone disease. This is an important aspect that can possibly be optimized with less resource use. The additional costs after SAP showed a wide spread with a few patients requiring a great deal of resources. Reports on when the patients return to daily activity and work are also limited. Doepel *et al*[11] found that 84% of patients who were working the year before the onset of SAP returned to work. In the present study, corresponding figures are higher, but still the return to work in many cases took a long time after SAP, with the possibility that some patients, despite surviving the acute disease, are never able to return to a normal life and work again. The time until patients felt recovered varied between patients and groups, but overall it took a long time, with several patients not being subjectively recovered, but back in full time work. Patients not recovered at the time of follow-up were mainly, but not exclusively, found in the group recovering from SAP.

Generally, reports in the literature concerning follow-up after acute pancreatitis have limitations and have led to contradictory results. This is due to a number of factors existing in almost all studies, e.g. a limited total number of patients, absence of agreement on a classification system describing the severity of the disease, differences in the proportions concerning e.g. etiology, different criteria for IGT and DM, different tests used to evaluate exocrine

insufficiency and different instruments to evaluate quality of life. The follow-up time also varied widely[36].

In the present study, most of the patients with a history of SAP had a major need to talk and discuss what actually had happened during their hospital stay and during the follow-up period. In the group with mild acute pancreatitis, a number of patients even had to remind themselves that they really had been ill a few years previously. A structured follow-up plan for patients that have undergone SAP, dealing with physiological aspects, including information on signs of exocrine insufficiency and the possible benefits of controlling blood glucose levels, could be of great benefit.

A weakness in the present study is the limited number of patients. A strength, however, is the attempt to make a complete follow-up including several important factors, and that a strict definition of SAP has been used. Another strength is that the same surgeon evaluated all patients at the follow-up in the outpatient clinic.

In conclusion, the present study presents a thorough long-term evaluation concerning several aspects associated with severe and mild acute pancreatitis. The results point at an impairment in endocrine function and also a subtle exocrine dysfunction. Sick leave and time until the patients recover can be long and the disease is associated with high costs for society, especially after SAP. The quality of life both after severe and mild acute pancreatitis is, however, as good as in the normal population years after the disease.

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COMMENTS

Background

Acute pancreatitis is associated with a mild and uneventful course in 80% of patients. However, severe acute pancreatitis is associated with the risk of complications both in and around the pancreas. Long-term exocrine and endocrine pancreatic function has been studied with divergent results. Morphological changes may often remain and functional recovery is not always complete. Additional important outcome measures are quality of life and health economical aspects.

Research frontiers

Long-term evaluation of patients after acute pancreatitis has mainly been focused on the severe disease with divergent results concerning pancreatic function and quality of life. Endocrine and exocrine dysfunction usually occurs. Only a few cost analyses have been published hitherto.

Innovations and breakthroughs

In this study, the authors found that the incidence of endocrine dysfunction was high but clinically important exocrine dysfunction limited after both severe and mild acute pancreatitis. The recovery was long especially after the severe form, but the quality of life was excellent. The hospital costs were high after severe acute pancreatitis, with a large spread within the group.

Applications

It is important to be aware of the high incidence of endocrine dysfunction, and a structured follow-up plan can be of importance. A good long time quality of life is important information for the patients and relatives during the demanding severe disease.

Peer review

The present paper is interesting and well written. Number of admitted patients are not so numerous but well selected and stratified.

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BRIEF ARTICLE

Pulmonary involvement in inflammatory bowel disease

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Abstract

AIM: To determine the relationship of pulmonary abnormalities and bowel disease activity in inflammatory bowel disease (IBD).

METHODS: Thirty ulcerative colitis (UC) and nine Crohn's disease patients, and 20 control subjects were enrolled in this prospective study. Detailed clinical information was obtained. Extent and activity of the bowel disease were established endoscopically. Each patient underwent pulmonary function tests and high-resolution computed tomography (HRCT). Blood samples for measurement of C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), angiotensin converting enzyme and total IgE were delivered by the patients.

RESULTS: Ten (25.6%) patients had respiratory symptoms. A pulmonary function abnormality was present in 22 of 39 patients. Among all patients, the most prevalent abnormalities in lung functions were a decrease in forced expiratory volume in 1 s (FEV1), FEV1/forced vital capacity (FVC), forced expiratory flow (FEF) 25%-75%, transfer coefficient for carbon monoxide (DLCO), DLCO/alveolar volume. Increased respiratory symptoms score was associated with high endoscopic activity index in UC patients. Endoscopic and clinical activities in UC patients were correlated with FEV1, FEV1/FVC, and FEF 25%-75%. Smoking status, duration of disease and medication were not correlated with pulmonary physiological test results, HRCT abnormalities, clinical/endoscopic disease activity, CRP, ESR or total IgE level or body mass index.

CONCLUSION: It is important that respiratory manifestations are recognized and treated early in IBD. Otherwise, they can lead to destructive and irreversible changes in the airway wall.

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Key words: Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; High-resolution computed tomography; Pulmonary function tests; Lung diseases

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory disease that commonly involves the gastrointes-

tinal tract, and it is of unknown etiology. Crohn's disease (CD) and ulcerative colitis (UC) are the two main forms of chronic IBD. Extraintestinal manifestations are very common: dermatological manifestations, erythema nodosum and pyoderma gangrenosum; ocular manifestations, uveitis and episcleritis; hepatobiliary manifestations, primary sclerosing cholangitis and autoimmune hepatitis; musculoskeletal manifestations, peripheral arthritis and axial arthropathy[1]. In contrast, pulmonary involvement is rare. A relationship between pulmonary disease and IBD was suggested 40 years ago. Respiratory involvement in IBD is disclosed with some pathophysiological mechanisms: both the colonic and respiratory epithelia share embryonic origin from the primitive foregut, and both types of epithelial cells include goblet cells and submucosal glands; and the lungs and gastrointestinal tract contain submucosal lymphoid tissue and play crucial roles in host mucosal defense. The similarity in the mucosal immune system causes the same pathogenetic changes. The aberrations in both innate and acquired immunity that are involved in the pathogenesis of IBD are complex and still incompletely understood[2]. The patterns of involvement in IBD are[2,3]: (1) upper airway: glottic/subglottic stenosis, tracheal inflammation and stenosis; (2) bronchi: chronic bronchitis, bronchiectasis, and chronic bronchial suppuration; (3) small airways: bronchiolitis obliterans, bronchiolitis, and diffuse pan-bronchiolitis; (4) lung parenchyma: bronchiolitis obliterans-organizing pneumonia, nonspecific interstitial pneumonia, granulomatous interstitial lung disease, desquamative interstitial pneumonitis, pulmonary infiltrates and eosinophilia, and sterile necrobiotic nodules; (5) sarcoidosis, α1 antitrypsin deficiency; (6) pulmonary vascular disease; Wegener's granulomatosis, Churg-Strauss syndrome, microscopic polyangiitis, and pulmonary vasculitis; (7) venous thromboembolism; and (8) serositis: pleural and pericardial manifestations.

The aim of present study was to evaluate pulmonary involvement in IBD. For this, we examined frequency of respiratory symptoms, pulmonary function tests, bronchial hyperreactivity, high-resolution computed tomography (HRCT), serum angiotensin-converting enzyme (ACE), C-reactive protein (CRP) level and erythrocyte sedimentation rate (ESR).

MATERIALS AND METHODS

During a 2-year period from January 2007 to December 2009, 39 consecutive patients with the diagnosis of IBD, who were seen in a gastroenterology clinic, were referred to our outpatient clinic. Subjects with the following characteristics were included: age $\geqslant$ 18 years old, CD and UC with endoscopic examination performed in a week.

Subjects with the following characteristics were excluded: lack of compliance in performing lung function tests, age < 18 years old, history of previous lung disease, history of atopy or familial atopy, peripheral eosinophilia, and obesity [body mass index (BMI) > 30 kg/m^2].

Thirty UC and nine CD patients were enrolled in this prospective study. Age- and sex-matched normal controls (20 subjects) were recruited from healthy volunteers. The detailed anamnesis of the subjects (age, sex, cigarette pack/years, family history, occupational history) was gathered. Duration of disease from the date of first endoscopic diagnosis and maximal extent of endoscopic diagnosis were recorded. The extent of the bowel disease was defined as pancolitis when the entire colon was involved; left-side colitis when the bowel from the hepatic flexure to the rectum was involved; and distal colitis when the sigmoid colon and rectum were involved. Patients with CD were classified with colon involvement, small bowel involvement, or ileocecal involvement. In patients with UC, the clinical activity of the disease was assessed using the Truelove score[4]: mild was considered to be in remission, and patients with moderate and severe indices had active disease. Endoscopic activity was assessed by videocolonoscopy (Fujinon EC 450/WL, Tokyo, Japan). All colonoscopic examinations were performed by an experienced investigator. The Rachmilewitz endoscopic activity index for UC was used to assess disease activity[5]. CD activity was assessed on the basis of clinical and endoscopic features[6]. Smoking habit was also recorded, however, most of our patients were nonsmokers or former smokers. Symptoms of cough, sputum, wheezing and breathlessness were scored out of a maximum of 2: 0 = no symptoms; 1 = intermittent symptoms; and 2 = regular symptoms. The total symptom score (maximum of 8) for each patient was derived from the sum of the individual symptom scores. A total symptom score of $\geqslant$ 3 points was assessed as "respiratory symptom is present" or "symptomatic"[7]. Blood samples for measurement of CRP, ESR, ACE and total IgE were delivered by the patients prior to endoscopy.

Pulmonary function testing

Each patient underwent standard pulmonary function tests for forced expiratory volume in 1 s (FEV1), vital capacity, forced vital capacity (FVC), and transfer coefficient for carbon monoxide (DLCO) measured by means of the single-breath test. Account was also taken of the hemoglobin value when calculating the DLCO. The results were compared with those of age- and sex-matched controls and expressed as a percentage of predicted values. Pulmonary function test indices were measured with a Sensormedics V max 229 (Sensormedics, Yonda Linda, CA, USA) series flow-sensitive spirometer. The limitation of our study was that lung volumes could not be measured. Bronchial hyperresponsiveness (BHR) (PD_{20}, dose of methacholine that caused a 20% fall in FEV_1) was measured in the morning with the methacholine challenge test using the dosimeter method according to ERS task force in all IBD patients[8]. In patients with high IgE level, the existence of an atopic state was evaluated by skin prick test using common allergen extracts (grass, tree and weed pollens; house dust mites; molds; cat and dog extracts), and reactions at least 3 mm greater then negative control test were regarded as positive (Stallergenes, Antony cedex, France). Histamine was used as a positive control.

HRCT

All CT scans were obtained with a scanner (Siemens Somatom Emotion, Germany). Images were acquired during inspiration. CT scans were evaluated by an independent investigator who was blinded to the results of the pulmonary function tests and clinical data. The individual features evaluated included the following: bronchiectasis, bronchial wall thickening, ground-glass opacification, emphysema and cysts.

Ethical considerations

Informed consent was obtained from all patients and control subjects and the study was approved by the local ethical committee.

RESULTS

Patient description

The characteristics of the control group and the 39 patients with UC and CD are shown in Table 1. Twenty-three male and 16 female (59%, 41%) patients, as well as 20 healthy controls, with mean ages of 44.28 ± 12.85 years and 39.50 ± 12.47 years, respectively, were recruited to the study. The mean duration of disease was 39.07 ± 29.38 mo. Thirty individuals were never smokers, five were ex-smokers and four were smokers. Control patients were nonsmokers. None of our patients had an occupational history or family history of respiratory disease and atopy. Fifteen (50%) UC and four (44.4%) CD patients had clinically active bowel disease at the time of the study. Of the 39 patients, 33 were receiving sulfasalazine, one azathioprine, and five sulfasalazine plus azathioprine. None of the patients had extraintestinal manifestations other than pulmonary involvement.

Twenty-five (64.10%) patients had HRCT abnormalities (Table 2). Ten (25.6%) patients had respiratory symptoms. In 16 (41%) patients, CRP level was elevated, and in 26 (66.7%), ESR was increased. Four (10.3%) patients had high levels of total IgE, and in these patients, skin prick tests were negative, and in one patient, weak BHR was observed.

Pulmonary function tests

Three (7.69%) patients had obstructive dysfunction and small airway obstruction was reported in 17 (43.58%). Two patients (5.12%) had restrictive dysfunction. When comparing all IBD patients with controls, we found statistically significant differences for FEV1, FEV1/FVC, FEF 25%-75%, DLCO and DLCO/alveolar volume (VA) ($P < 0.05$) (Table 3).

Correlation between pulmonary function parameters, clinical characteristics and HRCT features

The correlation of pulmonary function and endoscopic and clinical disease activity is shown in Tables 4 and 5. The most prevalent abnormality was a decrease in FEF 25%-75% in patients with CD and endoscopically and clinically active UC. The impairment in FEV1 and FEV1/FVC was significant and more pronounced in patients with active UC *vs* controls. In 10 (33.3%) patients with UC, the endoscopic activity index was high and correlated significantly with pulmonary symptom scores ($P < 0.05$). There was no significant correlation between smoking status and pulmonary physiological test results, HRCT abnormalities or clinical/endoscopic disease activity. Also, no relationship was found between disease activity and HRCT abnormalities, respiratory symptoms, CRP, total IgE level, ESR or BMI. There was no relationship between duration of disease and pulmonary physiological test results, HRCT abnormalities, CRP, total IgE level or ESR. There was no correlation between BMI and pulmonary function.

Table 1 Characteristics of the control and patient groups with inflammatory bowel disease

	UC	CD	Controls
n	30	9	20
Sex (M/F)	22/8	1/8	10/10
Mean age (yr)	43 ± 3	46 ± 2	39.50 ± 12.47
Smoking (smoker/never/ex-smoker)	4/22/4	-	-
Duration and range of bowel disease (yr) (mean ± SD)	3 ± 0.5 (0-9)	3 ± 0.5 (0.5-4)	
Respiratory symptom (present/absent)	9/21	1/8	0/20
Disease activity (active/remission)	14/16	5/4	

UC: Ulcerative colitis; CD: Crohn's disease.

Table 2 Findings on high-resolution computed tomography in patients with inflammatory bowel disease

Findings on HRCT	*n*
Normal	14
Peribronchial thickness	15
Bronchiectasis	2
Ground-glass opacity	8
Emphysema	9
Air cysts	1
Reticulonodular opacity	1

HRCT: High-resolution computed tomography.

Table 3 Correlations of pulmonary function tests with inflammatory bowel disease and controls

	UC (*n* = 30)	CD (*n* = 9)	Controls (*n* = 20)
FEV1	86.87 ± 15.09[a]	85.89 ± 13.75	95.75 ± 11.56
FVC	88.37 ± 15.80	93.22 ± 8.90	96.40 ± 10.00
FEV1/FVC	79.67 ± 8.98[a]	78.56 ± 11.11	84.15 ± 4.21
FEF 25%-75%	73.93 ± 21.38	64.89 ± 21.23[a]	85.00 ± 11.85
DLCO	96.43 ± 12.84[a]	90.67 ± 19.88[a]	103.5 ± 11.90
DLCO/VA	104.83 ± 16.99	93.00 ± 17.85[a]	112.95 ± 10.22

[a]$P < 0.05$ *vs* control group statistically significant. UC: Ulcerative colitis; CD: Crohn's disease; FEV1: Forced expiratory volume in 1 s; FVC: Forced vital capacity; FEF: Forced expiratory flow; DLCO: Transfer coefficient for carbon monoxide; VA: Alveolar volume.

Table 4 Correlation of pulmonary function tests between endoscopically and clinically active Crohn's disease with controls

	Endoscopically active (n = 5)	inactive(n = 4)	Clinically active (n = 4)	inactive (n = 5)	Control (n = 20)
FEV1	82 ± 12.28	90.75 ± 7.04	81.25 ± 19.50	89.60 ± 7.37	95.75 ± 11.56
FVC	91.20 ± 7.33	95.75 ± 11.15	92.25 ± 7.68	94.00 ± 10.61	96.40 ± 10.00
FEV1/FVC	75.80 ± 14.60	82 ± 4.08	74.25 ± 16.38	82 ± 3.54	84.15 ± 4.21
FEF 25%-75%	61.80 ± 28.84	68.75 ± 7.54	58.50 ± 32.07	70 ± 7.52	85 ± 11.85[a,c]
DLCO	91.20 ± 15.83	90 ± 26.81	103 ± 13.64	80.80 ± 19.42	103.5 ± 11.90[c]
DLCO/VA	95.40 ± 20.38	90 ± 16.55	102.25 ± 16.15	85.60 ± 16.95	112.95 ± 10.22[a,c]

[a]P < 0.05 comparison between active and control groups statistically significant; [c]P < 0.05 comparison between inactive and control groups statistically significant. FEV1: Forced expiratory volume in 1 s; FVC: Forced vital capacity; FEF: Forced expiratory flow; DLCO: Transfer coefficient for carbon monoxide; VA: Alveolar volume.

Table 5 Correlation of pulmonary function tests between endoscopically and clinically active ulcerative colitis with controls

	Clinically active (n = 15)	inactive (n = 15)	Endoscopically active (n = 20)	inactive (n = 10)	Control (n = 20)
FEV1	86.53 ± 13.15	87.20 ± 17.28	78.10 ± 19.58	91.25 ± 10.26[c]	95.75 ± 11.56[a]
FVC	91.60 ± 14.07	85.13 ± 17.22	86.70 ± 16.49	89.20 ± 15.81	96.40 ± 10.00
FEV1/FVC	78.07 ± 6.22	81.27 ± 11.08	72.70 ± 10.95	83.15 ± 5.28[c]	84.15 ± 4.21[a]
FEF 25%-75%	67.73 ± 19.00	80.13 ± 22.43[c]	53.80 ± 22.30	84 ± 11.93[c]	85 ± 11.85[a,e]
DLCO	97.93 ± 12.88	94.93 ± 13.07	96 ± 13.42	96.65 ± 12.89	103.5 ± 11.90[e]
DLCO/VA	107.67 ± 14.25	102 ± 19.44	106.50 ± 17.28	104 ± 17.24	112.95 ± 10.22[e]

[a]P < 0.05 comparison between active and control groups statistically significant; [c]P < 0.05 comparison between active and inactive groups statistically significant; [e]P < 0.05 comparison between inactive and control groups statistically significant. FEV1: Forced expiratory volume in 1 s; FVC: Forced vital capacity; FEF: Forced expiratory flow; DLCO: Transfer coefficient for carbon monoxide; VA: Alveolar volume.

DISCUSSION

Extraintestinal manifestations of IBD are increasing in developed countries. In 1976, Kraft *et al*[9] described six patients in whom chronic bronchial suppuration had appeared between 3 and 13 years after the onset of IBD. Since then, all respiratory complaints in IBD patients that cannot be explained by other causes have been defined as pulmonary manifestations of the disease. Furthermore, reports of pulmonary manifestations of the disease are increasingly present in the literature. In our patient group, among all patients, the most prevalent abnormalities in lung functions were a decrease in FEV1, FEV1/FVC, FEF 25%-75%, DLCO, and DLCO/VA. Increased respiratory symptom score was associated with high endoscopic activity index in UC patients. The most prevalent abnormality was a decrease in FEF 25%-75% in patients with CD and endoscopically and clinically active UC. The impairment in FEV1 and FEV1/FVC was significant and more pronounced in patients with active UC compared with the controls.

Godet *et al*[10] have studied patients with UC, and pulmonary function test abnormalities were found in 55%, 15/66 subjects had an obstructive pattern, 19 had abnormal diffusion, one had a restrictive pattern, and five had both an obstructive pattern and abnormal diffusion; these alterations could not be predicted by current or past smoking status, family history of respiratory disease, occupational history or current medication use. In our study, 3/39 (7.69%) patients had obstructive dysfunction, two (5.12%) had restrictive dysfunction, and five (12.8%) had abnormal diffusion. These results were not correlated with smoking status. None of our patients had a family or occupational history of respiratory disease.

The influence of disease activity was studied. In a recent study with UC patients, small airway obstruction (as demonstrated by diminished FEF 25%-75%) was reported in the 15 patients (57.6%), restrictive dysfunction in eight (30.7%) and obstructive dysfunction in three (11.5%), and the impairment in pulmonary function tests was significant and more pronounced in patients with active UC compared with the controls[11]. In our study, the most prevalent abnormality was a decrease in FEF 25%-75%, and FEV1/FVC and FEF 25%-75% were significantly lower in patients with active UC. In 10 (33.3%) patients with UC, the endoscopic activity index was high and correlated significantly with pulmonary symptom scores (P < 0.05). These findings suggest a direct pathogenic link with IBD. Tzanakis *et al*[12] have found small airway dysfunction in patients with CD and UC despite their normal baseline spirometric values, and there was no difference between active and nonactive disease.

Chest radiography is often normal in patients with respiratory symptoms and IBD. Bronchiectasis is the classic pulmonary manifestation of IBD, and is noted in 66% of cases of IBD that involve the large airways[2]. Mahadeva *et al*[7] have found bronchiectasis in 13 of 17 patients with IBD, in whom sputum production was present in 10. In contrast, bronchiectasis was identified in only two patients in the present study. In our study, the most frequent find-

ing on HRCT was peribronchial thickness. The most common respiratory association of IBD is inflammation of the airways. 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[13]. This inflammation is perceived on HRCT as an increase in bronchial wall thickness or an increase in diameter of pulmonary artery branches. In these patients, bronchial dilatation is commonly present and results from traction by fibrous tissue on the bronchial walls and results in bronchiectasis[14]. Consequently, peribronchial thickness might reflect inflammation, which usually responds well to steroids[15]. In this way, bronchiectasis can be prevented. This finding suggests a direct pathogenic link to IBD as well.

The expiratory HRCT seems to be a limitation in our study and air trapping could have been underestimated. In our series, nine patients had upper lobe emphysema, which was probably related to smoking but there was no significant correlation between smoking status and pulmonary physiological test results, HRCT abnormalities or clinical/endoscopic disease activity.

It is important to consider whether therapy with sulfasalazine or mesalazine could have been responsible for the pulmonary changes. The most common abnormality described in association with sulfasalazine therapy is upper lobe peripheral opacity, although lower lobe opacity, eosinophilic pneumonia, interstitial pneumonitis, bronchiolitis obliterans organizing pneumonia and cavitating nodules have also been reported[16,17]. None of our patients had peripheral blood eosinophilia, which is usually present in lung disease caused by sulfasalazine.

Kuzela *et al*[18] have identified a high incidence of pulmonary function abnormalities (suspicious of interstitial lung disorder) in patients with IBD, despite the lack of radiological abnormalities; 56.7% of patients with UC and 57.7% of those with CD had reduced lung transfer factor. Tzanakis *et al*[12] have shown that DLCO is significantly lower among IBD patients with active gastrointestinal disease than those in remission. Marvisi *et al*[19] have studied 32 patients with UC and found a mild reduction in DLCO and FEF 25%-75%, and the incidence was higher in patients with active disease despite the lack of radiological alterations and pulmonary symptoms. Also, significant differences in mean FVC, FEV1, total lung capacity and FEV1\FVC values were found between patients with active and inactive UC. In our study, DLCO and DLCO/VA were significantly lower among IBD patients, but not correlated with disease activity.

Douglas *et al*[20] have studied 44 IBD patients and found that 48% had unspecified respiratory symptoms. Songür *et al*[21] have found that 16 of 36 IBD patients (44%) in a gastroenterology clinic had symptoms of wheezing, cough, sputum production, or breathlessness. In our study, 25.6% of 39 IBD patients had respiratory symptoms.

The true prevalence of airway inflammation and respiratory and atopic symptoms in IBD remains obscure. Ceyhan *et al*[22] have studied 30 consecutive IBD subjects; allergic symptoms were seen in 14 IBD patients, respiratory symptoms were found in 15, asthma and antiasthmatic drug treatment were noted in three, and BHR was determined in four. They have concluded that allergic symptoms, respiratory symptoms, abnormal lung function tests and skin prick test positivity are more common among IBD patients in comparison with controls, and airway dysfunction is accompanied by atopy. Louis *et al*[23] have shown no correlation between BHR and airway inflammation in IBD patients, in contrast to asthma. In our study, we excluded subjects with atopy and a familial history of atopy, subjects with peripheral eosinophilia. Four (10.3%) patients had high levels of total IgE, and in these patients, skin prick tests were negative and in one patient, weak BHR was observed.

Nutritional status has been shown to have a significant influence on the overall pulmonary function in patients with IBD. Christie and Hill have demonstrated a 35% loss of body protein stores and associated 40% physiological impairment (FEV1, FVC and maximal voluntary ventilation) in patients with acute exacerbations of CD, compared to controls. There was a significant immediate and delayed improvement in these parameters after 2 wk nutritional supplementation, and further improvement on restoration of body proteins during convalescence[24]. Similarly, BMI has been examined as an index of nutritional status in patients with UC and in controls, and a significant positive correlation has been found between BMI and pulmonary function[10]. In our study, 19 patients were overweight (BMI: 25-29.9 kg/m^2), and there was no correlation between BMI and pulmonary function.

In conclusion, both the colonic and respiratory epithelia share embryonic origin from the primitive foregut. The inflammatory lesions seen beneath the bronchial epithelium are similar to those observed beneath the colonic epithelium in IBD. This means that there is inflammation that can be detected early by HRCT and pulmonary function tests. Although most patients have subclinical disease, the pulmonologist must be aware of the multiple potential pulmonary manifestations that can occur in a patient with IBD. Otherwise, they tend to generate persistent and annoying symptoms, and can lead to destructive and irreversible changes in the airway wall, or the "end-stage lung"[3].

COMMENTS

Background

Crohn's disease (CD) and ulcerative colitis (UC) are the two main forms of chronic inflammatory bowel disease (IBD), with unknown etiology. Extraintestinal manifestations such as dermatological, ocular, hepatobiliary and musculoskeletal diseases are very common. In contrast, pulmonary involvement is rare.

Research frontiers

Respiratory involvement in IBD is seen with some pathophysiological mechanisms: both the colonic and respiratory epithelia share embryonic origin from the primitive foregut, both types of epithelial cells include goblet cells and submucosal glands, and both the lungs and gastrointestinal tract contain submucosal lymphoid tissue and play crucial roles in host mucosal defense. The similarity in the mucosal immune system causes similar pathogenic changes. The aberrations in both innate and acquired immunity that are involved in the pathogenesis of IBD are complex and still incompletely understood. In this study, pulmonary involvement in IBD was evaluated.

Innovations and breakthroughs

The most prevalent abnormalities in lung functions are a decrease in forced expiratory volume in 1 s (FEV1), FEV1/forced vital capacity, forced expiratory flow 25%-75%, transfer coefficient for carbon monoxide (DLCO), DLCO/alveolar volume. The most frequent finding on high-resolution computed tomography, unlike previous studies, was peribronchial thickness. The most common respiratory association of IBD is inflammation of the airways. Biopsy shows either severe nonspecific chronic inflammation or non-caseating tuberculoid granulomas. These appearances are associated with those in the bowel, and it is possible that the gut and the lung are both affected because they share common antigens. This inflammation is perceived on high-resolution computed tomography as an increase in bronchial wall thickness or an increase in diameter of pulmonary artery branches. In these patients, bronchial dilatation is commonly present and results from traction by fibrous tissue on the bronchial walls, which results in bronchiectasis. Consequently, peribronchial thickness might reflect inflammation, which usually responds well to steroids. In this way, bronchiectasis can be prevented.

Applications

Various pulmonary manifestations can occur in IBD. It is important that respiratory manifestations are recognized and treated early. Otherwise, they might lead to destructive and irreversible changes in the airway wall, or the "end-stage lung".

Peer review

This is a very interesting study and gives us further insight into a disease that may manifest in various systems. Pulmonary disease in IBD has not been extensively studied because the problem is often treated only by gastroenterologists. This is a timely study and could lead to further studies that will help us understand more about this disease.

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BRIEF ARTICLE

Association of p53/p21 expression with cigarette smoking and prognosis in esophageal squamous cell carcinoma patients

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Abstract

AIM: To investigate the expression of p53 and p21 and associations with possible risk factors, such as cigarette smoking, in esophageal squamous cell carcinoma (ESCC) in northeastern Iran, a region with a high incidence of ESCC.

METHODS: The expression of p53 and p21 proteins was investigated immunohistochemically in tumor tissue from 80 ESCC patients and in 60 available paraffin-embedded blocks of adjacent normal specimens from the cases, along with normal esophageal tissue from 80 healthy subjects.

