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EDITORIAL

Identification of Lynch syndrome: How should we proceed in the 21st century?

Antoni Castells, Francesc Balaguer, Sergi Castellví-Bel, Victòria Gonzalo, Teresa Ocaña

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Abstract

Lynch syndrome, also known as hereditary non-polyposis colorectal cancer (HNPCC), is the most common form of hereditary colorectal cancer. Although great advances in the understanding of its molecular basis have taken place in the last decade, optimal selection of individuals for HNPCC genetic testing remains controversial. This is especially relevant since colonoscopy has been proven effective for reducing colorectal cancer incidence and mortality in individuals at-risk for this disorder. In this manuscript, we summarize the most significant contributions to this important issue that have appeared in the last few years.

Key words: Hereditary non-polyposis colorectal cancer; Screening; Prevention; Microsatellite instability; Genetics

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INTRODUCTION

Lynch syndrome, also known as hereditary non-polyposis colorectal cancer (HNPCC), is the most common form of hereditary colorectal cancer (CRC), accounting for 2%-5% of all colorectal malignancies[1]. It is characterized by early onset of CRC and other related neoplasms including endometrial, ovarian, gastric and urinary tract cancer. This syndrome is inherited in a non-fully penetrant autosomal dominant pattern, and occurs as a result of germline mutations in mismatch repair genes, predominantly *MLH1* and *MSH2* (> 90% of cases), but also *MSH6* and *PMS2*. The abnormal function of these genes leads to the accumulation of errors during DNA replication, especially in repetitive sequences (microsatellites). As a result, tumors in patients with Lynch syndrome characteristically demonstrate microsatellite instability (MSI), as well as loss of expression of the affected protein[1].

Although great advances in the understanding of its molecular basis have taken place in the last decade, optimal selection of individuals for HNPCC genetic testing remains controversial[2]. This is especially relevant since colonoscopy has been proven effective for reducing CRC incidence and mortality in individuals at-risk for this disorder[3]. In 1991, the International Collaborative Group on HNPCC established clinical criteria, known as the Amsterdam criteria, which provided a pivotal definition of this syndrome and were critical in identifying its molecular basis[4]. In response to criticism that the Amsterdam criteria were too stringent, the extended Amsterdam Ⅱ criteria were developed to include extracolonic HNPCC-associated cancers[5].

The use of the Amsterdam criteria achieved the original purpose of classifying a family as having HNPCC, but their limited sensitivity hampered decisions about which patients should undergo genetic testing[2]. In 1996, an international workshop on HNPCC hosted by the National Cancer Institute outlined a set of recommendations, known as the Bethesda guidelines, for the identification of individuals with HNPCC who should be tested for MSI and/or genetic testing[6]. More recently, a second HNPCC workshop revised these criteria and proposed a new set of recommendations, the revised Bethesda guidelines[7].

As it was previously mentioned, tumor MSI is a phenotypic indicator of defective DNA mismatch repair[8]. The fact that more than 90% of HNPCC-related cancers exhibit MSI suggests that screening of tumors for MSI may be an efficient way of selecting individuals for HNPCC genetic testing[9-12]. On the other hand, most mutations in either *MSH2* or *MLH1* genes result in abnormal MSH2 or MLH1 protein expression[13,14]. As a consequence, immunostaining for these two proteins is associated with MSI[15,16], but this association is not without exceptions[17]. Indeed, a mutant protein product can be expressed and detected by immunostaining[18],

whereas germline mutations may occur in patients with MSI-negative tumors[19]. These conflicting results have precluded the establishment of a unique method for primary screening of mismatch repair deficiency.

Recently, the Epicolon study, a prospective, multicenter, nation-wide survey aimed at assessing the incidence and characteristics of hereditary and familial CRC in Spain[20], has demonstrated that the revised Bethesda guidelines constitute a very useful approach to select patients at risk for HNPCC[21]. Moreover, in patients fulfilling these criteria, both MSI testing and protein immunostaining were equivalent and highly cost-effective strategies to further select those patients who should be tested for *MSH2/MLH1* germline mutations. Considering this equivalence and the fact that immunostaining is more available than DNA analysis in a clinical setting, the use of immunohistochemistry may contribute to identify a larger proportion of patients with Lynch syndrome[21,22].

The combination of revised Bethesda guidelines with tumor molecular analysis, however, is not fully accepted since some gene mutation carriers do not fulfill these clinical criteria[23]. To overcome this limitation, a massive, universal tumor mismatch repair screening by MSI analysis and/or immunostaining in any given CRC patient has been proposed[23,24]. Nevertheless, this approach is much less efficient[21], a critical issue that could be somehow solved by improving tumor molecular analysis. In that sense, it has been recently demonstrated that the use of two microsatellite markers (combination of BAT25 or BAT26 with NR21 or NR24) performed as well as the entire pentaplex of mononucleotide repeats (BAT26, BAT25, NR21, NR22, and NR24 markers) and better than the recommended panel by the National Cancer Institute (BAT26, BAT25, D5S346, D2S123, and D17S250 markers) in identifying mismatch repair deficient tumors[25]. Similarly, the introduction of BRAF V600E mutation analysis as a step prior to germline gene testing in patients with mismatch repair deficiency improves the cost-effectiveness of this approach, especially in those with incomplete or unknown family history[26,27].

On the other hand, the revised Bethesda guidelines have also been criticized because of their broad and complex variables, their relatively low specificity, and their inability to establish the likelihood of carrying a mutation in a given patient[24,28]. In addition, the need of performing tumor molecular analyses in patients fulfilling these criteria by some means constitutes a restriction since tissue samples are not always available. In that sense, as in hereditary breast-ovarian cancer syndrome in the past, identification of Lynch syndrome is moving toward complex algorithms and multivariable models combining personal and family history[28-31].

The first approach to this goal was the Leiden model[29], a regression logistic model derived from CRC patients attended in a high-risk clinic and designed to identify *MLH1/MSH2* mutation carriers, which has represented the only predictive model for years. Variables included in this model were fulfillment of the Amsterdam criteria, mean age of CRC diagnoses, and presence of any endometrial cancer in the family. However, it still included rather complex variables, it was developed using a relatively small population in a high-risk setting, and it did not take into account tumor molecular.

More recently, a second model was developed in the United Kingdom in a large population-based cohort of early onset (< 55 years) CRC patients[30] and consists of two consecutive stages: stage 1, based exclusively on clinical variables (age, sex, tumor location, presence of synchronous or metachronous CRC, family history of colorectal and endometrial cancer, and age of the youngest relative with CRC) and available on the web[32]; and stage 2, based on tumor MSI or immunostaining data. The area under the ROC curve of this model, which predicts MLH1, MSH2 and MSH6 germline mutations, was 0.82 (95% CI, 0.72-0.91). However, its applicability to CRC patients older than 55 years or those with other Lynch syndrome-associated tumors has not been assessed yet[30].

The third approach is a Mendelian model for determining *MLH1*, *MSH2* and *MSH6* carrier probabilities based on published estimates of mutation frequencies and cancer penetrances in both mutation and non-mutation carriers, and including MSI data[31]. This Bayesian model uses the CancerGene software[33] and provides the likelihood of finding a mutation in both probands and relatives on the basis of clinical and molecular information (age at diagnoses of colorectal and endometrial cancer, age of healthy relatives, MSI analysis and genetic testing). The area under its ROC curve was 0.83 (95% CI, 0.78-0.88). The performance of this model on clinical practice and different population settings is still unknown[31].

Finally, the PREMM1,2 model (accessible at the Dana-Farber Cancer Institute web site[34]) has demonstrated an excellent ability to discriminate between risk groups (area under the ROC curve of 0.80; 95% CI, 0.76-0.84), categorized by the estimated risk for probability of a mutation[28]. This study provides a new model based on a logistic regression analysis from one of the largest cohorts published so far of patients at-risk for hereditary CRC with proved mutation in the *MSH2/MLH1* genes. The authors recommend using their model as an initial assessment for individuals at risk for this disorder, before molecular information is available to the clinician. Based on the risk estimate generated from the model and other factors (accessibility to genetic services, timelines of genetic information, insurance coverage, and availability of tumor block), the clinician may choose whether genetic evaluation should be pursued as well as the approach to testing (MSI analysis and/or immunostaining, versus direct germline testing)[28]. The model does not include tumor molecular data to further refine the estimated probability nor takes into account *MSH6* gene mutations, although updates of the model are planned.

In summary, at the beginning of the 21st century, there is no unique, universally accepted strategy for the identification of Lynch syndrome. However, the tremendous advanced experiences in recent years allow us to be optimistic. Indeed, besides the fact that ongoing investigations may eventually elucidate the most effective and efficient approach to select individuals for HNPCC gene testing, the attention paid by the whole medical

community to this disease in the last decade will definitely contribute to make Lynch syndrome recognition more widely accessible.

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REVIEW

Is there a changing trend in surgical management of gastroesophageal reflux disease in children?

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Abstract

Gastroesophageal reflux disease (GORD) is a pathological process in infants manifesting as poor weight gain, signs of esophagitis, persistent respiratory symptoms and changes in neurobehaviour. It is currently estimated that approximately one in every 350 children will experience severe symptomatic gastroesophageal reflux necessitating surgical treatment. Surgery for GORD is currently one of the common major operations performed in infants and children. Most of the studies found favour laparoscopic approach which has surpassed open antireflux surgery as the gold standard of surgical management for GORD. However, it must be interpreted with caution due to the limitation of the studies, especially the small number of subject included in these studies. This review reports the changing trends in the surgical treatment of GORD in children.

Key words: Children; Gastroesophageal reflux; Antireflux surgery; Laparoscopic fundoplication

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INTRODUCTION

Symptomatic gastroesophageal reflux (GOR) has been identified in children with increasing frequency over the last two decades[1]. The perceived increase in incidence since the late 1970s is due to an improved awareness of the condition, and to increasingly sophisticated diagnostic techniques for identifying the disorder[2]. The term GOR implies a functional or physiological process in a healthy infant with no underlying systemic abnormalities[3]. It is a self-limiting process in infants that usually resolves by 6 to 12 mo of age[4]. Gastroesophageal reflux disease (GORD) is a pathological process in infants manifesting as poor weight gain, signs of esophagitis, persistent respiratory symptoms and changes in neurobehaviour[3].

Conservative first line management involves thickened feeds, positioning of the infant and parental reassurance. Second line management is through medication and includes the use of antacids, H_2 antagonists, proton pump inhibitors (PPIs) and pro-kinetic agents. Surgical intervention should be considered after the patient has failed conservative and medical interventions. It is currently estimated that approximately one in every 350 children will experience severe symptomatic GOR necessitating surgical treatment[2].

Surgery for GORD is currently one of the common major operations performed in infants and children by paediatric surgeons in the United States[5]. Prior to 1991, the open Nissen fundoplication was the standard surgical procedure for severe GORD in adults[6]. The first laparoscopic Nissen fundoplication was performed by Dallemagne and colleagues in Belgium in April 1991[6]. It has been shown that the principles learned through open antireflux surgery can be applied to the laparoscopic approach, and this breakthrough has since been translated to pediatric surgery.

This study reviews the aetiology, risk factors, signs, clinical symptoms, diagnosis, and management of GORD in children. A Pubmed database search was performed. All abstracts were reviewed and all articles and prospective randomized clinical trials of GORD in children were further scrutinized. Further references were extracted by cross-referencing.

PATHOGENESIS AND RISK FACTORS

The pathogenesis of reflux is not completely understood. A combination of factors appears to contribute to the development of GORD in infants and children. It appears that a decrease of lower esophageal sphincter tone plays a role in contributing to reflux. Transient lower esophageal sphincter relaxation not associated with swallowing has been implicated as the major mechanism allowing the

Table 1 Clinical manifestations of GORD[11]

	GOR	GORD
Symptoms	'Happy sitter'	Regurgitation/persistent vomiting/feeding refusal/ hypersalivation
		Arching/irritability/persistent crying
	Regurgitation	Abdominal pain/heart burn/ hematemesis/chest pain
	Vomiting but thriving	Sleep disturbance
		Silent reflux- stridor, wheezing, cough
		Sandifer's syndrome - head turning episodes to lengthen the oesophagus and LES pressure; repetitive stretching and arching, which gives the appearance of seizure/dystonia
Complications	GORD	Esophagitis/failure to thrive
	Esophagitis	Reactive airway disease/ recurrent pneumonia
		Apnoea/bradycardia/acute life threatening events
		Barrett's esophagus/esophageal ulceration and perforation/ stricture formation
		Anaemia/seizure

Table 2 Disorders that have been associated with symptomatic GORD[2]

Neurological	Mental retardation from any cause
	Brain injury from any cause
	Cerebral palsy
	Down's syndrome
	Microcephaly
	Seizure disorders
	Mobius syndrome
	Cornelia-de lange syndrome
	Hydrocephalus
Gastrointestinal	Gastric outlet obstruction from any cause
	Esophageal atresia
	Pharyngeal swallowing uncoordination
	Congenital duodenal obstruction (Ladd's band, diaphragm)
	Congenital abdominal defects (omphalocele, gastroschisis)
	Short bowel syndrome
	Hirschsprung's disease
	Portal hypertension
	Ascites
Cardiac	Anomalies causing left heart failure
Respiratory	Congenital diaphragmatic hernia
	Tracheal or subglottic stenosis
	Cleft palate
	Pierre Robin syndrome
	Phrenic nerve palsy
	Bronchopulmonary dysplasia
Prematurity	
Multiple anomalies	

gastric contents to return into the esophagus[7].

Delayed gastric emptying has also been implicated as another mechanism in GORD in children[8]. It predisposes to gastric distension, increased acid secretion and esophagitis. Other factors associated with the mechanism of reflux include positional factors, neurological disease, stress manoeuvres and hiatus hernia[9].

While many factors contribute to reflux mechanism, the composition of the refluxate and the time spent with an acidic refluxate (pH < 4) are related to the development of GORD[10].

CLINICAL SYMPTOMS AND DIAGNOSIS

Clinical manifestations

Clinical manifestations of GORD cover a wide spectrum with truly physiological reflux at one end to complicated esophagitis at the other (Table 1)[11]. Complications such as respiratory symptoms and neurobehaviour may be present. In older children the most common symptoms are recurrent emesis, esophagitis, chronic respiratory infections or asthma caused by repeated aspiration, which seldom represent an immediately life-threatening condition[12]. However, in infants, GORD often occurs in association with other congenital anomalies, indicating that certain anatomical factors might influence the development of reflux[12]. There is recognition that severe GORD can cause life-threatening bradycardic and apnoeic spells and even sudden death in infants[13]. A number of disorders have been associated with symptomatic GOR[14]. A higher prevalence of GORD is present in children who have a history of esophageal atresia with repair[15]. Neurologically impaired children have an increased incidence of GORD and comprise the majority of pediatric patients who undergo antireflux surgery[16,17]. Hiatus hernia and respiratory diseases have also been associated with the occurrence of GORD in children[18,19]. Table 2 shows the disorders that have been associated with symptomatic GORD.

A complete history and clinical examination are still the mainstays in diagnosing GORD. Evaluation should pay particular attention to the occurrence and frequency of symptoms and associated complications. If the initial evaluation points toward GOR, a period of lifestyle modification and empirical pharmacotherapy may be used to confirm the diagnosis. At this stage, parental reassurance, education and anticipation are important[20]. If the history and clinical examination point towards symptomatic GOR or GORD, a variety of diagnostic studies are available to assess the extent of the reflux, severity of the complications and contributing factors.

Diagnosis

No single definitive investigation can diagnose GORD. Therefore the choice is based on the clinical context. A 24-h pH probe remains the gold standard in diagnosing GORD. This test will determine the extent of esophageal acid exposure by measuring the frequency and duration of acid reflux exposure[20]. Radiography and pulmonary scintiscan may be useful in identifying the severity of pulmonary infections due to aspiration. The barium contrast upper gastrointestinal study is also helpful in identifying the presence of hiatus hernia and stricture. It is useful to exclude anatomical abnormalities[20]. Gastric

emptying studies are used to assess gastric motility and identify patients who have increased gastric emptying in the absence of mechanical obstruction. Gastroscopy is helpful in detecting reflux esophagitis and biopsy is taken to assess the severity of esophagitis[20]. Esophagogastric manometry is an accurate method for quantifying the resistance of the lower esophageal sphincter to reflux of gastric juice. The esophageal motility study is used to evaluate peristaltic contractions in the esophageal body. The benefits and limitations of commonly used diagnostic tests are described in Table 3.

Table 3 Benefits and limitations of commonly used diagnostic tests

Study	Advantages	Disadvantages
Barium esophagram	Readily available Evaluates upper GI structure	Inadequate screen for GORD Results are operator dependent
24-h pH probe	Quantification of reflux Evaluates atypical symptoms Monitors medical treatment	Requires hospitalization Requires special equipment and trained personnel
Endoscopy with biopsy	Evaluates persistent GORD, PUD, *H Pylori* infection, allergic enteropathy and Barrett's oesophagus	Invasive and requires sedation/general anaesthesia

TREATMENT

The objectives of therapy include decreasing the symptoms, frequency and duration of reflux episodes, healing the injured mucosa and preventing complications[21]. The approach to the treatment of GORD is age-dependent[22]. The management of symptomatic disease often follows the line of conservative therapy which includes posture and feeding techniques, medication and antireflux surgery.

Conservative treatment

Frequent small feeds of thickened formula or food minimise gastric distension and reduce GOR. Elevation of the upper body at 60 degrees, maintained for 24 h a day, favours esophageal clearance and effectively reduces symptoms of reflux in two-thirds of infants while awake and during sleep[2]. Positional therapy is based on the gravitational phenomenon and when discontinued the reflux may reappear.

Medical treatment

If conservative measures do not improve symptoms, medical therapy is recommended. Pharmacological therapies are aimed at the various steps in the pathophysiology of GORD. These include the use of antacids, hydrogen ion-blocking drugs, PPIs and prokinetic agents. Antacids work by neutralising gastric acid. H_2-blockers and proton pump inhibitors work by decreasing the secretion of gastric acid. Prokinetic agents work by increasing esophageal peristalsis, increasing the lower esophageal sphincter pressure and enhancing gastric emptying.

Surgical treatment

Until the early 1990s, antireflux surgery was the main stay treatment for severe GORD, until the emergence of PPIs[23]. Surgical treatment of GORD has considerable appeal as it offers potential cure and avoids the need for long-term medication use. The primary indication for performing an antireflux operation is the control of intractable and symptomatic GOR which has been clearly demonstrated by 24-h pH probe and a barium study of the esophagus[2]. Operative treatment is usually undertaken after an unsuccessful trial of a few weeks of medical therapy; for patients with severe complications of reflux, such as aspiration, failure to thrive or esophagitis with stricture. Antireflux surgery may be performed shortly after diagnosis is established[2]. However, the majority of children appear to present for surgery after only a barium study; less than 25% undergo basic objective testing such as endoscopy and fewer have pH or gastric emptying studies[1].

The major objectives of operative repair are to increase the high pressure zone in the lower esophagus by accentuating the angle of His and increasing the length of the abdominal esophagus[2]. Surgical therapy is effective because it improves sphincter function, which is one of the main contributing factors in most cases of GORD[6]. The most widely used fundoplication procedure was originally described by Nissen and Rosette in 1959. Nissen fundoplication is still a commonly used technique, with intra-abdominal positioning of the distal esophagus, hiatus hernia repair, and a 360° fundal wrap[24]. The term 360° fundoplication refers to total fundoplication. The technique has been developed and we now have the option of a partial fundoplication wrapping technique which refers to any wrap less than 360°. For example, Thal fundoplication requires only a partial wrap (210°-270°) of the fundus around the anterior side of the oesophagus[24], Toupet fundoplication a 270° posterior partial fundoplication[25] and Watson fundoplication a 120° anterior partial fundoplication[26].

Pediatric surgeons have documented high rates of failure and morbidity for antireflux surgery[27]. The problems with antireflux surgery occur especially in children with neurological impairment, repaired esophageal atresia or chronic lung disease[28]. The combination of antro-duodenal dysmotility and a wrap at the proximal stomach often cause difficulty eructating or vomiting and raised intragastric pressure with discomfort ("gas bloat syndrome"), resulting in forceful vomiting or retching[23]. This can cause wrap disruption or slippage of the wrap into the chest, the main causes of operative failure. Martinez *et al*[16] reported that more than 30% of children with neurological impairment had major complications or died within 30 d of surgery. Within a mean follow up period of 3.5 years, 25% had documented operative failure and overall, 71% had recurrent symptoms of GOR.

In children, the level of experience of the surgeon and surgical centre and appropriate case selection are key factors for determining the surgical outcome. Hassall[28] suggested that children who are the best candidates for

fundoplication have no neurological impairment, have endoscopically-established GORD and have exhibited an improvement in symptoms with PPIs therapy.

Most fundoplication surgery in the pediatric population is done through an open abdominal approach. In recent years, many reports have been published on the advantages and effectiveness of the laparoscopic approach for the management of patients with GORD[27].

CONCLUSION

Globally, the surgical management of GORD in children has changed dramatically with the refinement and clinical acceptance of the laparoscopic approach for fundoplication. Retrospective studies have established the benefits of the laparoscopic approach including more rapid recovery, faster return to unrestricted activity and decreased hospital stay while maintaining low complication and recurrence rates[29,30]. A clear increase in the number of publications related to laparoscopic fundoplication was noted supporting the global emergence and place of this technique in the management of GORD in pediatric surgery. Therefore, it shows there has been a change in the way children with GORD are managed surgically.

Collins *et al*[31] reported studies involving 120 patients that showed laparoscopic fundoplication complication rates for children were similar to those reported for open fundoplication. Blucher *et al*[32] reported that hospital stays after laparoscopic fundoplication were considerably shorter and patients returned to school and regular activities sooner. Somme[30] showed, in studies of 55 infants less than one year old, that in the laparoscopic Nissen fundoplication group, the time to initiation of feeding was significantly shorter than in the open Nissen fundoplication group. Rothenberg's[33] single large prospective study of laparoscopic fundoplication in 220 infants and children further supported the benefits of laparoscopic fundoplication. It showed that although the learning curve for laparoscopic fundoplication may be steep, the procedure is safe and effective in the pediatric population. The clinical results were comparable to the traditional open fundoplication but with a significant decrease in morbidity and hospitalization. A more recent prospective comparative study by Mattioli *et al*[34] confirmed that a minimally invasive approach was safe and effective for the treatment of primary GORD in children. Several studies reported that laparoscopic fundoplication has good long term outcomes[28,35] irrespective of neurological impairment associated with GORD[36].

Four failure patterns after open fundoplication have been described: the slipped or misplaced fundoplication, the disrupted fundoplication, the herniated fundoplication and the fundoplication that is too tight or too long[37]. Since the introduction of laparoscopic fundoplication, two additional failure patterns have emerged: the twisted fundoplication and the two-compartment stomach[37]. Some reports have emphasized the high incidence of early post-laparoscopic complications and re-operation[38-40]. The long learning curve for all-laparoscopic technique has been identified as a confounding factor[38-40]. However, most of these studies are based on laparoscopic fundoplication in adults, and whether the results will translate to children remains to be seen.

The minimal trauma to the upper abdominal wall in the laparoscopic approach results in less impairment of respiration and minimizes the need for narcotics and sedatives postoperatively[41]. A prospective comparative study of the fundoplication approach on analgesia requirement by Dick *et al*[42] showed the benefit of laparoscopic approach over open approach in decreased duration of pain as indicated by the decreased duration of analgesia following surgery. Thanks to the reduction of trauma-related problems, the laparoscopic approach has improved cosmetic results[30].

Laparoscopic surgery has been perceived as having higher procedure costs but lower total costs, primarily because of reduced duration of hospital stay[43]. However, a more recent retrospective study on cost effectiveness by Blewett *et al*[44] reported that although laparoscopic surgery was associated with a shorter hospital stay, no effect on total hospital costs was seen. They concluded that laparoscopic procedures were comparable with open operations in terms of operative costs[44,45]. Therefore, from an economic point of view, the perception that laparoscopic procedures are more cost effective is still inconclusive and subject to further study.

In conclusion, laparoscopic antireflux surgery has surpassed open antireflux surgery as the gold standard in the surgical management of GORD in children. The next question to be addressed should be which operative technique can complement the laparoscopic approach to produce the best operative results. Longer term outcome studies also need to be done to confirm the status of laparoscopic antireflux surgery as the gold standard of surgical treatment for GORD in children.

COMMENTS

Background

Gastroesophageal reflux disease (GORD) is a pathological process in infants manifesting as poor weight gain, signs of esophagitis, persistent respiratory symptoms and changes in neurobehaviour. Surgery for GORD is currently one of the common major operations performed in infants and children by paediatric surgeons.

Research frontiers

This study reviews the aetiology, risk factors, signs, clinical symptoms, diagnosis, and management of GORD in children. A Pubmed database search of GORD in children was performed.

Related publications

Pubmed database search must be performed for finding related articles.

Innovations and breakthroughs

Most fundoplication surgery in the pediatric population is done through an open abdominal approach. In recent years, many reports have been published on the advantages and effectiveness of the laparoscopic approach for the management of patients with GORD. Retrospective studies have established the benefits of the laparoscopic approach including more rapid recovery, faster return to unrestricted activity and decreased hospital stay while maintaining low complication and recurrence rates. Laparoscopic antireflux surgery has surpassed open antireflux surgery as the gold standard of surgical management for GORD in children. The next question to be addressed should be which operative technique can complement the laparoscopic approach to produce the best operative results and more long term outcome studies need to be done to confirm the status of

laparoscopic antireflux surgery as the gold standard of surgical treatment for this disease.

Applications

The study results suggest that surgery for GORD is currently one of the common major operations performed in infants and children while the principles learned through open antireflux surgery can be applied to the laparoscopic approach, and this breakthrough has since been translated to pediatric surgery.

Terminology

GOR: implies a functional or physiological process in a healthy infant with no underlying systemic abnormalities. It is a self-limiting process in infants that usually resolves by six to 12 mo of age. Nissen fundoplication: is a commonly used technique, with intra-abdominal positioning of the distal esophagus, hiatus hernia repair, and a 360° fundal wrap. 360° fundoplication refers to total fundoplication. Thal fundoplication: requires only a partial wrap (210°-270°) of the fundus around the anterior side of the oesophagus. Toupet fundoplication: a 270° posterior partial fundoplication. Watson fundoplication: a 120° anterior partial fundoplication.

Peer review

This is a decent review on surgical options for pediatric reflux disease. It's well organized with a very good presentation and readability.

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REVIEW

Genetic alterations in pancreatic cancer

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Abstract

The diagnosis of pancreatic cancer is devastating for patients and their relatives as the incidence rate is approximately the same as mortality rate. Only a small percentage, which ranges from 0.4% to 4% of patients who have been given this diagnosis, will be alive at five years. At the time of diagnosis, 80% of pancreatic cancer patients have unresectable or metastatic disease. Moreover, the therapeutic alternatives offered by chemotherapy or radiotherapy are few, if not zero. For all these reasons, there is an imperative need of analyzing and understanding the primitive lesions that lead to invasive pancreatic adenocarcinoma. Molecular pathology of these lesions is the key of our understanding of the mechanisms underlying the development of this cancer and will probably help us in earlier diagnosis and better therapeutic results. This review focuses on medical research on pancreatic cancer models and the underlying genetic alterations.

Key words: Carcinogenesis; Telomerase; p21; p16; Oncogenes; Epidermal growth factor

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INTRODUCTION

The diagnosis of pancreatic cancer is devastating for patients and their relatives as the incidence rate is approximately the same as mortality rate. Only a small percentage, which ranges from 0.4% to 4% of patients who have been given this diagnosis, will be alive at five years[1,2]. At the time of diagnosis, 80% of pancreatic cancer patients have unresectable or metastatic disease[3]. Moreover, the therapeutic alternatives offered by chemotherapy or radiotherapy are few, if not zero. For all these reasons, there is an imperative need of analyzing and understanding the primitive lesions that lead to invasive pancreatic adenocarcinoma. Molecular pathology of these lesions is the key of our understanding of the mechanisms underlying the development of this cancer and will probably help us in earlier diagnosis and better therapeutic results. This review focuses on medical research on pancreatic cancer models and the underlying genetic alterations.

CARCINOGENESIS IN PANCREAS

Histologically the development of adenocarcinoma of the pancreas has its roots in cuboidal ductal epithelium alterations. These alterations are named PanIN (pancreatic intraepithelial neoplasia) and are classified into different progressive types (Figure 1). The PanIN-1A lesions present only minimal alterations from the normal epithelium, such as tall columnar cells with some crowding while the PanIN-1B lesions present increased crowding of columnar cells with papillary projections. The PanIN-2 lesions apart from previous alterations develop nuclear atypia. Finally, the PanIN-3 lesions present atypical ductal hyperplasia with severe atypia and are more likely to progress to invasive carcinoma[4].

With the example of proposed progression model for colorectal neoplasia in mind, scientists tried to propose a model of progression for pancreatic neoplasia using the multi-hit hypothesis. The concept is the following: the first hit seems to be the point when mutations in the K-ras oncogene and overexpression of the HER-2/neu gene product occur. If some of these altered cells survive, they are susceptible to the second hit which is the inactivation of the p16 tumor suppressor gene. The third hit is represented by the loss of the tumor suppressor genes p53, DPC4, and BRCA2[5-10]. This theory is supported by the experimental work of Rozenblum *et al*[11] who analyzed the DNA from 42 pancreatic adenocarcinomas for alterations in the K-ras, p53, p16, and DPC4. They found that all 42 (100%) carcinomas presented point mutations in the K-ras oncogene, 82% genetic alterations in p16, 76% in p53, and 53% in DPC4. Concomitant activation of K-ras gene with inactivation of all three suppressor genes was presented in 38% of the tumors studied. Moreover, all these mutations had their origin in somatic cells[11].

These genetic alterations are correlated with histological findings of metaplasia, hyperplasia, dysplasia, and neoplasia. In addition, they most likely represent the precursor lesions for pancreatic adenocarcinoma. We shall try to present the genetic alterations of pancreatic cancer in more detail with the aim of better understanding and thus, earlier intervention.

CELL-CYCLE REGULATORS

The cell division cycle in pancreatic carcinoma, as other tumors, is an extremely complicated process. It is regulated by three major protein players, which act at particular checkpoints and permit, or not, the progression of cell division: (1) The cyclin dependent kinases (CDKs); (2) The cyclins; (3) The cyclin-dependent kinase inhibitors (CKIs).

In general, CDKs form complexes with their regulatory subunits named cyclins in order to help the cell to enter the S-phase. CDKs phosphorylation and CKIs are inhibitory signals for the complex activation process and consequently, for cell division progression. When the cell is found at G_1 checkpoint, before starting DNA replication, it has two possibilities: the first two possibilities: the first to progress to cell division and the other to remain in a quiescence state. The activation of CDK4 by cyclinD with the formation of CDK4/cyclinD complex leads the cell beyond the restriction point. The next step is hyperphosphorylation of the retinoblastoma protein, Rb, catalyzed by CDK4/cyclinD or CDK2/cyclinE complex. The phosphorylation results in the dissociation of Rb from its complex with transcription factors such as E2F with immediate consequences on activation of target genes that are required for G_1/S transition[12,13].

The oncogene products (p21, p16, p27) act as CKIs by blocking the hyperphosphorylation of the Rb oncogene *via* inactivation of CDK4/cyclinD and CDK2/cyclinE complexes. The cell thus cannot traverse the G_1/S checkpoint. Moreover, the p53 tumor suppressor gene can activate CKIs. When DNA alterations or negative external signals are present, p53 gene product is increased and stimulates transcription of the p21 gene, as a CKIs[14-17].

TELOMERASE ACTIVITY

Enzymes like telomerase play pivotal roles in cell-cycle regulation and have important implications in cell immortality. Telomeres have the property of not being reattached once they have been cut off from their fellow chromosome. Chromosomes lose 50-100 nucleotides from their telomeric sequence with every division. In this way, chromosomal length is reduced and programmed cell death may ensue. The stabilization of telomeric sequences is attributed to telomerase activation.

All normal somatic cells, with the exception of proliferating cells of self-regenerating tissues, do not present telomerase activity compared to malignant tissues. Reactivation or upregulation of telomerase has been detected in many types of cancer such as breast, lung, and bladder, gastric and colorectal cancer. Hiyama *et al*[18] using the TRAP assay (telomeric repeat amplification protocol), a highly sensitive PCR-based telomerase assay, tried to detect if there was telomerase activity in pancreatic tissue cancer and if possible, to correlate these results with telomere length. The authors studied 43 pancreatic cancer tissues, 11 benign tumors tissues, 3 chronic pancreatitis tissues, and 6 metastatic lesions from patients with pancreatic cancer. Telomerase activity was detected in 41 out of 43 (95%) pancreatic cancer samples analyzed, in all metastatic lesions (100%), but in none of the benign lesions. Unfortunately, the range of telomere length was the same in the malign and the benign lesions. This study showed the future utility of this enzyme in pancreatic cancer diagnosis.

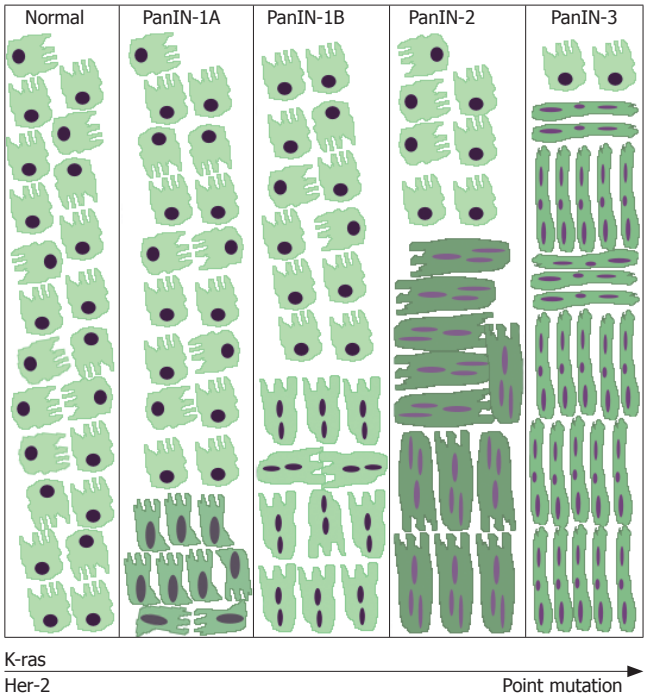

Figure 1 The most important genetic abnormalities associated with pancreatic cancer.

ONCOGENES

Oncogenes are genomic sequences that are activated under special conditions. Activation results in initiation of carcinogenesis either through encoding protein up-regulation or through encoding proteins with altered function.

The family of the ras protein is synthesized in the cytoplasm and arrives at the inner surface of cytoplasmic membrane with the role of transforming an inactive guanosine 5'-diphosphate (GDP)-bound form into an active guanosine 5'-triphosphate (GTP)-bound form. Ras oncogene, when it is found in its active form or under pressure of external signals, activates several downstream effectors such as Raf-1, Rac, Rho, or phosphatidynil-3 kinase (P13K) with important implications for cell differentiation and proliferation[19,20].

Mutations in K-ras oncogene are point mutations, a single amino acid change. They are located mainly on codon 12 and, rarely, on codon 13 and 61. K-ras gene mutations are found not only in 70%-95% of pancreatic carcinoma tissues but also in pancreatic juice, fine-needle aspirations of the pancreas, endoscopic retrograde

cholangiopancreatography brushings, duodenal fluid, and blood and stool of pancreatic cancer patients[21,22]. Wilentz *et al*[23] examined the duodenal fluid of patients with periampullary cancer for K-ras mutations. The results of this study showed a high specificity (100%) but a low sensitivity of K-ras mutations[23]. Of crucial clinical importance is the observation by Berthelemy *et al*[24] that pancreatic secretions may present cells with these genetic alterations even one year before the diagnosis of pancreatic cancer. Contrary to that, K-ras mutations may be present as benign condition in chronic pancreatitis without evidence of progression to pancreatic cancer even after 78 mo of follow-up[25].

K-ras mutations seem to be prerequisite for pancreatic carcinogenesis. The K-ras mutation rate increases with advancing cellular atypia. This mutation, in association with other genetic alterations, may help to identify precursor lesions in future.

p21

There is evidence that p21 acts in cyclinD1 synthesis, where overexpression is a marker of poor outcome in many human cancers including pancreatic cancer. Expression of p21 is regulated by other suppressor genes which are implicated in pancreas carcinogenesis. Biankin *et al*[26], using immunohistochemical methods, examined the expression of p21 in 451 PanIN lesions from 60 pancreatic cancer tissues and tried to correlate this expression with the histopathological grade of the lesions. Overexpression of p21 was present at 9% of the normal ducts, 16% of PanIN-1A, 32% of PanIN-1B, 56% of PanIN-2, 80% of PanIN-3 lesions and, finally, in 85% of invasive carcinoma. These observations suggest that p21 overexpression is an early event in this type of cancer and that there is a relationship between overexpression and progressive lesions. In addition, this study showed that overexpression of p21 is controlled by mutant K-ras and HER-2/neu genes rather than by p53 overexpression[26].

TUMOR SUPPRESSOR GENES

Tumor suppressor genes encode proteins with a protective role against malignant phenotypes. Their inactivation may lead to initiation and progression of carcinogenesis. When the balance between oncogenes and tumor suppressor genes is disrupted, the result is the initiation of carcinogenesis.

DPC4/SMAD4

SMAD4, known as DPC4 (deleted in pancreatic carcinoma locus4) and as a tumor suppressor gene, is located at 18q21.1. SMAD4 encodes a protein with major implications in signal transduction, through activating members of the TGF-β superfamily[27].

The SMAD family consists of nine members with a central role in the transduction of the TGF-β signaling from the cell surface to the nucleus. SMAD2 and SMAD3 are also named "receptor-regulated SMADs" because of their property of being phosphorylated by receptor kinases forming heteromeric complexes with SMAD4. These complexes enter the nucleus and bind to DNA - a prerequisite step for transcriptional activation of TGF-β responsive genes. Moreover, SMAD2/SMAD4 and SMAD3/SMAD4 complexes can downregulate c-myc proto-oncogene and upregulate p21 and p15 expression. p21 does not permit the formation of CDK4/cyclinD and CDK6/cyclinD complexes and their subsequent transcription[28-30].

TGF-β (transforming growth factor-β) is a member of the dimeric polypeptide growth factor family that regulates cell proliferation and differentiation, embryonic development, wound healing, and angiogenesis. In normal cells, TGF-β promotes differentiation and apoptosis and does not permit the cell to go beyond the G_1 phase. Contrary to that, tumor cells that encode for proteins participating in this signaling pathway are altered and the protective role of TGF-β against tumor phenotypes is abolished. The tumor cells begin to proliferate without restriction and with an increased production of TGF-β. A vicious cycle begins: an increased amount of TGF-β leads to increased invasiveness of tumor cells by destruction of extracellular matrix and promotion of molecular adhesive proceedings. The results of two studies show that 100% of pancreatic adenocarcinomas and 83% of colon cancers have a mutation which affects at least one gene involved in the TGF-β pathway[32].

Due to this process, SMAD4 expression is well-examined in human cancers. It is found that 50% of pancreatic cancers and 30% of colorectal and biliary cancers present mutant genes. It has been shown that in pancreatic adenocarcinomas, 30% present homozygous deletions while 20% present intragenic mutations in one allele coupled with loss of heterozygosity[33,34].

The protective character of SMAD4 expression against carcinogenesis was studied by Tascilar *et al*[35]. They examined the SMAD4 expression in patients with pancreatic carcinoma who had undergone surgical resection. Patients with positive SMAD4 expression survived 4.5 mo longer than patients with negative SMAD4 expression. For a patient with a very poor prognosis, this gain is significant.

Wilentz *et al*[36] studied the expression of SMAD4 gene in 188 PanIN lesions from 40 adenocarcinomas using immunohistochemical methods. All three "early" PanIN-1A, PanIN-1B, PanIN-2 lesions expressed DPC4 but it was only seen in one third of the PanIN-3 lesions. The conclusions from this study suggest that the loss of DPC4 gene expression occurs late in pancreatic carcinogenesis and, unfortunately, cannot be used for the differential diagnosis of the benign lesions from the malignant ones[36].

Finally, the last property of SMAD4 restoration is its influence on angiogenesis. It seems to decrease VEGF and to increase TSP-1 (trombospondin) expression, an angiogenesis inhibitor[37].

p16

On chromosome 9q21, there is a locus called $p16^{INK4A}$/$p14^{ARF}$, which encodes for two tumor suppressor genes. Genetic alterations of this locus through gene mutation, deletion, or promoter hypermethylation are found in 80%

to 95% of sporadic pancreatic cancers[38]. Additionally, expression of p16 has been studied in many types of cancer such as melanomas, gliomas, and leukemias.

p16 suppressor gene is also named cdkn2 (cyclin-dependent kinase-2) because it is a cyclin-dependent kinase 4 inhibitor. Loss of its expression results in an increasing activity of cyclin dependent kinase 4 with the direct consequence of Rb protein hyperphosphorylation and subsequent uncontrolled cell proliferation.

There are three different mechanisms for p16 inactivation: small mutations as seen in 40% of the cases, deletion of both alleles in the following 40%, and gene silencing through hypermethylation in the remaining 20% of the cases[38-40].

Genetic analyses have shown that p16 alterations are very common in pancreatic adenocarcinomas but these alterations are not necessarily seen in cultured cell lines. The question is whether p16 mutations and deletions are prerequisite for the establishment of such a cell line. Several studies on p16 expression in pancreatic adenocarcinomas have opposing results. Huang *et al*[41] report that only 26.7% of examined pancreatic cancers present deletions or mutations on this tumor suppressor gene. In a study by Bartch *et al*[42], this percentage increased to 34.4%. Later, Hu *et al*[43] studied 62 pancreatic cancer tissues using immunohistochemical methods and reported that 42% of the examined tissues did not express the gene at all. Moreover, loss of p16 expression could be correlated with less differentiated tumors, shorter overall survival, and the presence of metastatic disease[43].

It appears that there are at least two genetic alterations that must be present: K-ras mutations and p16 mutations. Human cancers hardly present simultaneous alterations in these two genes. This information may be useful in the future in differential diagnosis of adenocarcinomas of unknown origin.

p53

In human cancers, the most frequent mutant gene is the p53. It is located on the short arm of chromosome 17 and its mutations are either due to loss of heterozygosity in 95% of pancreatic adenocarcinomas or to sequence alterations in 75% of cases with small changes most likely in amino acid sequence such as G: C→A: T (transition)[44-46].

p53 is a nuclear phosphoprotein with the ability to bind to specific DNA elements and to activate gene transcription. It has a central position in cell cycle regulation through its role in inactivating a variety of genes and interrupting cell proliferation at G_1/S checkpoint.

Mutant status of p53 has been examined in pancreatic adenocarcinomas indirectly through p53 immunostaining and directly through molecular analyses using sequence analyses or polymerase chain reaction. The results of these studies show that mutant p53 correlates with shorter postoperative survival of patients and metastatic disease. However, all these studies have two main drawbacks. One is that the number of examined tissues was not adequate and the other is that the results obtained by the two methods - immunohistochemistry and molecular analyses - are not consistent. Using both techniques, Ruggeri *et al*[46] studied 126 cases of sporadic adenocarcinomas, 10 cases of familial adenocarcinomas, 77 cases of non-neoplastic but histologically abnormal pancreatic lesions, and 23 cases of metastatic lesions. The results of this published study show that p53 mutations were present at 56% of sporadic pancreatic adenocarcinomas, 33% of familial pancreatic adenocarcinomas. However, p53 alterations did not correlate with tumor grade, stage, or metastatic disease[46].

Generally speaking, genetic alterations of p53 tumor suppressor gene are an early event in pancreas carcinogenesis but not an initiating event.

Mdm-2

The mdm-2 gene encodes a protein with possible implications in appearance of malignant character of a cell. Its overexpression in absence of gene amplification has been studied in sarcomas and gliomas as well as in the presence of DNA-damaging agents. It was suggested that expression of mdm-2 gene is regulated by p53 tumor suppressor gene but Ruggeri *et al*[47] proved that there is no association between these two genes and moreover amplification and overexpression of mdm-2 is an infrequent event in the development of pancreatic adenocarcinomas.

MATRIX METALLOPROTEINASES (MMPS)

MMPs comprise a family of at least twenty members that act as zinc-dependent enzymes. The well-known collagenases, stromelysins, and gelatinases are members of this family. Their principal role is the degradation of extracellular matrix components. MMPs play a role only under special conditions such as tissue remodeling, embryonic development, and wound healing. Cytokines, growth factors and mechanical stress could be the triggers for MMPs production[48]. Abnormal expression of MMPs has been described in periodontitis, rheumatoid arthritis, tumor cell invasion, and metastasis[49].

At a structural level, MMPs consist of a signal peptide and a catalytic domain. At the functional level, proteolytic processes must be present in order to activate the enzymes.

MMPs have a pivotal position in carcinogenesis as well as in angiogenesis. Firstly, they degrade the basement membrane and the extracellular matrix components, offering tumor cells the best nutritive conditions for their establishment at the primary site and permitting the circulation of tumor cells and their extravasation at distant, metastatic sites[48]. In addition, MMPs are capable of removing sites of adhesion, exposing new binding sites, and releasing chemoattractants[50].

It seems MMPs play a role in as an "angiogenic switch", to facilitate the expression of proangiogenic factors such as VEGF and bFGF in order to overcome the negative signals of angiogenic inhibitors such as trombospondins, angiostatins, and INFs[48]. Due to these properties, the inhibition of MMPs represents the scientific rationale for the development of chemotherapeutic agents against pancreatic cancer.

Table 1 The most important pancreatic-prone syndromes

Syndrome	Mutation	Inheritance	Manifestations
Familial atypical mole-malignant melanoma syndrome (FAMMM)	CDKN2A	AD	Multiple atypical nevi Malignant melanoma Extracutaneous cancers
Hereditary pancreatitis	PRSS1 Kazal type 1 (SPINK1)	AD	Relapsing pancreatitis Young age of onset Associated pancreatic insufficiency, diabetes and pseudocysts
Hereditary non-polyposis colon cancer (Lynch Ⅱ)	HMSH2, HMLH1, HPMS2, p16 BRCA2	AD	Adenocarcinoma of the colon and extracolonic adenocarcinomas (endometrium, ovary)
Familial adenomatous polyposis	APS	AD	Innumerable colonic polyps with highly possible malignant transformation
Ataxia-telangiectasia	ATM	AR	Progressive cerebral ataxia, telangiectasias, sinopulmonary infections, oculomotor apraxia, immune deficiencies, 3-fold operative risk for PC
Li-Fraumeni	p53	AD	Predisposition to several neoplasms
Peutz-Jeghers	LKB1/STK11	AD	Multiple oromucosal and intestinal hamartomas

Epidermal growth factor receptors

The family of epidermal growth factor receptors (EGFR) consists of four types of receptors: HER-1, HER-2, HER-3, and HER-4, which have been studied in detail due to their implications in carcinogenesis. These four structurally similar receptor tyrosine kinase proteins are present on various domains: extracellular, transmembrane, and intracytoplasmic. Ligands of these proteins are EGF (betacellulin), TGFα (epiregulin), HB-EGF (amphiregulin) and three neuregulins (1, 2 and 3)[51,52].

Upon binding to ligands, these receptors undergo homo- or hetero-dimerization at the cell surface with subsequent phosphorylation of serine residues in the intracytoplasmic domain. This phosphorylation is translated into a downstream signal with resultant gene activation that leads to cell proliferation, decreased apoptosis, angiogenesis, and metastasis[53].

Overexpression of EGFR is a common characteristic in epithelial tumors such as breast, lung, and colorectal cancer. This expression has been associated with aggressive tumor growth and poor clinical outcome. Safran *et al*[54] studied 154 patients with metastatic pancreatic cancer for HER-2 overexpression by immunohistochemical means. They reported positive results for 21% of the cases studied.

All these important implications of HER-2 gene in carcinogenesis constitute the scientific rationale for new approaches in targeted therapy of pancreatic cancer.

FAMILIAL PANCREATIC CARCINOMA

It has been statistically observed that 5%-10% patients with pancreatic cancer have a close relative with the same cancer while this rate among controls is only about 0.6%[55]. Lynch *et al*[56] have shown that the risk for a person to develop pancreatic cancer is increased by 30% when there is a family history of any cancer among first-degree relatives. The European Registry of Hereditary Pancreatic Diseases (EUROPAC) identifies an individual at high risk for developing pancreatic cancer (PC) when he/she has two or more first-degree relatives with PC, or has three or more relatives of any degree with PC, or has any two relatives who have been given this diagnosis and the sum of their ages is under 110 years.

Studies of family histories might lead us to a better understanding of genetic alterations in human pancreatic adenocarcinoma. Patients with cancer in their families present an inherited germ-line genetic mutation in a cancer-causing gene. Among genes which have shown to be involved with familial pancreatic carcinogenesis are BRCA-2 and a large genetic area on locus 4q32-q34. Germ-line BRCA-2 mutations (mainly 6147delT) are present in approximately 17%-19% of familial pancreatic families in accordance with the results of recent studies[57-59].

Table 1 summarizes the most important pancreatic-prone syndromes. Much additional work needs to be done before the genetic basis of pancreatic cancer is completely understood in sporadic cases as well as in familial cases. This information will help us to identify the primary genetic factor and if possible to organize a counseling program for individuals at high risk.

NOVEL THERAPEUTIC AGENTS

Since 1997, the standard first-line chemotherapeutic agent for pancreatic adenocarcinoma has been gemcitabine (2' 2'-difluorodeoxycytidine), a difluorinated analogue of deoxycytidine, which is a member of the antimetabolites. The patient's benefit using this chemotherapeutic agent is an improvement in quality of life; however, the survival benefit is marginal. Antimetabolites cannot prolong the median survival time of patients with metastatic disease for more than six months.

The rationale for further understanding of genetic alterations of pancreatic cancer is based on the need for earlier diagnosis and development of more effective therapies. MMPs present very interesting links with extracellular matrix participating in its degradation and in the process of neovascularization. Marimastat, a MMPs inhibitor, was administrated in 414 patients with advanced pancreatic cancer as first-line chemotherapy in different doses (5, 10, and 25 mg orally twice a day) compared to the standard chemotherapy, gemcitabine, in a clinical study. Unfortunately, the study results are not encouraging. There

is no difference in the median survival interval between the two agents or with regard to marimastat dose escalation. The most important clinical information from this study is the longer overall survival time of patients with no metastatic disease *versus* patients with metastatic disease (200 *versus* 89 d). Thus, it is concluded that marimastat should be used in an adjuvant and not in a first-line setting[60,61].

Inhibition of EGFR by monoclonal antibodies (MoABs) that inhibit ligand binding or by tyrosine kinase inhibitors (TKIs) that bind to the adenosine triphosphate binding site of the growth factor receptor represents another therapeutic approach for pancreatic adenocarcinoma. Cetuximab (Erbitux) is the first human-mouse chimeric IgG1 antibody which has been approved for EGFR-positive expression in colorectal cancer. Currently, it is used in large clinical trials for EGFR-positive expression in pancreatic cancer. This novel agent presents more than one mechanism of action such as arrest of cell-cycle, activation of apoptosis, inhibition of angiogenesis, and inhibition of distant metastasis. It is interesting that EGFR inhibition contributes to angiogenic inhibition[62]. The next step is a clinical study comparing gemcitabine alone and in combination of an EGFR-inhibitor. Another novel agent which could be used as targeted therapy in pancreatic carcinoma is ABX-EGF, a fully humanized IgG2 monoclonal antibody that has a higher binding affinity to EGFR than the previous one. There is evidence that ABX-EGF, in combination with chemotherapy, could eradicate some tumors and prolong overall survival. Unfortunately, the number of patients with pancreatic cancer and EGFR overexpression is limited[63].

Several TKIs (gefitinib, erlotinib, PKI-166) have been tried as targeted therapies in pancreatic adenocarcinoma. Oral administration of PKI-166 in combination with intraperitoneal injections of gemcitabine in nude mice with implanted human pancreatic carcinoma cells into their pancreas showed significant regression of tumor growth and inhibition of metastasis. This inhibition was mediated directly by antitumor effect and indirectly by anti-angiogenic effects. Some clinical phase Ⅲ studies are in process, which compare a combination of TKIs and gemcitabine versus gemcitabine alone as a first-line treatment for pancreatic cancer[64,65].

Due to rapid cancer cell division, the tumor growth increases rapidly. The young cells need oxygen and nutrients supplied by newly made vessels, otherwise, they will die. This information represents the scientific rationale for the development of new drugs that will target several points along the angiogenic pathway. The targeted therapy advantage is that it applies only to new vessels, and will not present widespread toxicity. It acts by blocking vascular epithelial growth factor (VEGF) through monoclonal antibodies or through agents responsible for VEGF receptor tyrosine kinase inhibition. A multicentre phase Ⅱ trial, which studies the efficacy of bevacizumab plus gemcitabine for advanced pancreatic cancer, is currently taking place with satisfactory results: the median time to progression is 5.5 mo and the estimated 1-year survival rate is 54%[66].

Another therapeutic approach to pancreatic cancer is the antisense therapy. The mechanism of action is the inhibition of protein expression through trapping mRNA by specific RNA sequences. There are ongoing trials on murine xenografts on the human pancreatic cancer cell line, AsPC-1, where liposome-mediated gene transfer of antisense K-ras is used[67].

CONCLUSION

During the past decade, important steps have been made towards understanding the primary lesions that may lead to pancreatic adenocarcinoma. Molecular biology is the major key in this effort. Furthermore, a biologic and molecular staging of this disease may lead us to earlier diagnoses, efficient familial counseling, better management, and new therapeutic approaches.

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REVIEW

New therapeutic opportunities for Hepatitis C based on small RNA

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Abstract

Hepatitis C virus (HCV) infection is one of the major causes of chronic liver disease, including cirrhosis and liver cancer and is therefore, the most common indication for liver transplantation. Conventional antiviral drugs such as pegylated interferon-alpha, taken in combination with ribavirin, represent a milestone in the therapy of this disease. However, due to different viral and host factors, clinical success can be achieved only in approximately half of patients, making urgent the requirement of exploiting alternative approaches for HCV therapy. Fortunately, recent advances in the understanding of HCV viral replication and host cell interactions have opened new possibilities for therapeutic intervention. The most recent technologies, such as small interference RNA mediated gene-silencing, anti-sense oligonucleotides (ASO), or viral vector based gene delivery systems, have paved the way to develop novel therapeutic modalities for HCV. In this review, we outline the application of these technologies in the context of HCV therapy. In particular, we will focus on the newly defined role of cellular microRNA (miR-122) in viral replication and discuss its potential for HCV molecular therapy.

Key words: Hepatitis C virus therapy; miR-122; RNAi; Antisense oligonucleotides; Viral vectors

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INTRODUCTION

Hepatitis C virus (HCV), first identified in 1989, is a single-stranded positive-sense RNA flavivirus with 6 major genotypes and over 70 subtypes[1,2]. According to the estimation of the World Health Organization, approximately 170 million people, 3% of the world population, are HCV positive with 3 to 4 million de novo infections each year. Unfortunately, 55%-85% of those infected fail to clear the virus and progress to develop chronic infection. Over a period of 20 to 30 years cirrhosis develops in about 10% to 20% and hepatocellular carcinoma (HCC) develops in 1% to 7% of persons with chronic infection[3]. Currently, no safe and effective vaccine is available to prevent HCV infection. Conventional treatment, such as interferon taken alone or in combination with ribavirin, is only effective in part of the patients, but is often financially inaccessible for people in developing countries[4,5].

