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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.

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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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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.

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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, H₂ 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	Esophagitis/failure to thrive Reactive airway disease/recurrent pneumonia Apnoea/bradycardia/acute life threatening events Barrett's esophagus/esophageal ulceration and perforation/stricture formation Anaemia/seizure

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

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	

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.

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. H₂-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

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, <i>H. Pylori</i> infection, allergic enteropathy and Barrett's oesophagus	Invasive and requires sedation/general anaesthesia

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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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.

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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 G₁ 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 G₁/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 G₁/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

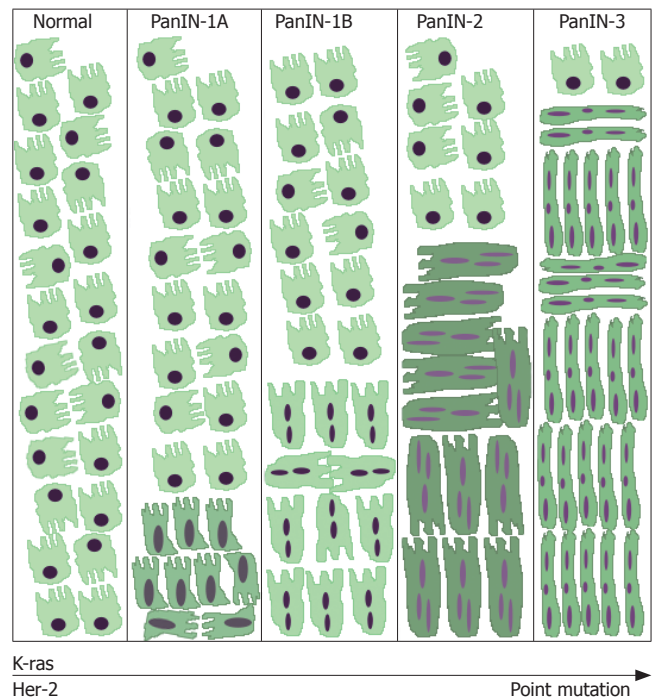


Figure 1 The most important genetic abnormalities associated with pancreatic cancer.

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.

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 phosphatidylin-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 G₁ 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 G₁/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, angiostatsins, 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
Hereditary pancreatitis	PRSS1 Kazal type 1 (SPINK1)	AD	Extracutaneous cancers 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	APC	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), TGF α (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 administered 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 (Erbix) 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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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, antisense 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.

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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 nucleic 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

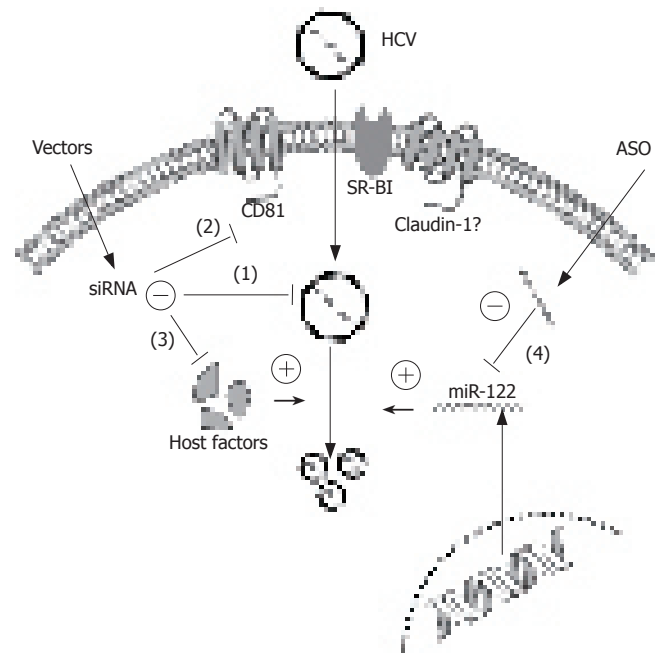


Figure 1 Novel anti-viral strategies based HCV life cycle. RNAi technology, inducing gene silencing at posttranscriptional level mediated by siRNA, can be applied to prevent HCV replication and infection by targeting either viral RNA (1) or host factors, such as surface receptor (2) or cellular molecules (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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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.

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

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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.

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 hereditary 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- μ m thick sections and mounted on polarized glass slides. After mounting, they were kept in an oven at 56°C 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°C 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

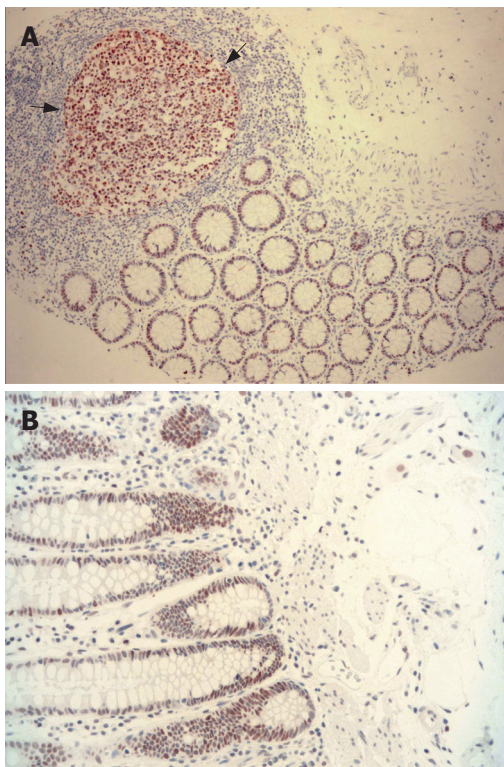


Figure 1 MLH1 and MSH2 expression. **A:** Nuclear MLH1 expression detected in germinal centre of lymphoid follicle (dark arrows) and in epithelia of normal colonic mucosa ($\times 100$); **B:** Crypt epithelia showing normal positive nuclear staining with MSH2 ($\times 200$).

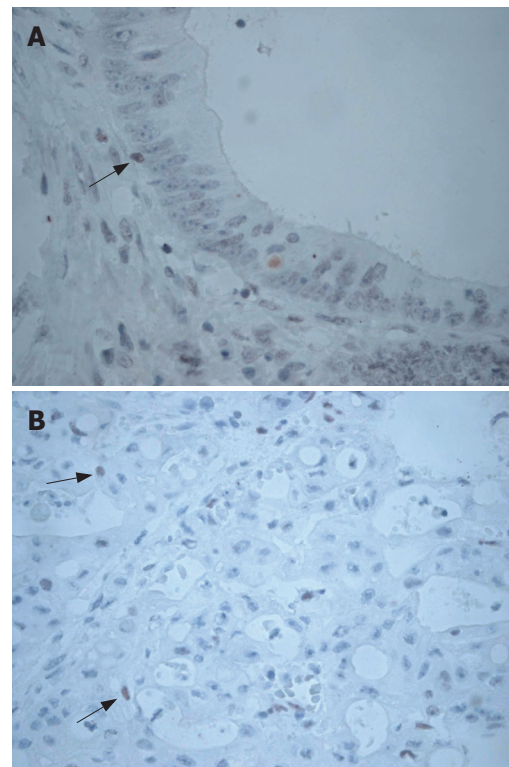


Figure 3 Loss of MLH1 and MSH2 expression in colorectal cancer. **A:** Loss of staining with MLH1 in cancer cells, although lymphocytes (arrow) show positive staining ($\times 400$); **B:** Adenocarcinoma with complete loss of MSH2 expression. Nuclear staining of lymphocytes (arrows) in the stroma served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

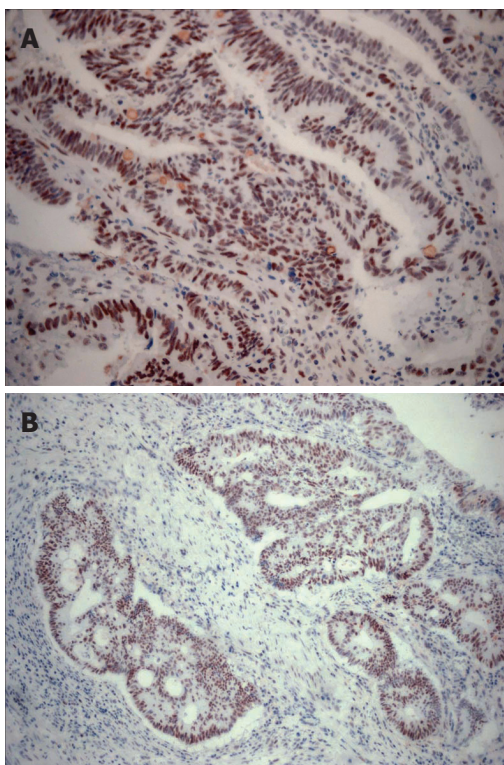


Figure 2 MLH1 and MSH2 expression. **A:** Extensive nuclear staining with MLH1 in adenocarcinoma of colon ($\times 200$); **B:** Tumor cells showing strong positive nuclear staining with MSH2 ($\times 100$).

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

(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 ≥ 50 years than did MSH2-positive ($P \geq 0.05$). 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 $\geq 10\%$ ($P < 0.05$). 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 (-)	<i>P</i>	MSH2 (+)	MSH2 (-)	<i>P</i>
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			0.660
-	7 (15.6)	3 (10.3)		9 (13)	4 (80)	
+	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)	

¹Peritumoral Crohn-like inflammation; ²Peritumoral lymphocytic inflammation; ³Perineural invasion; ⁴Lymph vessel invasion; ⁵Blood vessel invasion; ⁶American 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	

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

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

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-negative cases had peritumoral Crohn's-like lymphocytic infiltration and prominent intratumoral neutrophilic infiltration.

Although there was no statistical significance, 80% of MSH2-negative 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 ≥ 50 years. Tumor size was ≥ 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 polypsis 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.

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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)-1 β , IL6, and tumor necrosis factor (TNF)- α , appears to be enhanced by infection with *H. pylori*^[8,11]. Interleukin-1 β 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 *IL1B* -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 *IL6*-634C/G, the following MGB probes were prepared: a C allele-specific probe, 5'-FAM-CAACAGCCCCCTCACAG-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 *IL6*, 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°C for 2 min and 95°C for 10 min, followed by 50 cycles for *IL6* or 40 cycles for the other genotypes, denaturation at 92°C for 15 s, and annealing/extension at 60°C for 1 min. The FAM and VIC fluorescence levels of the PCR products were measured at 60°C for 1 min, resulting in the

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

	<i>H. pylori</i> -seropositive (<i>n</i> = 237)		<i>H. pylori</i> -seronegative (<i>n</i> = 173)		<i>P</i> -value
	<i>n</i>	(%)	<i>n</i>	(%)	
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

	<i>H. pylori</i> -seropositive (<i>n</i> = 237)		<i>H. pylori</i> -seronegative (<i>n</i> = 173)		<i>P</i> -value
	<i>n</i>	(%)	<i>n</i>	(%)	
<i>IL1B</i> -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	
<i>IL1B</i> -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	
<i>IL6</i> -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	
<i>TNF</i> -1031T/C					0.170
TT	152	64.1	115	66.5	
CT	80	33.8	51	29.5	
CC	5	2.1	7	4.0	
<i>TNF</i> -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	
<i>IL10</i> -1082A/G					0.852
AA	211	89.0	153	88.4	
AG/GG ¹	26	11.0	20	11.6	

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

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

	<i>n</i>	<i>Hp</i> (+)% ¹	Adjusted OR (95% CI)	<i>P</i> -value
<i>IL1B</i> -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
<i>IL1B</i> -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
<i>IL6</i> -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
<i>TNF</i> -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
<i>TNF</i> -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
<i>IL10</i> -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 (%); ²Only 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

	<i>IL1B</i> -511C/T						
	<i>n</i>	<i>Hp</i> (+)% ¹	C/C	C/T	<i>P</i> -value	T/T	<i>P</i> -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
	<i>IL1B</i> -31C/T						
	<i>n</i>	<i>Hp</i> (+)% ¹	C/C	C/T	<i>P</i> -value	T/T	<i>P</i> -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
	<i>IL6</i> -634C/G						
	<i>n</i>	<i>Hp</i> (+)% ¹	C/C	C/G	<i>P</i> -value	G/G	<i>P</i> -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

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

	<i>n</i>	<i>Hp</i> (+)% ¹	Adjusted OR (95% CI)	<i>P</i> -value
<i>TNF</i> -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
<i>IL1B</i> -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 (%). ²Each reference group represented all the other combinations of genotypes. ³ORs 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.

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.

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 -1031T/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 between 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*-1 β expression by whole blood leukocytes *in vitro* was lower in subjects with -31T and -511C^[37,38]. Since *IL*-1 β 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*-1 β expression studies.

The *IL*6-634C/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 *IL6*-174 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-environment 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 μ g/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- κ B (NF- κ B). 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.

INTRODUCTION

Proteasome inhibition is an emerging strategy to attenuate the inflammatory response^[1]. Inhibiting the proteasome blocks nuclear factor- κ B (NF- κ B) activation by detaining proteolysis of its inhibitory subunit, the I κ B. Preventing NF- κ B activation then decreases NF- κ B dependent proinflammatory gene expression, resulting in reduced inflammatory response. However studies also reveal that NF- κ B, one of the major initiators of pro-inflammatory pathways, has anti-inflammatory roles in the resolution of inflammation. Thus inhibiting NF- κ B 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- κ B inhibitory effects of MG132, it was also crucial to determine whether NF- κ B inhibition with MG132 after the onset of pancreatic 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 μ g/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°C until use.

Procedures

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

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

Western blotting: Western blot analysis of pancreatic heat shock protein 72 (HSP72) and I κ B α 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,

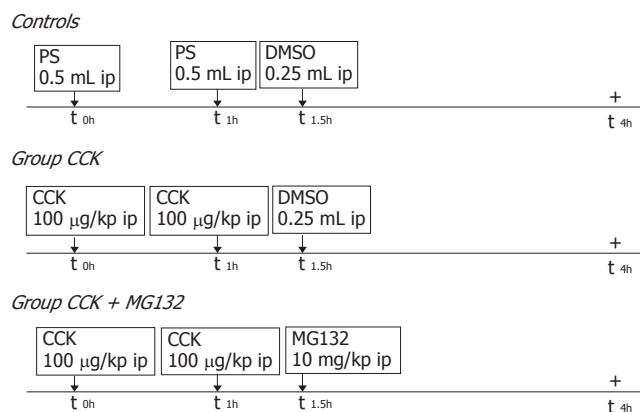


Figure 1 Experimental protocol of acute pancreatitis.

all blood samples were centrifuged at $2500 \times g$ 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'-CCTCTGCACGCATTAGTTGC-3', reverse primer, 5'-TGAAACAGGGCATAGCAGTAGG-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].

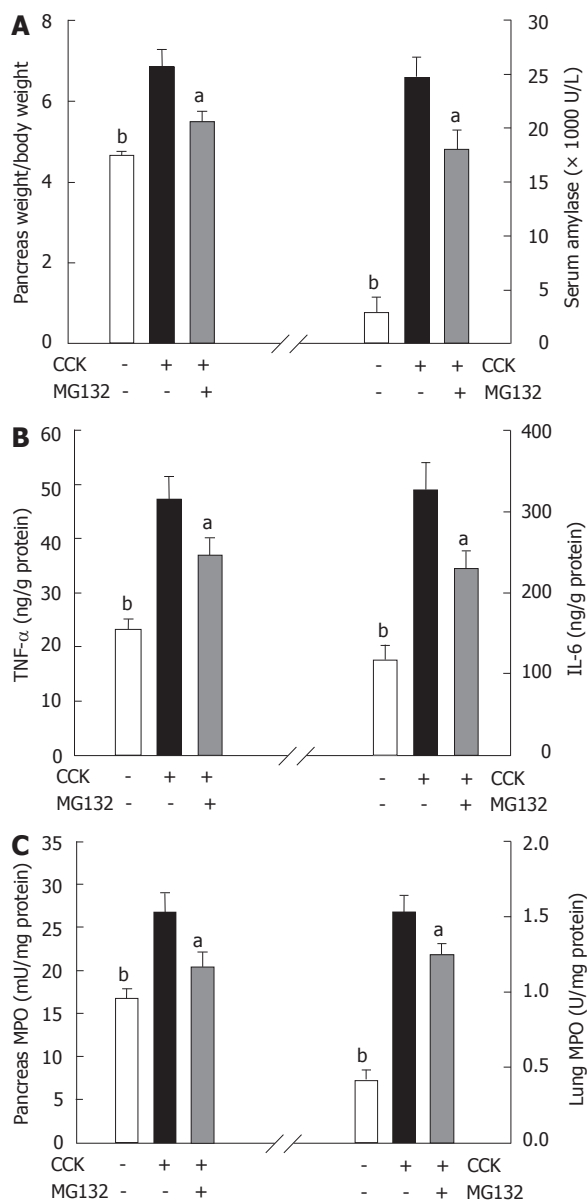


Figure 2 Effects of MG132 on laboratory parameters of acute pancreatitis. ^a $P < 0.05$; ^b $P < 0.01$.

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 μ m 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 \pm 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 μ g/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).

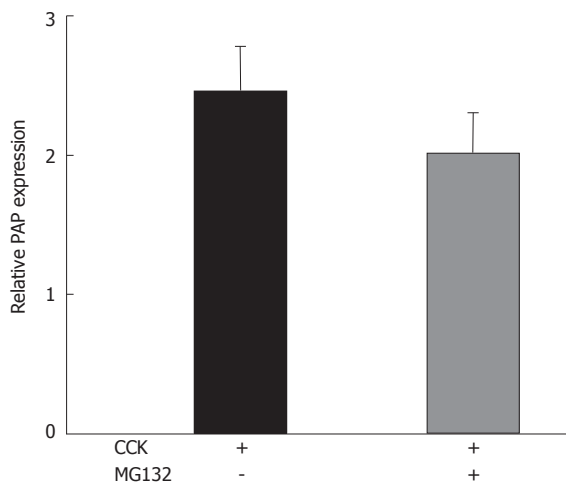


Figure 3 Effect of MG132 on mRNA expression of pancreatitis associated protein (PAP) in experimental acute pancreatitis.

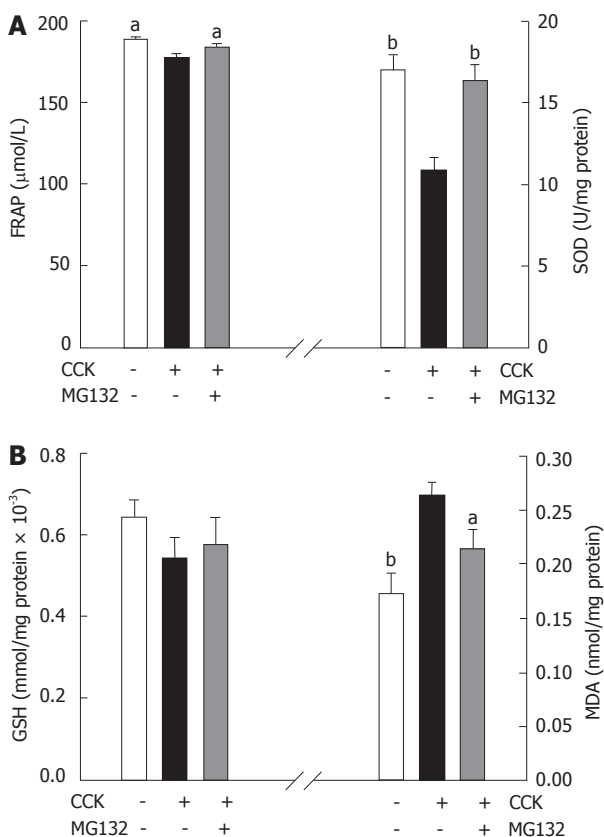


Figure 4 Effects of MG132 on measures of oxidative stress in experimental acute pancreatitis. ^a $P < 0.05$; ^b $P < 0.01$.

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- κ B activation

In the pancreas, supramaximal doses of CCK triggered the

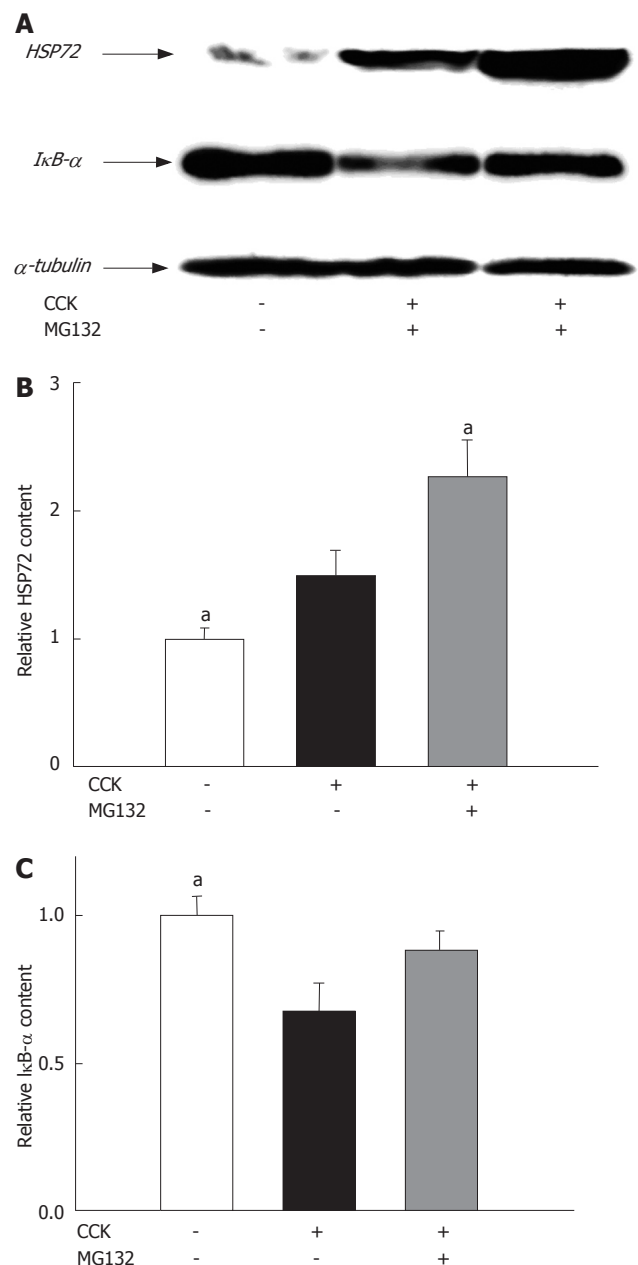


Figure 5 Effects of MG132 on HSP72 expression and IκB degradation in experimental acute pancreatitis. ^a $P < 0.05$.

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

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).

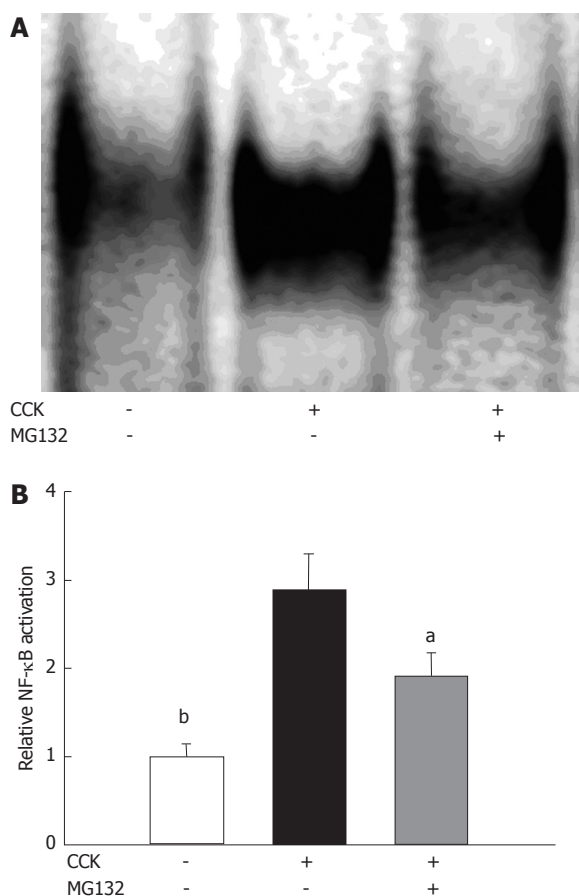


Figure 6 Effect of MG132 on NF- κ B activation in experimental acute pancreatitis. ^a $P < 0.05$; ^b $P < 0.01$.

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- κ B^[3-5]. Intrapaneatic activation of digestive enzyme zymogens can be prevented by the inhibition of lysosomal hydrolases like cathepsin B^[29-31]. NF- κ B activation can also be prevented by inhibiting the proteasome and other proteases (like calpains) that degrade the inhibitory I κ B 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- κ B 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 pancreatic inflammation characterized by intracellular activation of digestive enzymes and elevation of their serum levels, cytoplasmic vacuolization and death

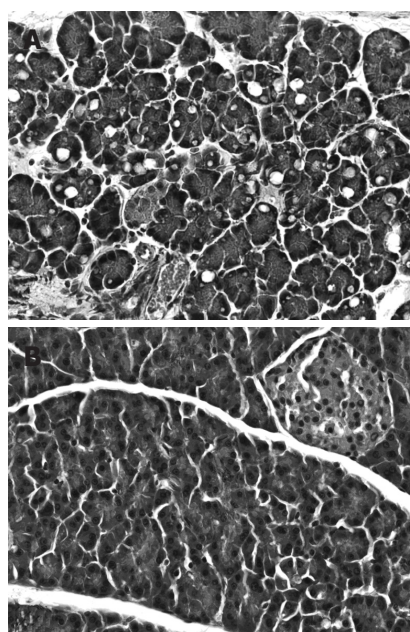


Figure 7 Effect of MG132 on pancreatic morphological damage in CCK-induced pancreatitis (HE, x40).

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 pancreatic 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- κ B. NF- κ B, 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- κ B activation peaks in the first phase of the disease^[39]. Since in MG132 treatment had more pronounced effects on HSP72 than on NF- κ B, 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- κ B 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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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.

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

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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 G₁/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.

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 (NexavarTM) or bortezomib (VelcadeTM). 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°C in a humidified atmosphere containing 50 mL/L CO₂ 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 (VelcadeTM) was bought from Millennium Pharmaceuticals, Inc. (Cambridge, MA, USA). The multi-kinase inhibitor sorafenib tosylate (NexavarTM) was a kind gift from Bayer Health Care (West Haven, CT, USA). Stock solutions were prepared in dimethyl sulfoxide (DMSO) and stored at -20°C until use. Gemcitabine hydrochloride (GemzarTM)

was bought from Lilly Pharma (Gießen, Germany). Doxorubicin hydrochloride was from Sigma (Deisenhofen, Germany) and also prepared in DMSO and stored at -20°C. 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 μ mol/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°C, 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

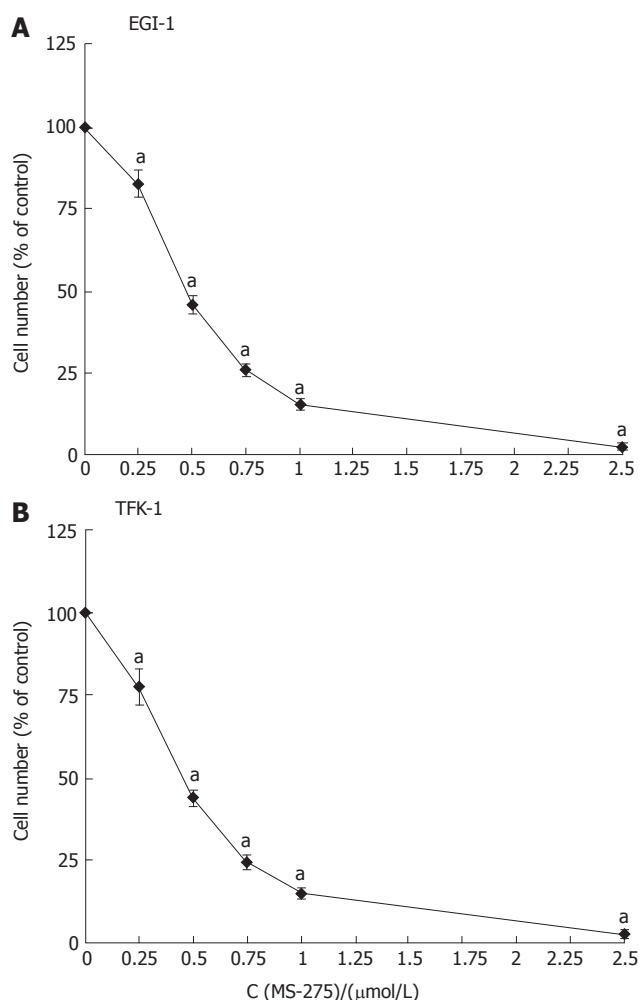


Figure 1 Anti-proliferative effects of MS-275 on human EGI-1 and TFK-1 cholangiocarcinoma cells. mean \pm SEM, $n > 5$, ^a $P < 0.05$ vs controls.

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 μ g 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°C 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 \pm SEM. Significance

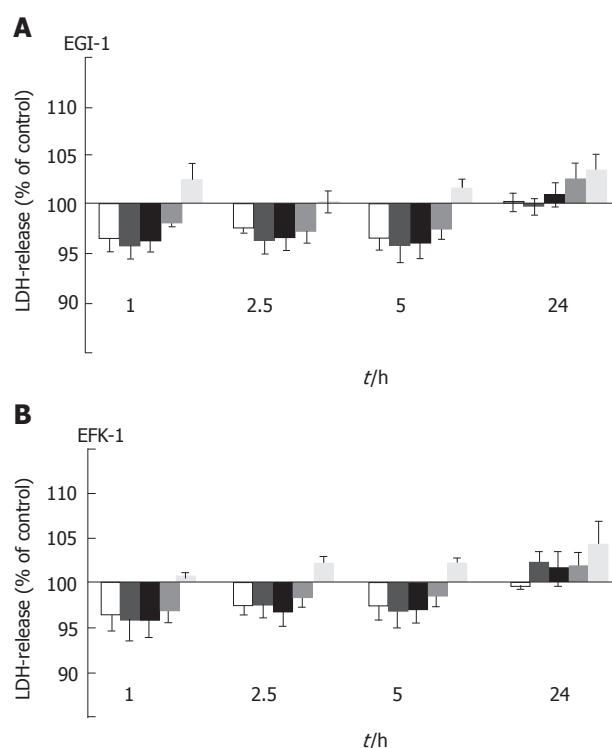


Figure 2 MS-275-induced LDH release into the supernatant of EGI-1 and TFK-1 cells. mean \pm SEM, $n > 3$ independent experiments; No significant increase in LDH release was observed.

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 μ mol/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 IC₅₀ value of MS-275, determined after 72 h of incubation, was 0.48 ± 0.02 μ mol/L for EGI-1 cells and 0.46 ± 0.02 μ mol/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 μ mol/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

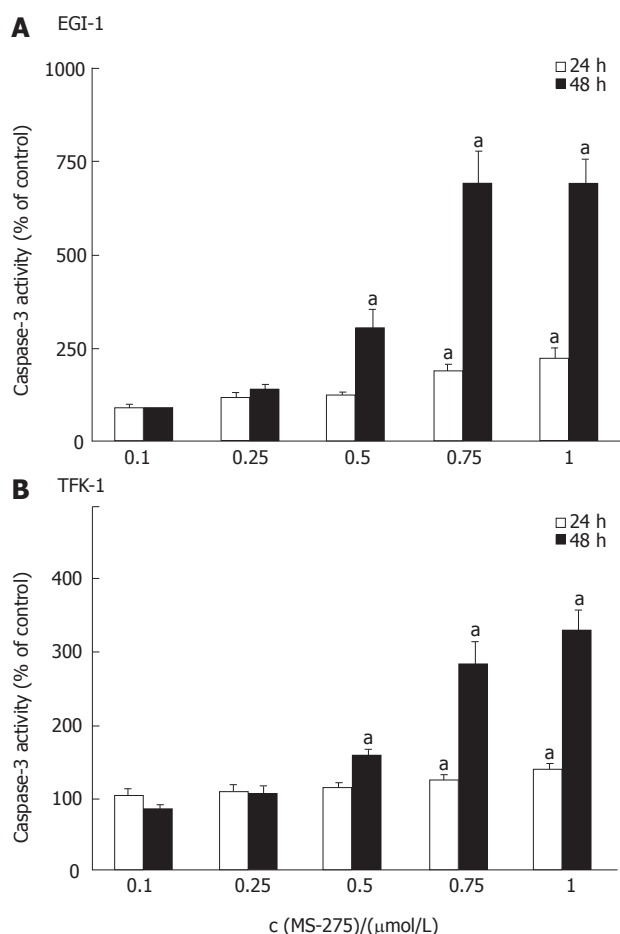


Figure 3 MS-275-induced apoptosis-specific caspase-3 activity increase in EGI-1 and TFK-1 cells. mean \pm SEM, $n > 3$ independent experiments, $^aP < 0.05$ vs controls.

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 $\mu\text{mol/L}$ 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 $\mu\text{mol/L}$ 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 $\mu\text{mol/L}$ in TFK-1 and 0.25 $\mu\text{mol/L}$ 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

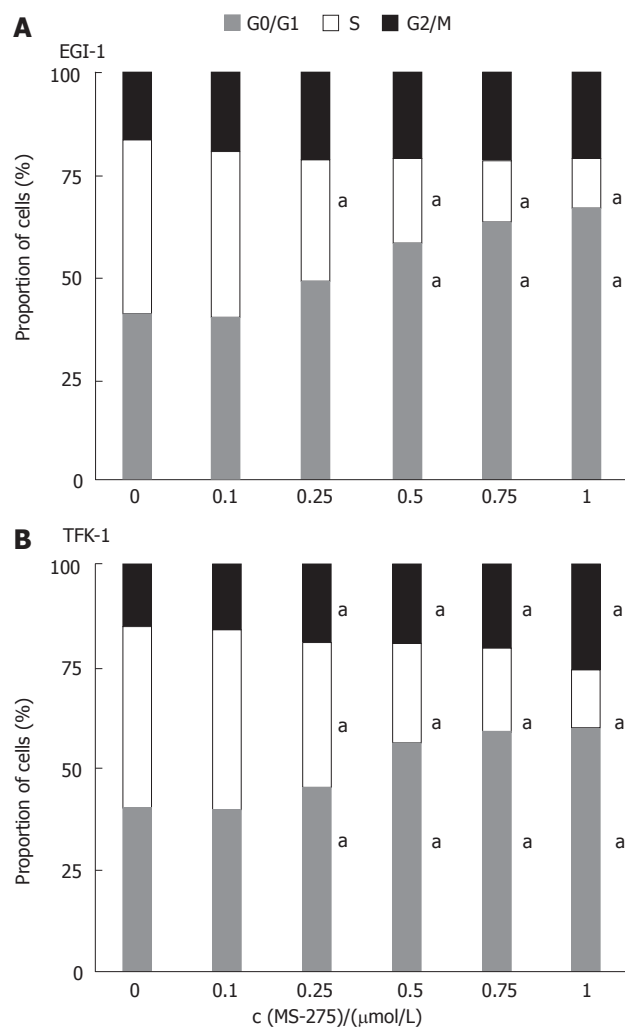


Figure 4 Induction of cell cycle arrest by MS-275 on EGI-1 and TFK-1 cells. means of > 3 independent experiments, $^aP < 0.05$ vs controls.