RESULTS: Positive expression of p53 protein was detected in 56.2% (45/80) of ESCC cases, and in none of the normal esophageal tissue of the control group ($P < 0.001$). Furthermore, 73.8% (59/80) of ESCC cases and 43.8% (35/80) of controls had positive expression of p21 protein ($P < 0.001$). Cigarette smoking was significantly associated with p53 over-expression in ESCC cases ($P = 0.010$, OR = 3.64; 95% CI: 1.32-10.02). p21 over-expression was associated with poorer clinical outcome among the ESCC patients ($P = 0.009$).

CONCLUSION: Over-expression of p53 in association with cigarette smoking may play a critical role in ESCC carcinogenesis among this high-risk population of northeastern Iran. Furthermore, p21 over-expression was found to be associated with poor prognosis, specifically in the operable ESCC patients.

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Key words: Esophageal squamous cell carcinoma; p53; p21; Immunohistochemistry; Survival; Smoking

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INTRODUCTION

Esophageal cancer has been reported as the eighth most

common cancer worldwide, with a great variation in the incidence all around the world[1]. A high-risk area for this cancer is known as the so-called "Asian esophageal cancer belt", which stretches from north central China into northern Iran, where esophageal squamous cell carcinoma (ESCC) predominates[2-4].

High incidence and mortality rates of ESCC have been reported in northeastern Iran, due to some distinct, but not well-known, environmental and genetic factors; however the complex network of molecular alterations underlying the development and progression of ESCC have not been clearly elucidated in this region[5,6].

Several studies have revealed that esophageal cancer, as with many other malignancies, is associated with cigarette smoking. However, the specific molecular targets affected by cigarette-derived carcinogens have not been thoroughly identified[7].

It has been shown that the p53 tumor suppressor gene is involved in the control of the cell cycle[8], and it is employed to protect cells exposed to DNA-damaging agents such as environmental risk factors including cigarette smoking[9]. The p53 inactivation in human cancer may result through binding to viral proteins, as a result of *MDM2* or *p19ARF* gene alteration or indirectly by p53 protein localization in the cytoplasm[10]. Furthermore, it has been shown that *p53* mutation is the most common aberration in human cancers, including esophageal carcinoma[11]. These mutations can lead to an increase in the stability of the protein, so it can accumulate in nuclei and be detected by immunohistochemistry methods. Therefore, it has been suggested that all cancer cells have some *p53* aberrations resulting in over-expression of p53[12].

Furthermore, it has been shown that p53 over-expression appears to have a central role in the progression of esophageal cancer in patients who have a positive history of tobacco consumption[7,13-15].

It is generally accepted that esophageal cancer develops through a multi-step process of genetic and epigenetic changes leading to a sequence of histological changes in the epithelia, including esophagitis, basal cell hypertrophia, dysplasia, carcinoma *in situ*, and finally advanced ESCC[13,16-19].

In normal cells, wild-type *p53* up-regulates the expression of several downstream genes to arrest the cell cycle so that damaged DNA either is repaired or apoptosis is promoted in response to DNA damaging agents[20,21]. The product of the mutated *p53* gene has a much longer half-life compared to the wild-type protein. Because of some conformational changes, it is more stable; thus, the accumulation of this protein in the early steps of carcinogenesis is easily detected by immunohistochemical techniques. Previous reports have shown a significant association between *p53* mutations and immunohistochemical p53 nuclear reactivity[19,22-24].

The p21 protein, which is encoded by the *p21*$^{WAF1/Cip1}$ gene, is regulated by wild type, not the mutant, *p53*[25,26]. It inhibits DNA synthesis, as well as the G1/S phase transition, by forming a complex with proliferating cell nuclear antigen and cyclin dependent kinase[27,28]. However, in addition to p53-dependent expression, p21 can be regulated in a p53-independent manner[29,30]. Unlike p53, mutation of the *p21* gene is a rare event in human cancers; therefore, alterations of this gene, involved in carcinogenesis, may be due to some abnormal changes at the expression level rather than genetic coding and epigenetic alterations[31]. Aberrant expression of p21, detected by immunohistochemical staining, has been shown in several cancers, including esophageal cancer, in which both the decrease and increase in p21 expression are reported to be associated with poor prognosis[32-34]. Concerning the clinical relevance of p21 and p53 expression in cancer patients, several studies have indicated that analyzing the combined immunohistochemical expression of these proteins may be more useful in interpreting the favorable and unfavorable clinical outcome than investigating each of them separately[35,36].

The current study was conducted to investigate the immunohistochemical expression of p53 and p21 in 80 ESCC patients in relation to possible risk factors, such as cigarette smoking, and to evaluate whether their expression is a prognostic factor with regard to p53-dependent and -independent pathways.

MATERIALS AND METHODS

Study population

A total of 80 consecutive patients with histologically-confirmed invasive squamous cell carcinoma of the esophagus (45 males and 35 females; mean age 61.39 ± 11.42 years, ranging from 35 to 83 years) were recruited from the two main referral oncology centers in northeastern Iran: Atrak clinic, the main specialized center for upper gastrointestinal (GI) disorders in Golestan province, and Omid Oncology Hospital, referral oncology hospital of northeastern Iran. All eligible subjects were recruited between September 2006 and September 2007 and written informed consents were obtained. The study was approved by the Ethics Committee of Tehran University of Medical Sciences.

The eligibility criteria for the enrolled ESCC patients were: (1) presence of a primary ESCC with no history of concurrent cancer in other organs or history of previous cancer in any organ; and (2) recent diagnosis of ESCC in the patients. Patients who had received any adjuvant therapy (radiotherapy or chemotherapy) were excluded.

Eighty eligible healthy subjects were randomly selected among individuals who were referred to Atrak clinic for upper GI health examination and diagnosed as normal based on physical examination and were histologically proven not to have a cancerous lesion. They were genetically unrelated to the cases and they had no previous cancer history. The control group was matched to the case group by age (± 6 years) and gender. According to a standard questionnaire, the demographic data of each patient and information about social habits, including cigarette smoking and opium use, were collected by an expert member of the research group.

With respect to social habits, never-users were defined as subjects who never or rarely used cigarettes or opium, and ever-users were defined as subjects who had used cigarettes or opium at least weekly for a period of 6 mo or more.

All patients were staged with radiological contrast (barium swallow) and computed tomography (CT) scans of the chest, abdomen, and pelvis. Endoscopic ultrasound and magnetic resonance imaging were performed when available. After the initial staging, patients with potentially operable conditions (defined as stages Ⅱ or ⅢA) proceeded directly to esophageal resection. The rest were categorized as an inoperable subgroup of patients based on their clinicopathological conditions. For those patients who were candidates for surgical resection, pathological stage was determined by histopathology at the time of esophagectomy, on the basis of the Union International Cancer TNM classification guidelines[37].

All 80 ESCC cases were followed up every 3 mo by office visits for clinical evaluation or *via* telephone contact. Deaths caused by ESCC were taken as outcome events, whereas others were considered censored. Survival duration was defined as the time interval from diagnosis to either death or the time of the last clinical evaluation of the patients. The cause of death was determined from the patient's records and death certificate.

Tissue collection

Tumor tissue and corresponding adjacent normal esophageal tissue specimens were obtained from the ESCC patients. All untreated specimen-proven carcinoma of the esophagus in ESCC cases, as well as esophageal normal epithelia of healthy controls, were obtained by esophagectomy or endoscopy procedure. All specimens were fixed and stored in 70% ethanol and embedded in paraffin. Esophageal squamous tumors were comprised of > 70% malignant cells with minimal necrosis, and normal esophageal specimens with no contaminating tumor cells were confirmed as noncancerous tissue by histological examination of a representative hematoxylin and eosin stained slide. Tumors were histologically verified as ESCC and sub-typed based on the grade of differentiation as well differentiated, moderately differentiated or poorly differentiated. Tumor tissue samples were selected so that all adjacent normal esophageal tissues were obtained from the macroscopically normal esophageal epithelium, distant from the cancerous lesion.

Immunohistochemical staining

Tissue sections 4-μm in thickness were obtained from archival alcohol-fixed paraffin-embedded tissues of the esophageal squamous tumor and normal esophageal specimens and mounted on poly-L-lysine-coated slides for immunohistochemistry study. After being dewaxed in xylene and rehydrated in a series of graded alcohols, they were placed in 10 mmol/L citrate buffer pH 6.0 to unmask the epitopes. After microwave antigen retrieval (20 min, 120 W; 3 × 5 min, 450 W), the sections were allowed to cool down to room temperature (approximately 20 min), and then incubated with 3% H_2O_2 for 10 min to quench the endogenous peroxidase activity. After blocking the nonspecific protein binding with serum-free protein block (Dako, Inc.) for 5 min, slides were incubated for 45 min at 37℃ with either anti-human $p21^{waf1/cip1}$ monoclonal antibody (clone SX118, DAKO, CA, USA; dilution 1:50) or anti-p53 monoclonal antibody (clone DO-7; DAKO, CA, USA; dilution 1:50) DO-7 which was raised against an epitope between amino acids 1 and 45 in the C-terminal domain of human wild-type and mutant *p53* recognizing both mutant and wild-type p53 protein, followed by phosphate buffered saline wash. Finally the primary antibody was detected, using EnVision™ + System/HRP, rabbit/mouse (DAB+) (Dako, Denmark), a secondary antibody. Staining was visualized using 3,3'-diaminobenzidine chromogen for 10 min, followed by acidified hematoxylin counterstaining for 1 min. Thereafter, the sections were mounted with mounting medium.

Control sections of known p53-positive and p21-positive cases of ESCC were included in each run, and the negative control section was carried out by omitting the primary antibody. Two expert pathologists who were blinded to the clinical and molecular results evaluated the tissue slides, independently. The final result was obtained through the consensus between the pathologists. Only staining of the cell nucleus was considered as a positive reaction for both $p21^{WAF1/CIP1}$ and p53 proteins (Figures 1 and 2). For p21 protein, the expression of p21 was graded as negative staining, < 10%; intermediate staining or low-expression, 10%-49%; high staining or over-expression, ⩾ 50%. The p53-negative expression was defined as less than 5% of p53 immunoreactivity, and p53-positive expression was classified into two groups according to the percentage of positive nuclei (5%-49%, intermediate staining or low-expression; strong staining or over-expression, ⩾ 50%). The median value for each p53 or p21 immunostaining (50%) was used as the cut off point for over-expression[24,38-40]. We also considered the adjacent non-neoplastic squamous epithelia to compare the positive staining in tumors.

Statistical analysis

The Statistical Package for the Social Sciences software version 16.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. The associations between p53 or p21 expression, clinicopathological parameters, and related risk factors were evaluated by the χ^2 test and Fisher's exact test in univariate analysis, and by logistic regression modeling in multivariate analysis. Prognostic factors were evaluated at the univariate level using the Kaplan-Meier method with log-rank test, and in multivariate analysis using the Cox's proportional hazards model of relevant prognostic variables. A 2-sided P value < 0.05 was considered as significant statistically.

RESULTS

A total of 80 ESCC cases (45 males and 35 females; mean age 61.39 ± 11.42 years, ranging from 35 to 83 years) and 80 healthy controls (48 males and 32 females; mean age

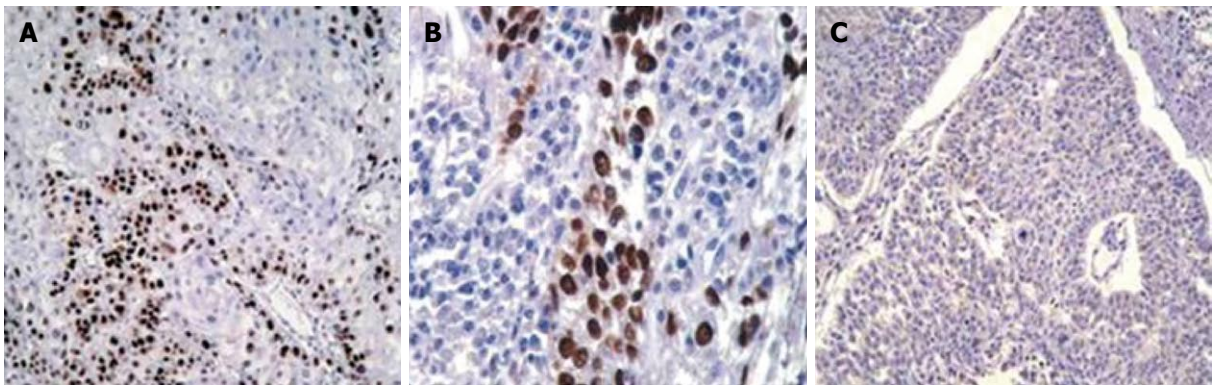

Figure 1 Immunohistochemical staining for *p53* in an esophageal squamous cell carcinoma exhibiting expression. A: With primary antibody, showing reactivity (brown nuclear staining of some tumor cells) (× 100); B: With primary antibody, showing reactivity (× 400); C: With primary antibody, no reactivity (× 100). Sections were counter-stained with hematoxylin.

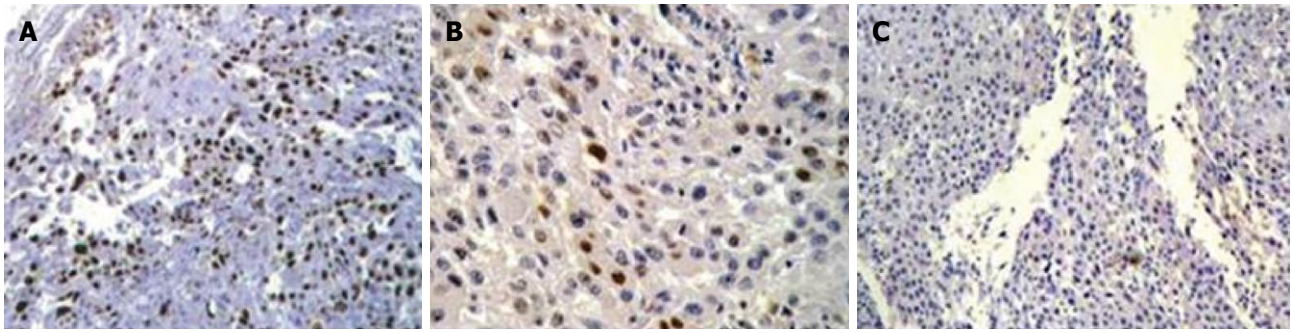

Figure 2 Immunohistochemical staining for *p21* in an esophageal squamous cell carcinoma exhibiting expression. A: With primary antibody, showing reactivity (brown nuclear staining of some tumor cells) (× 100); B: With primary antibody, showing reactivity (× 400); C: With primary antibody, no reactivity (× 100). Sections were counter-stained with hematoxylin.

Table 1 Distribution of the demographic variables for esophageal squamous cell carcinoma cases and healthy subjects *n* (%)

	Case	Control	*P* value[1]
Age (yr)			
< 60	36 (45)	27 (33.8)	NS
⩾ 60	44 (55)	53 (66.2)	
Gender			
Male	45 (56.2)	48 (60)	NS
Female	35 (43.8)	32 (40)	
Smoking			
Never	52 (65)	28 (35)	0.03
Ever	64 (80)	16 (20)	
Opium use			
Ever	21 (26.2)	18 (22.5)	NS
Never	59 (73.8)	62 (77.5)	
p53 expression			
Positive	45 (56.2)	0 (0)	< 0.001
Negative	35 (43.8)	80 (100)	
p21 expression			
Positive	59 (73.8)	37 (46.2)	< 0.001
Negative	21 (26.2)	43 (53.8)	

[1]Not statistically significant by χ^2 test. NS: Not significant.

62.81 ± 10.36 years, ranging from 32 to 84 years) were examined in this study. The distribution of demographic variables for the cases and controls are summarized in Table 1.

Analysis of protein expression by immunohistochemical staining

Expression of p53 protein in ESCC: Positive expression of p53 protein (Figure 1) was detected in 56.2% (45/80) of ESCC cases, and in none of the normal esophageal tissues of the control group ($P < 0.001$). Among the 35 ESCC tumors with p53-negative expression, 31 tumors showed no expression at all. The percentage of p53-positive cells ranged from 0% to 100%, with a mean of 54.6% and a median value of 50%. Of the p53-positive specimens, low-expression of p53 was detected in 21 of 80 cases (26.2%) and p53 over-expression was found in 24 of 80 cases (30%).

In the group of normal adjacent tissue, positive expression of p53 was observed only in the nuclei of basal cells. p53-negative expression was detected in 90% (54/60) of the normal adjacent tumor tissues; whereas, we detected the positive expression of p53 in 10% (6/60) of the normal adjacent tumor tissue samples including 50% (3/6) with dysplastic lesions, one graded as esophageal tissue with moderate to severe esophagitis, and the remaining two were histopathologically normal adjacent tissues.

There was no significant association between tumor and normal adjacent tissues based on p53-positive expression.

Expression of p21 protein in ESCC: Of the 80 ESCC

Table 2 Correlation between p53 and p21 expression in esophageal squamous cell carcinoma cases *n* (%)

p53 expression	p21 expression			Total number
	Negative (< 10%)	Positive		
		Low-expression (10%-49%)	Over-expression (≥ 50%)	
Negative (< 5%)	10/80 (12.5)	15/80 (18.7)	9/80 (11.2)	34
Positive				
Low-expression (5%-49%)	7/80 (8.75)	7/80 (13.6)	9/80 (11.2)	23
Over-expression (≥ 50%)	4/80 (5)	7/80 (13.6)	12/80 (15)	
Total number	21	29	30	80

There was no significant association between p53 and p21 expression among esophageal squamous cell carcinoma cases.

cases assessed in this study, positive expression of p21 protein (Figure 2) was detected in 73.8% (59/80) of ESCC cases, whereas only 43.8% (35/80) of controls had positive expression for p21 protein ($P < 0.001$). In the group of esophageal tumors, the percentage of cells within a section showing definite immunoreactivity varied from 0%-90%. Positive expression of p21 was detected with the average of 42.5% of cells in cases, and 17.5% of cells in controls. The corresponding median values of positive cells were 50% and 15% in the case and control groups, respectively. Twenty-one cases (26.2%) were detected as p21-negative, 29 of 80 (36.3%) cases had intermediate staining (low-expression) of p21 and in 30 of 80 (37.5%) cases, we detected p21 over-expression (high staining). Conversely, the corresponding values were 45 of 80 (56.2%), 35 of 80 (43.8%), and none (0%), for the control group, respectively ($P < 0.001$).

p21-positive nuclei were detected in 45% (27/60) of normal adjacent tissue in ESCC cases. This was significantly lower than in the tumor tissues in the case group ($P = 0.001$).

Comparison of p21 and p53 protein expression in ESCC: Immunohistochemical expression of p53 and p21 varied in the proportion of stained cells and the distribution of positive cells was heterogeneous between cancer nests. Overall, there was no significant correlation between p21 and p53 expression, at all cut off values, among ESCC cases (neither in tumors, nor in the normal adjacent tissues). Combined analysis of p21 and p53 expression has been summarized in Table 2.

Relationship between the expression of p53 and p21 proteins and clinicopathological parameters, including cigarette smoking

The relationship between p53 and p21 protein expression (at any cut off value) and different demographic and clinicopathologic parameters has been analyzed in the whole series of patients, in the p53-negative and p53-positive subgroups, and in the subgroups of patients who did or did not undergo esophagectomy, separately.

In the whole series of ESCC patients, our results showed that p53 or p21 expression was not related to age category, opium use, tumor location, histology of the tumor, depth of tumor invasion, lymph node involvement, or disease stage, when they were simply dichotomized to positive and negative groups, whereas over-expression of the p53 protein was observed in 46.4% (13/28) of ever-smokers but in only 19.2% (10/52) of never-smokers; the difference was statistically significant ($P = 0.01$, OR = 3.64; 95% CI: 1.32-10.02). After controlling for the potential confounding effects of age, sex, opium use, tumor size, tumor location, depth of tumor, lymph node involvement, disease stage, histology of the tumor, and p21 expression, multiple logistic regression analysis showed similar results ($P = 0.03$, OR = 3.89; 95% CI: 1.09-13.89). We did not find any statistically significant association between cigarette smoking and p21 protein expression at any cut off value of 10% or 50%.

In addition, combined analysis of p53 and p21 expression showed that there was no significant correlation between p21/p53 expression and pathological stages or other parameters when we used different cut off values; however, the esophageal tumors only expressing high levels of p21 protein (≥ 50%) (without p53 over-expression), were significantly associated with deep invasion ($P = 0.01$).

The relationship between clinicopathological findings and p53/p21 over-expression is shown in Table 3. We did not detect any significant association between p53 or p21 over-expression with different parameters among ESCC patients, when we compared all the cut off values.

Clinical outcome

All patients were followed up, and survival analysis was performed at the end of the study period in September 2009 (Figure 3). Among the entire patient population, mean survival was 8.21 ± 4.92 mo, with a median of 7.5 mo; ranging from 4 to 24 mo. Of the 80 ESCC patients, 56.2% (45/80) underwent curative esophagectomy, including 73.3% and 26.7% with stage Ⅱ and ⅢA of ESCC, respectively (mean survival, 9.49 ± 5.02 mo; median, 8 mo; ranging from 4 to 24 mo). On the other hand, 43.8% (35/80) of the cases were categorized as inoperable ESCC patients (mean survival, 6.57 ± 4.33 mo; median, 5 mo; ranging from 4 to 17 mo).

Prognosis of ESCC patients according to clinicopathological parameters and p21 and/or p53 protein expression

The overall 6-mo, 1- and 2-year survival rates of the entire group (80 ESCC patients) were 56.7%, 26.7% and 18.6%, respectively.

Table 3 Correlation between clinicopathological parameters and p53 and p21 over-expression in esophageal squamous cell carcinoma patients *n* (%)

	Number	p53 over-expression			p21 over-expression		
		Yes	No	*P* value	Yes	No	*P* value[1]
Age (yr)							
< 60	36	10 (27.8)	26 (72.2)	NS	14 (38.9)	22 (61.1)	NS
⩾ 60	44	13 (29.5)	31 (70.5)		16 (36.4)	28 (63.6)	
Gender							
Male	45	15 (33.3)	30 (66.7)	NS	20 (44.4)	25 (55.6)	NS
Female	35	8 (22.9)	27 (77.1)		10 (28.6)	25 (71.4)	
Smoking							
Ever-user	28	13 (46.4)	15 (53.6)	0.01	14 (50)	14 (50)	0.09
Never-user	52	10 (19.2)	42 (80.8)		16 (30.8)	36 (69.2)	
Differentiation							
Well	46	12 (26.1)	34 (73.9)		20 (43.5)	26 (56.5)	
Moderate	23	8 (34.8)	15 (65.2)		6 (26.1)	17 (73.9)	NS
Poor	11	3 (27.3)	8 (72.7)	NS	4 (36.4)	7 (63.6)	
Tumor site							
Middle	59	19 (32.2)	40 (67.8)	NS	26 (44.1)	33 (55.9)	NS
Lower	20	4 (20)	16 (80)		4 (20)	16 (80)	
Size of tumor (cm)							
< 3	23	8 (34.8)	15 (65.2)		5 (21.7)	18 (78.3)	0.07
⩾ 3	53	15 (28.3)	38 (71.7)	NS	23 (43.4)	30 (56.6)	
Operability							
Operable	45	12 (26.7)	33 (73.3)	NS	17 (37.8)	28 (62.2)	NS
Inoperable	35	11 (31.4)	24 (68.6)		13 (37.1)	22 (62.9)	

[1]Not statistically significant by χ^2 test. NS: Not significant.

Results of the univariate analysis for the whole series of patients showed no influence of p53 protein expression on survival duration, even if different cut off values were considered (5% and 50%). Similarly, no significant association was found between p21 expression and survival duration, using the cut off value of 10%, whereas the 50% cut off value revealed a significant association between p21 over-expression and poor clinical outcome ($P = 0.009$). In a univariate survival analysis for the entire group of cancer patients, there was no significant survival effect for all available clinicopathologic factors for ESCC patients, except for the patients who were aged above 60 years ($P = 0.006$), or those who underwent surgical operation ($P = 0.001$); factors which were significantly associated with poorer prognosis. Our findings also revealed a significantly reduced survival period among the cases with both p21 and p53 over-expressing tumors compared to patients with p21 over-expressing tumors alone (without p53 over-expression), or in those without over-expression of both p21 and p53 proteins ($P < 0.001$).

Furthermore, to analyze the factors related to prognosis according to p53 protein expression (for both 5% and 50% cut off values), univariate and multivariate analysis were performed separately. Among the p53-positive cases, the factors related to poorer clinical outcome consisted of patients with p21 over-expressing tumors ($P = 0.009$) and those who were aged above 60 years ($P = 0.03$).

Additionally, when analyzing clinical outcome according to p53 and p21 expression in 45 patients who underwent surgery, patients with p21 over-expressing tumors showed poorer clinical outcome ($P = 0.01$). This adverse effect was still significant when the study population was restricted to the operable patients with p53 over-expression ($P = 0.004$).

The Cox proportional hazards regression model showed that age categories, surgical operation status (operable or inoperable), and p21 over-expression were independent prognostic factors (Table 4).

DISCUSSION

The significant positive expression of p53 and p21 in the ESCC patients of this studied population, compared with the healthy subjects, revealed that these proteins play an important role in ESCC development in northeastern Iran. Furthermore, we found that p53 over-expression, but not p21, was associated with cigarette smoking habit in the ESCC patients. Contradictory results have been reported regarding the association of p53 protein expression and cigarette smoking. Our finding is consistent with the studies published by Mizobuchi *et al*[7], Montesano *et al*[41] and Cruz *et al*[39], but discordant with the observations of Lam *et al*[42]. Recent studies have shown that various kinds of carcinogens produced by smoked cigarettes might be responsible for different *p53* gene mutations and p53 over-expression; thus, they may play a role in carcinogenesis, including esophageal cancer development[7,43-45]. In this regard, recent evidence from Golestan province (in northeastern Iran) inhabitants showed that moderate to high exposure to polycyclic aromatic hydrocarbon (PAH) components, one of the substances related to cigarette smoke, may be associated with esophageal carcinogenesis[46]. Therefore, it has been hypothesized that continuous exposure to specific carcinogenic components

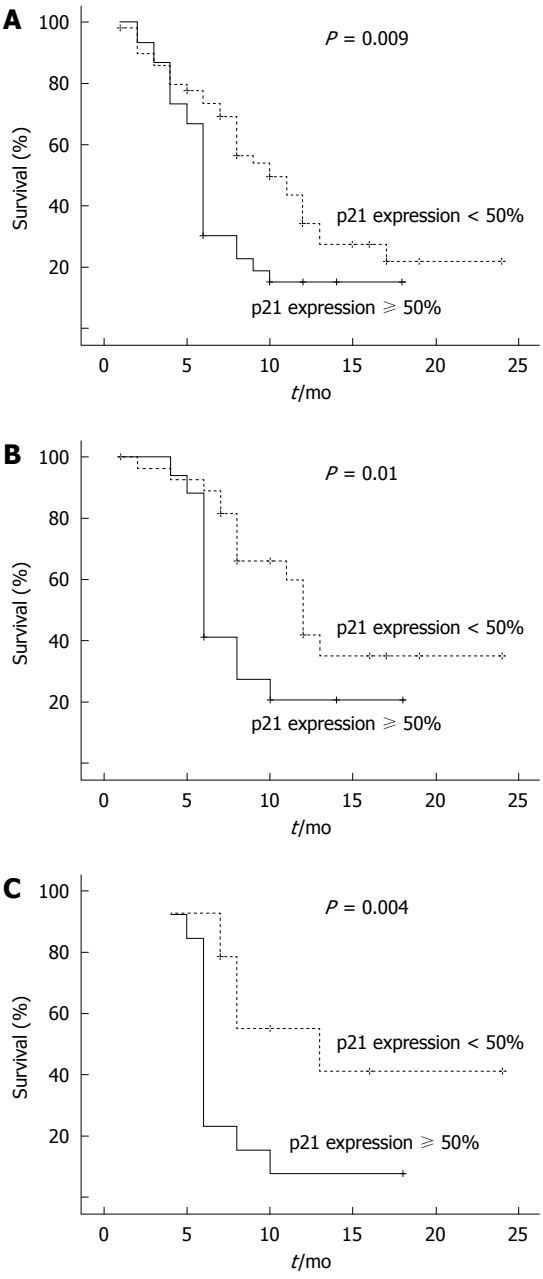

Figure 3 Kaplan-Meier survival curves for esophageal squamous cell carcinoma patients with or without p21 over-expression. A: Overall survival curves were classified by p21 over-expression in the whole series of ESCC patients; B: Survival curves in the operable group of ESCC patients, stratified according to p21 over-expression; C: Survival curves in the operable group of patients with positive expression of p53, stratified according to p21 over-expression.

of tobacco smoke in the studied area, such as PAH, may cause mutations in some important cell cycle genes, such as *p53*, leading to over-expression and abnormal accumulation of the translated proteins. This specific mutation may result in the formation of a dysfunctional protein, which is sequestered and accumulated in the cell, leading to cancer development. These observations provide support for further studies to evaluate the effect of possible carcinogen components of tobacco smoke, such as PAH, in ESCC patients residing in this region.