To explore the potential of new therapeutic strategies, it is critical to better understand the viral and host factors involved in virus cell entry, replication and virus-cell interaction. An apparent two-way dialogue exists in which the virus apparently takes advantage of the cells' own signal transduction systems to facilitate virus entry and support replication[6]. Indeed, remarkable progress has been achieved in understanding the properties of the HCV genome and viral proteins. Contributions have come through several different sources, including vaccination of chimpanzees, structural studies, binding studies with recombinant envelope proteins, and the use of clinical isolates, HCV-like particles (HCV-LPs), HCV pseudotyped particles (HCVpp), and cell culture-derived HCV particles (HCVcc) in infectivity assays[7,8]. Cellular pathways or molecules involved in viral entry, such as CD81, scavenger receptor class B type I (SR-BI), LDL receptor, L-SIGN, DC-SIGN and asialoglycoprotein receptor (ASGPR) could be putative therapeutic targets[9-12].

New technologies, particularly RNA interference (RNAi) induced by small interfering RNA (siRNA), are gaining favour as effective therapeutic entities for HCV infections. RNAi works at a posttranscriptional level by

degrading cognate mRNA. As HCV is a single-stranded RNA that functions as both a messenger RNA and a template for replication, it is a prime candidate for RNAi. Moreover, previous reports have shown that by blocking cellular determinants of viral entry and replication, such as CD81, HSP90, or p68, either by RNAi, antisense oligonucleotides or chemically engineered "antagomirs", leads to significant reduction of viral invasion[13-15]. In this review, we outline the novel small RNA based technologies in designing therapeutic approaches for HCV treatment, according to the mechanism of viral entry, replication and virus-cell interaction. In particular, we will discuss emerging evidence that a liver-specific, small non-coding microRNA (miRNA) is involved in replication of HCV through a novel mechanism and outline its therapeutic potential.

MOLECULAR CHARACTERISTICS OF HCV ENTRY AND REPLICATION

HCV, contains a single-stranded RNA genome of about 9400 nucleotides in length, composed of a 5' and 3' non-coding region (NCR) with a single open reading frame encoding a polyprotein precursor of approximately 3000 amino acids that is cleaved into three structural (core, E1, E2) and seven non-structural (p7, NS2-NS5B) proteins[16,17].

Since the discovery of HCV, numerous studies have demonstrated its mechanism of cell entry, but it is still unclear how the virus penetrates cell membranes. In order to elucidate the infection pathway, it is first required to identify and understand both the putative viral and cell factors involved in this process. The viral envelope glycoproteins E1 and E2, cleaved from the polyprotein by the endoplasmic reticulum (ER)-resident host enzymes signal peptidase and signal peptide peptidase, have been widely regarded as the critical determinants for virus cell entry. To date, several models have been designed to investigate E1/E2 function. These include HCV-LPs expressing E1-E2 heterodimers instead of glycosylated individual E1 and E2[18-21], HCVpp consisting of unmodified HCV envelope glycoproteins E1 and E2 assembled onto retroviral or lentiviral core particles[22-26], vesicular stomatitis virus (VSV)/HCV pseudotypes expressing HCV E1 or E2 chimeric proteins containing transmembrane and cytoplasmic domains of the VSV G glycoprotein, or HCVcc neutralization assays with E1 or E2 antibody[27-30]. These models have shown that both envelope glycoproteins E1 and E2 are essential for host cell entry. The lack of either E1 or E2 significantly decreases HCV infection activity whereas deletion of the whole envelope protein coding sequence abolishes the particle infectivity. Additionally, several cell surface molecules have been identified using these models and are now considered as critical components in mediating HCV attachment and entry.

Similar to viral entry, HCV replication requires both viral and cellular factors. Although our current knowledge of the HCV life cycle is still mainly at the hypothetical level, several minimum viral components and host cell factors have been proposed. The HCV 5' NCR, in particular the IRES sequence, plays an important function in ribosomal assembly and the NS3 to NS5B coding region are necessary for function of the replicase complex[31-35]. Found as interaction partners of NS5A and NS5B, human vesicle-associated membrane protein-associated proteins VAP-A and VAP-B were first identified from the host cell[36,37]. More recently, the geranylgeranylated protein FBL-2, the immunophilins cyclophilin B and FKBP8 have been identified as important host factors for HCV replication[38-40]. Furthermore, the host enzyme IMPDH, essential for the *de novo* synthesis of GTP nucleotides, may be involved in HCV replication as the IMPDH inhibitors ribavirin and mycophenolic acid suppresses replication[41,42]. Interestingly, the mammalian liver-specific miRNA (miR-122) has been recently defined to facilitate HCV replication, indicating that this small RNA may present a novel target for antiviral intervention[43].

miR-122 AND HCV REPLICATION

miRNAs are approximately 22 nucleotide noncoding RNAs that can downregulate various gene products by inducing either cleavage or a reduction in the translational efficiency of the target mRNA[44,45]. In the last 5 years, over 3000 miRNAs have been identified in vertebrates, flies, worms, plants and even viruses. Most miRNAs have been shown to participate in essential biological processes, such as cell proliferation, apoptosis, differentiation and metabolism[46]. The 22 nucleotide mature miR-122, derived from a noncoding polyadenylated RNA transcript of the hcr gene, is a liver-specific developmental regulator. It can be detected as early as 12.5 d post-gestation and reach a plateau immediately before birth, then slowly increase up to 70% of the total miRNA population in adult liver[47-49]. miR-122 is the first identified host miRNA linked to HCV viral replication. A further novelty to these findings is the fact that miR-122 upregulates, rather than downregulates, viral RNA by interaction with the 5' NCR of the viral RNA. Previous work had suggested that miRNA can only negatively regulate gene expression through targeting the 3' NCR of mRNA.

Interestingly, Jopling *et al*[43] have observed that though both Huh7 and HepG2 cells are derived from human hepatocytes, HCV RNA can only replicate in Huh7 cells. This may link to the fact that Huh7 is miR-122 positive, while HepG2 is miR-122 negative. To determine if miR-122 is required to regulate HCV replication, they transfected antisense oligonucleotides into Huh7 liver cells to suppress miR-122 function. The results showed that the amount of viral RNA was reduced by about 80% when miR-122 was silenced, but it is still unclear whether it is simply a direct or indirect interaction through cellular factors. Thus, to further address this issue, two putative binding sites, located in each of the viral NCR, were tested as possible targets for miR-122. It was found that only the binding sequence located in the 5' NCR was responsible for miR-122 targeting. This is notably very different from the common observation that miRNA target the 3' NCR, leading to suppression or degradation of target mRNA. Recently, a study in mice has shown synthesized antisense single-stranded 23-nucleotide RNA molecules

can effectively inhibit production of miR-122 *in vivo*[50]. Therefore, miR-122 seems a potential target for HCV treatment, although the mechanism for this new miRNA role is still very much unclear.

THERAPEUTIC STRATEGIES BASED ON GENE SILENCING TECHNOLOGY

As current antiviral regimens have proven largely unsatisfactory, particularly for patients with genotype 1 infection, it is important to explore novel therapeutic strategies. Small interfering RNAs and antisense oligonucleotides (ASO) have emerged as efficient nuclei acid-based gene silencing tools to target highly conserved or functionally important regions within the HCV genome or essential host cell factors for entry or replication (Figure 1).

RNAi, induces gene silencing at a post-transcription level by double-stranded small interference RNA (siRNA) and represents an exciting new technology that could have applications in the treatment of viral diseases. Particularly, HCV could be an attractive target for RNAi therapy, as it is a RNA virus. The HCV genome is a positive single-stranded RNA that functions both as the viral messenger RNA and a template for RNA replication *via* a negative-strand intermediate. Instead of a 5' cap, the IRES, located at the 5' NCR, plays an essential role to bind eukaryotic ribosomal subunits and initiates the assembly of the translationally active 80S complex. Consequently, this sequence is more conserved than any other part of the viral genome, at least among the six known HCV genotypes[51,52]. Thus, IRES seems an ideal target for RNAi mediated anti-HCV therapy and several groups have demonstrated efficient inhibition of HCV replication by designing siRNAs toward this region[53-55]. In addition, RNAi directed against the viral core, NS3, NS4B, NS5A and NS5B regions can suppress HCV infection. McCaffrey *et al*[56] was the first to demonstrate feasibility of siRNA targeting HCV NS5B *in vivo*. By co-expression of an NS5B-luciferase fusion gene with an anti-NS5B siRNA expression plasmid they found a significant reduction of luciferase expression in the mouse liver indicating selective degradation by the NS5B siRNA. Additionally, several other groups have observed suppression of HCV replicon by siRNA-mediated targeting either NS5B or NS3 region[57-59].

Besides these viral elements, numerous host cellular factors, such as CD81, SR-BI, HSP90, p68 or USP18, could be typical targets for potentiating RNAi antiviral therapy. CD81, expressed in most human cells, is able to bind to HCV E2 protein and is, therefore, considered an essential receptor for HCV entry. Further investigation, by either ectopic expression of CD81 in Huh7-Lunet cells (low expression of CD81) or modulation of CD81 cell surface density in Huh-7.5 cells (high expression of CD81) by RNAi, revealed that density of cell surface-exposed CD81 is a key determinant for HCV entry into host cells[60]. SR-BI, primarily expressed in the liver and steroidogenic tissues, was identified as another potential HCV receptor based on coprecipitation with recombinant E2. A 90% down-regulation of SR-BI expression in Huh7 cells by

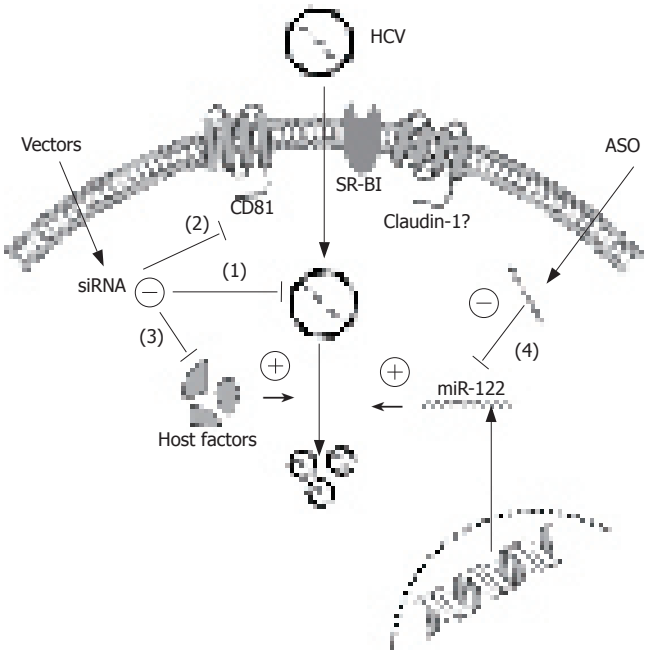

Figure 1 Novel anti-viral strategies based HCV life cycle. RNAi technology, inducing gene silencing at posttranscriptional level mediated by siRNA, can be applied to prevent HCV replication and infection by targeting either viral RNA (1) or host factors, such as surface receptor (2) or cellular molecules (3). miR-122 is a liver specific miRNA that is involved in HCV replication and therefore silencing of miR-122 by antisense oligonucleotides (ASO) could be considered as a potential therapeutic modality (4).

RNAi caused a 30%-90% inhibition of HCVpp infection, depending on the HCV genotype[61,62]. However, either CD81 or SR-BI alone is not capable of virus binding indicating that at least one additional host protein, possibly the recently identified co-receptor, Claudin-1[63], is required for cell entry of enveloped virions *via* the CD81/SR-BI pathways.

Although using siRNA to target either viral or host factors could be considered effective tools to significantly block HCV infection and replication, an advanced method by knockdown both viral and cellular factors may further improve the therapeutic efficacy. Work by our group has shown that both entry and replication can be simultaneously targeted using shRNAs directed against two regions of the HCV RNA and one region of the host cell receptor, CD81. The triple shRNA expression vector was effective in concurrently reducing HCV replication, CD81 expression, and E2 binding, comparable to conventional single shRNA anti-HCV vectors[64].

Antisense oligonucleotides represent an alternative gene-silencing tool that can be employed as HCV therapy. ASO-based inhibition of HCV has been demonstrated extensively in the past[65-71]. Currently, ASO is the most promising method to block the function of miRNA, such as miR-122. For instance, a 2'-O-methylated RNA oligonucleotide with exact complementarity to miR-122 was introduced to inactivate its function in Huh7 cells, in order to determine the relationship between miR-122 and HCV replication. Subsequently, Krutzfeldt *et al*[50] developed a pharmacological approach for silencing miRNA *in vivo*, by chemically modified,

cholesterol-conjugated single-stranded RNA analogues to complementarily target miR-122. By injection of these 'antagomirs' into the tail veins of mice, efficient and specific suppression of endogenous miR-122 was observed. Hence, designing ASO based molecular medicines would provide new agents for human major diseases, because upregulation of certain miRNAs linked to a set of diseases such as cancer, diabetes or HCV.

LIVER-TARGETED VIRAL DELIVERY SYSTEMS

Obviously, RNAi or ASO technologies could be regarded as potentially effective novel modalities for anti-HCV treatment. Nevertheless, the success depends on developing effective delivery systems, to target therapy to the liver. Regarding to treat a liver-hosted and long-term persistent hepatitis virus, an ideal vector would be able to transfer genetic material efficiently and specifically into the target cells/tissues, resulting in high level, properly regulated and prolonged expression, without toxic and immunogenic side effects. Since viruses have many advantages as transgenic vehicles, we will discuss two of the most promising delivery systems: lentiviral and adeno-associated viral (AAV) vectors.

Lentiviral vectors, are mainly based on human immunodeficiency virus type 1 (HIV-1) and have been shown to effectively transduce liver, muscle, and hematopoietic cells. These vectors integrate their payloads into the host genome ensuring transmission to progeny cells[72]. Although lentiviral-mediated short hairpin RNA (shRNA, precursor of siRNA) delivery has been widely developed for therapeutic application, there are few reports referring to HCV treatment[57,64]. There are currently some limitations for the use of lentiviral vectors: (1) production efficiency limits *in vivo* transfection; (2) possibility of insertional mutagenesis or generation of wild-type virus leading to safety considerations. To circumvent these drawbacks the following strategies may be required to achieve further improvement: firstly, newer generations, such as the gutted third generation, relatively high titers of VSV-G pseudotyped HIV-1 vectors, other types such as HIV-2 and simian immunodeficiency virus (SIV) vectors, or even immunodeficiency viruses derived from nonprimates, including felines and equines, are also being developed to overcome conventional problems[73-76].

Analogically, with the superiority of low pathogenicity and long-term gene expression, AAV could be another ideal viral vector for siRNA delivery, although no reference of AAV-mediated anti-HCV RNAi therapy has been reported so far. Particularly AAV serotype 8, a new member of the AAV family isolated from rhesus monkeys, is an attractive candidate for hepatic-directed shRNA transfer because of 10- to 100-fold increased transduction efficiency in mouse liver models, compared with the previous AAV2 based vectors[77]. Since derived from nonhuman primate, AAV8 is less prone to recognition by prevailing antibodies that generate side immunological effects in human[78]. Moreover, the safety and transgenic delivery efficacy could be further improved by conjugating other strategies, such as utilizing liver-specific promoters, hybridization of AAV8 with other serotypes, or modification of viral capsids.

Furthermore, since miRNA context based siRNA cassette (second-generation shRNA) can be driven by a regulated pol Ⅱ promoter instead of conventional pol Ⅲ promoters[79], liver-targeted expression of shRNA could be achieved by employing a liver-specific pol Ⅱ promoter in viral delivery system.

CONCLUSION

The treatment of HCV remains a challenge that requires further elucidating the process of viral life cycle and developing novel therapeutic approaches. In fact, recent progress has provided the possibilities of identifying novel antiviral targets and designing new therapeutic strategies. According to the previous description, miR-122 is one of the most emergent targets for HCV therapy that is commonly abundant in human livers and thus promotes viral replication. Therefore, downregulation of miR-122 by antisense based 'antagomirs' or oligonucleotides significantly suppressed viral replication. However, before such a method can be applied in the clinic, the role of miR-122 in maintaining normal hepatic function must be further investigated. Krutzfeldt *et al*[50] have demonstrated that silencing of miR-122 by 'antagomirs' do not show any apparent toxicity to mice, but the more recent study has shown that miR-122 is downregulated in the rodent and human hepatocellular carcinomas (HCC). Using the animal model of diet-induced hepatocarcinogenesis, Kutay *et al*[80] have observed that the reduced expression of miR-122 probably occurs between 36 and 54 wk when neoplastic transformation occurs. These findings suggest that the downregulation of miR-122 might be associated with hepatocarcinogenesis and, therefore, further investigation into the function of miR-122 is required before therapeutic application can be commenced. In conclusion, the recent progress of understanding the viral life cycle and identification of novel targets, in combination with the newly developed ASO and RNAi technology, may pave the way for new anti-HCV therapy.

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Importance of MutL homologue MLH1 and MutS homologue MSH2 expression in Turkish patients with sporadic colorectal cancer

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Abstract

AIM: To assess the incidence of MLH1 (the human MutL homologue) and MSH2 (the human MutS homologue) protein expression in Turkish patients with sporadic colorectal cancers and to compare their survival and clinicopathological features.

METHODS: We validated the tissue microarray technology in 77 colorectal carcinomas by analyzing the immunohistochemical expression of proteins involved in two main pathways of colorectal carcinogenesis: p53 protein for loss of heterozygosity tumors; MLH1 and MSH2 proteins for microsatellite instability (MSI).

RESULTS: Our analysis showed that 29 (39.2%) had loss of MLH1 expression, 5 (6.8%) had loss of MSH2 expression and 2 cases had loss of expression of both proteins. We found that 60% of MSH2-negative tumors were located in the right side of the colon; all MSH2-negative cases were women. In addition, the loss of MSH2 expression was correlated with low p53 expression. Neither MLH1 nor MSH2 expressions were associated with prognosis, although there seemed a tendency of longer survival (71.7 ± 8.65 mo *vs* 47.08 ± 5.26 mo) for the patients with MLH1-negative *versus* MLH1-positive carcinomas. There were not significant differences in overall and recurrence-free survival among MLH1/MSH2-positive and -negative cases.

CONCLUSION: Our data supports that Turkish patients with MLH1- and MSH2-defective tumors have some distinct features from each other. Although prognostic importance remains controversial, immunohistochemical analysis of mismatch repair genes may be used as a routine histopathological examination of sporadic colorectal carcinomas.

Key words: Colorectal carcinoma; MLH1; MSH2; Immunohistochemistry; Prognosis

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INTRODUCTION

In humans, mismatch repair (MMR) system is mediated by at least six genes, including human MutL homologue (hMLH1), human MutS homologue (hMSH2), hMSH3, hMSH6, hPMS1, and hPMS2[1]. MMR deficiency leads to the accumulation of base-base mismatches and short insertion/deletion mispairs, generated as a consequence of DNA replication errors and homologous recombinations. Most cell deficient in *hMLH1* and *hMSH2* genes often display a high level of genomic instability, characterized by changes in repeated numbers of simple repetitive sequences, microsatellite instability (MSI)[2,3]. The detection of MSI status is based on molecular analysis. According to this, three tumor phenotypes have been defined: Microsatellite stable (MSS), low-frequency MSI (MSI-L) and high-frequency MSI (MSI-H). A germline mutation in one of MMR genes, accompanied by somatic inactivation of the other allele, may result in high level of microsatellite instability (MSI-H)[4-6].

In patients with hereditery nonpolyposis colon carcinoma (HNPCC), MSI was detected in 80% of the tumor samples[7]. It has been reported that MMR genes

are involved in the 10%-15% of sporadic colorectal carcinomas[3,8]. In sporadic MSI-H colorectal carcinomas, the mechanism of development of mutator phenotype is inactivation of MLH1 by promoter hypermethylation. Sporadic MSI-H tumors tend to be poorly differentiated and/or mucinous subtype[9-11]. They are usually diploid; p53 mutations, loss of heterozygosity at 18q, APC mutations and K-ras mutations are found less frequently than in MSS tumors[12,13]. In literature, there are controversial results about the prognostic importance of MSI status in sporadic colorectal carcinoma.

Establishing the presence of MSI requires polymerase chain reaction-based technology, examining DNA sequences of intact and tumor tissue. This is an expensive and time-consuming procedure that is not readily available in all pathology laboratories. Immunohistochemically identifying MSI tumors is a less costly alternative procedure. There are some studies which have shown a high correlation of MLH1 and MSH2 immunohistochemical patterns of expression with the DNA analyses. MSI-H correlates with loss of immunohistochemical staining of either MLH1 or MSH-2 in the tumor nuclei[14-17]. The sensitivity of the immunohistochemical technique for detecting MSI-H tumors is about 80% to 100%[15-17].

To our knowledge, this is the first published study in Turkey concerning investigation of MSI status in sporadic colorectal carcinomas. Therefore, it is important to find out prognostic importance of MSI status in patients with colorectal carcinoma in Turkish population to perform their appropriate treatment and follow-up. In this study, we determined (1) the frequency of MLH1- and MSH2-deficient colorectal carcinomas in Turkish patients, (2) the relationship between MLH1/MSH2 expression and clinicopathological features, and (3) the predictive and prognostic relevance of loss of MLH1 and/or MSH2 expression in recurrence-free and overall survival.

MATERIALS AND METHODS

Tissue specimens

A total of 77 colorectal carcinoma specimens were obtained from the archives of the Department of Pathology of Cerrahpasa Medical College, Istanbul University. Patients with familial adenomatosis polyposis or inflammatory bowel disease were excluded. Tumors were staged according to the TNM staging system[18]. Tumor type and grade of differentiation were determined by criteria of the World Health Organization[19]. Peritumoral Crohn's-like reaction, pattern of growth and lymphocytic infiltration were also evaluated according to literature[20,21]. Tissue microarray (TMA) was applied to study normal colorectal mucosa and colorectal carcinomas. The patients had received neither chemotherapy nor radiation therapy before tumor resection. Their pathological specimens and slides were revised. After revision, two slides and corresponding two paraffin blocks were chosen, one for normal colorectal mucosa and one for colorectal carcinomas tissues for each case. Corresponding areas for normal mucosa and carcinomas were marked on chosen paraffin blocks. In TMA, 2-mm cores were taken from these selected areas, two for normal mucosa and two for cancer areas, and then they were embedded into microarray block. Each block containing 60 cores also contained other tissues (for example, sausage blocks included tissues from breast, thyroid, prostate, skin, gastric mucosa, lymph node, *etc*) for negative and positive controls.

Immunohistochemistry

Formalin-fixed paraffin-embedded TMA blocks were cut into 3-μm thick sections and mounted on polarized glass slides. After mounting, they were kept in an oven at 56℃ overnight. Sections were deparaffinized in xylene and rehydrated. They were incubated in a microwave containing 10 g/L EDTA solution 3 times for 5 min each, and then kept in room temperature for 20 min and placed in 10 mL/L hydrogen peroxide for 10 min. After being washed with distilled water and phosphate-buffered saline (PBS), sections were incubated overnight at 4℃ with primary antibodies of MSH2 (Ab-1, Clone 2MSH01, NeoMarkers; 1:25) and MLH1 (Clone 14, Zymed Lab; 1:50) with pre-antibody blocking solution (Immunovision-Sitogen). Primary antibody was replaced with PBS for a negative control. After being washed with PBS, the sections were incubated with primary antibody enhancer for 30 min and then with HRP polymer for 30 min. After being washed thrice with PBS for 10 min each, the sections were stained with a streptavidin-peroxidase detection system.

Immunostaining for p53 (p53 AB-5 clone DO-7, Neomarkers; 1:100) was almost the same as above-mentioned, except using citrate buffer solution instead of 10 g/L EDTA solution in microwave.

Immunohistochemical evaluation

Stained tissues in tissue microarray slides (TMS) were scored under the light microscope and the extent and intensity of staining with MLH1, MSH2 and p53 antibodies were evaluated independently by two pathologists (SE and EU) without knowledge of clinicopathological data. Tumors showing loss of nuclear MLH1 or MSH2 expressions were classified as MLH1- or MSH2-negative, respectively. Nuclear immunostaining of normal epithelial cells, lymphocytes and stromal cells served as internal positive controls. For p53, tumors showing a proportion of stained nuclei of > 10% were classified as p53-positive.

Statistical analysis

Spearman rank, Kendall correlation test, Cox regression and Kaplan-Meier test were used, when appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

There were positive staining of normal control mucosa in the lower third of the epithelium and nuclear staining of lymphocytes from a control germinal centre (Figure 1A and B). Three MLH1/MSH2-stained cases were excluded from the study because the quality of immunostaining was unsatisfactory. Of the remaining 74 colorectal

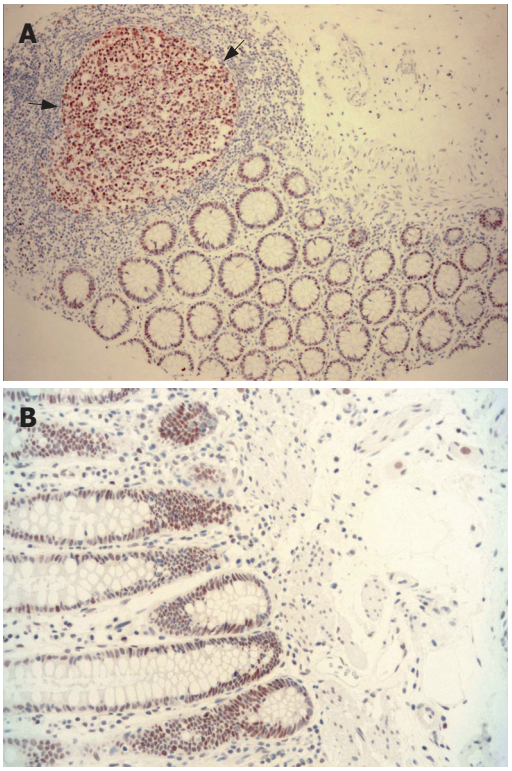

Figure 1 MLH1 and MSH2 expression. **A:** Nuclear MLH1 expression detected in germinal centre of lymphoid follicule (dark arrows) and in epithelia of normal colonic mucosa (**× 100**); **B:** Crypt epithelia showing normal positive nuclear staining with MSH2 (**× 200**).

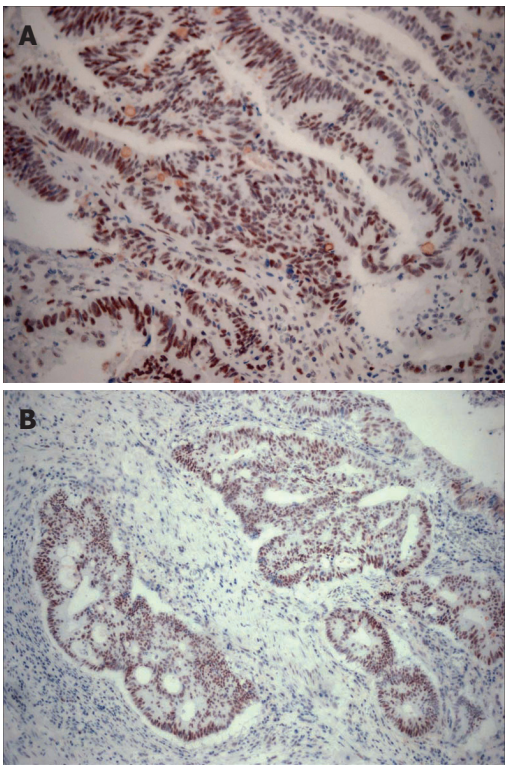

Figure 2 MLH1 and MSH2 expression. **A:** Extensive nuclear staining with MLH1 in adenocarcinoma of colon (**× 200**); **B:** Tumor cells showing strong positive nuclear staining with MSH2 (**× 100**).

adenocarcinomas, 42 (56.7%) cases demonstrated normal expression of both MLH1 and MSH2 gene products

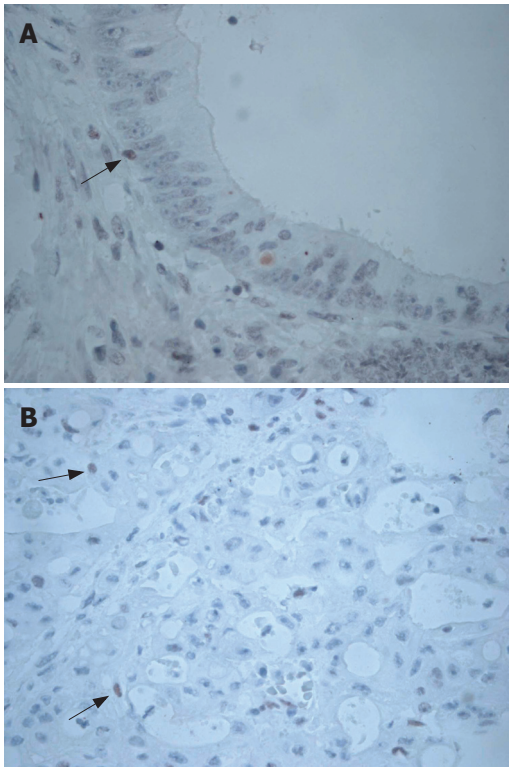

Figure 3 Loss of MLH1 and MSH2 expression in colorectal cancer. **A:** Loss of staining with MLH1 in cancer cells, although lymphocytes (arrow) show positive staining (**× 400**); **B:** Adenocarcinoma with complete loss of MSH2 expression. Nuclear staining of lymphocytes (arrows) in the stroma served as internal positive control (**× 200**).

(MLH1+/MSH2+) (Figure 2A and B). Loss of MLH1 or MSH2 expression was detected in 32 (43.2%) of all cases examined. Complete loss of MLH1 expression (Figure 3A) and normal immunoreactivity for MSH2 were observed in 27 (36.48%) cases of adenocarcinoma, while 5 (6.8%) cases displayed complete loss of MSH2 expression (Figure 3B) and normal immunoreactivity for MLH1. Two (2.07%) adenocarcinoma cases showed lack of both MLH1 and MSH2 expressions.

Immunohistochemical pattern of MLH1/MSH2 expressions was found to be related to some clinical and pathological variables (Tables 1 and 2). MSH2-negative carcinomas occurred only in women ($P = 0.049$). In addition, MSH2-negative carcinomas developed more frequently in patients $\geqslant$ 50 years than did MSH2-positive ($P \geqslant 0.05$). Majority of MSH2-negative tumors (60%) were located in the right colon ($P > 0.05$). However, no any preferential location for MLH1-negative cases was observed. There was no significant correlation between MLH1/MSH2 expression and tumor size and tumor type, while a significant relation was detected between tumor invasion and MSH2 expression (Table 2).

All MSH2-negative tumors had no or low p53 expression, while MSH2-positive cases had p53 expression $\geqslant$ 10% ($P < 0.05$). MLH1/MSH2 expressions were not significantly associated with other histopathological variables, such as perineural invasion, lymphatic/blood vessel invasion, peritumoral Crohn's-like lymphoid reaction. On the other hand, 80% of cases with loss of MSH2 expression had no perineural, lymphatic and blood vessel invasion (Table 2).

Our study included 32 (41.6%) females and 45 (58.4%)

Table 1 Clinicopathological parameters and MLH1 and MSH2 expressions *n* (%)

	MLH1 (+)	MLH1 (-)	*P*	MSH2 (+)	MSH2 (-)	*P*
Gender			0.943			0.049
Male	26 (57.8)	17 (58.6)		38 (55.1)	0 (0)	
Female	19 (42.2)	12 (4.4)		31 (44.9)	5 (100)	
Age (yr)			0.602			0.579
< 50	15 (33.3)	8 (27.6)		22 (31.9)	1 (20)	
⩾ 50	30 (66.7)	21 (72.4)		47 (68.1)	4 (80)	
Localization			0.630			0.244
Right colon	10 (22.2)	9 (31)		16 (23.2)	3 (60)	
Left colon	11 (24.4)	9 (31)		20 (29)	0 (0)	
Rectum	16 (35.6)	7 (24.1)		22 (31.9)	1 (20)	
Colon (NOS)	8 (17.8)	4 (13.8)		11 (15.9)	1 (20)	
Tumor size			0.757			0.969
< 5 cm	17 (37.8)	12 (41.4)		27 (39.1)	2 (40)	
> 5 cm	28 (62.2)	17 (58.6)		42 (60.9)	3 (60)	
Macroscopic type			0.479			0.144
Ulcerofungating	22 (48.8)	17 (57.1)		36 (52.1)	3 (60)	
Ulceroinfiltrative	22 (48.8)	12 (42.9)		33 (47.9)	1 (20)	
Polypoid	1 (2.4)	0 (0)		0 (0)	1 (20)	
Tumor type			0.206			0.219
Adenocarcinoma	35 (77.7)	27 (85.1)		58 (84)	4 (80)	
Mucinous AdenoCarcinoma	8 (17.9)	2 (14.9)		10 (14)	0 (0)	
Adeno+neuroendocrine	1 (2.2)	0 (0)		0 (0)	1 (20)	
Undifferentiated carcinoma	1 (2.2)	0 (0)		1 (2)	0 (0)	
Grade			0.805			0.743
1	4 (10.5)	4 (14.8)		7 (11.7)	1 (20)	
2	32 (84.2)	21 (77.8)		49 (81.7)	4 (80)	
3	2 (5.3)	2 (7.4)		4 (6.7)	0 (0)	
Tumor necrosis			0.451			0.660
-	5 (11.1)	5 (17.2)		9 (13)	1 (20)	
+	40 (88.9)	24 (82.8)		60 (87)	4 (80)	
Stromal desmoplasia			0.258			0.556
Mild	12 (26.7)	5 (17.2)		15 (21.7)	2 (40)	
Moderate	28 (62.2)	23 (79.3)		48 (69.6)	3 (60)	
Severe	5 (11.1)	1 (3.4)		6 (8.7)	0 (0)	
Stromal inflammatory reaction			0.512			0.352
Mild	9 (20)	9 (31)		18 (26.1)	0 (80)	
Moderate	31 (68.9)	18 (62.1)		45 (65.2)	4 (80)	
Intense	5 (11.1)	2 (6.9)		6 (8.7)	1 (20)	

MLH: MutL homologue; MSH: MutS homologue; NOS: Not otherwise specified.

males, with average age of 56 (range, 23-77) years. The mean follow-up in surviving patients was 34 (range, 3-97) mo. The patients with MSH2-negative/MLH-positive carcinomas more frequently died of disease (average 12.5 ± 1.06 mo post-operatively) than the patients with MLH1-positive/MSH2-positive carcinomas (47.08 ± 5.26 mo post-operatively) (Table 3). However, overall and disease-free survival analysis did not show significant differences among the four groups of patients

In COX regression analysis, only lymph node metastasis and stage were found as independent prognostic factors in all clinicopathological features. But loss of MLH1 and MSH2 expressions did not show prognostic significance (Table 4).

DISCUSSION

One of the two different pathogenetic pathways in colorectal carcinogenesis is mutator pathway which is characterized by explicit microsatellite instability (MSI). Mutator pathway covers DNA mismatch repair genes like MLH1 and MSH2. Identification of MSI status of large bowel adenocarcinomas is clinically important, since patients with MSI carcinomas demonstrated several distinct features compared to microsatellite stability (MSS) carcinomas[22-25]. It has also been suggested that MSI carcinomas might be particularly sensitive to 5-fluorouracil-based adjuvant chemotherapy[26,27]. Besides, patients with MSI tumors are considered to be at risk of developing metachronous colorectal cancers and need long-term colonoscopic surveillance[28].

As mentioned in previous studies, immunohistochemical analysis of MLH1 and MSH2 protein expression represents a rapid, easier and less costly alternative method for detection of colorectal tumors of the mutator phenotype[3,15,16,29-32]. This analysis could be performed in the histopathology laboratories as routine immunohistochemical staining, while genetic analysis of MSI status is time-consuming and expensive and requires specialized equipment[3,28]. This difference is important for developing countries like Turkey. Lindor *et al*[11] showed that absence of expression of MLH1 or MSH2 had a 100% specificity and 92.3% sensitivity for predicting a tumor with MSI-H phenotype. So, immunohistochemical

Table 2 MLH1/MSH2 expression and other clinicopathological parameters

	MLH1 (+)	MLH1 (-)	*P*	MSH2 (+)	MSH2 (-)	*P*
PT Crohn[1]			0.571			0.492
-	42 (93.3)	26 (89.7)		63 (91.3)	3 (60)	
+	3 (6.7)	3 (10.3)		6 (8.7)	2 (40)	
PT Lymph[2]			0.953			0.172
-	37 (82.2)	24 (82.8)		58 (84.1)	3 (60)	
+	8 (17.8)	5 (17.2)		11 (15.9)	2 (40)	
Tumor border			0.747			0.184
Expansive	7 (15.6)	3 (10.3)		8 (11.6)	2 (40)	
Infiltrating	17 (37.8)	13 (44.8)		29 (42)	1 (20)	
Both	21 (46.7)	13 (44.8)		32 (46.4)	2 (40)	
Invasion level			0.865			0.002
Submucosa	1 (2.2)	0 (0)		0 (0)	1 (20)	
Muscularis	7 (15.6)	4 (13.8)		10 (14.5)	1 (20)	
Subserosa	33 (73.3)	22 (75.9)		52 (75.4)	3 (60)	
Serosa	4 (8.9)	3 (10.3)		7 (10.1)	0 (0)	
PNI[3]			0.633			0.394
-	27 (60)	19 (65.5)		42 (60.9)	4 (80)	
+	18 (40)	10 (34.5)		27 (39.1)	1 (20)	
LVI[4]			0.522			
-	7 (15.6)	3 (10.3)		9 (13)	4 (80	0.660
+	38 (84.4)	26 (89.7)		60 (87)	1 (20)	
BVI[5]			0.114			0.927
-	38 (84.4)	20 (69)		54 (78.3)	4 (80)	
+	7 (15.6)	9 (31)		15 (21.7)	1 (20)	
LN metastasis			0.146			0.816
-	25 (58.1)	21 (75)		43 (65.2)	3 (60)	
+	18 (41.9)	7 (25)		23 (34.8)	2 (20)	
Survival			0.035			0.562
Disease-free	19 (45.2)	13 (50)		31 (48.4)	2 (40)	
Recurrence/metastasis	2 (4.8)	6 (23.1)		7 (10.9)	1 (20)	
Extend	21 (50)	7 (26.9)		26 (40.6)	2 (40)	
Stage (AJCC- 2002)[6]			0.119			0.520
1	5 (11.6)	3 (10.7)		6 (9.1)	2 (40)	
2	18 (41.8)	17 (60.7)		34 (51.5)	1 (20)	
3	16 (37.2)	5 (17.8)		20 (30.3)	1 (20)	
4	4 (9.4)	3 (10.7)		6 (9.1)	1 (20)	
p53 expression			0.227			< 0.05
≤ 10%	27 (60)	16 (57.1)		0 (0)	5 (100)	
> 10 %	18 (40)	12 (42.9)		74 (100)	0 (0)	

[1]Peritumoral Crohn-like inflammation; [2]Peritumoral lymphocytic inflammation; [3]Perineural invasion; [4]Lymph vessel invasion; [5]Blood vessel invasion; [6]American Joint Committee of Cancer.

Table 3 Relation of MLH1/MSH2 expression and survival time (mean ± SD, mo)

	n	Survival time	*P*
MLH1 (+)/MSH2 (+)	40	47.08 ± 5.26	0.065
MLH1 (+)/MSH2 (-)	3	12.50 ± 1.06	
MLH1 (-)/MSH2 (+)	24	71.71 ± 8.65	
MLH1 (-)/MSH2 (-)	2	51.00 ± 16.26	

Table 4 Results of COX regression analysis

	P
Loss of MLH1 expression	0.127
Loss of MSH2 expression	0.325
p53 over-expression	0.417
Stromal reaction	0.124
Stromal inflammatory reaction	0.407
Level of invasion	0.572
Perineural invasion	0.267
Lymphatic invasion	0.085
Blood vessel invasion	0.769
Peritumoral Crohn's-like inflammation	0.247
Peritumoral lymphocytic inflammation	0.952
Stage	0.000
Lymph node metastasis	0.000

analysis of MLH1/MSH2 proteins can be used as a prescreening method for mutation analysis of mismatch repair genes[33-35]. The inactivation of MLH1 and MSH2 genes is resulted in loss of expression of these proteins by immunohistochemistry.

We report here our first results of MMR (mismatch repair) analysis in Turkish sporadic colorectal carcinomas. To our knowledge, this TMA-based study about MSI status was completely performed for the first time in Turkey. We found loss of MLH1 expression in 29 (39.2%) and loss of MSH2 expression in 5 (6.8%) of cases. In addition, loss of either MLH1 or MSH2 expression was seen in 32 (43.2%) of cases, while loss of expression of both MLH1 and MSH2 was detected in 2 (3.1%) of cases. A previous study demonstrated that 351 (87.3%) cases

expressed either one or both of MLH1 and MSH2; MLH1 and MSH2 were not expressed in 35 (8.7%) and 19 (4.7%) cases, respectively; and 3 cases showed neither MLH1 nor MSH2 expression[33].

Lanza *et al*[28] found that 106 (80.3%) MSI-H carcinomas showed complete loss of MLH1 expression, 14 (10.6%) displayed complete loss of MSH2 expression, 12 (9.1%) MSI-H carcinomas demonstrated normal expression of both MLH1 and MSH2, but no MSI-H tumors showed lack of both MLH1 and MSH2 expression. In contrast, nuclear immunoreactivity for MLH1 and MSH2 proteins was observed in all MSS and MSI-L tumors analyzed[28]. The common finding of all these studies is that MLH1 extinction is more frequent than MSH2 extinction. This supports the hypothesis that involvement of the MLH1 gene is prevalent in the development of sporadic large bowel MSI cancers.

In our study, unlike previous studies, MLH1-negative cases had no gender, age or tumor localization predominance. On the other hand, loss of MSH2 seemed to be related to some parameters more than loss of MLH1; for example, all MSH2-negative cases were women, 60% of them were located in the right colon and no serosal involvement was detected in MSH2-negative cases. Other interesting but statistically insignificant findings were both MLH1-negative/MSH-nagative cases had peritumoral Crohn's-like lymphocytic infiltration and prominent intratumoral neutrophilic infiltration.

Although there was no statistical significance, 80% of MSH2-negaative cases did not show perineural invasion, lymphatic invasion and blood vessel invasion. Bernardo *et al*[36] found that only vascular invasion was significantly correlated with MSH2 expression. Similarly, Wright *et al*[14] found that in MLH1- and MSH2-negative carcinomas, extramural vascular, lymphatic and perineural invasion were all significantly less than the others.

In our study, 72.4% of MLH1-negative and 80% of MSH2-negative cases were $\geqslant$ 50 years. Tumor size was $\geqslant$ 5 cm in 58.6% of MLH1-negative cases and 60% of MSH2-negative cases. But there was no significant correlation between these parameters. Lanza *et al*[28] showed that MLH1- and MSH2-negative carcinomas were located in the proximal colon, more often of > 7 cm in diameter, poorly-differentiated, and had expanding pattern of growth and intense peritumoral Crohn's-like lymphoid reaction.

Several studies demonstrated that MSI tumors were correlated with clinicopathological features, such as right colon location, mucinous type, expansive borders, peritumoral Crohn's-like lymphoid reaction and peritumoral lymphoid response[10,29,31,37]. A Japanese study of a series of colorectal carcinomas did not find any correlation between MSI status and any clinicopathological features except for tumor location in the proximal colon[38].

The *p53* gene is mutated in 70% of colorectal carcinomas[39]. Over-expression of p53 has been used as an indicator of p53 mutational status in many studies[33]. In the present study, there was no significant difference between p53 expression and MLH1 expression, whereas a significant correlation between low p53 expression and loss of MSH2 expression was detected ($P < 0.05$). Park *et al*[33] demonstrated that there was a significant difference in p53 expression between the MLH1-positive group and MLH1-deficient group, indicating a correlation of loss of MLH1 or MSH2 expression with low p53 expression. We found that all MSH2-negative tumors showed low p53 immunostaining. This finding supports that different carcinogenic pathways, different molecular changes and different genes can be affected. Thus, an inverse correlation between genetic alterations of p53 and the mismatch repair system may simply reflect different carcinogenic pathways.

It has been reported that survival in patients with colorectal cancer with MMR gene defect is better than without one[24,27]. In our study, loss of MLH1/MSH2 expression had no significant correlation with the survival. It has been clearly elucidated that tumor stage and lymph node metastasis are the independent prognostic factors for colorectal carcinomas. Hameed *et al*[40] found similar results in their study. Chapusot *et al*[41] found that three independent factors were significantly associated with the loss of expression of MLH1 and MSH2: proximal location, the presence of Crohn's-like lymphoid reaction and poor differentiation.

Interestingly, in our study overall survival of the cases with loss of only MLH1 or both MLH1 and MSH2 expression was longer than those with both MLH1- and MSH2-positive or with only MSH2-negative expression. Although this finding is not statistically significant ($P = 0.065$), we think that this analysis should be repeated and combined with the result of genetic analysis which will be planned for near future. This finding showed some similarities with other studies. Lanza *et al*[28] found that cases with MLH1-negative carcinomas more often died of disease, but the survival of the cases was not statistically significant. However, Gafa *et al*[37] found that cases with both MLH1- and MSH2-negative carcinoma showed a better clinical outcome and survival.

In conclusion, our findings suggest that the assessment of MSI status using immunohistochemistry is important in genetic and biologic characterization of colorectal carcinomas. Turkish patients with colorectal cancers show some similarities with other populations in terms of histopathological features and MSI status. Although prognostic importance remains controversial, immunohistochemical analysis of MMR genes may be used as routine histopathological examination of colorectal cancer tissues. However, genetic analysis should be combined with these results.

COMMENTS

Background

Human colorectal cancer (CRC) is one of the leading cancers in most countries. Understanding pathogenetic pathways in colorectal carcinogenesis is important for diagnosis and treatment of these patients. DNA mismatch repair (MMR) genes like MLH1 and MSH2 identify of MSI status of large bowel adenocarcinomas as microsatellite stable (MSS) or MSI. MMR deficiency leads to the accumulation of base-base mismatches and short insertion/deletion mispairs, generated as a consequence of DNA replication errors and homologous recombinations.

Research frontiers

Simply, microsatellite instability (MSI) tumors are seen more frequently in

hereditary poliposis colon cancers. Last studies showed that MSI was found also in sporadic CRC. So, searching MSI status of CRC is clinically important, since patients with carcinomas demonstrated several distinct features compared to MSS carcinomas. In this point immunohistochemistry is useful method since it is easy, cheap and reliable method to detect these patients.

Innovations and breakthroughs

This analysis was performed for the first time in Turkey. Loss of MLH1 and/or MSH2 expression is important finding to predict their different morphology and behaviour in CRC cases.

Applications

Searching MSI status of CRC should be performed more commonly. Immunohistochemical analysis of MSI status of CRC cases may be used as screening method in developing countries.

Peer review

This is the first study of MMR deficiency and its prognostic importance in sporadic CRCs in Turkish population, which makes it valuable. The study assessed the incidence of MLH1 and MSH2 expression losses in Turkish sporadic CRCs and the data was compared with survival and clinicopathological features of the patients.

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H pylori seropositivity and cytokine gene polymorphisms

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Abstract

AIM: To investigate whether the pro- and anti-inflammatory cytokine gene polymorphisms, *IL1B*-511C/T, *IL1B*-31C/T, *IL6*-634C/G, *TNF*-1031T/C, *TNF*-857C/T, and *IL10*-1082A/G, interact with smoking and drinking habits to influence infection with *H pylori*.

METHODS: The subjects were 410 Japanese transit company employees. C-reactive protein and conventional cardiovascular risk factors were evaluated. Serum anti-*H pylori* antibodies were measured. The genotypes of *IL1B*-511C/T, *IL1B*-31C/T, *IL6*-634C/G, *TNF*-1031T/C, *TNF*-857C/T, and *IL10*-1082A/G polymorphisms were determined by allelic discrimination using fluorogenic probes and a 5´nuclease assay.

RESULTS: In gender- and age-adjusted logistic analyses, the subjects with *TNF*-857T/T had a significantly lower odds ratio (OR) for *H pylori* seropositivity (reference -857C/C; OR = 0.15, 95% CI: 0.03-0.59, P = 0.007). After stratification according to smoking and drinking status, among never-smokers, the subjects with *IL1B*-511C/T had a significantly lower OR (reference -511C/C; OR = 0.30, 95% CI: 0.10-0.90, P = 0.032). Among drinkers in the 1-5 times/wk category, the subjects with *IL1B*-511T/T had a significantly lower OR (reference C/C; OR = 0.38, 95% CI: 0.16-0.95, P = 0.039), and the subjects with *IL1B*-31C/T and T/T had a significantly higher OR (reference C/C; C/T: OR = 2.59, 95% CI, P = 0.042: 1.04-6.47; C/C: OR = 3.17, 95% CI: 1.23-8.14, P = 0.017). Among current smokers, the subjects with *IL6*-634C/G had a significantly higher OR (reference C/C; OR = 2.28, 95% CI: 1.13-4.58, P = 0.021). However, the interactions terms between the aforementioned genotypes and lifestyles were not statistically significant.

CONCLUSION: Contrary to previous findings, the results herein suggest that the *TNF*-857T/T genotype may be protective against chronic infection with *H pylori*. Drinking and smoking habits may influence the effect of cytokine gene polymorphisms. Further studies are required to clarify the effects of the pro- and anti-inflammatory cytokine polymorphisms and gene-environmental interactions on *H pylori* infection.

Key words: *H pylori* seropositivity; Cytokines; Polymorphisms

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INTRODUCTION

The prevalence of *H pylori* infection is generally higher in developing countries than in developed countries[1]; however, the Japanese population has a high prevalence of *H pylori* seropositivity[2]. Infection with *H pylori* represents a key factor in the etiology of various gastrointestinal diseases, including asymptomatic chronic active gastritis, peptic ulceration, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue lymphoma[3]. *H pylori* has also been implicated in a number of extra-gastrointestinal disorders, such as atherosclerosis[4], cerebral vascular disease[5], idiopathic thrombocytopenic purpura[6], and rosacea[7]. Because of the greater prevalence and various pathogenic activities of *H pylori* in Japanese, it is important to understand the basis for genetic susceptibility and identify the environmental factors that maintain chronic infection.

The mucosal production of pro-inflammatory cytokines, such as interleukin (IL)-1β, IL6, and tumor necrosis factor (*TNF*)-α, appears to be enhanced by infection with *H pylori*[8,11]. Interleukin-1β and TNF-α inhibit gastric acid secretion, providing a favorable condition for *H pylori* to survive in the stomach[12]. Although one study failed to show

inhibition of gastric acid secretion by IL-6[13], several studies have shown that gastric colonization with *H pylori* leads to elevated IL-6 levels in the gastric mucosa[14,15]. Thus, IL-6 may be one of the factors that maintain chronic infection with *H pylori*. Furthermore, IL-10, an anti-inflammatory cytokine, may reduce the inflammation associated with *H pylori* infection[16].

The host's ability to regulate cytokine production has been shown to be influenced by the presence of cytokine gene polymorphisms. Therefore, *H pylori*-susceptible cytokine gene backgrounds have recently been investigated. Regarding the *IL1B* gene, Japanese subjects with the -31T/T genotype have a significantly higher odds ratio (OR) for *H pylori* seropositivity as compared to subjects with the -31C/C or C/T genotypes[17]. A strong relationship involving *ILIB* -31T/T has been demonstrated in Japanese Brazilians[18]; however, such an association has not been shown to exist in Italians[19] or Jamaicans[20]. Regarding the *TNF* gene, Japanese subjects with the -1031C/C genotype have a significantly lower OR compared to those with the -1031T/T genotype[21]; however, an association was not found in Italians[19], Jamaicans[20], or Japanese Brazilians[22]. Thus, the effect *IL1B* and *TNF* polymorphisms on infection with *H pylori* remains controversial. Furthermore, among Jamaicans, the *IL6*-634C/G polymorphism (denoted -572G/C) was not associated with *H pylori* seropositivity[20], and the *IL10*-1082C/G polymorphism was not associated with *H pylori* infection among Jamaicans[20] or Italians[19]. Little is known regarding the effects of the *IL6* and *IL10* promoter polymorphisms on infection with *H pylori*.

Smoking cigarettes and drinking alcohol may have an effect on chronic infection with *H pylori*[23,26]. Therefore, interactions between the genome and lifestyle factors should be elucidated. An interaction between the *IL1B* genotype and one's cigarette smoking status on the eradication of *H pylori* has been reported[27]. It has also been reported that the effect of the *IL1B*-31T/T genotype on *H pylori* infection is modified by smoking cigarettes and drinking alcohol[18,28], but the interactions between other cytokine gene polymorphisms and lifestyle factors on *H pylori* infection have not been fully investigated.

The aim of this study was to investigate whether the pro- and anti-inflammatory cytokine gene polymorphisms, *IL1B*-511C/T, *IL1B*-31C/T, *IL6*-634C/G, *TNF*-1031T/C, *TNF*-857C/T, and *IL10*-1082A/G, interact with smoking cigarettes and drinking alcohol to influence infection with *H pylori* in Japanese.

MATERIALS AND METHODS

Subjects

The subjects were transit company employees (1255 men and 94 women, 35-60 years of age), who had their annual health checkup between April 2003 and March 2004. We used a self-administered questionnaire that included items regarding clinical history, smoking cigarettes, and consumption of alcohol. The questionnaire was distributed to the subjects prior to their annual health checkup, and was collected at the time of the checkup. Answers to the questionnaire and written informed consent to view pertinent health checkup data were obtained from 413 men and 5 women, for a response rate of 32.9% and 5.3%, respectively. Eight subjects were excluded due to inadequate blood samples. Ultimately, we analyzed a total of 410 employees (405 men and 5 women). No subject had a history of an internal malignancy or gastric surgery.

This study was conducted with written informed consent from all the subjects and approved by the institutional ethical board for epidemiological studies and human gene and genome studies of the Hokkaido University Graduate School of Medicine.

Data collection

Subjects were classified as current, never- or ex-smokers. Alcohol consumption habits were categorized as never/rarely, 1-5 times/wk, or 6-7 times/wk.

Blood samples were drawn from the antecubital vein of the subject after a 12 h fast while in a seated position and with minimal tourniquet use. The anti-*H pylori* antibody titer was measured using an enzyme immunoassay (E plate; Eiken Chemical, Tokyo, Japan)[29]; an assay value < 10 U/mL was considered negative and a value > 10 U/mL was considered positive.