MS-275 (0.1–1 $\mu\text{mol/L}$) 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 $\mu\text{mol/L}$ 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

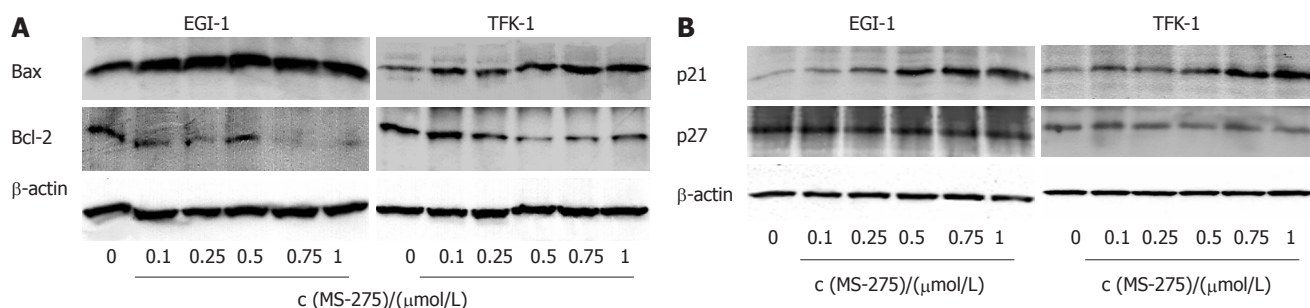


Figure 5 MS-275-induced modulation of apoptosis- and cell cycle-relevant proteins ($n = 3$ independent experiments).

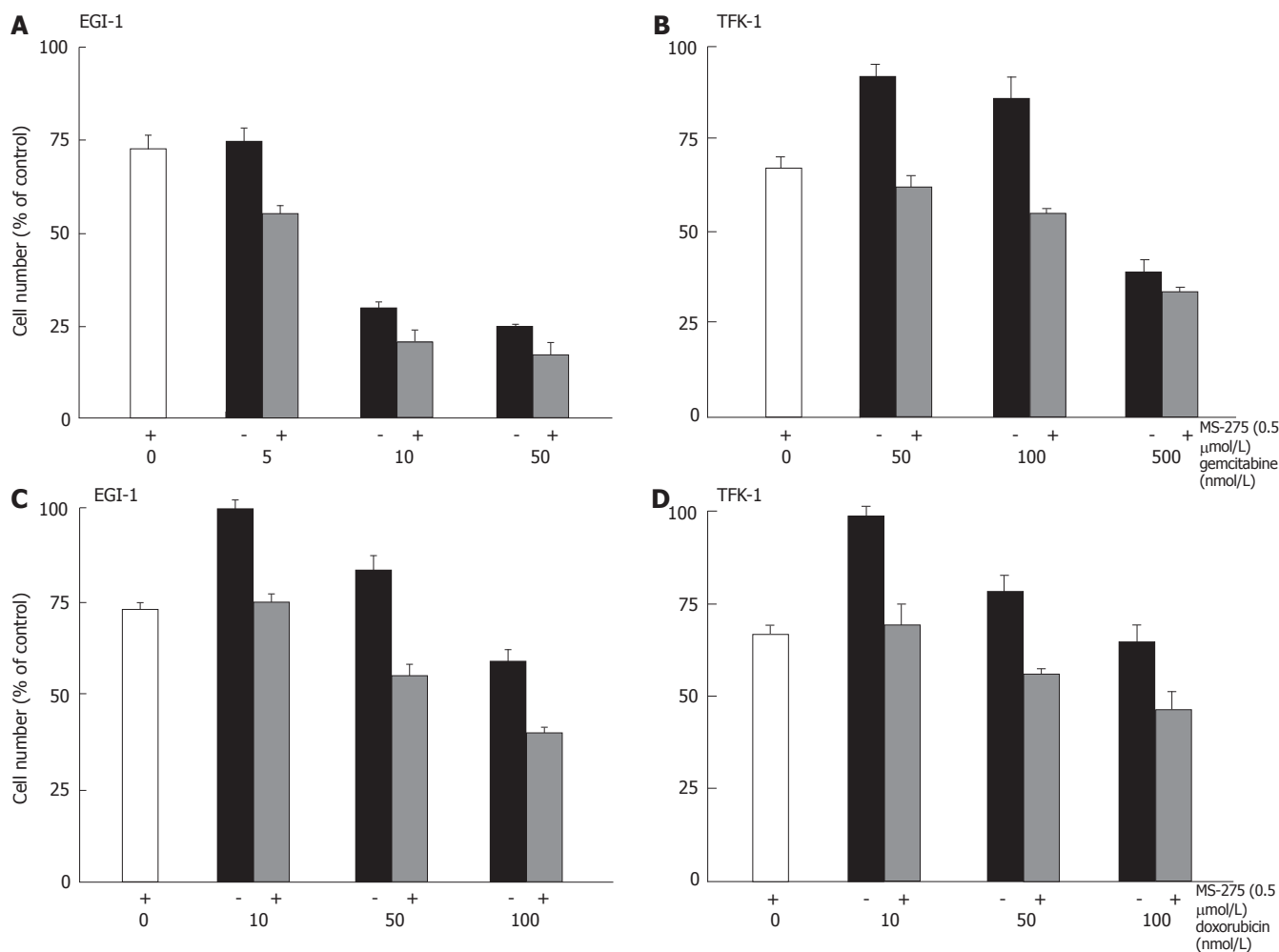


Figure 6 Anti-proliferative effects of MS-275 plus cytostatics on EGI-1 and TFK-1 cells. mean \pm SEM, $n = 3-4$ independent experiments.

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].

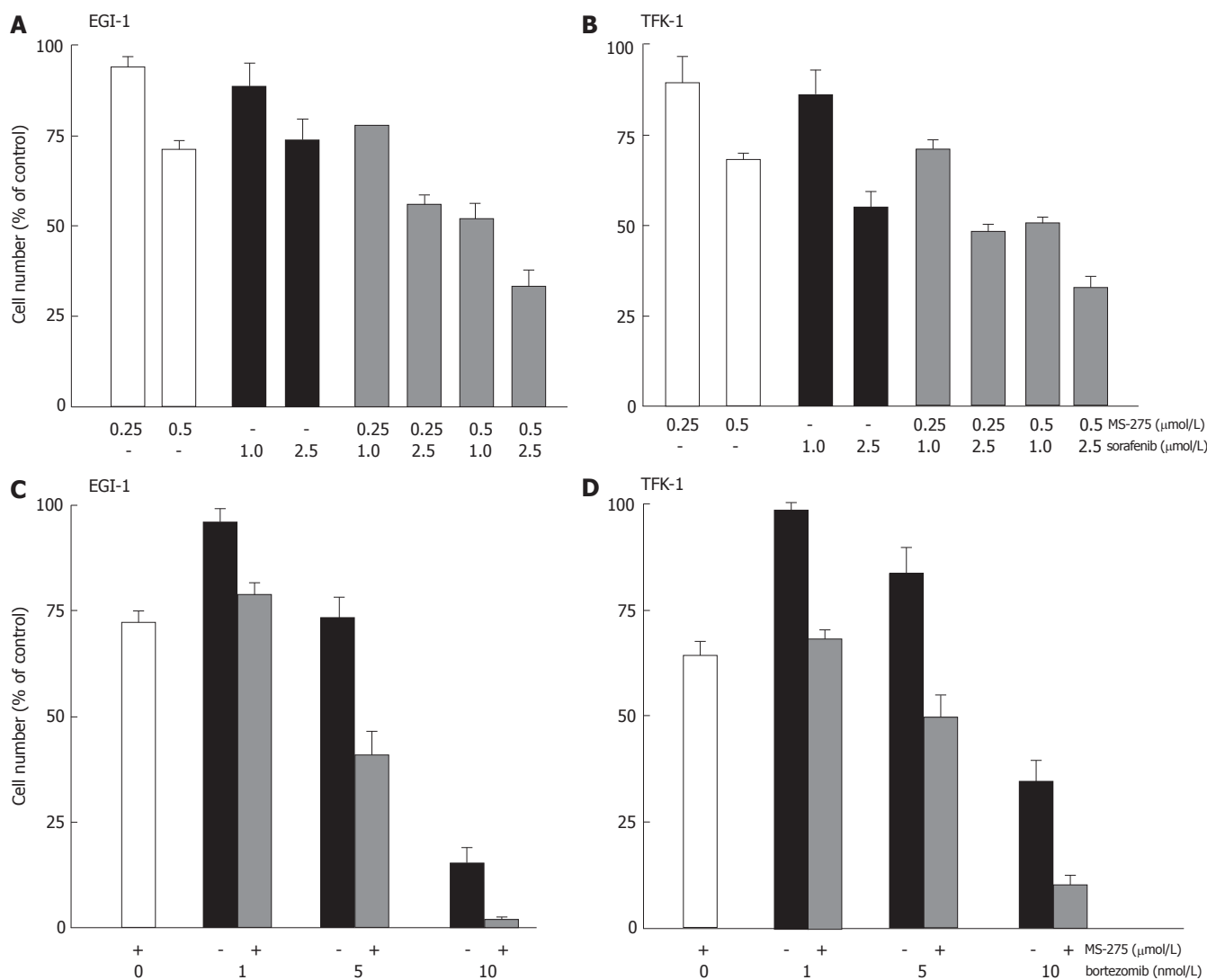


Figure 7 Anti-proliferative effects of MS-275 plus sorafenib or bortezomib on EGI-1 and TFK-1. mean \pm SEM, $n = 3-4$ independent experiments.

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 μ mol/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 G₀/G₁ and the G₂/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-1/Cip1}. p21^{Waf-1/Cip1} is a key component of cell cycle checkpoints, i.e., the G₁/S^[46] and the G₂/M checkpoints^[47,48]. Accordingly, HDAC inhibitors were found to inhibit both the G₁/S and G₂/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.

ACKNOWLEDGMENTS

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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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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.

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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 ≥ 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 <i>n</i> (%)					
-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 <i>n</i> (%)	194 (60.6)	43 (53.8)	48 (60.0)	50 (62.5)	53 (66.3)
<i>H. pylori</i> infection <i>n/n</i> (%)	202/306 (66.0)	52/76 (68.4)	61/76 (80.3)	51/77 (66.2)	38/77 (49.3)
NSAIDs/Aspirin use <i>n</i> (%)	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)

¹Pantoprazole vs Omeprazole: PP analysis: $P = 0.039$, ITT analysis $P = 0.022$; ²Rabeprazole 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	
	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%	<i>n</i>	%
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 com-

pared 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 not 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%, $P = \text{NS}$). 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%, $P = \text{NS}$) (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 (<i>n</i> = 71)	Lansoprazole (<i>n</i> = 71)	Pantoprazole (<i>n</i> = 74)	Rabeprazole (<i>n</i> = 72)	All (<i>n</i> = 288)
<i>H pylori</i> positive <i>n</i> = 188	38/49 77.6%	54/57 94.7%	45/48 93.8%	33/34 97.1%	170/188 90.4%
<i>H pylori</i> negative <i>n</i> = 100	19/22 86.4%	11/14 78.6%	24/26 92.3%	35/38 92.1%	89/100 89.0%
	Omeprazole (<i>n</i> = 49)	Lansoprazole (<i>n</i> = 57)	Pantoprazole (<i>n</i> = 48)	Rabeprazole (<i>n</i> = 34)	All (<i>n</i> = 188)
<i>H pylori</i> cured <i>n</i> = 149	24/32 75.0%	43/46 93.5%	39/42 92.6%	28/29 96.6%	134/149 89.9%
<i>H pylori</i> still-positive <i>n</i> = 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
	<i>n</i> = 301	<i>n</i> = 271	<i>n</i> = 30	<i>P</i> value
Heartburn (<i>n</i> , %)	131 (43.5)	9 (3.3)	6 (20.0)	0.0001
Acid regurgitation (<i>n</i> , %)	39 (13.0)	4 (1.5)	0 (0.0)	0.874
Epigastric pain (<i>n</i> , %)	143 (47.5)	10 (3.7)	2 (6.6)	0.781
Dysphagia (<i>n</i> , %)	10 (3.3)	0 (0.0)	0 (0.0)	--
Vomiting (<i>n</i> , %)	60 (19.9)	0 (0.0)	0 (0.0)	--
Anaemia (<i>n</i> , %)	28 (9.3)	0 (0.0)	0 (0.0)	--

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%): urticaria, 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 sub-

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
Anemia	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.

jects 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 esomeprazole 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%, $P = \text{NS}$)^[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%, $P = \text{NS}$)^[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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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.

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 heteromatrix 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 expressions 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, $\times 400$) 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 μ m thick sections, deparaffinized and preincubated with normal bovine serum to prevent non-specific binding. The sections were incubated overnight at 4°C with primary polyclonal antibody to human Ang-1, -2 or -4 ([N-18],[N-18],[L-18], respectively, 1 μ g/mL; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), Tie-1 or -2 ([C-18],[C-20], respectively, 1 μ g/mL; Santa Cruz Biotechnology, Inc.), followed by alkaline phosphatase-conjugated anti-goat IgG antibody (0.4 μ g/mL; Santa Cruz Biotechnology, Inc.) for Ang-1, -2 and -4, and anti-rabbit IgG antibody (0.4 μ g/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

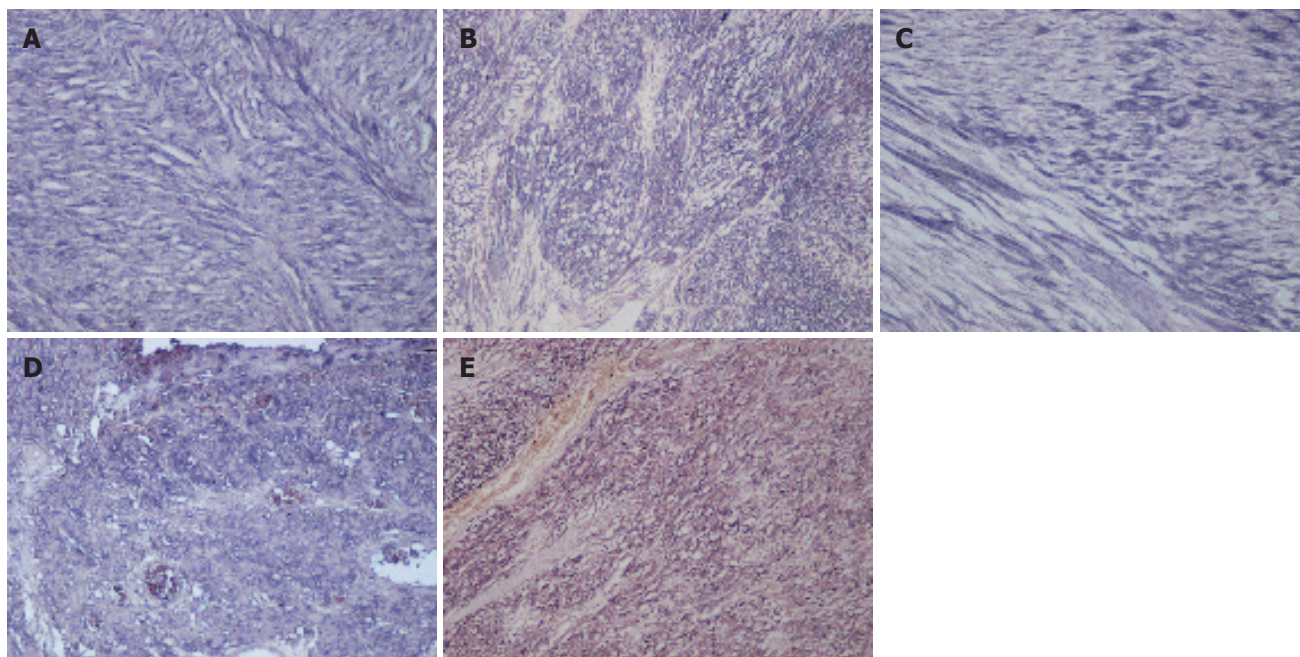
The Stat View II program (Abacus Concepts, Inc., Berkeley, CA) was used for statistical analysis. Analyses comparing the degree of Ang-1, -2 and -4 and Tie-1 and -2 expressions in GISTs, leiomyomas and schwannomas were performed using the Mann-Whitney's test.

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* (%)

	<i>n</i>	Ang-1		Ang-2		Ang-4		Tie-1		Tie-2	
		-	+	-	+	-	+	-	+	-	+
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

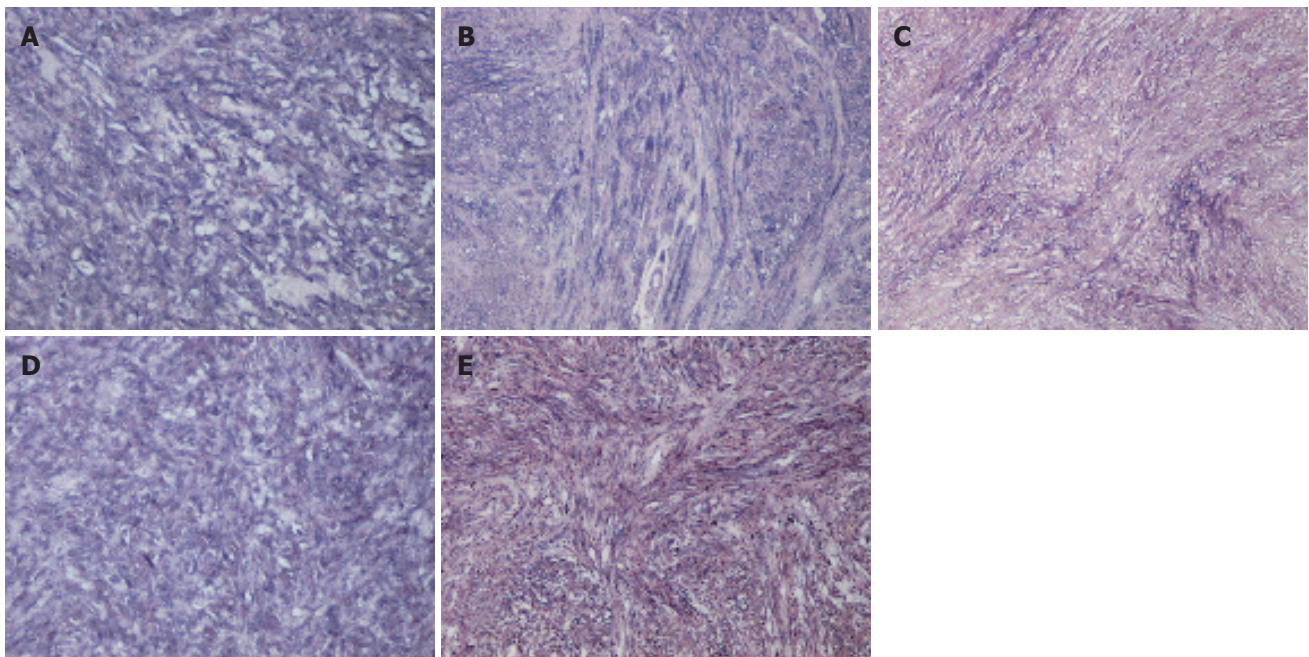


Figure 2 Immunohistochemical staining of human intestinal leiomyomas. Alkaline phosphatase reaction products demonstrating Ang-1 (A), Ang-2 (B), Ang-4 (C), Tie-1 (D) and Tie-2 (E) expression (x 200).

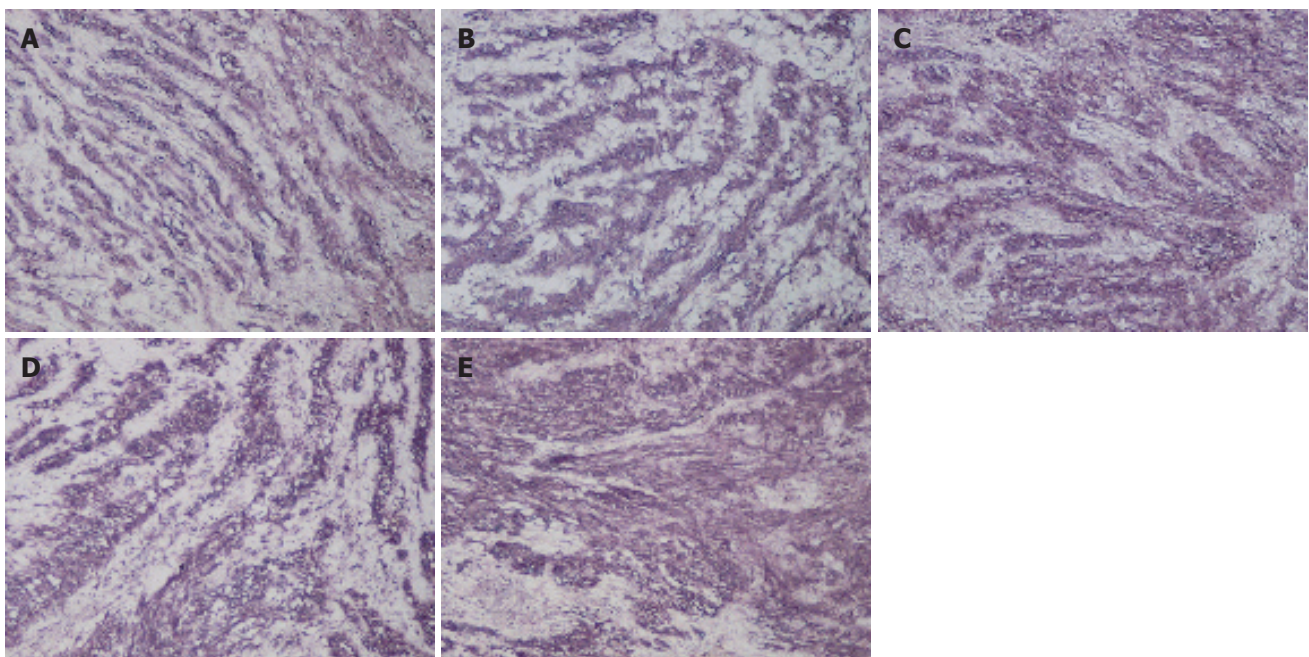


Figure 3 Immunohistochemical staining of human intestinal schwannomas. Alkaline phosphatase reaction products demonstrating Ang-1 (A), Ang-2 (B), Ang-4 (C), Tie-1 (D) and Tie-2 (E) expression (x 200).

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* (%)

	<i>n</i>	Ang-1		Ang-2		Ang-4		Tie-1		Tie-2	
		-	+	-	+	-	+	-	+	-	+
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 ($\times 200$) 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.

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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 large-

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 mm²; a high-power ($\times 200$) 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 μ m 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

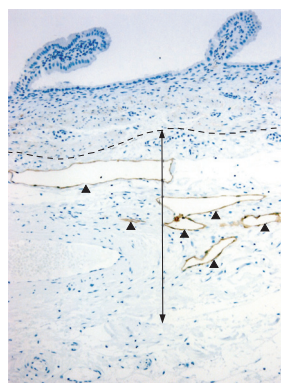


Figure 1 D2-40 antibody-positive lymphatic vessels in the wall of a human gallbladder. The perimuscular connective tissue contains more and larger lymphatic vessels (arrowheads), most of which are located within 500 μ m (two-headed arrow) of the lower border of the muscle layer (broken line), than the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

at 4°C 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.

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 ($\times 20$), 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 $\times 200$ 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 \pm SD, $n = 12$)

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

LVD: lymphatic vessel density; LVA: relative lymphatic vessel area; ^aKruskal-Wallis test, ^b $P < 0.001$ between mucosa, muscle and perimuscular connective tissue of the gallbladder for both LVD and LVA.

within 500 μ m 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 μ m) 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. Fur-

ther 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.

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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°C, 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 \pm SD: 54.94 ± 13.90 years) were definitively enrolled. They were previously given the following regimens: Proton pump inhibitor (PPI) + Clarithromycin + Amoxicillin in 49 cases; PPI + Clarithromycin + Tinidazole (or Metronidazole) in 30 cases; PPI + Levofloxacin + Amoxicillin in 13 cases; Ranitidine bismuth citrate + Clarithromycin 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,

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, <i>n</i>	2	1
False negative, <i>n</i>	18	10

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

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.

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, ¹³C-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 ¹³C-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 ¹³C-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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Crohn's disease in one mixed-race population in Brazil

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had an earlier diagnosis and appeared to have had a more severe disease presentation than white patients.

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

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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 immunosuppressors than white patients although this difference was not statistically significant.

CONCLUSION: Non-white patients with Crohn's disease

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

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

Vienna Classification	Total <i>n</i> = 65	White <i>n</i> = 21	Non-whites <i>n</i> = 44	<i>P</i>
Age at diagnosis				
A1 (< 40)	45 (69.2)	11 (52.4)	34 (77.3)	<i>P</i> < 0.05
A2 (\geq 40)	20 (30.8)	10 (47.6)	10 (22.7)	
Behavior				
B1-Nonsticturing, nonpenetrating	24 (36.9)	8 (38.1)	16 (36.4)	NS ^a
B2-Stricturing	5 (7.7)	2 (9.5)	3 (6.8)	
B3-Penetrating	36 (55.4)	11 (52.4)	25 (56.8)	
Localization ^b				
L1-Ileum terminal	15 (25.4)	7 (36.8)	8 (20.0)	NS ^c
L2-Colon	14 (23.7)	3 (15.8)	11 (27.5)	
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. ^aComparison between penetrating and non-penetrating; ^bData on 59 patients; ^cComparison between colonic and non-colonic; ^dComparison 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 <i>n</i> = 65	Whites <i>n</i> = 21	Non-whites <i>n</i> = 44	<i>P</i>
Age (mean \pm SD)	37.3 \pm 13.0	39.4 \pm 13.9	36.3 \pm 12.6	NS
Females <i>n</i> / %	40 (61.5)	12 (57.1)	28 (63.6)	NS
Smoking				NS
Non-smoker	54 (83.1)	16 (76.2)	38 (86.4)	NS
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)	NS
Equal to or more than 10 yr	13 (20)	3 (14.3)	10 (22.7)	

NS: no statistically significant difference. ^aData on 64 patients.

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.

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

NS: no statistically significant difference. ^aalmost 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 ileo-colonic 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 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 Crohn'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-factorial 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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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.

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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



Figure 1 Indigenous endotrainer used for training the staff.

operations, (3) coagulopathies, (4) and ASA grade III and onwards.

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	23
	Cholecystectomy	
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

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

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.

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 resharpended 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 verified 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 verified 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.

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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 verified 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 verified 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

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.

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.

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

Distance (mm)	Retrospective group (120 cases)			Prospective group (58 cases)		
	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

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 macrosatellites 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 macrosatellites 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 macrosatellites 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 macrosatellites 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

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

in 1, 56 microscopic tumor thrombi in 5 (1-18 per case), and 154 microscopic tumor thrombi in 8 (6-33 per case) and macrosatellites 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 $89.7\% \pm 5.6\%$.

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 macrosatellites 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

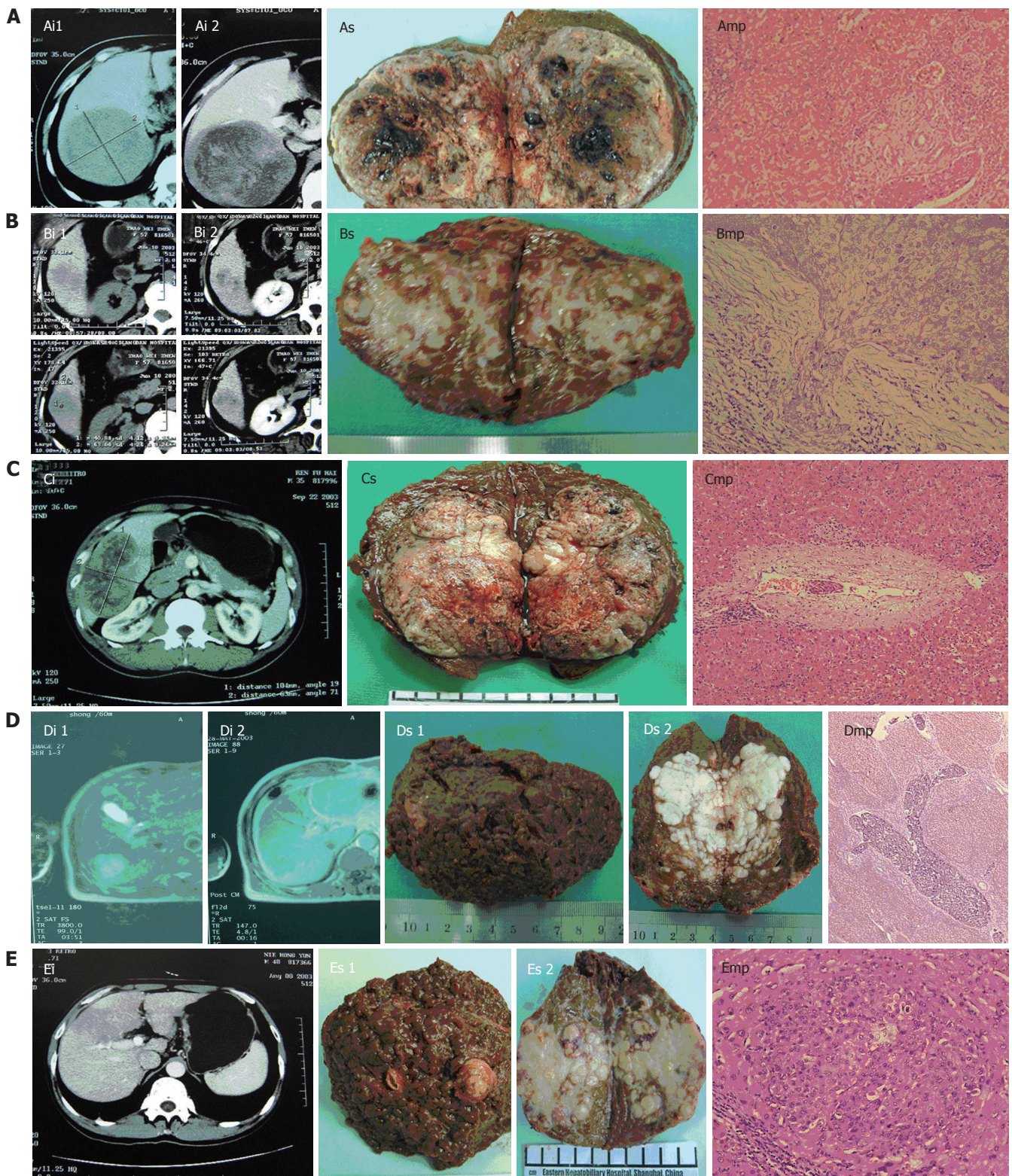


Figure 1 Imaging (i), specimens (s) and microscopic pathology (mp) of 5 PLC patients. **A:** case 24, tumor size 12.0 cm x 11.0 cm x 10.5 cm, with microscopic tumor thrombi (metastatic distance 6 mm, x 100); **B:** case 40, tumor size 4.0 cm x 3.8 cm x 3.5 cm, with microscopic capsular infiltration (x 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 microsatellites 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	0.08	9	0	0	9	< 0.01
AFP (μmol/L)	120					76				
< 20	44	0	7	37		21	0	4	17	
20-400	33	0	6	27		22	2	8	12	
> 400	43	4	9	30	0.1	33	15	10	8	< 0.01
Size of tumor (mm)	111					76				
≤ 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 ($1.21\% \pm 0.06\%$ vs $1.62\% \pm 0.07\%$, $P < 0.001$). 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.

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

Xu CF, Yu CH, Li YM, Xu L, Du J, Shen Z. Association of the frequency of peripheral natural killer T cells with

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/m²), 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/m²), or in the subjects with high waist circumference

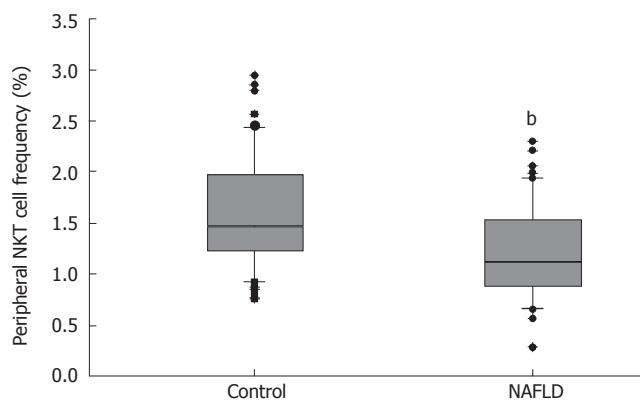


Figure 1 Frequency of peripheral NKT cells in NAFLD patients versus healthy controls. Boxes show values within 25th and 75th percentiles, horizontal bar represents the median, 80% of values are between the extremities of vertical bars (10th-90th percentiles), and extreme values are represented by individual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

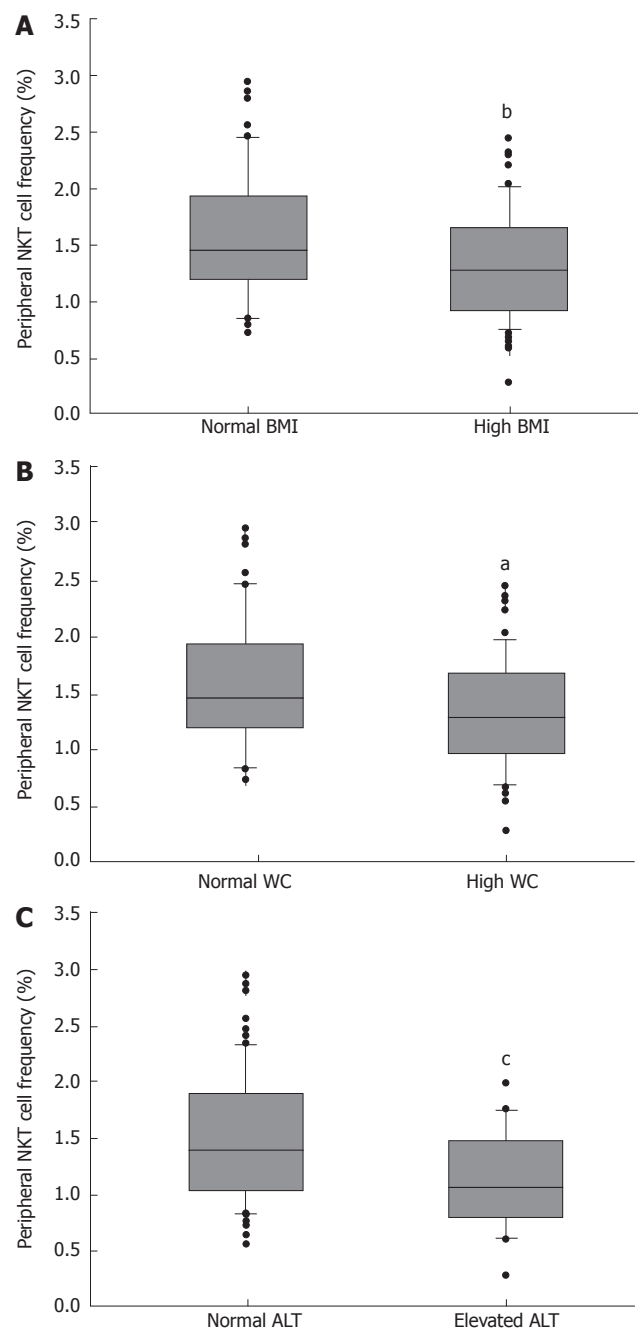


Figure 2 Relationships between the frequency of peripheral NKT cells and BMI, waist circumference and ALT. **A:** The frequency of peripheral NKT cells was compared between the subjects with BMI ≥ 24 kg/m² (*n* = 68) and the subjects with BMI < 24 kg/m² (*n* = 52), ^b*P* < 0.01 vs subjects with BMI < 24 kg/m²; **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. α GalCer/CD1d tetramers and V α 24/V β 11 double-staining are considered to be two standard methods for NKT cell identification^[27]. V α 24 staining alone as used in the present study may overestimate the numbers of V α 24 and V β 11 double-positive NKT cells. However, because NKT cells represent a subtype of T lymphocytes, staining NKT cells with V α 24 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 extensively 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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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 ($31.35\% \pm 10.85\%$ vs $44.43\% \pm 20.70\%$, $30.93\% \pm 18.10\%$ vs $44.43\% \pm 20.70\%$, respectively, $P < 0.05$). The PCNA protein expression was significantly lower in Groups C and D as compared with non-TACE group ($49.61\% \pm 15.11\%$ vs $62.92\% \pm 17.21\%$, $41.16\% \pm 11.83\%$ vs $62.92\% \pm 17.21\%$, respectively, $P < 0.05$). The Ki-67 protein expression was significantly higher in Group A as compared with non-TACE group ($55.44\% \pm 13.72\%$ vs $44.43\% \pm 20.70\%$, $P < 0.05$). The PCNA protein expression was significantly higher in Groups

A and B as compared with non-TACE group ($72.22\% \pm 8.71\%$ vs $62.92\% \pm 17.21\%$, $69.91\% \pm 13.38\%$ vs $62.92\% \pm 17.21\%$, 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.