In the present study, positive expression of p53 protein in the peripheral layers of cancer nests, which are representative of the most proliferative and invasive cells in the esophageal SCC (due to the inactivation by mutation or deregulation of other cancer-related genes in the cell cycle), may suggest that the p53-positive expression is a frequent genetic alteration and plays an important role in the carcinogenesis of esophageal carcinoma in the studied population.

Recent studies have indicated that alteration in the *p53* gene, as well as p53 protein accumulation, is frequently detected in dysplastic or precancerous lesions adjacent to ESCC tumors[47]. In the present study, 10% (6/60) of morphologically normal esophageal specimens adjacent to tumors showed p53 positive staining, including 3 samples with dysplastic lesions, one sample with moderate to severe esophagitis, and another two normal samples with no pathologic change. Although all of these specimens were positive for p21 immunostaining, the frequency and intensity of p21 expression were greater in dysplastic lesions than in the others. The observation of p53-positive expression in the adjacent dysplastic lesions (with or without p53 positivity in corresponding tumor) in this study supports the concept that potentially multiple origins, through similar or independent genetic alterations, may result in the development or recurrence of esophageal tumor in this site (either from the same clone or a different one as a consequence of other involved molecular alterations and pathways). Therefore, it is important to note that in patients, who have had the primary tumor removed, p53 accumulation may be a risk factor for tumor recurrence. This finding may be an important factor for the screening of the ESCC patients in a high-risk population. Therefore, immunohistochemical staining of p53 protein in the remaining unresected normal-appearing esophagus, beyond the normal margin, may be a valuable tool in these patients, to evaluate the risk of developing a secondary ESCC after an esophagectomy. Further prospective, large-scale studies are required as a validation set to support this concept.

Regarding the association between p21 and p53 protein expression in cancer patients, several studies have shown that one of the important ways to investigate the functional status of p53 is to evaluate some of its downstream effectors such as $p21^{Waf1/Cip1}$[48]. Unlike p53, the positive expression of p21 is most often representative of the wild-type protein since no mutations in this gene have been detected in a large number of human tumors[38,49]. p21 protein may be regulated either in a p53-dependent or -independent manner. In our study we found no significant correlation between p53 and p21 proteins, and co-expression of p21 and p53 proteins in a proportion of ESCC cases supports the hypothesis that activation of p21 was regulated through a p53-independent pathway in this series of esophageal tumor samples, in agreement with a previous report by Seta *et al*[50], who showed there was no correlation between the expression of these proteins in esophageal or gastric cancer. Similar results were also reported by Yasui *et al*[51] and Gomyo *et al*[52] for gastric cancer.

Table 4 Log-rank and proportional hazard regression analysis (Cox method) for clinicopathological parameters in esophageal squamous cell carcinoma patients

	Mean survival time (mo)	Log rank *P* value	Cox-regression		
			HR	95% CI	*P* value[1]
Operability					
Operable	12.68 ± 2.56	0.001	2.27	1.30-3.97	0.004
Inoperable	7.08 ± 1.72				
p21 over-expression					
Yes	7.41 ± 1.78	0.009	1.82	1.02-3.25	0.04
No	11.71 ± 2.36				
p53 over-expression					
Yes	8.00 ± 2.06	0.30	1.23	0.67-2.26	NS
No	10.69 ± 2.08				
Tumor size (cm)					
< 3	12.58 ± 3.28	0.06	1.75	0.92-3.32	0.08
⩾ 3	9.43 ± 2.18				
Age (yr)					
< 60	12.61 ± 2.46	0.006	2.30	1.29-4.09	0.005
⩾ 60	8.40 ± 2.14				

[1]Not statistically significant by χ^2 test. NS: Not significant; HR: Hazard ratio; CI: Confidence interval.

Several studies have investigated the significant prognostic impact of p21 over-expression in different cancers, including esophageal carcinoma[35,36,52,53]. However, the results are contradictory[54,55]. The discrepancy in the findings might be due to lack of a standard classification for p21 immunostaining interpretation, or it may depend on different characteristics of malignant cells or different molecular markers regulating p21 expression in a specific tissue or tumor type. In the present study, we adopted the cut off value of 50% nuclear staining to indicate p21 over-expression, as it was applied in some of the previous studies[53]. Using these criteria, our results showed that the prognosis of esophageal cancer patients deteriorates with p21 over-expression (in both univariate and multivariate survival analysis). This is consistent with the study by Sarbia *et al*[53] who showed an adverse survival effect of p21 over-expressing esophageal tumors when they considered the cut off value of 50% as p21 over-expression. Goan *et al*[36] also indicated that p21 over-expression was associated with adverse prognosis in ESCC patients. However, this result contradicts the result of Shimada *et al*[56]. In addition, the adverse survival effect of p21 over-expression in the present study was still significant when the study population was restricted to the patients with p53-positive expression who underwent surgical operation. Some studies have shown that combined analysis of p53 and p21 expression may provide more prognostic information than evaluation of either variable alone[57,58]. In this regard, our findings also revealed a significantly reduced survival period among the cases with both p21 and p53 over-expressing tumors than in patients with p21 over-expressed tumors alone (without p53 over-expression), or than in those without over-expression of both p21 and p53 proteins. This may suggest a possibly more malignant behavior of the tumors when they over-express both p53 and p21 proteins. In other words, patients who harbor p21 over-expressing tumor have a compromised survival that could be superimposed by the adverse effect of non-functional accumulated p53, leading to poorer prognosis.

Concerning the adverse prognostic effect of p21 over-expression, recent studies have shown that despite the role of p21 in cell cycle arrest, this protein could contribute to the inhibition of DNA repair and mitotic control. In the presence of p53 mutation, the adverse survival effect of p21 over-expression could be increased, leading to uncontrolled high expression of p21, as well as sustained genomic instability, leading to facilitation of the progression of the tumor[36,59]. Therefore, this phenomenon may also be responsible for the adverse survival effect of p21 over-expression among the studied population in the present study.

In conclusion, this is the first study focused on evaluating the prognostic effect of p21 and p53 protein expression, as well as their role as a target for cigarette smoking, in ESCC patients in northeastern Iran which is a high-incidence area for this type of cancer. Our results showed that (1) p53 and p21 expression play an important role in ESCC development in northeastern Iran; (2) p53 as a target of cigarette smoking plays a critical role in ESCC development among this high-risk population; (3) the presence of abnormally accumulated p53 in the morphologically normal tissue adjacent to the resected tumor may be a predictor of future recurrence of tumor, thus evaluating the remaining normal esophageal tissue after resection of tumor could help to indicate a population who are at higher risk for tumor recurrence at this site; and finally (4) we indicated the adverse survival effect of p21 over-expression in the ESCC patients of northeastern Iran. Therefore, our data suggest that the immunohistochemical assessment of p21 over-expression, in relation to p53 over-expression, in esophageal cancer patients may provide useful prognostic markers for identifying the subgroup of high risk patients with poor clinical outcome who need closer postoperative follow up, and probably a more intensive therapeutic protocol.

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COMMENTS

Background

The incidence of esophageal squamous cell carcinoma (ESCC) is high in northeastern Iran. This is due to environmental and genetic risk factors. *p53* gene mutation appears to have a central role in the progression of esophageal cancer in patients who have a positive history of cigarette smoking. The current study was conducted to investigate the immunohistochemical expression of p53 and p21 among ESCC patients from northeastern Iran in relation to possible risk factors, such as cigarette smoking, and to evaluate whether their expression is a prognostic factor according to p53-dependent and -independent pathways.

Research frontiers

This study showed that the presence of abnormally accumulated p53 in the morphologically normal tissue adjacent to a resected tumor may be a predictor of future recurrence of the tumor. Also, results show the adverse survival effect of p21 over-expression on the ESCC patients of northeastern Iran.

Innovations and breakthroughs

This is the first study describing progression of ESCC in patients who are resident in northeastern Iran with a positive history of cigarette smoking.

Applications

The results of this study may provide useful prognostic markers, such as p53 and p21 over-expression, for identifying the subgroup of high risk patients with poor clinical outcome who need closer follow up, and probably more intensive therapeutic protocol, during postoperative management.

Peer review

As this author mentioned, over-expression of p53 in immunohistochemistry is generally thought to be mutated p53. Sometimes it is difficult to say whether these all originate from the mutation, because there are other ways of inactivation of p53. The author should try to clarify how the p53 overexpression happened or give more scientific evidence or reference about this point.

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BRIEF ARTICLE

Etiology and long-term outcome of extrahepatic portal vein obstruction in children

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Abstract

AIM: To study the management and outcome of children with extrahepatic portal vein obstruction (EHPVO) in a whole country population.

METHODS: A nationwide multicenter retrospective case series of children with EHPVO was conducted. Data on demographics, radiographic studies, laboratory workup, endoscopic and surgical procedures, growth and development, were extracted from the patients' charts. Characteristics of clinical presentation, etiology of EHPVO, management and outcome were analyzed.

RESULTS: Thirty patients, 13 males and 17 females, 19 (63.3%) Israeli and 11 (36.7%) Palestinians, were included in the analysis. Age at presentation was 4.8 ± 4.6 years, and mean follow-up was 4.9 ± 4.3 years. Associated anomalies were found in 4 patients. The incidence of EHPVO in Israeli children aged 0-14 years was 0.72/million. Risk factors for EHPVO were detected in 13 (43.3%) patients, including 9 patients (30%) with perinatal risk factors, and 4 patients (13.3%) with prothrombotic states: two had low levels of protein S and C, one had lupus anticoagulant, and one was homozygous for methyltetrahydrofolate reductase mutations. In 56.6% of patients, no predisposing factors were found. The most common presenting symptoms were an incidental finding of splenomegaly (43.3%), and upper gastrointestinal bleeding (40%). No differences were found between Israeli and Palestinian children with regard to age at presentation, etiology and clinical symptoms. Bleeding occurred in 18 patients (60%), at a median age of 3 years. Sclerotherapy or esophageal banding was performed in 20 patients. No sclerotherapy complications were reported. Portosystemic shunts were performed in 11 patients (36.6%), at a median age of 11 (range 3-17) years: splenorenal in 9, mesocaval in 1, and a meso-Rex shunt in 1 patient. One patient underwent splenectomy due to severe pancytopenia. Patients were followed up for a median of 3 (range 0.5-15) years. One patient died aged 3 years due to mucopolysaccharidase deficiency type Ⅲ. None of the patients died due to gastrointestinal bleeding.

CONCLUSION: EHPVO is a rare disorder. The etiological factors are still mostly unknown, and the endoscopic and surgical treatment options ensure a good long-term prognosis.

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Key words: Children; Extrahepatic; Obstruction; Outcome; Portal; Vein

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INTRODUCTION

Extrahepatic portal vein obstruction (EHPVO), although rare in children, is an important cause of portal hypertension and upper gastrointestinal (UGI) bleeding in the pediatric age group[1,2]. The etiology of EHPVO is diverse and risk factors are usually detected in less than half of patients, and include perinatal events such as umbilical catheterization and sepsis, and prothrombotic disorders[2-4].

We aimed to study the evaluation, management and outcome of children with EHPVO in a whole country population.

MATERIALS AND METHODS

This study is a multicenter retrospective case series of children with EHPOV diagnosed and followed in all pediatric gastroenterology and hepatology divisions in Israel during the period January 1, 1993 to December 31, 2008. All pediatric gastroenterologists and hepatologists registered in the country were approached by mail and telephone and asked to participate in the study. The patients were allocated *via* the hospital or outpatient clinic archive registrations, including searches for hospitalizations, outpatient visits, radiological, endoscopic and surgical procedures with one or more of the following diagnoses: portal vein, thrombosis, splenomegaly, hepatomegaly, gastrointestinal bleeding, varices, sclerotherapy, ligation, liver biopsy, elevated liver enzymes, and shunt. Patients with portal vein obstruction and portal hypertension due to chronic liver disease were excluded after chart reviews. The referral population included both Israeli and Palestinian children referred to a medical center in Israel from the Gaza Strip and West Bank.

Data on demographics, radiographic studies, laboratory workup, endoscopic and surgical procedures, growth and development, were extracted from the patients' charts.

Table 1 Demographic data, clinical presentation and risk factors for extrahepatic portal vein obstruction in the study group

	n (%)
Gender	
Male	13 (43.3)
Female	17 (56.7)
Ethnicity	
Jewish	15 (50)
Arab	14 (46.6)
Russian	1 (3.3)
Referral	
Israeli	19 (63.3)
Palestinian	11 (36.7)
Gestational age	
Term	23 (76.7)
Preterm	7 (23.3)
Clinical presentation	
Splenomegaly	13 (43.3)
UGI bleeding	12 (40.0)
Cytopenia	5 (16.6)
Elevated liver enzymes	2 (6.6)
Risk factor for EHPVO	
Perinatal events	
Umbilical catheterization	6 (20)
Omphalitis	2 (6.6)
Sepsis, NEC	1 (3.3)
Hypercoagulable state	
APLA syndrome	2 (6.6)
Protein S and C deficiency	1 (3.3)
MTHFR mutation homozygosity	1 (3.3)
Unknown	17 (56.6)

EHPVO: Extrahepatic portal vein obstruction; UGI: Upper gastrointestinal; MTHFR: Methyltetrahydrofolate reductase; APLA: Anti phospholipid antibodies.

Characteristics of clinical presentation, etiology of EHPVO, management and outcome were analyzed.

Statistical analysis

The data were analyzed using Bio-medical P-series[5]. Discrete variables were compared using Fisher's exact test. Continuous variables were compared using the Mann-Whitney *U*-test, since the sample sizes were relatively small. A P-value $\leqslant 0.05$ was considered significant.

RESULTS

Thirty-two children were identified. Two patients were excluded due to missing clinical data, and 30 patients, 13 males and 17 females, were included in the analysis. The demographic data of the patients are presented in Table 1. Fourteen patients (46.6%) were Jewish, 15 (50%) were Arab, and 1 (3.3%) was of Russian descent. Nineteen (63.3%) patients were Israeli and 11 (36.7%) were Palestinians. The age at presentation was 4.8 ± 4.6 years (median 3.5 years, range 1 mo-14 years), and the mean follow-up was 4.9 ± 4.3 years (median 3 years, range 1-15 years). Associated congenital anomalies were found in 4 patients, including: cardiac anomalies (3) and mucopolysaccharidase deficiency type Ⅲ (1).

The diagnosis of EHPVO was based on clinical signs and symptoms of portal hypertension, as well as ultrasonographic findings of portal vein cavernous transformation, in the absence of any chronic liver disease. Additional radiographic evaluations were performed in 8 patients at diagnosis and during follow-up, including computed tomography (CT) with angiography (5), magnetic resonance imaging (MRI) (4), and angiography + venography (2).

Liver biopsy was performed in 20 (66.6%) patients. The histology of 10 biopsies was within normal limits. In 6 biopsies, minor changes including hepatocyte ballooning with mild sinusoidal dilatation were reported, and in 4 biopsies regenerative nodular hyperplasia was found. In these 4 patients EHPVO obstruction was diagnosed by US and CT-angiography or MRI. The mechanism of regenerative nodular hyperplasia may be similar to other hepatic findings resulting from deprivation of portal flow to the liver[4].

Incidence of EHPVO

The calculated incidence in Israeli children aged 0-14 years was 0.72/million. This calculation was based on an average number of children at this age in Israel during the years 1993-2008 (1.85 million, range 1.7-2.0 million). However, it cannot be ruled out that children with no symptoms and no complications of EHPVO were not diagnosed, and that the incidence may be higher.

The incidence in Palestinian children could not be calculated, due to referral bias. Some children may have been referred to other countries for evaluation, or followed-up in local hospitals if no bleeding or other complications occurred.

Etiology of EHPVO

Risk factors for EHPVO were detected in 13 (43.3%) patients (Table 1). Nine patients (30%) had perinatal risk factors including umbilical catheterization (6), omphalitis (2), and neonatal sepsis with necrotizing enterocolitis (1). Detailed prothrombotic profiles were available in 28 patients, including: protein S, protein C, antithrombin Ⅲ, lupus anticoagulant, factor V Leiden and factor Ⅱ mutations and methyltetrahydrofolate reductase (MTHFR) mutations. Janus kinase 2 (JAK2) V617F mutation of the prothrombin gene was assessed in 5 patients. Prothrombotic states were found in 4 patients (13.3%): two had low levels of protein S and C, lupus anticoagulant was positive in one, and one was homozygous for MTHFR mutations. In addition, 2 patients had mildly reduced activity of protein C levels, however, such levels were thought to be secondary.

In the majority of patients, 17 (56.6%), no predisposing factors for EHPVO were found.

Clinical course

The most common presenting symptoms were an incidental finding of splenomegaly on physical examination (43.3%), and UGI bleeding manifested as hematemesis and/or melena (40%). In two patients, failure to thrive with a weight under the 3rd percentile was noted at presentation in addition to the presenting symptoms.

Table 2 Clinical characteristics and course of Israeli and Palestinian children with extrahepatic portal vein obstruction *n* (%)

	Israeli	Palestinian	*P*
Gender (M/F)	11/8	2/9	0.06
Age at diagnosis (yr)			
mean ± SD	6 ± 5.1	3.5 ± 3.8	
Median (range)	7 (0.1-16)	2 (0.75-12)	NS
Etiology			
Perinatal events	4 (21)	4 (36.3)	NS
Hypercoagulability	2 (10.5)	2 (18.2)	
Variceal bleeding	9 (47.3)	9 (81.8)	NS
Follow-up (yr, mean ± SD)	6.5 ± 5.3	3.0 ± 2.2	NS
Surgery	7 (36.8)	4 (36.3)	NS

NS: Not significant.

Table 3 Endoscopic and surgical treatment of children with extrahepatic portal vein obstruction

Procedure	*n* (%)
Sclerotherapy	13 (43.3)
Variceal banding	7 (23.3)
Surgery	
Splenorenal shunt	9 (30.0)
Mesocaval shunt	1 (3.3)
Meso-Rex shunt	1 (3.3)
Splenectomy	1 (3.3)

A comparison between Israeli and Palestinian children did not reveal any significant differences with regard to the age at presentation, etiology and clinical symptoms (Table 2).

Outcome

Overall, 18 patients (60%) had bleeding: 12 (40%) at presentation and an additional 6 patients (20%) during follow-up. The median age at the first bleeding episode was 3 (range 0.75-13) years. Twenty-two patients, who had esophageal varices on upper endoscopy, received propranolol for secondary or primary bleeding prevention. Sclerotherapy or esophageal banding was performed in 20 patients. In 18 of these patients, the procedures were performed during and after bleeding, and in 2 patients banding was performed as primary prevention (Table 3). No complications of sclerotherapy were reported.

Shunt operation was performed in 11 patients (36.6%), at a median age of 11 (range 3-17) years. The indication was uncontrolled bleeding despite variceal banding in 10 patients, and emergency shunt for failure of bleeding control in one. The types of shunt were splenorenal in 9, mesocaval in 1, and meso-Rex in 1 patient. One patient underwent splenectomy due to severe pancytopenia (Table 3).

Patients were followed up for a median of 3 (range 0.5-15) years. One patient died aged 3 years due to mucopolysaccharidase deficiency type Ⅲ. None of the patients died due to gastrointestinal bleeding.

DISCUSSION

The present study summarizes a national experience of EHPVO in children with over 15 years of follow-up. The average incidence of EHPVO in children age 0-14 years in the current study was very low at 0.72 per million. Although EHPVO is an important cause of portal hypertension and gastrointestinal bleeding in children, the exact incidence of the disorder is unknown[6]. The available case series are mostly retrospective, summarizing the experience of one or multiple referral centers[2,7]. The current study is the only nationwide study including all patients diagnosed over a 15-year period, enabling the calculation of EHPVO incidence.

The results are in agreement with previous studies reporting an unknown etiology for EHPVT in over 50% of children[2-4]. Neonatal events, including umbilical catheterization and sepsis, were possible causes of EHPVO in a small number of patients in the current study, as reported by others[2]. In a retrospective study of 133 infants diagnosed with portal vein thrombosis (PVT) within the first month of life, an umbilical catheter was inserted in 73%[8]. Of 29 infants with grade Ⅲ PVT, 62% progressed to portal hypertension. It is, therefore, surprising that umbilical catheterization accounts for a minority of cases of EHPVO in children in different studies[2,7]. Since the mean follow-up period of the infants in the study by Morag *et al*[8] was only 79 d, this may indicate that most of the PVT seen post-umbilical catheterization either resolve or remain without clinical significance.

Venous thrombosis has been associated with thrombophilia[3]. Factor V Leiden mutation, which is the most common inherited cause of thrombophilia, has been described in association with hepatic vein thrombosis, but its association with PVT is questionable[3,9]. A lack of association between factor V Leiden mutation and PVT was reported in 3 studies[7,10,11]. In contrast, a high prevalence of this mutation in children with PVT (6/23 and 12/40 patients) was found in 2 other studies[12,13]. In the current study, one child had factor V Leiden mutation and an umbilical catheter, thus, the exact cause of EHPVO cannot be determined. Inherited deficiencies in protein C, protein S, antithrombin Ⅲ and prothrombin, increase the risk of venous thrombosis and are associated with EHPVO in adults[10,14,15]. Gurakan reported 5 of 12 pediatric patients with EHPVO to have protein C, S, anti thrombin Ⅲ or combined deficiencies[7]. Higher rates were found in Egyptian children - 27.5% had protein C and 2.5% had antithrombin Ⅲ deficiency[13]. However, in a study of 14 patients and their parents, the frequency of protein C, S and antithrombin Ⅲ deficiency was 43% in the PVT patients but none were inherited[16]. Mack *et al*[17] showed, that coagulation defects are pathophysiologic consequences of EHPVO, the consequence of depriving the liver of portal venous flow, and that surgical restoration of intrahepatic portal venous flow corrects the abnormalities. In the current study, 2 patients (6.6%) had low protein C values, one of them combined with protein S deficiency. Antiphospholipid antibody syndrome, identified in one of our patients, may also manifest as arterial or venous thrombosis[18]. An acquired JAK2 mutation (JAK2V617F) was recently reported in the majority of patients with polycythemia vera and essential thrombocytosis[19], and in 36% of adults with EHPVO[20]. In children, JAK2V617F screening was negative in one study[2], and was negative in the 5 patients screened in the current study.

The outcome of children with EHPVO depends on the control of variceal bleeding. Sclerotherapy and banding are effective therapies for bleeding esophageal varices[2,21,22], and may achieve long-term variceal eradication in 50% of patients[2]. Similarly, in the current study, long-term bleeding control was achieved in 50% of bleeding children. Portosystemic shunts were performed in 36.6% of patients, a higher rate than that of 8%-17% reported by others in recent studies[2,6,7]. The rate of surgery was similar for Israeli and Palestinian patients, demonstrating that there was no selection of patients living in remote areas for shunt operation. Most of the patients were followed in large centers, in which all endoscopic techniques are available, and the high surgical rate may reflect patients with more severe disease. The prognosis of patients in the current study was good, with no bleeding or liver related mortality, in agreement with other studies reporting mortality in less than 10% of patients[2,7].

The current study has a few limitations. One is the possibility of under detection in children with no symptoms and no complications of EHPVO, resulting in a lower than actual incidence. Another limitation is the shorter patient follow-up compared with other studies. The median follow-up in the current study was 3 years with a mean of 4.9 years, compared to a median of 6 years in one study[2] and a mean of 7.4 years in another study[7]. As a result, the long-term outcome may be worse than that found in the current study.

In conclusion, although EHPVO is an important cause of portal hypertension in children, it is a rare disorder. The etiological factors are still mostly unknown, and the endoscopic and surgical treatment options ensure a good long-term prognosis.

COMMENTS

Background

Extraintestinal portal vein obstruction (EHPVO), although rare in children, is an important cause of portal hypertension and upper gastrointestinal bleeding from varices in the pediatric age group. It accounts for almost 70% of children with portal hypertension. The etiology of EHPVO is diverse and risk factors are usually detected in less than half of patients and include congenital abnormalities and perinatal events such as exchange transfusions, umbilical catheterization and sepsis, and hypercoagulable states.

Research frontiers

Improvements in the definitions and tests for hypercoagulable states allow more extensive studies on the etiology of EHPVO. Medical control of acute variceal bleeding and long-term endoscopic control of varices by sclerotherapy/ ligation may improve the long-term outcome of children with EHPVO. Surgical options for shunts in patients who fail endoscopic control of bleeding include the recent introduction of the meso-Rex bypass, which results in restoration of normal blood flow to the liver.

Innovations and breakthroughs

Calculation of the incidence of EHPVO in this first national study revealed a low incidence of 0.72/million.

Applications

The long-term outcome of EHPVO in children is good, with low mortality. Variceal bleeding can be controlled in most patients, and shunt surgery is needed in about a third of patients.

Terminology

Portal hypertension caused by extrahepatic portal vein obstruction occurs when the site of the blockage is the portal vein before the blood reaches the liver. A portal cavernoma is usually formed. This disease entity is distinct and not primarily associated with primary liver disease.

Peer review

The study, though it is a retrospective analysis, is informative, well written and gives a good overview on the incidence, underlying causes and treatment of EHPVO in children in Israel.

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BRIEF ARTICLE

Glycemic index, glycemic load and insulinemic index of Chinese starchy foods

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Abstract

AIM: To determine the glycemic index (GI), glycemic load (GL) and insulinemic index (II) of five starchy foods that are commonly used in Chinese diets.

METHODS: Ten healthy subjects aged between 20-30 years were recruited. Each subject was asked to consume 50 g of available carbohydrate portions of test foods and reference food. Finger capillary blood samples were collected at the start of eating and 15, 30, 45, 60, 90 and 120 min after consumption. The GI and II of foods were calculated from the ratio of incremental area under the glucose/insulin response curves of test and reference foods. The GL for each test food was determined from its GI value and carbohydrate content.

RESULTS: The results showed that brown rice elicited the highest postprandial glucose and insulin responses, followed by taro, adlay, yam and mung bean noodles, which produced the lowest. Among the five starchy foods, brown rice evoked the highest GI and GL at 82 ± 0.2 and 18 ± 0.2, followed by taro (69 ± 0.4, 12 ± 0.2), adlay (55 ± 0.4, 10 ± 0.2), yam (52 ± 0.3, 9 ± 0.0) and mung bean noodles (28 ± 0.5, 7 ± 0.2), respectively. The II values of the test foods corresponded with GI values. Similarly, brown rice gave the highest II at 81 ± 0.1, followed by taro (73 ± 0.3), adlay (67 ± 0.3), yam (64 ± 0.5) and mung bean noodles (38 ± 0.3). All five starchy foods had lower GI, GL and II than reference bread ($P < 0.05$).

CONCLUSION: The GI, GL and II values of starchy foods provide important information for the public to manage their diet and could be useful for the prevention of lifestyle-related diseases such as diabetes mellitus.

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Key words: Glycemic response; Glycemic index; Glycemic load; Insulinemic response; Insulinemic index

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INTRODUCTION

Insulin resistance increases the risk of type 2 diabetes[1-3]. One characteristic that can be associated with insulin resistance is hyperinsulinemia that may result in deterioration of β-cell function, which is involved in the pathogenic process of diabetes[4]. In the context of current dietary strategies to prevent hyperinsulinemia and insulin resistance, it is imperative to consider diets/foods in terms of their ability to reduce the degree of postprandial glycemia

and insulinemia[5]. These issues have important public health implications. Any diet to counteract diabetes should be evaluated for its effects on glucose response and insulin secretion. To do this, it is urgent and necessary to continuously determine the glycemic index (GI) and insulinemic index (II) values of foods in different countries, especially the GI of agricultural foods.