Genomic DNA was extracted from each subject's peripheral blood lymphocytes using an EZ1 DNA blood kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. We genotyped the *IL1B*-511C/T (dbSNP: rs16944), *IL1B*-31C/T (dbSNP: rs1143627), *IL6*-634C/G (rs1800796), *TNF*-1031T/C (dbSNP: rs1799964), *TNF*-857C/T (dbSNP: rs1799724), and *IL10*-1082A/G (dbSNP: rs1800896) polymorphisms by allelic discrimination using fluorogenic probes and the 5´nuclease (TaqMan) assay, as previously described[30,31]. To detect a polymorphism in IL-6-634C/G, the following MGB probes were prepared: a C allele-specific probe, 5'-FAM-CAACAGCCCCTCACAG-MGB-3', and a G allele-specific probe, 5'-VIC-CAACAGCCGCTCACAG-MGB-3'. Each of the reporters was quenched with MGB, which was typically located at the 3' end. The primers for the PCR involving the promoter region, including the -634C/G polymorphism of IL-6, were as follows: forward, 5'-GGATGGCCAGGCAGTTCTA-3', and reverse, 5'-CCAGTCATCTGAGTTCTTCTGTGTT-3'. The reaction mixture contained approximately 40 ng of template DNA, 5.0 μL of TaqMan Universal PCR master mixture, and 0.3 μL of 40 × assay mixture, in a volume of 10 μL. The *IL1B*-511C/T, *IL1B*-31C/T, *TNF*-1031T/C, *TNF*-857C/T, and *IL10*-1082A/G polymorphisms were similarly genotyped using the TaqMan® SNP genotyping products: C_1839943_10, C_1839944_10, C_7514871_10, C_11918223_10, and C_1747360_10, respectively (Applied Biosystems, Foster City, CA, USA). Real-time PCR was performed on a 7500 Real-time PCR System (Applied Biosystems) using a protocol consisting of incubation at 50℃ for 2 min and 95℃ for 10 min, followed by 50 cycles for *IL6* or 40 cycles for the other genotypes, denaturation at 92℃ for 15 s, and annealing/extension at 60℃ for 1 min. The FAM and VIC fluorescence levels of the PCR products were measured at 60℃ for 1 min, resulting in the

Table 1 Characteristics of *H pylori*-seropositive and -seronegative subjects

	H pylori-seropositive (*n* = 237)		*H pylori*-seronegative (*n* = 173)		*P*-value
	n	(%)	*n*	(%)	
Gender					0.645
Male	234	98.7	171	98.8	
Female	3	1.3	2	1.2	
Age (yr)					< 0.001
< 45	30	12.7	63	36.4	
45-49	62	26.2	53	30.6	
50-54	85	35.9	43	24.9	
⩾ 55	60	25.3	14	8.1	
Smoking					0.281
Never	59	24.9	32	18.5	
Former	76	32.1	57	32.9	
Current	102	43.0	84	48.6	
Drinking					0.023
Never or rarely	35	14.8	43	24.9	
1-5 times/wk	131	55.3	91	52.6	
6-7 times/wk	71	30.0	39	22.5	

clear identification of all six genotypes of *IL1B*, *IL6*, *TNF*, or *IL10* on a two-dimensional graph.

Statistical analysis

The differences in the frequency of each characteristic between the *H pylori*-seropositive and -seronegative groups were examined by the chi-square test. Hardy-Weinberg equilibrium analyses were performed to compare the observed and expected genotype frequencies using the chi-square test. A logistic regression analysis was used to evaluate the associations between each cytokine genotype and *H pylori* seropositivity, with adjustment for age and gender, to obtain the OR and 95% confidence intervals (CI). After stratification according to cigarette smoking and alcohol consumption status, the adjusted OR for each genotype of *H pylori* seropositivity was calculated. The interaction term for the genotype/lifestyle factors was included in the logistic model with the main effect.

The haplotype was analyzed using Haploview, version 3.32[32], and linkage disequilibrium between loci was measured using Lewontin's D'[33]. The adjusted OR for the *IL1B* and *TNF* haplotypes was analyzed by logistic regression models. Statistical analyses were conducted with SPSS software for Windows, version 14.0 (SPSS; Chicago, IL, USA).

RESULTS

The characteristics of the groups according to *H pylori* seropositivity are shown in Table 1. Two hundred thirty-seven subjects (57.8%) were *H pylori*-seropositive. The *H pylori*-seropositive group was older and drank alcohol more frequently than the *H pylori*-seronegative group.

H pylori seropositivity, according to the genotypes of *IL1B*, *IL-6*, *TNF*, and IL-10, are shown in Table 2. The distribution of genotypes in each group was in the Hardy-Weinberg equilibrium. *TNF*-857C/T genotypes were significantly different between the *H pylori*-seropositive and -seronegative subjects.

Table 2 *H pylori* seropositivity according to cytokine genotypes

	H pylori-seropositive (*n* = 237)		*H pylori*-seronegative (*n* = 173)		*P*-value
	n	(%)	*n*	(%)	
IL1B-511C/T					0.243
CC	93	39.2	54	31.2	
CT	109	46.0	89	51.4	
TT	35	14.8	30	17.3	
IL1B-31C/T					0.434
CC	33	13.9	29	16.8	
CT	112	47.3	87	50.3	
TT	92	38.8	57	32.9	
IL6-634C/G					0.753
CC	138	58.2	104	60.1	
CG	88	37.1	59	34.1	
GG	11	4.6	10	5.8	
TNF-1031T/C		0.0			0.170
TT	152	64.1	115	66.5	
CT	80	33.8	51	29.5	
CC	5	2.1	7	4.0	
TNF-857C/T					0.018
CC	170	71.7	115	66.5	
CT	64	27.0	47	27.2	
TT	3	1.3	11	6.4	
IL10-1082A/G					0.852
AA	211	89.0	153	88.4	
AG/GG[1]	26	11.0	20	11.6	

[1]Only one subject had the *IL10*-1082 GG genotype.

Table 3 Age, gender-adjusted ORs for *H pylori* seropositivity according to cytokine genotypes

	n	*Hp* (+)%[1]	Adjusted OR (95% CI)	*P*-value
IL1B-511C/T				
CC	147	63.3	1.00	
CT	198	55.1	0.69 (0.43-1.10)	0.121
TT	65	53.8	0.70 (0.37-1.32)	0.270
IL1B-31C/T				
CC	62	53.2	1.00	
CT	199	56.3	1.11 (0.61-2.05)	0.726
TT	149	61.7	1.47 (0.78-2.78)	0.234
IL6-634C/G				
CC	242	57.0	1.00	
CG	147	59.9	1.06 (0.68-1.66)	0.785
GG	21	52.4	0.63 (0.24-1.62)	0.335
TNF-1031T/C				
TT	267	56.9	1.00	
CT	131	61.1	1.24 (0.78-1.95)	0.361
CC	12	41.7	0.48 (0.13-1.70)	0.253
TNF-857C/T				
CC	285	59.6	1.00	
CT	111	57.7	0.93 (0.58-1.49)	0.760
TT	14	21.4	0.15 (0.03-0.59)	0.007
IL10-1082A/G				
AA	364	58.0	1.00	
AG/GG[2]	46	56.5	1.08 (0.56-2.09)	0.811

[1]*H pylori* seropositivity (%); [2]Only one subject had the *IL10*-1082 GG genotype.

The age- and gender-adjusted ORs of the genotypes for *H pylori* seropositivity are shown in Table 3. The subjects with *TNF*-857T/T had a significantly lower OR for *H pylori* seropositivity (reference -857C/C; OR = 0.15, 95% CI 0.03-0.59).

After stratification according to cigarette smoking and

Table 4 Age, gender-adjusted ORs for *H pylori* seropositivity according to cytokine genotypes and lifestyle factors

			IL1B–511C/T			
	n	*Hp* (+)%[1]	C/C	C/T	*P*-value	T/T	*P*-value
All subjects	410	57.8	1.00	0.69 (0.43-1.10)	0.121	0.70 (0.37-1.32)	0.270
Smoking							
Never	91	64.8	1.00	0.30 (0.10-0.90)	0.032	0.40 (0.10-1.62)	0.200
Former	133	57.1	1.00	0.90 (0.39-2.10)	0.819	0.74 (0.26-2.15)	0.583
Current	186	54.8	1.00	0.83 (0.42-1.63)	0.582	0.80 (0.30-2.16)	0.658
Drinking							
Never or rarely	78	44.9	1.00	0.58 (0.20-1.66)	0.310	1.38 (0.31-6.23)	0.676
1-5 times/wk	222	59.0	1.00	0.81 (0.43-1.55)	0.531	0.38 (0.16-0.95)	0.039
6-7 times/wk	110	64.5	1.00	0.66 (0.25-1.69)	0.383	1.11 (0.33-3.77)	0.862
			IL1B–31C/T			
	n	*Hp* (+)%[1]	C/C	C/T	*P*-value	T/T	*P*-value
All subjects	410	57.8	1.00	1.11 (0.61-2.05)	0.726	1.47 (0.78-2.78)	0.234
Smoking							
Never	91	64.8	1.00	0.79 (0.22-2.29)	0.723	2.25 (0.56-9.08)	0.257
Former	133	57.1	1.00	1.34 (0.50-3.62)	0.560	1.55 (0.55-4.42)	0.409
Current	186	54.8	1.00	1.20 (0.44-3.24)	0.722	1.22 (0.44-3.40)	0.705
Drinking							
Never or rarely	78	44.9	1.00	0.44 (0.11-1.82)	0.258	0.68 (0.15-3.12)	0.624
1-5 times/wk	222	59.0	1.00	2.59 (1.04-6.47)	0.042	3.17 (1.23-8.14)	0.017
6-7 times/wk	110	64.5	1.00	0.72 (0.23-2.29)	0.579	0.81 (0.24-2.73)	0.735
			IL6–634C/G			
	n	*Hp* (+)%[1]	C/C	C/G	*P*-value	G/G	*P*-value
All subjects	410	57.8	1.00	1.06 (0.68-1.66)	0.785	0.63 (0.24-1.62)	0.335
Smoking							
Never	91	64.8	1.00	0.54 (0.22-1.38)	0.200	2.79 (0.28-27.73)	0.382
Former	133	57.1	1.00	0.63 (0.28-1.41)	0.259	0.00 (0.00)	0.999
Current	186	54.8	1.00	2.28 (1.13-4.58)	0.021	0.84 (0.22-3.15)	0.796
Drinking							
Never or rarely	78	44.9	1.00	1.07 (0.41-2.82)	0.886	0.00 (0.00)	1.000
1-5 times/wk	222	59.0	1.00	1.02 (0.55-1.91)	0.940	0.40 (0.11-0.41)	0.153
6-7 times/wk	110	64.5	1.00	1.61 (0.63-4.12)	0.325	1.60 (0.28-9.28)	0.599

[1]*H pylori* seropositivity (%).

alcohol consumption status, the age- and gender-adjusted ORs of *IL1B* and *IL-6* genotypes for *H pylori* seropositivity are shown in Table 4. Among never-smokers, subjects with *IL1B*-511C/T had a significantly lower OR for *H pylori* seropositivity (reference -511C/C; OR = 0.30, 95% CI: 0.10-0.90). Among the 1-5 times/wk drinkers, *IL1B* -511T/T had a significantly lower OR (reference C/C; OR = 0.38, 95% CI: 0.16-0.95), and *IL1B*-31C/T and -T/T had significantly higher ORs (reference C/C; C/T: OR = 2.59, 95% CI: 1.04-6.47; C/C: OR = 3.17, 95% CI: 1.23-8.14). Among current smokers, *IL6*-634C/G had a significantly higher OR (reference C/C; OR = 2.28, 95% CI: 1.13-4.58); however, the interaction terms between the aforementioned genotypes and lifestyles were not statistically significant. The remaining genotypes revealed no statistically significant ORs after stratification (data not shown).

Complete linkage disequilibrium existed between the two *IL1B* promoter lesions (D' = 1, r^2 = 0.048) and strong linkage disequilibrium existed between the two *TNF* promoter lesions (D' = 0.953). The estimated haplotype frequency of *TNF* (-1031T/C and -857C/T) was as follows: TC = 64.1%, CC = 18.9%, TT = 17.0%, and CT = 0%. The estimated haplotype frequency of *IL1B* (-511C/T and -31C/T) was as follows: CT = 58.9%, TC = 38.3%, TT = 1.7%, and CC = 1.1%.

The adjusted ORs of the combination of the two *IL1B* promoter genotypes and the *TNF* genotypes for *H pylori* seropositivity are shown in Table 5. The subjects with *TNF*-1031T/T and -857T/T had significantly lower ORs for *H pylori* seropositivity (reference, all the remaining combinations of genotypes; OR = 0.15, 95% CI: 0.04-0.60); however, subjects with *TNF*-1031T/T and -857T/T were similar to the subjects with -857T/T.

Table 5 Age, gender-adjusted ORs for *H pylori* seropositivity according to the combination of the two promoter genotypes of *TNF* and *IL1B*

	n	*Hp* (+)%[1]	Adjusted OR (95% CI)	*P*-value
TNF-1031/-857				
TT/CC[2]	169	58.6	1.07 (0.70-1.64)	0.743
TC/CC[2]	104	63.5	1.38 (0.85-2.25)	0.195
CC/CC[2]	12	41.7	0.44 (0.13-1.57)	0.207
TT/CT[2]	84	59.5	1.06 (0.63-1.78)	0.830
TC/CT[2]	27	51.9	0.89 (0.39-2.02)	0.781
TT/TT[2]	14	21.4	0.15 (0.04-0.60)	0.007
IL1B-511/-31[3]				
CC/TT[2]	141	61.7	1.29 (0.83-2.01)	0.259
CT/CT[2]	191	54.5	0.74 (0.48-1.13)	0.159
TT/CC[2]	59	52.5	0.77 (0.43-1.39)	0.384

[1]*H pylori* seropositivity (%). [2]Each reference group represented all the other combinations of genotypes. [3]ORs of the groups with < 7 subjects were not analyzed: CT/TT = 4, TT/TT = 4, CC/CT = 6, TT/CC = 2, and CT/CC = 3.

DISCUSSION

In the current study, the *TNF*-857T/T genotype had a significantly reduced OR for *H pylori* seropositivity. Because the subjects with both *TNF*-1031T/T and -857T/T genotypes were similar to the subject who was classified with the *TNF* -857T/T genotype only, the combination of genotypes also had a reduced OR.

It has been reported that Japanese subjects with the -1031C/C genotype have a significantly lower OR for *H pylori* infection when compared to those with the -1031T/T genotype, and that subjects with -857T/T and -1013T/T have significantly higher ORs for *H pylori* infection when compared to those with -1031C/C and -857C/C[21]. However, neither -1031T/C nor -857C/T polymorphisms were associated with *H pylori* infection in Italians[19] or Jamaicans[20], and neither the genotypes nor the combination of genotypes were associated with Japanese Brazilians[22]. The genotype distributions of -1031T/C and -857C/T among the aforementioned Japanese subjects were quite similar to the distributions in the subjects enrolled in our study. However, the subjects between the studies differed as follows: (1) our subjects were younger than in the previously published study, (2) nearly all of our subjects were male, while approximately one-half of the previous study subjects were female, and (3) our study subjects were healthy workers, unlike the subjects in the previous study that included outpatients participating in a *H pylori* eradication program, outpatients with chronic diseases, as well as health checkup examinees; these differences may have been the basis for the discrepant results. Further studies are needed to elucidate age and sex specific effects of *TNF*-857C/T polymorphism on *H pylori* infection.

In the current study, the subjects with the *TNF*-857T/T genotype had the highest level of *TNF*-α secretion, resulting in low gastric acid secretion, and they were resistant to chronic *H pylori* infection. Higuchi *et al*[34] reported that the level of TNF-α and the transcription promoter activity produced by concanavalin A-activated peripheral blood mononuclear cells in subjects with -1031C or -857T alleles were higher than in those subjects with the -1031T or -857C alleles. Skoog *et al*[35] reported that subjects with -863A tightly linked with -1031C had a significantly lower serum TNF-α level. Moreover, in another study it was shown that *ex vivo* lipopolysaccharide-stimulated whole-blood TNF production was higher in healthy *TNF*-857C homozygotes[34]. Thus, further studies will be needed to clarify the effect of the TNF genotype on susceptibility to infection with *H pylori* and production of TNF-α.

The *TNF* gene has more than three relatively frequent bi-allelic single-nucleotide polymorphisms in the promoter region: -863C/A, -308G/A, and -238G/A[35]. It has been reported that the *TNF*-308A allele is highly associated with *H pylori* infection in Italy[19], but the -308A allele is rare in Japan, and the other major allele, -238A, is also rare in Japan (1.7% and 2.0%, respectively)[35]. Moreover, the -863C/A allele is tightly linked with -1031C/T[36]. Therefore, we investigated the two promoter region polymorphisms of *TNF*. However, since the *TNF*-857 T/T genotype is not frequent in the population, simple and easy methods for genotyping are required for practical use.

The two *IL1B* promoter genotypes were not associated with *H pylori* infection in our entire group of subjects. In like manner, no association was found in Italians[19] or Jamaicans[20]. However, a previous Japanese study showed that subjects with the -31T/T genotype had a significantly higher OR (1.74, 95% CI: 1.15-5.63) for *H pylori* infection as compared to those subjects with the -31C/T or -31CC genotypes[17]. Furthermore, a study of Japanese Brazilian subjects found an association (OR of T/T = 1.45, 95% CI: 1.02-2.07)[28]. The subjects in the two previous studies involved an adequate number of female subjects and the Japanese study subjects were older than our study subjects. These differences may have accounted for our inability to obtain statistically significant results. In addition, the sample size of the previous Japanese study was nearly the same as that of our study (n = 437), but the sample size of the Japanese Brazilian study was almost twice as large as that of our study (n = 963). If a real OR of the T/T genotype was approximately 1.5, a smaller sample size as in our study may have failed to reach statistical significance.

Smoking cigarettes and drinking alcohol augment the T/T genotype effect on *H pylori* infection[18,28]. In our study, 1-5 times/week drinkers with T/C and T/T genotypes had significant ORs. In previous studies, the subjects were divided into drinkers or non-drinkers[18,28] and the pattern of drinking enhanced the T/T genotype effect on chronic *H pylori* infection. The drinkers in the previous study involved moderate and heavy consumption of alcohol, but the difference in T/T genotype augmentation between moderate and heavy consumption of alcohol was not analyzed. In our study, the results suggested that moderate drinking enhanced the T/T genotype effect on chronic *H pylori* infection. Therefore, further studies are needed to elucidate the interactions betweens the volume of alcohol consumption and genotypes on *H pylori* infection.

In our study, 1-5times/wk drinkers with the -511T/T genotype had a significantly lower OR since -31C and -511T were tightly linked (-511T/C and -31C/T combinations: 59.8% for T-C, 1.7% for T-T, 38.3% for C-T, and 1.1% for C-C). Non-smokers with -511C/T had a significantly lower OR. Chance may have influenced the significance of the result. Moreover, because this study was cross-sectional, changes in cigarette smoking and alcohol consumption habits were not involved in the analyses. Thus, the changes from previous habits may have affected the ORs.

Lipopolysaccharide (LPS)-stimulated IL-1β expression by whole blood leukocytes *in vitro* was lower in subjects with -31T and -511C[37,38]. Since IL-1β inhibits gastric acid secretion, thereby providing a favorable environment for *H pylori* to survive in the stomach[12], the results of the previous Japanese study and the moderate drinkers of our study were compatible to the *in vitro* IL-1β expression studies.

The *IL6*-634C/G (denoted -572G/C in reference 20) polymorphism was not associated with *H pylori* seropositivity among Jamaicans[20]. In our study, the polymorphism was also not associated with *H pylori* seropositivity among our entire group of subjects. However, current smokers with

the -634C/G genotype had a significantly higher OR for *H pylori* infection.

Persons with the C allele of the *IL6*-174G/C polymorphism are common among Caucasians, but extremely rare among East Asians[39,40]. However, persons with the G allele of the *IL6*-634C/G polymorphism are common among East Asians, and this genotype significantly relates to recurrent pregnancy loss[39], bone mineral density[41], and diabetic nephropathy[42]. Additionally, the -634G allele is associated with an elevated production and secretion of IL-6 by peripheral blood mononuclear cells *in vitro*[42].

In a study of young and healthy Caucasians, the IL-6 polymorphism was not associated with the *IL6*-174 genotypes in non-smokers, but in smokers where the -174C allele was associated with a higher number of leukocytes, lymphocytes, and monocytes[43]. In our study, the smokers with the -634G/G genotype had no significant results, perhaps because of a smaller sample size. In contrast, we found that the impact of the -634G allele on CRP elevation was greater in non-smokers than in current smokers (in press at Hypertens Res). Thus, the effect of *IL6* gene polymorphisms and the gene-environment interactions on *H pylori* infection should also be further elucidated.

In our study, the *IL10*-1082C/G polymorphism was not associated with *H pylori* infection. As previously mentioned, negative results were reported for Jamaicans[20] and Italians[19]. Other *IL10* promoter polymorphisms, such as -819C/T and -592C/A, have been reported[19] and a Japanese study showed that the combination of the *IL8*-251T/A and *IL10*-819C/T polymorphisms was significantly associated with *H pylori* infection, but the *IL10*-819C/T polymorphism alone did not have a statistically significant effect[44]. Furthermore, associations between *IL10*-1013A/A[45] and -819C/T[16] genotypes on non-cardia gastric cancer were reported. An experiment in mice showed that increased IL-10 levels may reduce the inflammation of *H pylori* infection[16]. Unfortunately, we did not evaluate other *IL10* promoter polymorphisms or the *IL8*-251T/A polymorphism; further studies are required to clarify how these polymorphisms effect *H pylori* infection.

Because this study examined IgG antibodies to *H pylori*, which can reflect a previous infection, IgG seropositivity to *H pylori* may not reflect active infection. However, the relative sensitivity, specificity, and rates of agreement between the results obtained using the enzyme immunoassay employed in the present study (i.e., the E plate) and those obtained by the culture/rapid urease test have been reported to be 100%, 80.0%, and 97.1%, respectively[29]. Strains isolated from Japanese gastric ulcer patients were used as antigens to prepare the E plate. Thus, this serological method to detect *H pylori* infection in Japanese is a suitable method for this type of genotype-associated study.

In summary, we observed the *TNF*-857T/T genotype significantly reduced the OR for *H pylori* seropositivity. Because the ORs for the subjects with both *TNF*-1031T/T and -857T/T genotypes were the same as the subject who was classified with the TN-857T/T genotype alone, the combination of genotypes also revealed a reduction in the OR. In the entire group of subjects analyzed, the promoter region polymorphisms of *IL1B*, *IL6*, and *IL-10* had no association with *H pylori* infection. After stratification according to cigarette smoking and alcohol consumption, never-smokers with the *IL1B*-511C/T genotype and 1-5 times/week drinkers with the *IL1B*-511T/T, *IL1B*-31C/T, and -31T/T genotypes, had a significant association with *H pylori* infection. Among current smokers, the *IL6*-634C/G genotype also had a significant association. However, interactions terms between the aforementioned genotypes and lifestyles were not statistically significant. Further studies are required to clarify the effects of the pro- and anti-inflammatory cytokine polymorphisms and the gene-environmental interactions on *H pylori* infection.

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BASIC RESEARCH

Therapeutic proteasome inhibition in experimental acute pancreatitis

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Abstract

AIM: To establish the therapeutic potential of proteasome inhibition, we examined the therapeutic effects of MG132 (Z-Leu-Leu-Leu-aldehyde) in an experimental model of acute pancreatitis.

METHODS: Pancreatitis was induced in rats by two hourly intraperitoneal (ip) injections of cholecystokinin octapeptide (CCK; 2 x 100 μg/kg) and the proteasome inhibitor MG132 (10 mg/kg ip) was administered 30 min after the second CCK injection. Animals were sacrificed 4 h after the first injection of CCK.

RESULTS: Administering the proteasome inhibitor MG132 (at a dose of 10 mg/kg, ip) 90 min after the onset of pancreatic inflammation induced the expression of cell-protective 72 kDa heat shock protein (HSP72) and decreased DNA-binding of nuclear factor-κB (NF-κB). Furthermore MG132 treatment resulted in milder inflammatory response and cellular damage, as revealed by improved laboratory and histological parameters of pancreatitis and associated oxidative stress.

CONCLUSION: Our findings suggest that proteasome inhibition might be beneficial not only for the prevention, but also for the therapy of acute pancreatitis.

Key words: Acute pancreatitis; Cholecystokinin octapeptide; Proteasome inhibition; Nuclear factor-κB; Heat shock proteins

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INTRODUCTION

Proteasome inhibition is an emerging strategy to attenuate the inflammatory response[1]. Inhibiting the proteasome blocks nuclear factor-κB (NF-κB) activation by detaining proteolysis of its inhibitory subunit, the IκB. Preventing NF-κB activation then decreases NF-κB dependent proinflammatory gene expression, resulting in reduced inflammatory response. However studies also reveal that NF-κB, one of the major initiators of pro-inflammatory pathways, has anti-inflammatory roles in the resolution of inflammation. Thus inhibiting NF-κB during the resolution of inflammation has been shown to protract the inflammatory response *in vivo*[2].

Acute pancreatitis is a severe inflammatory disease characterized by intrapancreatic activation of digestive enzyme zymogens that leads to acinar cell injury and subsequent inflammatory response[3-5]. The inflammatory response is first localized only to the pancreas, but due to the release of inflammatory mediators, later overspreads and becomes systematic affecting other organs including the lung and kidney. This exacerbation of pancreatitis results in multiple organ failure and systemic inflammatory response syndrome that is responsible for the mortality of acute pancreatitis. There have been many experimental attempts for the treatment of acute pancreatitis, however most failed to succeed in the clinics[6,7]. This might stem from the fact that many studies aim to examine only the prophylactic effects of compounds. One thing is clear however, the therapeutic potential of a compound in acute pancreatitis can only be established if it is given after onset of the disease[8,9]. In our previous study the peptide aldehyde proteasome inhibitor MG132 prevented the development of pancreatic inflammation when administered

before the induction of the disease[10]. In order to estimate the clinical potential of proteasome inhibition, we also had to examine the therapeutic effects of the compound administered after the onset of pancreatitis. Given the NF-κB inhibitory effects of MG132, it was also crucial to determine whether NF-κB inhibition with MG132 after the onset of pancreatitic inflammation might worsen or ameliorate pancreatitis.

The following paper will summarize the observed effects of therapeutic administration of MG132 in this experimental model of acute pancreatitis and suggest that proteasome inhibition might be beneficial for the therapy of the disease.

MATERIALS AND METHODS

Experimental protocol

For the *in vivo* studies male Wistar rats (provided by the Animal Center of the University of Szeged) weighing 250-300 g were used. The animals were kept at constant room temperature with a 12-h light-dark cycle, and were allowed free access to water and standard laboratory chow (Biofarm, Zagyvaszántó, Hungary). Animal experiments performed in this study were approved by the Animal Care Committee of the University and complied with the European Communities Council Directive of 24 November 1986 (86/609/EEC). In each experimental group eight rats were used (n = 8). Acute pancreatitis was induced by injecting 100 μg/kg of CCK (synthesized in the Department of Medical Chemistry, Szeged, Hungary as described by Penke *et al*[11]; dissolved in physiological saline) twice with an interval of 1 h (Figure 1). Ninety minutes after the first CCK injection, the animals were injected intraperitoneally (ip) either with 10 mg/kg of MG132 [Z-Leu-Leu-Leu-aldehyde; Sigma; dissolved in 0.25 mL dimethyl sulfoxide (DMSO)] or with an equal volume of DMSO (Sigma) alone. Controls received physiological saline (PS) and DMSO in the same manner. Four hours after the first CCK or saline injections, the animals were anesthetized (with pentobarbital sodium 50 mg/kg, ip) and killed by exsanguination through the abdominal aorta. Pancreases and lungs were quickly removed, the former were cleaned of fat and lymph nodes, weighed, frozen in liquid nitrogen and stored at -80℃ until use.

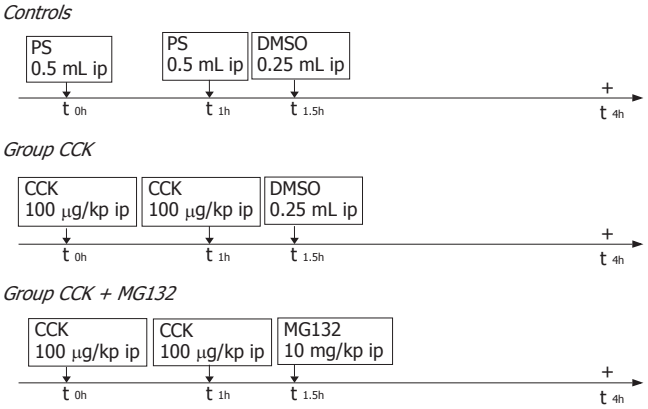

Figure 1 Experimental protocol of acute pancreatitis.

Procedures

Nuclear protein extraction: Nuclear protein extracts were prepared as described previously[12].

Electrophoretic mobility shift assay (EMSA) of NF-κB: EMSA of NF-κB was carried out as described previously[12,13].

Western blotting: Western blot analysis of pancreatic heat shock protein 72 (HSP72) and IκBa was performed as described by Rakonczay *et al*[12,14]. α-tubulin was used as a loading control.

Serum amylase activity assay: The pancreatic weight/body weight ratio was utilized to evaluate the degree of pancreatic edema. To measure the serum amylase activities, all blood samples were centrifuged at 2500 × *g* for 20 min. The serum levels of amylase were determined by a colorimetric kinetic method (Dialab, Vienna, Austria).

Pancreatic tumor necrosis factor-α and interleukin-6 levels: Tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) concentrations were measured in the pancreatic cytosolic fractions with ELISA kits (Bender Medsystems, Vienna, Austria) according to the manufacturers' instructions.

Pancreatic and lung myeloperoxidase activity: Pancreatic and lung myeloperoxidase (MPO) activity, as a marker of tissue leukocyte infiltration, was assessed by the method of Kuebler *et al*[15].

Real time quantitative polymerase chain reaction (RT-qPCR): RT-qPCR was performed on a RotorGene 3000 instrument (Corbett Research, Australia) with gene-specific primers (designed with the software PrimerExpress, Applied Biosystems, USA) and SYBRGreen I protocol as described previously[10]. Relative expression ratios were normalized to cyclophilin and calculated with the Pfaffl method[16]. The PCR primers used were as follows: cyclophilin, forward primer, 5'-TCTCTTCAAGGGACAAGGCTG-3', reverse primer, 5'-TGGCAAATCGGCTGACG-3'; pancreatitis-associated protein (PAP), forward primer, 5'-CCTCTGCAC GCATTAGTTGC-3', reverse primer, 5'-TGAAACAGGG CATAGCAGTAGG-3'.

Lipid peroxidation, reduced glutathione levels and activities of superoxide dismutase and catalase: Lipid peroxides may undergo metal- or enzyme-catalyzed decomposition to form multiple products, including malondialdehyde (MDA). Pancreatic MDA levels were measured according to the MDA/TBA-high performance liquid chromatographic (HPLC) method of Wong *et al*[17] and were corrected for the protein content of the pancreas. Reduced glutathione (GSH) levels were determined spectrophotometrically with Ellman's reagent[18]. Pancreatic total superoxide dismutase (SOD) activity was determined on the basis of the inhibition of epinephrine-adrenochrome autoxidation[19].

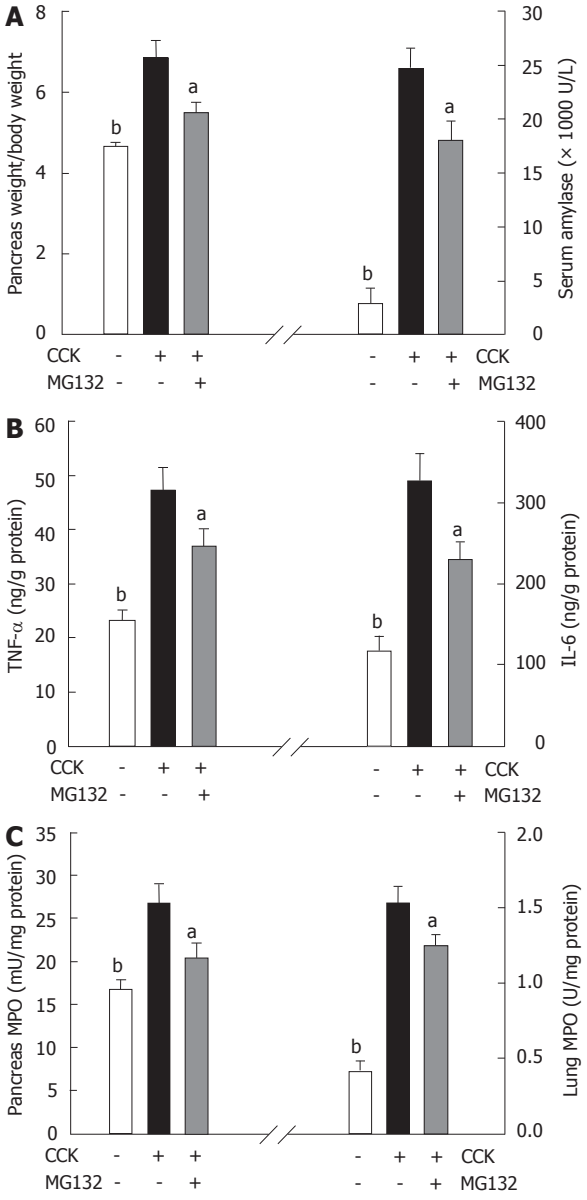

Figure 2 Effects of MG132 on laboratory parameters of acute pancreatitis. [a]$P < 0.05$; [b]$P < 0.01$.

Ferric reducing ability of plasma (FRAP): The total antioxidant activity of the plasma was determined with the method of Benzie and Strain[20]. Ferric to ferrous ion reduction-in a complex with tripyridyl-triazine - at low pH causes the development of an intense blue color, which has an absorption maximum at 593 nm. FRAP values are obtained by preparing a calibration curve with a solution of known Fe (Ⅱ) concentration.

Histological evaluation of CCK-induced acute pancreatitis

A portion of the pancreas was fixed in 8% neutral formaldehyde solution and subsequently embedded in paraffin. Sections were cut at 4 μm thicknesses and stained with hematoxylin and eosin (HE). The slides were coded and read for the traditional histological markers of pancreatic tissue injury by two independent observers who were blind to the experimental protocol. Semiquantitative grading of intestinal edema, inflammation, hemorrhage, vacuolization and acinar cell necrosis was performed on a scale of 0 to 3 (0-absent, 1-mild, 2-moderate, 3-severe).

Statistical analysis

Results were expressed as mean ± SD. Differences between experimental groups were evaluated by using analysis of variance (ANOVA). Values of $P < 0.05$ were accepted as significant.

RESULTS

Pancreatic weight/body weight ratio and serum amylase activity

Injecting 2 × 100 μg/kg body weight of CCK resulted in elevated serum amylase levels and pancreatic weight/body weight ratio, signs of acinar injury and pancreatic inflammation[21,22]. These actions of CCK were interfered by MG132 treatment (Figure 2A).

Intrapancreatic proinflammatory cytokine levels

Inflammatory mediators, like TNF and IL-6 couple the local pancreatic inflammation with systemic complications such as pancreatitis-associated lung and renal-injury[23,24]. In our study CCK significantly increased the expression of TNF and IL-6 in the pancreas compared to controls. MG132 treatment reduced intrapancreatic TNF and IL-6 levels (although, compared to Group CCK, the effect of MG132 on pancreatic TNF levels were not statistically significant, as shown in Figure 2B).

Pancreatic and lung myeloperoxidase activity

Neutrophils produce an enzyme called myeloperoxidase that can be used to identify the amount of neutrophils infiltrating a tissue after inflammation[25]. CCK hyperstimulation increased MPO activity in both the pancreas and lung, reflecting the elevated levels of neutrophil infiltration within these organs. Proteasome inhibition with MG132 decreased MPO activity in the lung and pancreas (Figure 2C).

Expression of pancreatitis-associated protein

Pancreatitis-associated protein (PAP), the acute-phase protein of the pancreas, is overexpressed in acute pancreatitis[26]. Supramaximal CCK doses significantly increased the expression of PAP mRNA. MG132 treatment could interfere markedly with this effect of CCK (Figure 3).

Parameters of oxidative stress

Two hourly injections of CCK induced pancreatic inflammation and underlying oxidative stress. Thus, the ferric reducing ability of plasma (FRAP), as an index of total antioxidant capacity was reduced four hours after the induction of pancreatitis. Moreover CCK stimulation depleted SOD activity and GSH, the two important antioxidant defense systems and increased malondialdehyde content (the marker of lipid peroxidation) in the pancreas. MG132 treatment inhibited the production of reactive oxygen species due to CCK hyperstimulation, as judged by the improvements of above mentioned laboratory parameters of antioxidant power and oxidative stress (Figure 4A and B).

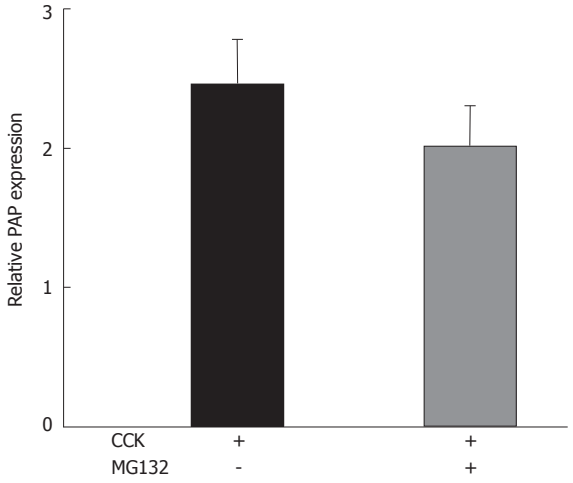

Figure 3 Effect of MG132 on mRNA expression of pancreatitis associated protein (PAP) in experimental acute pancreatitis.

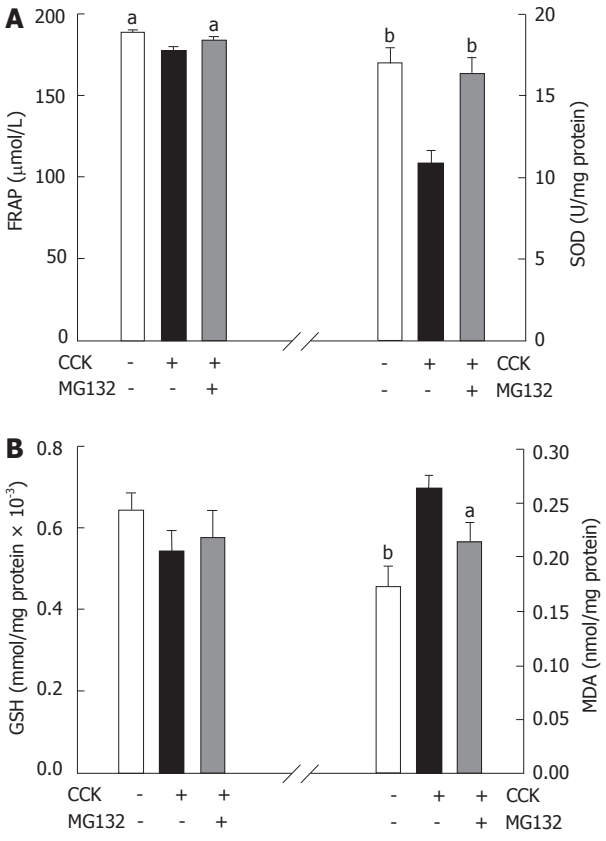

Figure 4 Effects of MG132 on measures of oxidative stress in experimental acute pancreatitis. $^{a}P < 0.05$; $^{b}P < 0.01$.

Pancreatic heat shock protein 72 (HSP72) levels

Induction of heat-shock proteins is a useful tool to increase cellular tolerance against stress[27,28]. Injections of CCK elevated the levels of pancreatic HSP72 four hours after the first CCK injection. MG132, the well-known inducer of heat-shock proteins, further increased the expression of HSP72 in the pancreas (Figure 5A and B).

Pancreatic NF-κB activation

In the pancreas, supramaximal doses of CCK triggered the degradation of IκBα and subsequent activation of NF-κB, based on Western blots and EMSAs carried out on pancreatic samples of animals involved in our study. Inhibiting the proteasome decreased IκBα degradation (Figure 5A and C) and DNA-binding of NF-κB (Figure 6A and B) (The effects of MG132 on IκBα degradation were not significant statistically).

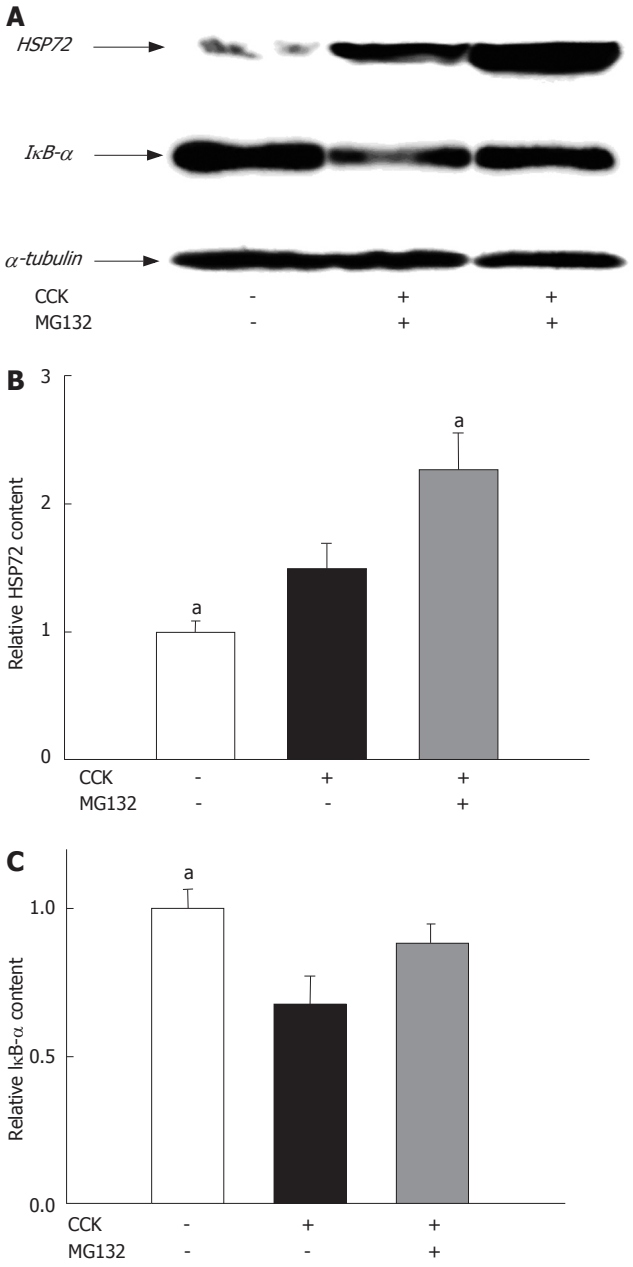

Figure 5 Effects of MG132 on HSP72 expression and IκB degradation in experimental acute pancreatitis. $^{a}P < 0.05$.

Histological findings

CCK hyperstimulation resulted in cytoplasmic vacuolization and death of acinar cells, edema formation, and infiltration of inflammatory cells in the pancreas samples of CCK-treated animals (Figure 7A). Treating the animals with the proteasome inhibitor MG132 inhibited the cellular damage and inflammatory response due to CCK, as reflected by milder histopathological changes in the pancreas (Figure 7B).

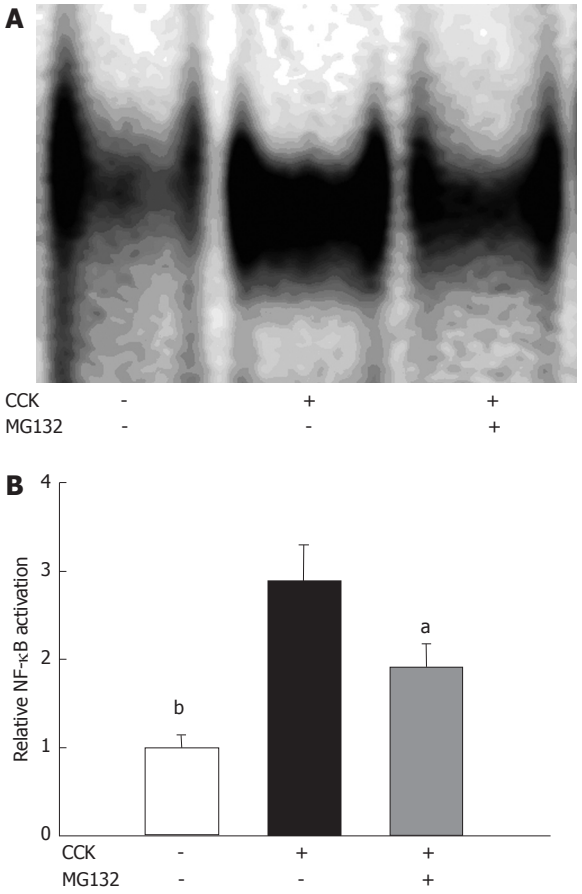

Figure 6 Effect of MG132 on NF-κB activation in experimental acute pancreatitis. [a]$P < 0.05$; [b]$P < 0.01$.

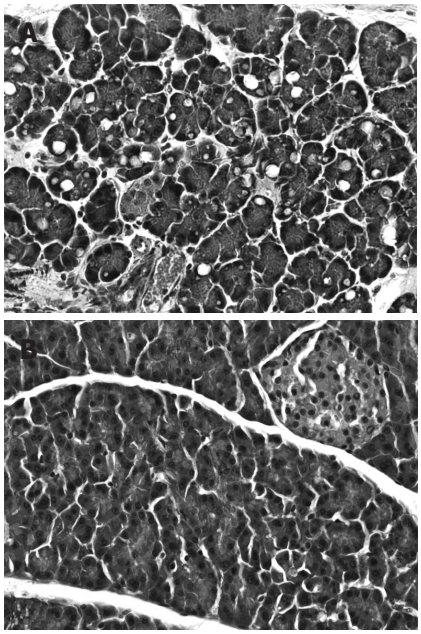

Figure 7 Effect of MG132 on pancreatic morphological damage in CCK-induced pancreatitis (HE, x 40).

DISCUSSION

Acute pancreatitis is a severe inflammatory disease triggered by abnormal activation of intrapancreatic proteases and enhanced transcriptional activity of stress-responsive transcriptional factors like NF-κB[3-5]. Intrapancreatic activation of digestive enzyme zymogens can be prevented by the inhibition of lysosomal hydrolases like cathepsin B[29-31]. NF-κB activation can also be prevented by inhibiting the proteasome and other proteases (like calpains) that degrade the inhibitory IκB subunit[32-35]. MG132 is a peptide aldehyde proteasome inhibitor with a broad inhibitory range, showing selectivity towards both serine and cysteine proteases including cathepsins and calpains[1,36]. To make it more complex, MG132 has the ability to induce heat shock proteins (including HSP72), which increases cellular tolerance to stress[37,38].

In our earlier study we have shown that pretreatment of rats with MG132 protected against acute pancreatitis by preventing NF-κB activation and inducing the expression of HSP72[10]. However the therapeutic value of prophylactic treatment in acute pancreatitis is indeed very doubtful. In order to validate the therapeutic potential of proteasome inhibition in pancreatitis, we also tested the effects of therapeutic administration of MG132 in an experimental model of the disease. Pancreatitis was induced by two hourly injections of the cholecystokinin octapeptide (CCK). In this model of the disease, CCK hyperstimulation resulted in pancreatitic inflammation characterized by intracellular activation of digestive enzymes and elevation of their serum levels, cytoplasmic vacuolization and death of acinar cells, edema formation, infiltration of inflammatory cells and oxidative stress. Thus severity of pancreatitis could be very accurately detected by monitoring the laboratory parameters of the disease.

Administering MG132 90 min after the onset of pancreatitic inflammation could still ameliorate the severity of the disease. So MG132 treatment could decrease cellular damage, inflammation and subsequent oxidative stress associated with pancreatitis. These beneficial effects of MG132 can be explained by its ability to induce the expression of HSP72 that protects cells against stressful conditions. MG132 also decreased the transcriptional activity of NF-κB. NF-κB, however, has a dual role in inflammatory diseases, because besides triggering proinflammatory cellular events during first phase of the inflammatory response, it has also anti-inflammatory role during the resolution of inflammation[2]. In CCK-induced pancreatitis, NF-κB activation peaks in the first phase of the disease[39]. Since in MG132 treatment had more pronounced effects on HSP72 than on NF-κB, thus it is likely that in our case the induction of heat shock proteins made larger contribution to the observed beneficial effects of MG132 in acute pancreatitis and than NF-κB inhibition.

Our observation that MG132 could ameliorate the severity of acute pancreatitis when administered 90 min after the induction of the disease is indeed very promising. Considering this, we have to note that although supramaximally stimulating doses of CCK cause the inflammatory response that underlies many of the features of human pancreatitis, still CCK-induced pancreatitis is a mild model of the disease[40]. Thus MG132 and other proteasome inhibitors should be further tested in other, more severe models of pancreatitis in order to accurately determine the clinical potential of proteasome inhibition for the treatment of acute pancreatitis.

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BASIC RESEARCH

Histone deacetylase inhibitor MS-275 alone or combined with bortezomib or sorafenib exhibits strong antiproliferative action in human cholangiocarcinoma cells

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Abstract

AIM: To investigate the antiproliferative effect of the histone deacetylase (HDAC) inhibitor MS-275 on cholangiocarcinoma cells alone and in combination with conventional cytostatic drugs (gemcitabine or doxorubicin) or the novel anticancer agents sorafenib or bortezomib.

METHODS: Two human bile duct adenocarcinoma cell lines (EGI-1 and TFK-1) were studied. Crystal violet staining was used for detection of cell number changes. Cytotoxicity was determined by measuring the release of the cytoplasmic enzyme lactate dehydrogenase (LDH). Apoptosis was determined by measuring the enzyme activity of caspase-3. Cell cycle status reflected by the DNA content was detected by flow cytometry.

RESULTS: MS-275 treatment potently inhibited the proliferation of EGI-1 and TFK-1 cholangiocarcinoma cells by inducing apoptosis and cell cycle arrest. MS-275-induced apoptosis was characterized by activation of caspase-3, up-regulation of Bax and down-regulation of Bcl-2. Cell cycle was predominantly arrested at the G_1/S checkpoint, which was associated with induction of the cyclin-dependent kinase inhibitor $p21^{Waf/CIP1}$. Furthermore, additive anti-neoplastic effects were observed when MS-275 treatment was combined with gemcitabine or doxorubicin, while combination with the multi-kinase inhibitor sorafenib or the proteasome inhibitor bortezomib resulted in overadditive anti-neoplastic effects.

CONCLUSION: The growth of human cholangiocarcinoma cells can be potently inhibited by MS-275 alone or in combination with conventional cytostatic drugs or new, targeted anticancer agents.

Key words: Apoptosis; Cholangiocarcinoma; Bortezomib; Combination treatment; Histone deacetylase inhibitor; MS-275; Proteasome inhibitor; Sorafenib

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INTRODUCTION

Cholangiocarcinoma (CC) accounts for 3% of all gastrointestinal cancers[1] and is the second commonest primary hepatic tumor[1,2]. It is characterized by the malignant proliferation of cholangiocytes that line intra-hepatic and extra-hepatic bile ducts and ductules. Biliary tumors are highly malignant and have a poor prognosis[3]. The incidence of intra-hepatic CC is rising in North America, Australia and Asia[2,4,5]. In addition to the well described risk factors, such as primary sclerosing cholangitis, liver fluke infestations or hepatolithiasis[6], recent studies suggest that chronic hepatitis B and C infections, HIV, non-alcoholic steatohepatitis, especially when combined with cirrhosis, also contribute to intra-hepatic CC risk[7]. On the other hand, the incidence of extra-hepatic CC is declining[2,4,5,8] most likely as a result of increasing rates of cholecystectomy over the past decades[2,5].

Treatment options for cholangiocarcinoma are limited. Unfortunately, the majority of patients suffer from advanced CC at presentation. Therefore, curative surgical resection or liver transplantation can only be offered to a minority of CC patients, leaving biliary drainage, radiotherapy or conventional chemotherapy as unsatisfactory palliative treatment options for advanced CC[6], with marginal effect on survival or quality of life[9].

Histone deacetylase (HDAC) inhibitors receive growing interest as cancer therapeutics due to their

ability to induce cell differentiation, growth arrest and apoptosis[10]. Acetylation and deacetylation of histones play an important role in the regulation of gene transcription and in the modulation of chromatin structure[11,12]. The steady state of histone acetylation is tightly controlled by antagonistic effects of histone acetyltransferases (HAT) and HDAC. Aberrant gene expression resulting in functional inactivation of HAT activity or over-expression of HDAC can promote tumor cell proliferation and survival[13]. Moreover, deregulation of HDAC recruitment to transcriptional promoters is a mechanism by which these enzymes contribute to tumorigenesis[14].

HDAC inhibitor monotherapy can inhibit the growth of various tumors *in vitro* and *in vivo*[11,15,17]. Importantly, HDAC inhibitors are relatively non-toxic to non-transformed cells[18,19], leading to their evaluation in phase I / II clinical cancer trials[14,15,20].

The synthetic orally available HDAC inhibitor, MS-275, potently inhibits histone deacetylases of several human tumor cells[21]. With a benzamide backbone, MS-275 is structurally unrelated to previous HDAC inhibitors, while showing a 30-fold stronger HDAC inhibitory activity than other natural HDAC inhibitors like sodium butyrate[22]. Recently, we and others demonstrated strong anti-proliferative activity of MS-275 towards several human cancer cells *in vitro* and *in vivo*[21,23,24]. MS-275 has now entered clinical trials both for single and combination therapy in solid and haematological malignancies. Since HDAC inhibition has not yet been evaluated for its anti-neoplastic effects on cholangiocarcinoma, we characterized the anti-neoplastic potency of the HDAC inhibitor MS-275 in human CC cells. We showed that MS-275 potently inhibited growth of CC cells, especially in combination with conventional cytostatic drugs or new, targeted anticancer agents, such as sorafenib (Nexavar™) or bortezomib (Velcade™). Furthermore, we provided an insight into major underlying mechanisms of MS-275-induced growth inhibition of CC cells.

MATERIALS AND METHODS

Cell lines and drugs

The poorly differentiated human bile duct adenocarcinoma cell line EGI-1[25] (DSMZ # ACC385) and the human papillary bile duct adenocarcinoma cell line TFK-1[26] (DSMZ # ACC344) were derived from patient cells prior to any exposure to chemotherapy or radiotherapy. Both cell lines were cultured in RPMI 1640 medium supplemented with 100 mL/L fetal calf serum (FCS), 100 kU/L penicillin and 100 mg/L streptomycin (Biochrom, Berlin, Germany) and kept at 37℃ in a humidified atmosphere containing 50 mL/L CO_2 in air.