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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 G₁ and S phases. Ki-67 is expressed throughout the cell cycle (late G₁, S, G₂, and M phases) of proliferating cells, but is absent in quiescent (G₀) 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 \pm 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 reevaluated jointly on a second occasion, and an agreement was reached.

Statistical analysis

Data were expressed as mean \pm 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 $44.43\% \pm 20.70\%$ and $62.92\% \pm 17.21\%$ in non-TACE group, $55.44\% \pm 13.72\%$ and $72.22\% \pm 8.71\%$ in Group A, $45.26\% \pm 14.97\%$ and $69.91\% \pm 13.38\%$ in Group B, $31.35\% \pm 10.85\%$ and $49.61\% \pm 15.11\%$ in Group C, and $30.93\% \pm 18.10\%$ and $41.16\% \pm 11.83\%$ 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 $44.43\% \pm 20.70\%$ and $62.91\% \pm 17.21\%$

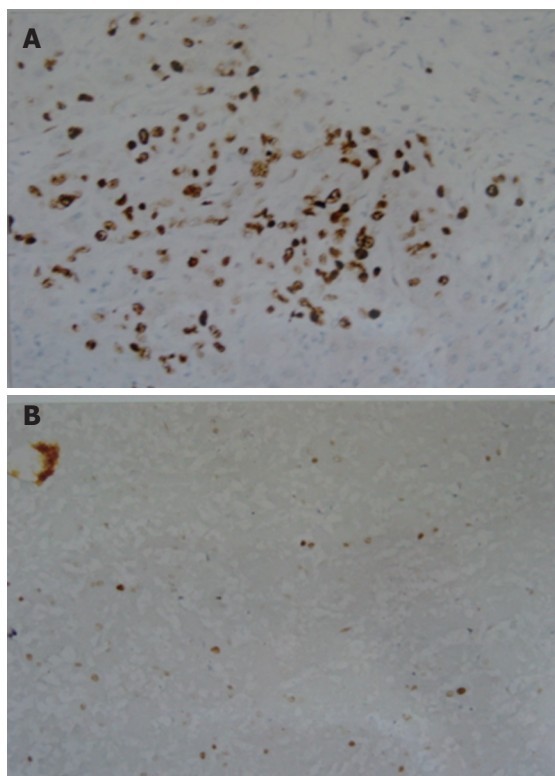


Figure 1 Ki-67 immunostaining of HCC cells ($\times 200$). **A:** Non-TACE group; **B:** Group D.

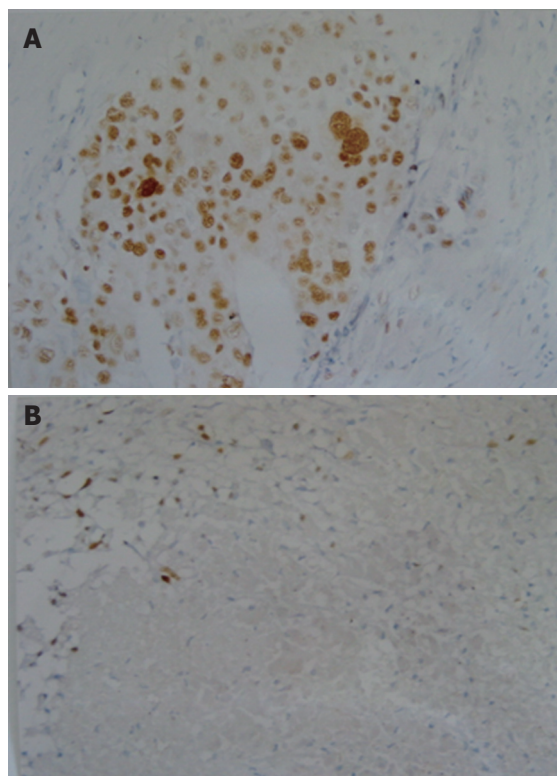


Figure 2 PCNA immunostaining of HCC cells ($\times 200$). **A:** Non-TACE group; **B:** Group C.

in non-TACE group, $41.34\% \pm 16.69\%$ and $62.00\% \pm 18.47\%$ in one-course of TACE group, $39.24\% \pm 14.48\%$ and $57.70\% \pm 15.54\%$ in two-courses of TACE group, and $38.33\% \pm 20.90\%$ and $53.97\% \pm 18.13\%$ 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 $44.43\% \pm 20.70\%$, $62.91\% \pm 17.21\%$ in non-TACE group, $35.42\% \pm 15.46\%$ and $53.19\% \pm 18.28\%$ in ≤ 1 mo interval of TACE group, $40.66\% \pm 18.53\%$ and $58.09\% \pm 15.90\%$ in 1-2 mo interval of TACE group, $42.22\% \pm 14.80\%$ and $66.83\% \pm 17.93\%$ in 2-3 mo interval of TACE group, and $50.62\% \pm 12.33\%$ and $72.53\% \pm 12.93\%$ 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 G₁, S, G₂, and M phases) of proliferating cells, but is absent in quiescent (G₀) 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 G₁ 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.

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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/mm³, 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 aphthous 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

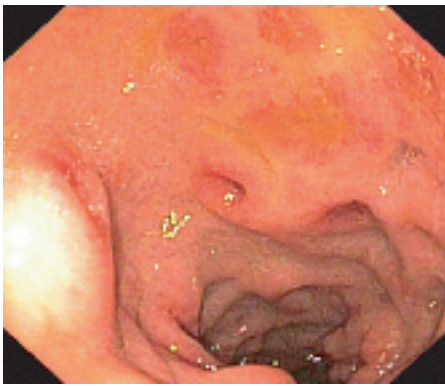


Figure 1 Inflammation of the duodenal mucosa with ulcerous lesions can be seen.

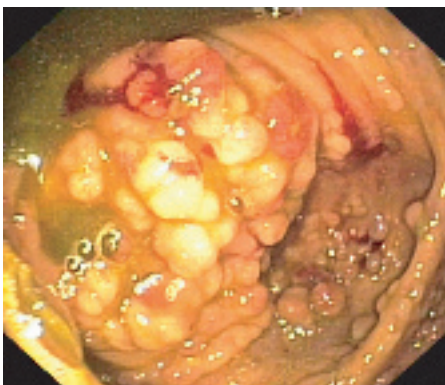


Figure 2 Diffuse polypoid lesions are present in the colon.

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

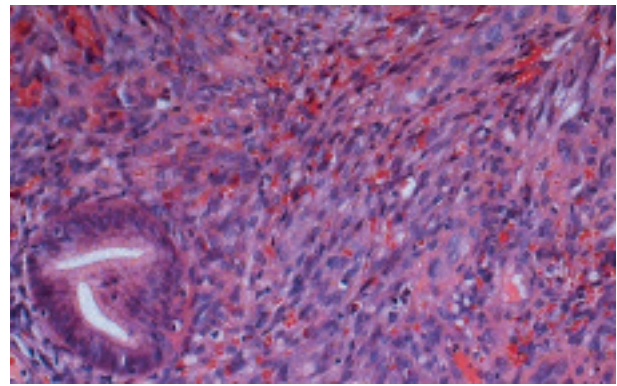


Figure 3 Proliferation of spindle cells is seen in the lamina propria. Slit-like spaces are present, with red blood cells between them.

evidence of Kaposi's sarcoma in the colon, with only small residuals remaining in the duodenum.

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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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.

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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 normocytic 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.

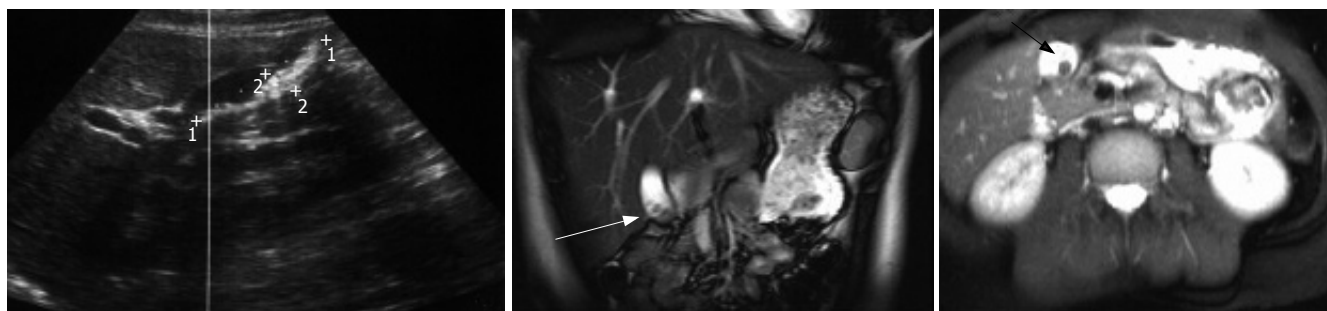


Figure 1 Computed tomography (CT) of the abdomen showed a radiopaque tissue in the wall of the gallbladder, with concomitant inflammation. US examination showed continual expansion of the suspected tissue. After 12 d and maximum extension of the tissue, a second MRI of the liver was performed, and the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

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Key words: Hepatocellular carcinoma; Brain metastasis; Radiotherapy

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most
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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

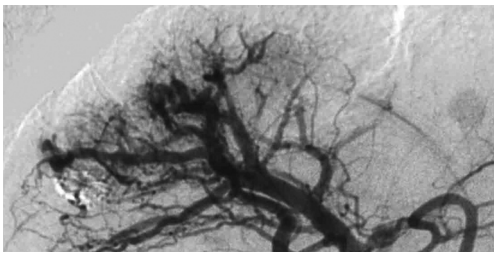


Figure 1 Hepatic arteriography at the time of the third session of TACE shows arterial tumor vessels with arterioportal shunting.

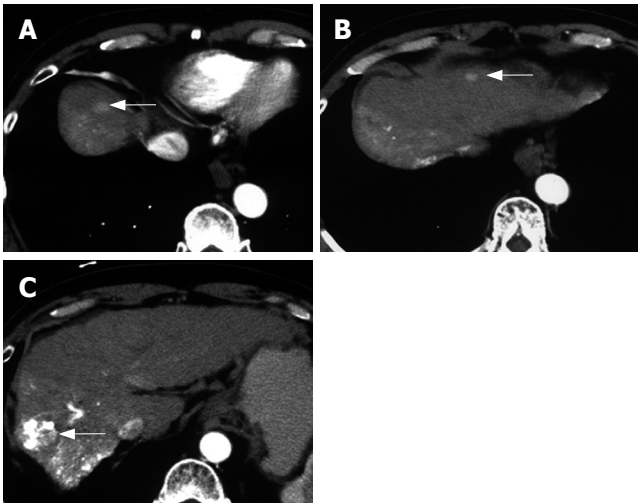


Figure 2 Contrast-enhanced CT at the time of the diagnosis of brain metastases shows multiple hypervascular HCCs (arrows) (A, B, C). Lipiodol retention in the main tumor is seen (C).

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

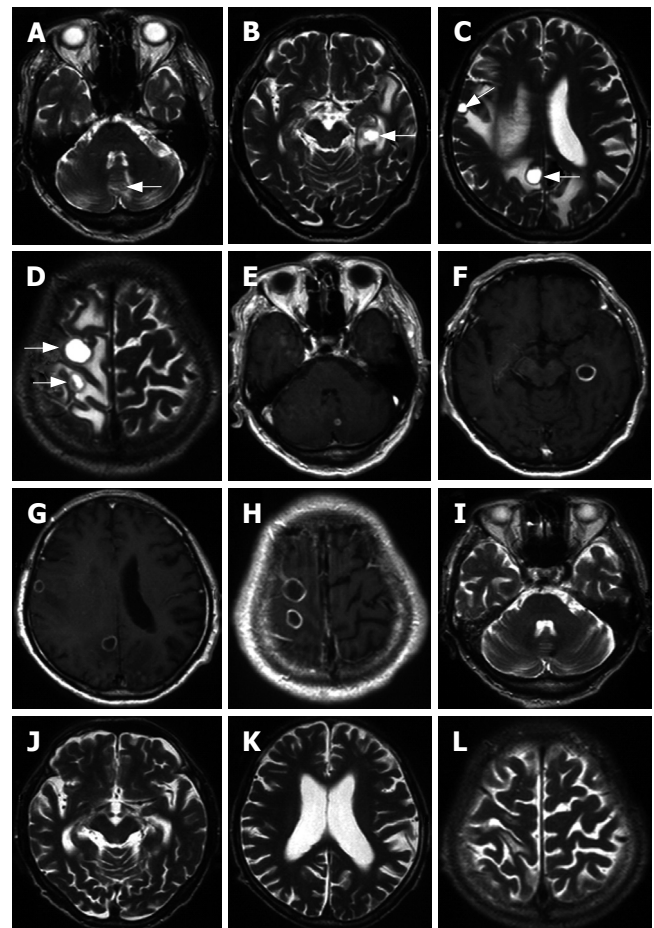


Figure 3 Unenhanced T2-weighted MR images show hyperintense tumors in the cerebrum and cerebellum (arrows) (A, B, C, D). Contrast-enhanced T1-weighted MR images show ring-shaped enhancement of the tumors (E, F, G, H). On T2-weighted, unenhanced MR images, captured 3 mo after the completion of radiotherapy,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

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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metastases and evaluating the therapeutic effects of radiotherapy.

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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.

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

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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 melaena, 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.

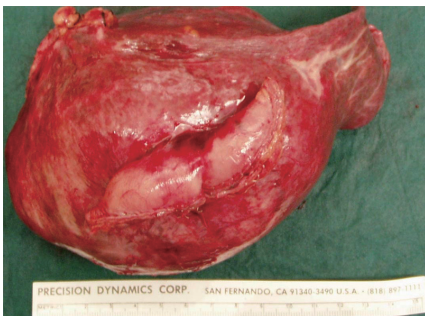


Figure 2 Inferior view of the resected tumor specimen with adherent stomach wall.

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

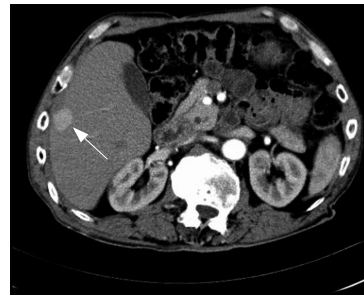


Figure 3 HCC recurrence in the right lobe. Enhanced axial hepatic arterial-phase CT performed on follow-up after initial resection shows a hypervascular lesion in segment 5/6 suspicious for HCC recurrence (arrow). Incidental note is made of a small cyst in the right kidney.

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

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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 $179 \times 10^3/\mu\text{L}$. 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

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.

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

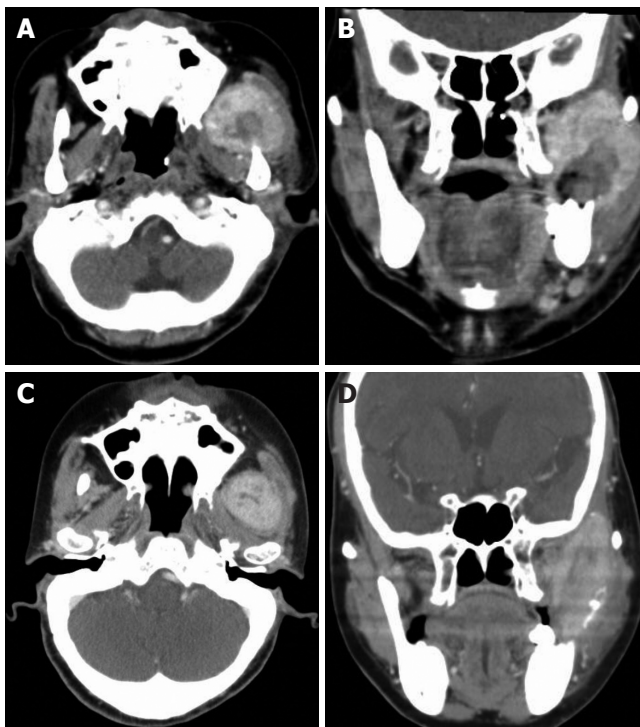


Figure 1 Axial and coronal CT scan of the neck with intravenous contrast showing a 6.2 cm x 5.0 cm, heterogeneously enhancing mass, which appears to be a left parapharyngeal mass involving the pterygoid muscle and temporal muscle (A and B); axial and coronal CT scan of the head and neck showing a 4.0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

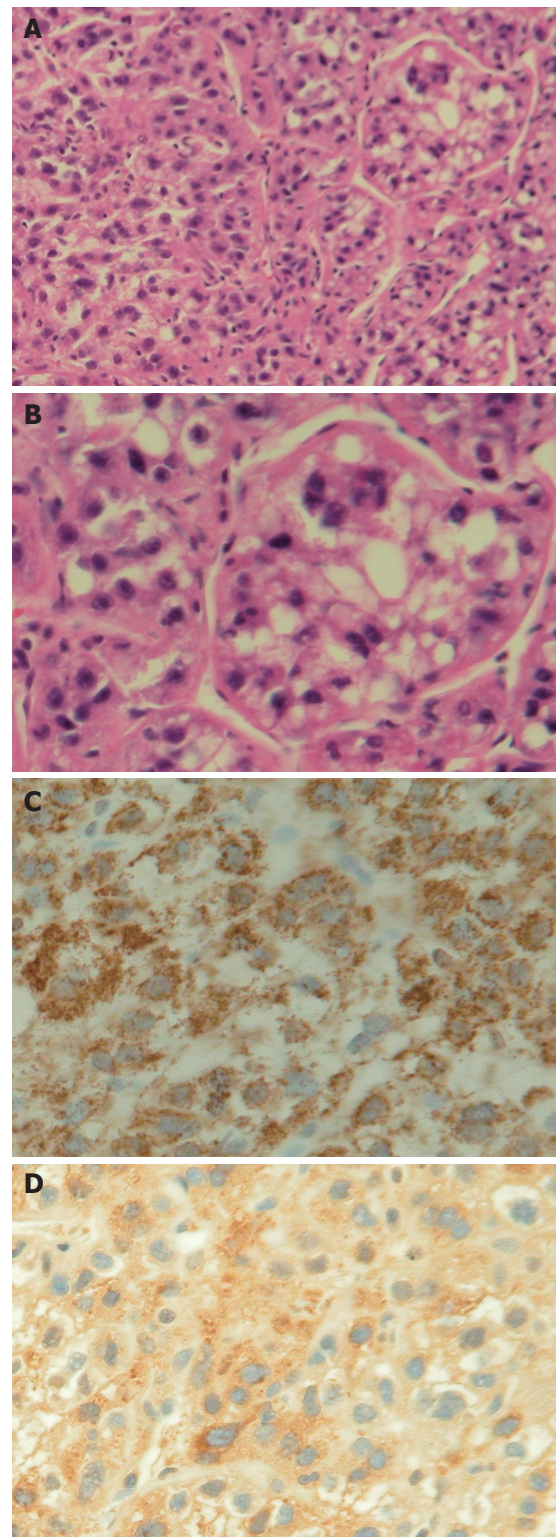


Figure 2 Histopathology of the metastatic lesion. A: The tumor is composed of polygonal cells featuring abundant clear to eosinophilic cytoplasm and microvesicular fatty change, and the tumor cells are arranged in broad trabeculae surrounded by sinusoid spaces which are characteristic of hepatocellular 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 SurgicelTM (Johnson & Johnson, New Brunswick, NJ) and bone wax packing^[7]. In our patient, three methods were utilized to stop bleeding: electrocauterization, SurgicelTM, 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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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.

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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

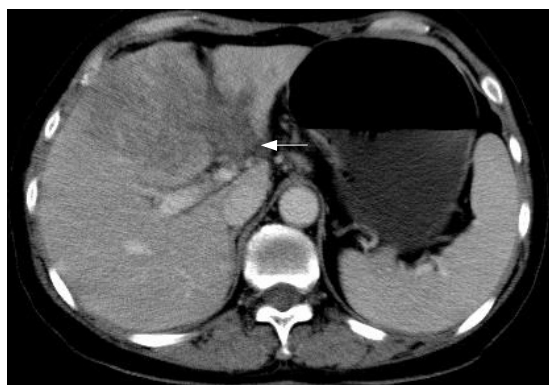


Figure 1 Portal phase of contrast-enhanced CT demonstrating the left portal vein tumor thrombus (white arrow).

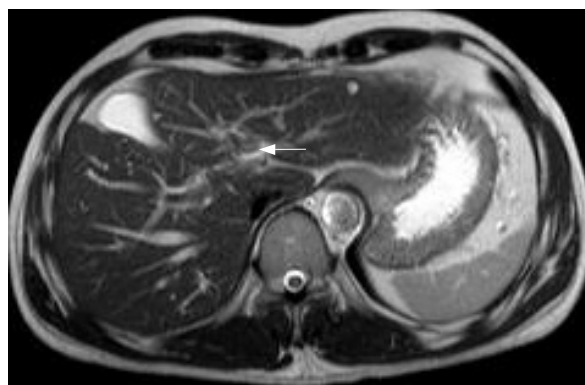


Figure 3 MRI T2 imaging showing a thrombus in the left portal vein.

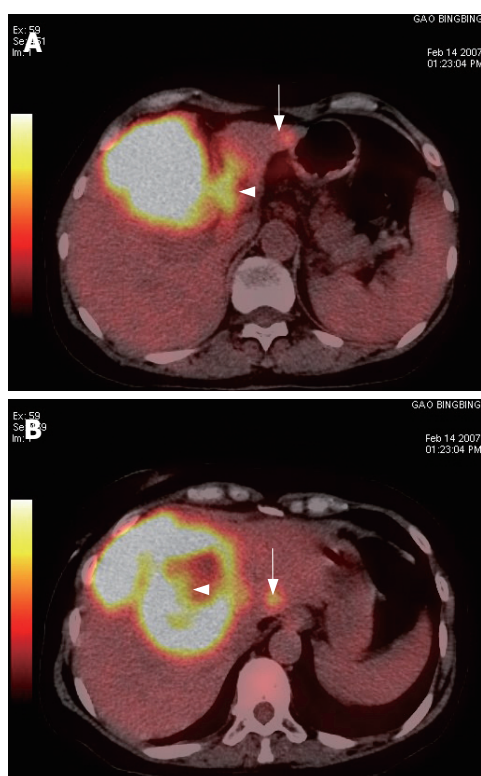


Figure 2 A: Integrated PET/CT image showing high FDG uptake by tumor thrombus in the left portal vein (arrow head) and a liver mass with a satellite lesion (arrow); B: Fused PET/CT imaging showing necrosis in the center of the mass (arrow head) with a satellite lesion (arrow).

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

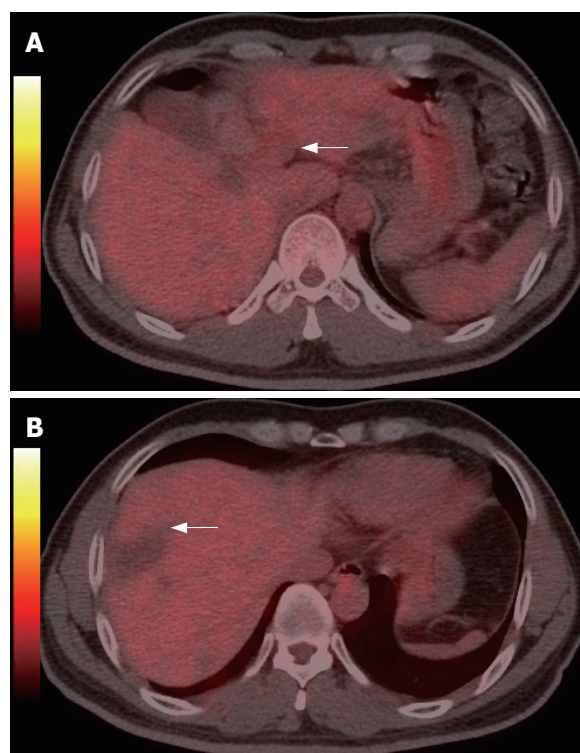


Figure 4 Fused PET/CT imaging showing no hypermetabolism (arrow) in the region of the left portal vein (A) and in the region of previous hepatic resection (B).

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 ¹⁸F-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 ¹⁸F-FDG PET/CT in HCC patients have not been reported so far. Compared with conventional CT and MRI, PET/CT with ¹⁸F-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 Beadsmoore *et al*^[7] found that ¹⁸F-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 ¹⁸F-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 ¹⁸F-FDG to various degrees, a high positive rate of ¹⁸F-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 ¹⁸F-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. ¹⁸F-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]. ¹⁸F-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 ¹⁸F-FDG uptake whose characterization can be interpreted as malignant or benign. ¹⁸F-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 ¹⁸F-FDG PET/CT. Thus it demonstrates the complimentary value of morphological and functional imaging in achieving a correct differentiation. PET/CT with ¹⁸F-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.

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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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Meetings

MAJOR MEETINGS COMING UP

Meeting Falk Research Workshop: Morphogenesis and Cancerogenesis of the Liver
25-26 January 2007
Goettingen
symposia@falkfoundation.de

Meeting Canadian Digestive Diseases Week (CDDW)
16-20 February 2007
Banff-AB
cagoffice@cag-acg.org
www.cag-acg.org/cddw/cddw2007.htm

Meeting Falk Symposium 158: Intestinal Inflammation and Colorectal Cancer
23-24 March 2007
Sevilla
symposia@falkfoundation.de

Meeting BSG Annual Meeting
26-29 March 2007
Glasgow
www.bsg.org.uk/

NEXT 6 MONTHS

Meeting 42nd Annual Meeting of the European Association for the Study of the Liver
11-15 April 2007
Barcelona
easl2007@easl.ch
www.easl.ch/liver-meeting/

Meeting Falk Symposium 159: IBD 2007 - Achievements in Research and Clinical Practice
4-5 May 2007
Istanbul
symposia@falkfoundation.de

Meeting European Society for Paediatric Gastroenterology, Hepatology and Nutrition Congress 2007
9-12 May 2007
Barcelona
espghan2007@colloquium.fr

Digestive Disease Week
19-24 May 2007
Washington Convention Center, Washington DC

Meeting Gastrointestinal Endoscopy Best Practices: Today and Tomorrow, ASGE Annual Postgraduate Course at DDW
23-24 May 2007
Washington-DC
tkoral@asge.org

Meeting ESGAR 2007 18th Annual Meeting and Postgraduate Course
12-15 June 2007
Lisbon
fca@netvisao.pt

Meeting Falk Symposium 160: Pathogenesis and Clinical Practice in

Gastroenterology
15-16 June 2007
Portoroz
symposia@falkfoundation.de

Meeting ILTS 13th Annual International Congress
20-23 June 2007
Rio De Janeiro
www.ils.org

Meeting 9th World Congress on Gastrointestinal Cancer
27-30 June 2007
Barcelona
meetings@imedex.com

EVENTS AND MEETINGS IN 2007

Meeting Falk Research Workshop: Morphogenesis and Cancerogenesis of the Liver
25-26 January 2007
Goettingen
symposia@falkfoundation.de

Meeting Canadian Digestive Diseases Week (CDDW)
16-20 February 2007
Banff-AB
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Pathogenesis and management issues for non-alcoholic fatty liver disease

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Abstract

Nonalcoholic fatty liver disease (NAFLD) has, although it is a very common disorder, only relatively recently gained broader interest among physicians and scientists. Fatty liver has been documented in up to 10 to 15 percent of normal individuals and 70 to 80 percent of obese individuals. Although the pathophysiology of NAFLD is still subject to intensive research, several players and mechanisms have been suggested based on the substantial evidence. Excessive hepatocyte triglyceride accumulation resulting from insulin resistance is the first step in the proposed 'two hit' model of the pathogenesis of NAFLD. Oxidative stress resulting from mitochondrial fatty acids oxidation, NF- κ B-dependent inflammatory cytokine expression and adipocytokines are all considered to be the potential factors causing second hits which lead to hepatocyte injury, inflammation and fibrosis. Although it was initially believed that NAFLD is a completely benign disorder, histologic follow-up studies have showed that fibrosis progression occurs in about a third of patients. A small number of patients with NAFLD eventually ends up with end-stage liver disease and even hepatocellular carcinoma. Although liver biopsy is currently the only way to confirm the NAFLD diagnosis and distinguish between fatty liver alone and NASH, no guidelines or firm recommendations can still be made as for when and in whom it is necessary. Increased physical activity, gradual weight reduction and in selected cases bariatric surgery remain the mainstay of NAFLD therapy. Studies with pharmacologic agents are showing promising results, but available data are still insufficient to make specific recommendations; their use therefore remains highly individual.

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Key words: Non-alcoholic fatty liver disease; Metabolic

INTRODUCTION AND EPIDEMIOLOGY

Nonalcoholic fatty liver disease (NAFLD) is an increasingly recognized cause of liver-related morbidity and mortality. It represents a spectrum of hepatic disorders characterized by macrovesicular steatosis that occur in the absence of alcohol consumption in amounts generally considered to be harmful to the liver (less than 40 g of ethanol per week). That spectrum ranges from simple hepatic steatosis without concomitant inflammation or fibrosis to hepatic steatosis with a necroinflammatory component that may or may not have associated fibrosis (non-alcoholic steatohepatitis-NASH) and can progress to cirrhosis.

Although the association of macrovesicular steatosis of the liver with inflammatory changes and fibrosis in obese subjects has been known for several decades, it was largely ignored as a clinical entity. The term "nonalcoholic steatohepatitis" was first introduced in 1980 by Ludwig *et al* and is used to describe the distinct clinical entity in which patients have liver biopsy findings indistinguishable from alcoholic hepatitis, but lack a history of significant alcohol consumption^[1].

The true incidence and prevalence of NAFLD are unknown. Population-based studies most often use imaging modalities or serum alanine aminotransferase levels to diagnose NAFLD^[2-4]. These studies are limited by the inability to make a definitive diagnosis of NAFLD or to distinguish between NAFLD and NASH, which requires liver biopsy. Studies that have used strict definitions for diagnosis including biopsies were most often based on specific subsets of the population (e.g. diabetics, obese individuals, in-hospital patients) and they cannot be applied to the general population^[5-7]. Despite the limitations of the published data, several facts are consistently present. Fatty liver and NASH have been reported in all age groups, including children^[4,8]. The prevalence increases with increasing body weight^[6,9]. Fatty liver has been documented in up to 10% to 15% of normal individuals and 70% to

80% of obese individuals. Correspondingly, about 3% of normal individuals and 15% to 20% of morbidly obese subjects ($\text{BMI} > 35 \text{ kg/m}^2$) have steatohepatitis^[6,10]. These findings are of particular concern given the increasing prevalence of obesity in virtually all age groups. The highest prevalence is in those between 40 and 60 years of age^[7,11,12]. Although earlier studies found higher prevalence of NASH in women (65 to 85 percent of all patients), more recent studies have shown that NASH occurs with equal frequency in both sexes^[1,7,11,13]. In the United States, there appear to be ethnic differences in the prevalence of NASH. A higher prevalence of hepatic steatosis was found in Hispanics (45%) compared with Caucasians (33%) or Afro-Americans (24%)^[14].

There is increasing evidence that NAFLD represents the hepatic component of a metabolic syndrome characterized by obesity, hyperinsulinemia, peripheral insulin resistance, diabetes, hypertriglyceridemia, and hypertension. Type 2 diabetes mellitus is a major component of the metabolic syndrome and is associated with both obesity and NASH^[1,12,13,15]. It has been described in 34% to 75% of patients with NASH. Diabetes is not only associated with NAFLD, but also may be a risk factor for development of progressive liver fibrosis^[16]. Obesity has been reported in 70 to 100 percent of cases of NASH, and most patients are 10% to 40% above ideal body weight^[1,12,13,15]. Numerous reports have documented resolution of fatty liver following gradual weight loss. Subjects with abdominal obesity are more prone to developing diabetes and hypertension as well as fatty liver. Hyperlipidemia (hypertriglyceridemia and/or hypercholesterolemia), which is frequently associated with both obesity and type 2 diabetes, has been reported in 20% to 80% of patients with NASH^[1,12,13,15].

In addition, NAFLD has been associated with several rare disorders of lipid metabolism and insulin resistance (e.g. abetalipoproteinemia, lipotrophic diabetes, Mauriac and Weber-Christian syndrome), as well as with total parenteral nutrition, acute starvation, intravenous glucose therapy, abdominal surgery (e.g. extensive small bowel resection, biliopancreatic diversion, and jejunal bypass), use of several drugs (e.g. amiodarone, tamoxifen, glucocorticoids, and synthetic estrogens) and several types of chemicals (e.g. organic solvents and dimethylformamide)^[17-31]. The incidence, mechanism, and natural history of these forms of NAFLD are unknown.