GI was introduced to describe the extent to which different foods elicit varying degrees of postprandial blood glucose. It is defined as the incremental area under the 2 h blood glucose response curve (IAUC) after consuming a test food compared to the corresponding area after a carbohydrate-equivalent amount of a reference food (either glucose or white bread)[6,7]. Expanding this theory to the postprandial insulin levels evoked by foods, the II of foods can also be determined from the corresponding incremental blood insulin areas[8]. Because insulin is the hormone that maintains blood glucose homeostasis, a food or diet high in II could induce a higher degree of postprandial insulin concentration and thus result in higher insulin demand in the long term[9,10]. Therefore, it is compulsory to grade foods based on their GI, along with the II, to prevent both postprandial glycemia and insulinemia in humans. Glycemic load (GL), on the other hand, is a concept that summarizes both GI and the carbohydrate content and is considered to represent the overall glycemic effects of a food[11]. Recent studies have shown that increased dietary GL resulted in predictable increases in glycemia and insulinemia in humans[12,13]. Therefore, it is important to evaluate the concept of GI value of foods together with their concurrent II and GL values.

Tubers and cereals have been considered as the main carbohydrate sources in Chinese diets since the early 1960s. They are not only rich in starch, but also contain vitamins, minerals, phytoestrogens, and trace elements. In the agricultural epoch of Taiwan, where rice and grains are considered rare and expensive, people often consume tubers, such as taro and yam, as a main meal or as a rice substitute to help them harness energy for endurance farm work. In the book, "Ben Chou Gun Mu"[14], a very famous Chinese ancient medical book, they were even described as having medical purposes. With rapid development of the economy, however, eating habits and lifestyle in Taiwan are changing. There is some concern that people think it is detrimental to consume tubers and some cereal products because they are high in starch and regular eating may cause hyperpostprandial glucose responses. Some people even avoid grains or tubers in their diets, particularly diabetic patients. Therefore, it is necessary to evaluate these foods according to their glycemic and insulinemic responses, since they are involved in diet management that helps maintain normoglycemia (possibly also maintaining insulin demand). The five most available starchy foods that are controversial regarding their glycemic effects on humans were chosen for this study. The proximate nutritional components and indigestible starch [dietary fiber (DF) + resistant starch (RS)] were also evaluated in this study.

MATERIALS AND METHODS

Ethics

The study was approved by the Institutional Review Board of Kaohsiung Medical University. Informed consent was obtained from each subject before the enrollment.

Study subjects

Ten healthy subjects were selected for the study. The subjects were six females and four males, aged between 20-30 years, with a mean body mass index (BMI) of 20.6 ± 0.6 (BMI ± SE, in kg/m^2). Subjects were recruited based on the following criteria: (1) healthy weight, stable for 6 mo prior to the study; (2) not being on a diet; (3) non-smoker; (4) not taking prescription medication; (5) normotensive; and (6) normal fasting glucose[7]. All subjects were asked to avoid alcohol, legumes and fried foods, eat a regular meal the night before each test, and refrain from unusual eating habits and activity the day before each test. Subjects were also required to complete a food questionnaire before each test to ensure that they had no irregular eating habits. The procedures of the study were orally explained to the subjects, and by written notification.

Test foods

Five starchy foods and one reference food were tested in 50 g available carbohydrate portions. The test foods examined included adlay (*Coix lachryma-jobi L.*), brown rice (variety, Tai Ken #9) (*Oryza sativa L. japonica*), mung bean noodles (glass or cellophane noodles), taro (*Colocasia esculenta L. Schott*) and yam (Chinese sweet potato) (*Ipomoea batatas L. Lam*). Brown rice was manufactured by the Union Rice Company (Taipei, Taiwan). Mung bean noodles were produced by the Longkow Company (Taipei, Taiwan). Taro and yam were purchased from a local farm (Kaohsiung County, Taiwan). Regarding food preparation, brown rice was prepared by a preliminary soaking (the ratio of rice to water was 1:1.5) overnight, and cooked by a rice cooker (Tatung Co., Ltd. Taiwan) right before the tests. Mung bean noodles were boiled. Taro and yam were skin peeled, cut into 5 cm cubes and steamed by the rice cooker (Tatung Co., Ltd. Taiwan). The reference food, white bread, was laboratory made the day prior to the tests.

Experimental procedures

This study was conducted using internationally recognized GI methodology[6,7,15,16]. All subjects were blind to the name of the food being tested. White bread was the reference food (GI = 100%) against which all test foods were compared. Subjects arrived at the laboratory at eight to nine o'clock in the morning after 10-12 h overnight fast. Each subject was fed equivalent 50 g available carbohydrate of test foods or reference food in random order. To minimize day to day variation of glucose tolerance, the reference food was tested in triplicate in each subject. All test and reference foods were served with 220 mL of water. An automatic lancet device (Safe-T-Pro; Roche Diag-

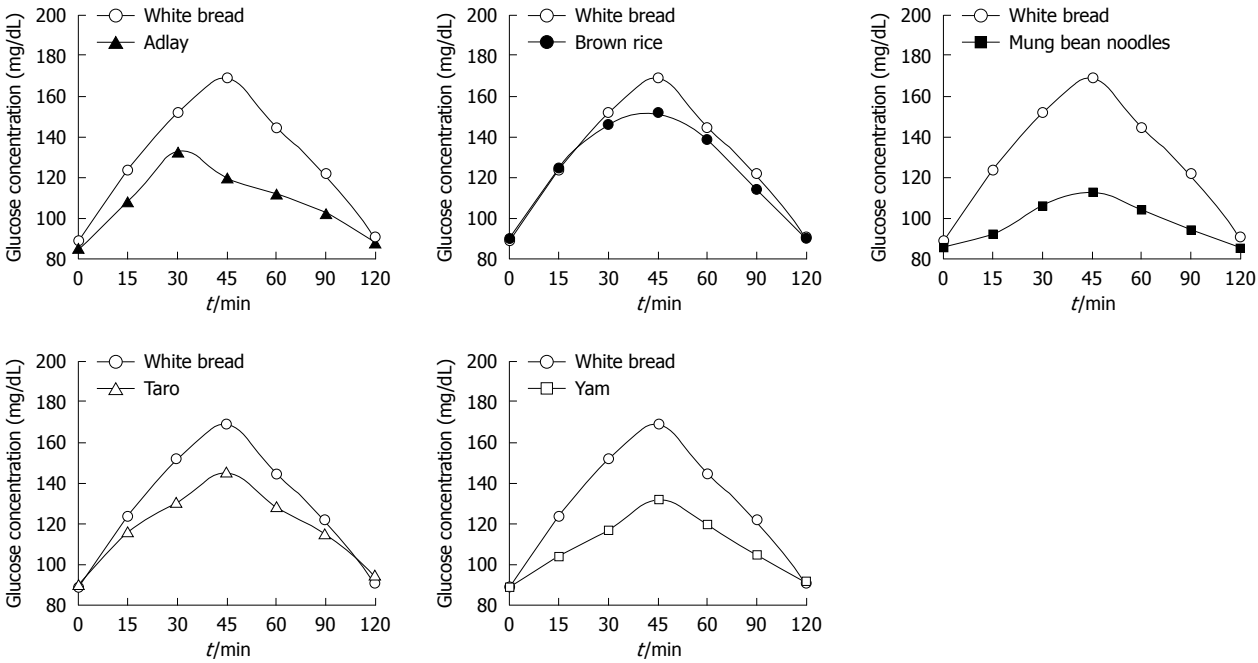

Figure 1 Mean glucose concentrations elicited by five different starchy foods in healthy subjects. Data are expressed as the change in plasma glucose concentration from the fasting baseline concentration.

nostics GmbH Mannheim, Germany) was used to collect finger capillary blood samples (1.5 mL). Blood samples were taken immediately before the start of the study (0 min) and 15, 30, 45, 60, 90 and 120 min after the start of eating. The blood samples were collected in heparinized tubes and centrifuged at 10 500 × *g* for 3 min at 4℃ to obtain plasma. Plasma was spotted onto a slide which contained a reagent layer (glucose oxidase and peroxidase) (Fuji Dri-Chem 3000; Fuji Film, Kanagawa, Japan) and analyzed with an automatic biochemistry analyzer (Fuji Dri-Chem 3000s, Fuji Film, Kanagawa, Japan) for glucose concentrations on each test day. Plasma insulin concentrations were analyzed in duplicate using an enzyme-linked immunosorbent assay (ELISA) with immunoassay kit (Insulin ELISA, Mercodia AB, Uppsala, Sweden) and microplate spectrophotometer (PowerWave XS, Bio Tek, Winooski, VT, USA).

Glycemic and insulin index determinations

The GI/II was calculated from the ratio of the IAUC of the blood glucose/insulin response curve of test food containing 50 g of available carbohydrate and the same amount of reference food (mean IAUC of three reference white bread samples) expressed as a percentage. Because the GI value of white bread is 71 (measured in advance), therefore, the resulting values need to be multiplied by 0.71 in order to convert them to GI values based on glucose[17-19].

Proximate composition analysis

The fat, protein and carbohydrate contents of test foods were analyzed according to AOAC methods[20]. Crude fat was estimated by solvent extraction in a soxhlet apparatus for 14-16 h with petroleum ether. Crude protein was analyzed by determining the total nitrogen in dried food samples using micro-kjeldahl procedures. A factor of 6.25 was used to convert 'N' (nitrogen) value into protein[20]. The analyses of RS + DF were carried out by the method of Onyango and others[21,22] with a slight modification. All measurements were in triplicate.

Statistical analysis

Results are presented as mean ± SE. Insulin concentrations were multiplied by a factor of 6.0 to convert the concentration from mU/L to pmol/L (scientific units). Analysis of variance was performed by using SPSS Windows Release 13.00 (Standard Version, Germany) to determine significant differences. A value of $P < 0.05$ was considered significant.

RESULTS

Postprandial glucose and insulin responses

The study protocol was well tolerated. All 10 subjects completed the study. The mean plasma glucose responses curves for the reference and five test foods are displayed in Figure 1. The reference food produced a large rise in blood glucose during the first 45 min and the greatest overall glycemic response. All test foods had similarity in their peak blood glucose concentrations, except adlay which reached a glycemic peak at 30 min. All test foods, however, varied in their overall glycemic responses. Among the test foods, the brown rice elicited the highest glycemic responses followed by the taro, adlay and yam, and the mung bean noodles produced the lowest. Figure 2 shows the mean plasma insulin response curves for the reference and five test foods. The reference food

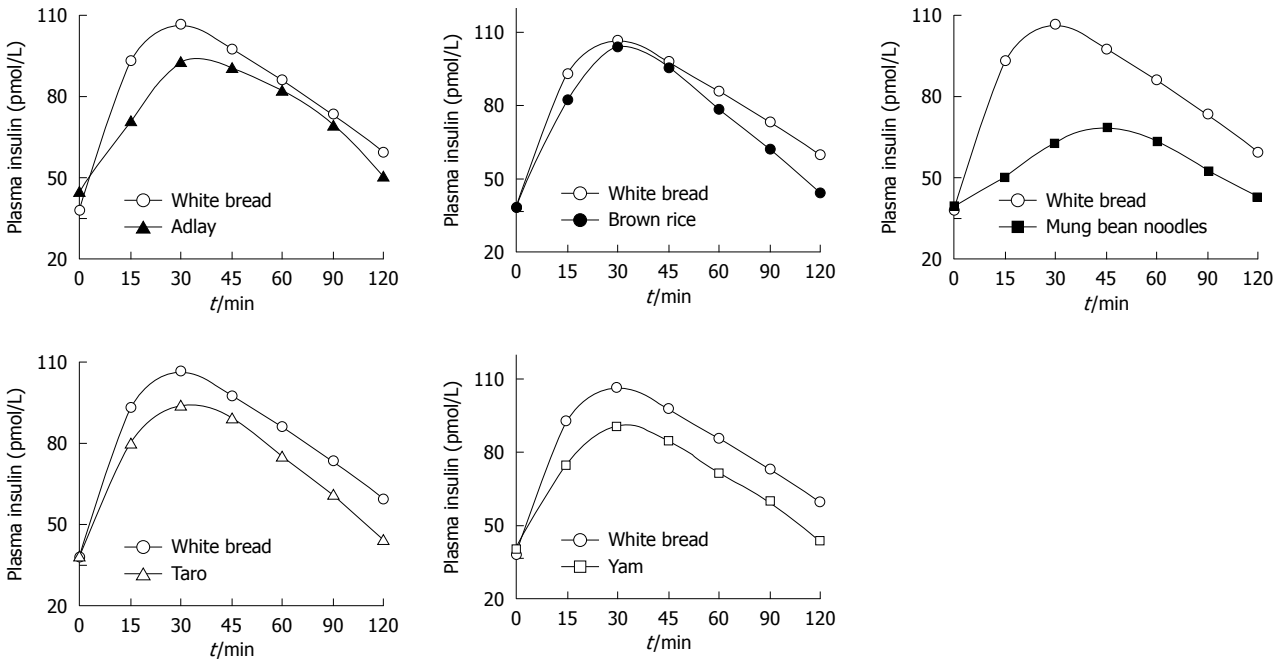

Figure 2 Mean insulin concentrations elicited by five different starchy foods in healthy subjects. Data are expressed as the change in plasma insulin concentration from the fasting baseline concentration.

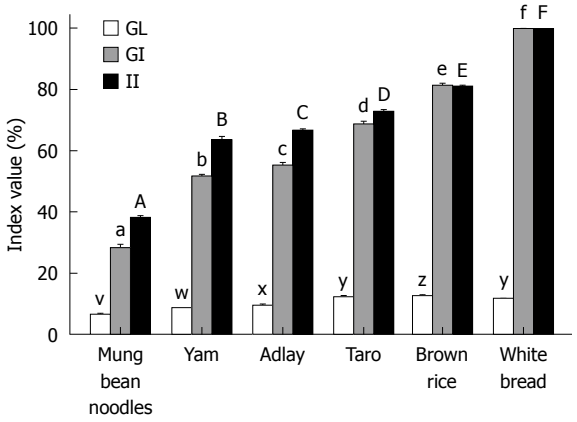

Figure 3 Glycemic index, glycemic load and insulinemic index values of the starchy foods. The mean glycemic index (GI), glycemic load (GL) and insulinemic index (II) for the reference food (white bread) and the five tested starchy foods. For the GI values, columns with different superscripts (a, b, c, d, e, f) are significantly ($P < 0.05$) different. Columns representing the GL values with different superscripts (v, w, x, y, z) are significantly different ($P < 0.05$). Columns representing the II values with different superscripts (A, B, C, D, E, F) are significantly different ($P < 0.05$).

produced the highest peak plasma insulin concentration and the largest overall plasma insulin responses, followed by the brown rice, and the mung bean noodles elicited the lowest plasma insulin responses. All five test foods and the reference food reached their highest response peak at 30 min, except mung bean noodles which reached a peak at 45 min. The plasma insulin responses observed for the five test foods showed a similar profile to their concurrent glycemic responses.

GI, GL and II

The GIs, GLs and IIs of all the test foods are presented in Figure 3 and the classifications of GIs and GLs are showed in Table 1. The mean GI and GL values of the white bread reference were significantly greater ($P < 0.001$) than the mean GI and GL values of each of the test foods. The II values of the test foods corresponded with the GIs. The mean II value of the white bread was significantly higher ($P < 0.05$) than the mean II values of each of the five test foods.

Proximate nutrition components

The nutrient levels and RS + DF are listed in Table 2. The RS + DF content of the yam, mung bean noodles, and adlay was intermediate (15-20 g), whereas the taro and reference white bread was low (9-10 g). We further estimated the caloric values of the test foods from their carbohydrate, fat and protein contents. All five test foods had caloric values ranging from 330 to 384 kcal (= 1379-1605 kJ) per 100 g.

DISCUSSION

The present study evaluates the GI, GL and II of five starchy foods that are traditionally used in the Chinese diet. The results suggest that brown rice produces the highest glycemic and insulinemic responses and has a GI lower than white rice cooked in a rice cooker (i.e. GI = 99-156)[11]. This result is surprising as a characteristic of brown rice is that the thick bran layer retained in brown rice is often composed of higher fiber content than white counterparts. As judged by several reports[23,24], the rate of gastric emptying of starch and digestibility of starch influence the glucose responses and GI values. The effects of fiber and RS on gastric emptying and digestibility have been evaluated in previous studies, showing that fiber and

Table 1 Glycemic index, glycemic load and insulinemic index of the test foods

	Glycemic index[1,3] (%)		Glycemic load[2,3] (g)		Insulinemic index (%)
	mean ± SE	Classification	mean ± SE	Classification	mean ± SE
Adlay	55 ± 0.40	Low	9 ± 0.15	Low	67 ± 0.27
Brown rice	82 ± 0.22	High	18 ± 0.15	Medium	81 ± 0.13
Mung bean noodles	28 ± 0.50	Low	7 ± 0.15	Low	38 ± 0.26
Taro	69 ± 0.35	Medium	12 ± 0.16	Medium	73 ± 0.30
Yam	52 ± 0.25	Low	9 ± 0.00	Low	64 ± 0.45
White bread	100[3]	High	12	Medium	100[3]

[1]Level of glycemic indexs (GIs) were classified according to high (> 69), medium (56-69) and low (< 56) GI; [2]Level of glycemic loads (GLs) were classified as high (> 20), medium (11-19), and low (< 10) GL; [3]White bread was used as reference food and was defined as 100.

Table 2 Major nutrient components and resistant starch content of the test foods (mean ± SE)

	Carbohydrate[1] (g/100 g)	Protein[1] (g/100 g)	Fat[1] (g/100 g)	RS + DF[1] (g/100 g)	Calories (kcal/100 g)
Adlay	85.9 ± 0.5	6.7 ± 0.1	2.5 ± 0.0	15.1 ± 0.5	329.9
Brown rice	86.2 ± 0.1	5 ± 0.1	1.7 ± 0.1	30.8 ± 0.6	380.1
Mung bean noodles	93.5 ± 0.1	0.0 ± 0.0	0.0 ± 0.1	15.7 ± 0.5	374.0
Taro	89.9 ± 0.1	3.25 ± 0.0	0.1 ± 0.0	9.8 ± 0.3	373.5
Yam	89.1 ± 0.1	2.12 ± 0.2	2.1 ± 0.2	20.2 ± 0.5	383.8
White bread	49.9 ± 0.5	9.4 ± 0.1	7.5 ± 0.0	8.8 ± 0.4	304.7

[1]Analyzed by dry weight. DF: Dietary fiber; RS: Resistant starch.

RS are indigestible and could delay gastric emptying[25-27]. Therefore, lower blood glucose responses and GIs are expected in brown rice. The present results, however, indicated that the brown rice we tested is considered as high GI and medium GL food. This information appears to coincide with clinical observations of a significant rise in postprandial blood glucose after consuming brown rice in both diabetic patients and healthy consumers. Traditionally, when cooking brown rice, it has often been soaked in cold water before cooking to reduce the hardness and chewy mouthfeel after cooking. A possible explanation for the high GI is that the process of soaking allows starch granule expansion and performance of better gelatinization, leading to improved digestibility and consequently a higher GI level is observed.

The result regarding mung bean noodles showed lower glucose and insulin responses than bread and produced the lowest GI and GL among the five starchy foods, although higher carbohydrate content was observed. Generally, mung bean noodles are made of mung bean or pea starch, high in amylose, which has been reported to have the effect of lowering GI[28]. Taro and yam both have long been used in the Chinese diet. There have been times when rice was considered rare and expensive, so yam has often been eaten as a sweet dessert or used as a rice substitute in traditional diets. The postprandial glucose and insulin responses elicited by yam are slightly lower than taro, and thus gave lower GI values. These properties of yam and taro can be encouraging for people who are concerned about their postprandial blood glucose levels. It is interesting to note that taro and yam both have similar carbohydrate contents, however they produced variable GI and GL values. They also had lower GI and GL than bread (the reference food), despite the fact that carbohydrate level in bread was much lower than in taro and yam. An unexpected observation was the relationship between GI and II (i.e. II has usually been described as lower than the relative GI values). In our results, the IIs observed from the five starchy foods were higher than their relative GIs. For example, the II of adlay was 67 ± 0.3; its GI, however, was 55 ± 0.4**.** Previous studies indicated co-ingestion of fat and/or protein could increase insulin responses and potentially elicit higher insulinemic responses than relative glycemic responses[29]. In the present study, fat and protein contents were observed among the five test foods and insulin responses are higher than their relative glycemic responses, consequently higher II than the corresponding GI values were found. This result implies that co-ingestion of fat and protein in real foods may influence insulin secretion, despite similar amounts of carbohydrate in their contents. The effect may be viewed as increasing glycemic and insulin responses as higher protein and/or fat contents in the starchy foods are measured. With regard to calorie content, all five starchy foods contained calories of about 368 kcal (per 100 g), which did not reach statistical significance ($P < 0.05$) when compared with white bread (305 kcal). Accordingly, food with lower GI has better satiety than high GI items. Therefore, the actual calorie input may be much lower in mung bean noodles, adlay, taro and yam than in brown rice and white bread.

Based on the correlation analysis, our results suggested that the RS + DF were negatively correlated with the GI and II values (r^2 = -0.66 and -0.10, respectively), and positively correlated with GL (r^2 = 0.49). Although this result is in line with previous findings[30,31], showing

that indigestible starch reduces postprandial glucose and insulin responses, the study may overestimate the amount of RS and DF in the test foods. All the test foods were served hot (approximately 60℃) to the subjects for GI determination. In the RS + DF analysis, however, all the test foods needed to be cooled and dried before proceeding to analytical procedures. The performance of cooling and drying allows retrogradation to occur in amylose chains and may increase the production of RS (retrograded amylose)[32]; consequently higher RS was observed. In particular, this applied to brown rice and yam.

Comparing GI data from other nations, the GI values of starchy foods produced in Taiwan are slightly different to that of counterpart foods produced overseas[9,11,19]. Findings such as this reveal that GI and II values of foods from different countries need to be determined strictly following their own recipes. The GI values of a food could vary when food preparation, cooking methods, food processing, GI testing methods[19] and even geographical location are different. This is more applicable for raw agricultural products. Hence, food with equivalent carbohydrate does not induce similar glycemic and insulinemic responses. This means that GI and GL, along with II values of foods, need to be determined at the same time, in order to provide better understanding as to their postprandial glycemic and insulinemic effects. The present study emphasizes that mung bean noodles, adlay and yam are low GI and GL foods but have variable degrees of II values. The results of this study may provide important information for the public to manage their diet and may prove useful for the prevention of lifestyle-related diseases, such as diabetes mellitus. Continuously evaluating GI values of foods, along with their relative GL and II values, is necessary for individual countries.

ACKNOWLEDGMENTS

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COMMENTS

Background

The glycemic and insulinemic effects of foods are relevant to the development of some lifestyle-related diseases and are involved in the therapeutic dietary plan of chronic diseases. However, the glycemic index (GI) and insulinemic index (II) of Chinese traditional starchy foods have not yet been adequately examined.

Research frontiers

GI, one of the most talked about topics in nutrition today, has recently been recommended as a potential tool for both diabetic and individual use. Research has shown that food GI and II values are relevant to the degree of postprandial glycemia and insulinemia and are involved in the therapeutic dietary plan for some lifestyle-related diseases. The need to continuously determine the postprandial glycemic and insulinemic effects of foods is still valuable for health professionals and researchers, and in particular, the GI and II data of agricultural products carried out in individual countries. Although some studies have evaluated the GI of Chinese foods, the glycemic and insulinemic effects of Chinese starchy foods have not yet been examined in parallel. Therefore, it was informative to evaluate the GI, glycemic load (GL) and II of Chinese starchy foods, since they are beneficial for dietary therapy and meal planning.

Innovations and breakthroughs

The present study evaluated the GI and II of five starchy foods that are commonly used in the Chinese traditional diet. The results will provide some preliminary information on both postprandial insulinemic and glycemic effects of Chinese starchy foods and prove useful for consumers to manage their diets, particular for diabetic patients.

Applications

Since a dietary approach is involved in the prevention and management of some chronic diseases, the results of this study will assist the public and health professionals in their meal planning and dietary management.

Terminology

Glycemic effect is expressed as the incremental area under the curve (AUC) of blood glucose response (120 min). Insulinemic effect of food is referring to as the AUC of the blood insulin response. GI is defined as the incremental blood glucose area (120 min) after ingestion of 50 g of available carbohydrates in the test food as a percentage of the corresponding area after an equivalent amount of carbohydrate from a reference food (either white bread or glucose). II is defined as the incremental blood insulin area after eating of 50 g of available carbohydrates in the test food as a percentage of the corresponding area after an equivalent amount of carbohydrate from a reference food. GL is calculated from the GI value of a food multiplied by the amount of carbohydrate in a usual portion size, divided by 100.

Peer review

The authors provided clinically meaningful data for glycemic control of diabetic patients and this reviewer agrees that preventing hyperinsulinemia after feeding would also be important for that.

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BRIEF ARTICLE

CABYR RNAi plasmid construction and NF-κB signal transduction pathway

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Abstract

AIM: To construct the CABYR RNAi plasmid and study its relation with the nuclear factor (NF)-κB signal transduction pathway.

METHODS: Human CABYR mRNA sequence was obtained from GenBank. The structure of cDNA sequence for the short hairpin RNA was *Bbs* I + sense + loop + antisense + transcription terminator + *Kpn* I + *Bam*H I. A CABYR silencing plasmid was constructed and transfected into the human embryo cell line 293T. Quantitative real-time polymerase chain reaction was used to analyze CABYR and NF-κB gene expression.

RESULTS: The CABYR and NF-κB expressions were detected in 293T cells. The oligonucleotide (5'-GCTCAGATGTTAGGTAAAG-3') efficiently silenced the expression of CABYR. The expression of NF-κB was not significantly affected by silencing CABYR (P = 0.743).

CONCLUSION: CABYR can be found in the human embryo cell line 293T. Cabyrmid 2 can efficiently silence its target, CABYR, indicating that CABYR is not related with the NF-κB signal transduction pathway.

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Key words: CABYR; Plasmid; Nuclear factor-κB; Signal transduction; RNAi; Cabyrmid 2

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INTRODUCTION

Tumors are the result of multiple genetic mutations in cells. The mutated genes generally affect the signal transduction pathways, inducing changes in the bionomic and hereditary characteristics of tumor cells (TC)[1]. Many abnormalities in various signal transduction pathways of TC have been reported, including the ILK, AP-1, Wnt and nuclear factor (NF)-κB pathways[2,3]. The NF-κB signal transduction pathway is known to enhance the transcription of target gene related to apoptosis, proliferation and differentiation of lymphocytes. Abnormalities in this pathway have been found in many TC. Inhibition of the NF-κB pathway can suppress the growth and metastasis of pancreatic carcinoma, and decrease chemo-drug resistance[2-6]. The NF-κB signaling pathway contains a positive feedback mechanism[7,8] and has crosstalk with other signaling pathways, such as PI3K/AKt[4,9,10], NOTCH1[11], K-ras[12] and Hedgehog[13]. Dysfunction of the NF-κB pathway can contribute to the development of tumors. Some signal transduction pathways related to the phos-

phorylation of proteins are of the most important regulation mechanism present in cells. CABYR is a calcium-binding tyrosine phosphorylation-regulated protein that has been detected in testis and also in lung cancer[14], and its CR-A and CR-B contain 5 PXXP consensus motifs[15], the cognate sites for SH3 which is one of the signal transduction protein modular binding domains. IκBα molecule, a key regulatory subunit of the NF-κB signal transduction pathway, can be regulated by the PI3K/Akt pathway. CABYR spliceosome Ⅲ/Ⅴ can act as an ideal substrate for glycogen synthase kinase-3 (GSK3)β within the extensin-like domain. GSK3β is one of the most important transduction proteins involved in many signal transduction pathways, and plays a vital role in tumorigenesis. We hypothesize that CABYR may be related with the NF-κB signal transduction pathway, affecting basal expression of NF-κB subunit, phosphorylation of IκBα, and DNA binding ability.

MATERIALS AND METHODS

Cell culture

Human embryo cell line 293T, obtained from Department of Immunology at Shanghai Tongji University (Shanghai, China), was maintained by passing twice a week in Dulbecco's modified Eagle's medium (DMEM; Life Technologies Inc., Gaithesburg, MD, USA) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 10 mg/mL streptomycin at 37℃ in an atmosphere containing 5% CO_2. Each cell line was passed at 70%-80% confluence. The cells were subjected to 6 treatment regimens based on the following groups: CABYR1, CABYR2, CABYR3, empty vector, blank and transfection efficiency reference.