MS-275 (*N*-(2-Aminophenyl)-4-[*N*-(3-pyridineyl-methoxycarbonyl)aminomethyl]-benzamide) was purchased from ALEXIS Biochemicals (Lausen, Switzerland). The 26S proteasome inhibitor bortezomib (Velcade™) was bought from Millennium Pharmaceuticals, Inc. (Cambridge, MA, USA). The multi-kinase inhibitor sorafenib tosylate (Nexavar™) was a kind gift from Bayer Health Care (West Haven, CT, USA). Stock solutions were prepared in dimethyl sulfoxide (DMSO) and stored at -20℃ until use. Gemcitabine hydrochloride (Gemzar™) was bought from Lilly Pharma (Gieβen, Germany). Doxorubicin hydrochloride was from Sigma (Deisenhofen, Germany) and also prepared in DMSO and stored at -20℃. All drugs were diluted in fresh medium before each experiment. In all experiments, the final DMSO concentration was ⩽ 5 g/L, not affecting cell growth. To evaluate the effects of the drugs, cells were incubated with either control medium or medium containing rising concentrations of the respective drug(s). Cell culture material was from Biochrom (Berlin, Germany). All other chemicals were from Sigma if not stated otherwise.

Measurement of growth inhibition

Drug-induced changes in cell numbers of EGI-1 and TFK-1 cells were evaluated by crystal violet staining as previously described[27]. In brief, cells in 96-well microtiter plates were fixed with 10 g/L glutaraldehyde. Then cells were stained with 1 g/L crystal violet in phosphate buffer solution (PBS). The unbound dye was removed by washing with water. Bound crystal violet was solubilized with 2 mL/L Triton-X-100 in PBS. Light extinction which increases linearly with the cell number was analyzed at 570 nm using an ELISA-Reader. To check for possible overadditive anti-proliferative effects, combination treatments of MS-275 plus conventional cytostatics (gemcitabine or doxorubicin) or plus sorafenib or plus bortezomib were performed. Increasing concentrations of the respective drug were combined with 0.25 and/or 0.5 μmol/L MS-275. The anti-neoplastic effects of the combination therapies were compared to those of each drug alone. Concentration range and effectiveness of the respective drugs have been determined in prior experiments.

Determination of cytotoxicity

Cells were seeded at a density of 8000 cells/well into 96-well microtiter plates and incubated with rising concentrations of MS-275 for 1, 2.5, 5 or 24 h. Release of the cytoplasmic enzyme lactate dehydrogenase (LDH), indicating cytotoxicity, was measured by using a colorimetric kit from Roche Diagnostics (Mannheim, Germany) as described elsewhere[28].

Detection of apoptosis

Preparation of cell lysates and determination of caspase-3 activity were performed as described previously[27]. The activity of caspase-3 was calculated from cleavage of the fluorogenic substrate Ac-DEVD-AMC (Calbiochem, Bad Soden, Germany). Cell lysates were incubated with substrate solution (caspase-3 substrate Ac-DEVD-AMC 20 mg/L, HEPES 20 mmol/L, glycerol 100 mL/L, DTT 2 mmol/L, pH 7.5) for 1 h at 37℃, and substrate cleavage was measured with a VersaFluor fluorometer (excitation: 360 nm emission: 460 nm) from Biorad (Munich, Germany).

Cell cycle analysis

Cell cycle analysis was performed by the method of Vindelov and Christensen as described previously[29]. Cells were trypsinized, washed and the nuclei were isolated using the CycleTest PLUS DNA Reagent Kit (Becton Dickinson, Heidelberg, Germany). DNA was stained with propidium iodide according to the manufacturers' instructions. The DNA content of the nuclei was measured by flow

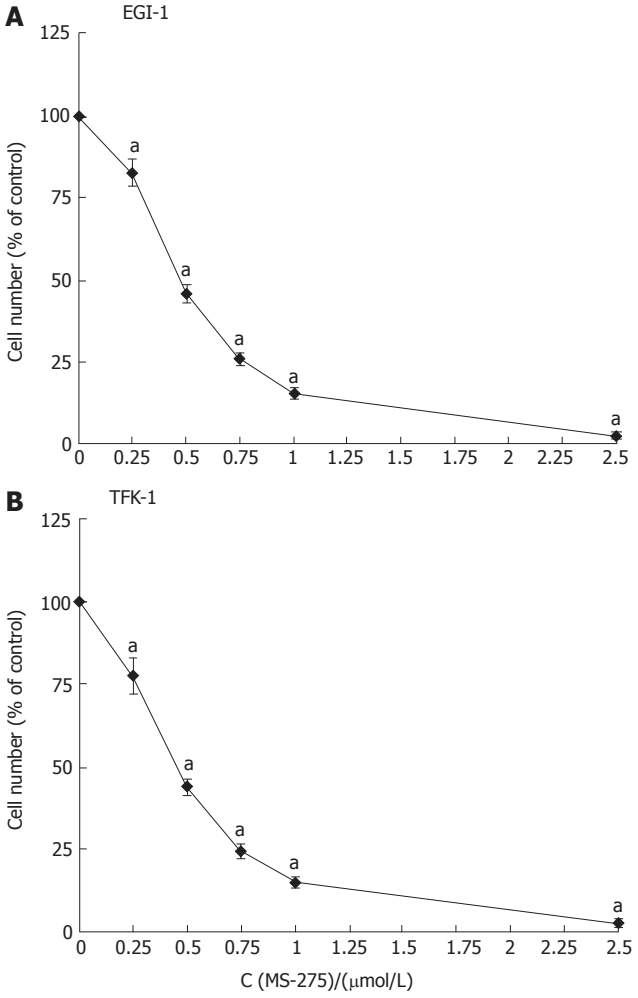

Figure 1 Anti-proliferative effects of MS-275 on human EGI-1 and TFK-1 cholangiocarcinoma cells. mean ± SEM, $n > 5$, $^{a}P < 0.05$ *vs* controls.

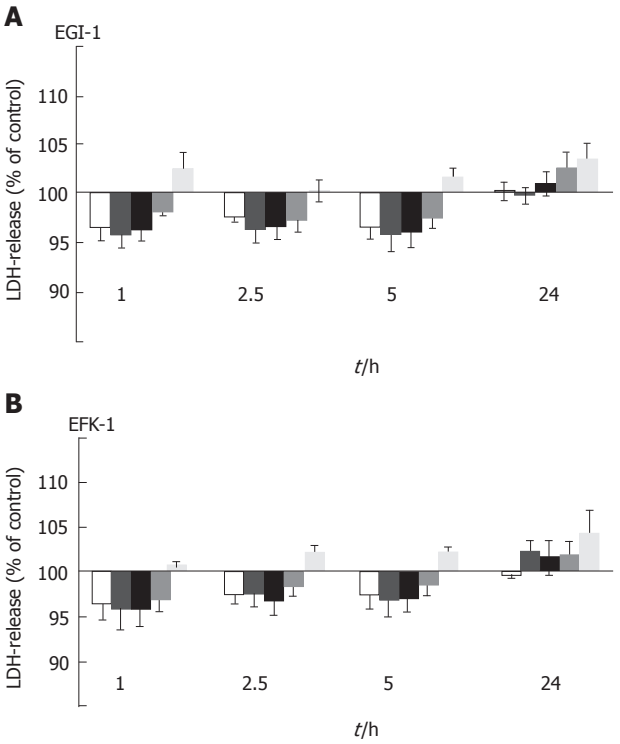

Figure 2 MS-275-induced LDH release into the supernatant of EGI-1 and TFK-1 cells. mean ± SEM, $n > 3$ independent experiments; No significant increase in LDH release was observed.

cytometry and analyzed using ModFit software (Becton Dickinson, Heidelberg, Germany).

Western blot analysis

Western blotting was performed as described previously[30]. In brief, whole-cell extracts were prepared by lysing cells. Lysates containing 30 μg protein were subjected to gel electrophoresis. Proteins were then transferred onto PVDF membranes by electroblotting for 90 min. Blots were blocked with 50 g/L non-fat dry milk in TBS-Tween-solution for 1 h at room temperature, and then incubated at 4℃ overnight with antibodies directed against anti-human Bax (1:1000), $p27^{Kip1}$ (1:200) (both from Santa Cruz Biotechnology, CA, USA), Bcl-2 (1:1000), or $p21^{Waf-1/CIP1}$ (1:1000) (both from Cell Signaling, MA USA). Anti-β-actin (1:5000) from Sigma (Deisenhofen, Germany) served as loading control. After incubation with horseradish peroxidase-coupled anti-IgG antibodies (1:10 000, GE Healthcare, Uppsala, Sweden) at room temperature for at least 1 h, the blot was developed using enhanced chemiluminescent detection (GE Healthcare) and subsequently exposed to Hyperfilm ECL film (GE Healthcare, Uppsala, Sweden).

Statistical analysis

If not stated otherwise, data were expressed as means of at least three independent experiments ± SEM. Significance between controls and treated samples was calculated by Student's two-sided *t*-test. Caspase-3 measurements were evaluated using the two-sided Welch *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Growth inhibitory effects of the HDAC inhibitor MS-275

The growth inhibitory effects of MS-275 on CC cells were studied by crystal violet staining. Treating EGI-1 and TFK-1 cells with 0.25-2.5 μmol/L MS-275 for 3 d reduced the growth of both cell lines in a dose-dependent manner by up to 100% (Figure 1). The IC_{50} value of MS-275, determined after 72 h of incubation, was 0.48 ± 0.02 μmol/L for EGI-1 cells and 0.46 ± 0.02 μmol/L for TFK-1 cells. Determination of growth inhibition after 24 h and 48 h of incubation revealed that MS-275 inhibited cell proliferation in a time-dependent manner (data not shown).

LDH release after MS-275 treatment

Cytotoxicity was evaluated by LDH release of the cells into the culture medium. Incubating EGI-1 and TFK-1 cells with 0.1-1 μmol/L MS-275 for up to 24 h did not result in a significant increase of LDH release (Figure 2), indicating that MS-275 does not directly affect cell membrane integrity and does not have immediate toxic effects even at higher concentrations.

Induction of apoptosis by MS-275

To determine the contribution of apoptosis to the observed growth inhibitory effect of MS-275, the activation of caspase-3, one of the key enzymes in the

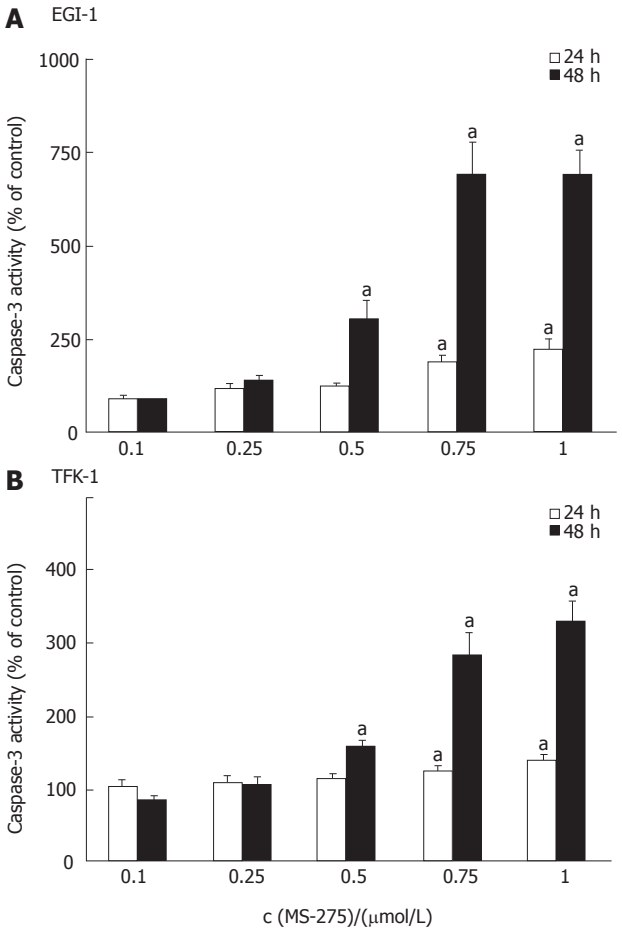

Figure 3 MS-275-induced apoptosis-specific caspase-3 activity increase in EGI-1 and TFK-1 cells. mean ± SEM, $n > 3$ independent experiments, $^aP < 0.05$ *vs* controls.

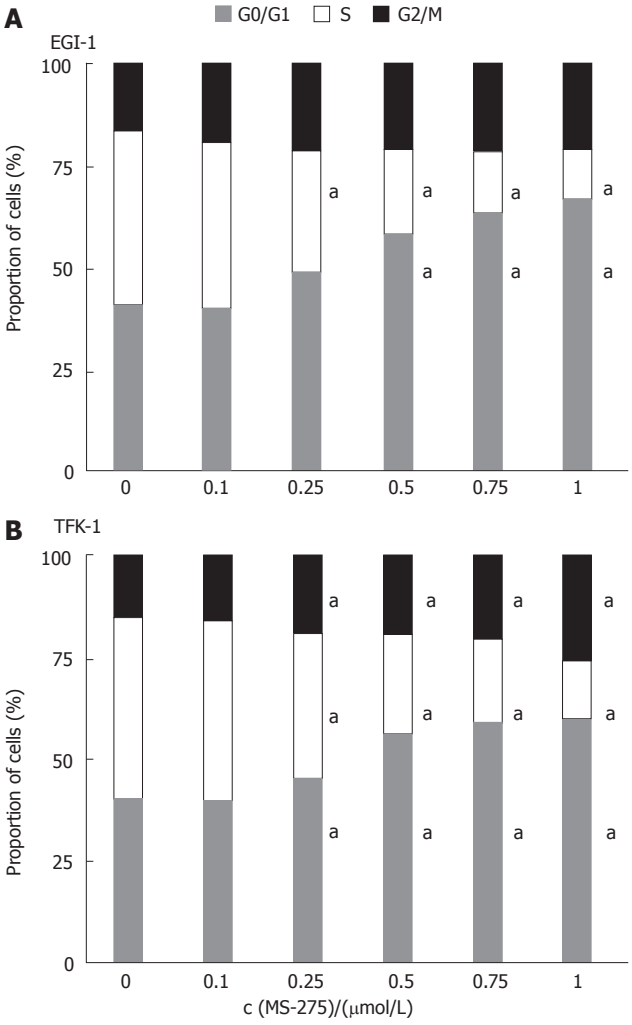

Figure 4 Induction of cell cycle arrest by MS-275 on EGI-1 and TFK-1 cells. means of > 3 independent experiments, $^aP < 0.05$ *vs* controls.

apoptotic signaling cascade, was determined after 24 h and 48 h of incubation. In both cell lines, HDAC inhibition by MS-275 resulted in a time- and dose-dependent increase of caspase-3 enzyme activity (Figure 3). After 48 h, 1 μmol/L MS-275 led to a 3-fold increase of caspase-3 activity in TFK-1 cells and even 7-fold increase in EGI-1 cells.

Cell cycle regulation

To test whether an induction of cell cycle arrest contributed to the antiproliferative potency of MS-275 in cholangiocarcinoma cells, we performed flow cytometric cell cycle analyses. Incubating EGI-1 and TFK-1 cells with 0.1-1 μmol/L MS-275 for 24 h resulted in a dose-dependent arrest in the G_0/G_1 phase of the cell cycle, whereas the proportion of cells in the S phase significantly decreased (Figure 4). The G_0/G_1-phase arrest by MS-275 was significant above concentrations of 0.1 μmol/L in TFK-1 and 0.25 μmol/L in EGI-1 cells. Moreover, the proportion of cells in the G_2/M phase also increased, indicating an additional block in the G_2/M phase. The G_2/M-phase arrest was more pronounced in TFK-1 cells.

Bax, Bcl-2, p21$^{Waf-1/CIP1}$ and p27^{Kip1} expression

To further characterize the apoptotic and cell cycle arresting effects of the HDAC inhibition, we performed Western blotting. Treating EGI-1 and TFK-1 cells for 48 h with MS-275 (0.1-1 μmol/L) resulted in a dose-dependent decrease of anti-apoptotic Bcl-2, whereas the expression of the pro-apoptotic Bax was increased in both cell lines (Figure 5A). Moreover, upon MS-275 treatment, a dose-dependent increase in the expression of p21$^{Waf-1/CIP1}$ was observed. By contrast, no regulation of the cyclin-dependent kinase inhibitor (CDKI) p27^{Kip1} was detected (Figure 5B).

Antineoplastic potency of MS-275 in combination with other drugs

To test potential (over-) additive anti-proliferative effects of MS-275-based combination treatment, EGI-1 or TFK-1 cells were treated with combinations of 0.5 μmol/L MS-275 plus either gemcitabine (0-500 nmol/L) or doxorubicin (0-100 nmol/L) for 2 d. In both combinations, an augmentation of the anti-proliferative effect was observed (Figure 6). Although the anti-proliferative efficacy of gemcitabine monotherapy differed in EGI-1 and TFK-1 cells, the addition of MS-275 caused an enhanced anti-proliferative efficacy in either cell line (Figure 6A and B). Similar results were obtained when co-administering MS-275 and doxorubicin, with additive growth inhibitory effects on both cell lines (Figure 6C and D).

Recently, we could demonstrate that the multi-kinase

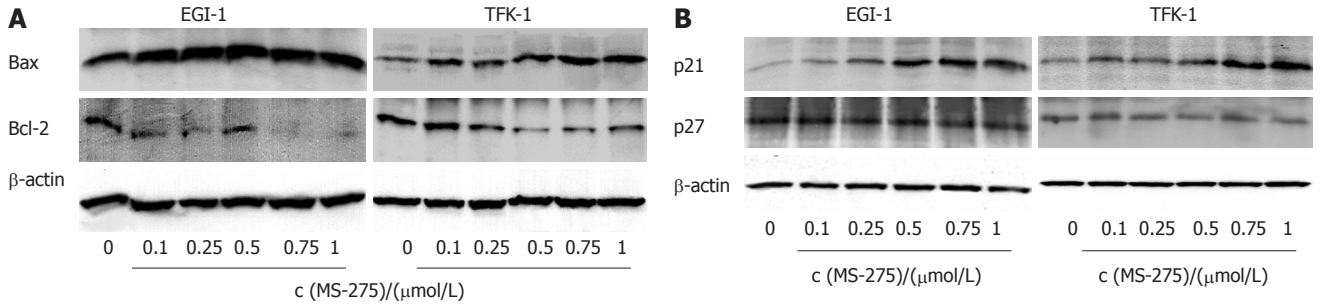

Figure 5 MS-275-induced modulation of apoptosis- and cell cycle-relevant proteins (n = 3 independent experiments).

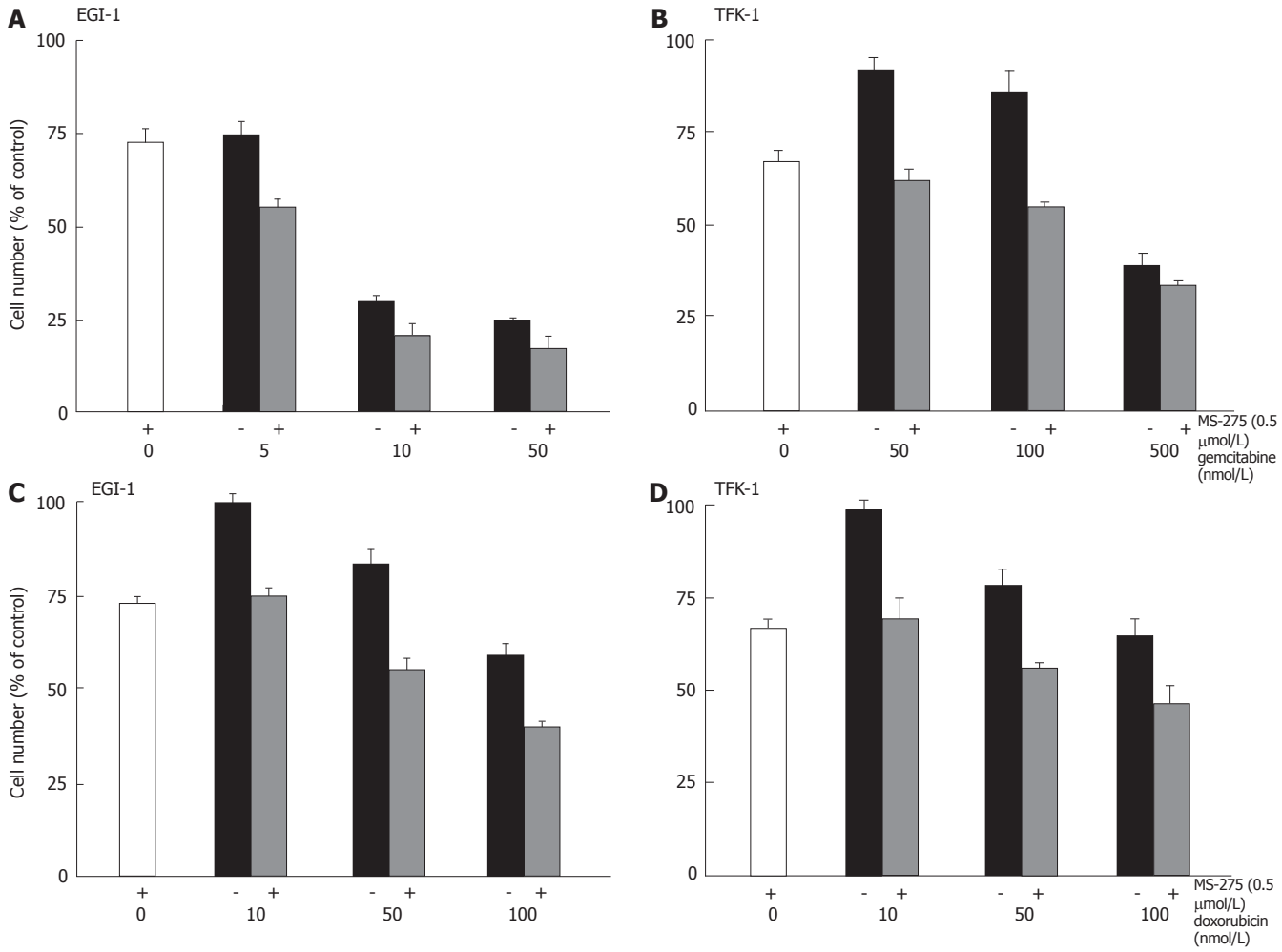

Figure 6 Anti-proliferative effects of MS-275 plus cytostatics on EGI-1 and TFK-1 cells. mean ± SEM, n = 3-4 independent experiments.

inhibitor sorafenib potently inhibited the proliferation of cholangiocarcinoma cells[31]. To check out the potency of inhibiting multiple targets of mitogenic signaling pathways for enhanced inhibition of cholangiocarcinoma cell growth, we treated EGI-1 and TFK-1 cells with MS-275 and sorafenib. After two days of incubation, a pronounced (over-)additive growth inhibitory effect of the combination was observed on both cell lines (Figure 7A and B).

Applying the proteasome inhibitor bortezomib alone (1-10 nmol/L) for two days reduced the growth of TFK-1 and EGI-1 cells by up to 65% and 84%, respectively. Again, additive and overadditive growth inhibitory effects were observed, when bortezomib was combined with MS-275 (Figure 7C and D).

DISCUSSION

Treatment options of advanced cholangiocarcinoma (CC) are unsatisfactory, and the prognosis of patients suffering from advanced CC is poor. New, effective and well-tolerated therapy strategies are urgently needed.

Aberrant gene expressions resulting in functional inactivation of histone acetyltransferase (HAT) activity or over-expression of histone deacetylases (HDAC) have been shown to contribute to tumor cell proliferation[13]. For this reason, HDAC inhibition has been regarded as a promising anticancer strategy to inhibit the multiple cellular processes that are dysregulated in various neoplastic cells[14].

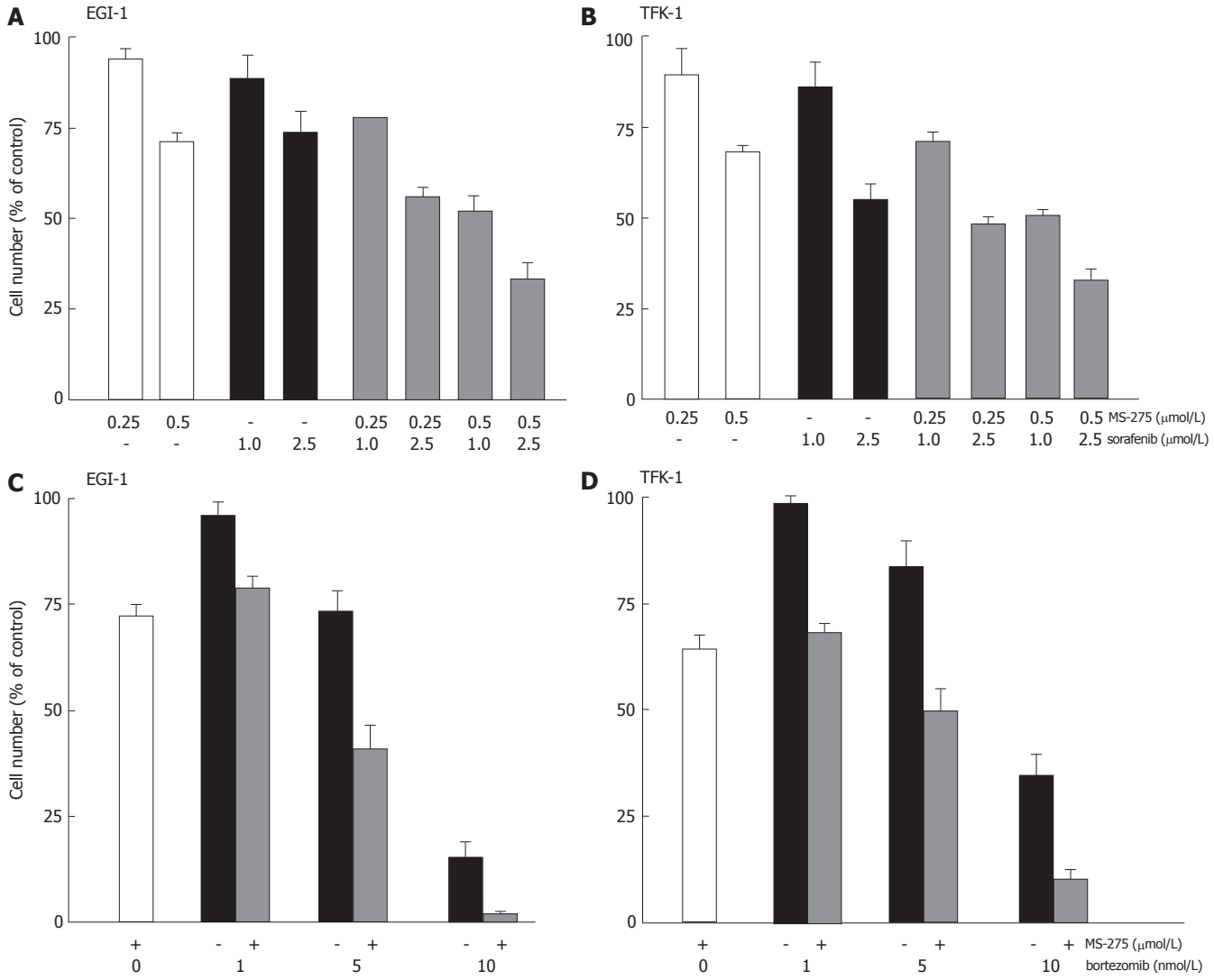

Figure 7 Anti-proliferative effects of MS-275 plus sorafenib or bortezomib on EGI-1 and TFK-1. mean ± SEM, *n* = 3-4 independent experiments.

The novel synthetic benzamide derivative MS-275 is an orally available HDAC inhibitor which has been shown to exert strong anti-proliferative effects on a variety of human cancers[21], but its potential suitability for the treatment of cholangiocarcinomas have not been tested so far. Here, we provide evidence that inhibition of HDAC activity by MS-275, alone or in combination with conventional chemotherapeutics or new, targeted anticancer drugs, may be a promising approach for the treatment of cholangiocarcinoma.

MS-275 potently inhibited cell growth of the cholangiocarcinoma cells EGI-1 and TFK-1 in a time- and dose-dependent manner. Submicromolar concentrations of MS-275 were already sufficient to significantly inhibit the proliferation of EGI-1 and TFK-1 cells, and the concentration needed to induce halfmaximal anti-neoplastic effects (IC_{50} value) was approximately 0.5 μmol/L in both cell lines. This is comparable to our previous findings on the effects of MS-275 on the growth of gastrointestinal neuroendocrine tumor cells[24].

Recent studies have shown that inhibition of HDAC activity induces apoptosis in a variety of cancers, including breast and prostate cancer, neuroblastoma, hepatoma, gastrointestinal neuroendocrine tumor cells and some types of hematologic malignancies[24,32-34].

The mechanisms involved in the HDAC inhibitor-induced apoptosis are complex and differ among cell types[35]. They can involve both intrinsic pathways and extrinsic pathways of apoptosis. MS-275 can induce mitochondrial permeability transition with a subsequent release of pro-apoptotic cytochrome c into the cytosol, resulting in the activation of caspase-9 and caspase-3, thus triggering the intrinsic apoptotic pathway[36-38]. Additionally, other HDAC inhibitors have been shown to upregulate pro-apoptotic Fas, a member of the tumor necrosis factor receptor superfamily and the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL-) receptors/death receptors DR4 and DR5, thereby triggering the extrinsic pathway, paralleled by down-regulation of the caspase-8 inhibitor c-FLIP, leading to caspase-8 and subsequently to caspase-3 activation[39,40]. Moreover, an altered expression of several pro- and anti-apoptotic intracellular genes by HDAC inhibitors has been reported. Up-regulation of pro-apoptotic Bak and induction of conformational changes of the pro-apoptotic protein Bax are some of the HDAC inhibitor-induced upstream events that trigger the

mitochondrial pathway of apoptosis[33,41,42]. HDAC inhibitors can also down-regulate anti-apoptotic proteins, such as Bcl-2, Bcl-XL, XIAP, Mcl-1, and survivin[37,43], further tilting the balance in favor of apoptosis. Here, we demonstrated that the pro-apoptotic effect of HDAC inhibition by MS-275 in CC cells is mediated by the activation of caspase-3 and a shift in the balance of pro-apoptotic Bax over anti-apoptotic Bcl-2.

HDAC-inhibition-induced changes in the expression pattern of apoptosis-related proteins seem to be cell type-specific. For example, in hepatoma and breast cancer cells, HDAC inhibition decreased the expression of Bcl-2, while expression of pro-apoptotic Bax was increased[33,44], in line with the present study. On the other hand, no changes in the expression pattern of Bax and Bcl-2 were found in HDAC-inhibitor-treated glioma cells[45], and in gastrointestinal neuroendocrine tumor cells, Bcl-2 expression was down-regulated upon MS-275 treatment, while Bax remained unchanged[24]. Especially for an estimation of suitable combination treatment regimens being based on the enhanced induction of apoptosis, it is necessary to check out cell type-specific changes in the expression pattern of HDAC-induced pro- and anti-apoptotic proteins.

To further characterize the growth inhibitory activity of MS-275, we performed cell cycle analyses. Upon treatment with MS-275 we found that cell cycle progression was blocked both in the G_0/G_1 and the G_2/M phase. The induction of cell cycle arrest was associated with an increase in the expression of the cyclin-dependent kinase inhibitor (CDKI) $p21^{Waf\text{-}1/Cip1}$. $p21^{Waf\text{-}1/CIP1}$ is a key component of cell cycle checkpoints, i.e., the G_1/S[46] and the G_2/M checkpoints[47,48]. Accordingly, HDAC inhibitors were found to inhibit both the G_1/S and G_2/M transition[49-51].

The combination of HDAC inhibitors with existing chemotherapeutic agents appears to be a promising approach to reduce the dose of other anti-neoplastic drugs and to overcome drug resistance. For non-cholangiocarcinoma tumors (e.g. glioblastoma and breast cancer cells), a potentiation of anti-neoplastic effects has already been described for HDAC inhibitors combined with cytostatic drugs[52]. However, except for the interaction of HDAC and topoisomerase inhibitors, in which HDAC-inhibitor-mediated increases in topoisomerase II levels appear to contribute to lethality[53], little is known about the mechanisms by which HDAC inhibitors increase the anti-neoplastic efficacy of cytostatic agents. Maggio *et al*[37] reported that combination of MS-275 with nucleoside analogues (e.g. Fludarabine) produced high synergy in triggering mitochondrial dysfunction and caspase activation in human leukemia cells. In our study, the nucleoside analogue gemcitabine and the topoisomerase-II-inhibitor doxorubicin were chosen for the evaluation of synergy with MS-275 in CC cells. While both combinations resulted in additive anti-proliferative effects, further studies are needed to clarify the exact mechanisms underlying the observed potentiation by MS-275. Nevertheless, the fact that MS-275-based combination therapies with cytostatics lead to an enhanced growth inhibition of CC cells gives rise to the hope that a HDAC-inhibitor-based combination therapy may hold a promise for more effective treatment of CC.

The same holds true for the combination of HDAC inhibition and multi-kinase inhibition by sorafenib, which mainly targets raf and ras kinases that are members of the MAPK (mitogen-activated protein kinase) pathway. The MAPK pathway is centrally involved in the regulation of multiple cellular functions, such as cell growth, apoptosis and cell cycle progression[54]. Recently, we could demonstrate a potent growth inhibition of CC cells by sorafenib monotherapy[31]. HDAC inhibitors can also down-regulate the MAPK-signaling pathway, as exemplified by HDAC-induced growth inhibition of transformed hepatocytes due to the suppression of oncogenic ras and ERK1/2 (extracellular regulated kinase)[55]. Here we found that coapplication of MS-275 and sorafenib resulted in an additive to overadditive growth inhibition of CC cells, indicating that HDAC-based dual-targeting of MAPK and pro-apoptotic pathways may have promising clinical implications for the future treatment of CC.

Finally, we also studied how far combination treatment of MS-275 together with the proteasome inhibitor bortezomib could enhance the anti-neoplastic effects of the monotherapies. Bortezomib has recently been shown to enhance the anti-proliferative effects of HDAC inhibitors on several non-CC cancer cells[56-58]. The sensitizing effect of bortezomib has been attributed to a blockade of the cytoprotective nuclear factor kappa B (NFκB)[59,60]. There is accumulating evidence that NFκB activation status plays an important role in regulating the response of cells to HDAC inhibitors[61]. Thus, in non-small cell lung cancer, HDAC inhibitors are only modestly effective due to high steady state levels of NFκB[11,57], but addition of bortezomib dramatically increased the pro-apoptotic effect of the HDAC inhibitor sodium butyrate[57]. Our findings are in line with these data in clearly demonstrating that bortezomib overadditively enhanced the anti-proliferative effect of MS-275 on CC cells. Thus, the combined treatment with HDAC inhibitors and bortezomib (and possibly sorafenib as a third agent) appears to be a promising approach for the treatment of cholangiocarcinomas.

In conclusion, our study provides first evidence that the HDAC inhibitor MS-275 potently inhibits the growth of human CC cells by inducing both cell cycle arrest and apoptosis. Importantly, we could demonstrate that combination treatment of MS-275 with gemcitabine, doxorubicin, and especially sorafenib or bortezomib leads to (over-)additive growth inhibitory effects. Our study may provide a rationale for future *in vivo* evaluations of MS-275 in combination therapies for growth control of advanced cholangiocarcinomas.

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COMMENTS

Background

Cholangiocarcinomas (CC) are highly malignant and have a poor prognosis. Since there is no satisfactory therapy for advanced CC, we studied the effects of the

histone deacetylase inhibitor MS-275 alone or in combination with bortezomib or sorafenib on the proliferation of cholangiocarcinoma cells.

Research frontiers

Histone modifications have been identified to play prominent roles in the epigenetic regulation of gene transcription. Accumulating evidence indicates that dysregulation of epigenetic processes causes transcriptional repression of a subset of genes, contributing to the pathogenesis of many human diseases, including cancer. In the past decade, substantial progress has been made in understanding the relationship between aberrant epigenetic changes and tumorigenesis. Enzymes involved in these epigenetic events include histone acetyltransferases (HAT) and histone deacetylases (HDAC), which tightly control the steady state of histone acetylation. Recently, histone deacetylase inhibitors receive growing interest as cancer therapeutics, due to their ability to induce growth arrest, differentiation and/or apoptosis.

Innovations and breakthroughs

Previous studies already demonstrated that targeting the histone deacetylase (HDAC) activity by specific HDAC inhibitors is an effective treatment option for cell growth control of haematological malignancies and of several solid tumors. Especially the novel, orally available HDAC inhibitor MS-275 has been shown to exert strong anti-proliferative effects on a variety of cancers.

Applications

In this paper, we studied the anti-proliferative effect of the potent histone deacetylase inhibitor MS-275 on cholangiocarcinoma cells for the first time. MS-275 significantly inhibits the proliferation of cholangiocarcinoma cells by inducing apoptosis and cell cycle arrest. Importantly, we could further demonstrate that combination treatment using MS-275 together with doxorubicin, gemcitabine, sorafenib or bortezomib, respectively, leads to (over-)additive growth inhibitory effects, thus providing a rationale for future *in vivo* evaluations.

Terminology

Histone acetyltransferases (HAT) and histone deacetylases (HDAC): HAT and HDAC are two sets of histone-modifying enzymes, which control the steady state of histone acetylation. The equilibrium of acetylation (HAT) and deacetylation (HDAC) of histones plays an important role in the modulation of chromatin structure and in the regulation of gene transcription.

Peer review

This manuscript is a good suggestion to be considered and it is true that the treatment options for advanced cholangiocarcinomas are still unsatisfactory. New therapeutic approaches are urgently needed.

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CLINICAL RESEARCH

Comparison of four proton pump inhibitors for the short-term treatment of esophagitis in elderly patients

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Abstract

AIM: To compare efficacy and tolerability of four proton pump inhibitors (PPIs) commonly used in the short-term therapy of esophagitis in elderly patients.

METHODS: A total of 320 patients over 65 years with endoscopically diagnosed esophagitis were randomly assigned to one of the following treatments for 8 wk: (1) omeprazole 20 mg/d; (2) lansoprazole 30 mg/d; (3) pantoprazole 40 mg/d, or (4) rabeprazole 20 mg/d. Major symptoms, compliance, and adverse events were recorded. After 8 wk, endoscopy and clinical evaluation were repeated.

RESULTS: Per protocol and intention to treat healing rates of esophagitis were: omeprazole = 81.0% and 75.0%, lansoprazole = 90.7% (P = 0.143 *vs* omeprazole) and 85.0%, pantoprazole = 93.5% (P = 0.04 *vs* omeprazole) and 90.0% (P = 0.02 *vs* omeprazole), rabeprazole = 94.6% (P = 0.02 *vs* omeprazole) and 88.8% (P = 0.04 *vs* omeprazole). Dividing patients according to the grades of esophagitis, omeprazole was significantly less effective than the three other PPIs in healing grade 1 esophagitis (healing rates: 81.8% *vs* 100%, 100% and 100%, respectively, P = 0.012). Pantoprazole and rabeprazole (100%) were more effective *vs* omeprazole (89.6%, P = 0.0001) and lansoprazole (82.4%, P = 0.0001) in decreasing heartburn. Pantoprazole and rabeprazole (92.2% and 90.1%, respectively) were also more effective *vs* lansoprazole (75.0%, $P < 0.05$) in decreasing acid regurgitation. Finally, pantoprazole and rabeprazole (95.2% and 100%) were also more effective *vs* lansoprazole (82.6%, $P < 0.05$) in decreasing epigastric pain.

CONCLUSION: In elderly patients, pantoprazole and rabeprazole were significantly more effective than omeprazole in healing esophagitis and than omeprazole or lansoprazole in improving symptoms. *H pylori* infection did not influence the healing rates of esophagitis after a short-term treatment with PPI.

Key words: Elderly; Esophagitis; Proton pump inhibitors

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INTRODUCTION

Old age is known to be a significant risk factor for severe esophagitis[1,2], chronic relapses[3], as well as severe complications of the disease[1,4]. Clinical features of esophagitis in elderly patients are quite different from those of young or adult subjects. Indeed, elderly patients present less frequently the typical symptoms of heartburn, acid regurgitation and/or epigastric pain. Conversely, the prevalence of other non-specific symptoms, i.e. anorexia, weight loss, anaemia, and/or vomiting significantly increases with age[2]. Thus, the diagnosis of reflux esophagitis may be missed in the elderly, and a substantial number of patients may suffer subclinical relapses of the disease[5].

The treatment of esophagitis is based on gastric acid suppression with antisecretory drugs. Proton pump inhibitors (PPIs) are widely used and their effectiveness and safety have been demonstrated also in patients of old age[6]. Currently, five PPIs are available on the market:

omeprazole, lansoprazole, rabeprazole, pantoprazole, and esomeprazole. Some age-associated differences in pharmacokinetics and pharmacodynamics of the PPIs have been reported[7]. However, it is unknown if these differences are associated with different clinical effects, i.e. healing rates and/or symptom relief, particularly in older patients.

The aim of this study was to compare the clinical efficacy and tolerability of four PPIs used for the short-term therapy of esophagitis in elderly patients.

SUBJECTS AND METHODS

Study design

This was an open, single-centre, randomized study including elderly subjects that consecutively underwent an upper gastrointestinal endoscopy. It was conducted according to the Declaration of Helsinki and the guidelines for Good Clinical Practice. All patients gave their informed consent prior to participation in the study.

The inclusion criteria were: (1) age 65 years or over and (2) endoscopic diagnosis of esophagitis grade Ⅰ to Ⅳ according to the Savary-Miller classification[8]. Major exclusion criteria were: history of Zollinger-Ellison syndrome, pyloric stenosis, previous surgery of the esophagus and/or gastrointestinal tract (except for appendectomy and cholecystectomy), and gastrointestinal malignancy. Patients were excluded if they had received antacids, sucralfate, prokinetics, H2-blockers, and/or PPIs for more than 7 d in the four weeks prior to the start of the study.

Assessments

At the initial visit, demographic data, medical history, clinical symptoms, non-steroidal anti-inflammatory drug (NSAID) use, and antisecretory therapy were recorded. At study entry, an endoscopy was performed to diagnose acute esophagitis (inclusion criteria). After 2 mo of treatment, endoscopy was repeated to evaluate healing of acute esophagitis. All patients were examined during therapy to record side effects and to count tablets. Compliance was defined as "good" when more than 90% of the tablets had been taken by the patients. Adverse events were rated by the investigator as not related, unlikely, possibly related, or likely related to the medication.

Endoscopic diagnoses

Reflux esophagitis was endoscopically defined by epithelial defects according to the Savary-Miller criteria[8] and classified as grade Ⅰ: non-confluent erosions; grade Ⅱ: confluent erosions; grade Ⅲ: lesions extending to the entire circumference of the lower esophagus; and grade Ⅳ: deep ulcer or esophagitis with complications, i.e. stenosis and/or hemorrhagic lesions. Patients with diffuse erythema and/or fragility of the lower esophagus were not included. Hiatus hernia was diagnosed when the Z-line and the gastric folds extended 2 cm or more above the diaphragmatic hiatus[9]. Patients with Barrett's esophagus were not included unless erosive esophagitis was also present.

Histology and H pylori infection

During endoscopy, six gastric biopsies were taken from both the antrum (three biopsies), and from the body (three biopsies). Two antral and two body biopsies were used for histological analysis, while one from each site was used for the rapid urease test (CLO test, Delta West Pty Ltd, Western Australia). For histological examination, biopsy specimens were immediately fixed in buffered neutral formalin and embedded in paraffin. Sections were stained with hematoxylin-eosin and modified Giemsa for the detection of *H pylori* and evaluated according to the Sydney classification[10]. Patients were considered *H pylori* negative if both histology and the rapid urease test were negative; patients were considered *H pylori* positive if either their histology or rapid urease test, or both, were positive for Hp infection[11].

Symptomatology

Symptoms were assessed during a structured interview. The patient was questioned about the principal symptoms, i.e. acid regurgitation, heartburn, and other symptoms of reflux esophagitis, i.e. epigastric pain, dysphagia, vomiting, and anaemia (loss of $\geqslant$ 3 grams of haemoglobin during the last 3 mo) and expressed as absent/present.

Treatments

Patients included in the study were consecutively assigned to one of the following regimens for two months: omeprazole 20 mg once daily, lansoprazole 30 mg once daily; pantoprazole 40 mg once daily, or rabeprazole 20 mg once daily. Randomization was performed by a computer-generated list in blocks of four with a 1:1:1:1 ratio. All PPIs were taken in the morning fasting just before breakfast. Patients who resulted *H pylori* positive were treated with the PPI plus two antibiotics i.e., amoxicillin 1g twice daily and claritromycin 250 mg twice daily or metronidazole 250 mg four times daily for 7 d[12].

Statistical analysis

Statistical analysis was performed by means of the SPSS version 13. Results were evaluated using both "per protocol" (PP) and "intention-to-treat" (ITT) analyses; the 95% confidence intervals (95% CI) were also calculated. The ITT population was defined as all patients initially enrolled who had taken at least one dose of study medication. Statistical analysis was performed using the χ^2 test (comparison of outcomes with the treatments) and Fisher exact test (healing rates related to *H pylori* infection, symptoms). All p values were two-tailed with statistical significance indicated by a value of $P < 0.05$.

RESULTS

A total of 320 consecutive elderly (156 males and 164 females, mean age 77.4 ± 7.9 years, range from 65 to 93 years) with an endoscopic diagnosis of acute esophagitis, grades 1 to 4 according to the Savary-Miller classification, were included in the study. Demographic and clinical characteristics of patients are shown in Table 1.

Nineteen patients (5.9% of the total population) dropped-out from the study due to: adverse events (2 patients), low compliance (11 patients), and refusal of endoscopy after two months of treatment (6 patients).

Table 1 Demographic and clinical characteristics of the study population

	All patients	Omeprazole	Lansoprazole	Pantoprazole	Rabeprazole
Number of patients	320	80	80	80	80
Males/Females	156/164	44/36	36/44	39/41	37/43
Mean age (yr)	77.4 ± 7.9	77.9 ± 6.4	77.8 ± 9.2	76.8 ± 6.1	77.0 ± 9.5
Age Range (yr)	65-93	65-93	65-92	65-88	65-93
Esophagitis *n* (%)					
-Grade Ⅰ°	96 (30.0)	34 (42.5)	26 (32.5)	20 (25.0)	16 (20.0)
-Grade Ⅱ°	152 (47.5)	27 (33.8)	33 (41.3)	42 (52.5)	50 (62.5)
-Grade Ⅲ°-Ⅳ°	72 (22.5)	19 (23.8)	21 (26.2)	18 (22.5)	14 (27.6)
Hiatus hernia *n* (%)	194 (60.6)	43 (53.8)	48 (60.0)	50 (62.5)	53 (66.3)
H pylori infection *n/n* (%)	202/306 (66.0)	52/76 (68.4)	61/76 (80.3)	51/77 (66.2)	38/77 (49.3)
NSAIDs/Aspirin use *n* (%)	78 (24.4)	18 (22.5)	17 (21.3)	26 (32.5)	17 (21.3)

Table 2 Healing rates, drop-out patients, and side effects in elderly patients divided according to the different PPI regimens

Regimen	No. of patients	Per protocol analysis		Intention to treat analysis		Drop outs	Side effects
		Cure rates % (N° of patients)	95% CI	Cure rates % (N° of patients)	95% CI		
Omeprazole	80	81.0 60/74	72.0–89.9	75.0 60/80	65.0–84.0	6 (7.5)	1
Lansoprazole	80	90.7 68/75	84.1–97.2	85.0 68/80	77.0-92.8	5 (6.3)	1
Pantoprazole	80	93.5[1] 72/77	87.9–99.0	90.01 72/80	83.4-96.5	3 (3.8)	1
Rabeprazole	80	94.6[2] 71/75	89.4–99.7	88.82 71/80	81.5-95.6	5 (6.3)	1
Total	320	90.0 271/301	86.6-93.4	84.7 271/320	80.7-88.6	19 (5.9)	4 (1.4)

[1]Pantoprazole *vs* Omeprazole: PP analysis: $P = 0.039$, ITT analysis $P = 0.022$; [2]Rabeprazole *vs* Omeprazole: PP analysis: $P = 0.022$, ITT analysis $P = 0.040$.

Table 3 Healing rates of esophagitis after eight weeks of PPI treatment in elderly patients with esophagitis divided according to the grades of severity of esophagitis according to the Savary-Miller classification

Severity grades	Omeprazole		Lansoprazole		Pantoprazole		Rabeprazole	
	n	%	*n*	%	*n*	%	*n*	%
Ⅰ grade[a]	27/33	81.8	25/25	100	20/20	100	14/14	100
Ⅱ grade[c]	18/22	81.8	28/29	96.5	36/40	90.0	46/48	95.8
Ⅲ-Ⅳ grades[e]	15/19	78.9	15/21	71.4	16/17	94.1	11/13	84.6

[a]$P = 0.012$; [c]$P = 0.215$; [e]$P = 0.458$.

Among the 301 patients who completed the study, 271 had healed esophagitis and 30 were unhealed. The overall PP and ITT healing rates of esophagitis were 90.0% (95% CI = 86.6-93.4) and 84.7% (95% CI = 80.7-88.6), respectively. Dividing patients according to treatments, the PP and ITT healing rates of esophagitis were: omeprazole = 81.0% and 75.0%, lansoprazole = 90.7% ($P = 0.143$ *vs* omeprazole) and 85% ($P = 0.167$ *vs* omeprazole), pantoprazole = 93.5% ($P = 0.04$ *vs* omeprazole) and 90.0% ($P = 0.02$ *vs* omeprazole), rabeprazole = 94.6% ($P = 0.02$ *vs* omeprazole) and 88.8% ($P = 0.04$ *vs* omeprazole) respectively (Table 2). Dividing patients according to the grades of esophagitis, a significantly lower healing rate was observed in patients with grade 1 esophagitis treated with omeprazole compared to patients treated with lansoprazole, pantoprazole, or rabeprazole (healing rates: 81.8% *vs* 100%, 100% and 100%, respectively, $P = 0.012$). Omeprazole was less effective than the three other PPIs also in patients with grade 2 esophagitis (healing rates: 81.8% *vs* 96.5% *vs* 90% *vs* 95.8%, respectively) and than pantoprazole and rabeprazole in grade 3-4 esophagitis (healing rates: 78.9% *vs* 94.1% *vs* 84.6%, respectively); probably due to the low number of patients, however, the differences were no statistically significant (Table 3).

At baseline 188 of 288 patients (65.3%) were identified as infected with *H pylori* in the gastric mucosa. No differences were observed in the healing rates of esophagitis between *H pylori* positive and *H pylori* negative patients (90.4% *vs* 89.0%, P = NS). After two months, 149 of 188 (79.3%) who were treated with triple therapies for one week were *H pylori* negative while 39 patients (20.7%) remained *H pylori* positive after treatment. No significant differences in the healing rates of esophagitis were observed between successfully and unsuccessfully treated *H pylori* patients (negative *H pylori vs* still-positive after treatment: 89.9% *vs* 92.3%, P = NS) (Table 4).

After two months of PPI treatment, a significant reduction of symptoms as compared to baseline was observed both in healed and in unhealed patients. While heartburn improved significantly more effectively in healed patients than unhealed patients (rates of heartburn

Table 4 Healing rates of esophagitis in elderly patients divided according to *H pylori* infection

	Omeprazole (*n* = 71)	Lansoprazole (*n* = 71)	Pantoprazole (*n* = 74)	Rabeprazole (*n* = 72)	All (*n* = 288)
H pylori positive *n* = 188	38/49 77.6%	54/57 94.7%	45/48 93.8%	33/34 97.1%	170/188 90.4%
H pylori negative *n* = 100	19/22 86.4%	11/14 78.6%	24/26 92.3%	35/38 92.1%	89/100 89.0%
	Omeprazole (*n* = 49)	Lansoprazole (*n* = 57)	Pantoprazole (*n* = 48)	Rabeprazole (*n* = 34)	All (*n* = 188)
H pylori cured *n* = 149	24/32 75.0%	43/46 93.5%	39/42 92.6%	28/29 96.6%	134/149 89.9%
H pylori still-positive *n* = 39	14/17 82.4%	11/11 100%	6/6 100%	5/5 100%	36/39 92.3%

Table 5 Symptoms in elderly patients with esophagitis before and after two months of PPI therapy

Symptoms	Before therapy	After therapy		
	All	Healed	Unhealed	Healed *vs* unhealed
	n = 301	*n* = 271	*n* = 30	*P* value
Heartburn (*n*, %)	131 (43.5)	9 (3.3)	6 (20.0)	0.0001
Acid regurgitation (*n*, %)	39 (13.0)	4 (1.5)	0 (0.0)	0.874
Epigastric pain (*n* , %)	143 (47.5)	10 (3.7)	2 (6.6)	0.781
Dysphagia (*n*, %)	10 (3.3)	0 (0.0)	0 (0.0)	--
Vomiting (*n*, %)	60 (19.9)	0 (0.0)	0 (0.0)	--
Anaemia (*n*, %)	28 (9.3)	0 (0.0)	0 (0.0)	--

Table 6 Symptom disappearance after therapy in elderly patients divided according to PPI regimens %

	Omeprazole	Lansoprazole	Pantoprazole	Rabeprazole
Heartburn	86.9[a]	82.4[b,d]	100	100
Acid regurgitation	100	75.0[c,f]	92.2	90.1
Epigastric pain	95	82.6[e,h]	95.2	100
Dysphagia	100	100	100	100
Vomiting	100	100	100	100
Aenemia	100	100	100	100

[a]$P < 0.05$ Omeprazole *vs* Pantoprazole and Omeprazole *vs* Rabeprazole; [b]$P = 0.0001$ Lansoprazole *vs* Pantoprazole; [d]$P = 0.005$ Lansoprazole *vs* Rabeprazole; [f]$P = 0.0001$ Lansoprazole *vs* Omeprazole; [c]$P < 0.05$ Lansoprazole *vs* Pantoprazole, Lansoprazole *vs* Rabeprazole; [e]$P < 0.05$ Lansoprazole *vs* Omeprazole and Lansoprazole *vs* Pantoprazole; [h]$P = 0.0001$ Lansoprazole *vs* Rabeprazole.

disappearance = 96.7% *vs* 80%, $P = 0.001$), other symptoms improved significantly both in healed and unhealed patients (Table 5). The rates of symptom disappearance in the four treatment groups, i.e. omeprazole, lansoprazole, pantoprazole, and rabeprazole, were 86.9%, 82.4%, 100%, and 100% for heartburn, 100%, 75.0%, 92.9%, and 90.1% for acid regurgitation, and 95.0%, 82.6%, 95.2, and 100% for epigastric pain, respectively (Table 6). Comparisons between the four PPIs demonstrated that pantoprazole and rabeprazole were more effective than omeprazole (100% *vs* 86.9, and 100% *vs* 86.9%, respectively, $P < 0.05$) and than lansoprazole (100% *vs* 82.4%, $P = 0.0001$ and 100% *vs* 82.4%, $P = 0.005$, respectively) in decreasing heartburn. Lansoprazole was less effective in improving acid regurgitation and epigastric pain than omeprazole ($P = 0.0001$, $P = 0.033$, respectively), pantoprazole ($P = 0.005$, $P = 0.028$, respectively), and rabeprazole ($P = 0.026$, $P = 0.0001$, respectively) (Table 6).