Several studies reported that many patients with NASH have biochemical evidence of iron overload, with elevation of transferrin saturation and serum ferritin level. Patients with NASH were found to be homozygous or heterozygous for the Cys282Tyr mutation in the HFE gene in significantly higher percent than the general population. However, the hepatic iron index was < 1.9 in all patients. The presence of iron overload has been reported to be associated with increased hepatic fibrosis, but this finding was not confirmed by other reports. The significance of the HFE mutations in NASH remains to be fully established^[32-36].

is principally associated with the metabolic syndrome. Therefore, two types of NAFLD can be recognized: primary NAFLD (associated with metabolic syndrome) and secondary NAFLD (associated with other specific metabolic or iatrogenic conditions distinct from the metabolic syndrome).

Pathophysiology of primary NAFLD still hasn't been completely clarified. The so called 'two hit' model of the pathogenesis of NAFLD has been proposed since 1998^[37]. Liver fat accumulation is the suggested 'first hit' or the first step. It is a consequence of excessive triglyceride accumulation caused by discrepancy between influx and synthesis of hepatic lipids on one side and their β -oxidation and export on the other (Figure 1)^[38]. This imbalance occurs with all of the previously mentioned potential etiologic factors. The steatotic liver subsequently becomes vulnerable to presumed 'second hits' leading to hepatocyte injury, inflammation and fibrosis. The most widely supported theory implicates insulin resistance as the key mechanism in primary NAFLD, leading to hepatic steatosis, and perhaps also to steatohepatitis. The presumed factors initiating second hits are oxidative stress and subsequent lipid peroxidation, proinflammatory cytokines (principally $\text{TNF-}\alpha$), and hormones derived from adipose tissue (adipocytokines) (Figure 1).

Obesity, type 2 diabetes, hyperlipidemia and other conditions associated with insulin resistance are generally present in patients with NAFLD. Insulin resistance has also been observed in patients with NAFLD who are not obese and in those with normal glucose tolerance^[39-41]. The molecular mechanism leading to insulin resistance is complex and hasn't been elucidated completely. Several molecules (tumor necrosis factor alpha, PC-1 membrane glycoprotein, leptin, and fatty acids) appear to interfere with the insulin signalling pathway^[42]. Alterations in lipid metabolism associated with insulin resistance result from the interaction between the effects of insulin resistance located primarily in muscles and adipose tissue and impact of the compensatory hyperinsulinemia on tissues that remain insulin sensitive. These alterations include enhanced peripheral lipolysis, increased hepatic uptake of FFAs and increased hepatic triglyceride synthesis. FFA influx and neosynthesis outweigh FFA oxidation and triglyceride secretion, resulting in the net effect of hepatic fat accumulation. This can explain a key role of insulin resistance in the development of hepatic steatosis and, potentially, steatohepatitis (Figure 2)^[43-48].

The resulting accumulation of fat within the hepatocytes has several effects. FFAs impair insulin signalling and cause hepatic insulin resistance *via* mechanisms involving activation of PKC-3, JNK, I- κ B kinase β (IKK- β) and $\text{NF-}\kappa\text{B}$ ^[49,50]. Hepatic insulin resistance then increases mitochondrial fatty acids oxidation. Also, FFAs and their metabolites are ligands for peroxisomal proliferators-activated receptor- α (PPAR- α), the transcription factor that regulates the expression of different genes encoding enzymes involved in mitochondrial, peroxisomal and microsomal fatty acids oxidation. Finally, it appears that both consequences of fat accumulation within the liver (fat-induced hepatic insulin resistance and up-regulation of PPAR- α -regulated genes) result in increased FFA oxidation. Mitochondrial and peroxisomal fatty acids oxidation are both capable of producing

HOW DOES IT DEVELOP? - NAFLD PATHOGENESIS

A large body of evidence clearly indicates that NAFLD

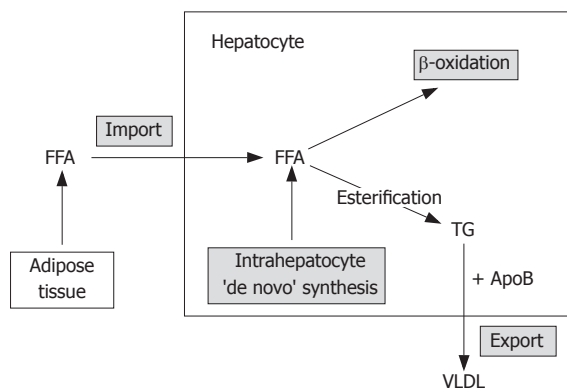


Figure 1 Lipid metabolism within the hepatocytes. Liver lipid content is determined by the equilibrium of several processes: import of free fatty acids (FFAs) from the adipose tissue, *de novo* FFA synthesis in hepatocytes, beta-oxidation of FFAs, esterification of FFAs into triglycerides and export of triglycerides as very low density lipoproteins (VLDL). Hepatic steatosis is a consequence of imbalance in those processes in favour of excessive triglyceride (TG) accumulation. FFA: free fatty acids; TG: triglycerides; VLDL: very low density lipoproteins; Apo B: apolipoprotein B.

hepatotoxic free oxygen radicals that contribute to the development of oxidative stress^[51-54]. Considering all these data, it appears that insulin resistance could in fact deliver both 'hits' in the pathogenesis of NASH. Considerable mitochondrial structural abnormalities were found in patients with NASH, but not in those with simple hepatic steatosis^[46,55-58]. It has also been found that several genes important for mitochondrial function were underexpressed in NASH patients^[59]. Aforementioned oxidative stress and subsequent lipid peroxidation are the factors supposed to alter both mitochondrial DNA and mitochondrial oxidative phosphorylation, leading to mitochondrial structural abnormalities and ATP depletion^[60,61]. However, it is also possible that these mitochondrial abnormalities are the preexisting conditions that enable excessive free oxygen radical species production in the setting of enhanced FFA beta-oxidation^[46]. This would mean that, in the absence of preexisting mitochondrial defects, insulin resistance will lead only to the development of simple hepatic steatosis.

Many studies demonstrated that oxidative stress is a prominent feature of NASH^[62-64]. Apart from hepatocytes, ROS production and oxidative stress in obese patients can also originate in adipose tissue (both in adipocytes and in macrophages infiltrating adipose tissue)^[65,66]. Inflammatory cells within the liver represent the third potential source of ROS and oxidative stress, especially in the setting of already developed steatohepatitis.

Hepatocyte reactive oxygen species accumulation (oxidative stress) could, at least to some extent, be responsible for further progression from steatosis to steatohepatitis and fibrosis. This could occur by three main mechanisms: lipid peroxidation, cytokine induction and Fas ligand induction. ROS-triggered lipid peroxidation of plasma or mitochondrial membranes causes cell necrosis or induces apoptosis. Lipid peroxidation also initiates release of malondialdehyde (MDA) and 4-hydroxynonenal (HNE) that can bind to hepatocyte proteins forming

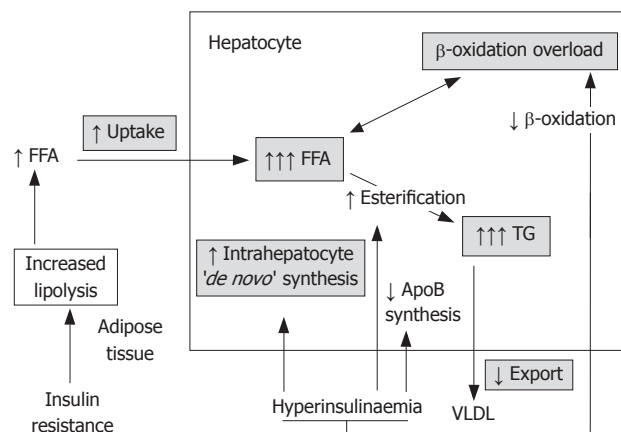


Figure 2 Effects of insulin resistance on lipid metabolism. Insulin resistance and resulting hyperinsulinemia lead to hepatocyte lipid accumulation in the liver by several mechanisms. In adipose tissue, insulin resistance enhances triglyceride (TG) lipolysis and inhibits esterification of free fatty acids (FFAs). The result are increased circulating levels of FFAs, which are then taken up by the liver. Additionally, in hepatocytes hyperinsulinemia increases the 'de novo' synthesis of fatty acids and inhibits their beta oxidation. The consequence is accumulation of FFAs within hepatocytes. Hepatic TG synthesis is driven by the increased hepatocyte FFA content and favoured by insulin-mediated upregulation of lipogenic enzymes, such as peroxisome proliferator-activated receptor gamma (PPAR- γ) and sterol regulatory element binding protein 1 (SREBP-1). Meanwhile, reduced very-low-density lipoprotein (VLDL) production and TG export may be impaired by decreased synthesis of apolipoprotein B (apo B) or reduced binding of TG to apo B by microsomal triglyceride transfer protein (MTP). The resulting accumulation of fat within the hepatocytes initiates further damage causing hepatic insulin resistance and reactive oxygen species production. (abbreviations: \uparrow -increased; \downarrow -inhibits; FFA: free fatty acid; TG: triglyceride; VLDL: very low density lipoprotein; Apo B: apolipoprotein B.

neoantigens and initiating a potentially harmful immune response, cross-link cytokeratins to form Mallory hyaline, or activate hepatic stellate cells promoting collagen synthesis and stimulate neutrophil chemotaxis^[62,67]. ROS also increases expression of Fas-ligand on hepatocytes that interacts with normally expressed membrane receptor Fas on the adjacent hepatocytes causing apoptotic cell death^[68]. ROS may also initiate the activation of the transcription factor NF- κ B, which leads to increased production of proinflammatory cytokines (TNF- α , TGF- β , IL-6, IL-8)^[69].

Furthermore, there are convincing data that inflammatory cytokines (TNF- α , IL-6 and IL-1 β) also play an important role in the pathogenesis of NAFLD^[70]. They may cause systemic and hepatic insulin resistance^[71]. They also cause hepatocyte injury and apoptosis, neutrophil chemotaxis, and hepatic stellate cell activation^[72-74]. Crespo *et al* have found that obese patients with NASH compared to those without it have significantly increased liver expression of TNF- α and its receptor p55, as well as increased expression of TNF- α in adipose tissue^[75]. This increased expression correlated with the degree of liver fibrosis. FFAs accumulated in hepatocytes stimulate NF- κ B-dependent inflammatory cytokine expression (TNF- α , IL-6, IL-1 β)^[76,77]. Kupffer cells are, as the liver specific macrophages, also a potent source of proinflammatory cytokines. Activating stimulus could be hepatocyte-derived cytokines, clearance of oxidated lipid deposits *via* scavenger receptors^[70], or gut-derived endotoxins in patients with small intestinal bacterial overgrowth^[78]. Finally, adipose

tissue is in obese people infiltrated by macrophages^[65], making it another possible source of proinflammatory cytokines^[75,79]. It appears that cytokines produced by adipose tissue macrophages (particularly TNF- α) could mediate systemic and hepatic insulin resistance^[71], as well as cause a reduction in the secretion of the protective adipocytokine adiponectin^[80].

Adipocytokines are peptides produced by visceral adipose tissue. Among them, adiponectin and leptin are directly involved in different metabolic and inflammatory pathways and could be particularly important in the pathogenesis of NAFLD. Adiponectin appears to have a pivotal role in improving fatty acid oxidation and decreasing fatty acid synthesis^[81]. Liver and muscle cells have adiponectin receptors. Stimulation of adiponectin receptors in the liver leads to the activation of PPAR- α and AMP-activated protein kinase (AMPK)^[82-84]. Consequently, adiponectin increases fatty acid β -oxidation and thereby decreases hepatic triglyceride content and hepatic insulin resistance. Adiponectin also has a direct anti-inflammatory effect, suppressing TNF- α production in the liver^[80,81]. Recent studies showed reduced serum levels of adiponectin and reduced hepatic expression of its receptor in patients with NASH compared to those with simple steatosis^[85,86]. It seems that increased production of TNF- α and the generation of ROS are the ones responsible for the reduction in adiponectin secretion^[66,80]. This again implicates that TNF- α and ROS-mediated suppression of adiponectin may play an important role in the pathogenesis of progressive NAFLD. A study in obese leptin-deficient mice demonstrated significant improvements in hepatic steatosis, hepatomegaly, and aminotransferase levels following administration of adiponectin^[81].

Leptin is another peptide produced in adipose tissue that may have an important role in the development of insulin resistance. Leptin inactivates insulin receptor substrate (dephosphorylation of insulin-receptor substrate 1) inducing peripheral and hepatic insulin resistance^[87]. Blood leptin levels correlate with the degree of fibrosis in patients with chronic hepatitis C^[88]; however Angulo *et al* have found no correlation between leptin levels and stage of liver fibrosis in a study of 88 patients with NAFLD^[89]. Cohen *et al* have found that leptin, at the levels comparable with those present in obese individuals, induces hepatic insulin resistance by dephosphorylation of insulin-receptor substrate 1^[87].

In the end, hepatocyte injury and associated inflammation will lead to the activation of hepatic stellate cells and synthesis of extracellular matrix proteins with liver fibrosis as the final consequence. In addition, apoptotic cell death is also of great importance in hepatic fibrogenesis^[68]. It leads to stellate cell activation by the means of ingestion of apoptosing hepatocytes by Kupffer cells and subsequent release of TGF- β ^[90,91]. There are several more mediators possibly involved in the pathogenesis of liver fibrosis in NAFLD. The adipocytokine leptin may play a role in fibrogenesis^[89,92]. The reduced production of adiponectin associated with obesity may also contribute to the development of liver fibrosis^[93]. Angiotensin II, which is also secreted by adipose tissue and is raised in the serum

of obese patients, stimulates hepatic stellate cells and thus has profibrogenic effect^[94]. Finally, hyperglycaemia and hyperinsulinaemia associated with insulin resistance are also suggested to be the key-factors in the progression of fibrosis through the up-regulation of the synthesis connective tissue growth factor by stellate cells^[95].

Despite all of the recent advances in understanding the pathogenesis of NAFLD, the reason why only a minority of patients with classical risk factors for NAFLD develop more than simple steatosis still remains largely unclear (Figure 2).

WHAT TO EXPECT FOR THE PATIENT?

- NATURAL HISTORY AND CLINICAL COURSE OF NAFLD

In spite of the increasing interest and significant progress in understanding of NAFLD, its natural history still hasn't been clearly defined. The reason for this is mostly the lack of large prospective histologic follow-up studies. However, some concepts are clear: NASH progresses to cirrhosis less frequently than alcoholic steatohepatitis, with significantly better long-term survival of NASH patients^[96,97].

Nevertheless, in a population-based study that used data from a large long-term epidemiology project, patients with NAFLD had a slightly higher mortality than the general population^[98]. Liver-related death was the third most common cause of mortality in those patients, compared to the thirteenth place of liver-related death in the general population^[98]. Another retrospective study of 132 patients found that poor outcomes (cirrhosis and liver-related mortality) occurred in 22 percent of patients in whom initial biopsies showed ballooning degeneration and Mallory hyaline or fibrosis, compared to 4 percent in patients with steatosis alone^[97].

There has been a lot of speculation about the rate of progression of disease. Initial studies with paired biopsies showed histologic progression in 30% to 50% of NASH patients, but they had limited conclusions due to small patient numbers^[12,15,99,100].

The largest reported series of NAFLD patients with sequential liver biopsies published in 2005 included 103 patients with a mean interval of 3.2 years between biopsies^[101]. Fibrosis stage progressed in 37%, remained stable in 34% and regressed in 29% of patients. Diabetes, low initial fibrosis stage and higher body mass index were associated with higher rate of fibrosis progression. Another study of 22 patients with a median 4.3-year interval between biopsies also found progression of fibrosis in about a third of patients, with obesity and higher body mass index being the only associated factors^[102].

According to these results it is obvious that NAFLD, and especially NASH, is not a completely benign condition as it was initially believed. Now it is clear that it may lead to end-stage liver disease, and small number of patients with NAFLD may eventually end up with liver transplantation. Interestingly, steatosis and steatohepatitis recurrence after liver transplantation has been described^[103]. Furthermore, several studies suggest that hepatocellular carcinoma (HCC)

might also be among possible NAFLD outcomes^[104-106]. Further studies are required before the risk of HCC in NAFLD can be more precisely defined.

HOW TO DIAGNOSE? - THE ROLE OF THE LIVER BIOPSY

Most patients with NAFLD come to medical attention due to an accidental finding of elevated liver function tests. Although aminotransferase levels are elevated in the majority of patients, normal values don't exclude the presence of necroinflammatory changes or fibrosis. That was best illustrated in a study of 51 subjects with normal ALT levels where 12 had bridging fibrosis and 6 had cirrhosis^[107].

In the longitudinal histologic study of 103 patients aminotransferase improvement correlated with improvement in activity grade, but change in aminotransferase levels did not correlate with a change in fibrosis stage. Interestingly, aminotransferase levels decreased significantly between biopsies both in patients with progressive fibrosis as well as in those without it^[101].

Imaging methods are of little value in diagnostic evaluation of NAFLD. Finding of hyperechoic liver on ultrasonography is frequent in NAFLD, but it is neither sensitive nor specific enough. Moreover, all of the three most commonly used imaging methods (US, CT, MRI) have been shown to be incapable of differentiating between NASH and other forms of NAFLD^[108].

The role of the liver biopsy in NAFLD has been widely discussed, and correlation between histologic findings and clinical features or disease prognosis extensively studied. Liver biopsy is currently the only way to confirm diagnosis of NAFLD and distinguish between fatty liver alone and NASH. It also permits determination of disease severity and possibly gives insight into prognosis. Nevertheless, no guidelines or firm recommendations can yet be made as for when and in whom is liver biopsy necessary.

Another problem with liver biopsy is that it has several significant limitations. First, the quality of liver biopsy specimens is variable. Furthermore, studies have shown significant inter- and intra-observer variability in biopsy specimen interpretation^[109,110]. Additionally, it has long been known that affection of liver parenchyma in various chronic liver diseases is not homogenous, and biopsy is, therefore, subject to sampling variability^[111]. This has also been proven for NASH in a study in which two liver biopsies were performed in 51 patients with NAFLD^[112]. None of the histological features displayed high agreement between two samples from the same patient. Six of 17 patients (35%) with bridging fibrosis on one sample had only mild or no fibrosis on the other and, therefore, could have been understaged with only one biopsy. The negative predictive value of a single biopsy for the diagnosis of NASH was at best 0.74, and discordance of one or more stages was 41%.

In order to overcome some of the frequently present difficulties in liver biopsy interpretation, Matteoni *et al* divided NAFLD into four categories or types, based on the presence of steatosis, lobular inflammation, hepatocyte

ballooning and Mallory bodies/fibrosis^[97]. Types 3 and 4 were associated with worse clinical outcomes. The first histological scoring system for NASH was proposed by Brunt *et al* and it was designed based on a model used in other chronic liver diseases and included 3 qualitatively assessed grades of necroinflammatory activity (based on degree of steatosis, ballooning and inflammation) and 4 stages of fibrosis^[113]. However, it didn't include the whole spectrum of NAFLD and it couldn't be used in assessment of response to therapy. Therefore, another scoring system has been developed, which specifically includes only features of active injury that are potentially reversible in the short term^[110]. The score, named NAS (NAFLD Activity Score), is the unweighted sum of the scores for steatosis (0-3), lobular inflammation (0-3), and ballooning (0-2), while fibrosis was not included. The primary purpose of NAS is to assess overall histological change; it wasn't designed to replace the pathologist's determination of steatohepatitis, nor to represent an absolute severity scale.

In spite of all the efforts, there is still no international consensus regarding the histopathological criteria that would firmly define non-alcoholic steatohepatitis and differentiate between NAFLD entities. Therefore, a large amount of confusion between pathologists and clinicians is still present. As an illustration, in NAS development study interrater agreement on the diagnosis of steatohepatitis was 0.61, and only 68% of liver biopsies with steatosis, ballooning and lobular inflammation all present were diagnosed as NASH^[110].

Several clinical and biochemical risk factors for progressive disease have been identified, as they could potentially facilitate patient selection for liver biopsy. In one study, age over 45, obesity, diabetes mellitus, and aspartate transaminase/alanine transaminase (AST/ALT) ratio greater than 1 were significant predictors of severe liver fibrosis^[116]. In another study, age over 50 years, BMI over 28, triglycerides over 1.7 mmol/L and alanine aminotransferase more than two times normal were independently associated with septal fibrosis^[114].

In conclusion, for the time being, the decision to perform liver biopsy remains highly individual, as well as the interpretation of the histopathologic finding.

AVAILABLE TREATMENT OPTIONS

NAFLD, a hepatic manifestation of metabolic syndrome, is emerging as the most common clinically important form of liver disease in obese patients^[9,14]. Obesity, along with insulin resistance, type 2 diabetes and dyslipidemia, is a central risk factor for NAFLD. Lifestyle modifications, which should include long-term weight management through induction of negative calorie balance (primarily reducing dietary carbohydrate and saturated fat intake), regular physical exercise with maintenance of weight loss and cognitive-behavior programs are the mainstays of the successful therapy of metabolic syndrome. Specific diet and exercise guidelines for NAFLD patients have not been established, but several types of diets have been proposed for treatment of obesity by different medical and commercial sources^[115-117]. Although the published

results of several studies in NAFLD population are mostly insufficient due to their small sample size, they have reported that short term weight loss with concomitant exercise leads to improvement in liver biochemical tests and to resolution of hepatic steatosis^[118-120]. Moderate weight loss with incorporated regular physical activity is advocated in contrast to rapid weight loss, which could aggravate the underlying liver disease. A recent study showed that weight reduction of 5 or more percent accompanied by regular exercise (at least twice a week) for one year were associated with improvement and normalization in ALT levels^[121]. Keeping this weight (< 5% gain) for two consecutive years was associated with maintaining normal ALT levels. Another recent study reported that intense dietary intervention can achieve a reduction in mean waist circumference, insulin resistance, levels of fasting glucose, triglycerides and liver function tests, as well as an improvement in liver histology in patients with NASH^[122].

Pharmacological treatment should be initiated only when there is no change in the course of disease after adequate lifestyle changes have been undertaken. The problem is that appropriate diet and physical activity, in addition to behavioral modifications, are not always successful, particularly in very obese NAFLD patients. A pilot study, which included ten obese NASH patients treated with weight-loss drug orlistat (inhibitor of pancreatic and gastric lipase) for 6 mo, showed that 10% or greater consequent body weight reduction leads to improvement in aminotransferase levels, liver steatosis and fibrosis^[123].

Bariatric surgery is considered to be the best therapeutic modality in NAFLD patients with severe obesity or with concomitant obesity-associated morbidities, like sleep apnea syndrome^[124]. There are different surgical approaches to morbidly obese patients: malabsorptive procedures, such as jejunio-ileal bypass and biliopancreatic diversions, and restrictive procedures, which include gastric bypass and gastroplasty (gastric banding). Jejunio-ileal bypass, as the more 'classical' procedure, has been virtually replaced by 'newer' procedures such as proximal gastric bypass and biliopancreatic diversion, mostly due to the high frequency of postoperative complications^[125,126]. Those included progression of liver disease, although, occasional cases of NASH progression and subacute liver failure have also been reported with 'newer' methods. Several recent trials reported that moderate or even massive weight loss with consequent mild to moderate malnutrition after gastroplasty in severe obese NASH patients resulted in improvement in diabetes mellitus, liver function tests and liver histology (inflammation and fibrosis)^[124,127-130]. Similar results were reported after gastric bypass surgery^[20]. Even though these promising results give a new perspective on treatment of severely obese NAFLD patients, the decision to perform surgery should still be individual.

The role of smoking and minimal to moderate alcohol intake in pathogenesis and potential progression of NAFLD has not been widely investigated; therefore, specific recommendations regarding those factors cannot be made.

Lipid-lowering agents

A one year pilot study, which evaluated a lipid-lowering

drug clofibrate in the treatment of 16 patients with hypertriglyceridemia and NASH, revealed no significant changes in levels of aminotransferases or liver histology^[132]. A controlled trial of a 4-wk treatment in 46 patients with NASH, using gemfibrozil 600 mg/d, resulted in a significant improvement in liver tests, although the study did not include liver biopsies^[133]. A total of 56 patients with NAFLD/NASH and hyperlipidemia was investigated in three separate studies and received 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase inhibitor atorvastatin (10-80 mg/d) for 6-12 mo, which lead to significant improvement or normalization in serum aminotransferase and lipids/cholesterol levels, as well as in the degree of inflammation, ballooning and Mallory bodies on liver histology^[134-136]. A small study using another HMG CoA reductase inhibitor pravastatin included 5 patients with biopsy proven NASH^[137]. After 6 mo of pravastatin treatment cholesterol levels were reduced, with normalization of liver function tests but without improvement in the fibrosis score.

Metformin

Metformin is an insulin-sensitizing agent that reportedly reversed hepatomegaly, steatosis and liver tests abnormalities in an animal model of fatty liver^[138]. First pilot study which investigated metformin treatment (500 mg three times daily) in patients with NASH suggested association of usage of this agent with improvement in liver tests and index of insulin sensitivity, as well as decrease in hepatic volume^[139]. A possible benefit of metformin in the induction of normalization of liver tests and loss of body weight was reported after 6 mo of therapy in a preliminary report of an open-label study of 15 patients^[140]. However, consequent results after 1 year of treatment showed no effect on aminotransferase levels, liver histology, or insulin sensitivity^[141]. Another randomized controlled study in patients with NAFLD reported that addition of metformin to a lipid and calorie-restricted diet *vs* diet alone for 6 mo lead to improvement in mean serum aminotransferase levels and insulin resistance. On the other hand, no statistically significant change in the severity of liver inflammation or fibrosis at the end of the treatment period was reported^[142]. Higher rates of aminotransferase normalization and improvement in liver histology and insulin sensitivity were associated with metformin treatment in comparison to vitamin E treatment or weight loss in an open-label randomized study of 110 non-diabetic patients^[143].

Thiazolidinediones

Thiazolidinediones (pioglitazone, rosiglitazone), peroxisome proliferator-activated receptor (PPAR) gamma ligands, are insulin-sensitizing drugs involved in glucose and lipid metabolism with suggested anti-inflammatory and anti-fibrotic properties^[144]. They are a new class of antidiabetic agents investigated recently as a potential treatment modality for patients with NAFLD/NASH. PPAR γ agonist pioglitazone prevents the activation of hepatic stellate cells *in vitro*, and improves hepatic steatosis and prevents liver fibrosis *in vivo*^[145]. In another study with animals 1 wk treatment with pioglitazone inhibited hepatic

fat accumulation and decreased levels of TNF α , while 4 wk treatment lead to improvement in hepatic fibrosis with a decrease in the expression of procollagen, α -smooth muscle actin, and TGF- β 1^[146]. In the first of prospective human studies, 48 wk of pioglitazone treatment (30 mg daily) of nondiabetic patients with biopsy-proven NASH improved the degree of insulin sensitivity with normalization of aminotransferase levels and histological improvement in hepatic steatosis, cellular injury, parenchymal inflammation, Mallory bodies, and fibrosis^[147]. MRI confirmed a marked decrease in liver fat and liver volume, but interestingly, increase in body weight and total body fat was observed. In another controlled study combination treatment with pioglitazone (30 mg daily) and vitamin E (400 IU daily) normalized aminotransferase levels, but the effect was also seen in the control group treated with vitamin E alone^[148]. However, only combination therapy induced a significant effect on histological findings (decrease in steatosis, hepatocyte ballooning, Mallory's bodies and pericellular fibrosis), with improvement in metabolic disturbances (increase in metabolic clearance of glucose and a decrease in fasting free fatty acid and insulin). A recent study proposed that improvements seen on liver histology after pioglitazone therapy may be the effect of change in adiponectin levels^[149]. In another study of rosiglitazone in 25 NASH patients of whom half had diabetes or impaired glucose tolerance, 1-year therapy was associated with improvement in liver function tests, insulin sensitivity and degree of fibrosis^[150]. Opposite to these promising results, the latest meta-analysis of 42 trials with rosiglitazone found that the drug was associated with significant increase in the risk of myocardial infarction^[151]. Considering all this, recommendations about use of thiazolidinediones in NAFLD treatment can not be yet established.

Losartan

A role for angiotensin II in pathogenesis of hepatic stellate cell activation, hepatic inflammation and fibrogenesis has been postulated^[152-156]. Given the potential therapeutic effects of angiotensin II receptor antagonists, studies with losartan were performed in patients with NASH and arterial hypertension^[157-159]. Reduction in levels of blood markers of hepatic fibrosis, transforming growth factor- β 1 (TGF- β 1) and ferritin concentration was reported, with improvement in serum aminotransferase levels. Reduction of hepatic necroinflammation, fibrosis and iron hepatocyte deposition was detected in the majority of liver biopsy specimens after 48 wk of treatment. Results suggest anti-fibrotic effect of losartan through inhibitory effect on hepatic stellate cell activation. These promising initial results justify further studies of angiotensin II receptor antagonists in treatment of NAFLD patients.

Antioxidants

Oxidative stress, along with the insulin resistance, plays one of the essential roles in NAFLD pathogenesis. Therefore, treatment with different antioxidants (vitamin E, vitamin C, betaine, iron depletion) has been studied in this group of patients. A study with vitamin E administration (300 mg/d during 1 year) confirmed improvement in liver tests

with reduction of plasma levels of TGF- β 1, but revealed no change in degree of steatosis, fibrosis and inflammation on follow-up liver biopsy^[160]. A placebo-controlled trial of a combination of vitamin E (1000 IU/daily) and vitamin C (1000 mg/d) during 6 mo showed no statistically significant difference between vitamin and placebo groups in liver enzyme levels, degree of steatosis and inflammatory activity, or fibrosis score, although an improvement in fibrosis was noted in the vitamin group^[161]. Given the suggested protective effect of betaine against steatosis in rats (through increase of S-adenosylmethionine levels), a pilot study was performed in 8 patients with NASH^[162]. After one year treatment with betaine (20 mg daily), a significant improvement or normalization in serum aminotransferase levels and liver histology was noted. Another placebo-controlled study included 191 patients with NAFLD treated for 8 wk with combination of betaine glucuronate (300 mg/d), diethanolamine and nicotinamide ascorbate^[163]. Significant improvement was noted in liver tests and degree of steatosis (evaluated by ultrasonography) in the treated group. Further controlled trials are needed for confirmation of these promising results. It has been hypothesized that iron induces oxidative stress by catalyzing production of ROS (reactive oxygen species) and increased hepatic iron deposition could be a part of the pathogenesis of NAFLD^[164]. In a few pilot studies in NASH patients, quantitative phlebotomies were performed with induction of iron depletion and reduction in serum ferritin levels^[165-167]. Results of these studies suggest that iron reduction therapy by phlebotomy can lead to improvement in aminotransferase levels; unfortunately, follow-up liver biopsies were not performed.

Ursodeoxycholic acid

Ursodeoxycholic acid (UDCA) could potentially act as a hepatoprotective agent by minimizing toxicity of hydrophobic bile acids and leading to a decrease in oxidative stress and hepatocyte injury. Initial small pilot-studies evaluated therapeutic effect of UDCA alone or in combination with low-fat diet or lipid-lowering agents in patients with NASH^[132,135,168-170]. Results suggested potential benefit of UDCA treatment with reported normalization/improvement in liver function tests, degree of hepatic steatosis and serum markers of fibrosis. However, consequent larger, randomized double-blinded placebo-controlled trial found no detectable benefit of UDCA treatment: similar degree of improvement in biochemistry and histology was detected in both study groups^[171]. Given the lack of effectiveness of UDCA alone as a therapeutic option in NASH, one recent study considered combination of UDCA with vitamin E. Significantly diminished levels of serum aminotransferases were observed, with improvement in histological activity index, mostly as a result of regression of steatosis^[172].

CONCLUSION

Non-alcoholic fatty liver disease is currently the object of significant scientific and clinical interest, and is to remain so in the following years. Larger studies with firm inferences are rather scarce, and their small number

reflects the difficulties in setting-up and performing clinical trials in NAFLD. Among the most important obstacles that researchers are confronted with are slowly progressive nature of the disease requiring long-term follow-up, variability in liver biopsy specimens and their interpretation, various associated conditions and multiple medication use that are common in these patients. Although clinicians dispose in theory with a wide array of possible therapies, few have been shown to have consistent effects and can therefore be firmly recommended in treatment of NAFLD.

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Capsule endoscopy: Current status in obscure gastrointestinal bleeding

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Abstract

Capsule endoscopy (CE) is a safe, non invasive diagnostic modality for the evaluation of small bowel lesions. Obscure gastrointestinal bleeding (OGIB) is one of the most important indications of capsule endoscopy. Capsule endoscopy has a very high diagnostic yield especially if the bleeding is ongoing. This technique appears to be superior to other techniques for the detection of suspected lesions and the source of bleeding. Capsule endoscopy has been shown to change the outcome in patients with obscure gastrointestinal (GI) bleed.

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Key words: Capsule endoscopy; Obscure gastrointestinal bleeding; Luminal endoscopy; Diagnostic yield; Small bowel study

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INTRODUCTION

Visualization of the small bowel presents a great challenge to the practicing physician and is considered the final frontier in luminal endoscopy. Newer technologies are constantly being developed towards the goal of better, safer, and complete evaluation of the small intestine. Capsule endoscopy (CE) is a major technological advance in this direction. Its non invasive nature, safety profile, capability of imaging the entire small bowel and its ability to store images makes CE the investigation of choice for the evaluation of small bowel lesions. Obscure gastrointestinal bleeding (OGIB) both in the overt and

occult forms is one of the most important indications for CE^[1]. The widespread acceptability and utility of CE in OGIB is evident from the fact that the number of publications in peer reviewed journals have steadily increased since the first publication in 2000^[2]. The present article attempts to review the progress made in the last decade with special emphasis on the use of capsule endoscopy in obscure gastrointestinal (GI) bleeding.

Diagnostic yield of CE

Evaluation of the current status of CE in OGIB requires assessment of the efficacy and accuracy of the procedure. The diagnostic yield of CE in OGIB is extremely variable^[3-9], from > 70% in small studies (< 20 patients) to < 60% in larger studies (> 50 patients). In reality, the overall positive diagnostic yield of CE in OGIB is around 50%. Subgroup analysis shows that the diagnostic yield is much higher, reaching 92.3% in patients with ongoing overt GI bleeding compared with 44.2% in obscure occult bleeding and 12.9% in past OGIB. Current data suggests that the timing of the procedure is very important in optimizing the yield of CE in OGIB^[10]. The ICCE consensus meeting on OGIB recommended that CE should be performed early (preferably within 2 wk) in the workup of patients with OGIB^[11].