Plasmids and transfection

Human CABYR mRNA sequence was obtained from GenBank (Accession number NM_153768). Three possible target sites to this sequence were chosen with the GenScript SiRNA target finder. BLAST was used to identify whether they are exclusive to CABYR. The target sequences are 5'-CCATCAAACATCAACCAGT-3' (nt 240-258), 5'-GCTCAGATGTTAGGTAAAG-3' (nt 627-645) and 5'-GCTCTCTGACACATCTTT-3' (nt 1256-1273). A short hairpin RNA (shRNA) was designed for use in RNA interference (RNAi) according to the three targets. The TTCAAGAGA sequence is part of the "loop" motif in the shRNA, and the transcription terminator is TTTTT. Restriction endonuclease sites for *Bbs*I, *Kpn* I + *Bam*H I were incorporated into the shRNA sequence, along with a sense and antisense sequence for each cDNA. Single-stranded oligonucleotide DNAs were synthesized by Shanghai Sangon Biological Engineering Technology and Service Company (Shanghai, China). Relative cDNAs are composed of a DNA double strand and a conjunct with pSilence1.0 plasmid. The plasmids were transformed into competent cells and possible transformants were identified, which are resistant to ampicillin. The plasmids were purified using the plasmid minipreps purification system B (BioDev, Beijing, China), then incubated with *Kpn* I to linearise and sequenced to confirm their identity. The 293T cells were transfected with CABYR-shRNA, shRNA control and pEGFP (reference of transfection efficiency) using the Effectene transfection kit (Qiagen, Hilden, Germany). CABYR-shRNA 1, CABYR-shRNA 2 and CABYR-shRNA 3 were designated as Cabyrmid 1, Cabyrmid 2 and Cabyrmid 3, respectively.

Semi-quantitative polymerase chain reaction

The β-actin gene was used as the reference gene when the results were quantified. The primers employed were β-actin (forward: 5'-ACAGAGCCTCGCCTTTGCC-3' and reverse: 5'-CATGTCGTCCCAGTTGGTG-3'), CABYR exon2 (forward: 5'-CAACCCATCAAACATCAACC-3' and reverse: 5'-TGCCATTGCTAACATCTGAG-3'), CABYR exon 4 (forward: 5'-CAGACACAGACGAGGACAATG-3' and reverse: 5'-TCC GTT TGC TCA GTG CCT-3'), NF-κB (forward: 5'-GAGACATCCTTCCGCAAACT-3' and reverse: 5'-TCCTTCCTGCCCATAATCA-3'). Total RNA was extracted from 293T cells using Trizol (Invitrogen, Shanghai, China) following its manufacturer's instructions, with quality and quantity determined by measuring the optical density at 260 nm and 280 nm. An $A_{260/280}$ ratio of approximately 1.8 indicated that the RNA sample was of sufficient purity. The RNA integrity was also checked by electrophoresis. Total RNA was reverse transcribed into cDNA using a RevertAid™ cDNA first strand synthesis kit (Fermentas, Ontario, Canada). Thirty cycles of semi-quantitative polymerase chain reaction (PCR) were conducted in a 25 μL volume, with an annealing temperature of 56℃. The PCR products were visualized by agarose gel electrophoresis.

Real-time analysis of gene expression

Changes in NF-κB expression were detected before and after CABYR RNAi treatment by quantitative real-time PCR (qPCR). Total RNA was extracted and reverse transcribed into cDNA using a RevertAid™ cDNA first strand synthesis kit (Fermentas, Ontario, Canada). The primers used in the qPCR are CABYR exon 4 (forward: 5'-CAGACACAGACGAGGACAATG-3' and reverse: 5'-TCCGTTTGCTCAGTGCCT-3'), β-actin (forward: 5'-GCACTCTTCCAGCCTTCCTT-3' and reverse: 5'-GGTCTTTGCGGATGTCCA-3'), NF-κB (forward: 5'-GAGACATCCTTCCGCAAACT-3' and reverse: 5'-TCCTTCCTGCCCATAATCA-3'). cDNA for the blank group was diluted at 1:1, 1:10, 1:100, 1:1000 and 1:10000 and used in over 40 cycles of two-step qPCR in a 25 μL volume, with an annealing temperature of 62℃. A SYBR Premix Taq kit from Takara Bio (Shiga, Japan) was used. The results were analyzed using the Rotor-gene real-time analysis software.

Statistical analysis

All the experiments were repeated three times and the results were analyzed using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). The statistical analytical method was one-way ANOVAD. $P < 0.05$ was considered statistically significant.

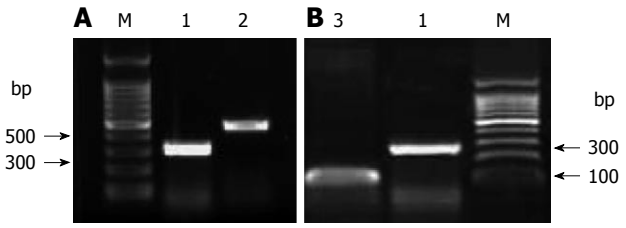

Figure 1 Basic expression of CABYR (A) and nuclear factor-κB (B) at mRNA level in 293T cells. Total RNA was extracted from 293T cells with Trizol and reverse transcribed to 293T cDNA with the primer Oligo(dt). The target fragment was amplified by semi-quantitative polymerase chain reaction and analyzed by agarose electrophoresis (1%). CABYR and nuclear factor (NF)-κB were detectable in 293T cells, indicating that 293T cells can be used to identify the efficient silence fragment for CABYR and study the relation between CABYR and NF-κB. 1: β-actin; 2: CABYR; 3: NF-κB; M: Marker.

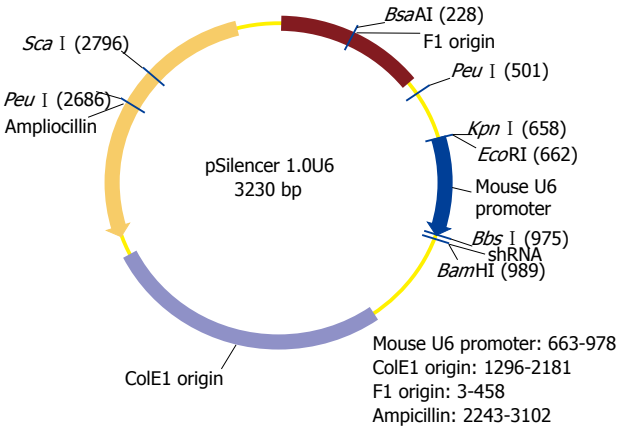

Figure 2 Construction of CABYR shRNA eucaryon expression vector by inserting the target gene fragment into pSlience1.0 (3.23 kb) between *Bbs* I and *Bam*HI.

RESULTS

Basal expression of CABYR and NF-κB mRNA in 293T cells

The transcripts of CABYR and NF-κB were detected in 293T cells (Figure 1).

Construction of CABYR shRNA eukaryotic expression vector

The gene fragment was inserted between the restriction sites of *Bbs* I and *Bam*H I in pSlience1.0 (Figure 2). The plasmid also contained a restriction site of *Kpn* I, and a *Kpn* I recognition sequence was incorporated into the ends of our gene fragment. Possible recombinant plasmids were identified by digesting with the appropriate restriction endonuclease and a 396-397 bp product would be liberated if the target fragment was correctly incorporated (Figure 3). The identified recombinant plasmids were sequenced by Shanghai Sangon Company for further verification (Figure 4).

CABYR shRNA expression in different groups

GFP was highly expressed in the reference group, indicating that a high efficiency of transfection can be achieved in the other groups under the same conditions (Figure 5). CABYR mRNA was also expressed in the blank control, vacant vector control and CABYR RNAi groups. The CABYR mRNA expression was decreased in CABYR RNAi group, indicating that 5'-GCTCAGATGTTAGGTAAAG-3' is an efficient silencing target for CABYR (Figure 6).

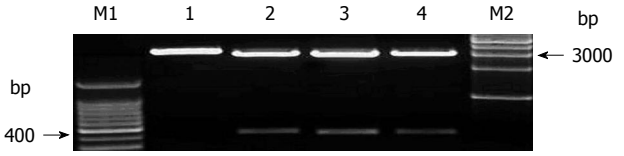

Figure 3 Construction of CABYR shRNA eucaryon expression vector by inserting the target fragment we constructed into the RNAi plasmid. 1:vacant vector; 2: Cabyrmid 1 vector; 3: Cabyrmid 2 vector; 4: Cabyrmid 3 vector; M1: Marker 1; M2: Marker 2.

Table 1 Relation between CABYR and nuclear factor-κB signal pathway (mean ± SD)

Group	CABYR [Δ(ΔCT) value]	NF-κB [Δ(ΔCT) value]
Control group	-0.06 ± 0.18	-0.38 ± 0.51
Cabyrmid 1	0.19 ± 0.23	-0.10 ± 0.25
Cabyrmid 2	2.11 ± 0.15[a]	-0.05 ± 0.79
Cabyrmid 3	0.76 ± 0.33	-0.15 ± 0.20
F value	51.928	0.381

[a]$P < 0.05$ *vs* control, cabyrmid 1 and cabyrmid 3 groups. NF: Nuclear factor; CT: Computed tomography.

Relation between CABYR and NF-κB signal pathway

According to the standard curve generated, CT exhibited a strong linear correlation between CABYR and NF-κB at different diluted concentrations, thus the precise results could be obtained using qPCR. The M value for β-actin, CABYR and NF-κB was approximately uniform, indicating that their amplification efficiency is similar. The concentration of target fragment was analyzed using the Δ(ΔCT) method. The results showed that the mRNA expression was obviously decreased in the siRNA2 group, indicating that 5'-GCTCAGATGTTAGGTAAAG-3' can silence the expression of CABYR mRNA transcript, while the expression of NF-κB was not affected by silencing CABYR ($P = 0.743$), displaying that CABYR has no significant effect on the expression of NF-κB (Table 1).

DISCUSSION

In this study, a CABYR silencing plasmid was constructed with its function observed. CABYR, first identified in the testis by Naaby-Hansen *et al*[16], plays a key role in protein tyrosine phosphorylation and increases the concentration of intracellular calcium. Its transcript variants encode multiple protein isoforms, but spliceosome Ⅲ/Ⅴ is not specific for testis[15]. CABYR can be found in pancreas, fetal brain, liver, motile cilia of human bronchus

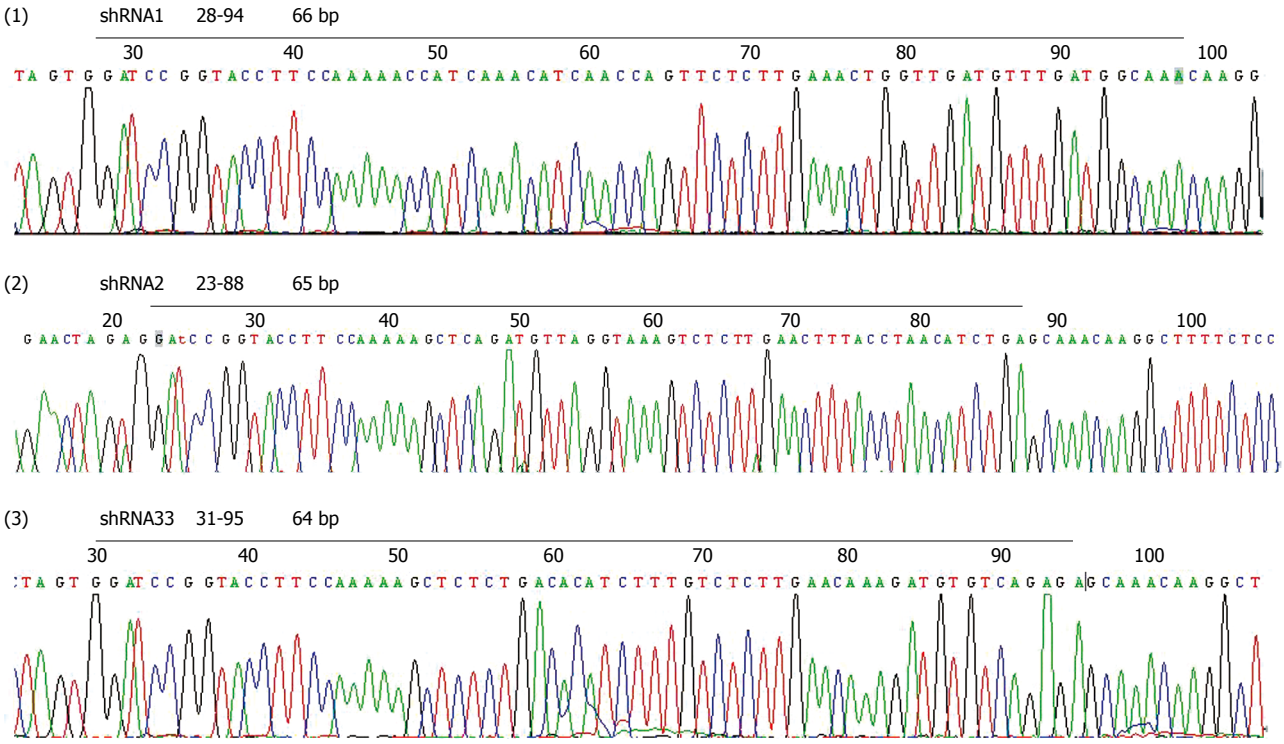

Figure 4 Construction of CABYR shRNA eucaryon expression vector by locating shRNA in plasmid.

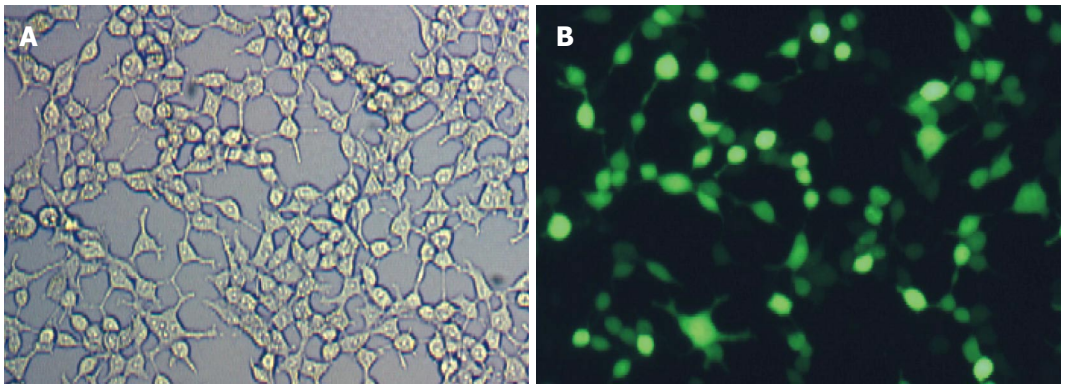

Figure 5 CABYR shRNA expression in reference group (A) and CABYR mRNA expression in other groups (B). A: HE stain, × 100; B: Blue fluorescent, ×100.

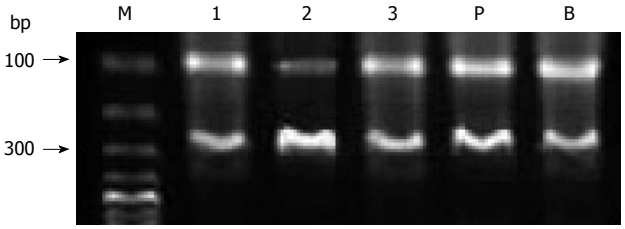

Figure 6 CABYR mRNA expressions in different groups after transfection. M: Marker; 1: Cabyrmid 1; 2: Cabyrmid 2; 3: Cabyrmid 3; P: Vacant vector; B: Blank.

and fallopian tubes[17]. In this study, CABYR was identified in the human embryo cell line 293T.

Cabyrmid 2 is an effective CABYR silencing plasmid. In this study, 3 target fragments of CABYR were designed using the GenScript SiRNA target finder and compared with the corresponding sequences in GenBank to determine its specificity[18]. The CABYR shRNA we constructed, inserted downstream from the strong U6 promoter in pSilence 1.0 and transcribed, which became a functional siRNA with an ability to degrade CABYR mRNA exclusively. The effective CABYR shRNA was screened by transfecting 293T cells *via* lipofection as previously described[19,20]. In this study, a highly effective CABYR silencing site, 5'-GCTCAGATGTTAGGTAAAG-3', was found, and a short hairpin plasmid that could effectively silence CABYR expression was constructed, which was designated as Cabyrmid 2.

The expression or repression of CABYR had no effect on NF-κB signaling pathways in our study. It has been shown that CABYR spliceosome Ⅲ/Ⅴ can act as an ideal substrate for GSK3β in the extensin-like domain[21]. GSK3β is known to play a key role in tumorigenesis[22-26]

in conjunction with PI3K/Akt which plays a role in the regulation of the NF-κB transduction pathway. NF-κB plays an important role in embryo growth, differentiation and apoptosis of lymphocytes, immunological and inflammatory reactions[27-31]. Abnormal CABYR and NF-κB have been detected in the same cancers, and CABYR possesses a tyrosine kinase activity which is an important kinase in various signaling pathways, suggesting that CABYR may be related with NF-κB. However, no significant effect of CABYR was observed on the expression of NF-κB in this study.

Two reasons can explain why CABYR had no significant effect on the expression of NF-κB in this study. One is that the 293T cells were used while the NF-κB signaling pathway was normal. If their relation was detected in Bxpc3 (NF-κB dysfunction), other results may be observed. The other is that NF-κB exists as an inactive precursor (p50, p60, IκBα) in cytoplasm. After NF-κB is activated, IκBα is phosphorylated and detached from the conglomeration. The remaining molecules enter the nuclei and adhere to target DNA, thereby enhancing transcription. Phosphorylation of IκBα is a key step in the NF-κB pathway. Though no significant effect of CABYR was observed on the expression of NF-κB in this study, CABYR possesses a tyrosine kinase activity possibly affecting the NF-κB pathway by phosphorylating IκBα. It has been reported that G3BP2 (RasGAP SH3-binding protein 2) is able to discriminate between amino terminals of IκBα[32] related with the retention of IκBα/NF-κB conglomeration in cytoplasm. CABYR also contains a PXXP motif, similar to G3BP2 which is a core part of the SH3 aglucone. A study involving the influenza A virus demonstrated that the structure of SH3 plays a key role in determining the activity of PI3K/Akt[33]. The PI3K/Akt signaling pathway can also regulate the phosphorylation of IκBα, indicating that CABYR may take part in the regulation of the NF-κB signaling pathway.

In summary, CABYR is not exclusive to the testis and codes for a calcium-binding tyrosine-phosphorylation regulated protein that is intimately involved in calcium signaling. Cabyrmid 2 can efficiently silence CABYR expression rather than the expression of NF-κB in 293T cells.

COMMENTS

Background

Tumor is a polygene mutation disease. The mutation gene effects on the signal transduction pathway inducing tumor cell's (TC). Many abnormalities of signal transduction pathway in TC have been reported, such as ILK, AP-1, Wnt, and nuclear factor (NF)-κB. Recently NF-κB signal transduction pathway was hotly researched. It can enhance the transcription of the target gene relating to the apoptosis, proliferation and differentiation of lymphocyte. And its abnormality was also been found in many TC. CABYR is an capacitation related calcium binding tyrosine-(Y)-phosphorylation regulated gene, it has no absolute testis specificity, it was also reported that CABYR antigen was detected in many cancers such as lung cancer.

Research frontiers

NF-κB signal transduction pathway can enhance the transcription of the target gene relating to the apoptosis, proliferation, and differentiation of lymphocyte *etc*. The inhibition of NF-κB can depress growth and metastasis of pancreatic carcinoma, and decrease the chemo-drug resistance. NF-κB signal transduction pathway has many crosstalk with other signal pathway. This indicated that the dysfunction of NF-κB signal pathway contribute an important part of tumors development. CABYR is an capacitation related calcium binding tyrosine-(Y)-phosphorylation regulated gene. its CR-A and CR-B contain five PXXP consensus motifs, the cognate sites for SH3 ,one of the signal transduction protein Modular Binding Domains, interaction. On another side IκBα, the key regulated subunit of NF-κB signal transduction pathway, can been regulated by the PI3K/Akt signal pathway. CABYR splicesome Ⅲ/Ⅴ act as an ideal substrate for GSK3beta (glycogen synthase kinase-3) within the extensin-like domain. The GSK3beta is one of the most important transduction proteins involving in many signal transduction pathway which play a key role in tumorous genesis and development including PI3K/Akt signal pathway. So the authors hypothesised that CABYR may have some relationship with NF-κB signal transduction pathway.

Innovations and breakthroughs

The authors constructed CABYR silence plasmid (Cabyrmid 2) and proved that CABYR RNAi plasmid 2 is the efficient silence target to CABYR. They also found that CABYR may have no relationship with NF-κB signal transduction pathway.

Applications

CABYR silence plasmid (Cabyrmid 2) may help the authors in the future research of CABYR.

Terminology

CABYR is a calcium binding tyrosine phosphorylation regulator gene. It was first found in testis by Naaby-Hansen in 2002. CABYR plays a key role in capacitation involving protein tyrosine phosphorylation and increased intracellular calcium. Transcript variants of this gene encode multiple protein isoforms. And its spliceosome Ⅲ/Ⅴ wasn't seem to be absolute testis specificity. It also be found in the pancreatic tissue, fetal brain, sclerosis liver as well.

Peer review

The authors have presented a basic study with convincing data. It will be suitable for publication after revision.

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BRIEF ARTICLE

Up-regulation of PIK3CA promotes metastasis in gastric carcinoma

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Abstract

AIM: To explore expressions of PIK3CA in the progression of gastric cancer from primary to metastasis and its effects on activation of phosphatidylinositol 3-kinase (PI3K)/Akt pathway.

METHODS: mRNA and protein levels of PIK3CA were assessed, respectively, by real-time quantitative polymerase chain reaction and immunohistochemistry in specimens of normal gastric mucosa, primary foci and lymph node and distant metastasis of gastric cancer. Akt and phosphorylated Akt protein were also examined by Western blotting in these tissues, in order to analyze the effect of PIK3CA expression level changes on the activation of PI3K/Akt signaling pathway.

RESULTS: PIK3CA mRNA in lymph node metastasis were approximately 5 and 2 folds higher, respectively, than that in the corresponding normal gastric mucosa and primary gastric cancer tissues ($P < 0.05$), while no statistical significance was found compared with distant metastasis. Immunohistochemically, PIK3CA protein expression was discovered in 7 (35%) specimens of 20 primary foci *vs* 10 (67%) of 15 of lymph node metastasis or 11 (61%) of 18 of distant metastasis (35% *vs* 67%, $P = 0.015$; 35% *vs* 61%, $P = 0.044$). With the increased level of PIK3CA expression, the total Akt protein expression remained almost unchanged, but p-Akt protein was upregulated markedly.

CONCLUSION: Increased expression of PIK3CA is expected to be a promising indicator of metastasis in gastric cancer. Up-regulation of PIK3CA may promote the metastasis of gastric cancer through aberrant activation of PI3K/Akt signaling.

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Key words: PIK3CA; Phosphatidylinositol 3-kinase/Akt pathway; Metastasis; Gastric cancer; Akt

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Liu JF, Zhou XK, Chen JH, Yi G, Chen HG, Ba MC, Lin SQ, Qi YC. Up-regulation of PIK3CA promotes metastasis in gastric carcinoma. *World J Gastroenterol* 2010; 16(39): 4986-4991 Available from: URL: http://www.wjgnet.com/1007-9327/full/v16/i39/4986.htm DOI: http://dx.doi.org/10.3748/wjg.v16.i39.4986

INTRODUCTION

Gastric cancer, the most common gastrointestinal cancer, is the leading cause of cancer death. Invasion and metastasis are the main biological characteristics of malignant tumors, and also the important factors contributing to the death of gastric cancer patients and affecting their therapeutic efficacy. Currently, no method has been available to predict metastasis of gastric cancer. Therefore, studies on the molecular mechanism of metastasis are crucial for diagnosis, treatment and prognosis of gastric cancer.

Several molecular pathways are known to play a role in gastric cancer development and progression[1-3]. The most important pathway may be the recently discovered phosphatidylinositol 3-kinase (PI3K)/serine/threonine kinase (Akt) signaling pathway.

The PIK3CA gene, which is located on chromosome 3q26.3, encodes the key enzymatic subunit p110α of PI3K[4]. It has been shown that mutations of the PIK3CA gene are highly prevalent in a variety of human solid tumors including colon, gastric, breast and pituitary cancer[5-9], which can lead to dysregulation of PI3K/Akt signaling pathway at several levels[10]. Guo *et al*[11] reported that mutant PIK3CA-bearing colon cancer cells displayed increased enzymatic activity of PI3K compared with wild-type PIK3CA-bearing colon cancer cells. In addition, the former showed a significantly enhanced level of phosphorylation of Akt as well as cell invasion and metastasis, which is consistent with the findings from Samuels *et al*[12]. Although many studies have implicated PIK3CA mutations with features of transformation[13,14], relationship between PIK3CA expression and metastasis of gastric cancer and aberrant activation of PI3K/Akt signaling pathway has not been elucidated to date.

Our previous work showed that higher expression levels of PIK3CA were associated with lower differentiation of gastric cancer cells and stronger ability of invasion and metastasis, suggesting that PIK3CA gene may contribute to differentiation, invasion and metastasis in gastric cancer cells[15]. To further explore the correlation between PIK3CA expression and metastasis of gastric cancer, expression levels of PIK3CA were detected by real-time quantitative polymerase chain reaction (RT-qPCR) and immunohistochemistry in different gastric cancer tissues. In addition, we investigated the effects of changes of PIK3CA expression levels on activation of PI3K/Akt signaling in order to provide important experimental evidences for the molecular mechanism of invasion and metastasis in gastric cancer.

Table 1 Primers used in real-time quantitative polymerase chain reaction

Primer	Nucleotide sequence (5'→3')
PIK3CA (+)	TGCTAAAGAGGAACACTGTCCA
PIK3CA (-)	GGTACTGGCCAAAGATTCAAAG
β-actin (+)	CTGAGCAGATCATGAAGAC
β-actin (-)	CTTGGTGGACGCATCCTGAG

"+" and "-" mean sense and antisense oligos, respectively.

MATERIALS AND METHODS

Gastric cancer specimens

From March 2008 to April 2010, 53 gastric carcinoma patients (45 with intestinal and 8 with diffuse gastric carcinoma) consisting of 29 males and 24 females, with a median age of 48 years (range from 20-72 years) admitted to the Department of Gastroenterology of our hospital and Guangdong Armed Police Hospital were assessed. Informed consent was obtained before operation from each patient for research use of the resected cancer lesions. Gastric cancer tissue samples including 20 primary (stage Ⅰ-Ⅱ), 15 lymph node metastasis (stage Ⅲ) and 18 distant metastasis (stage Ⅳ) in gastric cancer were verified by pathological diagnosis. Ten normal tissue samples were obtained from around tumor tissues. All samples were immediately frozen in liquid nitrogen or fixed in 5% formaldehyde solution for subsequent analysis. None of the gastric cancer patients had received preoperative radiotherapy, chemotherapy or biotherapy. This study was approved by the Ethics Committee of the Guangzhou Medical College.

RT-qPCR assays

Transcript abundance of PIK3CA and β-actin (internal control) was quantified by RT-qPCR on total RNA isolated from normal gastric mucosa, and gastric cancer tissues of primary foci, lymph node and distant metastasis. Briefly, 1 μg of total RNA was reversely transcribed in a reaction volume of 25 μL using oligodT(15) primers and M-MLV reverse transcriptase (Promega). The primers used for amplification are shown on Table 1. The PCR amplification and fluorescence detection were carried out in 20 μL solution, with 200 nmol/L of each primer and 5 μL of cDNA serving as templates, and SYBR Green PCR master Mix (ABI) using the ABI Prism 7500 Sequence Detection System was conducted following the manufacturer's instructions. Each cDNA was analyzed in triplicate for both target genes and β-actin housekeeping genes. The cycling conditions were 50℃ for 2 min, 95℃ for 10 min followed by 40 cycles with each cycle consisting of 30 s at 95℃, and 1 min at 60℃. For each sample, a standard quantity was calculated using the $2^{-\Delta\Delta C(T)}$ method according to previously described protocol[16].