All four PPIs were well tolerated. Adverse events were reported only by four patients (1.3%): orticaria, glossitis, nausea, and headache. Two patients discontinued therapy due to treatment-related side effects. No significant differences were found in the prevalence of adverse events among the four treatment groups.

DISCUSSION

This study demonstrates that in patients over 65 years PPI therapy for 2 mo is very effective in healing acute esophagitis. The pooled ITT and PP healing rates were 84.7% and 90.0%, respectively: These are comparable to previous data from double-blind studies carried out in non-elderly subjects treated for 8 wk with omeprazole 20 mg or lansoprazole 30 mg daily[13], pantoprazole 40 mg daily[14], or rabeprazole 20 mg daily[15]. In this population of older patients, pantoprazole and rabeprazole were significantly more effective in healing esophagitis than omeprazole. Moreover, pantoprazole and rabeprazole were more effective than lansoprazole and omeprazole in improving heartburn, and than lansoprazole in improving acid regurgitation and epigastric pain.

Previous studies were focused on potential discrepancies in efficacy among the different PPIs used for treatment of reflux esophagitis. While some previous reports suggest that acid-suppressive effect of the four PPIs is different on the basis of equivalent molecular dose, clinical studies that support such a different efficacy in healing esophagitis or improving symptoms of GERD on a PPI-equivalent molecular doses are lacking. A meta-analysis of 38 studies evaluating acute therapy of esophagitis reported that the PPIs were superior to ranitidine and placebo in healing erosive esophagitis, without significant differences in efficacy between omeprazole 20 mg daily and lansoprazole 30 mg daily, or pantoprazole 40 mg daily, or rabeprazole 20 mg daily[16]. Similarly, in another meta-analysis, no differences in healing rates of esophagitis were reported between standard doses of lansoprazole, pantoprazole, rabeprazole, and omeprazole[17]. More recently, a meta-analysis of eleven studies with 23 treatment arms reported no significant difference in the two-month healing rates of esophagitis between omeprazole 20 mg daily (*n* = 3.137

patients, pooled healing rate = 84.5%) and other PPIs, including lansoprazole, pantoprazole, rabeprazole, and esomeprazole at standard doses (n = 3.397 patients, pooled healing rate = 89.4%)[18]. However, none of the studies included in these meta-analyses were carried out specifically in elderly patients. Indeed, to our knowledge, this is the first study that compared the efficacy of different PPIs in curing esophagitis and improving symptoms in elderly patients.

Why pantoprazole and rabeprazole were more effective than omeprazole in healing esophagitis and than omeprazole and lansoprazole in improving symptoms in elderly patients is not clear. Very recently it was suggested that omeprazole has considerable potential for drug interactions since it has high affinity for the cytochrome CYP2C19 and a lower affinity for the cytochrome CYP3A4, while pantoprazole, and maybe rabeprazole, appear to have lower potential for interactions with other drugs[19]. Data from this study cannot confirm this hypothesis since no information was collected on concomitant treatments, with the exception of NSAID and aspirin. Interestingly, a previous multicentre study, carried out in 164 elderly patients with esophagitis reported that a 2-month therapy with pantoprazole 40 mg/d was highly effective in healing reflux esophagitis (81.1% and 93.7% by ITT and PP analyses, respectively), although the majority of patients received other drugs for concomitant illnesses (76.2% of patients), without that the presence of concomitant treatments adversely affected the efficacy or tolerability of pantoprazole[20].

Very recently, a systematic review of randomized controlled trials in patients with reflux esophagitis reported that esomeprazole demonstrated higher short-term healing rates when compared with standard dose PPIs[21]. While no data were reported comparing rabeprazole 20 mg to esomeprazole 40 mg, two studies included in the analysis compared pantoprazole 40 mg daily to esomperazole 40 mg daily. This comparison found no differences in healing rates between the two treatments both in patients with moderate-severe esophagitis, i.e. Los Angeles grades B and C (healing rates with pantoprazole = 83.2% *vs* esomeprazole = 80.7%, P = NS)[22] and in the subgroup of 550 patients aged 65 years or over included in the large multicenter EXPO study (healing rates with pantoprazole = 87.4% *vs* esomeprazole = 90.4%, P = NS)[23]. Unfortunately, information on esomeprazole was not available for the present study.

In this elderly population, *H pylori* infection did not influence the response to short-term treatment with PPIs. This finding confirms the data of previous studies performed in elderly populations showing that *H pylori* infection does not have a negative effect on healing of esophagitis, nor does it worsen reflux symptoms at two-month follow up[24]. It is also evident from our study that *H pylori* eradication does not affect the cure rate of esophagitis during a two-month course of PPI, in agreement with a recent multicentre randomized study also performed in elderly patients[25].

In conclusion, PPIs are highly effective and well tolerated in curing gastroesophageal reflux disease in elderly patients. Pantoprazole and rabeprazole were significantly more effective than omeprazole in healing esophagitis and than omeprazole or lansoprazole in improving symptoms. *H pylori* infection does not influence the healing rates of esophagitis after a short-term treatment with PPI.

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CLINICAL RESEARCH

Expression of Angiopoietin-1, 2 and 4 and Tie-1 and 2 in gastrointestinal stromal tumor, leiomyoma and schwannoma

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Abstract

AIM: To investigate the role of angiopoietin (Ang) -1, -2 and -4 and its receptors, Tie-1 and -2, in the growth and differentiation of gastrointestinal stromal tumors (GISTs).

METHODS: Thirty GISTs, seventeen leiomyomas and six schwannomas were examined by immunohistochemistry in this study.

RESULTS: Ang-1, -2 and -4 proteins were expressed in the cytoplasm of tumor cells, and Tie-1 and -2 were expressed both in the cytoplasm and on the membrane of all tumors. Immunohistochemical staining revealed that 66.7% of GISTs (20 of 30), 76.5% of leiomyomas (13 of 17) and 83.3% of schwannomas (5 of 6) were positive for Ang-1. 83.3% of GISTs (25 of 30), 82.4% of leiomyomas (14 of 17) and 100% of schwannomas (6 of 6) were positive for Ang-2. 36.7% of GISTs (11 of 30), 58.8% of leiomyomas (10 of 17) and 83.3% of schwannomas (5 of 6) were positive for Ang-4. 60.0% of GISTs (18 of 30), 82.4% of leiomyomas and 100% of schwannomas (6 of 6) were positive for Tie-1. 10.0% of GISTs (3 of 30), 94.1% of leiomyomas (16 of 17) and 33.3% of schwannomas (2 of 6) were positive for Tie-2. Tie-2 expression was statistically different between GISTs and leiomyomas ($P < 0.001$). However, there was no correlation between expression of angiopoietin pathway components and clinical risk categories.

CONCLUSION: Our results suggest that the angiopoietin pathway plays an important role in the differentiation of GISTs, leiomyomas and schwannomas.

Key words: Gastrointestinal stromal tumors; Leiomyomas; Schwannomas

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INTRODUCTION

Gastrointestinal stromal tumors (GISTs) are rare mesenchymal tumors of the gastrointestinal tract that may occur from the oesophagus to the anus, including the omentum[1,2]. Despite their rarity, GISTs are the most common primary mesenchymal tumors of the gastrointestinal tract[1-3]. The mechanisms of tumorigenesis, progression and differentiation of GISTs are unknown. Traditionally, all primary mesenchymal spindle cell tumors of the gastrointestinal (GI) tract were uniformly classified as smooth muscle tumors (e.g., leiomyomas, cellular leiomyomas or leiomyosarcomas). Tumors with epithelioid cytologic features were designated leiomyoblastomas or epithelioid leiomyosarcomas[4]. Recently, Sircar *et al*[5] postulated that GISTs originate from Cajal cells in the gastrointestinal tract and differ from leiomyomas and schwannomas, which are of mesenchymal cell origin. Cajal cells are thought to be gastrointestinal pacemaker cells that regulate intestinal motility[6]. GISTs are characterized by frequent expression of the bone marrow leukocytic progenitor cell antigen CD34[7] and the c-kit proto-oncogene[2,3,5].

Some GISTs have mutations in the genes encoding C-kit and platelet-derived-growth factor alpha (PDGFRA) that cause constitutive tyrosine kinase activation[3,8-10]. Tumors expressing C-kit or PDGFRA oncoproteins were indistinguishable with respect to activation of downstream signaling intermediates and cytogenetic changes associated with tumor progression. C-kit and PDGFRA mutations appear to be alternative and mutually exclusive oncogenic mechanisms in GISTs[9,10].

Recently, there has been a growing interest in understanding the role of receptor tyrosine kinases (RTK), such as vascular endothelial growth factor

receptor (VEGFR), platelet-derived growth factor receptor (PDGFR) and stem cell factor receptor (KIT) in promoting tumor growth and metastasis[3,9,11]. As both Tie-1 and Tie-2 possess unique multiple extracellular domains, they are thought to represent a new subfamily of RTKs[12,13]. Tie signaling is involved in multiple steps of the angiogenic remodeling process during development, including destabilization of existing vessels, endothelial cell migration, tube formation and the subsequent stabilization of newly formed tubes by mesenchymal cells[14-17].

The angiopoietin (Ang) family has been identified as a key regulator of angiogenesis[18] and is composed of subtypes Ang-1, Ang-2, Ang-3, and Ang-4[18-21]. They are the ligands for Tie receptors and the major mediators of the mitogenic and permeability-enhancing effects in endothelial cells[17,22]. In addition, angiopoietins are survival factors for endothelial cells, and a marked dependence on angiopoietins has been shown in newly formed, but not established tumor vessels[23,24].

Ang-1 has been shown to act as an obligatory agonist promoting structural integrity of blood vessels[18,20], whereas Ang-2 has been found to function as a naturally occurring antagonist, promoting either vessel growth or regression depending on the levels of other growth factors, such as VEGF-A[19,25]. The effect of Ang-3 and Ang-4 have been less characterized, but they also show context-dependent actions as antagonistic and agonistic ligands, respectively[21,26]. Signaling through Tie-2 has been extensively studied, and the results suggest that signaling involving phosphatidylinositol 3' kinase (PI3K) activation is a major pathway[27-29]. The ligand-independent function of Tie-1 involves shedding of the receptor[30,31] and heterometrix complex formation with Tie-2[30,32]. Recently, it has been found that Ang-1 and Ang-4 can activate Tie-1[33].

Coexpression of angiopoietin and its receptor, either Tie-1 or Tie-2, has been reported in tumor cells, suggesting the presence of an autocrine and/or a paracrine angiopoietin/Tie growth pathway in solid tumors[34-36]. Further, the expression levels of angiopoietin and its receptors have been shown to correlate with progressive tumor growth and development of metastasis by many types of carcinomas[35-38].

These studies suggest that the angiopoietin pathway is involved in tumor cell growth and differentiation. However, there are no data detailing angiopoietin expressions and Tie receptor expresions in GIST, leiomyoma or schwannoma, or the role of angiopoietin in the etiology of these tumors. The purpose of this study was to investigate the expression of angiopoietins and Ties in GISTs.

MATERIALS AND METHODS

Samples

A total of thirty GISTs included 26 cases from the stomach and four from the small intestine. Seventeen leiomyomas included four from the oesophagus, five from the stomach and eight from the large intestine. Six schwannomas included five from the stomach and one from the large intestine. Specimens were selected from surgical pathology archival tissues at Nagasaki University Hospital between 2001 and 2006. The GISTs were 0.8-12.0 cm in diameter, the leiomyomas were 0.1-4.5 cm, and the schwannomas were 0.6-5.0 cm. In this study, GISTs were defined as tumors expressing both c-kit and CD34 surface antigens. GISTs were classified by risk categories, mitosis counts and tumor size[39]. The number of mitoses was determined by counting 50 high-power fields (HPF, × 400) using a Nikon (Tokyo, Japan) E400 microscope. Leiomyomas were defined as expressing α-smooth muscle cell actin (SMA) but not c-kit, CD34 or S100-protein. Schwannomas were defined as expressing S100-protein but not c-kit, CD34 or SMA. Two independent pathologists (T. Nakayama and I. Sekine) determined tumor identification/classification.

Immunohistochemical staining

The subcellular localization of Ang-1, -2 and -4 and Tie-1 and -2 was determined in GISTs using polyclonal antibodies directed against unique sequences. These antibodies were devoid of any cross-reaction with other proteins in the angiopoietin family. Formalin-fixed and paraffin-embedded specimens were cut into 4 μm thick sections, deparaffinized and preincubated with normal bovine serum to prevent non-specific binding. The sections were incubated overnight at 4℃ with primary polyclonal antibody to human Ang-1, -2 or -4 ([N-18],[N-18],[L-18], respectively, 1 μg/mL; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Tie-1 or -2 ([C-18],[C-20], respectively, 1 μg/mL; Santa Cruz Biotechnology, Inc.), followed by alkaline phosphatase-conjugated anti-goat IgG antibody (0.4 μg/mL; Santa Cruz Biotechnology, Inc.) for Ang-1, -2 and -4, and anti-rabbit IgG antibody (0.4 μg/mL; Santa Cruz Biotechnology, Inc.) for Tie-1 and -2. The reaction products were visualized using a mixture of 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride (BCIP/NBT; Roche Diagnostic Corp., Indianapolis, IN). Negative controls replaced the primary antibody with non-immunized rabbit or goat serum, and the hemangioma tissue of human skin served as the positive control[40]. Ang-1, -2 and -4 and Tie-1 and -2 expressions were classified into three categories depending upon the percentage of cells stained and/or the intensity of staining: -, 0 to 10% tumor cells positive; +, > 10% tumor cells positive.

Statistical analysis

The Stat View Ⅱ program (Abacus Concepts, Inc., Berkeley, CA) was used for statistical analysis. Analyses comparing the degree of Ang-1, -2 and -4 and Tie-1 and -2 expressions in GISTs, leiomyomas and schwannomas were performed using the Mann-Whitney's test.

RESULTS

The results of immunohistochemical stainings for Ang-1, -2 and -4 and Tie-1 and -2 are summarized in Table 1. Ang-1, -2 and -4 and Tie-1 and -2 were heterogeneously expressed in GISTs, leiomyomas and schwannomas and

Table 1 Tie and Ang immunohistochemistry in intestinal stromal tumors *n* (%)

		Ang-1		Ang-2		Ang-4		Tie-1		Tie-2	
	n	-	+	-	+	-	+	-	+	-	+
GIST	30	10 (33.3)	20 (66.7)	5 (16.7)	25 (83.3)	19 (63.3)	11 (36.7)	12 (40.0)	18 (60.0)	27 (90.0)	3 (10.0)[a]
Leiomyoma	17	4 (23.5)	13 (76.5)	3 (17.6)	14 (82.4)	7 (41.2)	10 (58.8)	3 (17.6)	14 (82.4)	1 (5.9)	16 (94.1)
Schwannoma	6	1 (16.7)	5 (83.3)	0 (0.0)	6 (100)	1 (16.7)	5 (83.3)	0 (0.0)	6 (100)	4 (66.7)	2 (33.3)

[a]$P < 0.001$ *vs* leiomyoma.

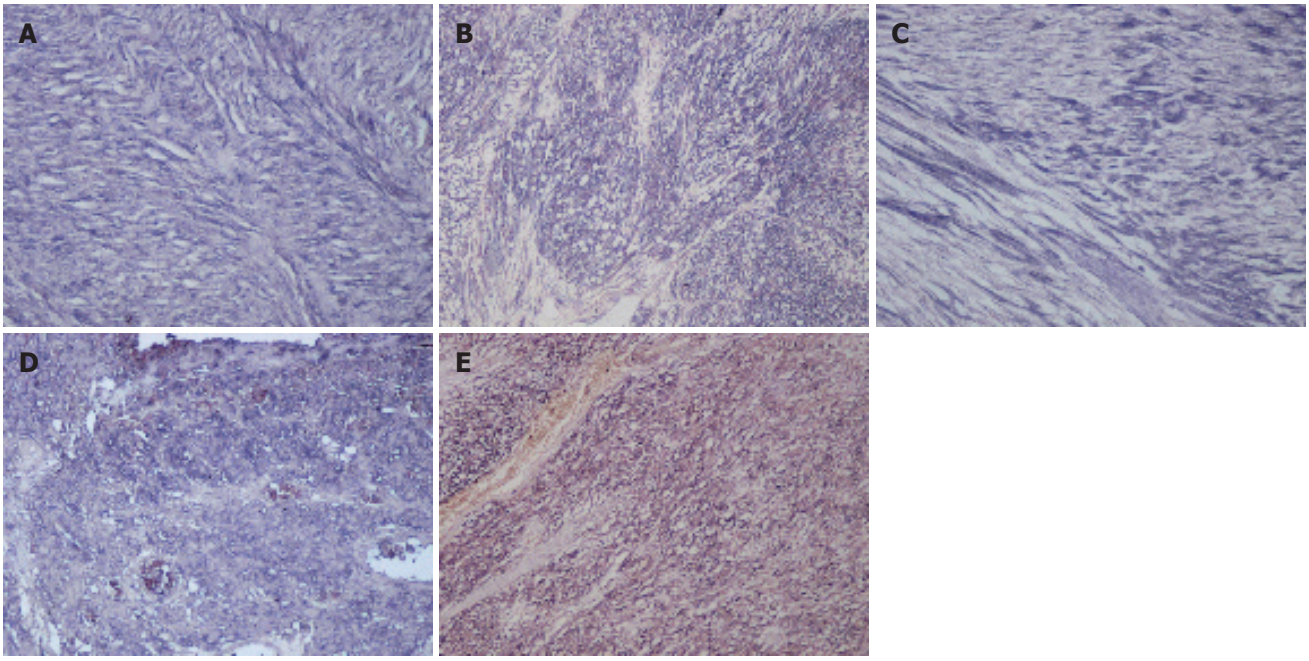

Figure 1 Immunohistochemical staining of Angiopoietin pathway components. Alkaline phosphatase reaction products demonstrating Ang-1 (**A**), Ang-2 (**B**), Ang-4 (**C**), Tie-1 (**D**) and Tie-2 (**E**) expression. Ang-1, -2 and -4 were expressed in the cytoplasm, and Tie-1 and -2 were expressed in both the cytoplasm and the cell membrane of GIST cells (x 200).

localized to the cytoplasm and/or membrane of tumor cells. Immunohistochemical staining revealed Ang-1, -2 and -4 in the cytoplasm of GIST (Figure 1A-C), leiomyoma (Figure 2A-C) and schwannoma (Figure 3A-C) cells. Tie-1 and -2 were found in the membrane and cytoplasm of GIST (Figure 1D and E), leiomyoma (Figure 2D and E) and schwannoma (Figure 3D and E) cells. Immunohistochemical staining revealed that 66.7% of GISTs (20 of 30), 76.5% of leiomyomas (13 of 17) and 83.3% of schwannomas (5 of 6) were positive for Ang-1. 83.3% of GISTs (25 of 30), 82.4% of leiomyomas (14 of 17) and 100% of schwannomas (6 of 6) were positive for Ang-2. 36.7% of GISTs (11 of 30), 58.8% of leiomyomas (10 of 17) and 83.3% of schwannomas (5 of 6) were positive for Ang-4. There were no statistical differences in Ang-1, -2 or -4 expression between GISTs and leiomyomas or schwannomas. 60.0% of GISTs (18 of 30), 82.4% of leiomyomas and 100% of schwannomas (6 of 6) were positive for Tie-1. 10.0% of GISTs (3 of 30), 94.1% of leiomyomas (16 of 17) and 66.7% of schwannomas (2 of 6) were positive for Tie-2. Tie-2 expression was statistically different between GISTs and leiomyomas ($P < 0.001$). However, there was no correlation between Tie-1 expression and histological differences.

GISTs were classified by risk category, mitosis counts and tumor size in Table 2. All six cases within the high risk category expressed Ang-1 and -2 and Tie-1 and -2 proteins. All three cases with over 10 mitoses per 50 HPFs strongly expressed Ang-1, -2 and -4 and Tie-1 and -2. Finally, only two tumors that measured over 10 cm strongly expressed Ang-1, -2 and -4 and Tie-1 and -2. However, there was no correlation between Ang-1, -2 and -4 and Tie-1 and -2 expression and each classification.

DISCUSSION

The coexpression of Ang-1, -2 and -4 and Tie-1 and -2 has been reported in tumor cells, suggesting the presence of an autocrine and/or a paracrine angiopoietin/Tie growth pathway in solid tumors[35-38]. Angiopoietins (Angs) also have been shown to play a role in the proliferation and/or differentiation of stromal tumors and normal mesenchymal cells[41,42]. Angiopoietin expression in GISTs has not been reported yet. Further, there have been no studies of Tie receptor expression in GISTs, leiomyomas and schwannomas or of the potential roles of angiopoietins and its receptors in the growth of these tumors. This is the first study to determine the expression of Tie receptors

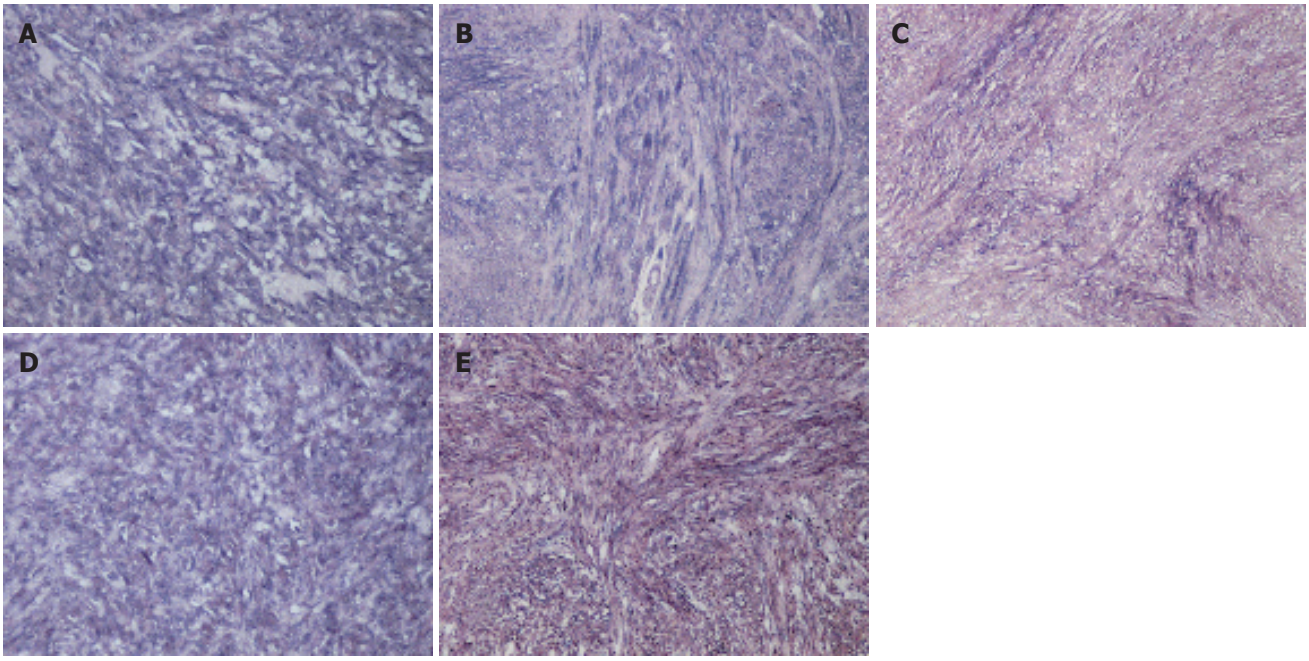

Figure 2 Immunohistochemical staining of human intestinal leiomyomas. Alkaline phosphatase reaction products demonstrating Ang-1 (**A**), Ang-2 (**B**), Ang-4 (**C**), Tie-1 (**D**) and Tie-2 (**E**) expression (x 200).

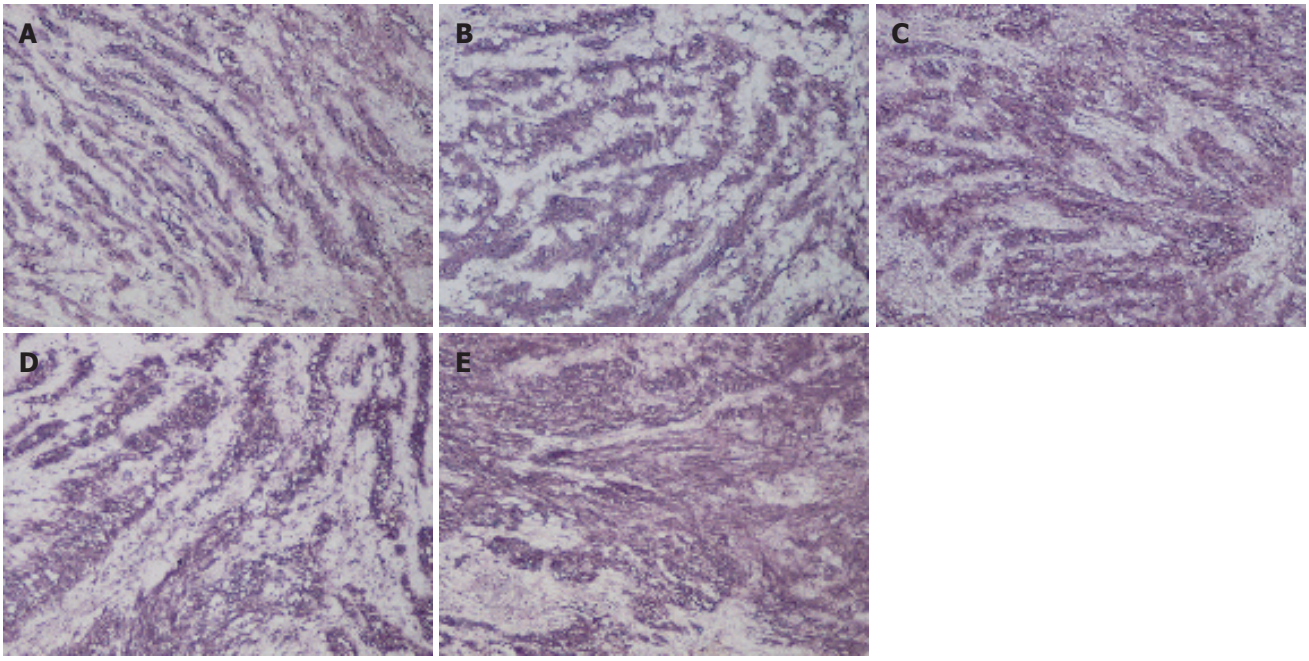

Figure 3 Immunohistochemical staining of human intestinal schwannomas. Alkaline phosphatase reaction products demonstrating Ang-1 (**A**), Ang-2 (**B**), Ang-4 (**C**), Tie-1 (**D**) and Tie-2 (**E**) expression (x 200).

in GIST and stromal tumors. Our results demonstrate substantial levels of angiopoietins and Tie receptors in the cytoplasm of GIST, leiomyoma and schwannoma cells. Therefore, we suggest that angiopoietins and its receptors may play an important role in growth and/or differentiation of intestinal stromal tumors via autocrine and/or paracrine pathways.

We did not find any statistical correlation between risk grade and the expression of Angs or Ties for GISTs. However, all four GISTs in the high risk category expressed Angs and Ties (Table 2). Further, all four GISTs that had higher mitosis counts (over ten per 50 HPFs) were positive for Angs and Ties. Our data suggest that high risk GISTs might express Angs and Ties at higher than normal levels. Future studies will examine angiopoietin pathway components in high risk GISTs.

Ang-2 induces a variety of enzymes and proteins important in the degradation process, including matrix-degrading metalloproteinases, metalloproteinase interstitial collagenase, and serine proteases, such as urokinase-

Table 2 Tie and Ang expression and risk categories for GISTs (30 cases) *n* (%)

		Ang-1		Ang-2		Ang-4		Tie-1		Tie-2	
	n	-	+	-	+	-	+	-	+	-	+
GIST	30	10 (33.3)	20 (66.7)	5 (16.7)	25 (83.3)	19 (63.3)	11 (36.7)	12 (40.0)	18 (60.0)	27 (90.0)	3 (10.0)
Risk categories		NS		NS		NS		NS		NS	
High	6	1 (16.7)	5 (83.3)	2 (33.3)	4 (66.7)	6 (100)	0 (0.0)	1 (16.7)	5 (83.3)	5 (83.3)	1 (16.7)
Intermediate	4	2 (50.0)	2 (50.0)	1 (25.0)	3 (75.0)	2 (50.0)	2 (50.0)	2 (50.0)	2 (50.0)	4 (100)	0 (0.0)
Low	15	6 (40.0)	9 (60.0)	2 (13.3)	13 (86.7)	8 (53.3)	7 (46.7)	5 (33.3)	10 (66.7)	13 (86.7)	2 (13.3)
Very low	5	1 (20.0)	4 (80.0)	0 (0.0)	5 (100)	3 (60.0)	2 (40.0)	4 (80.0)	1 (20.0)	5 (100)	0 (0.0)
Mitosis counts (per 50 fields, HPF)		NS		NS		NS		NS		NS	
< 2	19	7 (36.8)	12 (63.2)	3 (15.8)	16 (84.2)	11 (57.9)	8 (42.1)	9 (47.4)	10 (52.6)	17 (89.5)	2 (10.5)
2-5	7	2 (28.6)	5 (71.4)	1 (14.3)	6 (85.7)	4 (57.1)	3 (42.9)	2 (28.6)	5 (71.4)	6 (85.7)	1 (14.3)
6-10	2	1 (50.0)	1 (50.0)	1 (50.0)	1 (50.0)	2 (100)	0 (0.0)	0 (0.0)	2 (100)	2 (100)	0 (0.0)
10 <	2	0 (0.0)	2 (100)	0 (0.0)	2 (100)	2 (100)	0 (0.0)	1 (50.0)	1 (50.0)	2 (100)	0 (0.0)
Tumor size (cm in length)		NS		NS		NS		NS		NS	
< 2	4	1 (25.0)	3 (75.0)	0 (0.0)	4 (100)	3 (75.0)	1 (25.0)	3 (75.0)	1 (25.0)	4 (100)	0 (0.0)
2–5	16	6 (37.5)	10 (62.5)	2 (12.5)	14 (87.5)	9 (56.3)	7 (43.8)	6 (37.5)	10 (62.5)	15 (93.8)	1 (6.3)
5–10	7	3 (42.9)	4 (57.1)	2 (28.6)	5 (71.4)	4 (57.1)	3 (42.9)	2 (28.6)	5 (71.4)	6 (85.7)	1 (14.3)
> 10	3	0 (0.0)	3 (100)	1 (33.3)	2 (66.7)	3 (100)	0 (0.0)	1 (33.3)	2 (66.7)	2 (66.7)	1 (33.3)

NS: not significant.

type plasminogen activator (u-PA)[43,44]. In this study, we did not evaluate the invasive activities of GIST cells because all of the GISTs were solitary and showed clear margins. However, the activation of these various factors by angiopoietins may allow for the development of a prodegradative environment that facilitates migration and invasion of tumor cells.

There has been a growing interest in understanding the role of receptor tyrosine kinases (RTK), such as vascular endothelial growth factor receptor (VEGFR)[11], platelet-derived growth factor receptor (PDGFR)[9] and stem cell factor receptor (KIT)[3] in promoting tumor growth and metastasis. Joensuu *et al*[45] reported a patient in whom Imatinib (STI-571, Gleevec), a tyrosine kinase inhibitor, was effective against a GIST. Imatinib has proven to be remarkably efficacious in heavily pretreated GIST patients with advanced disease[46]. Further, anti-angiopoietin reagents are being used in clinical trials for the therapy of gastric, lung and breast cancer[47,48].

Sunitinib (sunitinib malate; SU11248; SUTENT®; Pfizer Inc, New York, NY, USA) is a novel multi-targeted tyrosine kinase inhibitor with high binding affinity for VEGFR and PDGFR that recently has shown anti-tumor and anti-angiogenic activities[49]. This drug recently received approval from the US Food and Drug Administration (FDA) for two applications: advanced gastrointestinal stromal tumors (GISTs)[50] and renal cell carcinoma[51], in patients who are resistant or intolerant to treatment with Imatinib. Since the Tie receptor is an RTK, Sunitinib may be suitable to down-regulate the activity of the angiopoietin pathway. In fact, this study presents data that supports the clinical validity of Sunitinib in GISTs.

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RAPID COMMUNICATION

Perimuscular connective tissue contains more and larger lymphatic vessels than the shallower layers in human gallbladders

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Abstract

AIM: To clarify whether perimuscular connective tissue contains more lymphatic vessels than the shallower layers in human gallbladders.

METHODS: Lymphatic vessels were stained immunohistochemically with monoclonal antibody D2-40, which is a specific marker of lymphatic endothelium, in representative sections of 12 normal human gallbladders obtained at the time of resection for colorectal carcinoma liver metastases. In individual gallbladder specimens, nine high-power (× 200) fields with the highest lymphatic vessel density (LVD), termed "hot spots", were identified for each layer (mucosa, muscle layer, and perimuscular connective tissue). In individual hot spots, the LVD and relative lymphatic vessel area (LVA) were measured microscopically using a computer-aided image analysis system. The mean LVD and LVA values for the nine hot spots in each layer were used for statistical analyses.

RESULTS: In the mucosa, muscle layer, and perimuscular connective tissue, the LVD was 16.1 ± 9.2, 35.4 ± 15.7, and 65.5 ± 12.2, respectively, and the LVA was 0.4 ± 0.4, 2.1 ± 1.1, and 9.4 ± 2.6, respectively. Thus, both the LVD and LVA differed significantly ($P < 0.001$ and $P < 0.001$, respectively; Kruskal-Wallis test) among the individual layers of the wall of the gallbladder, with the highest LVD and LVA values in the perimuscular connective tissue. Most (98 of 108) of the hot spots within the perimuscular connective tissue were located within 500 μm of the lower border of the muscle layer.

CONCLUSION: The perimuscular connective tissue contains more and larger lymphatic vessels than the shallower layers in the human gallbladder. This observation partly explains why the incidence of lymph node metastasis is high in T2 (tumor invading the perimuscular connective tissue) or more advanced gallbladder carcinoma.

Key words: Gallbladder; Lymphatic vessels; Monoclonal antibody D2-40; Gallbladder neoplasms; Lymphatic metastasis

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INTRODUCTION

Gallbladder carcinoma is a highly lethal disease[1,2]. Lymph node metastasis occurs early and is a major reason for the dismal prognosis for this disease[3-5]. The incidence of lymph node metastasis increases with the depth of penetration of the primary tumor; it ranges from 0% to 8%[5-9] for tumors that are limited to the mucosa or muscle layer (T1 disease according to the TNM staging system[10]), whereas it ranges from 33% to 62%[5-7,9,11,12] for tumors that have invaded the perimuscular connective tissue (T2 disease). This suggests that lymph node metastasis is frequent once the primary tumor has invaded the perimuscular connective tissue of the gallbladder.

The architecture of the lymphatic system of the gallbladder has been examined in various experimental animals, and a well-developed set of lymph channels has been described in the perimuscular connective tissue[13,14]. In 1989, Kambayashi reported similar findings for canine gallbladders[15]. The current study was conducted to clarify immunohistochemically whether the perimuscular connective tissue contains more lymphatic vessels than the mucosa or muscle layer of normal human gallbladders, using monoclonal antibody D2-40, which is a specific marker of the lymphatic endothelium[16-19], and a computer-aided image analysis system. The goal of the present study was to suggest the hypothesis that the presence of more and larg-

er lymphatic vessels in the perimuscular connective tissue is linked to the reported high incidences of lymph node metastasis in T2 (tumor invading the perimuscular connective tissue) or more advanced gallbladder carcinoma[5-7,9,11].

MATERIALS AND METHODS

Twelve patients with colorectal carcinoma liver metastases underwent partial hepatectomy combined with cholecystectomy between January and December, 2005. The patient group included eight men and four women, with a median age of 68.5 years (range, 42-80 years). None of the patients had a history of biliary disease or chronic liver disease. The gallbladder specimens obtained from the patients, all of whom gave informed consent for histologically examining the specimens, were included in the present study.

Individual resected specimens were submitted to the Department of Surgical Pathology in our hospital for gross or histologic examination, which revealed that none of the liver tumors involved the gallbladders. The gallbladders (n = 12) were opened and examined grossly by experienced surgical pathologists, who found neither mucosal lesions nor gallstones in any of the viscera. The gallbladder specimens were then fixed in formalin. A single longitudinal representative section, which passed through both the tip of the fundus of the gallbladder and the cystic bile duct, was cut from each gallbladder specimen and embedded in paraffin. Routine histologic examination with hematoxylin and eosin staining detected no abnormalities in any of the representative sections.

Anatomy of the gallbladder

The gallbladder is divided into three equal parts: the fundus, body, and neck. Histologically, the wall of the viscus comprises three layers: the mucosa, muscle layer, and perimuscular connective tissue (subserosal layer)[10].

Lymphatic vessel parameters

Lymphatic vessel density (LVD) was defined as the number of lymphatic vessels per mm^2; a high-power (× 200) field with the highest LVD in an area was referred to as a "hot spot", in line with earlier studies[20,21]. Relative lymphatic vessel area (LVA) was defined as the percentage of positively stained lymphatic vessel area in a hot spot[20,21].

Immunohistochemistry

The paraffin-embedded blocks of the representative sections (n = 12) were used for immunohistochemistry. Three serial sections (3 μm thickness) were cut from each block: one for routine histologic examination using hematoxylin and eosin staining, one for immunohistochemical staining with monoclonal antibody D2-40, and one as a negative control.

The mouse monoclonal antibody D2-40 (Signet Laboratories, Inc., Dedham, MA) was used at a dilution of 1:200. The streptavidin-biotin immunoperoxidase method was used for detecting immune complexes. The sections were deparaffinized and rehydrated, then microwaved at 500 W for 7 cycles of 3 min each in 10 mmol/L citrate buffer (pH 6.0) to retrieve antigenic activity. After blocking of endogenous peroxidase, the sections were incubated overnight at 4℃ with D2-40. The sections were then incubated at room temperature for 30 min with goat anti-mouse immunoglobulin conjugated to a peroxidase-labeled amino acid polymer, as provided in the SAB-PO (M) Kit (Nichirei Biosciences Inc., Tokyo, Japan). Diaminobenzidine was used as the chromogen, and the sections were counterstained with hematoxylin. Negative controls were treated in the same manner, except that incubation with the primary antibody was omitted. Sections of normal human tonsil were used as positive controls.

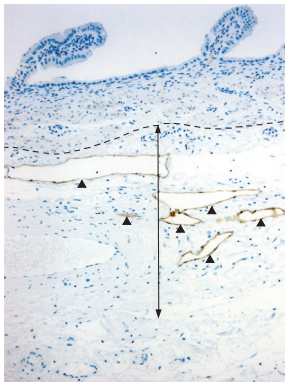

Figure 1 D2-40 antibody-positive lymphatic vessels in the wall of a human gallbladder. The perimuscular connective tissue contains more and larger lymphatic vessels (arrowheads), most of which are located within 500 μm (two-headed arrow) of the lower border of the muscle layer (broken line), than the mucosa or muscle layer (Original magnification, × 100).

Computer-assisted morphometry of lymphatic vessels

The representative sections stained with the D2-40 antibody (n = 12) were examined for LVD and LVA using an Olympus FX 380 microscope (Olympus Co. Ltd., Tokyo, Japan). By scanning each representative section at low power (× 20), a total of nine hot spots, three for each part of the gallbladder (fundus, body, and neck), was identified per layer (mucosa, muscle layer, and perimuscular connective tissue) of the gallbladder. In individual hot spots, the outlines of individual immunohistochemically stained lymphatic vessels were identified and traced using a computer-aided image analysis system (FlvFs, ver 1.10; Flovel Co. Ltd., Tokyo, Japan) at × 200 magnification, to measure LVD and LVA.

Statistical analysis

The mean LVD and LVA for the nine hot spots per each layer (or per each part) of the gallbladder were subjected to statistical analyses. The Kruskal-Wallis test was used to compare the lymphatic vessel parameters (LVD and LVA) among individual layers (or individual parts). Statistical evaluations were performed using the SPSS 11.5J software package (SPSS Japan Inc., Tokyo, Japan). A P value < 0.05 was considered to be statistically significant.

RESULTS

D2-40 antibody-positive lymphatic vessels were found in all 12 gallbladder specimens (Figure 1). Computer-assisted morphometric analysis of the lymphatic vessels showed that both the LVD and LVA values differed significantly (P < 0.001 and P < 0.001, respectively) among the individual layers of the wall of the gallbladder, with the highest LVD and LVA values found in the perimuscular connective tissue (Table 1). Most (98 of 108) of the hot spots within the perimuscular connective tissue were located

Table 1 LVD and LVA values for individual layers of the wall of the gallbladder (mean ± SD, n = 12)

Layer of the wall of the gallbladder	LVD (per mm^2)	P value[a]	LVA (%)	P value[a]
Mucosa	16.1 ± 9.2	< 0.001[b]	0.4 ± 0.4	< 0.001[b]
Muscle layer	35.4 ± 15.7		2.1 ± 1.1	
Perimuscular connective tissue	65.5 ± 12.2		9.4 ± 2.6	

LVD: lymphatic vessel density; LVA: relative lymphatic vessel are; [a]Kruskal-Wallis test, [b]P < 0.001 between mucosa, muscle and perimuscular connective tissue of the gallbladder for both LVD and LVA.

within 500 μm of the lower border of the muscle layer (Figure 1). The LVD and LVA values did not differ among the fundus, body, and neck (data not shown; P = 0.837 and P = 0.756, respectively).

DISCUSSION

The monoclonal antibody D2-40, which was generated against an oncofetal antigen that is expressed in fetal testes and in testicular germ cell tumors, is recognized as a highly specific lymphatic marker that does not react with the vascular endothelium[16-19]. Although it is difficult or sometimes impossible to distinguish morphologically lymphatic vessels from venules or capillaries on routine histologic examination, immunohistochemical staining using D2-40 enables easy identification of lymphatic vessels[16-19]. Using this method, we have clearly demonstrated that the LVD and LVA values are significantly higher in the perimuscular connective tissue than in the mucosa or muscle layer of normal human gallbladders.

A tumor that invades the deep layers of the gallbladder generally shows a higher histologic grade (less differentiation) than a superficial tumor[5,7]. It is very likely that a less-differentiated tumor that invades the perimuscular connective tissue (T2 disease) causes a higher incidence of nodal involvement than a differentiated tumor within the superficial layers (T1 disease). Another explanation for the high incidences of nodal disease in T2 gallbladder carcinoma is the higher abundance of vessels in the perimuscular connective tissue than in the shallower layers[15,22], as clearly demonstrated in the present study. The presence of more and larger lymphatic vessels in the perimuscular connective tissue (Figure 1) may increase the likelihood that the tumor will permeate the lymphatic vessels. The hot spots were prominent in the shallow (≤ 500 μm) zone of the perimuscular connective tissue of the gallbladder (Figure 1); this observation may partly explain why lymph node metastasis often occurs even in tumors with shallow (≤ 2 mm) subserosal invasion[12].

The results of the current study may provide a basis for the investigation of lymphangiogenesis in gallbladder carcinoma. Recent evidence suggests that lymphangiogenesis plays a key role in the development of nodal metastases from various human malignancies, including head and neck squamous cell carcinoma[21], cutaneous melanoma[20,23], breast carcinoma[24], and colorectal carcinoma[25]. However, to the best of the knowledge, no investigations of this type have been conducted on gallbladder carcinoma. Further investigations into the role of lymphangiogenesis in gallbladder carcinoma are warranted.

The limitations of the present study include the retrospective nature of the analysis, the low number of specimens examined, and the gallbladder specimens taken from patients with colorectal carcinoma liver metastases. Despite these limitations, the present work more clearly defines than earlier studies the abundance of lymphatic vessels in the perimuscular connective tissue of the human gallbladder.

In conclusion, the perimuscular connective tissue (subserosal layer) has more and larger lymphatic vessels than the shallower layers of the human gallbladder. This observation partly explains why the incidence of lymph node metastasis is high in T2 (tumor invading the perimuscular connective tissue) or more advanced gallbladder carcinoma.

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RAPID COMMUNICATION

Diagnostic accuracy of a rapid fecal test to confirm *H pylori* eradication after therapy: Prospective comparison with a laboratory stool test

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Abstract

AIM: To investigate the clinical performances of rapid stool test (ImmunoCard STAT HpSA, Meridian Diagnostic Inc.) in the evaluation of eradication therapy of *H pylori* and to compare it with a well-known and validated laboratory stool test (Amplified IDEA Hp StAR, Dako).

METHODS: Stool samples of 122 patients were evaluated after eradication therapy of *H pylori*. *H pylori* status was assessed by 13C-urea breath test (UBT). Stool specimens were tested using either the rapid immunoassay kit or the laboratory immunoassay kit.

RESULTS: Forty-three patients were infected and 79 non-infected. Sensitivity and specificity of ImmunoCard STAT and Hp StAR were 58.14% and 76.4%, and 97.47% and 98.73%, respectively ($P > 0.05$). Overall agreement between the two tests was 92.6% (113 of 122 cases).

CONCLUSION: ImmunoCard STAT seems to have rather low performances, and it cannot be regarded as a reliable tool in the post-treatment setting. Also Hp StAR cannot be recommended to confirm *H pylori* eradication after treatment.

Key words: *H pylori*; Diagnosis; Feces

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INTRODUCTION

Nowadays, there is an increasing interest in non-invasive methods to diagnose *H pylori* infection. Indeed, they can profitably replace endoscopy in predicting the diagnosis and determining the management of some types of patients, according to a "test and treat" strategy[1]. Post-therapy testing to confirm eradication is also growing in importance, as resistant strains of *H pylori* are now widely prevalent in both USA and Europe, with a failure rate of current eradication regimens ranging from 10% to 20%[2,3]. Furthermore, in patients with bleeding peptic ulcer, the risk for rebleeding is greatly increased if *H pylori* infection persists[4].

Until some years ago, the only non-invasive test that reliably demonstrated whether eradication was successful was the urea breath test (UBT)[5]. It is easy to perform and does not need special transport conditions, but requires expensive and specific instruments. In the last years, several stool antigen tests have been put on the market and approved by the U.S. Food and Drug Administration for detection of *H pylori* before and after therapy. They are considered reliable in either pre-treatment or post-treatment settings[1,6,7], even though some controversial results have been reported after eradication therapy[8-12]. These tests are based on an enzyme immunoassay carried out in laboratory, and this limit delays the diagnostic report. Moreover, if stool samples are not frozen immediately after receipt and stored frozen until titration at a temperature lesser than -20℃, sensitivity of the test can drop[13]. More recently, some rapid stool tests not requiring laboratory assay have been put on the market. These near-patient tests are cheap, easy and quickly performed, and have good diagnostic accuracy in the pre-treatment setting[14-19]. For these reasons, they could represent a valid alternative to both UBT and traditional stool tests. However, at present there are few data about their clinical usefulness in the post-treatment setting.

In this prospective pilot study, we investigated the clinical performances of a rapid stool test in the evaluation

of eradication therapy and compared it with a well-known and validated *H pylori* stool test requiring laboratory assay.

MATERIALS AND METHODS

Patients

One hundred thirty consecutive outpatients undergoing ^{13}C-UBT to determine their post-treatment *H pylori* status at least 6 wk after the end of antimicrobial therapy, were asked to deliver a stool specimen the day after ^{13}C-UBT was performed. Eight of them did not deliver it and were excluded from the study; the remaining 122 patients (46 males and 76 females; mean age ± SD: 54.94 ± 13.90 years) were definitively enrolled. They were previously given the following regimens: Proton pump inhibitor (PPI) + Clarythromycin + Amoxycillin in 49 cases; PPI + Clarythromycin + Tinidazole (or Metronidazole) in 30 cases; PPI + Levofloxacin + Amoxycillin in 13 cases; Ranitidine bismuth citrate + Clarythromycin in 4 cases; PPI + Bismuth citrate + Metronidazole + Tetracycline in 2 cases; and undetermined in 24 cases. Exclusion criteria were: use of antibiotics, histamine-2 receptor antagonists, bismuth or PPIs in the last 6 wk; chronic use of corticosteroids or non-steroidal anti-inflammatory drugs; previous gastric surgery; severe concomitant diseases; pregnancy or lactation. All patients gave their informed consent, and the study was approved by our Ethical Committee.

Methods to assess H pylori

The post-treatment *H pylori* status was assessed by ^{13}C-UBT that was assumed as the gold standard according to previous reports[1,10,20,21]. ^{13}C-UBT was carried out at least 6 wk after the end of antimicrobial therapy according to previously validated protocols[20]. In brief, the patients were fasted overnight, and the baseline breath sample was collected. Afterwards, they drank a 200-mL water solution containing 75 mg of ^{13}C-urea and 1.4 g of citric acid (BREATHQUALITY-UBT ^{13}C-UREA, AB Analitica, Padova, Italy), and a further breath sample was collected 30 min later. Samples were analyzed by an infrared spectrometer and positive result was defined by a cut-off of 2.5‰. The doctor who performed the ^{13}C-UBTs was blinded to the results of all the other tests.

A portion of each fresh stool sample was tested by using a rapid immunochromatographic assay commercial kit (ImmunoCard STAT HpSA, Meridian Diagnostic Inc., OH, USA) (ImmunoCard STAT) for the detection of *H pylori* antigens in stools. All tests were performed by a single unique observer (L.T.) who was unaware of the *H pylori* status. He evaluated each sample according to the method previously described[18]. A positive result was defined if both a pink-red band (test line) and a blue-colored band (control line) appeared in the reading window. If only a blue band appeared in the reading window, the result was considered negative.

The remaining portion of each stool specimen was frozen and stored, and successively tested by using a commercial kit (Amplified IDEA Hp StAR, Dako, Glostrup, DK) (Hp StAR), widely validated in literature in both the pre-treatment and post-treatment settings[22-24]. This monoclonal enzyme immunoassay has been reported to be more accurate than polyclonal enzyme immunoassay in determining *H pylori* status after eradication treatment[25]. The Hp StAR test is an *in vitro* qualitative procedure for the detection of *H pylori* antigens in stool samples, and needs a laboratory to be performed. It is a sandwich-type enzyme immunoassay using immunoassay technology. The test was performed according to the manufacturer's instructions as previously described[18]. According to the manufacturer's guidelines, an absorbance 450 nm (A_{450}) ⩾ 0.190 was defined as a positive and A_{450} < 0.190 as a negative test result.

Table 1 Performances of Immuno Card STAT and HpStAR tests

	ImmunoCard STAT	*Hp* StAR
Sensitivity, % (95% CI)	58.14% (42-73)	76.74% (61-88)
Specificity, % (95% CI)	97.47% (91-100)	98.73% (93-100)
PPV, % (95% CI)	92.59% (76-99)	97.06% (85-100)
NPV, % (95% CI)	81.05% (72-88)	88.64% (80-94)
Global accuracy, % (95% CI)	83.61% (76-90)	90.98% (84-95)
False positive, *n*	2	1
False negative, *n*	18	10

CI: Confidence interval; PPV: Positive predictive value; NPV: Negative predictive value.

Estimation of sample size and statistical analysis

Sample size calculation was performed to obtain a good accuracy power, i.e. at about 95%, and the significance study threshold was chosen at 5% (type I error: 0.05). Stool tests sensitivity, specificity, predictive values of positive and negative results, diagnostic accuracy, and their 95% confidence intervals (95% CI) were calculated using standard methods. Differences in the test performances between the two methods were analysed by using Fisher's exact test. A P value < 0.05 was regarded as statistically significant.

RESULTS

According to the study protocol, UBT showed eradication in 79 (64.8%) patients, and persistency of *H pylori* infection in 43 (35.2%) patients.

ImmunoCard STAT was positive in 27 cases (two false-positives), and negative in 95 (18 false-negatives), with a sensitivity and specificity of 58.14% and 97.47%, respectively. Hp StAR was positive in 34 patients (one false-positive), and negative in 88 (10 false-negatives), with a sensitivity and specificity of 76.74% and 98.73%, respectively. The overall agreement between the two tests in the evaluation of *H pylori* status was 92.6% (113 of 122 cases).

The diagnostic performances of ImmunoCard STAT and Hp StAR are reported in Table 1. Despite Hp StAR seemed to work better than ImmunoCard STAT, no significant difference was observed between the two stool tests.

DISCUSSION

Rapid tests for the detection of *H pylori* antigens in stool can be very useful in clinical practice, as they are cheap, easy and quickly performed, and can be done in the doctor's office within 10 min. Several studies demonstrated their high diagnostic accuracy in untreated patients[14-18], and they can reliably replace UBT in this setting. Conversely, their clinical usefulness to evaluate *H pylori* status after eradication therapy has been scarcely investigated. Our study demonstrated that sensitivity, negative predictive value, and global accuracy of rapid stool test were rather low (58.1%, 81%, and 83.6%, respectively), so this test cannot be regarded as a reliable tool in the post-treatment setting. On the contrary, several authors reported that rapid stool tests have post-treatment performances similar to the pre-treatment ones, and are also indicated to assess the success of eradication therapy[26-33]. However, other authors reported lower performances of rapid stool tests after eradication therapy in either adults[34] or children[14], and Gisbert *et al*[35] suggested that they cannot be recommended to confirm *H pylori* eradication after treatment. In a multicenter trial investigating the same commercial kit used in our study, Kato *et al*[14] found a sensitivity of only 75% in the post-treatment setting, but in this study only frozen stools were tested. Conversely, we tested fresh stools, as this option better reflects what happens in the near-patient environment, such as the doctor's office. Using fresh stools, we expected results better than (or at least similar to) those obtained by Kato *et al*[14]. On the contrary, they were worse because sensitivity of Immuno Card STAT was 58.14%, resulting in wrong diagnosis in 20 of 122 patients, with a global accuracy of 83.6%.

In our study, all tests were read by a single unique observer, who in all cases was able to assess the positivity or negativity of the test. Indeed, the test was classified as positive even if the intensity of the pink-red band appearing in the reading window (test line) was very weak, according to the manufacturer's instructions. Conversely, other authors observed that the test line was so weakly visible that they judged the result as equivocal, in a percentage ranging from 5% to 11.9%[15,26].

We compared the rapid stool test with another, well-known, monoclonal enzyme immunoassay (Hp StAR), which has to be performed in laboratory. No significant difference was observed between the two tests, and their concordance was 92.6%, similar to that reported in our prior study investigating the same commercial kits in the pre-treatment setting[18]. It follows that in our study the performances of the laboratory monoclonal test provided unsatisfying also results, with a sensitivity lower than 80% and a global accuracy of 90.98%, and we think it cannot be recommended to confirm *H pylori* eradication after treatment. Our opinion is supported by a recent systematic review on the role of stool antigen test for the diagnosis of *H pylori*, which showed relatively low accuracy in some post-treatment studies with polyclonal stool antigen test, and suggested that its use in clinical practice is yet to be defined[23]. Indeed, the Maastricht 2-2000 Consensus Conference also recommended UBT as the most reliable, non-invasive test to assess eradication efficacy[1].