Comparison of CE with other modalities

The second issue is whether CE is superior to other diagnostic tests in the evaluation of OGIB^[12-16]. A meta-analysis compared CE with other modalities in patients with OGIB^[17], and showed that the diagnostic yield of CE was superior to push enteroscopy, small bowel radiography, CT enteroclysis, mesenteric angiography and small bowel MRI. Recently, several studies have compared CE with Double Balloon Enteroscopy (DBE). The detection rate of potential bleeding source was significantly better with CE than DBE^[18]. However these two procedures should be considered complimentary and not competitive. The usefulness of repeat CE in OGIB has been reported. In a retrospective study, repeat CE in patients with OGIB showed additional findings in 75% patients^[19]. However this data needs further validation with prospective studies.

The role of CE in iron deficiency anaemia

The role of CE in the evaluation of iron deficiency anaemia is still evolving. In two recent studies, the yield of CE in iron deficiency anemia using strict diagnostic criteria varied from 57% to 80%^[20,21]. These results

are encouraging and suggest a definite role of CE in documented iron deficiency anaemia.

Impact of CE on clinical outcome

Although several studies have assessed the yield of CE in OGIB, the exact significance of the lesions identified and their impact on clinical outcome has not been adequately examined. When we consider outcome in clinical practice, the emphasis should be on meaningful and positive results. In the case of OGIB, a positive outcome should either be stoppage of bleeding or resolution of anemia. The majority of studies on CE in OGIB discuss change in management rather than a change in the outcome. Pennazio *et al* determined the outcome in 56 patients, with a mean follow up of 18 mo. Complete resolution of bleeding was seen in 86.9% patients with ongoing overt GIB, 69.2% in occult OGIB and 41.4% in past OGIB. Other studies have assessed the change in clinical decision making after CE, with figures varying from 22% to 88% in patients with OGIB. In a multicentre study, Alberts *et al* assessed the impact of CE on clinical outcomes based on 247 capsule studies^[22]. A specific intervention or change of management was implemented in about 2/3rd of the patients who had a definite diagnosis on CE. In another recent study, 70% patients underwent definitive treatment based on CE results. On the other hand, Rastogi *et al* reported a positive clinical outcome in only 16% patients.

It is difficult to draw definite conclusions from these conflicting results. The variations in outcome may be explained by the differences in study population, the lack of a standardized approach to management, and different policies at different medical centers. However there is no doubt that CE plays a definite role in planning the management of patients with OGIB.

Cost effectiveness of CE

Not many studies have addressed the issue of cost effectiveness of CE in OGIB. One study examined the cost effectiveness of several approaches including initial DBE, CE followed by DBE if a lesion was detected, push-enteroscopy, intraoperative-enteroscopy, angiography and no treatment for the diagnosis and management of small bowel angiectasia, in patients with transfusion dependent obscure/occult bleeding. DBE was found to be the most cost effective strategy; however CE followed by DBE was more cost effective if the probability of angiectasia at DBE was less than 59%^[23]. Prospective clinical studies are needed to clarify as to when to use DBE or CE as the initial study.

CONCLUSION

Despite its limitation of being a purely diagnostic modality, CE is an important tool in the evaluation of OGIB. The technology is improving at a fast pace. The development of new software has reduced considerably the reading time of CE images. Many new capsule endoscopes are under development. CE is clearly a giant technological leap in GI endoscopy. In the near future, the technological qualities of capsule endoscope are likely to improve. A capsule

endoscope capable of not only localizing, but also treating a suspected lesion is a distinct possibility.

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LIVER CANCER

Expression of PTEN, PPM1A and P-Smad2 in hepatocellular carcinomas and adjacent liver tissues

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Abstract

AIM: To investigate the expressions of PTEN, PPM1A and P-Smad2 in hepatocellular carcinoma (HCC) and their significance.

METHODS: The expressions of PTEN, PPM1A and P-Smad2 in 31 HCC tissues, 25 adjacent liver tissues and 13 non-tumor liver tissues were detected by using Envision immunohistochemical technique.

RESULTS: The positive expression (64.52%) and staining intensity (4.19 ± 3.31) of PTEN in the cytoplasm of HCC were significantly lower and weaker than those in the adjacent or non-tumor liver tissues (97.37%, 7.88 ± 0.93 ; 100%, 7.77 ± 0.93 , respectively) ($P < 0.05$), and its staining intensity in the cytoplasm of HCC, which belongs to Edmondson pathologic grades II-III and above, was also lower than that of grade I and I-II. Furthermore, its location in the nucleus or cytoplasm of liver cells was negatively correlated with the progression of liver disease ($r = -0.339$, $P = 0.002$); most of PPM1A might be only expressed in the nucleus of adjacent liver tissues, non-HCC tissues or Edmondson grade I and I-II HCC, but it was mainly expressed in the cytoplasm of HCC with Edmondson grade \geq II, weakly or negatively expressed in the nucleus ($P < 0.05$), and its location was negatively correlated with the progression of liver disease ($r = -0.45$, $P = 0.0000$). P-Smad2, which was

mostly located in the nucleus and cytoplasm of grade I and I-II HCC, surrounding or non-tumor liver tissues, was only in the nucleus of HCC with Edmondson grade II and above ($P < 0.001$), and its location was positively correlated with the disease progression ($r = 0.224$, $P = 0.016$). Spearman correlation analysis revealed that P-Smad2 was significantly negatively correlated with PTEN and PPM1A ($r = -0.748$, $P = 0.000$; $r = -0.366$, $P = 0.001$, respectively); and PTEN and PPM1A were positively correlated with HCC carcinogenesis ($r = 0.428$, $P = 0.000$).

CONCLUSION: The aberrant location of expression and staining intensity of PTEN, PPM1A and P-Smad2 in HCC and their relationship might have an impact on the pathogenesis of HCC.

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Key words: Hepatocellular carcinoma; Phosphorylated Smad2; PTEN; PPM1A

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the 5th most common solid tumor worldwide and accounts for about 110 000 deaths each year in China, with the 2nd most common cause of mortality among all malignant tumors^[1]. The pathogenesis of HCC is not clearly elucidated so far; hence, the exploration of the key genes and pathways involved in hepatocarcinogenesis and novel therapeutics strategies is very crucial. Many data indicated that TGF- β /Smads pathway significantly acted on the pathogenesis of HCC^[2,3]. Recent studies showed persistent accumulation of phosphorylated Smads (P-Smads) in the nucleus of HCC may be associated with the development of HCC. But its dephosphorylation regulation remains unknown^[4]. Lin *et al*^[5] identified PPM1A as a protein serine/threonine phosphatase which can dephosphorylate and promote nuclear export of TGF- β -activated Smad2/3, and implied that dual specific phosphatases (DUSPs) also catalyze dephosphorylation of pS/Ts. Phosphatase and tensin

homolog deleted on chromosome ten (PTEN) is the first tumor suppressor gene which can encode a dual specificity phosphatase (protein phosphatase and lipid phosphatase), its deletion or mutation may impact on the development and prognosis of HCC and other tumors^[6-8]. In the present study, we investigated the expressions of PTEN, PPM1A and P-Smad2 in HCC with different Edmondson pathological grades by using immunohistochemical technique and analyzed their possible relationship associated with the pathogenesis of HCC.

MATERIALS AND METHODS

Materials

This study included 31 HCC and 25 corresponding paracancerous tissues and 13 non-tumor liver tissues (5 liver cirrhosis, 3 hepatitis and 5 normal liver tissues) obtained from Tongji Hospital from 1997 to 1998 and 2003 to 2004. Among 31 HCC cases, 27 were males and 4 were females, with age ranged from 24-78 (mean, 43.45 ± 10.77) years. None of the cases had distant metastases and received any chemotherapy and radiotherapy. According to Edmondson Grading System, 10 cases belonged to grade I and I-II, 7 cases grade II and 14 cases > grade II. In addition, among 25 cases of paracancerous liver tissues, 11 cases had intrahepatic vascular embolism and 14 cases had not. All tissue samples were fixed in formalin and imbedded in paraffin, and cut into 4- μ m thick sections.

Antibodies

Rabbit anti-phosphorylated Smad2 (P-Smad2) monoclonal antibody (1:100 dilution), mouse anti-PPM1A monoclonal antibody (1:25 dilution) and rabbit anti-human PTEN polyclonal antibody (1:400 dilution) were purchased from CellSignaling Technology Company, Abcam and Zhongshan Biotechnology Company, Beijing. DAKO Envision kit was purchased from DAKO Company.

Immunohistochemistry

Immunohistochemical staining was performed by using DAKO Envision (DAKO, Carpinteria, CA) kit according to the manufacturer's instructions. Briefly, each section was deparaffinized, rehydrated and incubated with fresh 3 mL/L hydrogen peroxide in methanol for 30 min at room temperature and then washed in phosphate-buffered saline (PBS). Sections were incubated with normal goat serum for 30 min to block the nonspecific antibody binding. Sections were then incubated with primary antibody against P-Smad2 or PTEN or PPM1A overnight at 4°C, washed three times in PBS, followed by incubation with respective secondary antibody for 30 min at room temperature. Then 3, 3'-diaminobenzidine tetrachloride (DAB) was used for color development and the sections were counterstained with hematoxylin. Primary antibody was substituted by PBS for negative control.

Evaluation of PTEN, PPM1A and P-Smad2 expression

The degree of staining was categorized by the extent and intensity of the staining; the staining results were assessed according to Detre *et al*^[9]. Briefly, the cells staining positively throughout the section were assigned

scores from 1 to 6 as follows: 1 = 0%-4%; 2 = 5%-19%; 3 = 20%-39%; 4 = 40%-59%; 5 = 60%-79%; and 6 = 80%-100%. The average intensity, corresponding to the presence of negative, weak, intermediate, and strong staining, was given a score from 0 to 3, respectively. The positive cell score was added to the average intensity to form an additive quickscore. A positive cut-off quick score was ≥ 3 .

Statistical analysis

Data analyses were performed with the SPSS (version 12.0) statistical package on a personal computer. Significant differences between variables were assessed by Student's *t* test, Chi-square test or Fisher's exact probability test, when appropriate. The correlation between factors was evaluated by using Spearman rank correlation coefficient test. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Expression of PTEN protein in HCC, paracancerous and non-tumor liver tissues

The expression of PTEN was localized generally in the nucleus, cytoplasm and cell membrane of liver cells (Figure 1), but its distribution and staining intensity in HCC, paracancerous or non-tumor liver tissues (including normal liver tissues, hepatitis tissue and cirrhotic liver tissues) were different. As shown in Table 1, 20 of 31 (64.52%) HCCs tissues were positive for PTEN, mainly expressed in the cytoplasm and cell membrane. PTEN expression was strongly positive in 13 of 17 HCCs with Edmondson grade I and I-II, but weakly positive in 8 of 14 HCC with grade II-III and above. Furthermore, PTEN was stained strongly in the cytoplasm, nucleus and/or cell membrane of 24 of 25 paracancerous and 13 non-tumor liver tissues compared with HCC tissues ($P < 0.05$).

In addition, compared with the group without intrahepatic vascular embolism (85.71%, 12/14), PTEN was weakly positive in HCC with intrahepatic vascular embolism (45.45%, 5/11). Spearman rank correlation analysis showed that PTEN expression was negatively correlated with intrahepatic vascular embolism ($r = -0.428$, $P = 0.033$).

Expression of PPM1A in HCC, paracancerous and non-tumor liver tissues

We found that PPM1A staining was mainly localized in the nucleus and cytoplasm of different type liver tissues (Figure 2), but its distribution and staining intensity were different. PPM1A was strongly expressed in the nucleus of non-tumor liver tissues, paracancerous liver tissues and grade I and I-II HCC, with weak or negative expression in the cytoplasm; whereas strongly expressed in the cytoplasm of HCC with grades II-III and above, with weak expression in the nucleus, thereby showing a significant difference in distribution and staining intensity ($P < 0.001$). However, Spearman rank correlation analysis showed that the location and staining intensity of PPM1A in HCC were not correlated with intrahepatic vascular embolism ($r = -0.038$, $P = 0.821$) (Table 2).

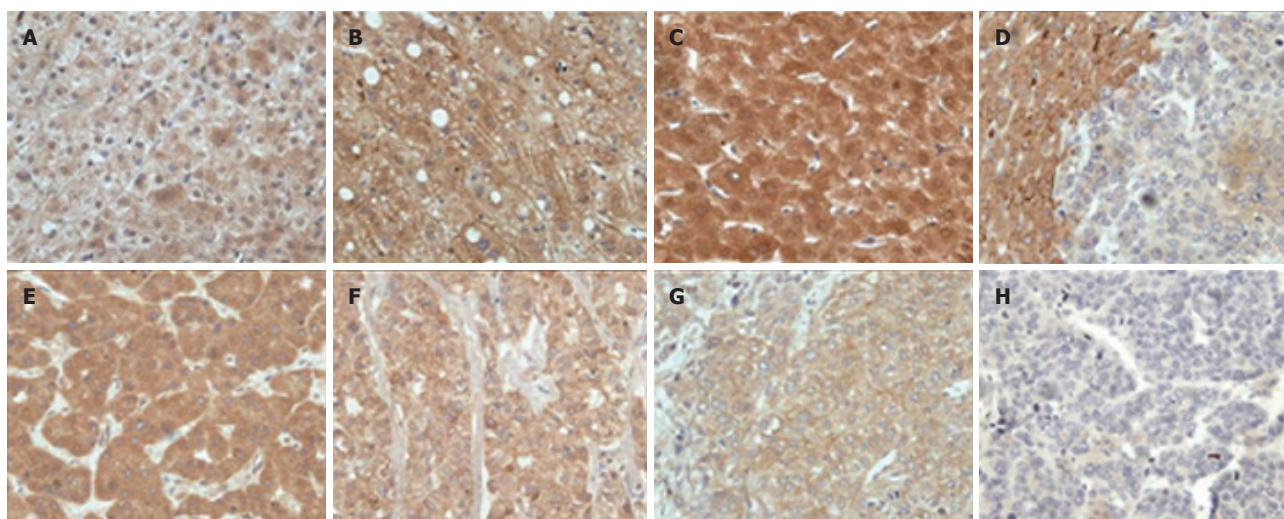


Figure 1 Expression of PTEN in hepatocellular carcinomas, paracancerous tissues and non-tumor liver tissues (Envision, x 200). **A:** Expression of PTEN in the nucleus, cytoplasm and membrane of normal liver tissues; **B:** Expression of PTEN in the cytoplasm and membrane of hepatitis tissue; **C:** Expression of PTEN in the nucleus, cytoplasm and membrane of cirrhotic liver tissues; **D:** Expression of PTEN weakly in the cytoplasm of HCC, strongly in the cytoplasm and membrane of paracancerous tissues; **E:** Strong expression of PTEN in the cytoplasm of HCC with Edmondson grade I; **F:** Expression of PTEN in the cytoplasm and membrane of HCC with grade II; **G:** Weak expression of PTEN in the cytoplasm and membrane of HCC with Edmondson grade II-III; **H:** Negative expression of PTEN in HCC with Edmondson grade III-IV.

Table 1 Expression of PTEN in hepatocellular carcinomas, paracancerous tissues and non-tumor liver tissues

Parameters	Cases (n)	Positive cases	Mean score of PTEN staining (mean \pm SD)		
			Nucleus	Cytoplasm	Cell membrane
HCC (Edmondson grading system)	31	20 ^{a,c}	0 ^{a,b,c}	4.19 \pm 3.31 ^{a,b,c}	4.77 \pm 3.92
Grade I and I-II	10	8	0	5.80 \pm 3.12 ^e	5.30 \pm 3.68
Grade II	7	4	0	4.14 \pm 3.89	4.86 \pm 4.56
Grade II-III and above	14	8	0	3.07 \pm 2.87 ^e	4.36 \pm 4.01
Paracancerous tissue	25	24	3.88 \pm 3.14 ^{a,b}	7.88 \pm 0.93 ^{a,b,d}	5.16 \pm 3.69 ^d
Non-tumor liver tissue	13	13	5.79 \pm 2.31 ^{b,c}	7.77 \pm 0.93 ^{b,c,d}	3.92 \pm 3.35 ^d
Cirrhotic liver tissue	5	5	7.40 \pm 1.14	8.40 \pm 0.55	4.40 \pm 4.10
Hepatitis tissue	3	3	4.33 \pm 3.79	8.00 \pm 1.00	4.33 \pm 3.79
Normal liver tissue	5	5	5.0 \pm 1.41	7.00 \pm 0.71	3.20 \pm 2.95
Intrahepatic vascular embolism					
Present	11	5	0 ^b	3.00 \pm 3.46 ^{b,e}	3.27 \pm 3.79
Absent	14	12	0 ^b	6.28 \pm 2.37 ^{b,e}	6.71 \pm 2.89

^a $P < 0.05$ HCC vs paracancerous tissue; ^b $P < 0.01$ nucleus vs cytoplasm group; ^c $P < 0.05$ HCC vs non-tumor liver tissue; ^d $P < 0.05$, inter-group comparison; ^e $P < 0.001$ cytoplasm vs cell membrane.

Expression for P-Smad2 in HCC, paracancerous and non-tumor liver tissues

P-Smad2 staining was mostly localized in the nucleus and its surrounding cytoplasm (Figure 3), with different pattern of its distribution in different types of liver tissues (Table 3). P-Smad2 was expressed in the nucleus and the surrounding cytoplasm of non-tumor liver tissues, paracancerous liver tissues and HCC $<$ grade II, and only in the nucleus of \geq grade II HCC. Although its staining intensity in the nucleus was not significantly different between HCC and paracancerous tissues, P-Smad2 expression was stronger in the nucleus and weaker in the cytoplasm of HCC than those of non-tumor liver tissues ($P < 0.05$). In addition, the expression of P-Smad2 in HCC was not associated with intrahepatic vascular embolism ($r = 0.052$, $P = 0.775$).

Relationship among PTEN, PPM1A and P-Smad2 in HCC

The expressions of PTEN, PPM1A and P-Smad2 in

HCC were not related with age and gender, but might be associated with progression of liver disease. With liver pathology developing from non-tumor liver tissues, adjacent cancerous tissues to HCC, the location of PTEN shifted from the nucleus to the cytoplasm or cell membrane, even some negative staining. Most of PPM1A also shifted from the nucleus to the cytoplasm. But P-Smad2 was translocated from the nucleus and cytoplasm to the nucleus accumulation. Spearman rank correlation analysis suggested that the staining location of PTEN and PPM1A in liver cells was negatively correlated with progression of liver disease ($r = -0.339$, $P = 0.002$; $r = -0.45$, $P = 0.0000$, respectively), but P-Smad2 was positively correlated ($r = 0.228$, $P = 0.015$) (Table 4). As shown in Table 5, with progression of tumor Edmondson grades, the expression of PPM1A in the nucleus was decreased ($r = -0.322$, $P = 0.029$), but P-Smad2, in more cases, was accumulated in the nucleus ($r = 0.459$, $P = 0.003$), implying that although the location of PTEN was

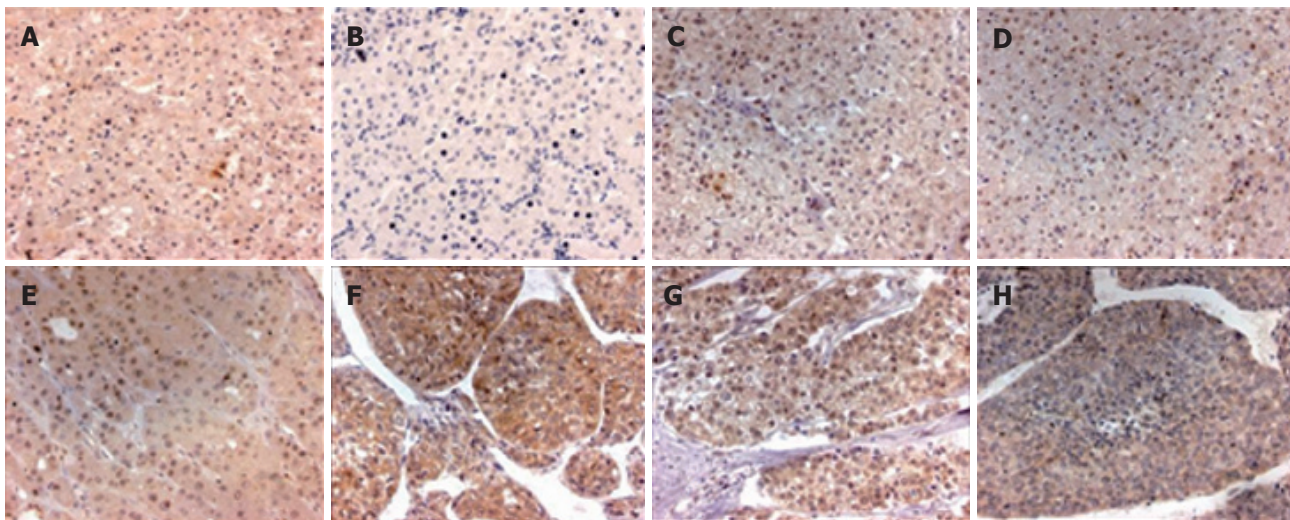


Figure 2 Expression of PPM1A in hepatocellular carcinomas, paracancerous tissues and non-tumor liver tissues (Envision, x 200). **A-E**: Expression of PPM1A in the nucleus of normal liver tissues, hepatitis tissues, cirrhotic liver tissues, paracancerous tissues and Edmondson grade I HCC, respectively; **F**: Expression of PPM1A in the nucleus and cytoplasm of grade II HCC; **G**: Weak expression of PPM1A in the nucleus and cytoplasm of grade II-III HCC, mainly in the cytoplasm; and **H**: Weak expression of PPM1A in the cytoplasm of grade III-IV HCC.

Table 2 Expression of PPM1A in hepatocellular carcinomas, para-cancerous tissues and non-tumor liver tissues

Parameters	Cases (n)	Mean score of PPM1A staining (mean \pm SD)	
		Nucleus	Cytoplasm
HCC (Edmondson grades)	31	3.45 \pm 3.00 ^{a,b,c}	6.32 \pm 3.00 ^{a,b,c}
Grade I and I - II	10	5.60 \pm 2.54 ^e	3.90 \pm 4.14 ^e
Grade II	7	3.57 \pm 2.15 ^b	7.86 \pm 0.69
Grade II-III and above	14	1.86 \pm 2.79 ^{d,b}	7.28 \pm 1.38 ^{b,e}
Paracancerous tissue	25	6.28 \pm 1.95 ^{a,b}	2.08 \pm 3.45 ^{a,b}
Non-tumor liver tissue	13	5.38 \pm 1.45 ^{b,e}	0 ^{c,b}
Liver cirrhosis	5	6.40 \pm 0.55	0
Hepatitis	3	3.33 \pm 0.58	0
Normal liver tissue	5	5.60 \pm 1.14	0
Intrahepatic vascular embolism			
Present	11	3.82 \pm 3.22	6.09 \pm 3.11
Absent	14	3.71 \pm 3.05	6.14 \pm 3.44

^a $P < 0.05$ HCC vs paracancerous tissue; ^b $P < 0.01$ nucleus vs cytoplasm; ^c $P < 0.05$ HCC vs non-tumor liver tissue; ^e $P < 0.05$, inter-group comparison.

positively correlated with that of PPM1A in HCC ($r = 0.428$, $P = 0.000$), the expression and location of P-Smad2 were significantly negatively correlated with those of PTEN and PPM1A ($r = -0.748$, $P = 0.000$; $r = -0.366$, $P = 0.001$, respectively).

DISCUSSION

Phosphatase and tensin homolog deleted on chromosome ten (PTEN) was discovered as a tumor suppressor gene in 1997. It could regulate normal cell growth by negatively regulating the phosphatidylinositol 3-kinase (PI3K) signaling pathway which is an important driver of cell proliferation and cell survival, and its deletion, mutation or otherwise inactivation was frequently correlated with the development and progression of many human tumors^[6,7,10]. In this study, we detected the expression of PTEN in 31 HCCs, 25 paracancerous tissues and 13 non-tumor liver tissues by using immunohistochemical technique. Our results showed that the positive rates of PTEN in HCC, paracancerous and non-tumor liver tissues were

61.29%, 97.37% and 100%, respectively. Furthermore, the staining intensity of PTEN in the cytoplasm of HCC was significantly weaker than that of paracancerous and non-tumor liver tissues. In addition, the staining intensity of PTEN in the cytoplasm of HCC with grade \geq II-III was weaker compared to HCC with grade I and I - II. Also, PTEN expression in HCC was correlated with intrahepatic vascular embolism. These results are in agreement with previous studies^[8,11] and imply that the expression and intensity of PTEN maybe related to the malignant and invasive potential of HCC, and its deletion and weak expression would result in tumorigenesis and development of HCC.

In this study, we also observed that PTEN was localized in the nucleus, cytoplasm or cell membrane of non-tumor or paracancerous liver tissues, but mainly localized in the cytoplasm or cell membrane of HCC, and the nuclei were almost negative, suggesting that its location was closely correlated with the progression of liver disease. However, whether this phenomenon is connected with the role of PTEN in tumor suppression needs to be further studied.

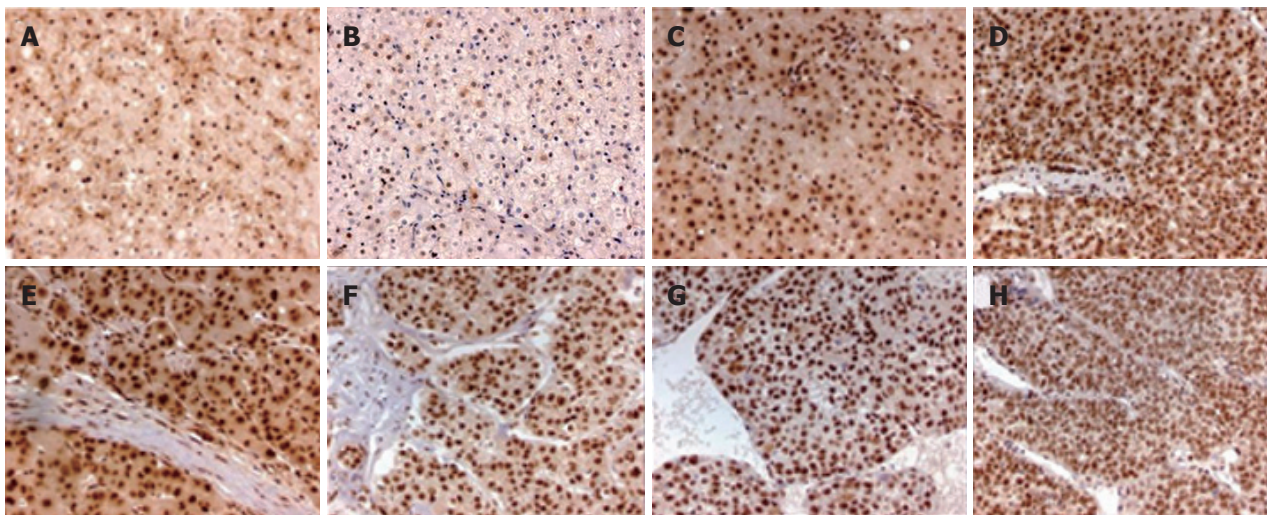


Figure 3 Expression of P-Smad2 in hepatocellular carcinomas, paracancerous tissues and non-tumor liver tissues (Envision, x 200). **A-E**: Expression of P-Smad2 in the nucleus and cytoplasm of normal liver tissues, hepatitis tissues, cirrhotic liver tissues, paracancerous tissues and Edmondson grade I HCC, respectively; **F-H**: Expression of P-Smad2 in the nucleus of Edmondson grade II, II-III and III-IV HCC, respectively.

Table 3 Expression of P-Smad2 in hepatocellular carcinomas, paracancerous tissues and non-tumor liver tissues

Parameters	Cases (n)	Mean score of P-Smad2 staining (mean \pm SD)	
		Nucleus	Cytoplasm
HCC (Edmondson grades)	31	8.61 \pm 0.68 ^{b,d}	1.74 \pm 3.08 ^{a,b,d}
Grade I and I - II	10	8.70 \pm 0.48	4.90 \pm 3.54 ^c
Grade II	7	7.85 \pm 1.07	0.71 \pm 1.89 ^c
Grade II-III and above	14	8.79 \pm 0.58	0 ^d
Paracancerous tissue	25	8.28 \pm 0.74 ^b	7.28 \pm 1.21 ^{a,b}
Non-tumor liver tissue	13	7.08 \pm 1.61 ^{b,d}	5.31 \pm 2.84 ^{b,d}
Cirrhosis tissue	5	8.40 \pm 0.55 ^c	7.40 \pm 1.81
Hepatitis tissue	3	5.33 \pm 0.58 ^c	1.33 \pm 2.31
Normal liver tissue	5	6.80 \pm 1.64	5.60 \pm 0.89
Intrahepatic vascular embolism			
Present	11	8.82 \pm 0.40	2.36 \pm 3.35
Absent	14	8.43 \pm 0.76	2.00 \pm 3.37

^a $P < 0.05$ HCC vs paracancerous tissue; ^b $P < 0.005$ nucleus vs cytoplasm; ^d $P < 0.001$ HCC vs non-tumor liver tissue; ^c $P < 0.05$, inter-group comparison.

Table 4 Relationship of PTEN, PPM1A and P-Smad2 with HCC, paracancerous and non-tumor liver tissues

Types	Cases (n)	PTEN		PPM1A		P-Smad2	
		Cytoplasm	Nucleus	Cytoplasm	Nucleus	Cytoplasm	Nucleus
HCC	31	20	0	26	20	8	31
Paracancerous tissue	25	24	17	7	25	25	25
Non-tumor liver tissue	13	13	12	0	13	11	13

The location of expression of PTEN vs PPM1A vs P-Smad2 in the cytoplasm or nucleus of different liver diseases was significantly different ($P < 0.001$).

PPM1A is a member of the PP2C family of Ser/Thr protein phosphatases which are known to be negative regulators of cell stress response pathways, such as p38 and JNK kinase cascades. This phosphatase can also dephosphorylate cyclin-dependent kinases, thus may be involved in cell cycle control. Over-expression of the phosphatase is reported to activate the expression of the tumor suppressor gene *TP53/p53*, which leads to G2/M cell cycle arrest and apoptosis^[12]. In this experiment, we examined the expression of PPM1A in HCC, paracancerous liver tissues and non-tumor liver tissues. The results showed

that PPM1A was located in the nucleus and cytoplasm of liver cells. With the progression of liver disease, most of PPM1A shifted from the nucleus to the cytoplasm and the staining intensity was obviously different, suggesting that its expression location was negatively correlated with liver pathological type ($r = -0.45$, $P = 0.0000$) and implying that the location shift of PPM1A may have impact on the carcinogenesis and progression of HCC.

TGF- β /Smads signaling is an important regulator of cell growth pathway, which modulates diverse cellular processes including cell proliferation, differentiation,

Table 5 Relationship of PPM1A and P-Smad2 with different Edmondson grades of HCC

Pathological type	Cases (n)	PPM1A		P-Smad2	
		Cytoplasm	Nucleus	Cytoplasm	Nucleus
HCC (Edmondson grade)	31	26	20	8	31
Grade I and I - II	10	5	9	7	10
Grade II	7	7	6	1	7
Grade II-III and above	14	14	5	0	14

The location expression of PPM1A vs P-Smad2 in the cytoplasm or nucleus of HCC of different Edmondson grades was significantly different ($P < 0.05$).

adhesion, extracellular matrix remodeling, apoptosis and immunomodulation. Smad2 is an essential intracellular transducer for TGF- β signals. In response to TGF- β stimulation, it is phosphorylated by TGF- β I receptor and forms a complex with Smad3, Smad4 and transported into the nucleus, where Smads cooperates with specific DNA-binding transcription factor CRB/P300 and activates TGF- β signals to regulate gene transcription, inhibit cell growth and promote apoptosis. Xu *et al*^[13] reported that Smad2 phosphorylation or dephosphorylation would play a key role in regulation TGF- β signaling^[13]. A recent study showed that phosphorylated R-Smad2 was accumulated in the nucleus of hepatoma cell which might be correlated with hepatocellular tumorigenesis and development^[4]. In this study, we found phosphorylated Smad2 was strongly expressed in the nucleus and cytoplasm of non-tumor liver tissues, paracancerous liver tissues and HCC with Edmondson grade I and I - II. However, P-Smad2 was only located in the nucleus of HCC \geq grade II, implying that accumulation of P-Smad2 in the nucleus was closely correlated with liver pathological type and progression of hepatocarcinogenesis and may play an important role in HCC development.

Spearman rank correlation analysis results suggested that the expression location of PTEN and PPM1A in the nucleus or cytoplasm of HCC was negatively correlated with P-Smad2. Lin *et al*^[5] demonstrated that PPM1A/PP2C can dephosphorylate the SXS motif of Smads, and nucleus-to-cytoplasm shift of PPM1A would result in P-Smad2 accumulation in the nucleus and TGF- β signaling termination^[5]. Hence, we presumed that PTEN, which is dual specific phosphatases (DUSPs), may also catalyze dephosphorylation of pS/Ts of Smad2 in the nucleus or cooperate with PPM1A, and its nucleus-to-cytoplasm shift, or weak or negative expression would block TGF- β signaling, deregulate hepatocellular growth and promote tumorigenesis and development of HCC. However, further studies are needed to clarify it.

In conclusion, deletion or weak expression or nucleocytoplasmic shift of PTEN and PPM1A and accumulation of P-Smad2 in the nucleus of hepatoma cells might be associated with the tumorigenesis and progression of primary hepatocellular carcinoma.

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VIRAL HEPATITIS

Different natural courses of chronic hepatitis B with genotypes B and C after the fourth decade of life

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comparable in patients infected with genotypes B and C when CH-B occurred at < 30 years old. However, CH-B patients infected with genotype C showed poor prognosis if they were 30-49 years old and were positive for HBeAg. Age-specific natural course of CH-B should be considered when patients with CH-B are treated with antiviral drugs.

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Key words: Hepatitis B, chronic; Hepatitis B virus; Hepatitis B e antigen; Genotypes; Molecular epidemiology

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Abstract

AIM: To investigate the different impact of genotypes B and C on the development of liver cirrhosis (LC) among different age groups of patients with chronic hepatitis B (CH-B).

METHODS: We examined the outcome of 121 patients with CH-B, divided by age and genotype. Univariate analyses were used to compare different groups. The Cox proportional hazard model was employed to evaluate factors affecting the development of LC.

RESULTS: In patients < 30 years old, there were no significant predictors for development of LC. However, in patients ≥ 30 years old, genotype C was the only significant predictor. In the genotype C group, 8 of 12 patients who progressed to LC were 30-49 years old at initial diagnosis of chronic hepatitis (7 patients were positive for HBeAg). In the genotype B group, 4 of 8 patients who developed LC were ≥ 50 years old at initial diagnosis and were HBeAg-negative.

CONCLUSION: The rate of development of LC was

INTRODUCTION

Hepatitis B virus (HBV) is a double-stranded DNA virus. Chronic HBV infection is associated with different forms of liver diseases, encompassing inactive carrier, chronic hepatitis (CH), liver cirrhosis (LC) and hepatocellular carcinoma (HCC)^[1].