Immunohistochemistry

Immunohistochemical analysis was performed using

DAKO Envision kits according to the manufacturer's protocol. Briefly, 4-μm sections were cut into coated slides and were deparaffined using routine techniques. After treatment with 3% hydrogen peroxidase for 10 min to block endogenous peroxidases, the sections were subsequently incubated with monoclonal antibodies (rabbit anti-human PIK3CA, Cell Signaling Technology) for 30 min at room temperature, washed with Tris Buffered Saline for 10 min and reacted with Envision TM (Dako) for 30 min. Labelling was then detected as above using 3,3'-diaminobenzidine. Negative control was obtained by omitting the primary antibody. Samples mixed with only Phosphate Buffered Saline (PBS) buffer were treated as negative controls. For the evaluation of immunostaining, at least 1000 cells were counted from randomly selected 10 fields of vision and staining intensity as well as number of positive cells were assessed according to Hara's method with minor modifications[17]. The staining was scored as follows: 0-1, < 20% cells with no or faint staining (negative); 2-4, 20%-50% cells with moderate staining (positive); and 5-6, ⩾ 50% with marked staining (strong positive).

Western blotting

The normal gastric mucosa, and gastric cancer tissues of primary foci, lymph node and distant metastasis were polished to powder in liquid nitrogen and then were lysed in protein extraction buffer [0.5 mmol/L Tris.Cl (pH 7.0), 0.1% β-mercaptoethanol, 0.5 mmol/L ethylenediaminetetraaceticacid (EDTA) (pH 7.0), 0.5 mmol/L ethyleneglycol-bis (2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) (pH 7.0), and 2 mmol/L leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 2.5 mg/mL Aprotinin, 1 mmol/L dithiothreitol (DTT), 0.5% Triton X-100]. The lysates were resolved by 10% SDS-PAGE and transferred to nitrocellulose membranes (Amersham Life Sciences). The membranes were blocked for 1 h, probed with the anti-phosphorylated Akt (Ser473), anti-Akt and anti-β-actin (Cell Signaling Technology) antibodies, respectively, and then reacted with a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody. Immunoreactive proteins were detected using an enhanced chemiluminescence detection reagent (BestBio).

Statistical analysis

Statistical analysis was performed using SPSS software (Version 17.0). Differences in the results between groups were analyzed by the Mann-Whitney *U*-test, and a *P* value of less than 0.05 was taken as significant.

RESULTS

Analyses of PIK3CA mRNA and protein expression

PIK3CA mRNA expression was detected by RT-qPCR in normal gastric mucosa, and gastric cancer tissues of primary foci, lymph node and distant metastasis. The primary gastric cancer specimens showed higher expression of PIK3CA mRNA in comparison with the normal gastric mucosa. The lymph node metastasis tissues displayed the strongest expression of PIK3CA mRNA, which was approximately 5 and 2 folds higher, respectively, than that in normal gastric mucosa and primary gastric cancer tissues ($P < 0.05$), whereas in contrast to distant metastasis, no statistically significant difference was found in PIK3CA mRNA expression (Figure 1A). In addition, the expression and localization of PIK3CA protein were studied immunohistochemically in resected tissues mentioned above. No or weak cytoplasmic staining for PIK3CA appeared in the normal gastric mucosa, while moderate staining was displayed in 7 (35%) of 20 of primary gastric cancer specimens, and moderate or intense staining in lymph node metastasis (10/15 or 67%) and distant metastasis (11/18 or 61%) (Figure 1B, Table 2), which is similar to the corresponding PIK3CA mRNA expression profile. Moreover, statistically significant correlation was discovered between PIK3CA expression and the presence of lymph node metastasis in gastric cancer, while all other clinicopathological factors such as gender, age and differentiation, were statistically irrelevant

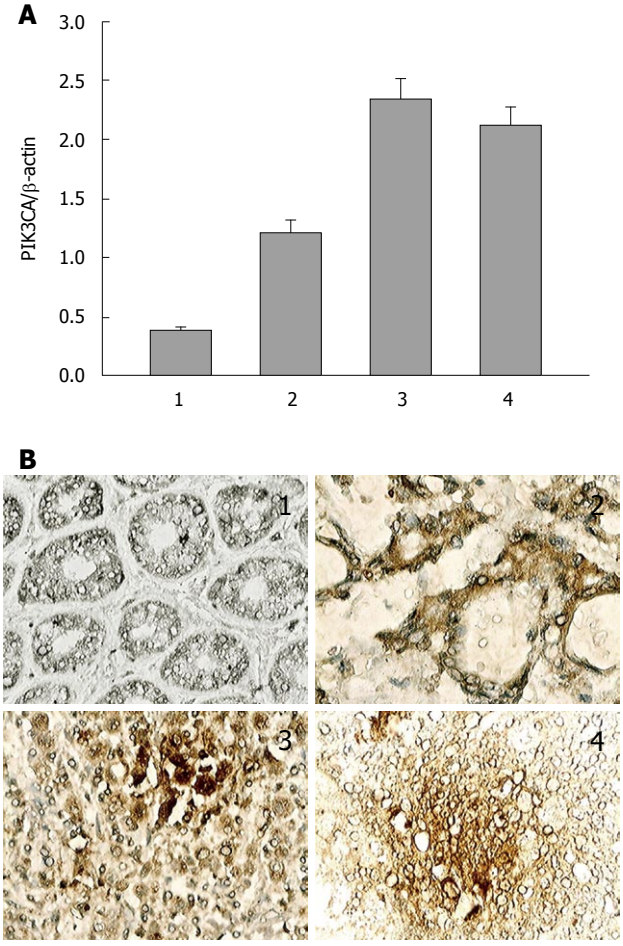

Figure 1 PIK3CA mRNA and protein expression in normal and gastric cancer tissues. A: Expression analyses of PIK3CA mRNA determined by real-time quantitative polymerase chain reaction, values are shown as mean ± SD; B: Immunohistochemical study of PIK3CA (original magnification: × 400). Negative or weak expression of PIK3CA in normal tissues around tumor; Positive expression of PIK3CA in primary gastric cancer tissues. Strong positive expression of PIK3CA was detected in lymph node metastasis and distant metastasis gastric cancer tissues. 1: Normal gastric mucosa; 2: Primary gastric cancer; 3: Lymph node metastasis in gastric cancer; 4: Distant metastasis in gastric cancer.

Table 2 Difference of PIK3CA protein expression among normal gastric mucosa, primary, lymph node metastasis and distant metastasis in gastric cancer

Tissues	PIK3CA staining			Total	*P*
	0-1	2-4	5-6		
Normal	10	0	0	10	0.036[a]
Primary	13	6	1	20	0.015[b]
Lymph node metastasis	5	3	7	15	0.044[c]
Distant metastasis	7	3	6	18	0.537[d]

[a]$P < 0.05$, normal *vs* primary; [b]$P < 0.05$, primary *vs* lymph node metastasis; [c]$P < 0.05$, primary *vs* distant metastasis; [d]$P > 0.05$, lymph node metastasis *vs* distant metastasis, all by Mann-Whitney *U* test.

Table 3 Correlation between PIK3CA expression and clinicopathological characteristics in gastric carcinoma

Variables	PIK3CA expression				
	n	Positive	Negative	χ^2	*P*
Gender					
Male	29	17	12	0.862	0.834[a]
Female	24	11	13		
Age (yr)					
≥ 60	32	16	16	0.256	0.968[a]
< 60	21	12	9		
TNM stage					
Ⅰ, Ⅱ	20	7	13	4.926	0.177[a]
Ⅲ, Ⅳ	33	21	12		
Differentiation					
Well	22	7	15	6.685	0.083[a]
Moderate and poor	31	21	10		
Lymph node metastasis					
Negative	26	8	18	9.982	0.019[a,b]
Positive	27	20	7		
Distant metastasis					
Negative	35	17	18	0.749	0.862[a]
Positive	18	11	7		

[a]χ^2 test; [b]The value is significant.

to the positive staining for PIK3CA (Table 3). These results suggested that up-regulation of PIK3CA expression was likely related to lymph node metastasis in gastric cancer.

Effects of PIK3CA expression on activation of PI3K/Akt signaling pathway

PIK3CA encoding the catalytic subunit p110α of PI3K, is an important signal molecule in PI3K/Akt signaling pathway, which was involved in tumor growth and metastasis[18]. Our studies indicated that normal gastric mucosa had almost no expression of PIK3CA, while lymph node metastasis and distant metastasis had significantly higher PIK3CA expression than the primary gastric cancer tissues, suggesting that PI3K/Akt pathway could be aberrantly activated in gastric cancer metastasis. To test this possibility, we examined the phosphorylation of Akt [p-Akt (Ser473)] and total Akt in normal gastric mucosa, primary foci, lymph node and distant metastasis in gastric cancer. The results revealed that p-Akt (Ser473) was not expressed (or loss) in normal gastric mucosa, and highly expressed in lymph node metastasis and distant metastasis compared

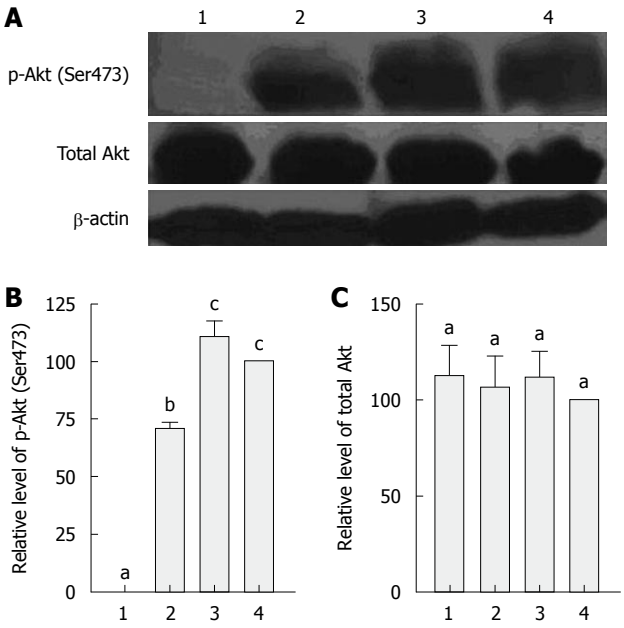

Figure 2 Western blotting analyses of Akt phosphorylation. A: Protein from the indicated tissues was Western blotting with anti-phospho-Akt (Ser473) and anti-Akt to analyze the effect on activation of phosphatidylinositol 3-kinase (PI3K)/Akt signaling. β-actin was used as a loading control; B: Relative expression level of p-Akt (Ser473) protein quantified by grey analysis; C: Relative expression level of total Akt protein quantified by grey analysis. p-AKT, phosphorylated Akt; Lane 1: Normal gastric mucosa; Lane 2: Primary gastric cancer; Lane 3: Lymph node metastasis in gastric cancer; Lane 4: Distant metastasis in gastric cancer. [a,b,c]Significant difference was indicated with different lower-case letters ($n = 3$).

with that in primary gastric cancer tissues (Figure 2A and B). However, total Akt expression levels remained nearly unchanged (Figure 2A and C). These findings indicated that up-regulation of PIK3CA expression level contributed to the increased catalytic activity of PI3K p110α, promoting phosphorylation of Akt and overactivation of PI3K/Akt signaling pathway, in which the latter promoted invasion and metastasis in gastric cancer cells.

DISCUSSION

In recent years, although the morbidity and mortality rates of gastric cancer have fallen worldwide, the therapeutic efficacy of advanced gastric cancer is still unsatisfactory. In order to clarify the molecular mechanism of invasion and metastasis in gastric cancer, we investigated the mRNA and protein expression of PIK3CA in different gastric cancer tissues, as well as the effect of PIK3CA expression on activation of PI3K/Akt signaling pathway.

Interestingly, Woenckhaus *et al*[19] pointed out that increased expression of PIK3CA was associated with progression of dysplasia to an invasive squamous cell carcinoma. Akagi *et al*[20] found that PIK3CA mRNA overexpression was highly prevalent in esophageal squamous cell carcinoma samples by quantitative RT-PCR. Additionally, the presence of node metastasis was significantly higher in the group with positive staining for PIK3CA compared with the negative staining group immunohistochemically. Similar to their studies, weak or no expression

of PIK3CA mRNA and protein was discovered in normal gastric mucosa, while strong expression was detected during the progression of primary gastric cancer to lymph node metastasis and distant metastasis in our study. Thus, up-regulation of PIK3CA was most likely linked to tumor invasion and metastasis.

In a previous study, deregulation of PI3K/Akt pathway frequently found in a great number of human malignant tumors was closely related to tumor development[21]. Akt, also known as protein kinase B, the major downstream effector of PI3K, is activated by phosphoinositide-dependent protein kinase 1, which is recruited and phosphorylated by activation of PI3K[22]. Increasing studies have shown that Akt plays an important role in many physiological processes, such as cell growth and proliferation, apoptosis, cell motility and invasion[23,24]. In this study, we found for the first time that p-Akt (Ser473) was lost or not expressed in normal gastric mucosa tissues through Western blotting analysis, while it was highly expressed in metastatic tissues compared with primary gastric cancer tissues. In addition, such observation is in good agreement with the expression profile of PIK3CA. This implied that up-regulation of PIK3CA could increase catalytic activity of PI3K, as suggested by Shayesteh *et al*[25] and Ma *et al*[26], and subsequently overactivated PI3K/Akt pathway to promote metastasis in gastric cancer.

These data, together with our previous findings, demonstrated that up-regulation of PIK3CA and resultant constitutive activation of PI3K/Akt signaling pathway are of primary importance in understanding the process of metastasis in gastric cancer. To date, because of no reliable clinical method available to predict metastasis in gastric cancer, increased expression of PIK3CA is expected to be a potential molecular marker for the early diagnosis of advanced gastric cancer. Meanwhile, PIK3CA, the most proximal pathway component, might be a better target for anticancer drug discovery than distal components such as Akt and mTOR.

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COMMENTS

Background

Invasion and metastasis are the main factors contributing to the death of gastric cancer patients. Currently, no ideal method was available to predict metastasis of gastric cancer in clinic. Many studies have implicated PIK3CA mutations with features of metastasis, but the correlation between PIK3CA expression and metastasis in gastric cancer and its effects on activation of phosphatidylinositol 3-kinase (PI3K)/serine/threonine kinase (Akt) remains unclear.

Research frontiers

Several molecular pathways are known to play a role in gastric cancer development and progression. Perhaps the most important pathway is the currently discovered PI3K/Akt pathway. Many studies have reported that mutations of the PIK3CA gene encoding the catalytic subunit p110α of PI3K, are contributed to dysregulation of PI3K/Akt pathway.

Innovations and breakthroughs

Previous reports have highlighted the importance of mutations of PIK3CA oncogene in various carcinogenesis. In this study, the authors reported for the first time that up-regulation of PIK3CA and resultant constitutive activation of PI3K/Akt pathway are of primary importance in understanding the process of metastasis in gastric cancer.

Applications

The data presented in this paper, together with the authors' previous findings, suggested that up-regulation of PIK3CA is expected to be a potential molecular marker for the early diagnosis of advanced gastric cancer. Meanwhile, PIK3CA, the most proximal pathway component, might be a better target for anticancer drug discovery than distal components such as Akt and mTOR.

Terminology

PIK3CA gene, encoding the key catalytic subunit p110α of PI3K, is located on chromosome 3q26.3. AKT, a serine/threonine kinase, serving as the major downstream effector of PI3K, regulates many biological processes, such as proliferation, apoptosis and growth.

Peer review

This descriptive study focuses on the important role of PIK3CA in PI3K/Akt pathway in gastric carcinogenesis. The authors demonstrated the data indicating that PIK3CA could be a promising indicator for metastasis from gastric carcinomas. The study is well written.

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BRIEF ARTICLE

Five-year long-term outcomes of laparoscopic surgery for colon cancer

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Abstract

AIM: To perform a meta-analysis to answer whether long-term recurrence rates after laparoscopic-assisted surgery are comparable to those reported after open surgery.

METHODS: A comprehensive literature search of the MEDLINE database, EMBASE database, and the Cochrane Central Register of Controlled Trials for the years 1991-2010 was performed. Prospective randomized clinical trials (RCTs) were eligible if they included patients with colon cancer treated by laparoscopic surgery *vs* open surgery and followed for more than five years.

RESULTS: Three studies involving 2147 patients reported long-term outcomes based on five-year data and were included in the analysis. The overall mortality was similar in the two groups (24.9%, 268/1075 in the laparoscopic group and 26.4%, 283/1072 in open group). No significant differences between laparoscopic and open surgery were found in overall mortality during the follow-up period of these studies [OR (fixed) 0.92, 95% confidence intervals (95% CI): 0.76-1.12, P = 0.41]. No significant difference in the development of overall recurrence was found in colon cancer patients, when comparing laparoscopic and open surgery [2147 pts, 19.3% *vs* 20.0%; OR (fixed) 0.96, 95% CI: 0.78-1.19, P = 0.71].

CONCLUSION: This meta-analysis suggests that laparoscopic surgery was as efficacious and safe as open surgery for colon cancer, based on the five-year data of these included RCTs.

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Key words: Colon cancer; Laparoscopic surgery; Open surgery; Randomized clinical trials; Meta-analysis

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INTRODUCTION

Colon cancer is the third most common malignancy in men and women[1]. Traditionally, cancers of the colon were removed through large abdominal incisions. Since the advent of laparoscopic surgery, it has become clear that patients benefit from a minimally invasive approach in a variety of ways[2]. In 1991, laparoscopic-assisted colectomy (LAC) was first reported[3,4]. Short-term advantages of laparoscopic colorectal surgery compared to conven-

tional surgery are well known, and include less pain, better pulmonary function, shorter duration of postoperative ileus, less fatigue and a better quality of life[5-8].

However, it was uncertain whether there would be a long-term survival difference. Several large trials have been reported which discuss the long-term survival difference. The primary aim of these trials is to test the hypothesis that disease-free survival and overall survival are equivalent, regardless of whether patients receive laparoscopic-assisted or open colectomy. The second aim is to assess the recurrence of cancer. The objective of this systematic review is to assess that in the long-term, laparoscopic-assisted colon resection for cancer is not inferior to open colectomy with respect to cancer survival and recurrence. The main outcome of concern is overall mortality and recurrence.

MATERIALS AND METHODS

Identification and selection of studies

A comprehensive literature search of the MEDLINE database, EMBASE database, and the Cochrane Central Register of Controlled Trials for the years 1991-2010 was performed. Searches were carried out using medical subject headings (MeSH) and free text words in combination with the search strategy for randomised controlled trials (RCT). The following search was adapted for each database: laparoscopy [MeSH], surgery [MeSH], colon [MeSH], colectomy [MeSH], restorative proctocolectomy [MeSH], and colonic neoplasms [MeSH]. Reference lists from the trials were hand-searched to identify further relevant trials. The following selection criteria were applied: (1) study design: RCTs reported with relevant information available; (2) study population: patients with colon cancer; (3) intervention: laparoscopic surgery *vs* open surgery; (4) samples more than 100 patients; and (5) follow-up more than five years.

Quality assessment

Two authors independently evaluated all included trials using a list of selected quality items assessing components of internal validity. Method of randomization, concealment of random allocation, blinding of outcome assessors and reporting of an intention-to-treat analysis were assessed. Trials were considered to be of good quality if they reported on three or four of these quality items, of moderate quality if they reported on one or two items, and of low quality if they reported none of the items. The reporting of this systematic review is in accordance with the QUOROM statement[9].

Statistical analysis

Treatment effects were expressed as risk ratios with corresponding 95% confidence intervals (95% CI). Where possible, outcomes were pooled with a fixed effects model and random effects model. Heterogeneity was assessed using the χ^2 statistic and the proportion of variation due to heterogeneity was expressed as I^2. In the absence of significant heterogeneity ($P > 0.05$ for χ^2), fixed-effects model (inverse variance method) was used, and in the presence of significant heterogeneity ($P < 0.05$ for χ^2) and random effects (DerSimonian-Laird method) models. If substantial heterogeneity was found in the included studies, the result was reported from the random effects model. A P value of 0.05 was used as the cut-off value to determine statistical significance. The meta-analyses were performed by using Review Manager 4.2 provided by The Cochrane Collaboration. If data for meta-analysis was considered inappropriate in the included studies, some outcomes were presented in a descriptive way.

Funnel plots were constructed to evaluate potential publication bias using the standard error and diagnostic odds ratio, however, because there are only three studies included, the funnel plots will not be very helpful and will not be shown in the article.

RESULTS

A total of 66 published articles of RCTs comparing laparoscopically assisted and open surgery for colon cancer were identified. Of these trials, there were 19 articles just with short-term outcomes available and another 17 articles with regard to the colorectal cancer. In addition, 21 articles had not reported relevant information. So there were 9 potential articles left to further review. Of these trials, 4 studies were excluded because there were less than 100 patients included[10-13]. Two studies were reported at a different time. So finally 3 studies involving 2147 patients reported long-term outcome data and were included in the analysis. All of the included studies were published as full articles. Baseline characteristics of included studies are described in Table 1. Quality assessment revealed that all studies were of good or moderate quality (Table 2), indicating that all studies were of reasonable methodological quality; none of the studies had any "fatal" methodological flaws.

In 2009, the COLOR trial[14] published its long-term outcomes after laparoscopic surgery *vs* open surgery. 1076 patients were eligible for analysis (542 assigned open surgery and 534 assigned laparoscopic surgery). Median follow-up was 53 mo (range 0.03-60). The combined 3-year disease-free survival for all stages was 74.2% in the laparoscopic group and 76.2% in the open surgery group ($P = 0.70$). The hazard ratio (HR) for disease-free survival (open *vs* laparoscopic surgery) was 0.92 (95% CI: 0.74-1.15). The combined 3-year overall survival for all stages was 81.8% in the laparoscopic group and 84.2% in the open-surgery group ($P = 0.45$).

In 2008, Lacy *et al*[15] reported the long-term results of a randomized clinical trial of laparoscopy-assisted *vs* open surgery (LAC *vs* OC) for colon cancer. Two hundred and nineteen patients entered the study. The median follow-up was 95 mo. There was a tendency towards higher cancer-related survival ($P = 0.07$, NS) and overall survival ($P = 0.06$, NS) for the LAC group. The regression analysis showed that LAC was independently associated with a reduced risk of tumor relapse (hazard ratio 0.47, 95% CI: 0.23-0.94), death from a cancer-relat-

Table 1 Characteristics of included studies (laparoscopic *vs* open group)

Ref.	Age (yr, median)	Localisation of the tumour	Follow-up	Analyzed (*n*)
COST 2007[16]	70/69	Right/left/sigmoid colon	5 yr	863
Lacy 2008[15]	68/71	Right/left/sigmoid colon	95 mo	219
COLOR 2009[14]	71/71	Right/left/sigmoid colon	53 mo	1076

Table 2 Quality assessment: internal validity of the included randomized trials

Ref.	Randomization allocation	Concealment of allocation	Blinding	Intention-to-treat analysis	Withdrawal and dropouts
COST 2007[16]	Yes	Not clear	Not clear	Yes	Clear report
Lacy 2008[15]	Yes	Adequate	Not clear	Yes	Clear report
COLOR 2009[14]	Yes	Not clear	Not clear	Yes	Clear report

ed cause (0.44, 0.21-0.92) and death from any cause (0.59, 0.35-0.98). So they concluded that LAC is more effective than OC in the treatment of colon cancer. In 2002, Lacy *et al*[5] reported the same study with a median length of follow-up of 43 mo, demonstrating that LAC was more effective for treatment of colon cancer in terms of morbidity, hospital stay, tumor recurrence, and cancer-related survival.

In 2007, Fleshman *et al*[16] published the 5-year data from the COST study group trial. Patients were followed a median of 7 years. Disease-free 5-year survival (OC 68.4%, LAC 69.2%, $P = 0.94$) and overall 5-year survival (OC 74.6%, LAC 76.4%, $P = 0.93$) were similar for the 2 groups. Overall recurrence rates were similar for the 2 groups (OC 21.8%, LAC 19.4%, $P = 0.25$). These recurrences were distributed similarly between the 2 treatment groups. Sites of first recurrence were distributed similarly between the treatment arms (OC: wound 0.5%, liver 5.8%, lung 4.6%, other 8.4%; LAC: wound 0.9%, liver 5.5%, lung 4.6%, other 6.1%). Likewise, in 2004, the three-year outcomes of COST[17] were also reported with the recurrence rate and the overall survival being similar for the two groups. So they concluded that laparoscopic colectomy for curable colon cancer is not inferior to open surgery based on oncologic endpoints.

Overall mortality and cancer-related mortality

All 3 studies reported overall mortality at maximum follow-up. 2147 patients were included in this meta-analysis. The overall mortality was similar in the two groups (24.9%, 268/1075 in the laparoscopic group and 26.4%, 283/1072 in the open group). No significant differences between laparoscopic and open surgery were found in overall mortality during the follow-up period of the study [OR (fixed) 0.92, 95% CI: 0.76-1.12, $P = 0.41$] (Figure 1A). Regarding the cancer-related mortality, only Lacy's study reported this result (16%, 17/106 in laparoscopic group and 27%, 28/102 in open group, $P = 0.07$, NS).

Overall 5-year disease-free survival and overall 5-year survival

Both COLOR and COST trials reported the overall 5-year disease-free survival and overall 5-year survival between laparoscopic and open groups. In the COLOR trial, the overall 5-year disease-free survival and overall 5-year survival were 66.5% *vs* 67.9% and 73.8% *vs* 74.2%, respectively; in the COST trial, the overall 5-year disease-free survival and overall 5-year survival were 69.2% *vs* 68.4% and 76.4% *vs* 74.6%, respectively. As seen in the two large randomized trials, these two outcomes were similar between the two groups.

Overall and local and distant recurrence

All 3 studies reported these outcomes. No significant difference in the development of overall recurrence was found in colon cancer patients, when comparing laparoscopic and open surgery [2147 pts, 19.3% *vs* 20.0%; OR (fixed) 0.96, 95% CI: 0.78-1.19, $P = 0.71$] (Figure 1B). The number of patients that developed a local recurrence at the maximum follow-up of the study was similar after laparoscopic and open surgery, showing that there is no significant difference between laparoscopic and open procedures [2147 pts, 4.0% *vs* 4.4%; OR (fixed) 0.91, 95% CI: 0.59-1.39. $P = 0.66$] (Figure 1C). Similarly, no significant difference in the development of distant metastases was found in colon cancer patients, when comparing laparoscopic and open surgery [2147 pts, 12.8% *vs* 14.0%; OR (fixed) 0.90, 95% CI: 0.70-1.16, $P = 0.41$] (Figure 1D).