In our study, we did not use invasive methods (such as histology or rapid urease test) to evaluate *H pylori* status, as we and our Ethical Committee judged it unethical that patients with no indications to gastroscopy had to undergo invasive procedures to assess *H pylori* eradication. However, ^{13}C-UBT was assumed as the gold standard to evaluate *H pylori* status, as it is considered the method of choice to monitor success of therapy in both adults and children, and is recommended by current guidelines[1,36,37]. Indeed, strong evidences of sensitivity and specificity of ^{13}C-UBT close to 100% emerge from some good reviews[20,38,39], and these performances remain very high (quite over 90%) also using low doses of ^{13}C-urea[40].

In conclusion, our study suggests that rapid stool test is not very accurate in the post-treatment setting, and it cannot be recommended to evaluate the success of eradication therapy, as well as the laboratory monoclonal test. However, the conflicting results reported in literature about this topic make the planning of wide multicenter trials quite necessary, to reach a definitive answer to this controversial question.

COMMENTS

Background

In the last years, several stool antigen tests have been put on the market and approved by the U.S. Food and Drug Administration for detection of *H pylori* before and after therapy. These tests are based on an enzyme immunoassay carried out in laboratory, and this limit delays the diagnostic report.

Research frontiers

Recently, some rapid stool tests not requiring laboratory assay have been put on the market. These near-patient tests are cheap, easy and fast to be performed, and have good diagnostic accuracy in the pre-treatment setting. Several authors reported that rapid stool tests have post-treatment performances similar to the pre-treatment ones, and are also indicated to assess the success of eradication therapy

Innovations and breakthroughs

Our study suggests that sensitivity, negative predictive value, and global accuracy of rapid stool test are rather low, so it cannot be regarded as a reliable tool in the post-treatment setting.

Applications

Our study suggests that rapid stool test is not very accurate in the post-treatment setting, and it cannot be recommended to evaluate the success of eradication therapy.

Peer review

This is a well-designed paper and the results are correctly presented. Through comparison with the well-known and validated laboratory stool test (Amplified IDEA Hp StAR, Dako), the authors suggest that rapid stool test cannot be regarded as a reliable tool in the post-treatment setting. Also Hp StAR cannot be recommended to confirm *H pylori* eradication after treatment.

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RAPID COMMUNICATION

Crohn's disease in one mixed-race population in Brazil

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Abstract

AIM: To evaluate the classification and severity of Crohn's disease in different racial groups.

METHODS: Patients with Crohn's disease from the outpatient clinic of the University Hospital Prof. Edgard Santos were enrolled in the study. This hospital is a reference centre for inflammatory bowel disease. Race was determined using self-identification. The Vienna's classification was applied for all subjects. The severity of Crohn's disease was determined according to the number of surgical procedures, hospital admissions in the last year and treatment with steroids and immunosuppressors. Statistical analysis was calculated using *t* test for means, χ^2 or F for proportions. A *P* value < 0.05 was considered to be significant.

RESULTS: Sixty-five patients were enrolled. Non-white patients were more frequently diagnosed with Crohn's disease in the age less than 40 years than white patients. The behaviour of disease was similar in both groups with a high frequency of the penetrating form. There was a tendency for non-white patients to have a greater frequency of hospital admissions in the last year compared to white subjects. Non-whites also had a higher rate of colonic and upper gastrointestinal involvement, and were also more frequently on treatment with immunossupressors than white patients although this difference was not statistically significant.

CONCLUSION: Non-white patients with Crohn's disease had an earlier diagnosis and appeared to have had a more severe disease presentation than white patients.

Key words: Racial group; Brazil; Race; Inflammatory bowel disease; Crohn's disease

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INTRODUCTION

Genetic and environmental factors appear to have a role in the pathogenesis of Crohn's disease. The incidence of the disease among first degree relatives of patients is greater than the incidence in the general population[1]. So far it is believed that the illness is more common among whites than Afro-descendants[2]. The greatest prevalence is described in Jews[1] and a low prevalence is described in the Asian population[3]. The prevalence of Crohn's disease in Brazil is not known however the illness is considered to be of low prevalence in developing countries[4]. Data from Latin America have found an incidence of 0.03 cases per 100.000 person-years in the period of 1987 to 1993[5].

In contrast to the increased number of studies about the behaviour of many other diseases according to the racial groups, there is relatively little knowledge about the influence of race in Crohn's disease[6]. Studies have noted the different characteristics of Crohn's disease among racial groups and the possibility of different pathogenetic mechanisms of this illness in different countries. A more severe disease on presentation and an earlier diagnosis in successive generations has been observed in some patients of Jewish ancestry[7]. A North American study has demonstrated a genetic heterogeneity between Afro-American patients and non-Jews Caucasians with Crohn's disease[8]. In the same study it was observed that the Crohn's disease is more common than ulcerative colitis in Afro-Americans with inflammatory bowel disease.

As there is a high miscegenation of races in the Brazilian population the characterization of Crohn's disease within each racial group has become an interesting topic. The evaluation of these aspects may contribute to the

knowledge of the importance of genetic and environmental factors to the development of this illness. The aim of the present study is to evaluate the classification and severity of Crohn's disease in different racial groups.

MATERIALS AND METHODS

This is a sectional study in which patients with diagnosis of Crohn's disease who are followed up in the outpatient clinic of the University Hospital Prof. Edgard Santos were enrolled from March to December of 2006. This clinic is a reference centre for Crohn's disease. We included patients older than 18 years after they had signed the informed consent. The study was approved by the Ethics Committee of the Institution.

The diagnosis criteria for Crohn's disease were based on clinical, laboratorial, radiological, endoscopic and pathologic evaluations[9].

The demographics variables analysed were: age, gender, self-identification of race and Jewish ancestry. The self-identification was based on the criteria of the Brazilian Governmental Statistics Agency (IBGE). The distribution of the population of Bahia according to the IBGE 2000 Demographic Census using these criteria is: whites, 23%; Afro-Brazilians, 13%; Asians, < 1%; mixed-race, 62%, and Indians, 5%. The state of Bahia has the largest number of self-identified Afro-Brazilians, as well as the one of the highest combined percentages (75%) of Afro-Brazilians and mixed-race residents[10]. In this study patients were classified according to race into whites and non-whites.

The characterization of the Crohn's disease has been made using the criteria of the Vienna classification: age at diagnosis, localization and behavior colonic disease was compared with non-colonic and penetrating disease with non-penetrating. Other variables analysed were: mean age, gender, smoking, family history of Crohn's disease, perianal and rectovaginal fistula, presence of granuloma in the biopsy and time of diagnosis.

Evaluation of severity of the disease included surgical procedures, hospitalization in the last year and use of immunosuppressors and steroids. Comparison between racial groups was carried out by the χ^2 or F when the expected value was less than 5. For comparison between means the *t* was used. The differences observed were considered significant when the probability of the alpha error *P* was < 0.05.

RESULTS

During the study period sixty-five patients were enrolled, one patient refused to participate. Table 1 shows the characteristics of Crohn's disease in whites and non-white patients according to the Vienna classification; 21 (32.3%) were white, 28 (43.1%) were mixed-race Brazilians and 16 (24.6%) were Afro-Brazilians. Three individuals had Jewish ancestry: two were women, all had presented with ileal disease, and two were classified as L1 (ileum terminal) and one as L3 (ileocolonic). Jewish ancestry was detected only in white patients.

Table 2 shows other variables of the disease including mean age, gender, history of smoking, perianal fistula, rectovaginal fistula, and presence of granuloma in the biopsy and time of diagnosis in all patients and in white and non-white patients. Only two white patients had family history of Crohn's disease and one of them had Jewish ancestry and a twin sister with the disease.

Table 3 shows the comparison of parameters of the severity of Crohn's disease between whites and non-white patients.

Table 1 Vienna Classification in whites and non-whites patients with Crohn's disease in Bahia-Brazil, 2006

Vienna Classification	Total *n* = 65	White *n* = 21	Non-whites *n* = 44	*P*
Age at diagnosis				
A1 (< 40)	45 (69.2)	11 (52.4)	34 (77.3)	$P < 0.05$
A2 (≥ 40)	20 (30.8)	10 (47.6)	10 (22.7)	
Behavior				
B1-Nonsticturing, nonpenetrating	24 (36.9)	8 (38.1)	16 (36.4)	
B2-Stricturing	5 (7.7)	2 (9.5)	3 (6.8)	
B3-Penetrating	36 (55.4)	11 (52.4)	25 (56.8)	NS[a]
Localization[b]				
L1-Ileum terminal	15 (25.4)	7 (36.8)	8 (20.0)	
L2-Colon	14 (23.7)	3 (15.8)	11 (27.5)	NS[c]
L3-Ileocolonic	23 (39.0)	8 (42.1)	15 (37.5)	
L4-Upper gastrointestinal tract (UGT)	7 (11.9)	1 (5.3)	6 (15.0)	NS[d]

NS: no statistically significant difference. [a]Comparison between penetrating and non- penetrating; [b]Data on 59 patients; [c]Comparison between colonic and non-colonic; [d]Comparison between UGT and non-UGT.

Table 2 Others parameters in whites and non- whites patients with Crohn's disease in Bahia-Brazil, 2006

Parameters	Total *n* = 65	Whites *n* = 21	Non-whites *n* = 44	*P*
Age (mean ± SD)	37.3 ± 13.0	39.4 ± 13.9	36.3 ± 12.6	NS
Females *n*/%	40 (61.5)	12 (57.1)	28 (63.6)	NS
Smoking				NS
Non-smoker	54 (83.1)	16 (76.2)	38 (86.4)	
Current smoker	4 (6.2)	1 (4.8)	3 (6.8)	
Ex-smoker	7 (10.8)	4 (19.0)	3 (6.8)	
Perianal fistula	30 (46.2)	10 (47.6)	20 (45.5)	NS
Rectovaginal fistula	3 (4.6)	1 (4.8)	2 (4.5)	NS
Granuloma in biopsy[a]	11 (17.2)	4 (19.0)	7 (16.3)	NS
Time of diagnosis				NS
Less than 10 yr	52 (80)	18 (85.7)	34 (77.3)	
Equal to or more than 10 yr	13 (20)	3 (14.3)	10 (22.7)	

NS: no statistically significant difference. [a]Data on 64 patients.

DISCUSSION

This study was carried out in the state from Brazil with the highest frequency of Afro-descendants. Perhaps this type of ancestry explains the low number of patients with Crohn's disease included in this study. This small number can limit the external validation of the result of the study, but it is important to note that this is a reference center and the study included almost all patients being followed up.

The comparison between whites and non-whites

Table 3 Severity criteria in whites and non-whites patients with Crohn's disease in Bahia-Brazil, 2006

Severity criteria	Total *n* = 65	Whites *n* = 21	Non-whites *n* = 44	*P*
Treatment with immunossupressors *n* (%)	28 (43.1)	8 (38.1)	19 (45.5)	NS
Treatment with steroids *n* (%)	46 (70.8)	13 (61.9)	33 (75)	NS
Hospitalisation in the last year *n* (%)	19 (29.2)	3 (14.3)	16 (36.4)	NS[a]
Surgery fistula *n* (%)	13 (20.0)	4 (19.0)	9 (20.5)	NS
Partial Colectomy *n* (%)	13 (20.0)	2 (9.5)	11 (25.0)	NS

NS: no statistically significant difference. [a]almost reached statistical significance ($P = 0.07$).

showed a statistically significant difference in the age at diagnosis. Similar results have been demonstrated by other studies that have detected a greater frequency of diagnosis of Crohn's disease before the age of 40 years in Afro-descendants[12]. This can be attributed to a more severe disease with earlier symptoms. We also found that the behaviour of the illness was similar in whites and non-whites, with a high frequency of patients with the penetrating form of the disease in both groups[11]. This result can be explained by the fact that this study has been carried out in a reference center for the treatment of Crohn's disease to where more complex cases are referred.

The colonic localization seemed to be more frequent among non-white patients. We believe that if we had evaluated a higher number of patients the comparison of this variable might have reached statistical significance. This finding is in agreement with an American study that found African American patients were more likely to have ileocolonic or colonic disease compared to white patients[13]. A large North American Cohort[14] observed that African American patients were more likely to develop upper gastrointestinal and colorectal disease than whites, but less likely to have ileum involvement. A Chinese study found a greater rate of upper gastrointestinal Crohn's involvement and less terminal ileum disease in Chinese patients[15]. This was also observed in the present study in non-white patients. Possibly a difference in the balance between genetic inheritance and environmental factors contribute to the differences in the location of the disease among white and non-white patients[1].

The presence of ileal disease in all cases with Jewish ancestry raises the possibility of a genetic contribution in the location of this illness. Jewish ancestry was observed only in white patients. The immigration of individuals from countries of Jewish culture might be one of the forms for insertion of this disease in the white patients in the studied population.

In general, there is a slight female predominance in patients with Crohn's disease, although this is not described in all studies. The highest frequency of female patients observed in our study has been described before especially among women in late adolescence and early adulthood, suggesting that hormonal factors may play a role in disease expression[4]. A large North American cohort study[14] described a slight female predominance of inflammatory bowel disease among African Americans compared to whites and Hispanics.

The presence of family history and the high frequency of a previous history of smoking among white patients point out to the importance of genetic aspects and smoking as possible risk factors for the illness in this group of patients. The presence of family history of Crohn's disease only in whites in comparison to non-whites has been previously demonstrated and the authors noted the probable influence of genetic factors in these patients in contrast to the non-white individuals[14]. Possibly, environmental factors are contributing to the development of the illness in the latter group. Likewise, in a study involving 65 Chinese patients with Crohn's disease the absence of mutation in the CARD15 gene raises raises the question of whether environmental factors, such as lifestyle, are important in the pathogenesis of this illness. One study that evaluated Western patients has proposed a new hypothesis in which ingestion of highly fermentable but poorly absorbed short-chain carbohydrates and polyols to the distal small intestinal and colonic lumen is an important factor underlying the susceptibility to Crohn's disease[17]. Further studies are needed in order to address the importance of lifestyle in these patients especially regarding food intake.

Studies about single-nucleotide polymorphisms of the TNF-α promoter gene have found some differences in these polymorphisms between racial groups. Caucasians appear to more frequently have the phenotype of high production of TNF[18]. Several studies that evaluated cytokine genes polymorphisms have concluded that they have a greater influence on the classification of Crohn's disease rather than in the susceptibility to the illness[19]. Possibly the evaluation of cytokine gene polymorphisms will be another important topic in the inheritance of Cronh's disease.

The non-white patients had an increased period of time from diagnosis, equal or greater than ten years. This result could be explained by the earlier age at diagnosis in non-white patients and similar ages in both groups at the moment of the study. The greater duration of disease in non-white patients did not influence any change in the behaviour of disease as it would be expected probably because this difference in time was not great enough.

The analysis of severity of the disease showed a greater frequency of partial colectomy and hospitalisation in the last year and treatment with steroids and immunosuppressors in non-white patients. Probably the small number of patients can explain the lack of statistical significance. Nevertheless, it is interesting to note that the variable hospitalisation in the last year almost reached statistical significance. As these patients were evaluated in a single center by the same group of physicians who use a similar therapeutic approach this reinforces the possibility for a more severe illness in non-white patients.

The importance of the description in medical charts of the racial group was emphasized in a former publication which concluded that ethnic and racial differences in the causes, expression and prevalence of some illnesses exist[20]. In countries with high level of miscegenation, the study of the characteristics of multi-factorials illnesses, as it is the case of Crohn's disease, in the different racial

groups will be able to contribute to the knowledge of its genetics and environmental determinants. The identification of groups at risk for high prevalence and greater severity of the illness may lead to an earlier diagnosis, better treatment response and better quality of life of these patients.

In conclusion, the presence of family history and Jewish ancestry in white patients with Crohn's disease reinforces the importance of the genetic inheritance in these subjects. In this study, non-white patients presented with an earlier diagnosis of the disease and seemed to have had a more severe illness without aspects that might strengthen the influence of genetic inheritance. These data suggest a possible role of environmental factors in the presentation of the disease in non-white patients. Further studies aiming to evaluate the genes associated with Crohn's disease may help in the comprehension of the pathogenetic mechanisms of this illness in this population.

COMMENTS

Background

Crohn's disease is a multifactorial disorder and its pathogenesis is not entirely understood. Environmental and genetic factors are involved, but so far is still not clear the definitive relevance of racial factors.

Research frontiers

There are many studies about genetics aiming to elucidate the factors involved in the pathogenesis of Crohn's disease. So far several studies have found that there are genetic markers differences between races. In addition the importance of environmental factors for some racial groups has been emphasized.

Innovations and breakthroughs

Some studies have showed differences between racial groups in the presentation and severity of Crohn's disease. This article found that non-white were more frequently diagnosed with Crohn's disease in the age less than 40 years than white patients in one population with high rate of miscegenation.

Applications

This article emphasizes the importance to give special attention to racial aspects during follow-up and therapeutics of patients with Crohn's disease because probably distinct racial groups have different aspects in the pathogenesis of the disease. Therefore studies that evaluate the role of genetic markers in Crohn's disease need to consider racial features when describing the studied population.

Peer review

This article included some interesting data for the researchers in this field.

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RAPID COMMUNICATION

Is intra-operative cholangiography necessary during laparoscopic cholecystectomy? A multicentre rural experience from a developing world country

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Abstract

AIM: To evaluate the feasibility and safety of performing laparoscopic cholecystectomy (LC) in non-teaching rural hospitals of a developing country without intra-operative cholangiography (IOC). To evaluate the possibility of reduction of costs and hospital stay for patients undergoing LC.

METHODS: A prospective analysis of patients with symptomatic benign diseases of gall bladder undergoing LC in three non-teaching rural hospitals of Kashmir Valley from Jan 2001 to Jan 2007. The cohort represented a sample of patients requiring LC, aged 13 to 78 (mean 47.2) years. Main outcome parameters included mortality, complications, re-operation, conversion to open procedure without resorting to IOC, reduction in costs borne by the hospital, and the duration of hospital stay.

RESULTS: Twelve hundred and sixty-seven patients (976 females/291 males) underwent laparoscopic cholecystectomy. Twenty-three cases were converted to open procedures; 12 patients developed port site infection, nobody died because of the procedure. One patient had common bile duct (CBD) injury, 4 patients had biliary leak, and 4 patients had subcutaneous emphysema. One cholecystohepatic duct was detected and managed intraoperatively, 1 patient had retained CBD stones, while 1 patient had retained cystic duct stones. Incidental gallbladder malignancy was detected in 2 cases. No long-term complications were detected up to now.

CONCLUSION: LC can be performed safely even in non-teaching rural hospitals of a developing country provided proper equipment is available and the surgeons and other team members are well trained in the procedure. It is stressed that IOC is not essential to prevent biliary tract injuries and missed CBD stones. The costs to the patient and the hospital can be minimized by using reusable instruments, intracorporeal sutures, and condoms instead of titanium clips and endobags.

Key words: Laparoscopic cholecystectomy; Intra-operative cholangiography

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INTRODUCTION

Laparoscopic cholecystectomy (LC) is the gold standard for the treatment of symptomatic gallstones and other benign diseases of the gallbladder. It is the commonest operation performed laparoscopically worldwide[1-3]. Initially there were some concerns about its safety owing to its rapid adoption by untrained surgeons. However, when a careful, correct technique is employed, the operation is extremely safe[1]. The advantages of LC over open cholecystectomy in terms of minimal postoperative pain and postoperative pulmonary dysfunction, reduction in hospital stay, recovery periods, and improved cosmetic results have been established in a number of studies[2,4-8]. Large series of LC have been reported with few complications[6,9-12] and most surgeons and patients prefer LC to open cholecystectomy now.

The first operative cholangiography was reported in 1936 by Micken. Mirrizi in 1937 performed the first cystic duct cholangiography and this procedure remains the most accepted method for performing intra-operative cholangiography (IOC) today[13]. IOC has high sensitivity in detecting CBD stones, but its routine use is associated with increased costs and operation room time. The routine

use of IOC in all cases of LC is still controversial[14] with some authors supporting routine IOC[2,15,16], some favoring selective IOC[17-19], while others reporting no advantage of IOC[20-24] in preventing biliary injuries and missed CBD stones.

Unfortunately, access to laparoscopic surgery is limited in most of the rural areas of the state of Jammu and Kashmir. There are only two teaching hospitals offering the facility of laparoscopic surgery to a population of nearly 60 lakhs since 1995. Due to the absence of trained laparoscopic surgeons and the non-availability of laparoscopic equipment, the concept of minimal access surgery could not reach the underprivileged rural population with incomes of less than two dollars a day. Another major hurdle that had to be overcome was the misinformation prevalent both among surgeons and patients regarding the costs and the complication rate of LC.

It was only in Jan 2001 that laparoscopic equipment was installed in three non-teaching hospitals in the rural areas of Kashmir to cater the underprivileged population. A strategy had to be evolved to train the staff and to perform LC safely without the use of costly disposables, titanium clips, and endobags as the hospitals could not bear the extra expenses of these equipments. Taking into consideration the financial status of the population, the facility was provided free of cost.

This multicentre prospective study was conducted from Jan 2001 till Jan 2007 with the following aims: To evaluate the feasibility of performing LC in rural health centers of Kashmir without resorting to IOC and to evaluate the means to decrease the costs incurred by the hospitals.

MATERIALS AND METHODS

Patients undergoing LC for benign disorders of gall bladder in three non-teaching rural hospitals of Kashmir valley from Jan 2001 to Jan 2007 were included in the study.

To begin, only few surgeons were trained in laparoscopic surgery. The other team members were made familiar with the electronic equipment and the hand instruments. Seminars including video clips of various laparoscopic procedures and proper ways of troubleshooting the equipment and managing complications were conducted as a part of training. Emphasis was laid on the safety of the patient and the equipment. An indigenous costless endotrainer (Figure 1) was designed from a cardboard carton of 5% Dextrose bottles, to train the team members including paramedical personnel in the theatre.

Patient selection for surgery was made pre-operatively on the basis of history, physical examination, and radiological and laboratory diagnostic evidence of gall bladder disease. Ultrasonography was focused on the characteristics of any gallstones (size, number, and location), gallbladder polyps, the status of the common bile duct, the size of the gallbladder, the thickness of the gallbladder wall, and assessment of the liver and pancreas.

Exclusion criteria: (1) cases of acute cholecystitis after 48 h of the attack, (2) multiple previous upper abdominal operations, (3) coagulopathies, (4) and ASA grade Ⅲ and onwards.

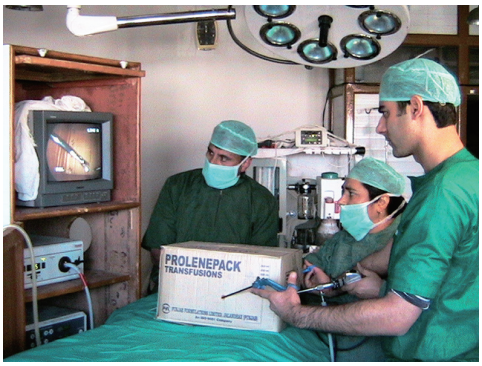

Figure 1 Indigenous endotrainer used for training the staff.

Those patients who had ultrasound documented choledocholithiasis or who had a history of jaundice with raised alkaline phosphatase and ultrasound documented CBD of more than 9 mm in diameter were sent to the nearest tertiary care centre for ERCP prior to taking them up for laparoscopic cholecystectomy.

At least one of the donor's and patient's blood samples was cross matched. Informed consent was obtained after a detailed discussion was held with the patient and attendants about the benefits and possible complications of LC.

To reduce the duration of the hospital stay, patients were admitted on the day of surgery and were allowed to have liquids up to 6 h before the operation.

Patients were asked to void urine before surgery and a Foley's catheter was not used.

Procedure

All operations were performed under general endotracheal anesthesia. In early cases standard four port LC was done. The Sulcus of Ruvier was used as a guide for location of Calot's triangle. The dissection of the cystic pedicle was initiated by lifting the posterior fold of peritoneum and creating a wide posterior window in the Calot's triangle. The gallbladder-cystic duct junction (the critical anatomical landmark) was identified. No attempts were made to dissect at cystic duct-CBD junction to avoid inadvertent injury. In patients in whom the Calot's triangle could not be clearly identified fundus first dissection was done by Berci's spatula. IOC facilities were not available so IOC was not performed. In cases having multiple small stones the cystic duct was partially opened and milking was done by a laparoscopic right angled forceps. Mostly 00 Vicryl sutures were placed both on cystic duct and cystic artery before cutting in between. This was done because of fear of internalization of clips into the common bile duct as reported by some authors[25] and to reduce the costs of the titanium clips. A fan retractor placed through an additional 5 mm port and a 30 degree telescope were used in grossly obese patients to obtain a clear view of anatomy. Gall bladder was removed through epigastric port after reducing the stone load. In case of infected or thick walled gallbladders specimens were removed in low cost condoms

Table 1 Patient characteristics and other observations

S. No.	Observation	Variable
1	Age (yr)	47.2 (13 to 78)
2	Female/Male	976/291
3	Previous abdominal surgeries	519
4	Preoperative ERCP	49
5	Mean operation time	39 min (11 min to 190 min)
6	Conversion to open Cholecystectomy	23
7	Drain (yes/no)	184/1083
8	Mean duration of analgesic requirement	3 d (1 d to 5 d)
9	Mean hospital stay	26 h (18 to 72 h)

Table 2 Intraoperative and postoperative complications

S. No.	Complications	No. of patients	%
1	Shoulder tip pain	213	17.12
2	Perforation of gallbladder with Stone spill	109	8.76
3	Port site infections	27	2.17
4	Cystic duct stones	17	1.37
5	Significant bleeding	12	0.96
6	Subcutaneous emphysema	4	0.32
7	Controlled biliary Leak	4	0.32
8	Undetected GB malignancy	2	0.16
9	Bile duct injury	1	0.08
10	Retained CBD stones	1	0.08
11	Cholecystohepatic duct	1	0.08
12	Drop in oxygen saturation	1	0.08

instead of costly endopouches.

Drains were placed selectively. All port wounds were infiltrated with long-acting local anesthetic. Antibiotic prophylaxis was ensured with 2 peri-operative doses of third generation cephalosporin intravenously. Post-operative analgesia was achieved with Diclofenac (p.o, 50 mg 3 times a day). All patients had oral liquids and were encouraged to have food in the evening after the operation, provided there was no nausea or vomiting.

The drain was usually removed after 24 h if drainage was minimal. The majority of patients were discharged on the first postoperative day if they lived in the area. Those living in outlying communities were encouraged to stay in town for 48 h. Patients were reviewed at weeks 1 and 4 postoperatively in the surgical OPD.

RESULTS

This series involved 1267 patients with symptomatic diseases of gall bladder from ages 13 to 78 years, (mean 47.2) who presented to Government Gousia hospital, District Hospital Baramulla and Ahmed's Hospital, Kashmir for LC from Jan 2001 till Jan 2007. The female to male ratio was 3.4:1. About 41% patients had undergone previous abdominal or pelvic surgery (commonest being lower segment cesarean section). Accordingly, the insertion point of the Veress needle and the first trocar was adjusted to avoid the risk of bowel perforation or injury.

The average operating time from insertion of Veress needle till closure of all ports was 39 minutes (ranging from 11 min to 190 min), and the mean length of postoperative hospital stay was 26 h (ranging from 18 h to 72 h). Two cases of incidental gallbladder malignancy were detected by histopathological examination of the specimens.

Table 3 Causes of conversion

S. No.	Causes of conversion	No. of patients	%
1	Dense adhesions at Calot's	11	0.86
2	Significant bleeding	6	0.47
3	CBD injury	1	0.08
4	CBD stone	1	0.08
5	Drop in oxygen saturation	1	0.08
6	Extensive subcutaneous emphysema	1	0.08
7	Inability to achieve working space due to dense intra-abdominal adhesions	1	0.08
8	Faulty equipment	1	0.08
	Total	23	1.82

The outcomes of this series are reported in Tables 1 and 2. There was no mortality in our series. Only one common bile duct injury was sustained during our 6 years experience which was identified peri-operatively, repaired and a T tube placed after conversion to open procedure. Twenty seven patients had port site infections but none had evidence of deep space or systemic infection. The most common post-operative complaint was right shoulder tip pain which usually lasted for 3 to 5 d. Twenty three cases were converted to open cholecystectomy after failed laparoscopic technique early in the series (Table 3).

DISCUSSION

The first LC was performed in 1986 by Muhe[26]. LC has become the operation of choice for benign disorders of gallbladder[2,6,27]. Numerous publications, mostly from large surgical centres, have exhaustively dealt with the operative technique, complications and the benefits of LC. The results of this case series of LC performed in non-teaching rural hospitals of a developing country are comparable to those from tertiary care settings and rural hospitals[2,4,5,8-13,28].

The low rate of morbidity and nosocomial infections may be due to reduced hospital stay, the favorable staff-to-patient ratio, attention to aseptic technique, and environmental sanitation. Surgeons and patients prefer LC to open cholecystectomy now and this procedure is cost-effective, cosmetically superior, and produces far less morbidity, as substantiated by other studies from rural hospitals of developing countries[4,8,27,28]. Access to LC is equally important for rural communities of the developing world.

Nonetheless, several limitations are worth noting. The relatively high start-up costs (the capital equipment and training of medical and nursing staff) have to be considered. These can be minimized by using a costless indigenous endotrainer. It is possible to decrease the costs of the procedure both for the patient as well as the hospital by using reusable trocars and cannulae, reusable instruments, intracorporeal ligatures instead of costly titanium clips, and condoms in place of endobags as

reported by other studies too[5]. To prevent injuries due to blunting of the tip, the trocars have to be sharpened after every 30-40 procedures.

Laparoscopy and LC are invasive procedures associated with a range of minor and major complications[29]. Bleeding is one of the most frequent and dangerous complications of LC. Clinically significant bleeding occurs in 0.5% of LC[6]. In our series, bleeding was observed in 12 (0.95%) patients, but in most cases it was controlled laparoscopically. Only 6 (0.47%) patients had significant bleeding that required conversion to the open procedure. Though bleeding is a potentially catastrophic complication inherent to the laparoscopic technique, it is also the most preventable one, as it is largely related to operator technique. In our study 1 (0.08%) patient suffered injury to the common bile duct. The frequency of this complication is 0%-0.8% in LC[9,10,11,12,15]. The low number of major bile duct injuries without resorting to IOC as reported in our study is comparable to results from other centres which recommend routine or selective IOC and questions the value of operative cholangiography during LC.

The reported rate of conversion to open cholecystectomy ranges between 1.88% to 10.1%[6,9,15,27,31]. In our series, 23 (1.82%) of all procedures were converted to the open technique (Table 3). In most cases, uncontrollable bleeding and dense adhesions at Calot's were the main reasons for conversion to the open procedure.

A controversial topic that was addressed in our study is whether IOC is helpful in preventing biliary tract injuries and missed CBD stones. Even though some authors are of the view that IOC is essential to detect biliary tract injuries and detect missed CBD stones[2,15,16], others feel that it is an unnecessary step[20-24]. Some authors recommend selective use of IOC[17,18,19]. Choledocholithiasis occurs in 3.4% of patients undergoing LC but more than one third of these pass the calculi spontaneously within 6 wk of operation[31]. Collins C concluded that treatment decision based on assessment by operative cholangiography alone would result in unnecessary intervention in 50% of patients who had either false positive studies or subsequently passed the calculi. The other arguments against IOC are that the biliary tract injury has already occurred before IOC can be performed. Routine IOC picks up unsuspected stones in 1%-4% of cases only, needs additional radiological personnel and more cost; hence routine IOC is not advisable[20].

The patient should be evaluated thoroughly for detection of any CBD stones before surgery. Pre-operative ERCP followed by immediate LC is the treatment of choice for such cases. High quality pre-operative ultrasound imaging is unlikely to miss any stones more than 3-4 mm in size. These stones usually migrate into the duodenum and may not require any immediate therapeutic approach[20]. In our series we had one case of retained CBD stones which were managed by post-operative ERCP. In our opinion proper case selection and sticking to the basic principals of LC like identification of Sulcus of Ruvier, making a wide posterior window, decompressing a tense gallbladder[32], proper traction, hydrodissection with saline, and using the fundus first technique in difficult cases[3,6] all can help to minimize the CBD injuries, need for IOC, and conversion to open procedure.

Cystic duct stones (CDS) should be suspected in all cases having a wide cystic duct in the presence of multiple small gall bladder calculi. Careful retraction and manipulation should therefore be done to minimize the risk of CDS slipping into the CBD[33]. The partial opening of cystic duct with milking of stones by a laparoscopic right angled forceps should be employed in such cases. After missing a stone in the cystic duct early in our series it has become a policy in our unit to routinely perform this maneuver in all cases having a wide cystic duct in the presence of multiple small gallbladder calculi.

Port site infection, usually involving the umbilical cannulation site through which the gallbladder is extracted, occurs in 0.3%-9.0% of cases[27,34,35]. Port site infection was seen in 12 (0.95%) of our patients, and all of these were treated successfully with local wound toilet and oral antibiotics.

Since the patients were admitted only on the day of surgery and early ambulation and feeding was instituted, the average duration of hospital stay was 26 h. Recent studies have demonstrated that laparoscopic cholecystectomy can be performed as one day-surgery[7,36]. In our series, this was true in most of the cases.

Successful performance of laparoscopic cholecystectomy requires proper training, discipline, skills and technology, and ongoing maintenance of competency. We believe this series demonstrates that procedural training and ongoing practice assessment can provide timely, safe, and appropriate access to this latest surgical technique even in rural hospitals of developing countries. The success and complication rate in this consecutive series of 1267 attempted LCs (23 conversions to open cholecystectomy, 1244 successfully completed LCs associated with minor complications) without IOC competes favorably with results achieved in tertiary care centres and rural hospitals[2,4,5,8-13,28].

CONCLUSION

The outcomes of this series of LCs conducted in three non-teaching rural hospitals of a developing state (Jammu and Kashmir) are similar to those of other case series from tertiary care centres and meet the published standards of care. It is hereby concluded that laparoscopic cholecystectomy can be performed safely even in rural health centres of a developing country provided proper equipment is available. The surgeons and other team members should be well trained in the procedure for which even an indigenously built costless endotrainer can be used. The case selection at the start should be stringent until enough experience is gained to manage difficult cases. IOC is not essential to prevent biliary tract injuries or missed CBD stones. This operation can be made more cost effective especially in rural sector of a developing country like India by using intracorporeal knotting in place of costly titanium clips and condoms in place of endobags. Using properly sterilized resharpened metallic trocars and cannulae can further reduce the costs without increasing the incidence of port site sepsis as substantiated by the results of our series.

The minimal hospital stay and early return to work with the resultant positive financial implications after LC for those patients who are bread earners for their families are significant. The authors strongly suggest that LC should be the surgical treatment of choice for patients of benign disorders of gallbladder. It is up to the governments of these underdeveloped countries to provide the facility free of cost to its citizens.

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RAPID COMMUNICATION

Micrometastasis in surrounding liver and the minimal length of resection margin of primary liver cancer

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Abstract

AIM: To describe the distribution of micrometastases in the surrounding liver of patients with primary liver cancer (PLC), and to describe the minimal length of resection margin (RM) for hepatectomy.

METHODS: From November 2001 to March 2003, 120 histologically verfied PLC patients without macroscopic tumor thrombi or macrosatellites or extrahepatic metastases underwent curative hepatectomy. Six hundreds and twenty-nine routine pathological sections from these patients were re-examined retrospectively by light microscopy. In the prospective study, curative hepatectomy was performed from November 2001 to March 2003 for 76 histologically verfied PLC patients without definite macroscopic tumor thrombi or macrosatellites or extrahepatic metastases in preoperative imaging. Six hundreds and forty-five pathological sections from these patients were examined by light microscopy. The resected liver specimens were minutely examined to measure the resection margin and to detect the number of daughter tumor nodules, dominant lesions, and macroscopic tumor thrombi inside the lumens of the major venous system. The paraffin sections were microscopically examined to detect the microsatellites, microscopic tumor thrombi, fibrosis tumor capsules, as well as capsule invasion and the distance of histological spread of the micrometastases.

RESULTS: In the retrospective study, 70 micrometastases were found in surrounding liver in 26 of the 120 cases (21.7%). The farthest distance of histological micrometastasis was 3.5 mm, 5.3 mm and 6.0 mm in 95%, 99% and 100% cases, respectively. Macroscopic tumor thrombi or macrosatellites were observed in 18 of 76 cases, and 149 micrometastases were found in the surrounding live in 25 (43.1%) of 58 cases with no macroscopic tumor thrombi. The farthest distance of histological micrometastasis was 4.5 mm, 5.5 mm and 6.0 mm in 95%, 99% and 100% cases, respectively. Two hundred and sixty-seven micrometastases were found in surrounding liver in 14 (77.8%) out of 18 cases with macroscopic tumor thrombi or macrosatellites. The farthest distance of histological micrometastasis was 18.5 mm, 18.5 mm and 19.0 mm in 95%, 99% and 100% cases, respectively.

CONCLUSION: The required minimal length of RM is 5.5 mm and 6 mm respectively to achieve 99% and 100% micrometastasis clearance in surrounding liver of PLC patients without macroscopic tumor thrombi or macrosatellites, and should be greater than 18.5 mm to obtain 99% micrometastasis clearance in surrounding liver of patients with macroscopic tumor thrombi or macrosatellites.

Key words: Primary liver cancer; Micrometastases; Resection margin; Hepatectomy

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INTRODUCTION

Primary liver cancer (PLC) is the fifth most common cancer in the world. The number of new cases is estimated to be 564000 per year. About 80% of all cases are found in Asia. Hepatocellular carcinoma (HCC) accounts for more than 80% of all PLCs, while intrahepatic cholangiocarcinoma (ICC) and hepatocellular-cholangiocarcinoma (HCCC) account for less than 20%. Most patients with PLC also suffer from concomitant cirrhosis, which is the major clinical risk factor for hepatic cancer. Overall, 80%

of PLCs can be attributed to chronic hepatitis B virus infection in Asia, especially in China. Hepatic resection and liver transplantation are considered the only curative treatment for PLC. For most cirrhotic patients who fulfill the Milan criteria, liver transplantation is the ultimate choice of treatment, but its application is limited due to the lack of donors[1]. Hepatic resection remains the treatment of choice for PLC despite unsatisfactory outcomes due to the high incidence of intrahepatic recurrence[2,3]. Resection margin (RM), which refers to the shortest distance from the edge of the lesion to the line of parenchymal transection margin[4], is vital to a safe operation and a complete clearance of micrometastases in surrounding liver. Because of underlying chronic liver diseases, the optimal RM in radical hepatectomy of PLC remains controversial and ambiguous[4-20] and has not been well illustrated theoretically. Although there were prospective studies on micrometastases in 55 patients[17], 36 patients[18] and 23 patients[19] and surgical margin in 40 patients[20] with PLC, they did not distinguish patients with macroscopic tumor thrombi or macrosatellites from those without macroscopic tumor thrombi or macrosatellites. To ensure a complete clearance of micrometastases in surrounding liver, the minimal length of RM depends on the farthest distance of histological micrometastasis. This study was to describe the distribution of micrometastases in the surrounding liver of patients with PLC, and the minimal length of resection margin for hepatectomy.

MATERIALS AND METHODS

Specimens

From November 2001 to March 2003, 120 histologically verfied PLC patients without macroscopic tumor thrombi or macrosatellites or extrahepatic metastases underwent curative hepatectomies (Table 1). Six hundred and twenty-nine routine pathological sections from these patients were re-examined retrospectively by light microscopy. In the prospective study, hepatectomy was performed from March to November 2003 for 76 histologically verfied PLC patients without definite macroscopic tumor thrombi or macrosatellites or extrahepatic metastases in preoperative imaging (Table 1). Six hundred and forty-five pathological sections from these patients, including 389 routine pathological sections, were examined by light microscopy. A computerized database was used to collect clinicopathological data of all patients in the prospective group, including the macroscopic width and histological involvement of surgical margin assessed by pathologists. Any postoperative recurrence was entered into the database immediately after diagnosis. No difference was found in age, sex, HBsAg (+) and tumor size between the two groups ($P > 0.05$).

All surgical procedures were performed by the same surgical team in Department of Hepatobiliary Surgery, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University. All pathology slides were reviewed independently by two hepatobiliary pathologists.

Preparation of fresh specimens

The resected liver specimens were photographed and then examined to measure the RM. Macroscopically, the number of daughter tumor nodules, besides the dominant lesion, were recorded, and the presence of macroscopic tumor thrombi inside the lumens of the major venous system and the level of its infiltrated venous branches were also noted. The size of tumors and vertical, transverse and anteroposterior dimensions of the specimens were documented according to their different shapes and photographed before 3-6 rectangle specimens were cut in the portal vein direction, hepatic vein direction and other directions, which measured approximately 2 mm by 10 mm in thickness and width, including 3-5 mm tumor and 10-25 mm liver parenchyma in length. The specimens were fixed in 10% formalin and stained with hematoxylin and eosin for microscopic examination.

In the presence of a multinodular lesion, the nodule with the largest diameter was taken as the dominant nodule except that 2-3 nodules were considered synchronous multicentric liver carcinogenesis if they located in different hepatic lobes with no significant difference in size, at a distance beyond 5 cm and had no macroscopic tumor thrombi. All the remaining macroscopically evident tumor nodules, or daughter nodules, macroscopic tumor thrombi and micrometastases were assumed to have radially disseminated from the dominant nodule without other preferred direction except for portal vein and hepatic vein directions.

Table 1 Perioperative data of 196 cases of primary liver cancer

	Retrospective group		Prospective group	
	Without Mt or Ms	Total	Without Mt or Ms	With Mt or Ms
Cases	120	76	58	18
Age (yr)	49.6 ± 11.5 (30-81)	50.8 ± 11.4 (24-78)	52.4 ± 11.0 (31-78)	45.4 ± 11.2 (24-65)
Sex (male/female)	100/20	63/13	47/11	16/2
TBIL (μmol/L)		12.8 ± 4.9	13.1 ± 4.5	11.9 ± 5.8
PALB (mg/L)		219 ± 59	222 ± 59	208 ± 58
PT (s)		12.5 ± 1.1	12.5 ± 1.0	12.6 ± 1.2
HBsAg (+)	98 (81.7%)	65 (85.5%)	49 (84.5%)	16 (88.9%)
Liver cirrhosis or fibrosis	103 (85.8%)	76 (100%)	58 (100%)	18 (100%)
Size of tumor (mm)	58.4 ± 42.6	54.9 ± 35.2	43.4 ± 23.9	92.0 ± 40.6
Tumor volume (cm^3)		107 ± 203	54 ± 111	275 ± 320

Mt: macroscopic tumor thrombus; Ms: macrosatellite. TBIL: total bilirubin; PALB: pre-albumin; PT: prothrombin time.

Correction for shrinkage

The tissue shrinkage rate secondary to the process of histological slide preparation was estimated by comparing the width of specimens from non-tumor liver in its final state on the slide and its fresh state before formalin immersion.

Documentation of pathological features

Various pathological features were studied, including the presence and absence of microsatellites, microscopic tumor thrombi, fibrosis tumor capsules, and capsule invasion (whether the tumor capsule was infiltrated partially or completely by the tumor), or liver invasion (whether the tumor infiltrated directly into the adjacent non-tumor liver), and the distance of micrometastases.

Table 2 Distribution of micrometastases in surrounding liver of 178 cases of primary liver cancer without macroscopic tumor thrombi or macrosatellites

	Retrospective group (120 cases)			Prospective group (58 cases)		
Distance (mm)	Cases	Percent	Accumulative percent	Cases	Percent	Accumulative percent
0	94	78.3	78.3	35	60.3	60.3
1	9	7.5	85.8	8	13.8	74.1
2	10	8.3	94.2	4	6.9	81
3	2	1.7	95.8	6	10.3	91.4
4	4	3.3	99.2	3	5.2	96.6
5	1	0.8	100.0	2	3.4	100.0

Table 3 Distribution of micrometastases in surrounding liver of 18 cases of primary liver cancer with macroscopic tumor thrombi or macrosatellites

Distance (mm)	Cases	Percent	Accumulative percent
0	5	27.78	27.8
3	7	38.89	66.7
6	3	16.67	83.3
9	0	0	83.3
12	1	5.56	88.9
15	1	5.56	94.4
18	1	5.56	100.0

Measurement of micrometastases

The size of all micrometastases detected in the adjacent non-tumor liver was estimated by the microscope scale. The shortest distance between the edges of the dominant nodule and the farthest micrometastasis was considered the distance of histological spread.

Statistical analysis

SPSS10.0 for Windows was used to compute the distribution of frequencies and SAS6.12 System to compute the statistical significance of difference for unpaired data. Time of recurrence and survival after recurrence were determined by Kaplan-Meier analysis, and the relation between micrometastases and clinicopathological characteristics was compared using the T stat test or Wilcoxon test. $P < 0.05$ was considered statistically significant.

RESULTS

Micrometastasis in liver parenchyma surrounding the lesion

Of the 120 cases, 24 (20%) had no encapsulation, 54 (45%) had incomplete encapsulation and 42 (35%) had almost complete encapsulation. Seventy micrometastases were found in the liver parenchyma surrounding the lesions in 26 cases (21.7%), among which 27 microsatellites were found in 16 (1-10 per case), 12 microscopic tumor thrombi in 6 (1-3 per case), and 26 microscopic tumor thrombi in 4 (3-15 per case) and microsatellites in 5 (1-2 per case). The farthest distance of micrometastasis was 3.5 mm, 5.3 mm and 6.0 mm in 95%, 99% and 100% cases, respectively (Table 2).

Of the 76 cases, 12 (15.8%) had no encapsulation, 55 (72.4%) had incomplete encapsulation and 9 (11.8%) had almost complete encapsulation. Among the 58 cases free of macroscopic tumor thrombi or macrosatellites, 25 (43.1%) exhibited 149 micrometastases in the liver parenchyma surrounding the lesions, among which, 9 microsatellites were found in 5 (1-3 per case), 69 microscopic tumor thrombi in 12 (1-20 per case), and 37 microscopic tumor thrombi in 8 (1-12 per case) and microsatellites in 34 (1-20 per case) (Figure 1A-C). The farthest distance of micrometastasis was 4.5 mm, 5.5 mm and 6.0 mm in 95%, 99% and 100% cases, respectively (Table 2). In 18 cases with macroscopic tumor thrombi or macrosatellites, 267 micrometastases were found in 14 (77.8%), 3 micrometastases in 1, 56 microscopic tumor thrombi in 5 (1-18 per case), and 154 microscopic tumor thrombi in 8 (6-33 per case) and microsatellites in 54 (2-11 per case) (Figure 1D and E). The farthest distance of micrometastasis was 18.5 mm, 18.5 mm and 19.0 mm in 95%, 99% and 100% cases, respectively (Table 3). As only 18 cases had macroscopic tumor thrombi or macrosatellites and it was impossible to obtain the liver parenchyma surrounding the lesion beyond 2 cm, the practical farthest distance should be greater than 18.5 mm. The tissue shrinkage rate was 89.7% ± 5.6%.

Relation between micrometastases and clinicopathological characteristics

The yield rate of micrometastasis among patients with incomplete encapsulation was statistically higher than that among patients with no or complete encapsulation ($P < 0.05$). The yield rate of micrometastasis in the liver parenchyma surrounding the lesion was positively correlated with the preoperative serum AFP level ($P < 0.01$), tumor size ($P < 0.01$) and presence of macroscopic tumor thrombi or macrosatellites ($P < 0.01$) in patients with PLC (Table 4).

DISCUSSION

Postoperative intrahepatic recurrence results either from residual intrahepatic metastasis or from de novo tumor due to the underlying hepatitis or liver cirrhosis[21-24]. The incidence of multicentric carcinogenesis in postoperative tumor is around 50%[24,25]. Theoretically, hepatectomy for PLC only resects the main tumor and surgical margin, the high risk area of intrahepatic metastasis[25]. Of the 6 PLC patients with only macrosatellites, 4 had no micrometastasis, which may be synchronously multicentric carcinogenic. In addition, of the 13 patients with 2-3 nodules who were clinically considered to be synchronously multicentric carcinogenic, only 4 had microsatellites without microscopic tumor thrombi, while 1 of them had microscopic tumor thrombi. Furthermore, treatment after postoperative recovery, aiming at the activity of hepatitis or liver cirrhosis, may decrease recurrence due to metachronously multicentric carcinogenesis[26-28]. Therefore, the aim of hepatectomy for PLC is not only to resect the main tumor and possible micrometastasis but also to decrease postoperative morbidity.

Up to date, prospective studies on micrometastases are

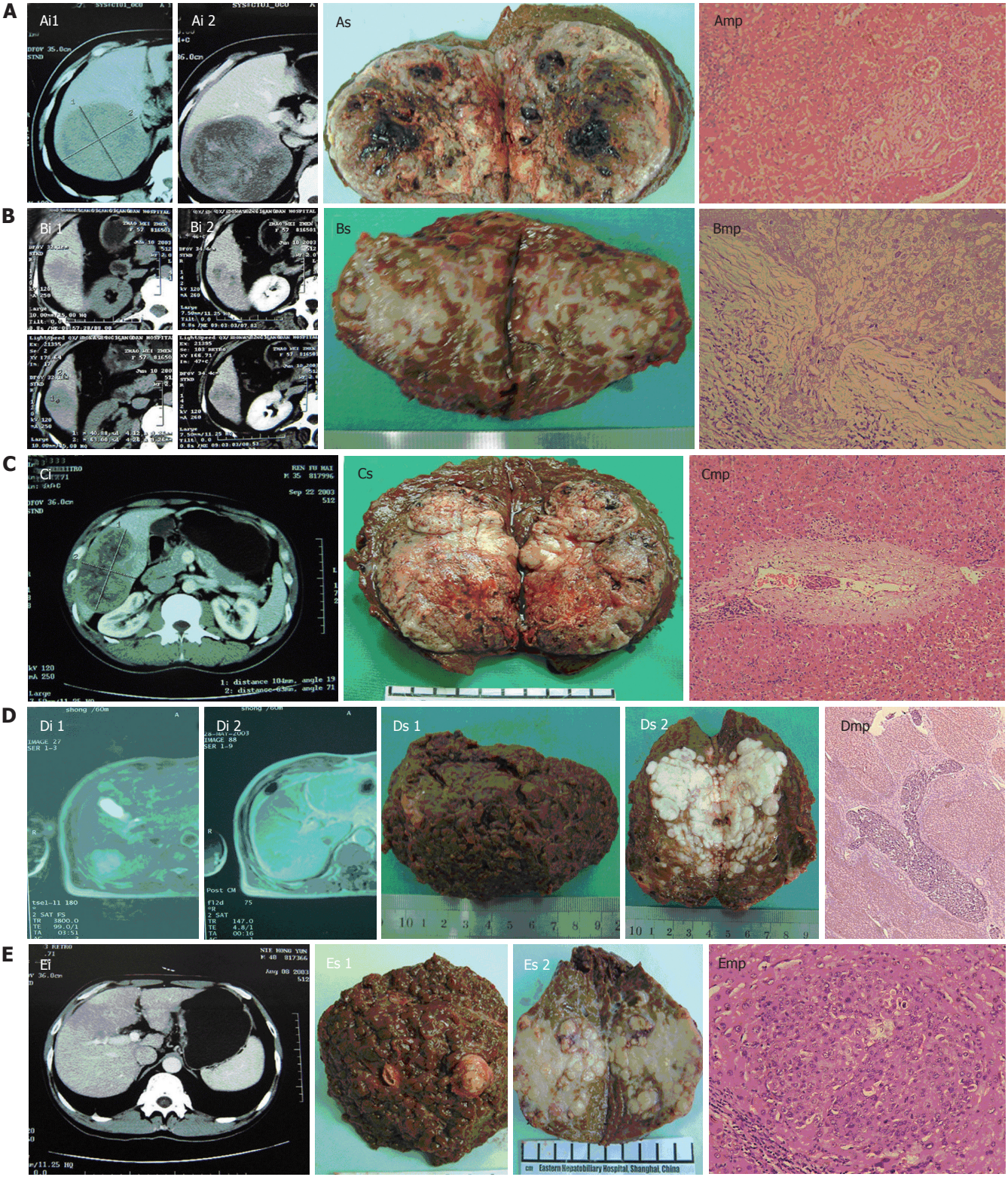

Figure 1 Imaging (i), specimens (s) and microscopic pathology (mp) of 5 PLC patients. **A**: case 24, tumor size 12.0 cm x 11.0 cm x 10.5cm, with microscopic tumor thrombi (metastatic distance 6 mm, x 100); **B**: case 40, tumor size 4.0 cm x 3.8 cm x 3.5 cm, with microscopic capsular infiltration (x 200); **C**: case 58, tumor size 10.5 cm x 6.5 cm x 6.0 cm, with microscopic tumor thrombi (metastatic distance 2 mm, x 100); **D**: case 38, tumor size 6.5 cm x 4.0 cm x 3.6 cm, with macroscopic tumor thrombi in the branches of right portal vein and microscopic tumor thrombi of arborization (x 16); **E**: case 52, tumor size 7.0 cm x 5.0 cm x 5.0 cm, with macroscopic tumor thrombi in the branches of right portal vein and microsatellites (metastatic distance 3.5 mm, x 100).

only available from 55[17], 36[18] and 23 patients[19] and surgical margin in 40 patients[20] with PLC, but they did not distinguish patients with macroscopic tumor thrombi or macrosatellites from those without them, and micrometastasis from synchronously multicentric micro-foci. The farthest distance of micrometastasis[17-20] was more than 1.0 cm.

Clinical follow-up studies showed that although safety margin at resection is not a prognostic factor, patients with

Table 4 Relation between micrometastases and clinicopathological characteristics in 196 cases of primary liver cancer

Parameters	Retrospective group					Prospective group				
	Cases	Both mt and ms	One of mt and ms	None of mt or ms	*P* value	Cases	Both mt and ms	One of mt and ms	None of mt or ms	*P* value
Encapsulation	120				< 0.01	76				< 0.01
No	24	1	2	21		12	1	5	6	
Incomplete	54	3	17	34		55	16	17	22	
Complete	42	0	3	39		9	0	0	9	
AFP (μmol/L)	120				0.08	76				< 0.01
< 20	44	0	7	37		21	0	4	17	
20-400	33	0	6	27		22	2	8	12	
> 400	43	4	9	30		33	15	10	8	
Size of tumor (mm)	111				0.1	76				< 0.01
⩽ 20	10	0	1	9		7	0	1	6	
20-30	23	1	3	19		14	2	4	8	
30-50	30	1	6	23		29	4	8	17	
50-100	27	0	3	24		15	6	6	3	
> 100	21	2	5	14		11	5	3	3	
Mt or Ms						76				< 0.01
Mt and Ms						3	3	0	0	
Mt						9	6	3	0	
Ms						6	0	2	4	
None of both						58	8	18	32	

Mt; microscopic tumor thrombus; ms: microsatellite. Incomplete encapsulation: part encapsulation or encapsulation breakthrough or with lesion inside encapsulation.

a surgical margin of over 1 cm[7-9] are free from tumor recurrence and a surgical margin of 0.5-1.0 cm[10-13] does not affect the prognosis and postoperative recurrence rate of hepatectomy for HCC after hepatectomy. These findings are not consistent with the reported results[17-20].