It has been recently reported that HBV genotypes influence disease severity and clinical outcome of HBV infection^[2-6]. HBV genotypes are classified into 8 groups, from A to H, based on a sequence difference greater than 8%, and are variously distributed in the world^[2,5,7-14]. In Japan, genotypes B and C are predominant, as in other Asian countries, but > 90% of chronic carriers have genotype C^[3,5,8,15].

We previously reported that HBeAg seroconversion rate in genotype B was significantly higher compared with genotype C^[6]. Moreover, it has been reported that progression to LC is more frequently seen in genotype C patients than in genotype B patients^[2-6]. Therefore, it seems that infection with genotype C is associated with a poor prognosis.

However, our previous study^[16] and another study^[17] showed that association of genotype C with advanced

liver disease was not seen in patients over 50 years old. Therefore, the influence of HBV genotype on the prognosis of CH may vary depending on the age groups.

The aim of the present study was to investigate the different impact of HBV genotypes B and C on the development of LC among patients with chronic hepatitis B (CH-B) belonging to different age groups.

MATERIALS AND METHODS

From January 1977 to January 2005, we enrolled 145 consecutive patients with CH-B who were admitted to our institute and related facilities followed for more than 6 mo, i.e. for 7 to 207 mo (mean \pm SD: 79.8 \pm 48.4). In 124 patients, diagnosis of CH was made by liver biopsy after written informed consent, and histological classification was carried out according to standard criteria^[18]. The degree of hepatic inflammation and the stage of fibrosis were scored by a modified Knodell histological index^[19]. The inflammation score (grading) was obtained by combining the scores for the first three components of the index: portal, periportal, and lobular inflammation. The score could range from 0 to 18. The degree of inflammation was graded as 4 to 8, mild; 9 to 18, moderate to severe. The fibrosis score (staging) was graded as 0 to 1, no-mild; 2 to 3, moderate-severe. In the remaining 21 patients, diagnosis of CH was made clinically and was characterized by persistent alanine aminotransferase (ALT) elevation over for at least 6 mo and by ultrasound findings using a cirrhosis score (< 8 points)^[20]. Diagnosis of LC was made mainly by clinical assessment including serial determination of serum ALT levels, platelet count, ultrasound examination (using the above mentioned cirrhosis score, ≥ 8 points)^[20] and computed tomography. For the diagnosis of HCC, we added the measurement of serum alpha-fetoprotein to the above diagnostic criteria for LC. Patients positive for antibodies to hepatitis C virus (HCV), antibodies to hepatitis D virus (HDV) and with a history of alcohol or drug abuse were excluded from the study. Patients treated with antiviral drugs (lamivudine or interferons) were also excluded from this study. Patients were observed every 1 to 3 mo or more frequently if required. Routine follow-up studies included clinical evaluation, conventional liver function tests for ALT, aspartate aminotransferase (AST), and γ -glutamyl transpeptidase (γ GTP) and serological markers of HBV infection including hepatitis B e antigen (HBeAg) and antibodies to HBeAg (anti-HBe). Ultrasound examination and computed tomography were done every 6-12 mo or more often if necessary. Hepatitis B surface antigen (HBsAg), HBeAg, and anti-HBe antibody were tested using commercially available enzyme immunoassays (EIA) (Enzygnost, Behring, Germany). Anti-HCV was assayed by a second-generation EIA (Ortho Diagnostics, Raritan, USA). Anti-HDV was examined by a commercially available EIA (Dinabot, Tokyo, Japan). HBV genotype was determined by the restriction fragment length polymorphism (RFLP) method on an S-gene sequence amplified by polymerase chain reaction (PCR) with nested primers, as previously described by Mizokami *et al.*^[21].

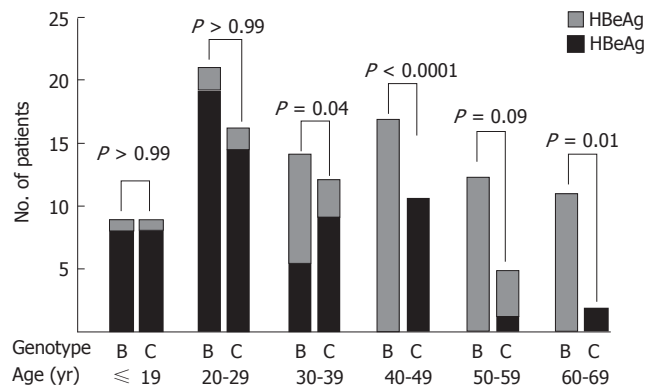


Figure 1 Age-specific HBeAg status at entry in the study in chronic hepatitis type B (CH-B)-infected patients with genotype B or C. Black box, HBeAg-positive patients; gray box, HBeAg-negative patients. Among the ≥ 30 yr old group, there are significantly more HBeAg-negative patients of genotype B than those of genotype C. In contrast, there are more HBeAg-positive patients in both groups, among the < 30 yr old group.

Statistical analysis

The chi-square test, Fisher's exact test, and Student's *t*-test were used to compare differences between groups, when appropriate. Cumulative developing rates of LC were calculated by the Kaplan-Meier method and differences were determined by the log rank test. In a multivariate analysis, the Cox proportional hazard model was employed to evaluate factors affecting development of LC. Statistical comparisons were done using SPSS for Windows version 11 package (SPSS Inc., Japan). All analyses were two sided, and *P*-values less than 0.05 were considered statistically significant.

RESULTS

Figure 1 shows age-specific HBeAg status at entry in the study for patients with CH-B infected with genotype B or C. For patients < 30 years old, most individuals were HBeAg-positive, and no difference was seen in HBeAg-positive rate between patients infected with genotype B and those infected with genotype C. However, for patients ≥ 30 years old, the majority of genotype C patients were positive for HBeAg, whereas most patients infected with genotype B were HBeAg-negative: no patient infected with genotype B showed a positive reaction for HBeAg in the age group greater than 40 years old. In addition, for patients > 50 years old, the percentage of patients infected with genotype B was increased. Since the proportion of patients positive for HBeAg was different between the group < 30 years old and that ≥ 30 years old, we divided the subjects into 2 groups (< 30 years old and ≥ 30 years old) to compare the natural course of CH-B in patients infected with genotypes B *vs* C (Table 1). There were no differences in LC development rates between genotypes B and C among the group < 30 years old using the Kaplan-Meier method and the log rank test ($P = 0.48$, Figure 2). In addition, when using the Cox proportional hazard model, which included HBeAg, gender, degree of fibrosis and genotype as associated variables, there was no significant

Table 1 Characteristics of patients with genotypes B and C

	< 30-yr-old group		≥ 30-yr-old group	
	Genotype B	Genotype C	Genotype B	Genotype C
Number of patients	27	23	41	30
Age (yr, mean ± SD)	21.6 ± 5.3	22.2 ± 4.4	45.5 ± 10.8	42.7 ± 8.7
Gender (Male/Female)	19/8	16/7	37/4	22/8
HBeAg (+/-)	24/3	21/2	4/37	19/11 ^b
Observation period (mo, mean ± SD)	69.0 ± 53.4	62.8 ± 45.9	77.9 ± 50.1	87.0 ± 50.6
ALT (IU/L, mean ± SD)	266.3 ± 262.1	245.8 ± 292.4	223.1 ± 251.7	265.8 ± 359.8
Degree of fibrosis (0/1/2/3)	4/11/5/1	2/13/2/2	6/13/10/4	2/13/6/7

^b $P < 0.0001$ vs genotype B in ≥ 30-yr-old group.

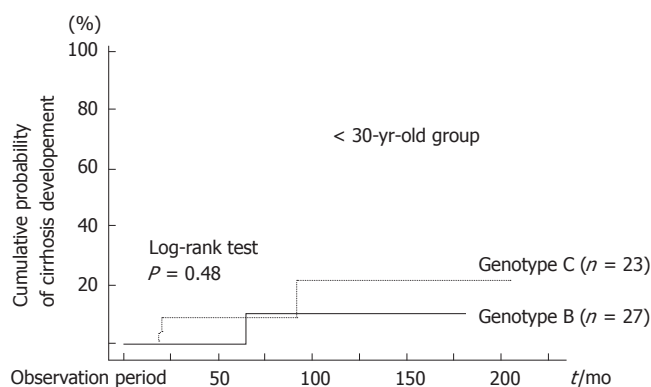


Figure 2 Comparison of cumulative probabilities of cirrhosis development between patients of genotypes B and C in the < 30 yr old group ($P = 0.48$, log-rank test). Multivariate analysis identified no significant predictors.

predictor for development of LC (Table 2). On the other hand, in the group ≥ 30 years old, only genotype was found to contribute to LC development by the Kaplan-Meier method: in fact, the rate was higher in patients infected with genotype C than in those infected with genotype B ($P = 0.002$, Figure 3). The Cox proportional hazard model also indicated that HBV genotype C was the only significant predictor of the development of LC (Relative risk 5.75; 95% CI: 0.033-0.916, $P = 0.039$, Table 2).

Patients who progressed to LC during the observation period are shown in Table 3. Eight patients progressed to LC in patients infected with genotype B. Five of these 8 patients were ≥ 40 years old at initial diagnosis, and all these 5 patients were HBe Ag-negative. Three patients < 30 years old who progressed to LC showed a positive reaction for HBeAg at enrollment. All 3 patients experienced a severe hepatic flare during the observation period and thereafter progressed to LC. However, following development of LC, they followed a benign clinical course of burn-out hepatitis: disappearance of HBeAg and persistently normal or nearly normal ALT levels. On the other hand, in the genotype C group, 8 of 12 patients who progressed to LC were 30-49 years old at initial diagnosis of CH, and 7 of the 8 patients were HBeAg-positive. These 8 patients rapidly progressed to LC within 2 to 8 years, and all of them were less than 50

Table 2 Cox proportional hazard analysis for development of LC

Variables	< 30-yr-old group			≥ 30-yr-old group		
	Relative risk	95% CI	P	Relative risk	95% CI	P
Gender (Male/Female)	0	-	0.972	0	0.000-3.993	0.969
HBeAg						
Positive	1	0.028-6.469	0.538	1	0.185-3.250	0.727
Negative	2.36			1.29		
Degree of fibrosis						
Mild-moderate	1	0.080-1.030	0.053	1	0.296-3.238	0.972
Severe	10.87			1.02		
Genotypes						
B	1	0.019-2.833	0.258	1	0.033-0.916	0.039
C	4.24			5.75		

LC: Liver cirrhosis.

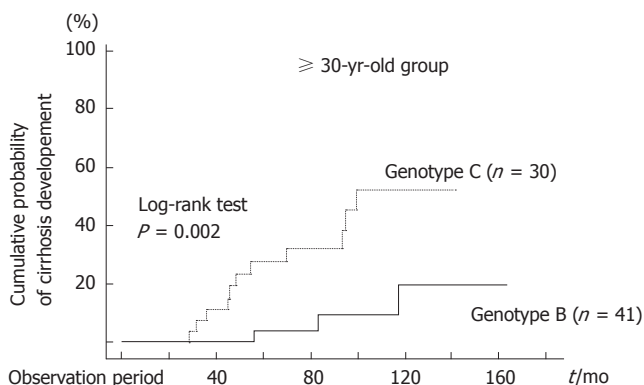


Figure 3 Comparison of cumulative probabilities of cirrhosis development between patients with genotypes B and C in the ≥ 30 yr old group ($P = 0.002$; log-rank test). A Cox proportional hazards analysis also indicated that genotype C was the only significant predictor (relative risk 5.75; 95% confidence intervals: 0.033-0.916, $P = 0.039$).

years old at the development of LC. Three of 8 patients died from HCC or liver failure shortly after development of LC, with periods ranging from 1 to 7 years.

DISCUSSION

In East Asia, most of HBV carriers are infected with genotype B or C^[2,5,22]. HBV genotype C is the predominant genotype in Japan, accounting for approximately 70% of HBV carriers living in Japan^[5].

The island Okinawa prefecture is located south-west of the main Japanese islands. Although people living in the Okinawa prefecture are Japanese, two-thirds of HBV carriers are infected with genotype B. In Okinawa prefecture, prevalence of HBsAg among blood donors is 3.5%, which is twice the average for all areas of Japan (1.5%), and is the highest in Japan (from the annual report of the Japanese Red Cross Center). In contrast, mortality rates due to cirrhosis and HCC associated with HBV infection are the lowest in Japan^[23,24]. This paradoxical phenomenon may be due to the fact that the majority of HBV carriers in Okinawa are infected with genotype B, and carriers of genotype B have a more benign clinical

Table 3 Patients progressing to liver cirrhosis during the observation period

Genotype B Age (yr)			Genotype C Age (yr)		
At entry	Development of LC	Outcome	At entry	Development of LC	Outcome
17 M e+	18 e+	24 e-	18 M e+	24 e+	31 e+ dead (HCC)
23 M e+	27 e-	36 e-			
28 M e+	30 e+	38 e-			
			31 M e+	33 e+	40 e+ dead (hepatic failure)
			31 M e+	39 e+	44 e-
			33 M e+	40 e+	48 e+
			36 M e+	40 e-	46 e-
			38 M e+	45 e-	48 e- dead (HCC)
41 M e-	48 e-	49 e-	41 M e+	43 e+	51 e-
			42 M e+	46 e-	48 e-
			45 M e-	48 e-	49 e- dead (HCC)
58 M e-	64 e-	70 e-	50 M e-	54 e-	57 e-
58 M e-	72 e-	73 e-	50 M e-	52 e-	53 e- dead (hepatic failure)
60 M e-	61 e-	66 e-			
66 M e-	70 e-	71 e-	73 F e+	79 e+	90 e+

M: Male; F: Female; e+: HBeAg-positive; e-: HBeAg-negative; LC: Liver cirrhosis; HCC: Hepatocellular carcinoma.

phenotype than those with genotype C (the predominant genotype in Japan).

In the present study, in the group < 30 years old, the majority of patients with CH-B were positive for HBeAg and the rate of positivity for HBeAg was not significantly different between patients infected with genotypes B and C. However, HBeAg was not detected in the majority of patients infected with genotype B in the group ≥ 30 years old. In contrast, most patients infected with genotype C were positive for HBeAg in the group ≥ 30 years old.

It has been reported that the seroconversion rate of HBeAg was higher in CH patients infected with genotype B than those infected with genotype C^[6,25,26]. However, in this study, LC development rate were not significantly different between patients with genotypes B and C in the group < 30 years old. Only one CH patient infected with genotype C progressed to LC during the observation period, whereas LC developed in 3 patients infected with genotype B. Therefore, prognoses of CH patients infected with genotypes B and C were comparable in the group < 30 years old. In contrast, LC development rate among CH patients infected with genotype C was significantly higher than that of genotype B in the group ≥ 30 years old. These patients progressed to LC within a short period of time after diagnosis of CH. In the group ≥ 30 years old, there was a significant difference in prevalence rates of HBeAg between patients infected with genotypes B and C. Therefore, it was presumed that difference in LC developing rate between genotypes B and C was responsible for differences in HBeAg positive rates. However, only genotype C was a significant predictor for development of LC using the Cox proportional hazard model, while HBeAg was not a significant predictor.

Among the 55 patients infected with genotype B in the group ≥ 30 years old, 5 were HBeAg positive at initial

diagnosis of CH. These 5 patients didn't progress to LC during the observation period (data not shown). Further stratification of the patients according to HBeAg status showed that development of LC was more frequently seen in patients infected with genotype C than in those with genotype B in the group ≥ 30 years old. As mentioned above, difference in prognosis between genotypes B and C was significantly different. In particular, prognosis was very poor if patients were infected with genotype C, were positive for HBeAg, and were ≥ 30 years old. Therefore, we believe that antiviral therapy (lamivudine, adefovir dipivoxil or entecavir) is beneficial if patients are ≥ 30 years old, positive for HBeAg and infected with genotype C.

Kao *et al.*^[26] reported that among 270 chronic HBV carriers in Taiwan, 53% belonged to genotype B and 32% to genotype C. Genotype C was significantly more common in patients with LC and/or HCC who were older than 50 years, when compared to age-matched inactive carriers, suggesting that genotype C was associated with more severe liver diseases. However, genotype B was more frequently seen in younger patients (< 35 years old) with HCC, most of whom did not have LC. While Kao *et al.* showed that genotype C was associated with poor prognosis in older age groups, as confirmed in our study, the predominance among younger patients with HCC in genotype B was quite different. These discrepancies may be responsible for differences in subgenotypes among patients infected with genotype B between Taiwan and Okinawa. Sugauchi *et al.* determined subgenotypes among 274 carriers of HBV infected with genotype B and reported that all 177 genotype B isolates from the carriers living in Asian countries other than Japan belong to the genotype Ba^[27,28]. On the contrary, they also reported that of the 97 carriers infected with genotype B living in Japan, only 7 (7%) possessed genotype Ba, and that the remaining majority was of genotype Bj without recombinations. In addition, it has been reported that loss of HBeAg occurs earlier and is more frequent in carriers of genotype Bj than genotype Ba or genotype C, particularly after they have reached 30 years or older^[27,28]. In this study, not all patients with genotype B were subgenotyped, but approximately 80% of them were infected with genotype Bj. Therefore, it is possible to speculate that differences in subgenotypes of HBV genotype B isolates may be responsible for differences in the natural course of patients infected with genotype B between Taiwan and Okinawa.

In this study, development of LC was commonly seen in patients with genotype B, and the majority of patients were HBeAg-negative at initial diagnosis of CH. Most of HBV carriers infected with genotype B seroconverted from HBeAg to anti-HBe under 30 years old and became HBeAg-negative inactive carriers^[5,16]. Status of HBeAg and anti-HBe antibody correlated with mutations in pre-core lesions^[29,30]. Most patients infected with genotype B showed the pre-core mutation before 30 years old, stopping HBeAg production, followed by reduction in HBV-DNA level^[5]. However, after clearance of HBeAg, CH occurred in some patients, and occurrence was associated with increase in serum HBV-DNA level. HBeAg-negative CH was more frequently seen in patients

infected with genotype B compared with genotype C. LC developed in some patients with genotype B, the majority of whom were ≥ 40 years old at diagnosis of CH. Therefore, CH-B has to be treated in consideration of the above described natural course of HBV infection. We believe that antiviral therapy (lamivudine, adefovir dipivoxil or entecavir) is highly recommended for the following patients: (1) HBeAg-positive CH infected with genotype C aged ≥ 30 years; (2) HBeAg-negative CH infected with genotype B aged ≥ 40 years, especially if showing advanced fibrosis (F2 or F3).

In conclusion, when HBeAg-positive CH-B was diagnosed in the group ≥ 30 years old, the rate of development of LC was higher in patients infected with genotype C than in those with genotype B. Therefore, we believe that these cases should be treated with antiviral drugs. In addition, development of LC is not rare in patients infected with genotype B if HBeAg-negative CH occurs at ≥ 40 years of age.

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COMMENTS

Background

Chronic infection by hepatitis B virus (HBV) causes various liver diseases. HBV genotypes are classified into 8 groups, from A to H. Genotypes B and C are predominant in Asia and > 90% of chronic carriers show genotype C, which is associated with poor prognosis. However, prior studies showed that association of genotype C with advanced liver disease was not seen in patients over 50 years old.

Research frontiers

HBV genotypes, distributed differently in the world, influence disease severity and clinical outcomes. Therefore, studies of genotypes have been thoroughly investigated to improve disease outcomes.

Innovations and breakthroughs

Prior studies showed that infection with genotype C was associated with poor prognosis. This study has focused on different age groups of chronic hepatitis B for different influences of genotype B and C and unveiled the age-specific natural course of the disease.

Applications

This study has clearly shown the age-specific natural course of the chronic hepatitis B with genotype B and C in Japan. These results should be helpful for determining the indication of antiviral drugs.

Terminology

HBV genotype: HBV genotypes are classified into 8 groups, from A to H, according to differences in base sequences greater than 8%, and are differently distributed in the world. In Japan, genotypes B and C are predominant as in other Asian countries, but > 90% of chronic carriers show genotype C.

Peer review

This population study by Maeshiro and colleagues describes the natural history of chronic hepatitis B of genotypes B and C. A moderate number of patients were seen over a long time period of 28 years and an even lower number of 121 patients was included in the analysis. Nevertheless, the study shows strong evidence for a higher progression rate towards cirrhosis for patients infected with HBV genotype C. This phenomenon was associated with a lower HBe conversion rate in genotype C patients.

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BASIC RESEARCH

Preparation method of an ideal model of multiple organ injury of rat with severe acute pancreatitis

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at 3, 6 and 12 h in ascites/body weight ratio; 1582 (284), 1769 (362), 1618 (302) (U/L) *vs* 5303 (1373), 6276 (1029), 7538 (2934) (U/L) at 3, 6 and 12 h in Amylase; 0.016 (0.005), 0.016 (0.010), 0.014 (0.015) (EU/mL) *vs* 0.053 (0.029), 0.059 (0.037), 0.060 (0.022) (EU/mL) at 3, 6 and 12 h in Endotoxin; 3.900 (3.200), 4.000 (1.700), 5.300 (3.000) (ng/L) *vs* 41.438 (37.721), 92.151 (23.119), 65.016 (26.806) (ng/L) at 3, 6 and 12 h in TNF- α , all $P < 0.01$]. Visible congestion, edema and lamellar necrosis and massive leukocytic infiltration were found in the pancreas of rats of model group. There were also pathological changes of lung, liver, kidney, ileum, lymphonode, thymus, myocardium and brain.

CONCLUSION: This rat model features reliability, convenience and a high achievement ratio. Complicated with multiple organ injury, it is an ideal animal model of SAP.

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Key words: Severe acute pancreatitis; Multiple organs; Injury; Animal model; Rats; Inflammatory mediator

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Abstract

AIM: To establish an ideal model of multiple organ injury of rats with severe acute pancreatitis (SAP).

METHODS: SAP models were induced by retrograde injection of 0.1 mL/100 g 3.5% sodium taurocholate into the biliopancreatic duct of Sprague-Dawley rats. The plasma and samples of multiple organ tissues of rats were collected at 3, 6 and 12 h after modeling. The ascites volume, ascites/body weight ratio, and contents of amylase, endotoxin, endothelin-1 (ET-1), nitrogen monoxidum (NO), phospholipase A₂ (PLA₂), tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6) in plasma were determined. The histological changes of multiple organs were observed under light microscope.

RESULTS: The ascites volume, ascites/body weight ratio, and contents of various inflammatory mediators in blood were higher in the model group than in the sham operation group at all time points [2.38 (1.10), 2.58 (0.70), 2.54 (0.71) *vs* 0.20 (0.04), 0.30 (0.30), 0.22 (0.10)

INTRODUCTION

Severe acute pancreatitis (SAP) is an acute clinical disease, featuring acute onset, rapid progression, high incidence of complications and high mortality. Its pathogenesis has still not been fully elucidated till now. There is also no ideal therapy. It is quite worthwhile to rely on a SAP animal model to strengthen the study of pathogenesis and treatment of SAP. There are many requirements on an ideal SAP experimental animal model. There should be simple and reliable methods, and a low cost and high achievement ratio. The pathological changes, progression and response to treatment of the model also should be similar to those of the human body. In this study, SAP animal models were induced by retrograde injection of 3.5% sodium taurocholate into the biliopancreatic duct of

Sprague-Dawley (SD) rats. The feasibility and significance of the model were studied. And the preparation methods for an ideal model of SAP rats with multiple organ injury were established.

MATERIALS AND METHODS

Experimental animals

Ninety clean grade healthy male SD rats with a body weight of 250-300 g were purchased from the Experimental Animal Center of Zhejiang Medical School.

Experimental drugs and reagents

Sodium taurocholate and sodium pentobarbital purchased from USA Sigma Company. Dexamethasone injection purchased from Zhejiang Xinchang Pharmaceutical Company. The full automatic biochemical analyzer was used to determine the plasma amylase level (U/L). Plasma endotoxin tachypleus amebocyte lysate kit was purchased from Shanghai Yihua Medical Science and Technology Corporation (Institute of Medical Analysis in Shanghai), the calculation unit for content is EU/mL. The serum nitrogen monoxidum (NO) kit was purchased from Nanjing Jiancheng Bioengineering Research Institute, the calculation units for content is $\mu\text{mol/L}$. TNF- α ELISA kit was purchased from Jingmei Bioengineering Corporation, the calculation unit for content is pg/mL (ng/L). The serum secretory phospholipase A₂ enzyme Assay ELA kit (phospholipase A₂) was purchased from R&D system Ins and the calculation unit for content is U/mL. The serum Endothelin-1 ELA kit (Endothelin-1) was purchased from Cayman chemical company (Catalog Number: 583151) and the calculation unit for content is ng/L (pg/mL). The above determinations were all operated according to the instructions of the kits.

Experimental methods and groups

The experimental rats were randomly assigned to the model group and control group (45 rats/group). The Aho's method was adopted and the SAP rats of model group were prepared by retrograde injection of 3.5% sodium taurocholate into biliopancreatic duct through segmental eqidural catheter *via* duodenal papilla. Another 45 rats were assigned to the sham operation group where only exploratory laparotomy was performed. The above-mentioned groups were then divided into 3, 6 and 12 h group, with 15 rats in each group. The following steps were performed at 3, 6 and 12 h after operation: (1) Rat mortality of all groups was observed. Then the rats were sacrificed in batches. The ascites/body weight ratio was observed. (2) The samples of pancreas, lung, liver, kidney, terminal ileum, ileocecal junction lymphonode, thymus, myocardium and brain were collected and their pathological changes were observed. (3) After collecting blood from the heart, the ascites volume, ascites/body weight ratio and contents of amylase and endotoxin in plasma and contents of endothelin-1 (ET-1), nitrogen monoxidum (NO), phospholipase A₂ (PLA₂), tumor necrosis factor- α (TNF- α) and IL-6 in serum were determined.

Animal model preparation

Fasting with water restraint was imposed on all rat groups 12 h prior to the operation. The rats were anesthetized by intraperitoneal injection of 2% sodium pentobarbital (0.25 mL/100 g) after which lay and fixed the rats, and performed the routine shaving, disinfection and draping.

Model group: after entering abdomen *via* median epigastrium incision, confirmed the bile-pancreatic duct and hepatic hilus common hepatic duct, disclosed the pancreas, identified the duodenal papilla inside the duodenum duct wall, and then used a No. 5 needle to drill a hole in the mesenterium avascular area. After inserting a segmental eqidural catheter into the duodenum cavity *via* the hole, inserted into the bile-pancreatic duct toward the direction of papilla in a retrograde way, used the microvascular clamp to nip the catheter head temporarily and meanwhile used another microvascular clamp to temporarily occlude the common hepatic duct at the confluence of hepatic duct. After connecting the eqidural catheter end with the transfusion converter, transfused 3.5% sodium taurocholate 0.1 mL/100 g by retrograde transfusion *via* the microinjection pump at the speed of 0.2 mL/min. Stayed for 4 min after injection and removed the microvascular clamp and epidural catheter. After checking for bile leakage, sutured the hole in the duodenum lateral wall. Used the disinfected cotton ball to absorb up the anesthetic in the abdominal cavity and close the abdomen.

Statistical analysis

The statistical analysis was conducted to the arranged experimental results by applying the SPSS11.5 software. The Kruskal-Wallis test or variance analysis (only applied to PLA₂) was applied to the two-group comparison. The Bonfferoni test was also applied to comparison. There are statistical significances when $P \leq 0.05$.

RESULTS

Survival

Model group: Mortality respectively was 0% (0/15), 0% (0/15) and 13.33% (2/15) at 3, 6 and 12 h. The sham operation group survived at all time points with 100% survival.

Comparison of ascites volume

The volume was higher in model group than in sham operation group at all time points ($P < 0.001$) (Table 1).

Comparison of ascites/body weight ratio

The coefficient was higher in model group than in sham operation group at all time points ($P < 0.001$) (Table 2).

Comparison of amylase and endotoxin in plasma and NO, PLA₂, TNF- α and IL-6 in serum

The contents were higher in model group than in sham operation group at all time points ($P < 0.001$) (Table 3).

Comparison of serum ET-1 contents

The content was higher in model group than in sham operation group at all time points ($P < 0.01$) (Table 3).

Table 1 Comparison of ascites volume in all groups [M(Q_R)]

Groups	3 h	6 h	12 h
Sham operation group	0.50 (0.00)	0.70 (0.50)	0.60 (0.30)
Model group	7.20 (2.00)	6.40 (2.30)	7.90 (1.70)

$P < 0.001$ at all time points between model group and sham operation group.

Table 2 Comparison of ascites/body weight ratio [M(Q_R)]

Groups	3 h	6 h	12 h
Sham operation group	0.20 (0.04)	0.30 (0.30)	0.22 (0.10)
Model group	2.38 (1.10)	2.58 (0.70)	2.54 (0.71)

$P < 0.001$ at all time points between model group and sham operation group.

Table 3 Comparison of different indexes level in blood [M (Q_R) or (mean \pm SD)]

Index	Sham operation group			Model group		
	3 h	6 h	12 h	3 h	6 h	12 h
Amylase (U/L)	1582 (284)	1769 (362)	1618 (302)	5303 (1373)	6276 (1029)	7538 (2934)
Endotoxin (EU/mL)	0.016 (0.005)	0.016 (0.01)	0.014 (0.015)	0.053 (0.029)	0.059 (0.037)	0.060 (0.022)
ET-1 (ng/L)	15.293 (4.231)	16.275 (3.180)	14.173 (2.556)	24.745 (1.011)	25.625 (7.973)	24.725 (3.759)
NO (μ mol/L)	7.500 (5)	7.500 (5)	10.000 (5)	65.000 (7.5)	62.500 (38.8)	74.100 (26.2)
TNF- α (ng/L)	3.900 (3.200)	4.000 (1.7)	5.300 (3.000)	41.438 (37.721)	92.151 (23.119)	65.016 (26.806)
IL-6 (ng/L)	1.846 (0.346)	1.743 (0.838)	2.036 (0.818)	5.437 (1.025)	6.817 (0.81)	5.356 (0.747)
PLA ₂ (U/mL)	18.70 \pm 4.40	16.70 \pm 3.83	18.52 \pm 11.32	103.69 \pm 20.82	119.85 \pm 17.74	121.29 \pm 17.00

$P < 0.01$ at all time points between model group and sham operation group.

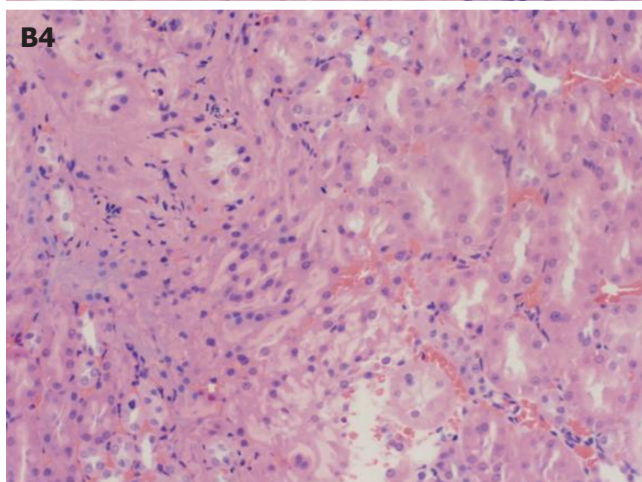
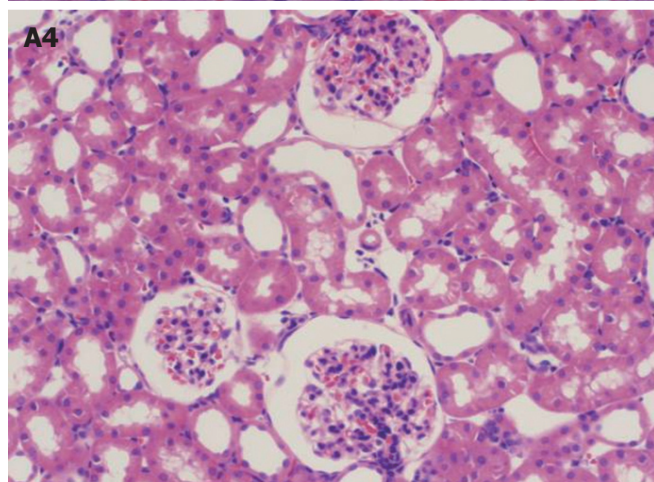
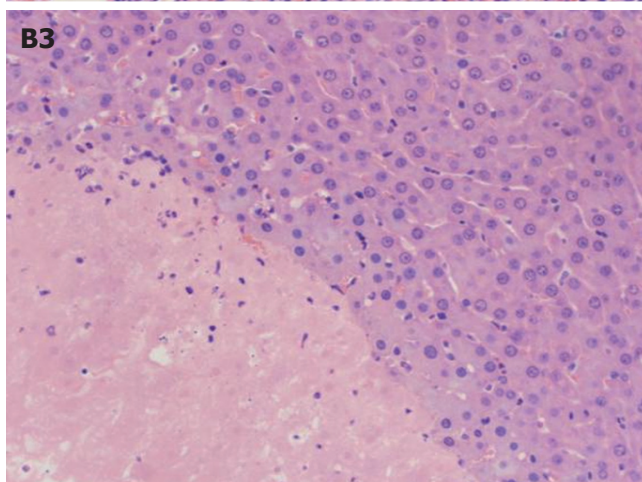
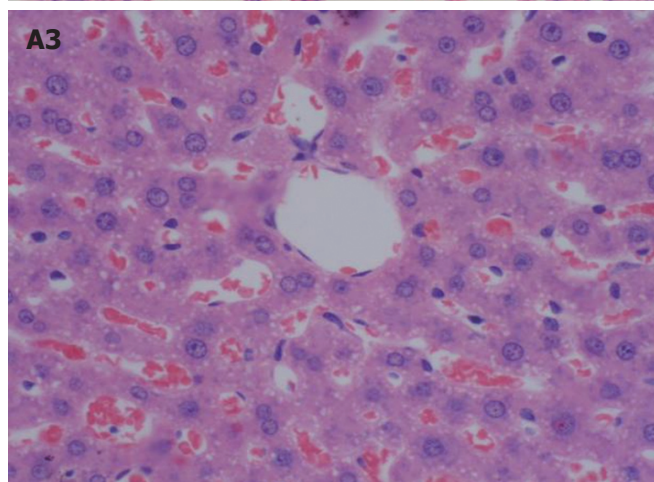
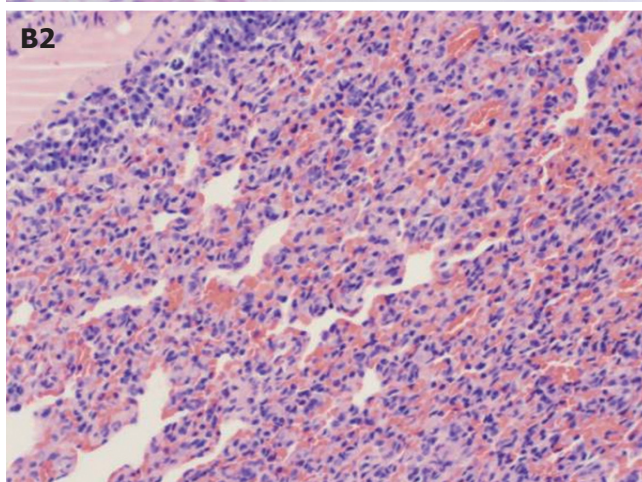
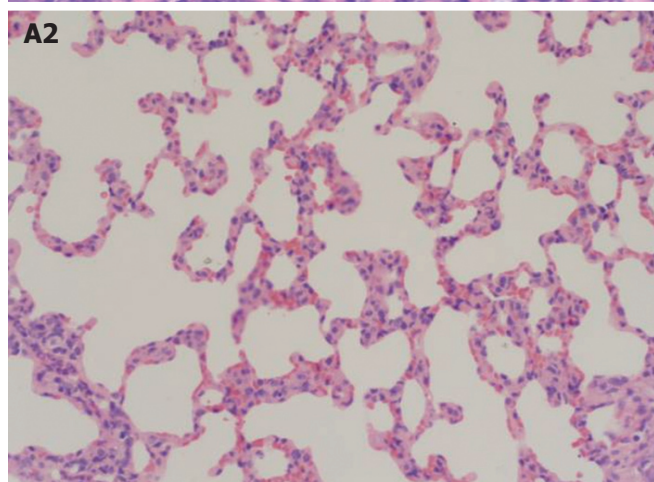
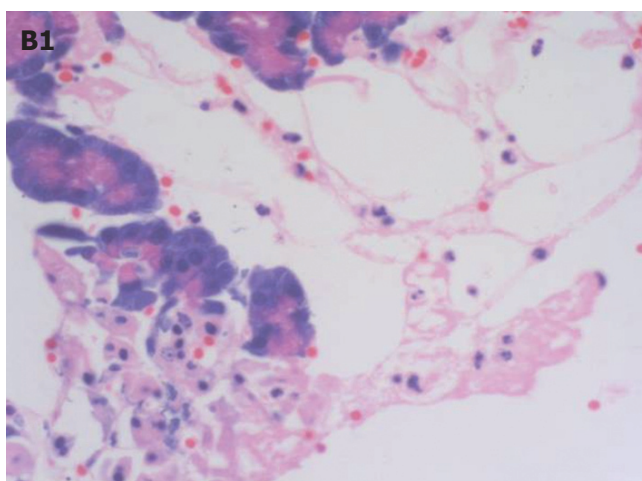
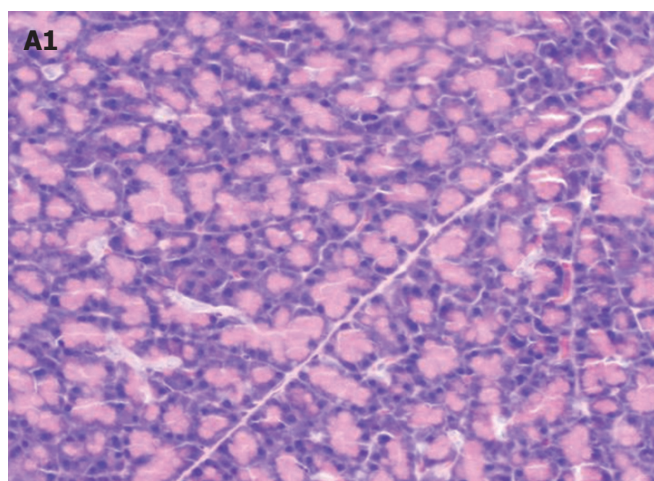
Pathological changes of multiple organs

Pathological changes of pancreas: (1) Sham operation group, Gross changes: Pancreas, fat surrounding pancreas and epiploon were normal at all time points after operation. Changes under light microscope: Most cases were normal. Mild interstitial edema was found in very few cases and neutrophil infiltration was seen by chance. There was no necrosis and hemorrhage of acinar cell and fat (Figure 1 A1). (2) Model group, Gross changes: The level of gross pathological changes and changes under light microscope was higher in pancreas tail than in pancreas head. The severity of all pathological changes aggravated with time after modeling. Edema, hemorrhage and necrosis appeared in the pancreas 5 min after inducing models. There were small amounts of ascites, slightly hemorrhagic in most cases, congestion and edema of the pancreas, and hemorrhage and necrosis in some cases at 3 h. The ascites was hemorrhagic in most cases at 6 and 12 h. And the average volume was higher at 6 and 12 h than that at 3 h. The severity of edema, hemorrhage and necrosis was higher at 6 and 12 h than at 3 h, with many saponification spots in epiploon and peritoneum surrounding pancreas, gel like change and contour disappearance of pancreas, and significant hemorrhage and necrosis (Figure 1 B1). Changes under light microscope: The severity of all pathological changes aggravated with time after modeling.