DISCUSSION

Colon cancer is one of the most common cancers in both female and male persons. Treatment involves surgical resection of the segment of the bowel containing the tumor and wide tumor-free margins. Lymph nodes in the area are also removed. Conventional surgery is the mainstream treatment of colorectal cancer and has good survival rates for stage-1 tumors. For many people it is now possible to use video-endoscopic surgery (laparoscopy), which may have short term advantages that include less pain, better pulmonary function, shorter time for return of bowel function (duration of postoperative ileus), less fatigue, and improved convalescence, as suggested in a Cochrane systematic analysis on short-term outcomes[18]. This meta-analysis also demonstrated that postoperative duration of hospital stay is less and quality of life may be improved in

A

Review: Long-term results of laparoscopic colon cancer resection
Comparison: 01 overall mortality at maxium follow-up
Outcome: 01 overall mortality

Study or subgroup	Laparoscopic *n/N*	Open *n/N*	Odds ratio (fixed) 95% CI	Weight (%)	Odds ratio (fixed) 95% CI
COST 2007[16]	102/435	108/428		39.62	0.91 (0.66-1.24)
Lacy 2008[15]	38/106	50/102		15.54	0.58 (0.33-1.01)
COLOR 2009[14]	128/534	125/542		44.84	1.05 (0.79-1.39)
Total (95% CI)	1075	1072		100.00	0.92 (0.76-1.12)
Total events	268	283			

Test for heterogeneity: $\chi^2 = 3.50$, $df = 2$ ($P = 0.17$); $I^2 = 42.9\%$
Test for overall effect: $Z = 0.82$ ($P = 0.41$)

0.1 0.2 0.5 1 2 5 10
Favours treatment Favours control

B

Review: Long-term results of laparoscopic colon cancer resection
Comparison: 02 recurrence at maxium follow-up
Outcome: 01 overall recurrence

Study or subgroup	Laparoscopic *n/N*	Open *n/N*	Odds ratio (fixed) 95% CI	Weight (%)	Odds ratio (fixed) 95% CI
COST 2007[16]	84/435	93/428		43.66	0.86 (0.62-1.20)
Lacy 2008[15]	19/106	29/102		14.00	0.55 (0.29-1.06)
COLOR 2009[14]	105/534	92/542		42.34	1.20 (0.88-1.63)
Total (95% CI)	1075	1072		100.00	0.96 (0.78-1.19)
Total events	208	214			

Test for heterogeneity: $\chi^2 = 5.13$, $df = 2$ ($P = 0.08$); $I^2 = 61.0\%$
Test for overall effect: $Z = 0.37$ ($P = 0.71$)

0.1 0.2 0.5 1 2 5 10
Favours treatment Favours control

C

Review: Long-term results of laparoscopic colon cancer resection
Comparison: 02 recurrence at maxium follow-up
Outcome: 02 local recurrence

Study or subgroup	Laparoscopic *n/N*	Open *n/N*	Odds ratio (fixed) 95% CI	Weight (%)	Odds ratio (fixed) 95% CI
COST 2007[16]	10/435	11/428		24.13	0.89 (0.37-2.12)
Lacy 2008[15]	7/106	10/102		21.20	0.65 (0.24-1.78)
COLOR 2009[14]	26/534	26/542		54.67	1.02 (0.58-1.77)
Total (95% CI)	1075	1072		100.00	0.91 (0.59-1.39)
Total events	43	47			

Test for heterogeneity: $\chi^2 = 0.58$, $df = 2$ ($P = 0.75$); $I^2 = 0\%$
Test for overall effect: $Z = 0.44$ ($P = 0.66$)

0.1 0.2 0.5 1 2 5 10
Favours treatment Favours control

D

Review: Long-term results of laparoscopic colon cancer resection
Comparison: 02 recurrence at maxium follow-up
Outcome: 03 distant recurrence

Study or subgroup	Laparoscopic *n/N*	Open *n/N*	Odds ratio (fixed) 95% CI	Weight (%)	Odds ratio (fixed) 95% CI
COST 2007[16]	74/435	82/428		52.86	0.86 (0.61-1.22)
Lacy 2008[15]	8/106	14/102		10.17	0.51 (0.21-1.28)
COLOR 2009[14]	56/534	54/542		36.97	1.06 (0.71-1.57)
Total (95% CI)	1075	1072		100.00	0.90 (0.70-1.16)
Total events	138	150			

Test for heterogeneity: $\chi^2 = 2.15$, $df = 2$ ($P = 0.34$); $I^2 = 7.0\%$
Test for overall effect: $Z = 0.82$ ($P = 0.41$)

0.1 0.2 0.5 1 2 5 10
Favours treatment Favours control

Figure 1 Meta-analysis on overall mortality (A), overall recurrence (B), local recurrence (C) and distant recurrence (D) at maximum follow-up.

the early postoperative course. Furthermore, the risk of postoperative morbidity is decreased by the laparoscopic approach, namely because of a reduced surgical morbidity.

However, the procedure is complex and for colon cancer the long-term results on survival are not known. There are several large RCTs published and several systematic reviews performed to assess the difference between the laparoscopic and open approach. In 2007, Bonjer and his colleague[19] performed a meta-analysis of trials randomizing patients with colon cancer to laparoscopically assisted or open colectomy to determine whether laparoscopic colectomy for cancer is oncologically safe. Patients included in this analysis had at least 3 years of complete follow-up data. Of 1765 patients, 229 were excluded, leaving 796 patients in the laparoscopically assisted arm and 740 patients in the open arm for analysis. Three-year disease-free survival rates in the laparoscopically assisted and open arms were 75.8% and 75.3%, respectively. The 3-year overall survival rate after laparoscopic surgery was 82.2% and after open surgery was 83.5%. Disease-free and overall survival rates for stages Ⅰ, Ⅱ, and Ⅲ evaluated separately did not differ between the 2 treatments. So they concluded that laparoscopically assisted colectomy for cancer is oncologically safe. Again in 2007, Kahnamoui and his colleagues[20] published a systematic review on laparoscopic surgery for colon cancer. The results of this review suggest that, although there is no definitive answer, overwhelming evidence presently indicates that laparoscopic colon cancer resection is as safe and efficacious as the conventional open technique. In 2008, Kuhry and his colleagues[21] published a Cochrane systematic review of randomised controlled trials on the long-term outcomes of laparoscopic surgery for colorectal cancer with the median follow-up from 19-59 mo. No significant difference in tumour recurrence after laparoscopic and open surgery for colon cancer was observed (3 RCTs, hazard ratio for tumour recurrence in the laparoscopic group 0.86; 95% CI: 0.70-1.08). Similarly, in colon cancer patients, no significant differences in overall mortality were found (2 RCTs, hazard ratio for overall mortality after laparoscopic surgery 0.86; 95% CI: 0.86-1.07). So they also concluded that laparoscopic resection of carcinoma of the colon is associated with a long-term outcome that is similar to that after open colectomy.

All 3 systematic reviews demonstrated that laparoscopic colon cancer resection is as safe and efficacious as the conventional open technique in terms of 3-year survival and 3-year disease-free survival and recurrence.

The main objective of this present meta-analysis is to demonstrate that in the long run, laparoscopic colon cancer resection is also as safe and efficacious as open surgery, especially for overall 5-year survival and mortality and recurrence. So the duration of follow-up of all included studies was more than 60 mo, as shown in the table of patient characteristics. There are several characteristics of the three included trials. First, all 3 included trials had a large sample size, especially COST trial (863) and COLOR (1076) trial. Second, the quality of the 3 trials is very high. Third, all of the patients only had colon adenocarcinoma. Additionally, the follow-up was very long. So the combined results from the 3 studies should be convincing. 2147 patients were included in this meta-analysis. The overall mortality was similar in the two groups (24.9%, 268/1075 in the laparoscopic group and 26.4%, 283/1072 in the open group) with no significance detected, as was the overall recurrence regardless of local or distant recurrence. Both the overall 5-year survival (about 74%) and 5-year disease-free survival (about 68%) were similar between the two groups, and no significant difference was demonstrated. From these results, laparoscopic colon cancer resection was demonstrated to be as safe and efficacious as the open surgery, as we expected.

In conclusion, compared to open surgery, laparoscopically assisted colectomy has been demonstrated to have short term advantages that include less pain, better pulmonary function, shorter time for return of bowel function (duration of postoperative ileus), less fatigue, improved convalescence, and more importantly reduced surgical morbidity and shorter duration of hospital stay. From the 3-year long-term data, laparoscopic surgery was also demonstrated to be as safe and efficacious as the conventional open approach. Our present work again demonstrated that, in terms of long-term outcomes of trials a the median follow-up more than 5 years, laparoscopic surgery is also as safe and efficacious as the conventional open approach. In addition, laparoscopic resection is associated with a modest additional cost, compared with open surgery. So regardless of the short-term outcomes or the long-term outcomes or even the cost-effectiveness, we could conclude that laparoscopic surgery is not inferior to the conventional open approach. From these analyses above, we think that for colon adenocarcinoma, laparoscopic assisted colectomy should be the preferred choice as appropriate.

COMMENTS

Background

Short-term advantages of laparoscopic colorectal surgery compared to conventional surgery are well known, however, it was uncertain whether there would be a long-term survival difference. Several large trials has been reported which report on long-term survival differences.

Research frontiers

The objective of this systematic review is to assess that in the long term, laparoscopic-assisted colon resection for cancer is not inferior to open colectomy with respect to cancer survival and recurrence.

Innovations and breakthroughs

This meta-analysis suggests that laparoscopic surgery was as efficacious and safe as open surgery for colon cancer, based on the five-year data of the included randomized clinical trials.

Applications

For colon adenocarcinoma, laparoscopic assisted colectomy should be the preferred choice as appropriate.

Peer review

The study was well structured and gives interesting information about the present status of laparoscopy and colon cancer. The main strength is the longer follow up of patients and the criteria to be included. I think this manuscript should be interesting for surgeons dealing with this disease.

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BRIEF ARTICLE

Diagnosis of bile duct hepatocellular carcinoma thrombus without obvious intrahepatic mass

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Abstract

AIM: To study the diagnosis of hepatocellular carcinoma (HCC) presenting as bile duct tumor thrombus with no detectable intrahepatic mass.

METHODS: Six patients with pathologically proven bile duct HCC thrombi but no intrahepatic mass demonstrated on the preoperative imaging or palpated intrahepatic mass during operative exploration, were collected. Their clinical and imaging data were retrospectively analyzed. The major findings or signs on comprehensive imaging were correlated with the surgical and pathologic findings.

RESULTS: Jaundice was the major clinical symptom of the patients. The elevated serum total bilirubin, direct bilirubin and alanine aminotransferase levels were in concordance with obstructive jaundice and the underlying liver disease. Of the 6 patients showing evidence of viral hepatitis, 5 were positive for serum alpha fetoprotein and carbohydrate antigen 19-9, and 1 was positive for serum carcinoembryonic antigen. No patient was correctly diagnosed by ultrasound. The main features of patients on comprehensive imaging were filling defects with cup-shaped ends of the bile duct, with large filling defects presenting as casting moulds in the expanded bile duct, hypervascular intraluminal nodules, debris or blood clots in the bile duct. No obvious circular thickening of the bile duct walls was observed.

CONCLUSION: Even with no detectable intrahepatic tumor, bile duct HCC thrombus should be considered in patients predisposed to HCC, and some imaging signs are indicative of its diagnosis.

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Key words: Hepatocellular carcinoma; Obstructive jaundice; Bile duct tumor thrombus; Diagnosis; Diagnostic imaging

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INTRODUCTION

Obstructive jaundice associated with hepatocellular carcinoma (HCC) is not common, with an incidence of 0.5%-13% in patients with HCC[1-11]. This type of HCC is also known as icteric type of HCC (IHCC)[5-7]. Bile duct tumor thrombus (BDTT) is the leading cause of obstruc-

tion in IHCC. It has been shown that HCC is smaller in patients with biliary tumor thrombi than in those without biliary tumor thrombi, with a mean tumor size of 3.8 ± 2.1 cm *vs* 6.7 ± 4.6 cm[8]. Several studies[9-11] concerning IHCC reported that primary hepatic parenchymal tumor is detectable in most patients with IHCC while no obvious intrahepatic tumor is detectable in only 2.9%-25.0% of patients with biliary HCC thrombi. We encountered 6 patients with this special type of IHCC. Postoperative pathologic examinations "surprisingly" proved that the bile duct nodules leading to obstructive jaundice were HCCs. However, neither intrahepatic mass nor portal vein thrombus was identified on the preoperative imaging or even during explorative surgery. This type of IHCC is very rare and difficult to diagnose, and only few cases have been occasionally reported[12-17], without their features summarized. We retrospectively analyzed the clinical and imaging data about 6 patients with emphasis laid on the diagnostic imaging correlated with pathologic and surgical data, and clinical features and imaging signs that might lead to the diagnosis.

MATERIALS AND METHODS

Ethics

This study was approved by our institutional review board. Informed consent was not obtained from the patients as this was a limited, anonymous retrospective review of patient data.

Patients

Six patients including 5 men and 1 woman at the age of 47-64 years were confirmed with bile duct HCC by surgery and histology between January 2000 and November 2008 at our hospital. Their medical records were thoroughly reviewed and cross-checked.

Jaundice was the predominant symptom of the patients, and presented as an initial symptom of 4 patients. The time from the onset of jaundice to admission was 7 d-3 mo (median 1 mo). The other main clinical symptoms were fatigue, right upper quadrant abdominal pain or upper abdominal pain, abdominal distension, loss of appetite and loss of weight. Liver function reserve was Child grade A and B in 4 and 2 patients, respectively.

Methods

Laboratory data about the patients before surgery were recorded and analyzed.

Each patient underwent two or more preoperative diagnostic imaging procedures, including transabdominal ultrasonography (US), computed tomography (CT) with plain scan and arterial and portal phase contrast enhanced scans, magnetic resonance imaging (MRI) with magnetic resonance cholangiopancreatography (MRCP), endoscopic retrograde cholangiopancreatography (ERCP) and percutaneous transhepatic cholangiography (PTC). All available imaging data including diagnosis reports and images were retrospectively reviewed by two radiologists with more than 5 years of experience in abdominal imaging. A consensus was reached with the main findings or signs recorded.

Surgical records and pathologic reports were also reviewed and correlated with the major findings or signs on comprehensive imaging.

RESULTS

Blood test

The levels of total serum bilirubin (TBIL), direct bilirubin (DBIL), alanine aminotransferase (ALT), hepatitis B surface antigen (HBsAg), α-fetoprotein (AFP), carbohydrate antigen 19-9 (CA19-9), and carcinoembryonic antigen (CEA) of each patient are listed in Table 1.

US

Transabdominal US was performed, showing dilatated intrahepatic ducts with nodules in hilar bile ducts but no intrahepatic mass. The intraluminal nodules were hypoechoic, slightly hyperechoic, and mixed echoic in 4, 1, and 1 patients, respectively. Three and 1 patients were diagnosed as hilar cholangiocarcinoma and choledocholithiasis, respectively. Further evaluation was needed in 2 patients.

CT

CT with pre-contrast scan and dual-phase contrast-enhanced scan was performed for 5 patients, showing dilated bilateral intrahepatic ducts with an intraductal nodule obstructing the hilar bile duct and/or common bile duct, but no tumor thrombus in the portal vein or systemic vein and no obvious mass in the hepatic parenchyma. These intraductal nodules were relatively mild hypodense to the hepatic parenchyma on pre-contrast images. During the arterial phase, they showed different degrees of enhancement and were relatively isodense or mildly hyperdense to the hepatic parenchyma. The enhancement of intraductal nodules was relatively lower in portal phase than that of hepatic parenchyma. In three lesions with the longest diameter greater than 3.0 cm, the intraluminal nodules appeared as cast moulds in the dilated ducts without obvious thickening of the walls. Non-enhanced sludge, which was mildly hyperdense in the bile, was observed in the common bile duct of 2 patients (Figure 1). The sludge was found to be tumor debris or hemorrhage of tumor at surgery. CT showed signs of liver cirrhosis in 4 patients, such as splenomegaly, varices, heterogeneously attenuated liver with lacelike fibrosis and regenerative nodules, and irregular or nodular liver surface. A small amount of ascites was present in 1 patient.

MRI combined with MRCP

Conventional non-enhanced MRI combined with MRCP was performed for 3 patients, showing no mass in the hepatic parenchyma or portal vein. MRCP images showed moderate-severe dilatation of bilateral hepatic ducts with columnar or plugged filling defect of bile ducts in the hilar area. The filling defects were hypointense on T_1-weighted

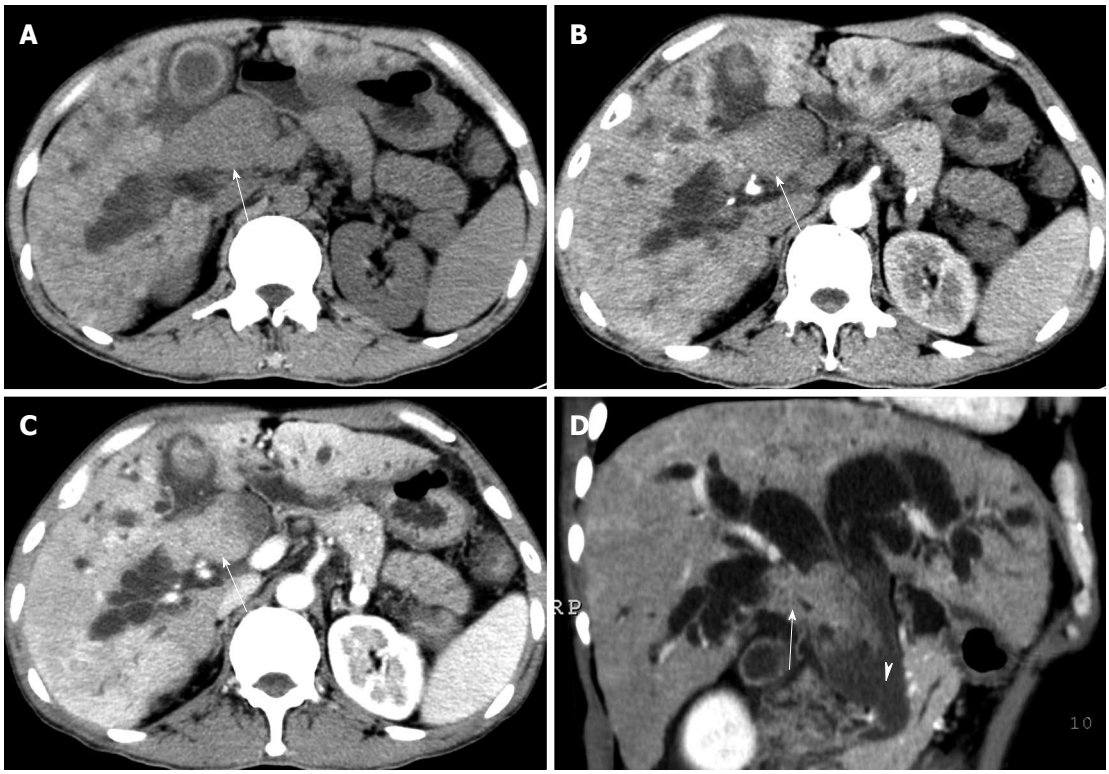

Figure 1 Axial computed tomography images of case 1 at plain scan (A), early arterial phase (B), portal phase (C), and coronal oblique plane reformation image at portal phase (D) showing irregular tumor thrombi in right hepatic, common hepatic and common bile ducts (arrows). The tumor thrombus was mild hypointense on plain scan with enhancement in early arterial phase. Neither portal vein thrombus nor hepatic parenchymal mass was identified. Heterogeneously attenuated liver with lacelike fibrosis and regenerative nodules due to cirrhosis could be observed. The common bile duct was filled with hemorrhage and debris (arrowhead), which was not enhanced and mild hyper-dense in the bile.

Table 1 Blood test

Patient No./sex/age (yr)	TBIL (μmol/L)	DBIL (μmol/L)	ALT (U/L)	HBsAg	AFP (ng/mL)	CA19-9 (U/mL)	CEA (μg/L)
1/M/48	364.4	145.7	99.4	Positive	159	123.1	2.82
2/F/64	265.1	110.2	190.9	Positive	12.4	> 575	3.24
3/M/47	268.6	146.4	127.4	Positive	263	465.7	7.10
4/M/51	277.3	156.1	137.2	Positive[1]	> 1210	Unknown	Unknown
5/M/53	373.9	153.6	133.8	Positive	Negative	183.1	1.52
6/M/55	344.2	199.3	204.0	Positive	10.7	164.9	3.90

Normal value is less than 8.1 ng/mL for α-fetoprotein (AFP), less than 37 U/mL for carbohydrate antigen 19-9 (CA19-9), and less than 5 μg/L for carcinoembryonic antigen (CEA), respectively. [1]Also positive for hepatitis E virus-IgG. HBsAg: Hepatitis B surface antigen; DBIL: Direct bilirubin; ALT: Alanine aminotransferase; TBIL: Total serum bilirubin.

images and iso or mild hyperintense on T_2-weighted images. The intraluminal nodule was originated from the left hepatic duct and extended downward into the common bile duct of 1 patient accompanying a short T_1 signal in surrounding bile duct and gallbladder due to intraluminal hemorrhage of tumor confirmed at surgery (Figure 2). Debris as a sludge-like filling defect was observed in the common bile duct of another patient.

ERCP

ERCP was performed for 1 patient, showing a smooth oval filling defect in the upper common bile, common and right hepatic ducts with dilated intrahepatic ducts (Figure 3A). The cup-shaped filling defect caused dilatation of the bile duct. Gallbladder was not visualized because of obstruction by the tumor.

PTC

PTC was performed for 1 patient, showing an oval smooth intraluminal filling defect in common and right hepatic ducts with dilated intrahepatic ducts (Figure 3B). Both ends of the filling defect were cup-shaped.

Findings during surgery

Tumor thrombi in bile ducts and evident hepatic cholestasis were found in 6 patients during surgery. Typical liver cirrhosis was found in 4 patients. Diffuse HCC was not considered because none of them had evidence of

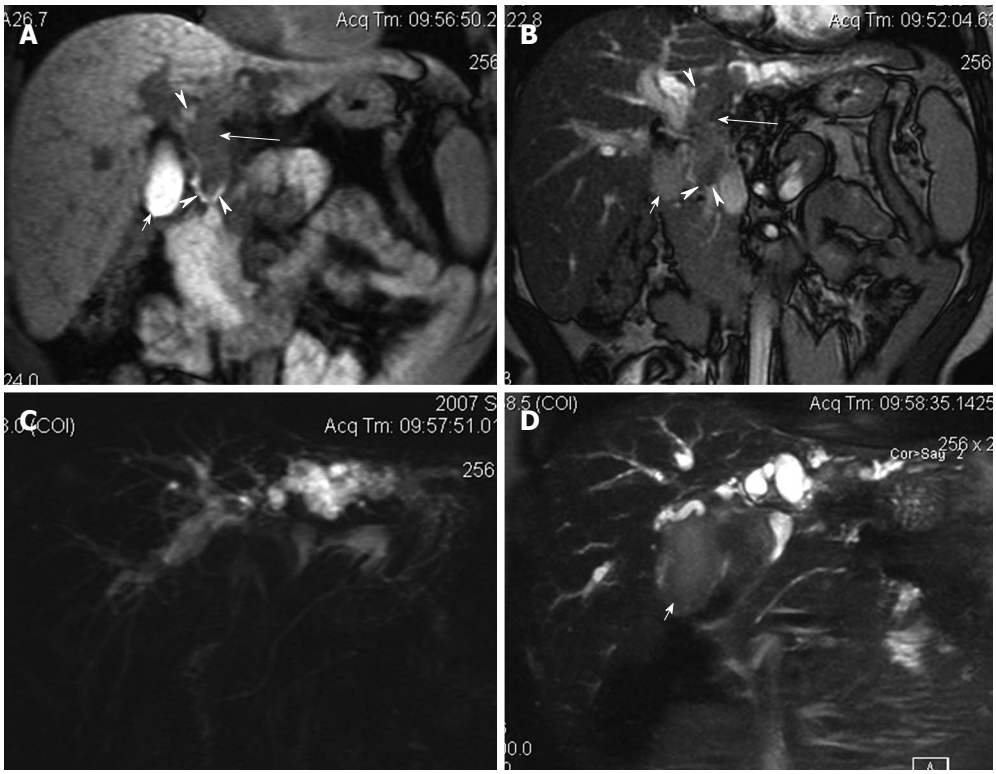

Figure 2 Magnetic resonance and magnetic resonance cholangiopancreatography images of Case 2. A: Coronal T1 weighted image showing hypo-intense tumor thrombus (large arrows) in left hepatic, common hepatic and common bile ducts; B: Coronal T2 weighted image showing the tumor thrombus as iso- to slight hyper-intense; C: Thick slice magnetic resonance cholangiopancreatography image showing bilateral dilated intrahepatic ducts with "vanished" common hepatic and common bile ducts; D: Thin slice magnetic resonance cholangiopancreatography image showing filling defect in the hilar. Neither portal vein thrombus nor hepatic parenchymal mass is identified. The signal intensity of liver is heterogeneous due to cirrhosis. Blood clots or hemorrhage can be observed in gallbladder (small arrows), common hepatic and common bile ducts (arrowheads), which are hyper-dense on T1 weighted image, and slight hyper-intense on T2 weighted image.

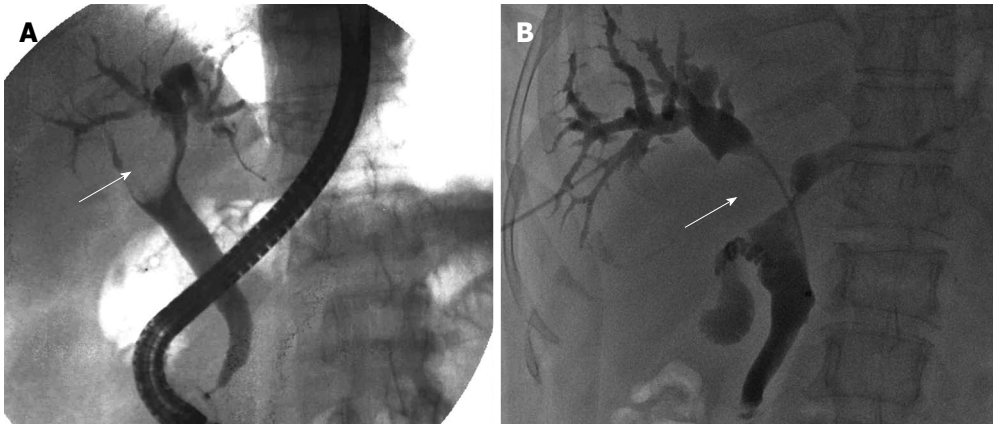

Figure 3 Endoscopic retrograde cholangiopancreatography image of case 3 (A) and percutaneous transhepatic cholangiography image of case 4 (B) showing tumor thrombus (arrows) in right hepatic duct and common hepatic duct as a smooth oval filling defect on endoscopic retrograde cholangiopancreatography image and a smooth oval filling defect on percutaneous transhepatic cholangiography image, respectively.

tumor invasion of the portal vein or system vein. No obvious intrahepatic mass was palpated in all patients. Diffuse miliary peritoneum metastasis was observed in 1 patient and thought to be infiltration of the tumor in hilar duct. The tumor thrombi were dark brown, dark red or yellowish brown in color, and soft or slightly elastic, relatively friable, and extremely vascular tending to bleed even on light touch. Most of them were easily separated from the bile duct walls. Sludge-like debris or small blood clots were found in the common bile ducts of 4 patients and blood-stained bile was found in the intrahepatic ducts of 2 patients. Removal of tumor thrombi was attempted in 6 patients and was successful without active hemorrhage in 4 patients. Partial hepatectomy was performed for 2 patients, and aborted in 4 patients due to the poor liver function reserve or peritoneal metastasis. Further exploration after clearance of thrombi revealed relatively smooth internal walls of common bile duct (CBD) and

common hepatic duct (CHD). The resected liver tissue revealed a small hepatic parenchymal tumor in each, 0.5 cm × 1.0 cm and 0.8 cm × 1.5 cm in size.

Pathology

The pathologic reports of intrabiliary thrombi revealed HCC but no variants of cholangiocarcinoma, mixed type of HCC or cholangiocarcinoma in 6 patients. Of the 6 patients, 2 had poorly-differentiated HCC, 3 had poorly-moderately differentiated HCC, and 1 had moderately-differentiated HCC, which accompanied hemorrhage or necrotic tissue in most of the 6 patients.

DISCUSSION

Portal vein tumor thrombus (PVTT) is frequently seen in HCC patients. However, HCC presented as biliary duct tumor thrombus (BDTT) is a relatively rare entity. Intrahepatic tumor or PVTT is evident in most of patients with HCC presented as BDTT, yet few patients with HCC thrombi in the bile duct but without any detectable intrahepatic mass or PVTT have been reported[9-17]. In this circumstance, it is difficult but still important to establish the correct diagnosis, especially to differentiate it from cholangiocarcinoma or diffuse-type HCC. Because the therapeutic plan for HCC presented as BDTT may be substantially different from that for cholangiocarcinoma and diffuse-type HCC, surgery can often be offered when the disease is still localized[1,2,10-20], while percutaneous transhepatic cholangial drainage (PTCD) combined with transcatheter arterial chemoembolization (TACE) serves as an effective alternative therapy especially when the tumor is unresectable[1,6,7]. We retrospectively analyzed the clinical and imaging data about these patients, and found that some features might be helpful for the diagnosis.

Jaundice is the predominant clinical presentation of this disease[1-21]. Causes of obstructive jaundice in this type of HCC include intraluminal growth of tumor leading to obstruction of intra or extrahepatic ducts, tumor tissue fragments and/or hemorrhages or blood clots due to necrosis, bleeding, and detachment of intraductal tumors, giving rise to the obstruction, which are similar to the reported findings in IHCC[3].

Apart from jaundice, there are also other non-specific symptoms such as fatigue, abdominal pain, abdominal distension and loss of appetite. A differential diagnosis between this and other common diseases causing obstructive jaundice such as cholangiocarcinoma and choledocholithiasis is essential.

In this study, all the 6 patients were positive for the markers of chronic viral hepatitis. The proportion of liver cirrhosis was relatively high with typical liver cirrhosis found in 4 patients. The elevated serum ALT level in our patients might be associated with the underlying disease. The majority of patients were middle-aged or old males. These features were also found in common types of HCC.