In the present study, the farthest distance of micrometastasis was 3.5 mm, 5.3 mm and 6.0 mm in 95%, 99% and 100% patients without macroscopic tumor thrombi or macrosatellites, respectively, which is different from the reported results of other prospective studies on micrometastasis[17-20], but is in agreement with clinical follow-up studies[10-13]. Because routine pathological sections, in which the liver parenchyma surrounding the lesion obtained is relatively less (0.2-1.0 cm), are mainly used to make diagnosis, it was impossible to achieve accurate record of resection margin and integrated clinical data for all PLC patients. The result of our prospective study on micrometastasis in PLC patients without macroscopic tumor thrombi or macrosatellites or extrahepatic metastases showed that the farthest distance of micrometastasis was 5.5 mm and 6 mm in 99% and 100% cases, respectively, which was in agreement with that of our retrospective study. These findings can explain the difference found in prospective studies on micrometastases[17-20] and clinical follow-up studies[7-13].

In conclusion, the farthest distance of micrometastasis is 18.5 mm and 19.0 mm in 99% and 100% of patients with macroscopic tumor thrombi or macrosatellites, respectively. The required minimal length of RM is 5.5 mm and 6 mm respectively to achieve 99% and 100% micrometastasis clearance in surrounding liver of PLC patients without macroscopic tumor thrombi or macrosatellites, and should be greater than 18.5 mm to obtain 99% micrometastasis clearance in patients with macroscopic tumor thrombi or macrosatellites.

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RAPID COMMUNICATION

Association of the frequency of peripheral natural killer T cells with nonalcoholic fatty liver disease

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Abstract

AIM: To investigate whether changes in the frequency of peripheral natural killer T (NKT) cells were correlated with liver disease in patients who had metabolic predispositions to nonalcoholic fatty liver disease (NAFLD).

METHODS: Peripheral blood samples were obtained from 60 Chinese NAFLD patients and 60 age and gender matched healthy controls. The frequency of peripheral NKT cells was detected by flow cytometry. Clinical and laboratory data were collected for further analysis.

RESULTS: NAFLD patients had a lower frequency of peripheral NKT cells than healthy controls (1.21% ± 0.06% *vs* 1.62% ± 0.07%, $P < 0.001$). Further analysis revealed that the frequency of peripheral NKT cells was negatively correlated with body mass index, waist circumference and serum levels of alanine aminotransferase. Logistic regression analysis revealed that elevated body mass index [hazard ratio (HR): 2.991], aspartate aminotransferase levels (HR: 1.148) and fasting blood sugar (HR: 3.133) increased the risk of NAFLD, whereas an elevated frequency of peripheral NKT cells (HR: 0.107) decreased the risk.

CONCLUSION: Changes in the frequency of peripheral NKT cells were correlated with NAFLD and a decreased frequency of peripheral NKT cells was a risk factor for NAFLD.

Key words: Nonalcoholic fatty liver disease; Natural killer T cells; Flow cytometry; Risk factor

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INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD), ranging from nonalcoholic steatosis to nonalcoholic steatohepatitis (NASH) and cirrhosis, may be the most common liver disease in western countries, with a prevalence of 20%-30% in the general population[1,2]. Nonalcoholic steatosis is considered to be a benign condition, but NASH can progress to cirrhosis and liver failure, and the 5-year survival rate for an individual diagnosed with NASH is estimated to be 67%[3]. Although NAFLD has been extensively studied in recent years, the exact pathogenesis of this disease remains unknown.

Natural killer T (NKT) cells were originally defined in mice in the last decade as a lymphocyte subtype that co-expresses natural killer receptors together with T cell receptors[4]. A striking characteristic of these T cells is the recognition of lipid antigens presented by the restrictive non-classical, non-polymorphic MHC class I-like CD1d molecule[5]. The capacity for rapid secretion of cytokines, such as interleukin-4, interferon-γ, interleukin-10 and interleukin-13, assures these cells an immuno-modulatory role in autoimmune, allergic, antimicrobial and anti-tumor immune responses[6].

Recently, several lines of evidence from animal experiments have suggested a link between NKT cell deficiency and NAFLD[7-10]. Hepatic NKT cells were reduced in leptin-deficient *ob/ob* mice[7] and in mice fed with a high fat diet[8]. Adoptive transfer of NKT cells[9] or oral administration of liver-extracted proteins[10] ameliorated steatosis and glucose intolerance in leptin-deficient *ob/ob* mice. These metabolic improvements were partly associated with an increase in hepatic NKT cell numbers[9,10].

These experimental results support a regulatory role for NKT cells; however, their role in the clinical setting of NAFLD remains unclear. This observation prompted this investigation of the possible role of NKT cells in NAFLD patients. The present study questions whether changes in the frequency of peripheral NKT cells are correlated with liver disease in patients with a metabolic predisposition to NAFLD.

MATERIALS AND METHODS

Subjects

This study was carried out at the First Affiliated Hospital of Zhejiang University School of Medicine. All subjects were volunteers attending their annual examination at our hospital from Sep 5 to Oct28, 2005. Informed consent was obtained from all subjects and the study protocol was approved by the hospital Ethics Committee.

The diagnosis of NAFLD was based on the criteria established by the Fatty Liver and Alcoholic Liver Disease Study Group of the Chinese Liver Disease Association[11]. The exclusion criteria specific to this study included persons with a self-reported history of acute infection or tissue injury in the previous 3 mo, patients with a history of a malignant tumor or autoimmune disease, and patients above 65 years old or below 20 years old. A total of 60 eligible NAFLD patients were enrolled (50 males and 10 females, median age 40.0 years, range from 24 years to 65 years).

For each NAFLD patient, one control was enrolled with matching gender and age (within 3 years). A total of 60 healthy controls were enrolled (50 males and 10 females, median age 42.0 years, range from 25 years to 65 years). All controls were free of viral hepatitis and autoimmune disease and had alcohol consumption within "sensible" limits (less than 30 g/d for men and less than 20 g/d for women). Exclusion criteria were the same as for the patient group.

Clinical examination

The clinical examinations were administered in the mornings after an overnight fast, and the subjects were also instructed to refrain from exercise during the day before their examination. The examination consisted of a physical examination by a physician, blood draw, blood pressure measurement, anthropometry and a health habit inventory. Body mass index (BMI, kg/m^2), used as an index of body fat, was calculated as weight in kilograms divided by height in meters squared. The waist to hip ratio was calculated as waist circumference divided by hip circumference.

Laboratory investigation

Blood samples were obtained from an antecubital vein and the samples were used for the analysis of biochemical values and NKT cells frequency. Biochemical values were measured by the Hitachi autoanalyzer model 7600 (Hitachi Corp, Japan). The biochemical values included alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglyceride, total and high-density lipoprotein cholesterol and fasting blood sugar (FBS).

The frequency of peripheral NKT cells was measured by flow cytometry as previously described[12,13]. In brief, two-color flow cytometric analysis was performed with an EPICS-XL flow cytometer (Beckman-Coulter Corp, USA) using System Ⅱ software. FITC-conjugated anti-Vα24 monoclonal antibody (Immunotech, France) was used to label NKT cells, and isotype matched controls were also used in all experiments. Vα24$^+$ T cells were considered to be NKT cells in the present study.

Table 1 Data comparison between NAFLD patients and healthy controls (mean ± SE)

Variable	Healthy controls	NAFLD patients
Age (yr)	43.0 ± 1.3	43.0 ± 1.3
Height (cm)	166.7 ± 6.2	167.1 ± 6.9
Weight (kg)	62.6 ± 7.6	75.3 ± 10.4 [b]
Body mass index (kg/m^2)	22.50 ± 0.29	26.91 ± 0.37 [b]
Waist circumference (cm)	80.2 ± 0.8	92.0 ± 1.1[b]
Hip circumference (cm)	93.7 ± 0.5	100.3 ± 0.7 [b]
Waist-hip ratio	0.856 ± 0.006	0.916 ± 0.006 [b]
Systolic blood pressure (mmHg)	119.5 ± 2.2	130.3 ± 2.0 [b]
Diastolic blood pressure (mmHg)	73.3 ± 1.4	81.0 ± 1.2 [b]
Alanine aminotransferase (U/L)	21.5 ± 1.8	46.9 ± 3.6 [b]
Aspartate aminotransferase (U/L)	24.4 ± 1.0	36.0 ± 2.8 [b]
Triglyceride (mmol/L)	1.712 ± 0.142	2.632 ± 0.144 [b]
Total cholesterol (mmol/L)	4.583 ± 0.111	4.973 ± 0.117 [d]
High density lipoprotein cholesterol (mmol/L)	1.264 ± 0.277	1.277 ± 0.033
Fasting blood sugar (mmol/L)	4.356 ± 0.068	5.567 ± 0.311[b]

[b]$P < 0.001$ *vs* healthy controls; [d]$P < 0.01$ *vs* healthy controls.

Statistical analysis

Dates are presented as mean ± SE. The data were analyzed by SPSS11.5 statistical software. The Mann-Whitney *U* test or Student's *t*-test were used for comparisons of the data. Spearman correlation analysis was used to estimate the relationship between the frequency of peripheral NKT cells and other variables. Logistic regression analysis (Backward: Wald; Entry: 0.05, Removal: 0.10) was used to evaluate the risk factors for NAFLD. $P < 0.05$ (2-tailed test) was considered statistically significant.

RESULTS

Clinical and laboratory data

NAFLD patients and healthy controls were different in terms of weight, BMI, waist circumference, hip circumference, waist to hip ratio, systolic blood pressure, diastolic blood pressure, ALT, AST, triglyceride, total-lipoprotein cholesterol and FBS, while there was no difference in terms of age, gender, height, or high-density lipoprotein cholesterol between the two groups (Table 1).

Frequency of peripheral NKT cells

NAFLD patients had a lower frequency of peripheral NKT cells, 1.21% ± 0.06% in NAFLD patients versus 1.62% ± 0.07% in healthy controls ($P < 0.001$) (Figure 1).

Relationship between the frequency of peripheral NKT cells and other variables

Spearman correlation analysis revealed that there was a poor correlation between the frequency of peripheral NKT cells and BMI ($r = -0.322$, $P = 0.001$), waist circumference ($r = -0.237$, $P = 0.021$) and ALT levels ($r = -0.217$, $P = 0.035$).

We further analyzed the relationship between the frequency of peripheral NKT cells and the three variables. As shown in Figure 2, the frequency of peripheral NKT cells was decreased in the subjects with high BMI (BMI ⩾ 24 kg/m^2), or in the subjects with high waist circumference

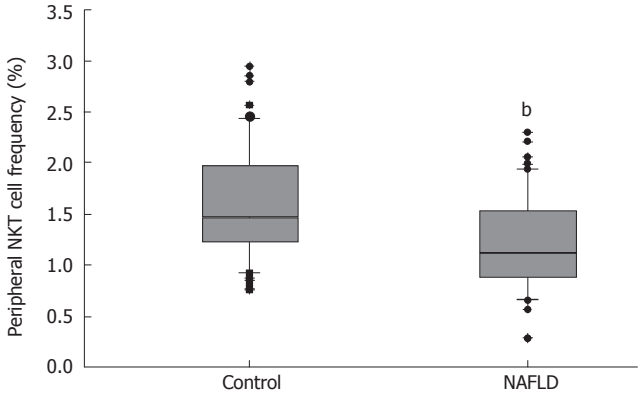

Figure 1 Frequency of peripheral NKT cells in NAFLD patients versus healthy controls. Boxes show values within 25th and 75th percentiles, horizontal bar represents the median, 80% of values are between the extremities of vertical bars (10th-90th percentiles), and extreme values are represented by individual symbols. $^{b}P < 0.001$ *vs* healthy controls.

(waist circumference ⩾ 90 cm for male and ⩾ 85 cm for female), or in the subjects with elevated ALT (ALT ⩾ 50 U/L). This result partially indicated that the frequency of peripheral NKT cells was negatively correlated with BMI, waist circumference and ALT.

The risk factors in NAFLD

Stepwise regression analysis was performed on the 11 variables that were different between the two groups (weight and hip circumference were excluded) using the dichotomous variable logistic regression model. The results showed that four features were closely associated with the risk for NAFLD, including the frequency of peripheral NKT cells, BMI, AST and FBS (Table 2).

DISCUSSION

These results indicate that changes in the frequency of peripheral NKT cells are correlated with liver disease in patients with a metabolic predisposition to NAFLD. This finding was supported by three main results. First, NAFLD patients had a lower frequency of peripheral NKT cells, a unique T lymphocyte subtype that shares some characteristics with natural killer cells[14]. NKT cells have been shown to play important regulatory roles in various liver diseases such as viral hepatitis[15], autoimmune liver disease[16], metabolic liver disease[17] and hepatic malignant tumor[18]. The frequency of peripheral NKT cells was decreased in patients with hepatitis C[15] and autoimmune hepatitis[16]. The decreased frequency of peripheral NKT cells may be caused by down-regulation of T cell receptors, apoptosis of the cells and/or compartmentalization into peripheral organs[15]. The decreased frequency of peripheral NKT cells in NAFLD patients in the present study indicates that NAFLD may represent another liver disease related to a decreased frequency of peripheral NKT cells.

The second result was that the frequency of peripheral NKT cells was negatively correlated with BMI, waist circumference and ALT. NAFLD has been shown to be strongly associated with excess body weight and central

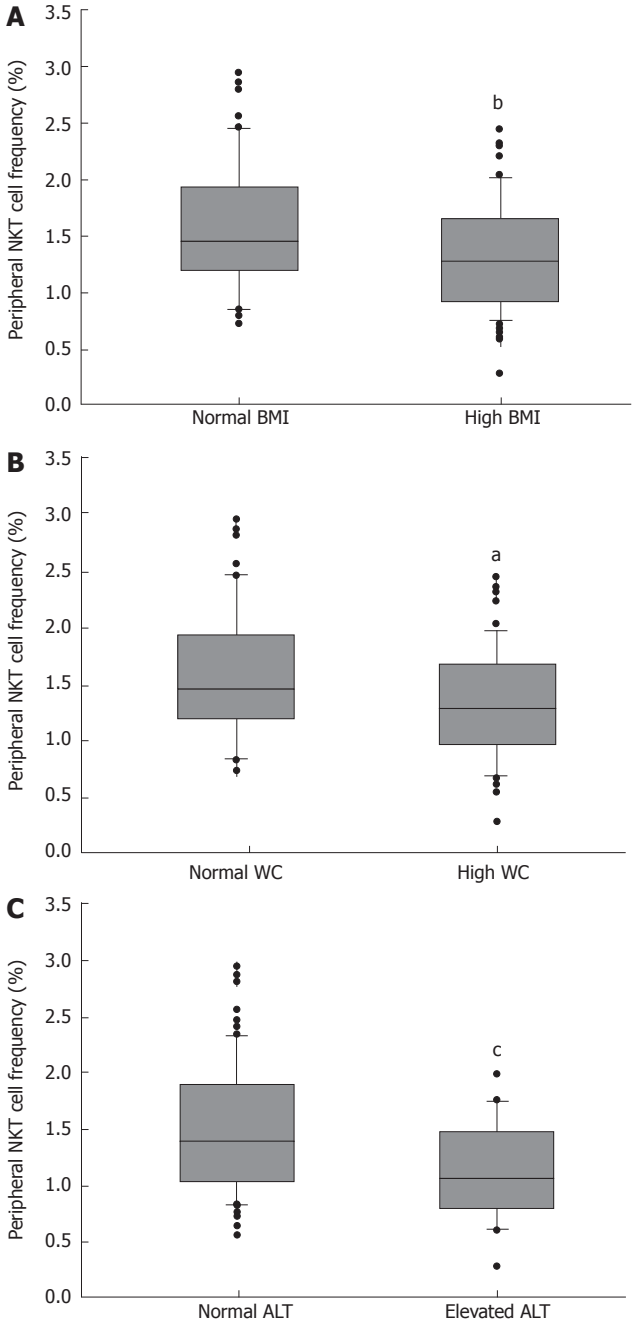

Figure 2 Relationships between the frequency of peripheral NKT cells and BMI, waist circumference and ALT. **A**: The frequency of peripheral NKT cells was compared between the subjects with BMI ⩾ 24 kg/m^2 (n = 68) and the subjects with BMI < 24 kg/m^2 (n = 52), $^{b}P < 0.01$ *vs* subjects with BMI < 24 kg/m^2; **B**: The frequency of peripheral NKT cells was compared between the subjects with waist circumference ⩾ 90 cm for male and ⩾ 85 cm for female (n = 47) and the subjects with normal waist circumference (n = 73), $^{a}P < 0.05$ *vs* subjects with normal waist circumference; **C**: The frequency of peripheral NKT cells was compared between the subjects with ALT ⩾ 50 U/L (n = 21) and the subjects with ALT < 50 U/L (n = 99), $^{c}P < 0.01$ *vs* subjects with ALT < 50 U/L.

adiposity in particular[19-21]. Most NAFLD patients are overweight, and the prevalence of NAFLD is much more common in obese than in non-obese individuals (76% *vs* 16%)[19]. BMI and waist circumference, used as indicators for obesity and central obesity, are closely related with NAFLD[19-22]. ALT is also closely related with NAFLD, and excluding causes such as chronic hepatitis and alcohol-induced liver disease, NAFLD explains 80% to 90% of the

Table 2 Results of the logistic regression analysis of risk factors for NAFLD

Variable	β	SE	Wald value	*P*-value	OR	95% CI of OR
NKT	-2.239	0.984	5.181	0.023	0.107	0.016-0.733
BMI	1.096	0.370	8.755	0.003	2.991	1.448-6.181
AST	0.138	0.062	4.960	0.026	1.148	1.017-1.296
FBS	1.142	0.459	6.186	0.013	3.133	1.274-7.707
Constant	-53.119	14.751	12.967	< 0.001	< 0.001	-

B: partial regression coefficient; SE: standard error of partial regression coefficient; OR: odds ratio; CI: confidence interval; NKT: peripheral natural killer T cell frequency; BMI: body mass index; AST: aspartate aminotransferase; FBS: fasting blood sugar.

remaining cases of elevated ALT[23]. Therefore, elevated ALT has been used as a noninvasive surrogate marker for NAFLD[21,24]. In the present study, the frequency of peripheral NKT cells was found to be significantly correlated with BMI, waist circumference and ALT, but the *r* values were very low, indicating that the correlation between these terms was very weak. However, as BMI, waist circumference and ALT were all strongly associated with NAFLD, the present findings might suggest that peripheral NKT cells are correlated with NAFLD.

The third result supporting the correlation of NKT cells with NAFLD was that the decreased frequency of peripheral NKT cells was a risk factor for NAFLD. Previous studies revealed that overweight, diabetes mellitus and elevated liver enzymes were risk factors for NAFLD in agreement with our results[1,19,25,26]. In addition, this study showed that the decreased frequency of peripheral NKT cells was another risk factor for NAFLD. This association may due, at least in part, to a diminished protective effect of the lower number of NKT cells against vulnerable factors such as lipopolysaccharide (LPS)[8]. This finding also suggests that NKT cells are associated with NAFLD, and could lead to immune manipulations of NKT cells as a therapeutic tool in NAFLD in the future. Interestingly, data from animal experiments confirmed the therapeutic role of NKT cells in NAFLD[9,10].

This study had some limitations, however. The first limitation was regarding the methodology used to evaluate peripheral NKT cells. Until recently, NKT cells could not be unambiguously identified. αGalCer/CDld tetramers and Vα24/Vβ11 double-staining are considered to be two standard methods for NKT cell identification[27]. Vα24 staining alone as used in the present study may overestimate the numbers of Vα24 and Vβ11 double-positive NKT cells. However, because NKT cells represent a subtype of T lymphocytes, staining NKT cells with Vα24 and CD3 reduces the possibility of overestimation, and the same method has also been used by previous studies[12,13]. The second limitation was that hepatic NKT cells were not studied in NAFLD patients or in healthy controls. NKT cells arise mainly in the thymus and migrate to peripheral tissues, such as the liver and pancreas, where they accumulate in large numbers[28]. It would be much more meaningful to study hepatic NKT cells in NAFLD patients, but it was not convenient to get enough liver tissue samples from NAFLD subjects, in part due to ethical reasons. The third limitation is that the involvement of peripheral NKT cells in NAFLD was not studied. NKT cells have several subtypes such as $CD4^+CD8^-$, $CD4^-CD8^-$ (DN) and $CD4^-CD8^+$, and different subtypes have different functions[6]. Further studies are needed to understand the relationship between the subtypes of NKT cells and NAFLD.

In conclusion, these results suggest that changes in the frequency of peripheral NKT cells were correlated with liver disease in patients who had a metabolic predisposition to NAFLD, and a decreased frequency of these cells was a risk factor for NAFLD. These findings suggest a potential role for peripheral NKT cells in the pathogenesis of NAFLD.

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COMMENTS

Background

Nonalcoholic fatty liver disease (NAFLD) has been extensive studied in recent years, but the mechanism of this disease remains unclear. The most popular two-hit hypothesis keeps that hepatic fat accumulation is the first hit and concomitant hepatic inflammation is the second hit. Several potential pathogenic factors including insulin resistance, mitochondrial dysfunction, lipid peroxidation and immunity abnormality have been intensively investigated.

Research frontiers

To explore the role of NKT cells in the clinical setting of NAFLD.

Related publications

Recent evidences from animal experiments indicated an association between NKT cells and NAFLD. It was observed that hepatic NKT cells were reduced in leptin-deficient *ob/ob* mice and in mice fed with a high fat diet. Adoptive transfer of NKT cells or oral administration of liver-extracted proteins ameliorated steatosis and glucose intolerance in leptin-deficient *ob/ob* mice.

Innovations and breakthroughs

Previous evidences supporting the association between NKT cells and NAFLD were all from animal experiments. Whether NKT cells also play important regulatory role in the clinical setting of NAFLD remains unclear. In this study, the authors found that changes in the frequency of peripheral NKT cells were correlated with NAFLD and a decreased frequency of peripheral NKT cells was a risk factor for NAFLD.

Applications

The results may provide new theoretic and experimental evidences for the study of the pathogenesis of NAFLD, and new therapeutic approaches for NAFLD by regulating the balance of NKT cells in the body.

Terminology

NKT cells were originally defined in mice in the last decade as a lymphocyte subtype that co-expresses natural killer receptors together with T cell receptors. A striking characteristic of these T cells is the recognition of lipid antigens presented by the restrictive non-classical, non-polymorphic MHC class I-like CD1d molecule. NKT cells play an important immuno-modulatory role in autoimmune, allergic, antimicrobial and anti-tumor immune responses.

Peer review

The paper by Xu *et al* describes that a reduced number of peripheral NKT cells is associated with NAFLD. The topic of the paper is highly interesting. The patients have to be much better characterized.

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RAPID COMMUNICATION

Effect of preoperative transcatheter arterial chemoembolization on proliferation of hepatocellular carcinoma cells

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Abstract

AIM: To evaluate the effect of preoperative transcatheter arterial chemoembolization (TACE) on proliferation of hepatocellular carcinoma (HCC) cells.

METHODS: A total of 136 patients with HCC underwent liver resection. Of 136 patients, 79 patients received 1 to 5 courses of TACE prior to liver resection (TACE group), who were further subdivided into four groups: Group A (n = 11) who received 1 to 4 courses of chemotherapy alone; Group B (n = 33) who received 1 to 5 courses of chemotherapy combined with iodized oil; Group C (n = 23) who received 1 to 3 courses of chemotherapy combined with iodized oil and gelatin sponge; and Group D (n = 12) who received 1 to 3 courses of chemotherapy combined with iodized oil, ethanol and gelatin sponge. The other 57 patients only received liver resection (non-TACE group). The expressions of Ki-67 and proliferating cell nuclear antigen (PCNA) protein were detected in the liver cancer tissues by immunohistochemical method.

RESULTS: The Ki-67 protein expression was significantly lower in Groups C and D as compared with non-TACE group (31.35% ± 10.85% *vs* 44.43% ± 20.70%, 30.93% ± 18.10% *vs* 44.43% ± 20.70%, respectively, $P < 0.05$). The PCNA protein expression was significantly lower in Groups C and D as compared with non-TACE group (49.61% ± 15.11% *vs* 62.92% ± 17.21%, 41.16% ± 11.83% *vs* 62.92% ± 17.21%, respectively, $P < 0.05$). The Ki-67 protein expression was significantly higher in Group A as compared with non-TACE group (55.44% ± 13.72% *vs* 44.43% ± 20.70%, $P < 0.05$). The PCNA protein expression was significantly higher in Groups A and B as compared with non-TACE group (72.22% ± 8.71% *vs* 62.92% ± 17.21%, 69.91% ± 13.38% *vs* 62.92% ± 17.21%, respectively, $P < 0.05$).

CONCLUSION: Preoperative multi-material TACE suppresses the proliferation of HCC cells, while a single material embolization and chemotherapy alone enhance the proliferation of HCC cells.

Key words: Hepatocellular carcinoma; Transcatheter arterial chemoembolization; Proliferating cell nuclear antigen; Ki-67

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies. Curative local resection is recognized as a safe and effective method for patients with HCC[1]. Unfortunately, only a minority of patients currently diagnosed with HCC may benefit from this radical option. Transcatheter arterial chemoembolization (TACE) has become one of the most popular and effective palliative methods for patients with HCC. Various mixtures of anticancer drugs, iodized oil and gelatin sponge have been used as TACE agents. There have been a few reports on comparison of the efficacy of different TACE regimens on patients with HCC[2].

Proliferating cell nuclear antigen (PCNA) is an auxiliary factor in DNA polymerase, and is expressed in the nuclei, particularly in the late G_1 and S phases. Ki-67 is expressed throughout the cell cycle (late G_1, S, G_2, and M phases) of proliferating cells, but is absent in quiescent (G_0) cells. Therefore, PCNA and Ki-67 are believed to be useful markers for proliferative activity[3]. Our previous study showed that expression of p53 can enhance expression of PCNA and Ki-67 after TACE[4].

As far as we are aware, the effects of different TACE regimens on proliferation of HCC cells have not been

investigated previously. In particular, it is unclear whether TACE can enhance or suppress proliferation of HCC cells by modulating the expressions of PCNA and Ki-67 proteins. In the present study, we examined the effects of the four main types of TACE used clinically (pure intra-arterial chemotherapy; chemotherapy plus iodized oil; chemotherapy plus iodized oil plus gelatin- sponge; chemotherapy plus iodized oil plus alcohol plus gelatin-sponge) on proliferation of HCC cells *in vivo*.

MATERIALS AND METHODS

Patients

From Feb 1992 to Feb 2001, a total of 136 patients with HCC were referred to our hospital for surgery. There were 122 men and 14 women with mean age of 45 (ranged from 20 to 70) years. A diagnosis of HCC was obtained for all patients by preoperative ultrasound (US) or/and computed tomography (CT) or/and magnetic resonance image (MRI) or/and digital subtraction angiography (DSA) and plasma AFP levels and confirmed by pathological biopsy.

Surgical procedure

The patients were divided into two groups according to treatment manners. In the TACE group, 79 patients underwent 1-5 courses of chemoembolization prior to liver resection. In the control group, 57 patients received initial liver resection without preoperative TACE. The extent of liver resection was carried out based on the location of tumor, the severity of concomitant liver cirrhosis and preoperative liver reserve function.

TACE methods

By Seldinger's technique, indirect portal-veinography through the superior mesenteric artery was firstly performed to observe portal vein flow, thrombus, mislocalized tumor-feeding artery. Then a catheter was inserted selectively and superselectively into the right or left hepatic artery or the tumor-feeding artery. The patients in TACE group were divided into four subgroups: one to four courses of only infusion of chemotherapeutic agents, including 5-fluorouracil (5-FU) 1000 mg (NanTong Pharmaceutical Factory, China), mitomycin-c (MMC) 10 mg (Kyowa Hakko Kogyo Co. Ltd., Japan), carboplatin 300 mg (QiLu Pharmaceutical Factory, China), or epirubicin (E-ADM) 60 mg (Zhejiang HiSun Pharmaceutical Co. Ltd., China), were performed in 11 patients (Group A); one to five courses of first infusion of the same chemotherapeutic agents as group A, then embolization with mixture composed by iodized oil (Lipiodol, Guerbet, France) 5-20 mL according to the tumor size and E-ADM were performed in 33 patients (Group B); one to three courses of chemotherapy combined with iodized oil, the same as group B, plus adequate gelatin sponge particle embolization were performed in 23 patients (Group C); one to three courses of chemotherapy combined with iodized oil, ethanol and gelatin-sponge, that is, firstly, the same chemotherapy as group A, secondly, embolization with mixture composed of iodized oil 5-20 mL and waterless ethanol 1-5 mL (two ratio 4:1), finally, embolization with adequate gelatin sponge particle, were performed in 12 patients (Group D). Of them, 50 patients underwent one course of TACE; 19 patients underwent two courses of TACE; 10 patients underwent three or more courses of TACE during an interval of 52.8 ± 12.2 d (mean ± SD). Of them, 25 patients had ⩽ 1 mo interval; 29 patients had ⩽ 2 mo interval; 16 patients had ⩽ 3 mo interval; and 9 patients had > 3 mo interval.

Immunohistochemical method

The formalin-fixed, paraffin-embedded specimens were examined immunohistochemically using anti-Ki-67 monoclonal antibody M7187 (1:50 dilution) and anti-PCNA monoclonal antibody M0879 (1:200 dilution) (LSAB kit Dako). Positive controls were normal lymph nodes. Negative controls were generated by substituting for the primary antibody with a non-specific IgG (normal rabbit IgG) and tris-buffered saline. Ki-67- and PCNA-positive cells showed brown-yellow staining in the nuclei of cancer cells (Figures 1 and 2). Rate of positive immunostaining for Ki-67 or PCNA was calculated as the ratio of the number of positively stained tumor cells to the total number of tumor cells counted per section. All slides were reviewed and scored in a blind fashion by two observers without knowledge of the corresponding clinical data. A few cases with discrepant scoring were revaluated jointly on a second occasion, and an agreement was reached.

Statistical analysis

Data were expressed as mean ± SD and analyzed by means of SPSS 10.0 software package (SPSS, Chicago, IL, USA, 1999) using Student's *t* test, Crosstabs (Chi-square and Fisher exact probability test) and K Independent Samples, when appropriate A P value < 0.05 was considered statistically significant.

RESULTS

Expression of Ki-67 and PCNA proteins

Ki-67 and PCNA protein expressions of HCC cells, respectively, were 44.43% ± 20.70% and 62.92% ± 17.21% in non-TACE group, 55.44% ± 13.72% and 72.22% ± 8.71% in Group A, 45.26% ± 14.97% and 69.91% ± 13.38% in Group B, 31.35% ± 10.85% and 49.61% ± 15.11% in Group C, and 30.93% ± 18.10% and 41.16% ± 11.83% in Group D. Ki-67 protein expression was significantly higher in Groups A and B as compared with Groups C and D, was lower in Groups C and D as compared with non-TACE group, and was higher in Group A as compared with non-TACE group (P < 0.05). PCNA protein expression was significantly higher in Groups A and B as compared with Groups C, D and non-TACE group, and was lower in Groups C and D as compared with non-TACE group (P < 0.05).

Correlation between courses of TACE and expressions of Ki-67 and PCNA proteins

Ki-67 and PCNA protein expressions of HCC cells, respectively, were 44.43% ± 20.70% and 62.91% ± 17.21%

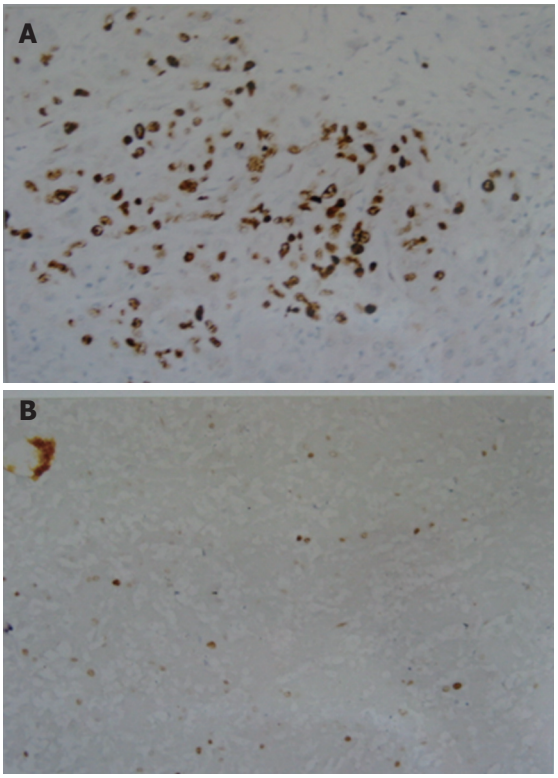

Figure 1 Ki-67 immunostaining of HCC cells (× 200). **A**: Non-TACE group; **B**: Group D.

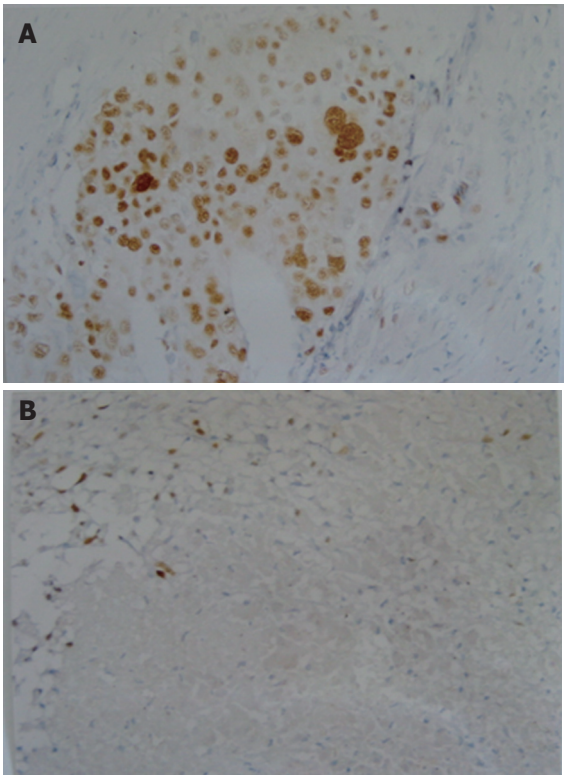

Figure 2 PCNA immunostaining of HCC cells (× 200). **A**: Non-TACE group; **B**: Group C.

in non-TACE group, 41.34% ± 16.69% and 62.00% ± 18.47% in one-course of TACE group, 39.24% ± 14.48% and 57.70% ± 15.54% in two-courses of TACE group, and 38.33% ± 20.90% and 53.97% ± 18.13% in three- or four- or five-courses of TACE group, indicating that Ki-67 and PCNA protein expressions were insignificantly decreased as the courses of TACE increased.

Correlation between interval of TACE and expressions of Ki-67 and PCNA protein

Ki-67 and PCNA protein expressions of HCC cells, respectively, were 44.43% ± 20.70%, 62.91% ± 17.21% in non-TACE group, 35.42% ± 15.46% and 53.19% ± 18.28% in ≤ 1 mo interval of TACE group, 40.66% ± 18.53% and 58.09% ± 15.90% in 1-2 mo interval of TACE group, 42.22% ± 14.80% and 66.83% ± 17.93% in 2-3 mo interval of TACE group, and 50.62% ± 12.33% and 72.53% ± 12.93% in > 3 mo interval of TACE group. Comparison between groups indicated that the Ki-67 protein expression was significantly lower in "≤ 1 mo" interval group as compared with "> 3 mo" interval and non-TACE groups ($P < 0.05$), while the expression of PCNA protein was significantly lower in the "≤ 1 mo" and "1-2 mo" interval groups as compared with "> 3 mo" interval and non-TACE groups ($P < 0.05$).

DISCUSSION

HCC is one of the most common malignant neoplasms. The majority of the HCC patients are treated with palliative approaches to improve the respectability rate and prolong survival. TACE has been one of the most common and effective palliative approaches. The prognosis of patients treated with TACE depends not only on use of an effective TACE regimen but also on tumor factors[5].

To our knowledge, few data currently are available regarding the molecular mechanism of TACE treatment for patients with HCC, and the current study is the first report detailing the correlations between the expressions of Ki-67 and PCNA protein and different TACE regimens.

The two proliferative indices assessed in our study were Ki-67 and PCNA. Ki-67 presents throughout the cell cycle (late G_1, S, G_2, and M phases) of proliferating cells, but is absent in quiescent (G_0) cells[6]. PCNA is a non-histone nuclear protein of 36 kDa, an auxiliary pro-DNA polymerase δ that plays a major role in synthesizing DNA, and is believed to be expressed in the nuclei, particularly in the late G_1 and S phases[6]. Univariate and multivariate analyses showed that the high labeling index of PCNA resulted in high tumor recurrence risk, more aggressive growth and poor survival[7-9].

The current study demonstrated that the effects of TACE on proliferation of HCC cells depended on its regimens. We found that the mean percentage of Ki-67 and PCNA protein expression was in a decreasing order as follows: Groups A and B > non-TACE group > Group C and D, which suggested that TACE using iodized oil, gelatin sponge particle and/or ethanol significantly inhibited proliferation of HCC cells, whereas TACE using iodized oil alone and chemotherapy alone increased proliferation of HCC cells, which is in agreement with

our previous reports that TACE using iodized oil, gelatin sponge particle and/or ethanol significantly decreased proliferative index (PI) and S-phase fraction (SPF) of HCC cells[10]; alone iodized oil TACE and chemotherapy alone increased PI and SPF of HCC cells[10]; PCNA protein expression of HCC cells was significantly higher in the TACE group which mostly consisted of iodized oil and anticancer drugs[11], and multi-material TACE easily resulted in decreasing of HCC volume[12].

The best interval of treatment for repeated TACE or second stage resection is controversial. Hsu *et al*[13] considered 3 to 21 d interval was adequate to prevent the regrowth of residual tumor cells. Zhu *et al*[14] considered 1 to 3 mo interval was best for resectable tumors, and longer interval for unresectable tumors. Moreover, 1 to 2 mo interval by Liang *et al*[15], 2 to 3 mo interval by Lai *et al*[16], 3 mo interval by Zhang *et al*[17], 3 to 4 mo interval by Kenji *et al*[18], and > 3 mo interval by Teng *et al*[19] were proposed as the best interval. The above-mentioned data were based on the clinical and pathological data. In this study, we found that there were significantly lower Ki-67 and PCNA protein expressions in the "≤ 1 mo" and "> 1 and ≤ 2 mo" interval Groups as compared with "> 3 mo" interval Group ($P < 0.05$). In other word, the remaining cancer cells after TACE treatment had significantly lower proliferative activity at 1 to 2 mo interval than > 3 mo interval. According to this molecular and genetic study and previous clinical and pathological study, we considered the best interval of treatment for repeated TACE or second stage resection is between 2 and 3 mo.

This study demonstrated that Ki-67 and PCNA protein expressions decreased as the courses of TACE increased. Taken collectively, the present data together with our previous study[12] and findings by Spreafico *et al*[20] and Lai *et al*[16] that tumor necrosis, shrinkage of the tumor mass, proliferation and encapsulation of perimass fibrous tissue were closely related to the courses of TACE and findings by Zhang *et al*[21] that the survival in patients with multi-times TACE was better than those with single one, suggest that TACE could be performed multi-times, provided the patients' condition is preferable. But the liver cirrhosis rate after TACE treatment had significant correlation with the courses of treatment[22]. The selective and superselective catheterization is the best way to avoid damaging the normal liver tissue.

In conclusion, the present study demonstrates that the proliferative activity of residual HCC cells after being treated by TACE using iodized oil, gelatin sponge, and/or ethanol is significantly decreased as compared with TACE using iodized oil alone or pure intra-artery chemotherapy. The effect of TACE on proliferation of HCC cells has negative correlation with number of course of TACE and positive correlation with the interval of TACE.

COMMENTS

Background

Transcatheter arterial chemoembolization (TACE) has become one of the most popular and effective palliative methods for patients with HCC. Various mixtures of anticancer drugs, iodized oil and gelatin sponge, have been used as TACE agents. There have been a few reports on comparison of the efficacy of different TACE regimens on patients with Hepatocellular carcinoma (HCC).

Research frontiers

The effects of different TACE regimens on proliferation of HCC cells had not been investigated previously.

Innovations and breakthroughs

In the present study, the effects of the four main types of TACE used clinically on proliferation of HCC cells *in vivo* have been examined.

Applications

Best mixtures and methods could be used for TACE.

Terminology

TACE: Transcatheter arterial chemoembolization.

Peer review

As mentioned TACE is one of the most popular and effective palliative method for patients with HCC. Various mixtures and methods had been used for TACE. In this study, the effectiveness of the TACE methods was compared. It is an interesting study.

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CASE REPORT

Intestinal Kaposi's sarcoma may mimic gastrointestinal stromal tumor in HIV infection

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Abstract

Diffuse intestinal Kaposi's sarcoma shares macroscopic and histopathologic features with gastrointestinal stromal tumors. Correct diagnosis may pose a clinical challenge. We describe the case of a young HIV-1-infected African lady without advanced immunodeficiency, who presented with a diffuse spindle cell tumor of the gut. Initial diagnosis was of a gastrointestinal stromal tumor, based on endoscopy and histopathology. Further evaluation revealed evidence for human herpesvirus 8 (HHV8) and the diagnosis had to be changed to diffuse intestinal Kaposi's sarcoma. Antiretroviral triple therapy together with chemotherapy was commenced, and has led to the rapid remission of intestinal lesions. With a background of HIV infection, the presence of HHV8 as the causative agent of Kaposi's sarcoma should be determined, as distinct treatment is indicated.

Key words: Gastrointestinal stromal tumor; Kaposi's sarcoma; HIV infection; Human herpesvirus 8; c-kit

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INTRODUCTION

Diffuse Kaposi's sarcoma bears a significant morbidity and mortality risk in individuals with HIV infection. It is commonly observed in untreated male homosexual HIV-1-infected individuals and is associated with advanced immune deficiency. Progressive disease is a very rare condition in HIV-infected women[1,2]. In Africa, Kaposi's sarcoma is one of the most frequently occurring tumors. It exists in epidemic and endemic forms, the latter not being associated with HIV infection[3]. Diagnosis remains a challenge, particularly in a setting where clinical findings could be readily explained by alternative diagnoses, which may be more common.

CASE REPORT

A 29-year-old African woman was admitted to our gastroenterology ward in December 2005 with persisting diffuse abdominal pains and abdominal bloating after returning from Nigeria. She also complained of swelling of the right leg for several months. She was known to be infected with HIV-1 and receiving no specific therapy because of only mild immunodeficiency (absolute CD4 count 304/mm^3, CD4/CD8 ratio 0.94 on admission) despite a high viral load (180 000 HIV-1-RNA/mL). Previous history included diabetes, hypothyroidism and arterial hypertension. On examination, mild swelling of the right leg was observed. Ultrasound suggested a deep venous thrombosis, which was treated initially with low molecular weight heparins. Pathologic findings in the blood test included normocytic anaemia, mild thrombocytosis and slightly raised LDH, amylase and lipase levels. Abdominal CT scan was normal. On endoscopy of the upper GI tract, candida oesophagitis, diffuse, non-erosive gastritis and duodenitis with several ulcerous lesions were observed (Figure 1). Colonoscopy revealed diffuse polypoid lesions throughout the entire colon, with aphtous lesions in the sigma (Figure 2). Histopathology showed ulcerative duodenitis. No evidence for T. whippeli, fungi, giardiasis, CMV or HSV infection was found in biopsies. However, a mesenchymal CD34-positive and weakly CD117-positive, SM actin-negative stroma-like tumor was found in all biopsies from the colon. The nuclear proliferation antigen Ki67 was positive in 10%-20% of tumor cells. The diagnosis of a gastrointestinal stromal tumor (GIST) was made by the pathologist. Figure 3 shows spindle cell proliferations in colon biopsies involving the lamina propria; these are readily observed in gastrointestinal stromal tumors.

Because of the diffuse involvement of the entire GI tract, the histological diagnosis was questioned and the patient was readmitted for further biopsies. The possibility

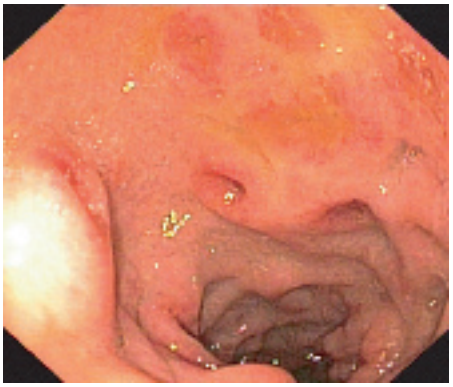

Figure 1 Inflammation of the duodenal mucosa with ulcerous lesions can be seen.

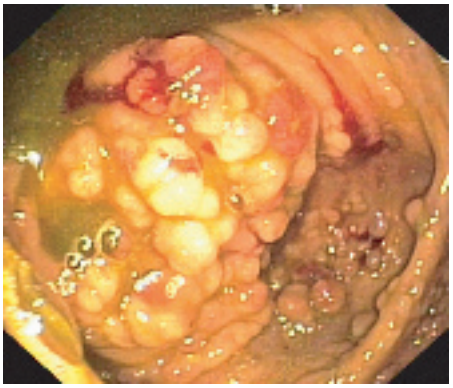

Figure 2 Diffuse polypoid lesions are present in the colon.

of an atypical clinical presentation of a gastrointestinal stromal tumor due to underlying HIV infection was discussed.

In the meantime, her clinical condition had deteriorated with marked weight loss and persistent abdominal pain. Examination of her right foot revealed several tumour-like lesions, which were diagnosed as dermatitis exsudativa by a consultant dermatologist, and a skin biopsy was taken. Ultrasound of the persistently swollen right leg revealed an enlarged lymph node in the right groin, but no signs of a DVT.

Again, gastrointestinal endoscopy was performed, showing a macroscopic appearance similar to the previous investigations, and several biopsies were taken. Finally, further histopathologic examination, including HHV-8 staining of these biopsies, now suggested diffuse Kaposi's sarcoma of the intestine. Retrospective evaluation of the biopsies previously taken from the colon also revealed HHV-8 reactivity.

The diagnosis of diffuse Kaposi's sarcoma was further supported by the detection of HHV-8 in the skin biopsies as well as in the blood. Antiretroviral combination therapy, including two nucleoside reverse transcriptase inhibitors and a boosted protease inhibitor together with 2-weekly liposomal Doxorubicin, was commenced. On her last follow-up visit 6 mo later, the abdominal symptoms and swelling of the leg had resolved completely. Endoscopy of the upper and lower intestinal tract showed no further evidence of Kaposi's sarcoma in the colon, with only small residuals remaining in the duodenum.

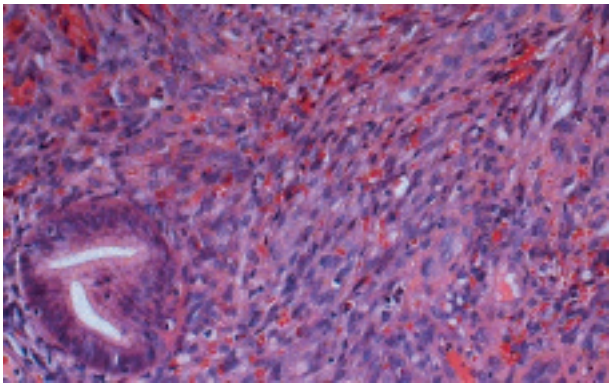

Figure 3 Proliferation of spindle cells is seen in the lamina propria. Slit-like spaces are present, with red blood cells between them.

DISCUSSION

GIST and Kaposi's sarcoma both present as diffuse intestinal lesions with spindle cells, and their diagnosis may be confused because of the presence of CD34-expressing spindle cells with only weak CD117 (c-kit) positivity. Most GISTs show positive immunostaining for CD117 and CD34, but are negative for HHV8. Recent data show the presence of CD117 positivity in up to 56% of Kaposi sarcomas, and overexpression occurs in HHV8-coinfected cells[4]. However, the original cells in Kaposi's sarcoma are of vascular origin, and the presence of human herpesvirus 8 can be found in at least 95% of cases[5]. HHV8 PCR should be performed early to confirm or exclude the diagnosis of Kaposi's sarcoma, especially in immunodeficient patients, because distinct treatment may be indicated[6].

In HIV-positive patients, highly active antiretroviral therapy (HAART) has clearly influenced the occurrence of Kaposi's sarcoma, and is the most important therapy to stop the progression and improve the prognosis of diffuse disease, as also demonstrated by our case[7]. In individual cases with widespread disease and multiple organ involvement, the use of liposomal anthracyclines may be beneficial for the induction of complete remission[8]. The presence of c-kit positivity may rather warrant the use of a selective tyrosine kinase inhibitor, such as imatinib mesylate. To date, however, clinical experience is limited to a few patients with Kaposi's sarcoma refractory to conventional therapy[9]. Further studies using this novel therapeutic approach clearly need to be performed.

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CASE REPORT

Gallbladder endometriosis as a cause of occult bleeding

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Abstract

A 17-year-old girl with colicky abdominal pain and chronic anemia presented to the gastrointestinal service of the University Hospital of Essen. In the routine workup, there were no pathological findings despite the anemia. Because of the fluctuation of symptoms with a climax at the time of menstruation, consecutive ultrasound studies were performed revealing a visible mass inside the gallbladder. This finding was confirmed by a magnetic resonance imaging (MRI) study performed at the same time. Because of the severe anemia by that time, a cholecystectomy was performed, and histology reconfirmed the diagnosis of isolated gallbladder endometriosis. The patient recovered well and has had no recurrence of the disease to date.

Key words: Gallbladder; Endometriosis; Bleeding; Menstrual cycle; Abdominal pain

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INTRODUCTION

Endometriosis is the presence of functioning endometrial tissue outside the uterine cavity. Indeed, when this hormonally active tissue implants in the gastrointestinal tract, it can cause cyclical bleeding, resulting in anemia and pain. Herein, we describe a very rare case of an endometrial tissue manifestation in the gallbladder.

CASE REPORT

A 17-year-old girl with colicky abdominal pain and chronic anemia of unknown origin was referred to the University Hospital of Essen. The right upper quadrant pain was most severe during menstruation. Similar symptoms had occurred one year prior to admission and led to further hospital stays; however, despite extensive diagnostic efforts, a satisfying diagnosis was lacking and the treatment was for symptoms only. The patient was treated with repetitive iron replacement and blood transfusions, which led to several complications including thrombophlebitis, with the need for surgical intervention. Physical examination revealed no pathological findings apart from a local tenderness to palpation in the right upper quadrant of the abdomen. Routine laboratory studies confirmed a normocytemic hypochromic anemia. No further abnormalities were seen in routine laboratory studies; liver function tests were within normal limits, as was hepatitis serology including tests for major hepatotropic viruses.

Several diagnostic means were used to identify the cause of symptoms, including oesophago-gastro-duodenoscopy and colonoscopy as well as radiologic studies. Endoscopic examinations showed physiologic findings in the upper and lower gastrointestinal tract. Radiographic examinations, including barium follow-through, initial ultrasound (US) and magnetic resonance imaging (MRI) of the abdomen, were normal. Computed tomography (CT) of the abdomen showed a questionable radiopaque tissue in the wall of the gallbladder, with concomitant inflammation. These findings, combined with the history of complaints, led to the presumed diagnosis of gallbladder endometriosis. US examination was performed once more on d 14 of the menstrual cycle and repeated every second day, showing continual expansion of the suspected tissue. After 12 d and maximum extension of the tissue, a second MRI of the liver was performed, and the diagnosis of endometriosis of the gallbladder could be confirmed (Figure 1).

According to the radiologic findings and the continued abdominal complaints, laparoscopic cholecystectomy was discussed with the Department of General Surgery and finally performed. The postoperative course was uneventful. Twelve months after surgery the patient was still without any complaints, and during the laboratory follow up her hemoglobin level remained within the normal range; no further transfusions have been necessary. Histopathological examination confirmed the clinical diagnosis of endometriosis of the gallbladder.

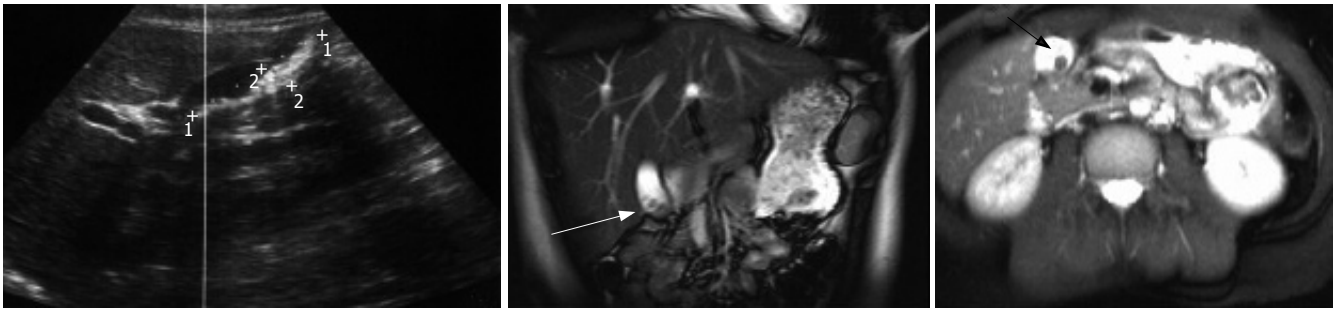

Figure 1 Computed tomography (CT) of the abdomen showed a radiopaque tissue in the wall of the gallbladder, with concomitant inflammation. US examination showed continual expansion of the suspected tissue. After 12 d and maximum extension of the tissue, a second MRI of the liver was performed, and the diagnosis of endometriosis of the gallbladder could be confirmed.

DISCUSSION

Endometriosis, defined as the presence of endometrial tissue outside the uterine cavity and musculature, was first described by von Rokitasky in 1860. Although it usually occurs in the pelvis, endometriosis has been found in almost every region of the human body. As a response to physiologic hormonal changes that occur during the menstrual cycle, this ectopic endometrium will invade, distort and occasionally destroy normal organs. The prevalence of this abnormality has been reported to be between 8% and 18% in young women[1,2]. The majority of extrauterine endometrial tissue invades ovaries and the pelvic peritoneum. Ectopic endometriosis in other sites of the female body usually involves the gastrointestinal tract, but ectopic tissue may also present within the urinary tract[1,3,4]. In addition, symptomatic mediastinal, bronchial and pleural endometriosis have been documented[3,5-8] and the presence of endometrial tissue has been identified in thigh muscle tissue[9,10], the inguinal canal[10], nasal mucosa[11], incisional scars[12] and, in very few cases, in the gallbladder[13]. The clinical diagnosis of intestinal endometriosis may be difficult to make because of non-specific symptoms and the missing relationship between symptoms and the menstrual cycle. However, endometriosis should always be considered in women with recurrent abdominal pain and intestinal symptoms, especially in young females with gynaecologic complaints. The high prevalence of irritable bowel syndrome increases the risk of misdiagnosis in these rare cases.

According to its localization, intestinal endometriosis is often an incident finding in laparoscopic procedures[14-18]. Recognition requires a high index of suspicion. Thus, physicians should be aware of endometriosis as a differential diagnosis in female patients with recurrent periumbilical or abdominal pain and other episodic bowel symptoms.

An important component of the evaluation is a bimanual pelvic examination that includes combined rectovaginal palpitation. Because findings may vary considerably throughout the menstrual cycle, all examinations should be performed immediately before and again after menses[3,4].