There were visible interstitial congestion and edema, mild inflammatory cell infiltration, focal necrosis and mild interstitial hemorrhage in pancreas at 3 h, with lamellar necrosis and hemorrhage in some cases. There were interstitial congestion and edema, focal or lamellar necrosis and hemorrhage, more inflammatory infiltration in pancreas at 6 h. There were interstitial edema and hemorrhage, massive necrosis and hemorrhage, damaged lobule contour and massive inflammatory cell infiltration in pancreas at 12 h.

Pathological changes of lung: (1) Sham operation group: Gross changes: The color and morphous of both sides of lung were normal without bleeding point on surface or effusion in thoracic cavity. Changes under light microscope: The structure was normal in most part of lung. There were mild interstitial edema and mild inflammatory cell infiltration in a very small part. (2) Model group: Gross changes: 3 h: There were congestion and edema in pulmonary lobes of both sides, madder red bleeding points on surface of local pulmonary lobes, mild amber effusion in thoracic cavity; 6 and 12 h: There were pathological changes of both sides of lung aggravating with time after molding, prunosus plaque on lung surface, more hemorrhagic effusion in thoracic cavity. Changes under light microscope: There was edema of the lung interstitium and alveolar space, broadened alveolar wall interstitium, inflammatory cell infiltration, telangiectasis and congestion of alveolar wall, and broadened alveolar septum; 6 and 12 h: There were increased range of pathological changes of pulmonary lobes and effusion in alveolar space, edema and hemorrhage of interstitium and alveolar space, broadened alveolar septum, inflammatory cell infiltration, and lucent kytoplasm of epithelial cell in local tunica mucosa bronchiorum (Figure 1 A2 and B2).

Pathological changes of liver: (1) Sham operation group: Gross changes: The color of liver was normal without visible swelling. Changes under light microscope: There were normal hepatic tissue, mild inflammatory cell infiltration in portal area, normal morphous of hepatic cell in most cases, acidophilia denaturation in some cases, and mild dilation and congestion of sinus hepaticus in few cases (Figure 1 A3). (2) Model group: Gross changes: 3 h: There were mild liver swelling, local gray plaque in local liver of individual rats, obscure boundary; 6 and 12 h: There were pale and muddy color and congestion of



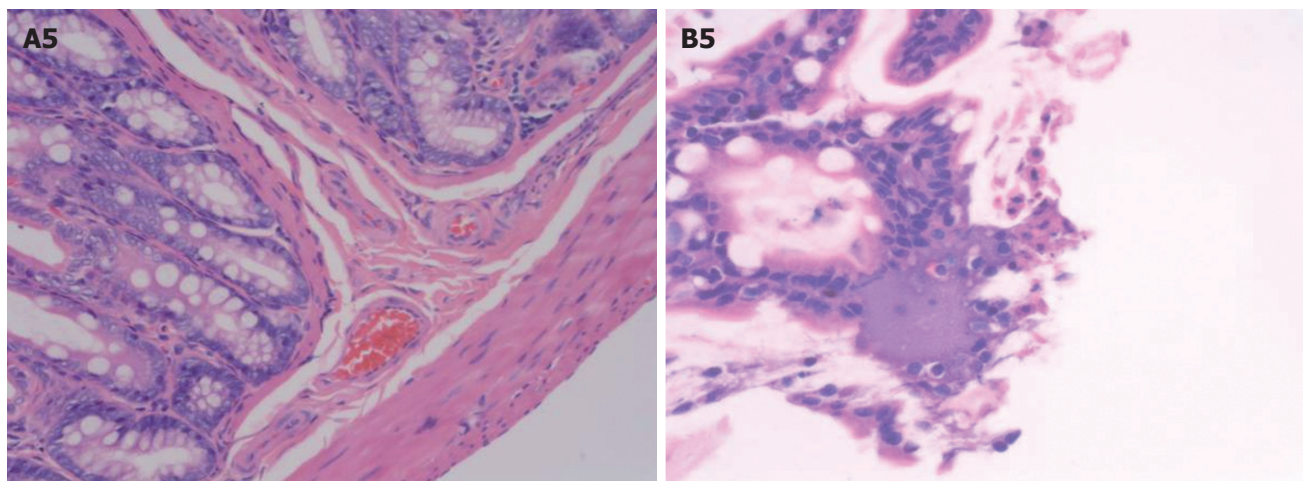


Figure 1 A1: Normal pancreas, HE, × 200; B1: Hemorrhage and necrosis of pancreatic acinus and fatty tissue, HE, × 400; A2: Normal lung in sham operation group at 12 h, HE, × 400; B2: Lung (effusion in alveolar space) in model group at 12 h, HE, × 200; A3: Normal liver, HE, × 400; B3: Massive necrosis of hepatic cell, HE, × 200; A4: Normal kidney, HE, × 400; B4: Scattered necrosis in epithelium of renal tubule, HE, × 200; A5: Normal ileum, HE, × 400; B5: Flossy necrosis of intestinal mucosa, HE, × 400.

the liver, irregular gray plaque or necrosis in local area. Changes under light microscope: 3 h: There were hepatic cell swelling or acidophilia denaturation, inflammatory cell infiltration in portal area, dilation and congestion in sinus hepaticus, scattered spotty necrosis in hepatic lobule; 6 h: There were visible hepatic cell swelling, increased range and area of hepatic cell necrosis, focal or massive hemorrhagic necrosis, inflammatory cell infiltration in necrotic focus, congestion in partial sinus hepaticus, bile duct proliferation in portal area and scattered necrosis of single cell (condensation and break of nucleus); 12 h: There were damaged hepatic lobule structure, further increased range and area of hepatic cell necrosis, inflammatory cell infiltration in lobule and/or portal area, congestion in sinus hepaticus (Figure 1 B3).

Pathological changes of kidney: (1) Sham operation group: Gross changes: The morphous of the kidney was normal without swelling, with no bleeding points on surface of renal cortex. Changes under light microscope: There were normal structure of renal glomerulus, tubule and interstitium in most rats, and blurry boundary of epithelial cell of renal tubule (especially proximal tubule), stegnosis and atresia of lumens, congestion of renal glomerulus and interstitial edema in few rats (Figure 1 A4). (2) Model group: Gross changes: There was no gross change in kidney at 3 h. There were kidney swelling, tension of renal envelope, scattered bleeding points on surface of renal envelope of rats, and slightly hemorrhagic urine in pelvis in severe cases at 6 and 12 h. Changes under light microscope: 3 h: There were capillary congestion of renal glomerulus, swelling, scattered necrosis and blurry boundary of renal tubule epithelial cell, stegnosis or atresia of lumens, visible protein cast, interstitial edema and inflammatory cell infiltration. 6 and 12 h: There were capillary congestion of renal glomerulus, swelling and scattered necrosis of epithelial cell of renal tubule, interstitial edema and inflammatory cell infiltration. The floss and red cell with eosinophilic staining were found in renal glomerulus and so was homogen or red cell cast with

eosinophilic staining in renal tubule. There were lumens dilation of medulla renal tubule and atrophica of epithelial cell. The pathological changes aggravated with time. There was lamellar necrosis of epithelial cell of renal tubule in few rats (Figure 1 B4).

Pathological changes of ileum: (1) Sham operation group, Gross changes: There was no intestinal dilation or congestion or edema of intestinal wall. The surface of intestinal mucosa was smooth without pathological changes such as hemorrhage and ulcer. Changes under light microscope: The structure of epithelium and microvillus of intestinal mucosa was integrated without exfoliation or necrosis. Edema of proper layer, submucous layer and placenta percreta was found in few cases. Focal necrosis of mucosa and inflammatory cell infiltration of proper layer, submucous layer and placenta percreta were found in very few cases (Figure 1 A5). (2) Model group, Gross changes: There was no intestinal change at 3 h. Visible intestinal dilation was found at 6 and 12 h with retention of certain amount of gas and liquid, congestion and edema of intestinal wall and bleeding points on surface of intestinal mucosa. Small focus of mucosal ulcer was found in severe cases. Changes under light microscope: Focal necrosis of ileum mucosa and inflammatory cell infiltration in various mucosa layers were found in most rats. There were visible congestion edema of proper layer of intestinal mucosa, dilation of central chyle vessel, exuviations of microvillus top of epithelium of intestinal mucosa and disordered structure arrangement at 6 and 12 h (Figure 1 B5).

Pathological changes of lymphonode: The morphous and structure of lymphonode were normal in sham operation group and swelled in model group with lymphonode swelling, dilation of follicle germinal center of lymphonode and lymphatic sinus, sinus cell hyperplasia, and frequently spotty necrosis in germinal center of folliculus lymphaticus. Infiltration of neutrophil and plasmocyte was found only in few cases.

Pathological changes of thymus: (1) Sham operation group: The histological findings of thymus at 3, 6 and 12 h were consistent. There were normal structure of thymus, clear boundary between cortex and medulla, about 2-1:1 of thickness ratio of cortex and medulla, visible lobule, integrated envelope, “starry sky” changes in few epithelial cells of cortex, and condensed dark purple blue nucleus. There were more epithelial reticular cell slightly stained in medulla, in star shape and with many prominences, large nucleus and rich kytoplasm. The structure was looser in medulla than in cortex. Slightly stained nucleus of epithelial cell was found only in few cases and epithelial cell was “vacuole” in individual cases. (2) Model group: The cortex was gradually thinned and expanded compared with medulla at 3, 6 and 12 h. There were “starry sky” epithelial cell, nuclear fragmentation and reduced amount of lymphocytes. The nucleus of epithelial cell in medulla was slightly stained. There were more “vacuole” epithelial cells in model group than in normal group.

Pathological changes of myocardium: (1) Gross changes: Normal manifestation and no visible change of appearance in all groups. (2) Changes under light microscope: The transverse striation of cardiac muscle fiber was clear in all cases. There was no abnormal cardiac muscle fiber in sham operation group. The fiber was normal in most cases of model group. But one case of blurry transverse striation of cardiac muscle fiber occurred at 3 h in model group. Granulation or lyses of muscle plasma of cardiac muscle fiber was found in 2, 3, 5 rats respectively at 3, 6 and 12 h. Mild inflammatory cell infiltration of myocardium interstitium and epicardium was found in one case of each group respectively at 3 h.

Pathological changes of brain: (1) Gross changes: Normal manifestation and no visible change of appearance in all groups. (2) Changes under light microscope: The pathological changes were almost invisible. The mild swelling of neuron was found in 2, 2, 3 rats of model group at 3, 6 and 12 h. Other rats were normal.

DISCUSSION

At present, preparation of SAP animal model is still the main method for study of SAP pathogenesis and evaluation of therapeutic effects of drugs. Therefore, many researchers have designed and carried out a great deal of animal experiment studies. The most commonly used living SAP animals are induced by puncture injection of biliopancreatic duct^[1-4], injection under capsula pancreatic^[5], ligation of pancreatic duct^[6], loop close of duodenum^[7], biliopancreatic duct injection in combination with intravenous infusion^[8] and intraperitoneal injection^[9-11]. In addition, the drugs most frequently used to induce SAP model are sodium taurocholate^[12,13] that is most popular, glycodeoxycholic acid, bombesin combined with lipopolysaccharide^[14,15], and L-arginine^[16-18]. The rat

is most frequently used. Although the above methods and drugs can successfully induce SAP model, they cannot sufficiently control the severity of pathological changes of pancreas and injury of non-pancreas organs. Therefore, it is difficult for them to accommodate the therapeutic effects and mechanism of drugs evaluated. In addition, the experimental result also will be influenced by the inconsistency of severity and range of pathological changes in pancreas and multiple non-pancreas organs in SAP models induced by the same method. Sometimes a completely opposite conclusion will be obtained. Therefore, it is necessary to establish a reliable and stable SAP model with high achievement ratio that should correctly reflect the pathological changes in pancreas and multiple non-pancreas organs during SAP and aid the evaluation of therapeutic effects.

Aho *et al*^[1] first reported the SAP rat induction by retrograde injection of 5% sodium taurocholate through puncture of biliopancreatic duct in 1980. The method is a classic model preparation method extensively applied^[19-21]. The principle of its preparation is based on the theory of bile reflux. The rat SAP will be induced directly by injuring pancreatic tissue or activating endogenous pancreatin after retrograde injection of sodium taurocholate into biliopancreatic duct. Compared with other models, this method can induce the pathological changes including blood supply disturbance of microcirculation, edema, hemorrhage and necrosis of pancreatic tissue, quite similar to the pathological features of human bile reflux pancreatitis. However, it was found in our experiment that this method could cause serious surgical trauma, blood loss and high mortality. There were also problems including unsatisfactory model induction and insensitivity to medicine intervention due to biliary fistula in biliopancreatic duct, *etc.*

Therefore, in our experiment, SAP rats were induced by retrograde minipump injection of 3.5% sodium taurocholate *via* duodenal papilla using segmental eqidural catheter, which was based on Aho method. The “reflux” of sodium taurocholate due to injection using common injector has been reduced in this experiment where segmental eqidural cathete with diameter consistent with that of biliopancreatic duct was applied. The degree and range of edema, hemorrhage and necrosis due to minipump injection of sodium taurocholate are similar to those of human since this method with even pressure can distribute sodium taurocholate in rat pancreatic tissue evenly. The pancreatic injury due to sodium taurocholate, a cytotoxic substance, is dose dependent. Acute pancreatitis models of various severities can be prepared by controlling the concentration of the drug. 5% concentration of inducing dosage was most frequently used in past reports. After years of study, we found relatively severe pancreatitis models would be induced and many pathological changes of multiple organs were irreversible under this concentration, resulting in short survival time, high mortality and insensitivity to SAP medications or other therapies. Therefore, 3.5% sodium taurocholate

(0.1 mL/100 g) was adopted in our study. Certain mortality was observed in model group. The contents of amylase, ascites volume and various inflammatory mediators in plasma were higher in model group than in sham operation group at 3, 6 and 12 h ($P < 0.01$). Various severities of multiple organ injury were also found in model group. The severity was time dependent. The pathological changes were most significant and severe at 12 h in model group but these reversible changes could be significantly changed after medication. It can be used to simulate the multiple organ injury complicated with SAP patients.

Therefore, this model with pathogenesis, pathological changes, etc similar to clinical SAP is helpful for studies on pathogenesis and therapy of multiple organ injury. This rat SAP inducing method that can induce ideal animal models for empirical studies features small trauma, convenient operation and high achievement ratio. The multiple organ injury occurring at early stage of rat SAP is not severe and reversible. It can be used for evaluation of therapeutic effect. We hope this method could be brought into full play by other researchers.

COMMENTS

Background

Severe acute pancreatitis (SAP) is an acute disease, but there is no ideal therapy. It is quite worthwhile to rely on SAP animal model to strength the study on pathogenesis and treatment of SAP.

Research frontiers

There are many requirements on an ideal SAP experimental animal model. There should be simple and reliable methods, low cost and high achievement ratio. The pathological changes, progression and response to treatment of the model also should be similar to those of human body.

Innovations and breakthroughs

In this study, the feasibility and significance of the SAP animal model were studied. And the preparation methods for an ideal model of SAP rats with multiple organ injury were established. This model with pathogenesis, pathological changes, etc similar to clinical SAP is helpful for studies on pathogenesis and therapy of multiple organ injury.

Applications

This rat SAP inducing method that can induce ideal animal models for empirical studies features small trauma, convenient operation and high achievement ratio. The multiple organ injury occurring at early stage of rat SAP is not severe and reversible. It can be used for evaluation of therapeutic effect. We hope this method could be brought into full play by other researchers.

Terminology

Severe acute pancreatitis (SAP) is a fatal systemic disease featuring acute onset, serious conditions, high incidence of complications and 20%-30% of mortality.

Peer review

This is a very interesting paper. Title reflects the major contents of the article. Abstract gives a clear delineation of the research background, objectives, materials and methods, results (including important data) and conclusions. Methods are innovative and systemic. The statistical methods used are appropriate. The results provide sufficient experimental evidences. Discussion is well organized and an overall theoretical analysis is given. The conclusions are scientifically reliable and valuable.

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CLINICAL RESEARCH

Infectious causation of chronic disease: Examining the relationship between *Giardia lamblia* infection and irritable bowel syndrome

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Abstract

AIM: To evaluate whether a higher prevalence of *Giardia lamblia* infection is associated with an increase in irritable bowel syndrome (IBS) prescriptions at the county level in Michigan.

METHODS: The Michigan Disease Surveillance System (MDSS) was used to ascertain both the numbers of *Giardia lamblia* infections as well as the total number of foodborne illnesses per population by county in Michigan during 2005. This was compared with Blue Cross Blue Shield (BCBS) of Michigan numbers of drug prescriptions for IBS per one thousand members per county in 2005. These data were also analyzed for associations with per capita income by county and the number of refugees entering each county in 2005.

RESULTS: There were a total of 786 confirmed cases of *Giardia lamblia* reported to MDSS in 2005. During the same time period, the number of prescriptions for IBS varied from 0.5 per 1000 members up to 6.0 per 1000 members per month. There was no trend towards higher numbers of IBS prescriptions in the counties with more *Giardia lamblia* infections. Per capita income was not associated with either IBS prescriptions or Giardiasis. There was a significant linear association between the number of refugees entering each county, and the number of *Giardia lamblia* cases per 100 000 population.

CONCLUSION: In this ecological study, there was no association found between BCBS prescriptions for IBS and *Giardia lamblia* infections in Michigan counties. Our findings may have been influenced by the disparate number of refugees admitted per county.

INTRODUCTION

Giardia lamblia is a multi-flagellate protozoa which can cause symptoms of abdominal discomfort, bloating, diarrhea and mucus, but not blood in the stools^[1]. The cyst form is resistant to cold weather and chlorination^[2]. It is spread by the fecal-oral route, and is most commonly diagnosed in young children, especially in day-care centers, where the prevalence has been found to be as high as 35%^[1]. The incidence is also increased in men who have sex with men, and in immigrants from developing countries^[3]. The FDA (Food and Drug Administration) estimates a probable 2% annual Giardiasis attack rate in the United States (US) population^[4] although substantially fewer infections are diagnosed. The Center for Disease Control (CDC) recorded 18 126 infections in 2005 in the US as a whole^[5], for an incidence of approximately 0.006%. This would imply that the vast majority of infections are asymptomatic, symptomatic but not brought to the attention of the medical community, or symptomatic and misdiagnosed.

Irritable bowel syndrome (IBS) is a diagnosis of exclusion. The Rome criteria specify three months of abdominal discomfort, unrelated to a physiologic or biologic cause, which can be associated with bloating, constipation, diarrhea or mucus^[6]. These symptoms overlap with many other gastrointestinal illnesses, such as inflammatory bowel disease, lactose intolerance, gastrointestinal cancers and parasitic diseases^[6], including Giardiasis, which also can become chronic.

By definition, patients with IBS do not have a physiologic cause for their illness, but some studies have shown that a significant number of patients who have been

given the diagnosis of IBS do indeed have infection with *Giardia lamblia*^[7-9]. Likewise, a study of 100 consecutive patients in an outbreak of Giardiasis in Italy found that 82% of these patients met the Rome criteria for IBS, including the chronicity of symptoms^[10]. Other authors have described a syndrome of post-infectious IBS due to either bacterial or parasitic etiologies^[11,12]. Recent research has shown a similar association between the initiation of celiac disease, and the number of previous rotavirus infections in children^[13]. Further evidence of a possible connection between parasitic disease and IBS is that treatment with metronidazole, an anti-parasitic medication, has been shown to decrease symptoms of IBS^[14-16].

The current study was undertaken to determine whether there might be a causal relationship between current or past *Giardia lamblia* infection and the prevalence of IBS. To test this, we assessed whether a higher county level prevalence of IBS prescriptions is associated with a higher incidence of *Giardia lamblia* infections in Michigan counties.

MATERIALS AND METHODS

Michigan initiated an electronic surveillance system for communicable diseases in 2004, called the Michigan Disease Surveillance System (MDSS). Local health departments, the Michigan Bureau of Laboratories, and some of the larger private laboratories enter data directly into the system over the internet. Giardiasis is a reportable disease in Michigan, and the local health departments use MDSS to track their cases. This system ensures that MDSS captures all of the reported cases of *Giardia lamblia* in Michigan. The aggregate information is made available to the CDC, as well as to the local health departments. The number of cases can be analyzed by time, demographics and geographic location. The date of onset of illness was used for inclusion when available, and the date of referral was used when date of onset was not known. Only the confirmed, completed cases in 2005 were counted. The rate per 100 000 people was obtained by dividing the number of cases per county, by population in each county, obtained from the 2005 United States census estimates.

Blue Cross Blue Shield (BCBS) of Michigan maintains a record of all prescriptions filled for its members, which includes the address and county of each member. Information on the number of members over a particular time period is also available, broken down by county. This was used to obtain a rate of prescriptions per 1000 members per month in 2005. Three medications were included in this study, dicyclomine (Bentyl), tegaserod (Zelnorm) and alosetron (Lotronex). These three medications were chosen because their only approved indication is the treatment of IBS. Prescription information was used in lieu of IBS diagnoses because there is no current registry of IBS patients in Michigan.

Per capita income data were obtained from the US census for 2000. Refugee numbers were provided by the Office of Refugee Services of the Michigan Department of Human Services.

A database was constructed containing information on *Giardia lamblia* cases by county. This was linked with

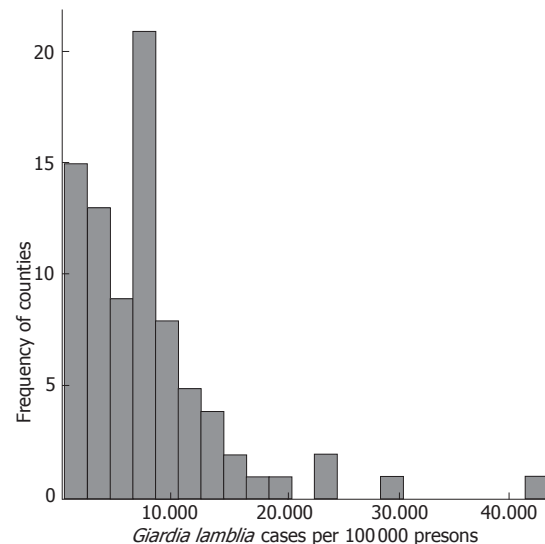


Figure 1 Frequency of Michigan counties by *Giardia lamblia* incidence in 2005.

information on the population of each county, to obtain a rate per 100 000 people. Next, the IBS prescription rates by county were added to the database, followed by per capita income and numbers of refugees admitted. Finally, the rate of foodborne illness by county was placed in the database, which was then cleaned and coded.

Descriptive statistics were used to assess frequencies and distributions of variables. Univariate regression was performed to assess relationships between IBS prescriptions and foodborne illness, IBS prescriptions and per capita income, and between *Giardia lamblia* and IBS prescriptions, refugees, foodborne illness, and per capita income. SPSS version 11.5 was used for all data analyses.

RESULTS

The total number of *Giardia lamblia* cases in Michigan, which has 83 counties, was 786 in 2005, which was down from 980 in 2000, but up from 733 in 2004, the year MDSS was introduced. Ingham County had 121 cases of Giardiasis in 2005, which gave it the highest incidence of 0.043%, which is about seven times the national average. Fourteen counties had no cases reported in 2005. The mean number of cases per county was 9.6, and the median was 3. The graph of incidence for each county by frequency was right-skewed (Figure 1). There were 362 (46%) female cases and 420 (53%) male cases with 4 unknown. One hundred and thirty-eight cases were children ≤ 4 years, and 89 were children between 5 and 9 years, for a total of 227 (29%) of the cases. There was another spike between ages 30 and 44 with a total of 184 (23%) cases. The number of cases gradually decreased after age 44 (Figure 2). The majority (51%) of cases were among 402 European Americans, with only 57 (7%) African Americans, 31 (4%) Asian Americans and 3 (< 1%) Native Americans. Race was not specified for 293 (37%) cases. Of the 393 whose ethnicity was known, 37 (9%) were Latino, and 356 (91%) were non-Latino.

The number of BCBS prescriptions for IBS per 1000 members per month in 2005 was much less variable

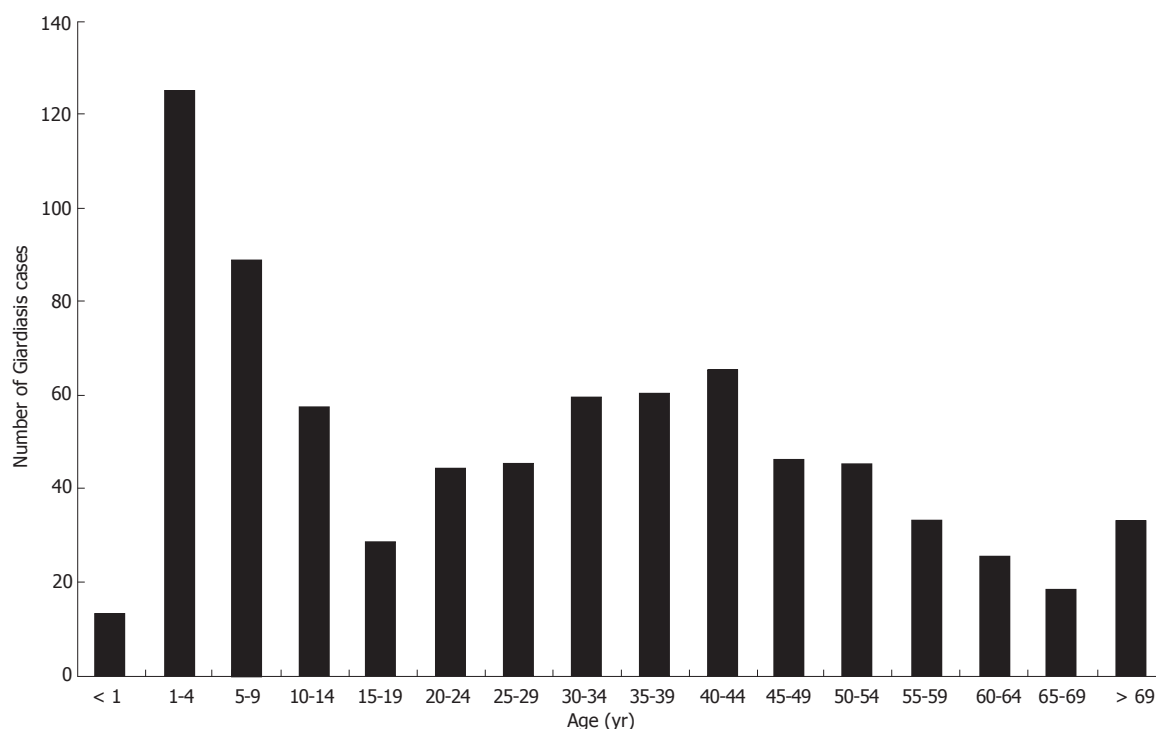


Figure 2 Michigan *Giardia lamblia* cases by age in 2005.

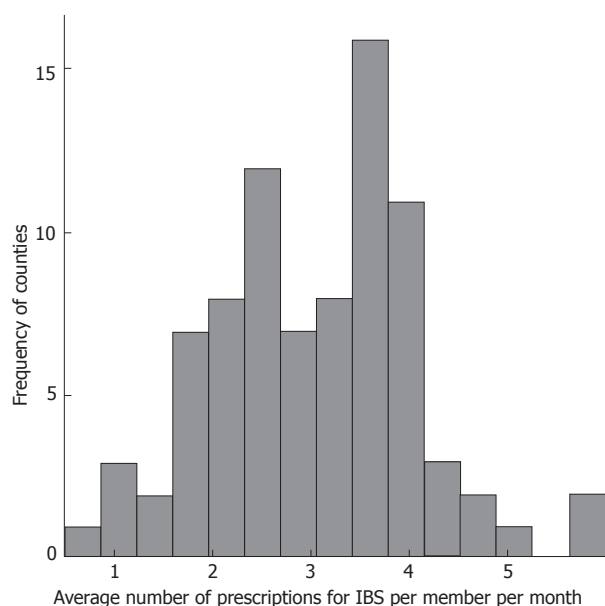


Figure 3 Frequency of counties by Michigan BCBS prescription incidence for 2005.

between counties. It ranged between 0.5 and 6.0, with a statewide total average of 3.3 (Figure 3). All six of the counties with rates above 4.3 were in the BCBS “western” region, and four of those counties were contiguous. There was no linear association between IBS prescriptions and per capita income by county.

There were 868 refugees admitted to Michigan in 2005. Of those, 342 were from Africa, 187 from the former Soviet Union, 167 from East Asia, 93 from the Middle East, 67 from Cuba, and 14 from Bosnia. Ingham County accepted 349 (40%) refugees. Thirteen other counties accepted at least one refugee. Sixty-nine counties did not

Table 1 *Giardia lamblia* incidence by refugees admitted per county in 2005

County	<i>Giardia lamblia</i> cases	Incidence per 100 000	Refugees admitted
Calhoun	7	5	12
Genesee	12	3	45
Ingham	121	43	349
Kent	86	15	236
Macomb	32	4	17
Oakland	72	6	117
Saginaw	15	7	11
Saint Clair	14	9	10
Wayne	77	4	53

receive any refugees that year. Of the nine counties that took at least 10 refugees, the number of *Giardia lamblia* cases ranged from 7 to 121, and the incidence was between 0.003% and 0.043% (Table 1).

Graphing the number of *Giardia lamblia* infections versus the number of IBS prescriptions revealed no linear relationship (Figure 4). The simple linear regression estimate provided an R-square of 0.001. Similarly, looking at total foodborne illness versus IBS prescriptions, no significant linear association was seen.

Per capita income was not significantly linearly associated with either Giardiasis or IBS prescriptions. The number of refugees admitted did show an association with the incidence of *Giardia lamblia* by county in 2005 (R-square = 0.221, $P < 0.001$).