Serum tumor markers may be helpful in the diagnosis of this disease[22]. Positive serum AFP supports the diagnosis of HCC, while CEA level is frequently elevated in patients with cholangiocarcinoma. In this study, the positive ratios for AFP and CA19-9 were high, suggesting that positive AFP and CA19-9 support the diagnosis. However, positive CA19-9 may also frequently be seen in cholangitis, bile duct stones and biliary or pancreatic tumors leading to obstructive jaundice[23]. Thus, its value for differential diagnosis has to be further evaluated in studies with more cases.

Several diagnostic imaging methods play an important role in the diagnosis of obstructive jaundice[24-31]. The location and extent of obstruction can be reliably demonstrated using proper techniques, and the cause of obstruction can often be inferred by analyzing the morphology of obstruction site and other relevant signs. Transabdominal US is the most widely used and usually the initial tool to evaluate obstructive jaundice. However, none of our patients was correctly diagnosed by transabdominal US, and most of them were misdiagnosed as cholangiocarcinoma. Tamada *et al*[29] reported that intraductal US (IDUS) can distinguish between tumor thrombi caused by HCC and polypoid type cholangiocarcinoma. However, IDUS has not been widely accepted because it is invasive and requires special equipments and specific expertise. CT, MRI combined with MRCP, ERCP and PTC are also widely used to evaluate obstructive jaundice. Jung *et al*[30] compared the CT features of HCC invading bile ducts with those of intraductal papillary cholangiocarcinoma, and found that the presence of parenchymal mass is the distinct difference between them. Since no parenchymal mass was detectable on cross-sectional images in our patients, we searched for other signs leading to the diagnosis. After analyzing the intrabiliary lesions on comprehensive images with surgical correlation, we found that the following features may be helpful in the diagnosis of this special type of IHCC.

First, the tumor thrombi are typically intraluminal polypoid lesions with irregular or smooth surface depending on the presence of necrosis. Lesions can be either small or larger. Small lesions are localized in 1 or 2 ducts while large lesions can form "biliary duct casts" and extend inferiorly. Because of rapid growth of the tumor with no apparent fibrosis of the duct wall, the ducts can often be expanded into a fusiform shape at the site of lesion. Cup-shaped filling defects can also be observed in dilated ducts around the lesion on cholangiographic images due to the expanded growth pattern of the lesion.

Second, the tumor thrombi are hypervascular. It is well known that HCC is a hypervascular tumor. Detection of vascularity in bile duct thrombi can reliably rule out the diagnosis of bile duct stones, and differentially diagnose between HCC and cholangiocarcinoma. The bile duct thrombi with the characteristic early enhancement pattern on dual-phase contrast-enhanced CT[30] or dynamic contrast-enhanced MRI[28] are important to differentiate HCC from cholangiocarcinoma. The results of this study support this finding. The hypervascularity of bile duct tumor thrombi was confirmed during surgery in this study. It was reported that color Doppler sonography can also effectively detect tumor vascularity of bile duct HCC thrombi[31].

Third, intraluminal fragments or blood clots may

present. Because the tumor thrombi are loose, fragile and prone to necrosis, detachment of parts of the lesions and hemorrhage may frequently occur, and those that are relatively large can lead to free thrombi in the ducts. CT can demonstrate irregular or sludge-like lesions in the ducts with no enhancement. MRI and MRCP may be even superior over CT in demonstrating such lesions[28]. Hemorrhagic lesions or blood clots have a high signal on T_1WI, and a low signal on T_2WI. Debris in bile appears as sludge in bile ducts and gallbladder.

Fourth, no apparent circular thickening of the duct walls or constriction of the ducts is present. Cholangiocarcinoma is often associated with the thickening of bile duct walls, often leading to constriction of nearby ducts. No apparent thickening of the duct walls, especially no circular thickening of the walls, was observed in our patients, and ducts were compacted rather than constricted or narrowed due to the tumor.

Fifth, no portal vein thrombus is present. It might be due to the relatively early stage of the disease in our patients, and it is critical for differentiating it from diffuse-type HCC.

Sixth, cirrhosis of the background liver may support the diagnosis. A relatively high percentage of cirrhosis was observed on preoperative images and during surgery in our patients. "Downstream duct dilatation", a sign standing for the dilatated bile duct below the level of intraluminal nodule, has been described by Jung *et al*[30] in patients with intraductal cholangiocarcinoma, which is thought to be related to mucin produced by the tumor. However, "downstream duct dilatation" was also present in 2 out of 6 patients in our case study, which was contributed to the obstruction by blood clots or fragments in the common bile ducts.

The reasons why HCC is present as intrabile duct tumor thrombi without detectable primary hepatic tumor are as follows. The tumor may originate from cancerization of ectopic hepatocytes in the bile duct wall[17], or the primary tumor is just too small to be identified, or the tumor located at the origin of or close to the intrahepatic duct grows intraluminally and stretchs inferiorly. Although no primary hepatic tumor was demonstrated on preoperative imaging or palpated during operation in our patients, the resected tissues revealed small hepatic tumors in 2 patients. Moreover, since deeply seated small hepatic tumor is hard to palpate during intraoperative exploration, especially in patients with marked cirrhosis, it is hard to rule out the potentiality of small primary intrahepatic HCC in the other 4 patients who did not undergo partial hepatic resection. So, it is recommended that intraoperative ultrasonography (IOUS) should be performed to find the potential intrahepatic tumor or to determine the resection level before the operator decides to perform the resection.

If this disease is suspected, it is still important to look for more sensitive techniques such as CT during arterial portography (CTAP) and superparamagnetic iron oxide (SPIO)-enhanced MRI to find possible primary tumors. CTAP is generally accepted as the most sensitive technique to detect small HCC, but it is only performed for selected patients due to its invasiveness. SPIO-enhanced MRI has emerged as another effective technique to detect small HCC, but its value in evaluating bile duct tumor has not yet fully investigated. Unless the clinicians or the radiologists take HCC into consideration, these techniques can be first adopted in the diagnosis of obstructive jaundice. Thus, our study may help the clinicians and radiologists to consider this disease before such techniques are applied.

There are some limitations in our study. First, it is a retrospective analysis of a limited number of cases. Second, although dynamic contrast-enhanced MRI is used as a conventional technique for the diagnosis of HCC in our hospital, it has not been routinely performed for the evaluation of obstructive jaundice. Third, contrast studies with other types of HCC or other tumors with intraluminal growth are not available due to the limited number of cases. Further study is needed to verify the diagnostic value of the features listed.

COMMENTS

Background

Hepatocellular carcinoma (HCC) thrombus in the bile duct is a rare cause of obstructive jaundice. Although it is rarely encountered, its correct diagnosis, especially differentiating it from other causes of biliary obstruction such as cholangiocarcinoma, is very important. Usually, the presence of primary intrahepatic tumors is the key to its diagnosis. However, since few cases of HCC thrombi in the bile duct with no detectable intrahepatic mass have been reported, its diagnosis is even difficult.

Research frontiers

Six patients with this rare disease were reported. Their clinical and imaging data were retrospectively analyzed with a review of the literature. Some clinical features and imaging signs that may favor the diagnosis were summarized. The study may be helpful for a better understanding of the disease, especially for its diagnosis.

Innovations and breakthroughs

Little is known about the diagnosis of this rare disease. More accurate diagnosis were introduced in this study by describing their clinical features and imaging signs.

Applications

This research may evoke the attention of clinicians to the diagnosis of bile duct hepatocellular carcinoma thrombi without an intrahepatic tumor demonstrated on the diagnostic imaging. If certain clinical features and imaging signs are presented, the diagnosis of the disease can be considered.

Peer review

The manuscript presents an interesting series of patients with HCC presented as biliary tract obstruction leading to jaundice. The clinical and imaging data help find the features that differentiate this tumor from cholangiocarcinoma.

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CASE REPORT

Laparoscopic wedge resection of synchronous gastric intraepithelial neoplasia and stromal tumor: A case report

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Abstract

Synchronous occurrence of epithelial neoplasia and gastrointestinal stromal tumor (GIST) in the stomach is uncommon. Only rare cases have been reported in the literature. We present here a 60-year-old female case of synchronous occurrence of gastric high-level intraepithelial neoplasia and GIST with the features of 22 similar cases and detailed information reported in the English-language literature summarized. In the present patient, epithelial neoplasia and GIST were removed *en bloc* by laparoscopic wedge resection. To the best of our knowledge, this is the first reported case treated by laparoscopic wedge resection.

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Key words: Laparoscopy; Stomach neoplasm; Gastrointestinal stromal tumor; Gastrectomy; Synchronous neoplasm

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INTRODUCTION

Synchronous occurrence of epithelial neoplasia and gastrointestinal stromal tumor (GIST) in the stomach is uncommon. Only few case reports can be found in the literature[1-16]. We present here a case of synchronous occurrence of gastric high-level intraepithelial neoplasia and GIST in the body of stomach, close to the cardia. Epithelial neoplasia and GIST were removed *en bloc* by laparoscopic wedge resection. To the best of our knowledge, this is the first reported case treated by laparoscopic wedge resection. In addition, we also summarized the features of 22 similar cases with detailed information reported in the English-language literature.

CASE REPORT

A 60-year-old woman was admitted to our department in June 2009 because of epigastric pain for three months. She had no fever, nausea or vomiting, hematemesis or melena, and weight loss. Physical examination showed no abnormalities. Blood biochemistry was within the normal range. Computed tomography (CT) of the abdomen with intravenous contrast demonstrated a soft tissue mass measuring 5 cm × 5 cm in size with a clear borderline near the lesser curvature of the gastric body, which was consistent with a GIST (Figure 1). Gastroscopy revealed a mucosal ulcer about 1 cm in diameter located in the lesser curvature of the stomach, 3 cm away from the cardia (Figure 2). Histological examination of the specimen from the ulcer showed high-level intraepithelial neoplasia with positive *Helicobacter pylori*.

Figure 1 Computed tomography scan demonstrating a soft tissue mass (arrow) near the lesser curvature of the gastric body.

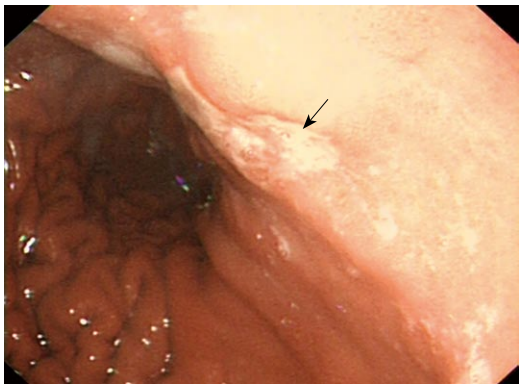

Figure 2 Gastroscopy revealing a mucosal ulcer (arrow) located in the lesser curvature.

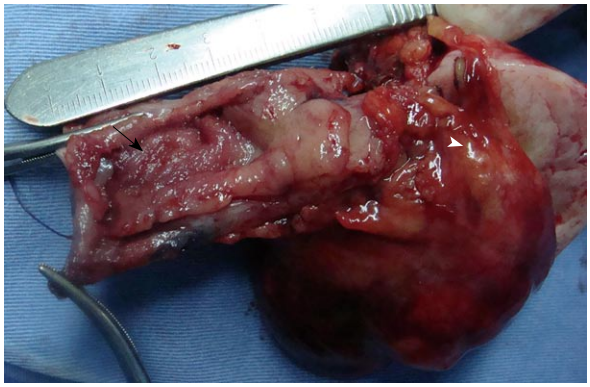

Figure 3 Resected specimens of mucosal ulcer (arrow) and GIST (arrowhead).

During laparoscopic exploration, an extramural pedunculated mass, approximately 5 cm in diameter, was located in the lesser curvature of the gastric body. By intraoperative gastroscopic injection of methylene blue, the mucosal ulcer was localized proximate to the extramural tumor, with 2 cm in between. Laparoscopic wedge resection of the two lesions was performed with triple endoscopic linear staplers (Endocutter 60 staple, green cartridge; Ethicon, Endo-Surgery, Cincinnati, OH, USA) (Figure 3). Intraoperative frozen section of the resected margins was

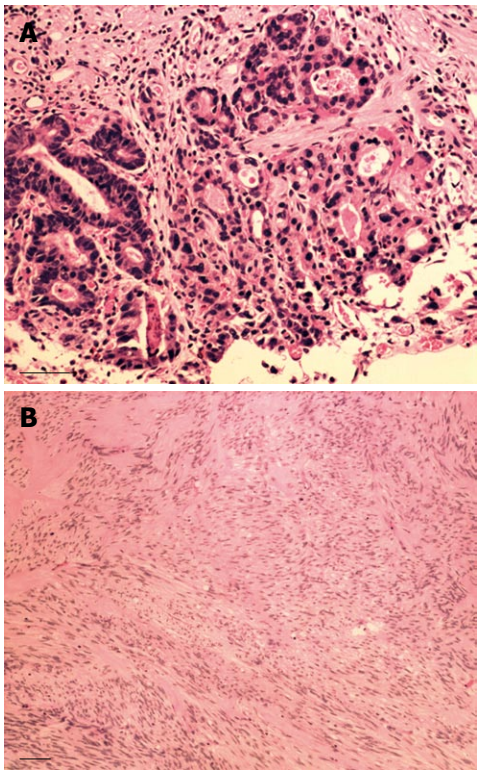

Figure 4 Histological features of high-level intraepithelial neoplasia (A) (HE stain, 200 ×) and gastrointestinal stromal tumor (B) (HE stain, 100 ×). Scale bar = 100 μm.

free of tumor. The operation time was 150 min and intraoperative bleeding was 50 mL. The postoperative course was uneventful, and the patient was discharged 4 days later. She was followed up and abdominal CT and upper gastrointestinal imaging 6 mo after operation showed no signs of recurrence.

Histopathological examination of the mucosal ulcer revealed high-grade intraepithelial neoplasia (Figure 4A) without lymph node metastasis (0/8), while the extramural mass was verified as a stromal tumor consisted of spindle to ovoid-shaped mesenchymal cells arranged in interlacing bundles or sheets (Figure 4B). The cells demonstrated eosinophilic cytoplasm and single elongated nuclei with a moderate level of mitotic activity (3 mitoses per 50 HPF, H&E stain). Immunohistochemical staining was positive for CD117 (Figure 5A) and CD34 (Figure 5B) but negative for SMA, S-100 and Desmin.

DISCUSSION

The term of GIST was introduced by Mazur *et al*[17] in 1983 in order to indicate a distinct heterogeneous group of mesenchymal neoplasms of spindle or epithelioid cells with varying differentiation. GIST occurs from the lower esophagus to the anus, with its most common site in the stomach. However, simultaneous occurrence of GIST and epithelial tumor in the stomach is uncommon. To the best of our knowledge, 44 cases have been reported in the English-language literature[1-16]. The largest published study consisted of 22 cases[16], but without detail information.

Table 1 Summary of previous synchronous gastric epithelial tumors and gastrointestinal stromal tumors in the stomach

No.	Source	Sex/ age (yr)	Epithelial tumor				GIST			Surgical procedure
			Location	Size (cm)	Appearance	Histology	Location	Size (cm)	Appearance	
1	Maiorana *et al*[1]	F/81	Cardia	4	Exophytic	AC	Fundus	5	Intramural mass	Partial gastrectomy
2	Maiorana *et al*[1]	F/79	Antrum	2	Erosion	AC	Pylorus	6	Submucosal mass	Partial gastrectomy
3	Maiorana *et al*[1]	M/75	Antrum	4	Ulcer	AC	Antrum	5	Submucosal mass	Total gastrectomy
4	Maiorana *et al*[1]	F/79	Pylorus	1.2	Ulcer	AC	Corpus	5	Subserosal nodule	Total gastrectomy
5	Maiorana *et al*[1]	M/79	Antrum	2	Ulcer	AC	Corpus	0.6	Subserosal nodule	Total gastrectomy
6	Maiorana *et al*[1]	M/69	Corpus	0.6	Sessile polyp	Carcinoid	Corpus	5	Submucosal nodule	Resection of submucosal nodule
7	Andea *et al*[2]	F/73	Antrum	0.6	Nodule	Carcinoid	Fundus	1.2	Intramural nodule	Antrectomy + wedge resection
8	Kaffes *et al*[3]	M/78	Antrum	Unknown	Slightly raised	AC	Corpus	1.5	Serosal nodule	Total gastrectomy
9	Liu *et al*[4]	M/70	Cardia + corpus	8	Ulcerative	AC (collision)	Cardia + corpus	8	Ulcerative tumor	Total gastrectomy
10	Bircan *et al*[5]	M/71	Antrum	5.7	Ulcerovegetative	AC	Corpus	0.5	Subserosal nodule	Total gastrectomy
11	Bircan *et al*[5]	M/77	Corpus	7.5	Exophytic	AC	Cardia	0.6	Submucosal nodule	Total gastrectomy
12	Wronski *et al*[6]	F/64	Antrum	5	Unknown	AC	Corpus	2	Unknown	Unknown
13	Wronski *et al*[6]	M/66	Antrum	1	Unknown	AC	Corpus	1	Unknown	Unknown
14	Lin *et al*[7]	F/70	Antrum	1.7	Depressed	AC	Fundus	1.1	Sessile polyp	Subtotal gastrectomy
15	Uchiyama *et al*[8]	M/74	Antrum	1.5	Elevated	AC	Corpus	0.8	Extramural nodule	LADG + wedge resection
16	Lee *et al*[9]	M/82	Corpus	1.5	Ulcer	AC	Corpus	9.5	Transmural tumor	Palliative wedge resection
17	Salemis *et al*[10]	F/78	Antrum	6.5	Ulcerative	AC	3 cm to AC	1	Nodular lesion	Total gastrectomy
18	Villias *et al*[11]	M/78	Antrum	Unknown	Ulcer	AC	3.5 cm to AC	0.9	Subserosal nodule	Subtotal gastrectomy
19	Kountourakis *et al*[12]	F/72	Unknown	Unknown	Unknown	AC	Unknown	1.8	Unknown	Subtotal gastrectomy
20	Hsiao *et al*[13]	M/75	GEJ	0.8	Polyp-like	AC	Near AC	3.3	Serosal nodule	Proximal gastrectomy + distal esophagectomy
21	Bi *et al*[14]	F/73	Fundus	4	Ulcerovegetative	AC (collision)	Fundus	4	Ulcerovegetative	Proximal subtotal gastrectomy
22	Ozgun *et al*[15]	M/78	Antrum	Unknown	Ulcer	AC	Opposite to AC	10	Extramural mass	Total gastrectomy

AC: Adenocarcinoma; LADG: Laparoscopic assisted distal gastrectomy; GEJ: Gastroesophageal junction; GIST: Gastrointestinal stromal tumor.

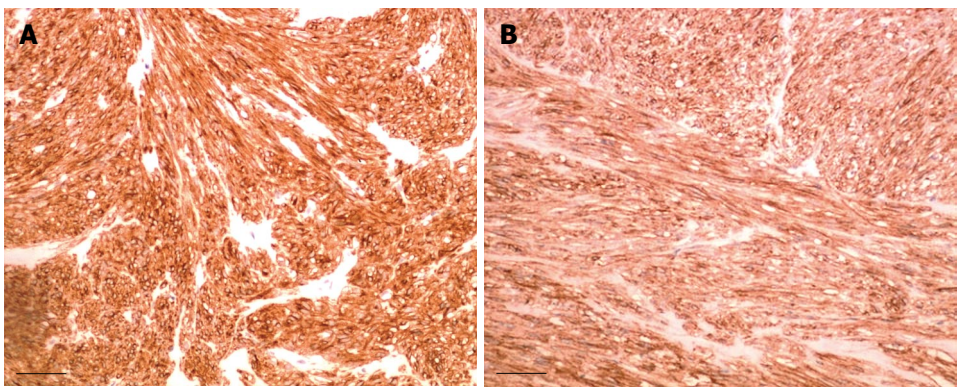

Figure 5 Over-expression of CD117 (A) and CD34 (B) (200 ×). Scale bar = 100 μm.

The remaining 22 cases (12 males and 10 females) at the age of 64-82 years (mean 74.6 years) are listed in Table 1. Of the 22 cases, 20 had adenocarcinoma and 2 had carcinoid.

The simultaneous development of gastric epithelial and stromal tumors, especially two cases of collision tumor composed of gastric adenocarcinoma intermingled with primary GIST[4,14], indicating that such an occurrence is intrinsically connected. An interesting hypothesis is that a single carcinogenic agent can interact with 2 neighboring tissues, inducing the development of tumors of different histotypes in the same organ, and experimental evidence for this possibility has been provided[18,19]. Oral administration of N-methyl-N9-nitro-N-nitrosoguanidine induces the development of gastric adenocarcinomas in rats[18]. When it is used in combination with other agents that alter the gastric mucosal barrier, such as aspirin or stress, leiomyosarcoma develops in conjunction with epithelial tumor[19].

Although many surgeons have realized the possibility of simultaneous development of gastric epithelial and stromal tumors, it is still difficult to diagnose it before operation. In our reviewed cases, simultaneous gastric adenocarcinoma and GIST were confirmed only in 1 case by histological examination before operation[7]. To increase the preoperative diagnostic rate of synchronous tumors, enhanced abdominal CT scan, gastroscopy and endoscopic ultrasonography have been recommended. Careful exploration of residual stomach intraoperatively is also important to avoid missing GIST when it is too small to be found by image examination.

It has been reported that laparoscopic surgery for early gastric cancer and GIST is safe, valid, and minimally invasive[20,21]. However, rare reports are available on laparoscopic resection of synchronous gastric epithelial tumor and GIST. In our reviewed cases, only 1 case was treated by laparoscopic procedure (laparoscopy-assisted distal gastrectomy + laparoscopic wedge resection)[8]. In our case, complicated lymphadenectomy was not needed for either gastric high-level intraepithelial neoplasia or GIST located in the same region with only 2 cm in distance, that makes laparoscopic wedge resection a optimal choice for the patient. Because of the close location of the lesions to the cardia, care should be taken not to injure the esophagocardial junction while firing the stapler. Intraoperative gastroscopy is a simple and effective procedure for the complete excision of tumors and intactness of esophagocardial junction.

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Haemodynamic and renal effects of tadalafil in patients with cirrhosis

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Abstract

A recent report introduced the phosphodiesterase-5 inhibition by vardenafil as a novel treatment of portal hypertension in patients with cirrhosis. In the herein presented "letter to the editor", the administration of tadalafil did not influence portal haemodynamics but impaired systemic haemodynamics in patients with cirrhosis. Our observations concur with the results of a report in a previous issue of *World Journal of Gastroenterology* (October 2008). Moreover, tadalafil adversely affected renal function in patients with decompensated liver disease.

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Key words: Tadalafil; Portal hypertension; Cirrhosis; Ascites; Phosphodiesterase-5 inhibition

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TO THE EDITOR

We read with interest the article by Clemmesen *et al*[1] in a previous issue of *World Journal of Gastroenterology* (October 2008) regarding the effects of sildenafil in patients with cirrhosis and hepatic venous pressure gradient (HVPG) above 12 mmHg. Sildenafil had no effects on HVPG but significantly reduced the mean arterial pressure. We would like to add our experience with the use of tadalafil, a long-acting phosphodiesterase-5 (PDE-5) inhibitor[2], in treatment of patients with cirrhosis.

Six patients with and 6 patients without ascites and oesophageal varices (Child-Pugh class A/B/C: 6/0/0 and 0/2/4, respectively) were studied at baseline and 2 h after oral administration of 10 mg of tadalafil. All patients were included after written informed consent was obtained from them and after the local scientific-ethical committee approved the study. The inclusion criteria were the same as in the study of Clemmesen *et al*[1]. Portal vein velocity (PVV) and portal flow volume (PFV) were evaluated as described by Deibert *et al*[3]. Cardiac output (CO) detected by Doppler ultrasound, mean arterial pressure (MAP) measured with an automatic sphygmomanometer, and systemic vascular resistance (SVR) expressed as the ratio MAP/CO were also evaluated. All patients received a continuous infusion of dextrose water at a rate of 2 mL/min for 4 h before and after the administration of tadalafil to

Table 1 Effects of tadalafil on portal and systemic haemodynamics and renal function in patients with cirrhosis (mean ± SE)

	Compensated (n = 6)		P^1	Decompensated (n = 6)		P^1	P^2
	Baseline	2 h		Baseline	2 h		
PVV (m/s)	0.103 ± 0.016	0.102 ± 0.019	0.9	0.186 ± 0.011	0.18 ± 0.015	0.8	0.6
PFV (L/min)	0.611 ± 0.116	0.543 ± 0.125	0.2	1.194 ± 0.169	1.175 ± 0.198	0.7	0.5
MAP (mmHg)	93.9 ± 3	87.5 ± 2.9	0.02	84.8 ± 2.4	76.9 ± 2	0.001	0.02
CO (L/min)	5.56 ± 0.23	5.7 ± 0.24	0.04	6.91 ± 0.3	7.35 ± 0.25	0.002	0.03
SVR (dynes.sec.cm^{-5})	1708 ± 116	1555 ± 101	0.02	1243 ± 84	1056 ± 59	0.001	0.03
ClCre (mL/min)	98.6 ± 8.1	95.6 ± 7.4	0.09	71.6 ± 2.4	64 ± 2.8	0.001	0.01
UNaV (μmol/min)	102 ± 15.9	97 ± 13.7	0.09	29.6 ± 7.3	20.6 ± 4.6	0.02	0.01

[1]*vs* baseline values; [2]*vs* basal and final results in two groups of patients. PVV: Portal vein velocity; PFV: Portal flow volume; MAP: Mean arterial pressure; CO: Cardiac output; SVR: Systemic vascular resistance; ClCre: Creatinine clearance; UNaV: Urinary sodium.

sustain diuresis, and urine was collected over the two periods of time for estimation of creatinine clearance (ClCre) and sodium excretion.

Tadalafil did not significantly change the PVV and PFV but significantly reduced the MAP and SVR and significantly increased the CO in both study groups (Table 1). More significant systemic haemodynamic changes together with a significant decrease in ClCre and natriuresis were noted in the patients with decompensated cirrhosis.

Our observations concur with the results of Clemmesen *et al*[1] and previous series of compensated[4] or mixed compensated and decompensated patients with cirrhosis[5], showing that PDE-5 inhibition by sildenafil has no effect on portal pressure and impairs systemic haemodynamics. Furthermore, the present results confirm those of Thiesson *et al*[6] in that PDE-5 inhibition may adversely affect renal function and natriuresis in patients with cirrhosis and ascites, possibly due to deterioration of the hyperdynamic state. Although a recent report introduced PDE-5 inhibition by vardenafil as a novel treatment of portal hypertension, the present and previous data[1,4,5] strongly question the portal hypotensive efficacy and safety of PDE-5 inhibitors in patients with cirrhosis.

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical Negligence and Litigation in Medical Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology Conference

January 28-30
Hong Kong, China
The 1st International Congress on Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of Gastroenterology & Endoscopy Meeting

March 09-12
Brussels, Belgium
30th International Symposium on Intensive Care and Emergency Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian National Association for Study of the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian Pacific Association for the Study of the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology and Hepatology Conference, EGHC 2010

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Landover, Maryland, United States
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April 14-18
Vienna, Austria
The International Liver Congress™ 2010

April 28-May 01
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3rd Central European Congress of surgery and the 5th Croatian Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual Meeting

May 06-08
Munich, Germany
The Power of Programming: International Conference on Developmental Origins of Health and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in the Research of Probiotics and Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver Transplantation Society ILTS Annual International Congress

June 20-23
Mannheim, Germany
16th World Congress for Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference on Antimicrobial Agents and Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on Controversies in Gastroenterology & Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International Symposium of Society of Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of Gastroenterology Annual Scienitfic Meeting

October 23-27
Barcelona, Spain
18th United European Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's 61st Annual Meeting

November 13-14
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Case-Based Approach to the Management of Inflammatory Bowel Disease

December 02-04
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The Medical Management of HIV/AIDS

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Chinese journal article (list all authors and include the PMID where applicable)

2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixudiarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

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

Organization as author

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]

Both personal authors and an organization as author

5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764 DOI:10.1097/01.ju.0000067940.76090.73]

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]

Volume with supplement

7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (**401**): 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

Books

Personal author(s)

10 **Sherlock S**, Dooley J. Diseases of the liver and billiary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

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

Author(s) and editor(s)

12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wieczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

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

Conference paper

14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

Electronic journal (list all authors)

15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http://www.cdc.gov/ncidod/eid/index.htm

Patent (list all authors)

16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

Statistical data

Write as mean ± SD or mean ± SE.

Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as χ^2 (in Greek), related coefficient as *r* (in italics), degree of freedom as υ (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

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