In many cases, radiologic findings are useful in raising the possibility of detection of endometriosis, providing supportive evidence for a preliminary diagnosis[19-22]. Intestinal endometriosis appears radiographically as a tapered, often eccentric, constricting deformity. Although CT scanning and US are often unable to differentiate between abscesses and hematomas from endometriotic lesions[4,18,23], such indirect imaging methods may be useful in defining the anatomic extent of pelvic endometriosis[4]. MRI is useful for monitoring the response to treatment, but it cannot be relied upon as a diagnostic substitute for laparoscopy[23-26].

As intestinal endometriosis is usually nonmucosal, enteroscopy is helpful in excluding other gastrointestinal disorders, especially neoplasia[27,28]. Unequivocal diagnosis relies upon histological confirmation of the presence of the endometrium within one or several organs of the gastrointestinal tract. It is of particular importance in such morphologic interpretations to avoid confusing endometrial tissue with carcinomatous glands. In postmenopausal women in particular, less prominent stromal elements leave scattered endometrial glands, which appear similar to well-differentiated adenocarcinomas. In general, when a diagnosis of intestinal endometriosis is made, hormonal therapy is often the first therapeutic option, similar to the standard approach to pelvic endometriosis[29-31]. Low-dose estrogen-progesterone compounds can cause pseudopregnancy states that result in the decidualization of endometrial tissue and often relieve symptoms like dysmenorrhea. However, their use in more severe diseases is questionable and generally not recommended for symptomatic intestinal diseases. The most effective agents currently available are the synthetic androgen danazol and the gonadotropin-releasing hormone (GnRH) agonists. New approaches tend to use add-back estrogen replacement to improve the quality of life and reduce the side effects of these treatments[32]. Although both are effective in decreasing pelvic pain associated with endometriosis and appear to decrease the size of endometrial implants, there are no studies of these agents in intestinal disease, and there is some concern that treatment can result in increased fibrosis[33]. In cases of mucosal endometriosis, laparoscopical ablation can be accomplished using a carbon dioxide laser[34,35]. In cases of endometriosis causing partial obstruction of the colon or small intestine, segment resection of the involved area is considered to provide the best results, and it also serves to exclude any underlying carcinoma[4,36]. In patients who have failed medical therapy and who have intractable symptoms,

hysterectomy and salpingo-oophorectomy can be performed at the time of resective surgery to minimize the risk of symptomatic disease in the future. Similar surgery also can be performed in postmenopausal patients[37-39].

Because of the isolated endometrial manifestation in the gallbladder and the age of the patient in this case, a surgical approach and laparoscopic cholecystectomy seemed to provide the best results. Considering the long history and suffering of this patient for over one year, it appears that familiarity with this nonneoplastic process and an appropriate index of suspicion is often lacking in physicians - even for patients with typical presentations.

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CASE REPORT

Radiotherapy for multiple brain metastases from hepatocellular carcinomas

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Abstract

A 78-year-old man with liver cirrhosis was found to have multiple hepatocellular carcinomas (HCCs) and underwent 3 sessions of transcatheter arterial chemoembolization. Fourteen months after diagnosis, the patient presented with left hemiparesis. Contrast-enhanced magnetic resonance imaging showed multiple metastases with ring-shaped enhancement in the cerebrum and cerebellum. There were no metastases to other organs. The metastatic lesions almost completely disappeared after whole-brain radiotherapy with a total dose of 50 Gy. Neurologic symptoms decreased, and the patient's quality of life improved. The patient underwent 2 more sessions of transcatheter arterial chemoembolization. Twelve months after the diagnosis of brain metastasis, the patient remains alive. The present case indicates that radiotherapy can improve quality of life and prolong survival in some patients with brain metastases from HCCs.

Key words: Hepatocellular carcinoma; Brain metastasis; Radiotherapy

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies in Asian countries, including Japan[1]. Although recent advances in treatment have helped prolong the survival of patients with HCC, they consequently increase the risk of intrahepatic recurrence and extrahepatic metastasis. The most frequent sites of extrahepatic metastasis are the lung, bone, lymph nodes and adrenal glands[2,4]. Brain metastasis is rare: it is found at autopsy in 2.2% of cases of HCC in Japan[5]. The extremely poor prognosis of patients with brain metastasis is a significant problem. A recent study of a large number of patients reported a median survival time of only 1 mo[6]; other studies have reported similar results[7,8]. Furthermore, quality of life (QOL) is decreased because of neurologic symptoms, such as headache, mental change and hemiparesis/hemiplegia, and many patients die of neurologic causes[6,7]. Radiotherapy is a possible treatment for brain metastasis, but its efficacy remains unclear. This paper reports a case of multiple brain metastases from HCCs, for which whole-brain radiotherapy had a dramatic therapeutic effect.

CASE REPORT

A 78-year-old man had visited a hospital for treatment of liver cirrhosis for more than 10 years. Tests for both hepatitis B surface antigen and antibodies to hepatitis C virus were negative. A routine contrast-enhanced computed tomography (CT) examination revealed multiple HCCs. The patient underwent transcatheter arterial chemoembolization (TACE). Four months later, he visited our hospital. The HCCs were not suitable for resection or ablation because of their number, size and location. Accordingly, the patient underwent 2 more sessions of TACE (Figure 1). Twelve months later, endoscopic sclerotherapy was performed for esophageal varices.

Fourteen months later, contrast-enhanced CT showed multiple enlarged HCCs (Figure 2). Laboratory data were as follows: aspartate aminotransferase, 28 IU/L (normal, 8-35 IU/L); alanine aminotransferase, 15 IU/L (normal, 5-43 IU/L); lactate dehydrogenase, 220 IU/L (normal, 106-211 IU/L); alkaline phosphatase, 191 IU/L (normal, 104-338 IU/L); γ-glutamyl transpeptidase, 35 IU/L (normal, 5-50 IU/L); total bilirubin, 0.8 mg/dL (normal, 0.3-1.1 mg/dL); albumin, 4.1 g/dL (normal, 3.8-5.1 g/dL); prothrombin time, 67% (normal, 70%-130%) (Child-Pugh score 5; class A). The serum levels of alpha-fetoprotein and des-gamma-carboxy prothrombin were 113.7 ng/mL (normal, < 10 ng/mL) and 217 mAU/mL (normal, < 40 mAU/mL), respectively. At that time, the patient was admitted to our hospital for the treatment of HCC. On

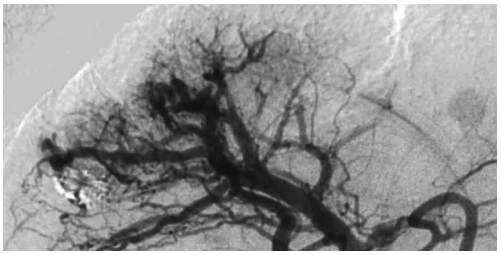

Figure 1 Hepatic arteriography at the time of the third session of TACE shows arterial tumor vessels with arterioportal shunting.

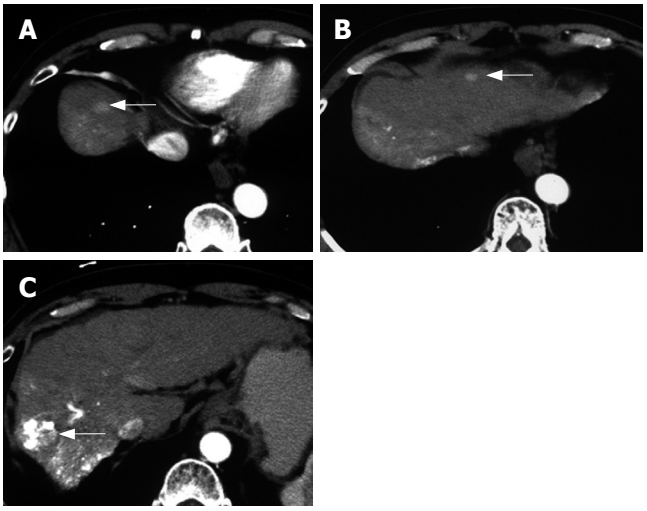

Figure 2 Contrast-enhanced CT at the time of the diagnosis of brain metastases shows multiple hypervascular HCCs (arrows) (**A**, **B**, **C**). Lipiodol retention in the main tumor is seen (**C**).

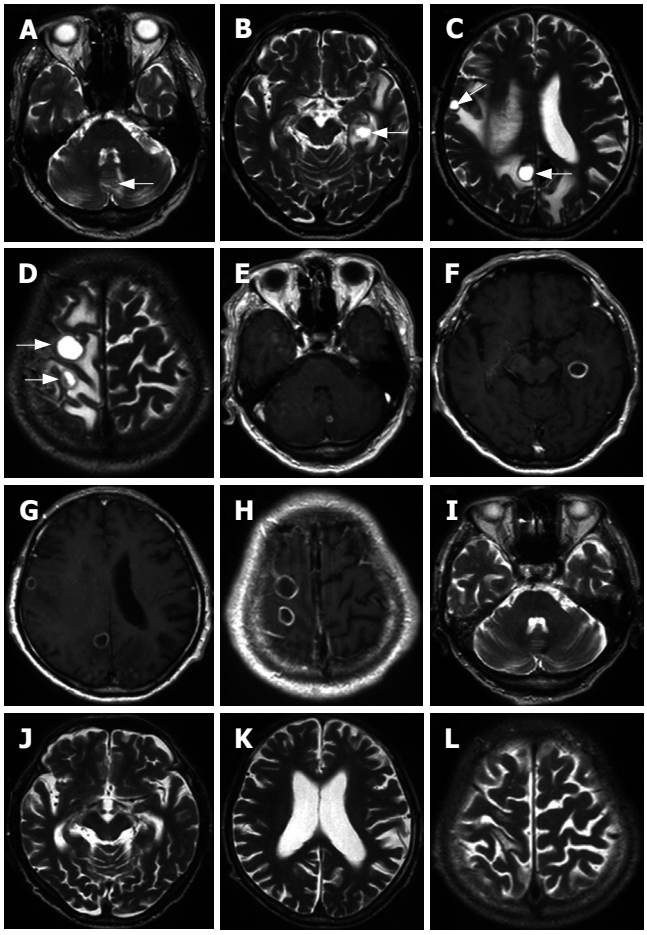

Figure 3 Unenhanced T2-weighted MR images show hyperintense tumors in the cerebrum and cerebellum (arrows) (**A**, **B**, **C**, **D**). Contrast-enhanced T1-weighted MR images show ring-shaped enhancement of the tumors (**E**, **F**, **G**, **H**). On T2-weighted, unenhanced MR images, captured 3 mo after the completion of radiotherapy, the brain tumors have almost completely disappeared (**I**, **J**, **K**, **L**).

admission, he complained of gradual and progressive left hemiparesis of 1 mo duration. Unenhanced magnetic resonance (MR) revealed 12 tumors, less than 2 cm in diameter, in the cerebrum and cerebellum; these tumors showed hypointensity on T1-weighted MR images and hyperintensity on T2-weighted MR images (Figure 3A-D). On contrast-enhanced MR with gadolinium-DTPA, the tumors showed ring-shaped enhancement (Figure 3E-H). On the basis of the results of chest and abdominal CT, endoscopic examinations, and bone scintigraphy, the brain tumors were diagnosed as metastases from HCCs; there were no metastases to other organs, including lung, bones, lymph nodes and adrenal glands. The patient received whole-brain radiotherapy with a total dose of 50 Gy in 25 fractions over 5 wk. Most metastatic lesions shrank immediately after radiotherapy. Three months after the completion of radiotherapy, the tumors had almost completely disappeared (Figure 3I-L). Neurologic symptoms had almost completely resolved and the patient's QOL had improved. No severe acute or late radiologic toxicity was observed. The patient underwent 2 more sessions of TACE. Hepatic functional reserve was good during the clinical course (Child-Pugh score 6 to 7). Twenty-three months after initial diagnosis, the patient stopped attending our hospital. Twenty-six months after the initial diagnosis of HCC, and 12 mo after the diagnosis of brain metastasis, the patient is alive and receiving supportive treatment at the original hospital.

DISCUSSION

Patients with brain metastasis from HCCs generally have advanced intrahepatic disease[7], and most patients have simultaneous metastases to other sites, usually the lung[7,8]. On the other hand, cases of brain metastasis from early-stage HCCs[9] or without other extrahepatic metastases[9,10] are occasionally reported. Clinicians should consider the possibility of brain metastasis from HCCs whenever neurologic symptoms develop in patients with HCC.

Recent studies have found that brain metastases from HCCs show homogeneous or ring-shaped enhancement on contrast-enhanced imaging[7]. The enhancement pattern of the present case was ring-shaped, which may be due to the central necrosis of tumors[7]. Brain metastases from HCCs tend to bleed in proportion to the size of the tumor[8]. MR is useful for distinguishing such tumoral hemorrhage from nontumoral hemorrhage[8].

Most patients with extrahepatic metastases from HCCs die of primary HCC or hepatic failure rather than the metastases because of the advanced stage of intrahepatic

disease or poor hepatic functional reserve, or both[4]. Such outcomes imply that active treatment for metastasis might not prolong survival unless the primary HCC is controlled and the hepatic functional reserve is good. In addition to shortening survival, extrahepatic metastasis often decreases QOL. It is therefore worth considering active treatment for metastasis in such cases regardless of prognosis.

There is no established therapeutic strategy for brain metastasis from HCCs. Craniotomy, radiotherapy or both are commonly performed. Chang *et al*[6] reported that patients with brain metastases who receive craniotomy, radiotherapy or both survive longer than patients who receive supportive care only (more than 4 mo compared with less than 1 mo). Chen *et al*[11] reported that clinical improvement associated with a stable lesion is observed in patients with brain metastasis who receive radiotherapy. On the other hand, Kim *et al*[12] reported that radiotherapy fails to stabilize the condition of patients with brain metastasis: the cause of death in most patients is progressive cachexia or hepatic failure. The applicability of radiotherapy should be carefully determined in each case from the viewpoints of prognosis and QOL. Recently, stereotactic radiosurgery has increasingly been used with or without whole-brain radiotherapy for the treatment of brain metastasis[13]. Future studies will be required to select an appropriate radiation method according to the number and sizes of metastatic lesions in the brain.

Possible reasons why the present patient survived for a long time after the diagnosis of brain metastases are as follows. First, the brain metastases did not cause life-threatening conditions, such as brain herniation or massive cerebral hemorrhage. Second, radiotherapy had a dramatic therapeutic effect on the brain metastases. Third, there were no other life-threatening extrahepatic metastases. Fourth, the primary HCCs could be controlled with repeated TACE, and hepatic functional reserve was good.

In conclusion, radiotherapy can improve QOL and prolong survival in some patients with brain metastases from HCCs. Further studies should be performed to establish a therapeutic strategy using radiotherapy for such patients.

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CASE REPORT

Hepatocellular carcinoma masquerading as a bleeding gastric ulcer: A case report and a review of the surgical management

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Abstract

Hepatocellular Carcinoma (HCC) is a common malignancy worldwide. While bleeding from the gastrointestinal tract (BGIT) has a well known association with HCC, such cases are mainly due to gastric and esophageal varices. BGIT as a result of invasion of the gastrointestinal tract by HCC is extremely rare and is reportedly associated with very poor prognosis. We describe a 67-year-old male who presented with BGIT. Endoscopy showed the site of bleeding to be from a gastric ulcer, but endoscopic therapy failed to control the bleeding and emergency surgery was required. At surgery, the ulcer was found to have arisen from direct invasion of the gastrointestinal tract by HCC of the left lobe. Control of the bleeding was achieved by surgical resection of the HCC en-bloc with the lesser curve of the stomach. The patient remains alive 33 mo after surgery. Direct invasion of the gastrointestinal tract by HCC giving rise to BGIT is very uncommon. Surgical resection may offer significantly better survival over non-surgical therapy, especially if the patient is a good surgical candidate and has adequate functional liver reserves. Prognosis is not uniformly grave.

Key words: Hepatocellular carcinoma; Gastrointestinal bleeding; Stomach invasion; Hepatectomy

Ong JCA, Chow PKH, Chan WH, Chung AYF, Thng CH, Wong WK. Hepatocellular carcinoma masquerading as a bleeding gastric ulcer: A case report and a review of the surgical management. *World J Gastroenterol* 2007; 13(33): 4523-4525

http://www.wjgnet.com/1007-9327/13/4523.asp

INTRODUCTION

Hepatocellular carcinoma (HCC) is an important malignancy and is responsible for more than 250 000 deaths worldwide each year[1]. Bleeding from the gastrointestinal tract (BGIT) is a common complication of HCC and a frequent cause of death from this disease. Most cases of BGIT are due to esophageal and gastric varices[2] and the rest are mainly due to peptic ulcers. However, invasion of the gastrointestinal tract by HCC, through metastasis or direct invasion, is found in only 4-12% of cases at autopsy[3-5,12]. These lesions tend to be asymptomatic and are thus mainly only discovered at post-mortem examination. Direct invasion of the stomach by HCC is extremely rare. A detailed literature review showed only 10 cases of HCC with direct or contiguous invasion of the stomach have been reported[2,6-12]. We describe the case of a 67-year-old Chinese man with this uncommon clinical entity, who presented with BGIT and was managed by surgical resection.

CASE REPORT

A 67-year-old Chinese man presented to the Accident and Emergency Unit with a 3 d history of non-specific epigastric pain associated with postural dizziness and exertional dyspnea. There was no history of significant alcohol consumption and his hepatitis B and C status were unknown. History was negative for malaena, hematemesis, and hematochezia. He had a history of one episode of mild hemoptysis subsequent to which he was diagnosed as having bronchietasis due to heavy smoking.

Physical examination revealed a cachectic patient who was alert but had marked pallor of the conjunctiva. He was mildly hypotensive with a supine blood pressure of 105/60 with no postural drop. His pulse rate was 78 beats per minute, and he was not tachypneic. Palpation of the abdomen revealed mild epigastric tenderness on deep palpation but no significant organomegaly. He did not exhibit any stigmata of chronic liver disease. Bowel sounds were normal as was the digital rectal examination. There was no clinical sign of cardiac failure.

Initial investigations showed a white cell count of 8.5×10^9 (normal range 4.0-10.0 $\times 10^9$/L), hemoglobin of 4.2 g/dL (normal range 14.0-18.0 g/dL), platelet count of 274×10^9 (normal range 140-440 $\times 10^9$) and amylase of 71 U/L (normal range 30-110 U/L), He was transfused with 3 units of packed red cells, and an urgent

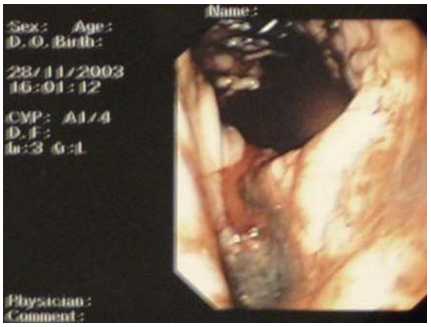

Figure 1 Endoscopic picture showing active bleeding from the lesser curve of the stomach.

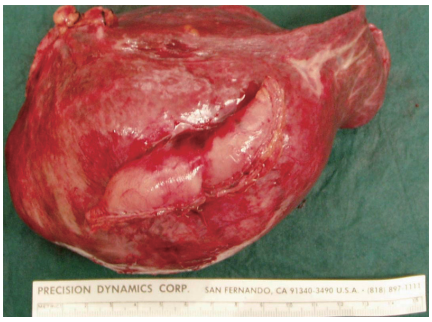

Figure 2 Inferior view of the resected tumor specimen with adherent stomach wall.

referral to the surgical service was made. Esophageal-gastroduodenoscopy and colonoscopy were suggested but the patient declined referral or admission and discharged himself from the hospital against medical advice.

One month later, he presented himself at the outpatient specialist surgical clinic. Hemoglobin level on arrival was 9.4 g/dL. He finally consented to endoscopic examination as an outpatient procedure. Esophageal-gastroduodenoscopy was carried out on the same afternoon, during which an actively bleeding ulcer was seen in the lesser curve (Figure 1). Repeated injection with adrenaline failed to stop the bleeding and he was immediately transfused with packed cells and prepared for emergency surgery.

At celiotomy, a 10 cm tumor arising from segments Ⅱ and Ⅲ of the liver was found; the tumor involved the lesser curve of the stomach and had eroded into the lumen. On-table referral to the hepato-biliary surgical service was made. Resection of segments Ⅱ and Ⅲ of the liver with en-bloc wedge resection of the lesser curve of the stomach was carried out both to control the hemorrhage and as a definitive therapy for the tumor (Figure 2).

The patient's post-operative recovery was uneventful. He was discharged from the surgical intensive care unit on the 2nd post-operative day. He tested positive for hepatitis B surface antigen and his serum hepatitis B antibody was found to be less than 10 IU/ml. The resected liver segments showed cirrhotic changes and a large necrotic tumor measuring 10 cm × 9 cm × 9 cm. Surgical margins were free of tumor. Histology revealed a trabecular hepatocellular carcinoma that was grade 2 by Edmondson's grading. He was discharged from the hospital in good

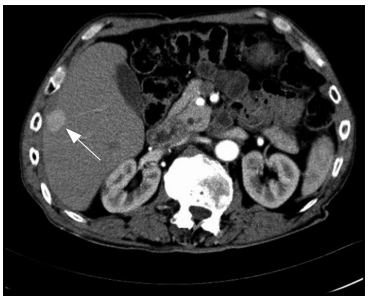

Figure 3 HCC recurrence in the right lobe. Enhanced axial hepatic arterial-phase CT performed on follow-up after initial resection shows a hypervascular lesion in segment 5/6 suspicious for HCC recurrence (arrow). Incidental note is made of a small cyst in the right kidney.

health on the 9th post-operative day. An early post-operative CT scan showed no residual tumor, but a repeat CT scan performed 9 mo later showed multiple new lesions of HCC in the liver (Figure 3). He opted for conservative management and was still alive 2 years and nine months after surgery.

DISCUSSION

HCC is a highly malignant type of tumor. Extrahepatic metastases occur in 30%-75% of patients, commonly affecting the lungs, regional lymph nodes, bones and adrenal glands[3]. Direct gastrointestinal tract involvement is rarely seen. In a clinical study, Chen *et al*[12] reported 8 out of 396 patients (2%) with HCC who developed gastrointestinal involvement during the course of the disease. Lin *et al*[2,6-12] similarly reported gastrointestinal metastases in 11 out of 2237 patients with HCC (0.5%). Only 10 cases of HCC invading directly into the stomach could be found in the literature. Eight of these presented with BGIT, and only one of the 10 subsequently underwent surgical resection of the HCC.

Of these 10 cases reported[2,6-12], 6 had received some form of regional therapy, such as trans-arterial chemoembolization (TACE), intra-arterial chemotherapy or radiotherapy, either alone or in combination, prior to the HCC invading the gastro-intestinal tract. This includes 4 of the 5 patients with direct invasion of the gastrointestinal tract by contiguous HCC reported by Chen *et al*[12]. Chen *et al*[12] postulated a relationship between regional therapy and the development of direct invasion of the gastrointestinal tract by HCC. It was proposed that when a large, subcapsular, massive-type HCC adjacent to the gastrointestinal tract is treated with TACE, the wall of the gastrointestinal tract could be affected by the inflammatory response secondary to TACE and become adherent to the tumor capsule. Viable tumor tissue could then invade the GI tract.

Our patient presented with non-specific symptoms and only a very low hemoglobin level, suggesting gastrointestinal bleeding. He was not known to be a hepatitis B carrier or a patient with HCC at initial presentation and, thus had no history of regional therapy for HCC. However the resected tumor was

large, measuring 10 cm × 9 cm × 9 cm. In the published literature, of the 19 cases of HCC with gastrointestinal tract involvement (both direct invasion and distant metastasis) reported by Lin *et al*[7] and Chen *et al*[12], 14 tumors were considered to be large and 17 were larger than 6 cm, with the tumor sizes of the remaining 2 cases not being described. A casual relationship between tumor size and the probability of direct invasion to the surrounding viscera should therefore be considered.

In previously reported cases of HCC with gastrointestinal tract involvement, treatments have included surgery, TACE and local injection with ethanol. Results of treatment have been poor, with almost all patients dying within 5 mo except for 1 case described by Nicoll *et al*[11]. In this reported case, the HCC invaded the stomach and the patient was treated with surgical resection. He was reported to be still alive 7 mo after resection. Our patient similarly had surgical resection with clear margins and remains alive 33 mo after surgery. Thus, such contiguous invasion of the stomach does not always represent terminal disease. Surgery should be considered wherever feasible as other modalities of treatment for HCC, including chemotherapy, are poorly efficacious[13].

Large HCCs may have a predisposition towards contiguous involvement of the gastro-intestinal tract, including the stomach. Diagnosis is difficult as most patients are asymptomatic and diagnosis by endoscopy is rarely achieved as these lesions have no special or characteristic endoscopic features. In our patient, diagnosis was made during emergency surgery to control bleeding from a supposed gastric ulcer. Based on limited case reports, the accepted paradigm is that an HCC directly invading the gastrointestinal tract is associated with very poor prognosis and that it may not be worthwhile to pursue an aggressive treatment policy. However, the scientific basis of this belief is limited, and this case report shows that aggressive resection can both resolve the acute clinical problem (gastrointestinal bleeding) as well as result in a relatively long-term survival. Surgery remains a viable option in patients with HCC and presenting with bleeding from the upper gastrointestinal tract as a result of direct invasion by a tumor.

Invasion of the upper gastrointestinal tract by HCC is extremely uncommon. In our case, the patient did not present with any signs and symptoms of chronic liver disease or hepatomegaly suggestive of hepatocellular carcinoma. The discovery of hepatocellular carcinoma invading directly into the stomach was an incidental finding from laparotomy performed for a bleeding peptic ulcer. Resection of segments Ⅱ and Ⅲ of the liver with en-bloc wedge resection of the lesser curve of the stomach was performed as a definitive procedure. This report demonstrates that the prognosis is not necessarily dismal in such cases if there are no distant metastases. Surgical resection may offer a significantly better survival over non-surgical therapy, especially if the patient is a good surgical candidate and has adequate functional liver reserves.

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CASE REPORT

Intractable bleeding from solitary mandibular metastasis of hepatocellular carcinoma

Shiang-Fu Huang, Ren-Chin Wu, Joseph Tung-Chieh Chang, Sheng-Chieh Chan, Chun-Ta Liao, I-How Chen, Chun-Nan Yeh

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Abstract

Hepatocellular carcinoma (HCC) metastasizes to the mandible is infrequently seen. Solitary bony metastasis to the mandible is rarer. The intractable bleeding caused by rupture of the metastatic HCC is challenging to clinicians. We present a case of a 74-year-old woman with HCC under control without progression for 3 years. Left facial swelling and episodes of bleeding developed recently and biopsy revealed a metastatic HCC. Computer tomography showed a large tumor in parapharyngeal space with evident mandibular ramus destruction. Bleeding occurred from the metastatic tumor but could not be controlled by electrocauterization, Surgicel™, tissue glue, and bone wax and angiographic embolization. Palliative radiotherapy (2400 cGy in 6 fractions) was tried and the intractable bleeding was successfully stopped after the radiotherapy. Because of the hypervascular and osteolytic nature of the solitary mandibular metastatic lesion, the bleeding was troublesome. Radiotherapy provided successful control of intractable bleeding from the metastatic tumor.

Key words: Hepatocelluar carcinoma; Metastasis; Mandible; Radiotherapy; Bleeding

Huang SF, Wu RC, Chang JTC, Chan SC, Liao CT, Chen IH, Yeh CN. Intractable bleeding from solitary mandibular metastasis of hepatocellular carcinoma. *World J Gastroenterol* 2007; 13(33): 4526-4528

http://www.wjgnet.com/1007-9327/13/4526.asp

INTRODUCTION

Hepatocellular carcinoma (HCC), with an estimated 1 million cases per year, is the 5th most common cancer in the world, particularly in the Southeast Asia[1]. HCC with extrahepatic metastasis has been reported in approximately 50% of cases; lungs, abdominal lymphatics, adrenal glands, great veins adjacent to the liver, the diaphragm, or the skeleton could be involved. Bone metastasis in HCC has been reported in 10.1% of patients, with the vertebrae being the most frequently affected, followed by (in decreasing order) ribs, sternum, and pelvis[2]. The mandible is a rare site of HCC metastasis. Here we report a rare case of mandibular metastasis of HCC with intractable bleeding and the bleeding was controlled successfully by radiotherapy.

CASE REPORT

A 74-year-old woman was suffering from chronic hepatitis C-related HCC for 6 years. Segmental hepatectomy was done initially. She had undergone transcatheter arterial chemoembolization (TACE) 9 times, and percutaneous ethanol injection (PEI) 3 times for tumor recurrence. She had 3 years of progression-free survival.

She was referred to our clinic due to a 1 cm × 1 cm ulcerative mass in the left buccal region, with preauricular swelling for 2 wk. Biopsy was done over the buccal mass. Two days later, she presented in the emergency room with the chief complaint of progressive facial swelling and persistently blood oozing from the left buccal tumor. The laboratory tests were AST (GOT) 58 U/L, ALT (GPT) 49 U/L and platelet count 179 × $10^3/\mu L$. Her liver disease was in the status of Child-Pugh class A. CT scan demonstrated a 6.2 cm × 5.0 cm osteolytic lesion in the left parapharyngeal space with destruction of the left mandible (Figure 1A). The lesion extended from the mandibular ramus to temporal muscle space (Figure 1B). Diffuse and massive bleeding occurred from the ulcerative

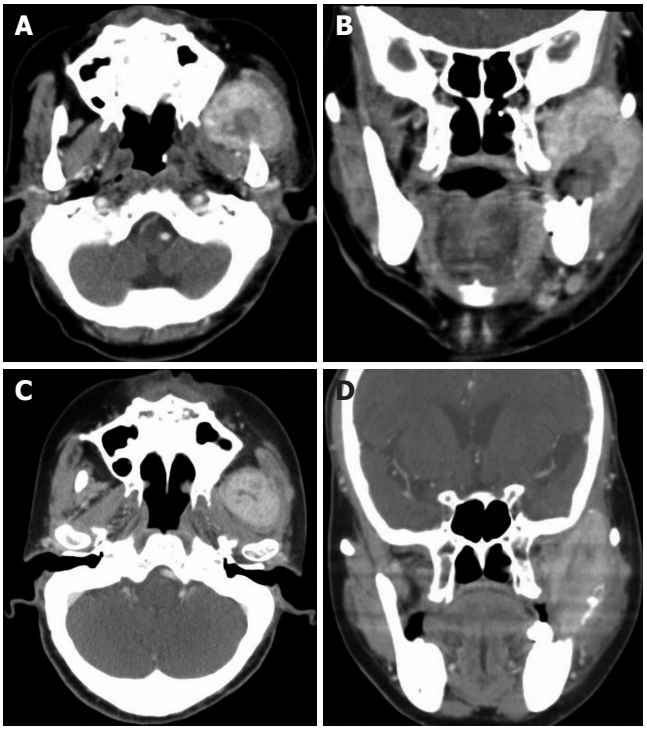

Figure 1 Axial and coronal CT scan of the neck with intravenous contrast showing a 6.2 cm x 5.0 cm, heterogeneously enhancing mass, which appears to be a left parapharyngeal mass involving the pterygoid muscle and temporal muscle (**A** and **B**); axial and coronal CT scan of the head and neck showing a 4.0 cm x 2.5 cm mass which shrunk after completion of radiotherapy (**C** and **D**).

mass the next morning after admission. Hemorrhage could not be stopped by electrocauterization or suture ligation alone, but it was stopped temporarily by the use of electrocauterization, Surgicel™, tissue glue, and bone wax concomitantly after 700 mL blood loss. The mandibular lesion was proved pathologically to be a metastatic HCC. Bone scan showed the mandibular lesion was a solitary metastasis of HCC. CT scan of the brain, chest, abdomen and pelvis failed to reveal any evidence of metastatic disease.

Unfortunately, a second episode of hemorrhage occurred 5 d later. Due to antecedent experience of difficult hemostasis, we tried vascular embolization. Under angiography, the injection of contrast medium through the left common carotid artery and external carotid artery showed a hypervascular stain about 3.8 cm in the left masticator space. The left maxillary artery and the main feeding artery were occluded by the injection of permanent embolizer (500 to 700 U of polyvinyl alcohol particles, PVA). Over 90% of tumor stain was obliterated after embolization and the main feeding artery was occluded. However, slow but profuse oozing from the left buccal wound persisted. After discussion with radiation oncologist, palliative radiotherapy was suggested in the handful treatment options. We arranged radiotherapy for the left mandibular metastatic lesion (2400 cGy in 6 fractions). Oozing decreased about 3 d after the start of radiotherapy and stopped 5 d after the completion of radiotherapy and the tumor shrunk after radiotherapy (Figure 1C and D). Now the woman was stable with disease at a follow-up period of 5 mo.

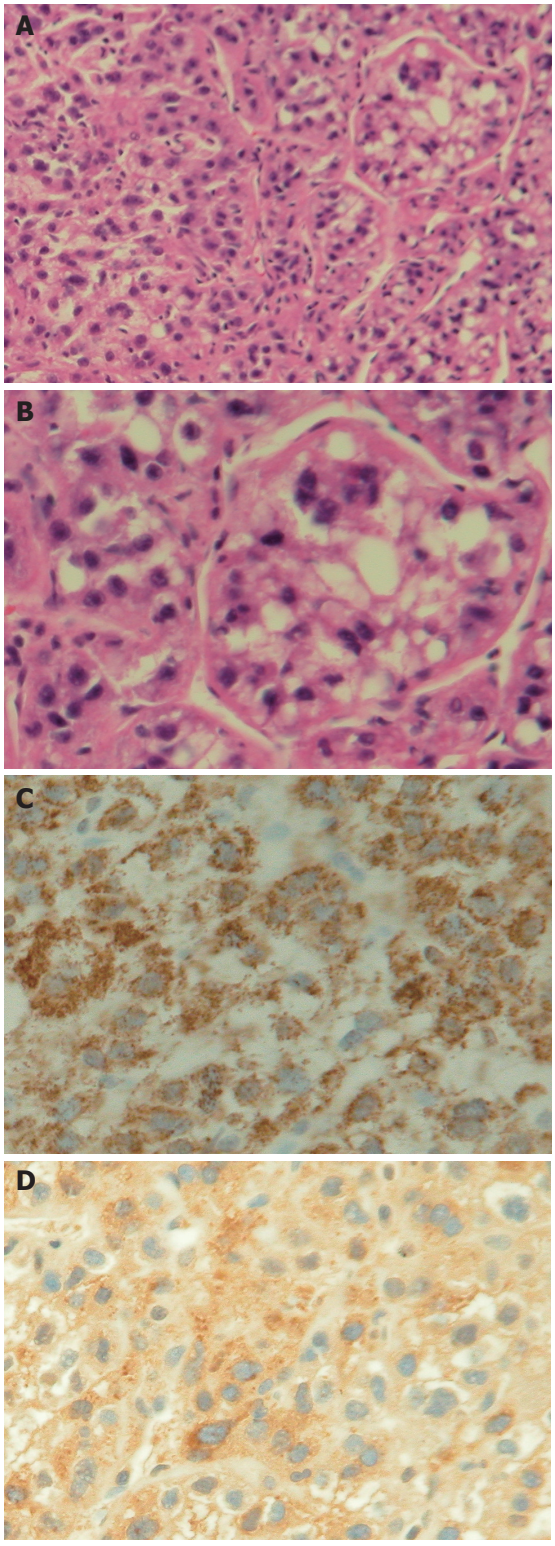

Figure 2 Histopathology of the metastatic lesion. **A**: The tumor is composed of polygonal cells featuring abundant clear to eosinophilic cytoplasm and microvesicular fatty change, and the tumor cells are arranged in broad trabeculae surrounded by sinusoid spaces which are characteristic of hepatocellular carcinoma (HE, x 100); **B**: Histology of the metastatic tumor (HE, x 200); **C**: The tumor cells showing immunoreactivity to Hep Par-1 with a cytoplasmic granular staining pattern (x 200); **D**: The tumor cells showing focal immunoreactivity to AFP (x 200).

Histopathological examination of the ulcerative mass at the mandible revealed the metastasis was from well-differentiated HCC (Figure 2A and B). Neoplastic cells

were diffusely stained by hepatocyte-paraffin-1 (Hep Par-1) (Figure 2C) and focally positive for α-fetoprotein (AFP) (Figure 2D).

DISCUSSION

With advances in surgical techniques and an improvement in perioperative care, surgical mortality rates in HCC patients receiving hepatectomy have reduced significantly. Postoperative recurrence is universally high and remains the main cause of late deaths. The development of PEI and TACE contributes to the better local control of HCC. Nevertheless, distant metastasis of HCC is more and more frequently seen after the better control of primary disease. The most common sites of HCC metastasis are the lungs, bones, brain, and skin[3]. The mode of extrahepatic spread from HCC is usually hematogenous metastasis. The incidence of bone metastasis from HCC accounts for approximately 1.6%-16% and the most common sites are the vertebrae, followed by pelvis and ribs[4]. Mandible is rarely metastasized by HCC. In addition, bone metastasis usually manifests as multiple lesions. Solitary bony metastasis in HCC is infrequently met, as in our patient.

Two modes of spread were ever proposed for the tumor spreading from the liver to the maxillofacial territory. The metastatic dissemination reaches the lungs first, and possibly the maxillofacial area later through the communication between the hepatic artery and portal vein. It has been postulated that there is a connection between the azygos and hemiazygos veins and the vertebral venous plexus (Batson's plexus)[2,5]. There is the existence of free communication between the neck, thorax, abdomen and pelvis venous systems with the non-valve vertebral venous plexus that extends from cranial base to coccyx. Any pressure increment inside the abdomen can create an ascendant flow through the vertebral venous plexus. The HCC cells could reach maxillofacial territory through these two hematogenous connections and grow into a metastatic lesion in the mandibular region.

Any malignancy in head and neck, either primary or metastatic lesions, can manifest as bleeding. However, HCC metastatic lesions, as seen in our case, are hypervascular and osteolytic in nature. It may rupture spontaneously to cause hemorrhage and could be devastating. Chen *et al*[6] reported a case of life-threatening hemorrhage from a sternal metastasis of HCC. The acute hemorrhage in mandibular metastasis of HCC was ever reported to be managed by Surgicel™ (Johnson & Johnson, New Brunswick, NJ) and bone wax packing[7]. In our patient, three methods were utilized to stop bleeding: electrocauterization, Surgicel™, tissue glue and bone wax in the 1st episode, but failed. Embolization was used in the 2nd hemorrhagic episode and failed again. Hypervascularity of the tumor and no single identifiable bleeder partly explained the reason of failure in two earlier methods. Palliative radiotherapy (2400 cGy) successfully stopped the bleeding and shrunk the tumor. The mechanisms behind hemostasis after radiation therapy were decrease of the tumor size, the tumoral vascularity, and induced fibrosis in the peripheral osseous structure. Surgery for the solitary mandibular metastasis of HCC was not considered in this patient. The metastatic lesion in our patient was osteolytic and invaded into the temporal muscle space. The lesion located in proximity of the skull base and was considered inoperable.

In conclusion, we reported a rare case of solitary mandibular metastasis of HCC and the intractable bleeding from the metastatic lesion was controlled successfully by palliative radiation therapy. Palliative therapy could be an option when coping with refractory bleeding in head and neck malignancies.

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CASE REPORT

Positron emission tomography/computed tomography with ^{18}F-fluorodeoxyglucose identifies tumor growth or thrombosis in the portal vein with hepatocellular carcinoma

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Abstract

Patients suffering from hepatocellular carcinoma (HCC) with tumor thrombus in the portal vein generally have a poor prognosis. Portal vein tumor thrombus must be distinguished from portal vein blood thrombus, and this identification plays a very important role in management of HCC. Conventional imaging modalities have limitations in discrimination of portal vein tumor thrombus. The application of positron emission tomography (PET) with ^{18}F-fluorodeoxyglucose (^{18}F-FDG) for discrimination between tumor extension and blood thrombus has been reported in few cases of HCC, while portal tumor thrombosis and portal vein clot identified by ^{18}F-FDG PET/CT in HCC patients has not been reported so far. We present two HCC cases, one with portal vein tumor thrombus and one thrombosis who were identified with ^{18}F-FDG PET/CT. This report illustrates the complimentary value of combining the morphological and functional imaging in achieving a correct diagnosis in such clinical situations.

Key words: ^{18}F-fluorodeoxyglucose; Positron emission tomography; Computed tomography; Portal vein tumor thrombus; Portal vein thrombosis

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INTRODUCTION

Hepatocellular carcinoma (HCC) has a dismal prognosis and carries a high risk of invasion of the portal vein[1]. Contrast CT and MRI markedly increase the detection rate of portal vein tumor thrombus, especially in small branches of the portal vein by its contrast enhancement features in cross-sectional images. However, all the noninvasive techniques currently used are imperfectly able to differentiate portal vein tumor thrombus from portal vein blood thrombus, and relying exclusively on contrast enhancement characteristics to make a conclusion runs the risk of misdiagnosis due mainly to the intrinsic limitations of the imaging modalities themselves[2]. This pattern of positron emission tomography/computed tomography (PET/CT) has been reported in cases of several types of malignant tumors[3], while portal vein tumor growth and portal vein thrombosis identified by PET/CT in hepatocellular carcinoma patients have not been reported so far. We herein present two cases identified by PET/CT with ^{18}F-fluorodeoxyglucose (^{18}F-FDG), which differentiates portal vein tumor thrombus from blood clot by recognizing different metabolic neoplastic activities and macromorphological characteristics.

CASE REPORT

Case 1

A 60-year-old woman with a five-year history of non-Hodgkin's lymphoma and hepatitis B virus infection was detected with a liver mass by ultrasound. She reported no vomiting, fever, diarrhea, or weight loss. On physical examination, abdomen was normal except for tender in the left liver area on palpation. Serum alpha fetoprotein (AFP) level was more than 1000 ng/mL, and HBsAg, HBeAb and HBcAb all were positive. Contrast-enhanced CT showed a mass with satellite lesions, and the left portal vein was not revealed clearly on the portal phase (Figure 1). A distinct lesion with high ^{18}F-FDG uptake was demonstrated in the region of the left portal vein (Figure 2A). The ^{18}F-FDG PET/CT showed a mass of high ^{18}F-FDG uptake with satellite lesions and lower metabolism in the central necrotic area (Figure 2B). HCC was confirmed by liver biopsy. It

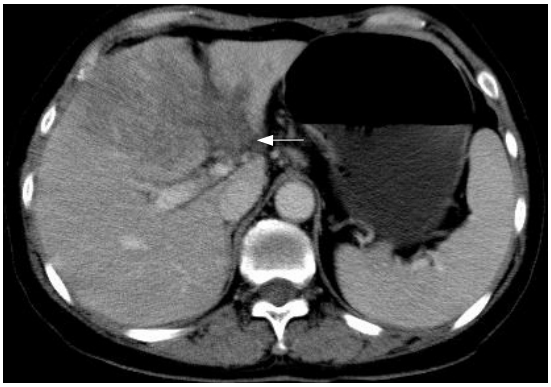

Figure 1 Portal phase of contrast-enhanced CT demonstrating the left portal vein tumor thrombus (white arrow).

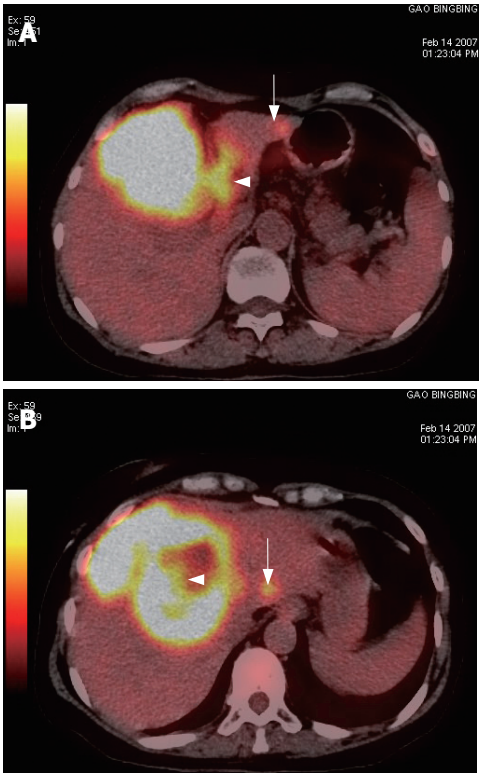

Figure 2 **A**: Integrated PET/CT image showing high FDG uptake by tumor thrombus in the left portal vein (arrow head) and a liver mass with a satellite lesion (arrow); **B**: Fused PET/CT imaging showing necrosis in the center of the mass (arrow head) with a satellite lesion (arrow).

is suggested that ^{18}F-FDG PET/CT may have a great potential value to discriminate tumor thrombus from blood thrombus and to detect satellite or metastatic lesions in patients suffering from HCC. The patient underwent once transcatheter arterial chemoembolization (TACE) and her conditions were relatively stable at the 2-mo follow-up.

Case 2

A 50-year-old man with hepatitis B virus infection for 20 years was detected by ultrasound to have a portal block 6 mo after HCC resection. Blood thrombus was suspected on contrast CT and MRI. Physical examination showed no abnormal abdominal findings. AFP level was less than

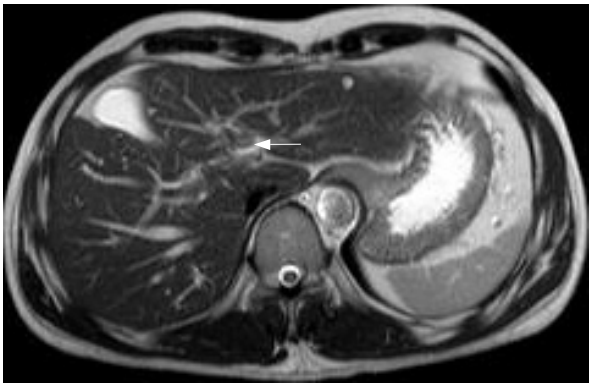

Figure 3 MRI T2 imaging showing a thrombus in the left portal vein.

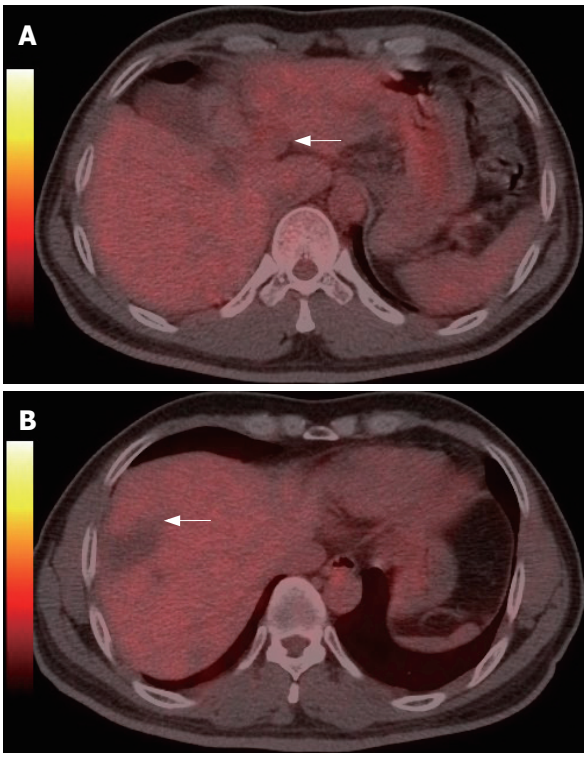

Figure 4 Fused PET/CT imaging showing no hypermetabolism (arrow) in the region of the left portal vein (**A**) and in the region of previous hepatic resection (**B**).

20 ng/mL and contrast-enhanced MRI demonstrated a thrombus in the left portal vein (Figure 3). Thus a portal vein tumor growth was suspected, with the necessity to differentiate it from portal vein blood thrombosis. Subsequently, the ^{18}F-FDG PET/CT revealed neither a lesion with high FDG uptake in the left portal vein (Figure 4A), nor such a lesion at the margin of previously resected hepatic region (Figure 4B). No distant metastatic lesions were found in the whole-body PET/CT scan. The patient underwent 5-mo follow-up of ultrasound examination, which produced normal results when checking the region for interpretation of the previously abnormal findings. The patient remained well during another 5-mo follow-up period and the thrombus disappeared on the latest ultrasound examination. Thus a diagnosis of the left portal vein blood thrombus was established at last.

DISCUSSION

Patients suffering from HCC with portal vein tumor thrombus generally have a poor prognosis, as tumor proliferation is often rapid and accompanied by intrahepatic metastases, liver dysfunction, portal hypertension and esophageal varices[4]. Conventional imaging modalities have limitations in discriminating portal vein tumor thrombus. The application of ^{18}F-FDG PET for discrimination between tumor extension and blood thrombus has been reported in few cases of HCC, while portal vein tumor thrombus and portal vein blood clot identified by ^{18}F-FDG PET/CT in HCC patients have not been reported so far. Compared with conventional CT and MRI, PET/CT with ^{18}F-FDG as a radiotracer has some advantages in evaluation of hepatic malignancies, including diagnosing, staging and restaging tumors, evaluating biologic characters, making up treatment plan and monitoring tumor response, early detecting of recurrence and providing prognosis assessment. PET/CT is getting more and more widely applicable in clinical practice with combined functional and anatomical images. With lower false-positive and false-negative rate, it is more sensitive and specific than PET alone[5].

Hanajiri *et al*[6] and Beadsmooreet *et al*[7] found that ^{18}F-FDG PET was more sensitive than conventional CT and MRI in detecting suspected vein tumor thrombus in patients with HCC, although portal vein tumor thrombus and portal vein blood thrombus identified by PET/CT in HCC patients have not been reported thus far. Tumor thrombus differentiates itself from blood thrombus by its intense uptake of ^{18}F-FDG as a result of its high metabolic neoplastic activity. Histological grade of differentiation and macroscopic vascular invasion are strong predictors of both survival and recurrence of tumor in patients with cirrhosis who have received transplants because of HCC. Cillo *et al*[8] reported that using HCC grades (grade 1 and 2) based on preoperative fine-needle aspiration biopsy to select candidates for liver transplantation was associated with an extremely low rate of tumor recurrence which was comparable with that of incidentally detected HCC. Although HCC accumulates ^{18}F-FDG to various degrees, a high positive rate of ^{18}F-FDG accumulation has been reported in patients with high-grade HCC[9] and in those with markedly elevated AFP levels. The standardized uptake value (SUV) ratio is related significantly to disease-related deaths as well as other predictive factors, including the number, size and stage of tumors, involvement of vessels and involvement of the capsule, and provides information about prognostic relevance in patients with HCC before surgery[10].

As to our first case, the PET/CT examination not only confirmed the diagnosis of portal vein tumor thrombus, but also suggested the low grade of cell histology that was confirmed by the results of liver biopsy. Histological examination using percutaneous needle biopsy could be the most definite assessment of HCC grades. However, it is invasive and the specimen retrieved does not always represent the entire lesion owing to sampling errors. Histological grade and vascular invasion cannot be determined preoperatively[11]. Case 1 showed that ^{18}F-FDG PET/CT might have a great potential in discriminating tumor thrombus and blood thrombus and in detecting satellite or metastatic lesions in patients suffering from HCC. It may play an important role in evaluating biologic characters of tumors.

In the second patient, there was no high metabolic lesion in the left portal vein area and in the region of previous hepatic resection. In addition, the whole body PET/CT examination found neither intra-hepatic nor extra-hepatic lesions; an overall 10-mo follow-up confirmed the diagnosis of blood thrombus. ^{18}F-FDG PET has been proved an effective whole-body imaging technique that detects metabolic changes preceding structural findings. Unexplained rise of serum AFP levels in HCC patients after the treatment of their malignancy is an early indicator of tumor recurrence or extra-hepatic metastases[12]. ^{18}F-FDG PET/CT provides fused images that demonstrate the complementary roles of functional and anatomic assessment in the diagnosis of cancer recurrence by the precise localization of suspected foci with ^{18}F-FDG uptake whose characterization can be interpreted as malignant or benign. ^{18}F-FDG PET/CT is better than conventional CT in judging tumor residue of HCC after treatment, and in guiding further treatment of HCC.

We reported two cases of HCC, one with a portal vein tumor thrombus and the other with a portal vein blood thrombus; both were identified by ^{18}F-FDG PET/CT. Thus it demonstrates the complimentary value of morphological and functional imaging in achieving a correct differentiation. PET/CT with ^{18}F-FDG as a radiotracer may further enhance the HCC diagnostic algorithm by accurate diagnosis, staging, restaging and evaluating its biological characters.

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Frequently overlooked and rarely listened to: Music therapy in gastrointestinal endoscopic procedures

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Abstract

To elucidate the role of music therapy in gastrointestinal endoscopic procedures following the conflicting outcomes reported in two recent studies. The findings of our recent meta-analysis that examined this matter were discussed in the context of later studies. Our meta-analysis illustrated the beneficial effects of music therapy on patient anxiety levels when used as a single measure of relaxation and analgesia. Beneficial effects were also shown on analgesia and sedation requirements and procedure duration times when used as an adjunct to pharmacotherapy. These findings are in agreement with those of both studies excluded from analysis and those that followed it. Music therapy is an effective tool for stress relief and analgesia in patients undergoing gastrointestinal endoscopic procedures.

Key words: Music; Endoscopy; Colonoscopy; Meta-analysis

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TO THE EDITOR

I greatly enjoyed reading the well-conducted, well-written studies by Ovayolu *et al*[1] and Bechtold *et al*[2] exploring the affects of music therapy on patients undergoing gastrointestinal endoscopic procedures. Those studies reported conflicting outcomes, which we aimed to resolve in our meta-analysis[3].

Our meta-analysis involved 641 patients undergoing esophagogastroduodenoscopy, flexible sigmoidoscopy or colonoscopy, with or without intervention through music therapy. The intervention was conducted by patient exposure to patient or researcher selected music, delivered with/without headphones, before and/or during the procedure. For patients that did not receive pharmacotherapy, anxiety levels were used as efficacy measures. For patients that did receive pharmacotherapy, medications were not uniformly administered within studies, and thus anxiety levels could not be used for that purpose. Alternately, medication requirements and procedure durations were noted. Our meta-analysis yielded significantly lower anxiety levels for the former group, whereas the latter group exhibited significant reductions in analgesia requirements and procedure duration times, while reductions in sedation requirements approached significance. Our findings are in agreement with those of both studies excluded from analysis[3] and those that followed it[2,4]. Furthermore, these findings are of particular importance, as sedation, analgesia use and procedures of prolonged duration are linked to cardiopulmonary complications. Further, patients undergoing intervention reported greater satisfaction rates and were more willing to have the procedures repeated[3]. Additionally, while our meta-analysis was insufficiently sized to determine a preferable intervention protocol, we suggested that patient selected music, delivered through headphones, may provide maximal benefits while circumventing potentially undesirable exposure of the medical staff to that particular music. Accordingly, despite only minor benefits reported by some[2], we suggest that this safe and cost-effective measure not be overlooked[3].

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Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: http//www.cdc.gov/ncidod/EID/eid.htm

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