DISCUSSION

This study examined the evidence for an association between the parasite *Giardia lamblia* and IBS, but found

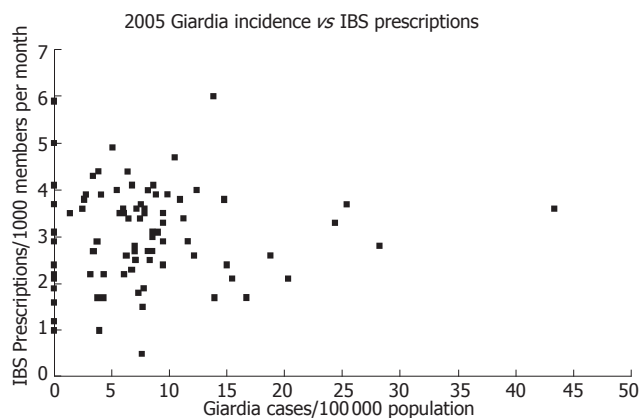


Figure 4 Michigan *Giardia lamblia* incidence versus BCBS prescriptions for IBS.

none. This lack of association is contrary to multiple previous studies linking acute infectious gastroenteritis with IBS and is therefore notable. There were 786 cases of Giardiasis reported in Michigan in 2005, which is a rate of 7.8 per 100 000 people. In comparison, in 2005, Ohio had a rate of 7.1, and Wisconsin had 13.0 per 100 000^[5]. The rate of cases among different counties in Michigan varied from zero up to 43 per 100 000 (Figure 5). In the US as a whole, the incidence in 2005 was 6.1 per 100 000 people^[5]. Ingham County had the highest activity in Michigan, and also the highest number of refugees admitted in 2005, when it received 349 of the 868 total refugees that entered the state (Personal communication, Al Horn, Director of the Office of Refugee Services of the Michigan Department of Human Services). There was a positive linear association between the incidence of Giardiasis by county and the number of refugees admitted in 2005 ($P < 0.001$). This supports the previously described increased risk of Giardiasis in immigrants from developing countries^[3].

Previous studies have posited a causal link between *Giardia lamblia* and IBS through inflammation of the mucosal lining of the gut^[10,12]. *Giardia lamblia* is also known to cause disaccharide intolerance for up to 6 mo after infection^[4]. This increases intestinal gas, causing painful distention of the colon, which could be misdiagnosed as IBS. These theoretical mechanisms need not be restricted to *Giardia lamblia*. Other parasites have been studied in relation to IBS, including *Entamoeba histolytica*^[17], *Dientamoeba fragilis*^[18], and *Blastocystis hominis*^[19-21], but none of these are reportable diseases in Michigan. Likewise acute bacterial gastroenteritis epidemics with *Salmonella enteritidis*, *Escherichia coli*, and *Campylobacter jejuni* have been shown to increase subsequent levels of IBS in those populations exposed^[22,23]. It might be expected, however, that Giardiasis alone, or foodborne illnesses in total, could act as a proxy for the myriad of parasitic and bacterial illnesses which are associated with IBS, since conditions which lead to high prevalence of one foodborne illness are likely to lead to increases in others as well. This study does not support such an association between *Giardia lamblia*, or foodborne illness in general, and IBS. It is possible that this lack of association is due to the relationship between high refugee numbers and the incidence of *Giardia lamblia* in Michigan.

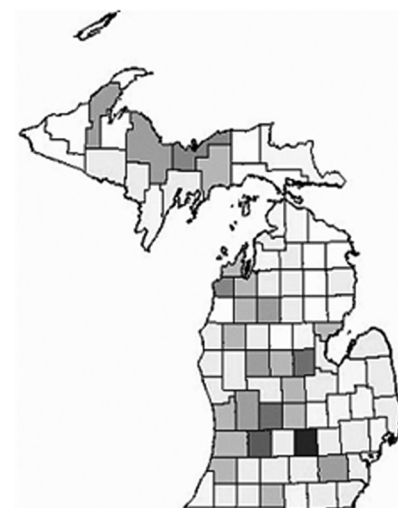
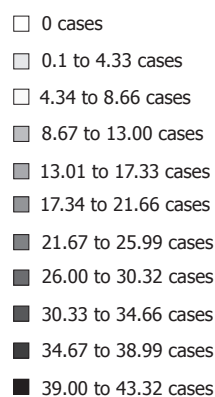


Figure 5 Map of Michigan counties by incidence of *Giardia lamblia* cases per 100 000 persons: Michigan Department of Community Health, unpublished data.

The refugee population may not have full access to regular health care, and so may be unlikely to receive a diagnosis of IBS, even if they meet the diagnostic criteria. There may also be a lag time between the onset of IBS and the use of disease-specific medication, and if this is true, then the large influx of refugees with Giardiasis in 2005 might not show up as an increase in IBS prescriptions until 2006 or later.

One limitation of this study is that BCBS prescription numbers may not accurately define the prevalence of IBS, nor do they distinguish between recent onset IBS and chronic disease. Also, although Giardiasis is a reportable disease, the collection of cases is by passive and not active surveillance, and the percentage of infections diagnosed and reported may vary greatly between counties. It seems reasonable, however, that these under-estimates in prevalence of IBS and incidence of *Giardia* might average out over the state as a whole. Finally, the 2005 Michigan population data, used to determine incidence, was necessarily an estimate, since the US census is only done every ten years. Despite these limitations, this ecological study is a first step in attempting to go beyond case reports and small clinical studies to define the true association between a parasite and IBS.

The lack of a known etiologic factor for IBS, the similarity of symptoms between IBS and Giardiasis, and the large number of undiagnosed *Giardia lamblia* infections make it tempting to look for a causal link between these two diseases, but at present, there is not enough evidence to prove one exists. Prospective studies, in a young, healthy, population at risk for IBS, with routine examination of stools for parasites, and collection of IBS symptom data, would make a tremendous contribution in this area.

COMMENTS

Background

This article concerns the possible link between an acute parasitic illness, *Giardia lamblia*, and subsequent development of a chronic disease, irritable bowel syndrome (IBS). *Giardia* is a common parasite, transmitted through ingestion of

the cyst form, which causes symptoms of diarrhea, bloating, mucus and abdominal discomfort in its victims.

Innovations and breakthroughs

Previous research has suggested associations of Giardia, other parasites, and some bacteria, with IBS. Mearin *et al* (*Gastroenterology* 2005; 129: 98-104) found a relative risk of IBS at one year after acute infection with *Salmonella enteritidis* to be 7.8 times that of the unexposed population. Two or three years after presumed infection with *Escherichia coli* 0157:H7 or *Campylobacter jejuni* gastroenteritis, Marshall *et al* (*Gastroenterology* 2006; 131: 445-450) found a rate of IBS 4.8 higher than expected. An earlier study of newly diagnosed Giardia cases in Italy by D'Anchino, Orlando and DeFeudis (*Journal of Infection* 2002; 45:169-172) noted that 82% of the patients had had at least six months of IBS symptoms before Giardia was diagnosed, suggesting that IBS predisposes patients to being symptomatic during Giardia infestation, and not that Giardia causes IBS. Whether Giardia and other intestinal infections actually cause IBS, or whether patients with IBS are simply more likely to be symptomatic during episodes of gastrointestinal infection has important implications for the diagnosis and treatment of IBS.

Applications

This study looked at rates of prevalence of IBS by county in Michigan, and compared that to incidence of newly diagnosed Giardia cases. No association was found, which implies that Giardia does not cause a significant number of cases of IBS.

Peer review

This is an important and interesting topic but were concerned that the ecological methodology of the study made the conclusions weaker than they would otherwise be.

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Prognostic value of ^{13}C -phenylalanine breath test on predicting survival in patients with chronic liver failure

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Abstract

AIM: To evaluate the prognostic value of percentage of ^{13}C -phenylalanine oxidation (^{13}C -PheOx) obtained by ^{13}C -phenylalanine breath test (^{13}C -PheBT) on the survival of patients with chronic liver failure.

METHODS: The hepatic function was determined by standard liver blood tests and the percentage of ^{13}C -PheOx in 118 chronic liver failure patients. The follow-up period was of 64 mo. Survival analysis was performed by the Kaplan-Meier method and variables that were significant ($P < 0.10$) in univariate analysis and subsequently introduced in a multivariate analysis according to the hazard model proposed by Cox.

RESULTS: Forty-one patients died due to progressive liver failure during the follow-up period. The probability of survival at 12, 24, 36, 48 and 64 mo was 0.88, 0.78, 0.66, 0.57 and 0.19, respectively. Multivariate analysis demonstrated that Child-Pugh classes, age, creatinine and the percentage of ^{13}C -PheOx (HR 0.338, 95% CI: 0.150-0.762, $P = 0.009$) were independent predictors of survival. When Child-Pugh classes were replaced by all the parameters of the score, only albumin, bilirubin, creatinine, age and the percentage of ^{13}C -PheOx (HR 0.449, 95% CI: 0.206-0.979, $P = 0.034$) were found to be independent predictors of survival.

CONCLUSION: Percentage of ^{13}C -PheOx obtained by ^{13}C -PheBT is a strong predictor of survival in patients with chronic liver disease.

INTRODUCTION

The identification of patients with poor prognosis is of crucial importance, especially since liver transplantation has emerged as an important therapy for patients with advanced cirrhosis^[1]. The exact prediction of survival for an individual patient with cirrhosis is not easy. This may be one of the reasons to explain why so many studies have investigated factors which predict survival of these patients^[2-4].

In recent years, growing interest has been devoted to the quantitative liver function tests, as they are expected to increase the accuracy of estimating the severity of liver disease; however, several studies on their prognostic value have shown contradictory results^[5-8].

C-phenylalanine oxidation (^{13}C -PheOx) is a valuable indicator of liver function. It represents the cytosolic enzyme activity, is a non-invasive test, easy to perform and distinguishes patients with various degrees of liver disease from otherwise healthy persons. Some studies have shown that the severity of liver cirrhosis correlates with the suppression of $^{13}\text{CO}_2$ recovery after a dose of phenylalanine; other studies have reported that as liver function worsens, as defined by the Child-Pugh (CP) score, so does the phenylalanine metabolism^[9-14]. Nowadays, the CP score is still the most widely used tool to estimate the severity of liver disease in patients with cirrhosis and to predict survival.

The prognostic value of ^{13}C -phenylalanine breath test (^{13}C -PheBT) for survival in patients with chronic liver disease is yet to be established. Therefore, the aim of this study was to evaluate the prognostic value of percentage of ^{13}C -PheOx obtained by ^{13}C -PheBT for the survival of patients with chronic liver failure.

MATERIALS AND METHODS

Patients

Consecutive patients with chronic liver failure were studied at the Laboratory of Gastro-Hepatology of Centro Médico Nacional Siglo XXI. The study was approved by the Ethical Committee of the hospital. Patients were included according to the following criteria: age above 18 years, both genders; diagnosis of chronic liver failure based on history, clinical and biochemical findings combined with ultrasonographic results, plus liver biopsy when possible; and written informed consent of patients when entering the study. Exclusion criteria were pulmonary alterations, neurologic diseases different to encephalopathy; participation in other studies during the preceding thirty days of this study; and presence of other diseases conditioning a short prognosis by themselves (e.g. carcinoma). According to these criteria, 121 patients were selected from a group of 136 patients with chronic liver disease after having excluded 9 patients with hepatocellular carcinoma, 5 patients because of their unwillingness to participate and one patient with a percentage of ^{13}C -PheOx > 17 .

The etiology was defined on the basis of the history obtained from patients and their relatives and serological tests for viruses. The cause of liver disease was chronic alcohol consumption (≥ 30 g/d for 2 years or more) in 23 (19.5%) patients, chronic type-C hepatitis/HVC in 56 (47.5%), mixed (viral + alcohol) in 8 (6.8%) and other causes (cryptogenic, autoimmune, Budd Chiari syndrome, primary biliary cirrhosis, chronic type-B hepatitis/HBV-related cirrhosis and idiopathic) in 31 (26.3%). No patients had hemochromatosis or Wilson's disease.

Methods

All patients were studied following the same protocol with data collected by the laboratory staff and were followed up either as outpatients or inpatients when necessary, according to general medical practice. Survival time was accounted until March 2005. The hepatic function was evaluated by ^{13}C -PheBT and standard liver blood tests. All patients were classified by the CP score as class A (5-6 points), class B (7-9 points) or class C (10-15 points)^[15]. This classification comprises albumin, total bilirubin (TB), prothrombin time (PT), ascites and encephalopathy. Ascites was classified as "absent" or "present" according to clinical examination and ultrasonographic findings. The clinical diagnosis and degree of encephalopathy were determined according to the West-Haven criteria^[16]. Other tests were alanin transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (AP), creatinine and glucose. Biochemical data were evaluated by standard clinical chemical methods (Dimension, ARXL-Max DADE[®], Boehringer, Germany).

^{13}C -PheBT was measured following an overnight fast without control of prior dietary intake. A 100-mg oral dose of L-[1- ^{13}C] phenylalanine-isotopic purity 99% ^{13}C (Isotec[®] Inc, Ohio, USA)-dissolved in 50 mL water was administered. Alveolar breath samples were collected while in resting position and following a normal exhalation. At each sample time, patients were asked to blow directly into a 10-mL exstainer tube (Labco Limited[®], Buckinghamshire,

U.K.) through a straw. Duplicate breath samples were taken before administration of the ^{13}C -phenylalanine dose (basal), and every 10 min thereafter until completion of 1 h. Enrichment of $^{13}\text{CO}_2$ was determined by isotope ratio mass spectrometry (BreathMat-plus[®] Finnigan Bremen, Germany). The rate of hepatic ^{13}C -phenylalanine oxidation at each time point was calculated from the appearance of $^{13}\text{CO}_2$ on exhaled air, assuming a CO_2 production rate of 300 mmol/m² body surface area per hour, as described by Shneider *et al*^[17]. The analytical data were expressed as percentages of the ^{13}C -phenylalanine dose metabolized per hour (percentage of ^{13}C -phenylalanine oxidation/ ^{13}C -PheOx)^[10-12]. The day when patients first presented for the registration of clinical and laboratory data and the measurement of ^{13}C -PheBT was considered as "zero time" for the follow-up period of observation.

Statistical analysis

Results were expressed as percentages and mean \pm SD. Receiving operating characteristic (ROC) analysis was used to define the optimal percentage of ^{13}C -phenylalanine oxidation cut-off point with the highest sensitivity and specificity among thresholds. The analysis was carried out in two steps. To identify independent prognostic variables, a univariate analysis was performed with the Kaplan-Meier statistics. The curves were compared using the log-rank test. During the first stage, covariates analyzed for inclusion in the model were age, sex, etiology, pharmacologic treatment, previous hemorrhage, creatinine, glucose, ^{13}C -phenylalanine oxidation, CP score and complications. Variables that were significant ($P < 0.10$) in the univariate analysis were subsequently introduced in a multivariate analysis. Then each chosen covariate was reconsidered and eliminated if $P > 0.05$. The procedure was performed stepwise until no further covariates could be added or removed according to the afore-mentioned criteria.

In a second step, the same Cox model analysis was performed substituting the CP score with the five variables (albumin, bilirubin, prothrombin time, ascites and encephalopathy) that define the score.

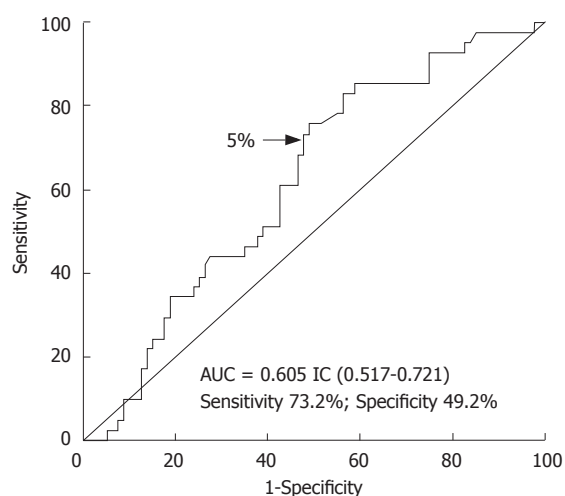
To check the proportionality of the hazard in time for the different functional classes, log of the cumulative hazard was plotted against time, demonstrating a parallel behavior in patients with low and high values for the selected predicting covariates when inspected. The goodness of fit of the model was investigated by a partial likelihood function and the Akaike's information criterion (AIC). The decision to include or to exclude the respective regressor variables was based on a χ^2 test^[18-20]. Statistical analysis was carried out by using the STATA V 8.0 statistical package (StataCorp LP, College Station, Tex).

RESULTS

One hundred and twenty-one consecutive patients with chronic liver failure were included. Cirrhosis was confirmed by liver biopsy in 35 (28.9%) cases, and the constellation of typical physical signs, such as ascites, oesophageal varices, upper gastrointestinal bleeding, and

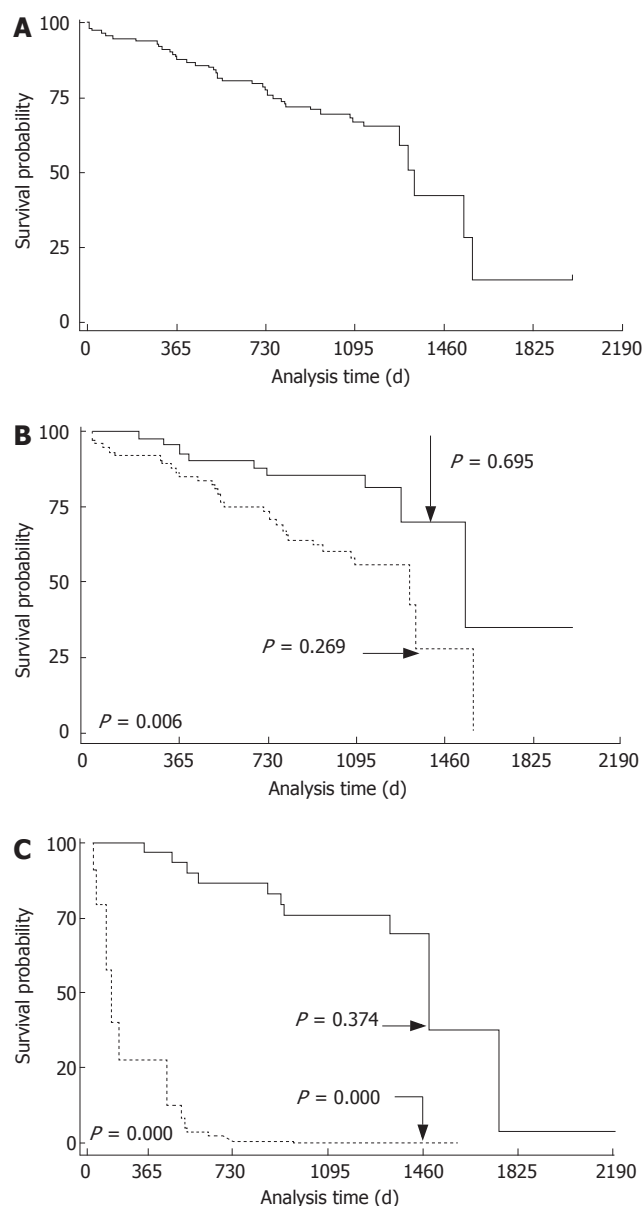
Table 1 Characteristics of 118 patients with chronic liver failure ($n = 118$, mean \pm SD)

Variables	Surviving $n = 77$	Non-surviving $n = 41$	<i>P</i> value
Age (yr)	51.8 \pm 10.6	55.8 \pm 9.6	0.043
Sex (F/M)	49/28	22/19	0.292
Etiology			
Alcoholic/others	15/62	8/33	0.997
Alcohol consumption	38	18	0.573
Pharmacological treatment	71	41	0.067
History of upper GI hemorrhage	53/24	28/13	0.952
Ascites	26	24	0.010
Encephalopathy	7	6	0.360
Albumin (g/L)	34 \pm 6.0	29 \pm 4.6	0.000
Total bilirubin (mmol/L)	27.4 \pm 17.1	58.1 \pm 99.2	0.059
Prothrombin time (%)	76.2 \pm 18.9	67.6 \pm 25.1	0.058
ALT (nkat/L)	1.2 \pm 1.14	0.99 \pm 0.81	0.247
AST (nkat/L)	1.3 \pm 0.99	1.63 \pm 0.81	0.370
AP (nkat/L)	4.18 \pm 3.38	4.50 \pm 3.41	0.623
Creatinine (mmol/L)	13.7 \pm 3.4	22.2 \pm 30.8	0.100
Glucose (mmol/L)	6.3 \pm 2.4	5.8 \pm 1.2	0.216
Child Pugh class			
Class A	45	6	0.000
Class B	23	26	
Class C	9	9	
Child-Pugh score (points)	6.7 \pm 1.9	8.3 \pm 2.1	0.000
¹³ C-phenylalanine oxidation (%)	4.9 \pm 2.9	3.8 \pm 2.2	0.027

**Figure 1** (ROC) curve for percentage of ¹³C-phenylalanine oxidation in predicting mortality (The optimal cut off point with the highest sensitivity and specificity for survival in 118 patients with chronic liver failure was 5.0%).

typical laboratory findings, was accepted as evidence of chronic liver failure in the other 86 (71.0%) patients. During the follow-up period, 44 (20 males, 24 females) patients died. Causes of death were liver failure in 9 (7.6%) patients, upper gastrointestinal bleeding in 10 (8.5%), ascites in 6 (5.1%) and encephalopathy in 8 (6.8%) associated to liver failure, hepatorenal syndrome in 4 (3.4%) and hepatocellular carcinoma in 4 (3.4%), whereas 3 patients died of other causes (accident, transplant surgery and gastric cancer). These three patients were excluded from the further analysis.

Table 1 depicts the demographic and clinical data, biochemical features and liver function tests of patients. The ROC curves showed that the optimal percentage of

**Figure 2** Kaplan-Meier statistics for survival probability. **A:** All patients; **B:** Percentage of ¹³C-phenylalanine oxidation; **C:** Percentage of ¹³C-phenylalanine oxidation adjusted for covariables (age < 60 years and \geq 60 years, albumin, total bilirubin and creatinine). *P* values are given by nonparametric log-rank test.

¹³C-PheOx cut-off value for predicting survival in liver disease patients was 5.0% (sensitivity 73.2%; specificity 49.3%) (Figure 1). Serum albumin, total bilirubin and PT were clustered on the basis of the CP score. Ascites and encephalopathy were clustered as “absent” or “present”. The median probability of survival after entering the study in all the included patients was 1316 d (Figure 2A). The correlation between the percentage of ¹³C-PheOx (< 5.0% and \geq 5.0%) and survival was highly significant ($P < 0.006$; Figure 2B). Likewise, the results of this study confirmed that percentage of ¹³C-PheOx was correlated with the CP score ($r = -0.255$, $P = 0.005$).

Eleven of the twenty investigated variables showed an independent association to poor prognosis in the univariate analysis. The variables: albumin ($P < 0.000$), total bilirubin ($P < 0.000$), prothrombin time (%) (PT) ($P < 0.000$), creatinine ($P < 0.000$), ascites at follow-up ($P < 0.000$),

Table 2 Variables with an independent prognostic value, as indicated by two Cox's models in patients with chronic liver failure ($n = 118$)

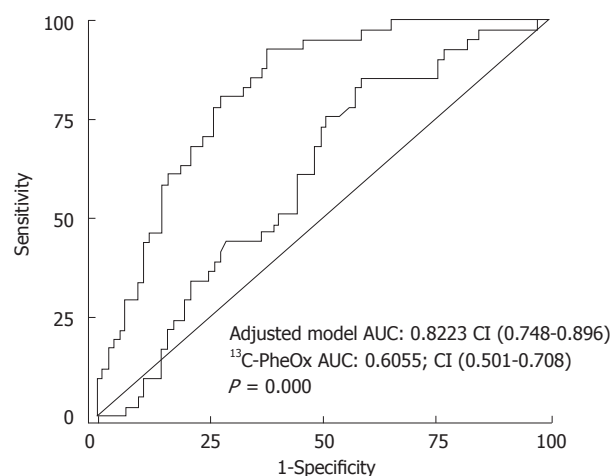
Variables	Coefficient	Hazard ratio ¹	SE	95% CI	P value
1					
Albumin (g/L)	-1.254	0.285	0.085	0.158-0.512	0.000
Total bilirubin (mmol/L)	0.149	1.161	0.051	1.065-1.265	0.001
Creatinine (mmol/L)	0.510	1.666	0.263	1.222-2.270	0.001
Age					
≤ 60 yr	1.000 ²				
> 60 yr	0.765	2.150	0.779	1.056-4.377	0.035
¹³ C cumulative dose					
< 5.0%	1.000 ²				
≥ 5.0%	-0.798	0.449	0.178	0.206-0.979	0.044
2					
Child Pugh class					
A	1.000 ²				
B	1.629	5.099	2.382	2.040-12.743	0.000
C	2.078	7.993	4.627	2.570-24.859	0.000
Creatinine (mmol/L)	0.417	1.518	0.200	1.173-1.966	0.002
Age,					
≤ 60 yr	1.000 ²				
> 60 yr	1.029	2.799	1.044	1.347-5.816	0.006
¹³ C cumulative dose					
< 5.0%	1.000 ²				
≥ 5.0%	-1.081	0.338	0.140	0.150-0.762	0.009

¹Hazard ratio of Cox's proportional hazard regression; ²Reference category.

complications at follow-up ($P < 0.000$), encephalopathy at follow-up ($P = 0.012$), upper gastrointestinal bleeding at follow-up ($P = 0.015$), age ($P = 0.034$) AST ($P = 0.081$) and ascites when entering the study ($P = 0.042$). The variables with no independent association to prognosis: ALT ($P = 0.135$), patients' entry to the hospital at follow-up ($P = 0.146$), AP ($P = 0.198$), treatment of portal hypertension ($P = 0.213$), history of encephalopathy ($P = 0.424$), gender ($P = 0.437$), history of upper gastrointestinal bleeding ($P = 0.464$), etiology ($P = 0.478$) and alcohol consumption ($P = 0.804$).

The multivariate analyses demonstrated that the Child-Pugh classes, age (< 60 years and ≥ 60 years), creatinine and the percentage of ¹³C-PheOx were independent predictors of survival. When the Child-Pugh classes were replaced by all the parameters of the score, only albumin, bilirubin, creatinine, age < 60 years and ≥ 60 years, and the percentage of ¹³C-PheOx were found to be independent predictors of survival. Etiology of liver disease, gender, history of gastrointestinal bleeding, ascites, encephalopathy and prothrombin time were not significant predictors of survival after adjusting for the other explanatory variables on the model. Statistical parameters for the variables included in the final Cox model are depicted in Table 2. The first Cox's model included significant variables contained in Child-Pugh score and the second model included Child-Pugh classes.

A prognostic index (PI) predicting death was derived from the best model as: $PI = \exp(-0.798 \times ^{13}C\text{-PheOx}) + (0.765 \times \text{age}) + (-1.25 \times \text{albumin}) + (0.149 \times \text{bilirubin}) + (0.510 \times \text{creatinine})$. We attributed a value of 0 for ¹³C-PheOx < 5% and 1 for ¹³C-PheOx ≥ 5%, 0 for male sex and 1 for female sex, 0 for age < 60 years and 1 for

**Figure 3** (ROC) curve for percentage of ¹³C-phenylalanine oxidation and percentage of ¹³C-phenylalanine oxidation adjusted for age (age < 60 years and ≥ 60 years), albumin, total bilirubin and creatinine in predicting mortality ($n = 118$).

age ≥ 60 years. The relationship between PI due to the best adjusted model and risk of death were compared with a PI for percentage of ¹³C-PheOx (Figure 2C). The ROC curve for PI of adjusted model for predicting death from liver failure always depicted a better performance than that obtained from percentage of ¹³C-PheOx alone (Figure 3). Areas under the curve were also significantly larger (AUC = 0.822, 95% CI: 0.748-0.896 *vs* AUC = 0.605; 95% CI: 0.501- 0.708, $P = 0.000$).

DISCUSSION

This study shows that the percentage of ¹³C-PheOx is an important prognostic factor of long-term survival in patients with chronic liver failure. In fact, values lower than 5.0% for the percentage of ¹³C-PheOx were associated with an elevated probability of dying and values over 5.0% were associated with a better outcome (probability 0.26 *vs* 0.69 at 48 mo and 0.00 *vs* 0.37 at 64 mo). In addition, predictive value was conserved even after adjusting for covariates (Figure 2B-C). Furthermore, percentage of ¹³C-PheOx added new prognostic information to that obtained by CP classification or the common clinical and biochemical data included in the CP score. The results of this study additionally confirm that percentage of ¹³C-PheOx correlates inversely with the CP score ($r = -0.2550$, $P = 0.0053$). These data are consistent with other studies showing that ¹³C-PheBT correlates with parameters reflecting the severity of hepatic diseases, including albumin, total bilirubin, PT and CP score^[12,21-24].

Additionally, some studies have documented that percentage of ¹³C-PheOx values are significantly lower in patients with liver cirrhosis than in healthy adults^[12,14,23]. It has also been suggested that the decreased ability of decompensated livers to oxidize phenylalanine may be the result of progressive liver damage, individual cellular function; low activities of phenylalanine hydroxylase (PAH) and p-hydroxyphenylpyruvate hydroxylase; the severity or the course of liver disease that produced a decrease

in the total number of cells and the functioning liver cell mass with a consequent reduction in phenylalanine metabolism^[10,12,14,23]. Likewise, the percentage of ^{13}C -PheOx in patients with cirrhosis was estimated to be 20% of the normal value, suggesting that reduced enzyme levels account for a decreased metabolism in phenylalanine^[13]. Hehir *et al.*^[9] described that the fractional clearance rates of aromatic amino acids in plasma of patients with acute fulminant liver disease are 2 to 10 times lower than those in normal subjects.

Other authors have demonstrated that ^{13}C -PheBT is used to monitor the clinical course of patients with chronic liver failure^[13,25], as a clinical predictor to assess postoperative early complications in patients undergoing hepatectomy^[26] or to evaluate the restoration of the plasmatic phenylalanine clearance to normal during the postoperative period that is attributed to the ability of the new liver to catabolize this amino acid^[9]. In an experimental model, ^{13}C -PheBT was used to monitor the hepatic dysfunction associated with obstructive jaundice^[27]. However, there is scarce information on the prognostic value of ^{13}C -PheBT with regard to the survival of patients with cirrhosis. Our study suggests that the percentage of ^{13}C -PheOx adds new prognostic information to that obtained by the CP score or the common clinical and biochemical data included in the CP score. Nevertheless, discrepant results were recently obtained by Koeda *et al.*^[24] who could not demonstrate a correlation of ^{13}C -PheBT with mortality. They examined 23 patients with liver cirrhosis, 6 of them died of hepatic dysfunction in a follow-up period of 816 d. The lack of significance of ^{13}C -PheBT in their study may have probably related, at least in part, to the fact that less end-points were analyzed. It has been clearly established that the number of end-points heavily influences the ability to detect significant effects^[24,2].

In contrast to previous reports, ^{13}C -PheOx constituted an important prognostic index in our series. In fact, mortality due to liver-related causes was best predicted by a prognostic index containing albumin, bilirubin, creatinine, age, and ^{13}C -PheBT than by a prognostic index containing the CP score, creatinine, age and ^{13}C -PheBT, as assessed by a partial likelihood function and the Akaike's information criterion (AIC). However, there are no more data available on the prognostic value of ^{13}C -PheOx.

A limitation in our study could be that it was not possible to calculate a model for end-stage liver disease (MELD)^[28] because we did not perform the international normalized ratio of prothrombin time (INR) in all patients. MELD score is superior to CP score as a predictor of intermediate mortality is unclear^[29].

Even when the use of ^{13}C -breath tests with various substrates is not a novelty in hepatology, the clinical usefulness of these tests that explore the hepatocellular subfunctions (microsomal, cytosolic or mitochondrial) as well as the superiority of some quantitative prognostic liver function tests compared with the CP score are still unclear^[30,31].

Aminopyrine breath test (ABT) has been used to predict short-term prognosis and mortality in patients with alcoholic hepatitis. There are contradictory results of ABT

for predicting survival in patients with cirrhosis. Some studies showed that ABT is better than the Child-Turcotte score, whilst others demonstrate that ABT is not better. However, some other studies seem to provide additional prognostic information to the CP score^[31-34].

Galactose elimination capacity (GEC), a valuable indicator of liver function, is dependent on hepatic blood supply and inhibited by alcohol consumption. It has been demonstrated that GEC adds some more prognostic information when the CP score is not included; whereas, other studies reported that, GEC is unable to improve the prognostic ability of GEC^[8,35-37].

The caffeine breath test (CBT) represents a valid indicator of plasma caffeine clearance (CC) and correlates with the varying degrees of liver dysfunction. However, both tests do not appear to add prognostic information beyond that provided by the CP classification. CBT and CC may decrease with increasing age, cigarette smoking and disease state^[38-40].

Indocyanine green elimination (IGC) provides some prognostic information related to survival of patients with cirrhosis, but does not improve the predictive ability of clinical information considered for the CP score. It is also sensible to hepatic blood flow, and adversely affected by cardiovascular drugs (calcium channel blockers) and is significantly reduced after portosystemic shunting^[6,8,41,42].

On the other hand, the interest in search for accurate prognostic tests for patients with chronic liver disease is still of utmost importance since the emergence of liver transplantation is an important therapy for patients with advanced cirrhosis^[1].

In conclusion, ^{13}C -PheBT is a strong predictor of survival in patients with chronic liver failure that adds information which may not be available from the common clinical and biochemical data included in the CP score for the assessment of the risk of death due to liver disease. Should these data be confirmed in other studies and different settings, the ^{13}C -PheBT could prove to be a useful clinical tool to routinely evaluate the prognosis of patients with chronic liver disease in addition to the common clinical and biochemical data, because of its non-invasiveness and safety.

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COMMENTS

Background

In recent years, there has been a growing interest in quantitative liver function tests, including breath tests, since they are expected to increase the accuracy of estimating the severity of liver disease. L-[1- ^{13}C] phenylalanine breath test ^{13}C -PheBT distinguishes patients with various degrees of liver disease from healthy persons. However, the prognostic value of ^{13}C -PheBT for survival of patients with chronic liver disease is yet to be established.

Research frontiers

Even when the use of ^{13}C -breath tests with various substrates is not a novelty in hepatology, the clinical usefulness of these tests that explore the hepatocellular subfunctions (microsomal, cytosolic or mitochondrial) and measure of hepatocyte

functional capacity in liver diseases as well as the prognostic value of ^{13}C -breath tests compared with the Child-Pugh (CP) or model for end-stage liver disease (MELD) are still unclear.

Innovations and breakthroughs

The current study provides evidence that ^{13}C -PheBT is a strong predictor of survival in patients with chronic liver failure and adds information which may not be available from the common clinical and biochemical data included in the CP score for the assessment of the risk of death due to liver disease.

Applications

^{13}C -PheBT could prove to be a useful clinical tool to routinely evaluate the prognosis of patients with chronic liver disease in addition to the common clinical and biochemical data, because of its non-invasiveness and safety.

Terminology

L-[1- ^{13}C] phenylalanine breath test measures hepatocyte functional capacity by estimating the oxidation of L-[1- ^{13}C] phenylalanine and represents the hepatic cytosolic enzyme activity.

Peer review

This manuscript describes the prospective evaluation of ^{13}C -phenylalanine breath test in the evaluation of prognosis in cirrhotic patients.

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RAPID COMMUNICATION

Hypoestoxide inhibits tumor growth in the mouse CT26 colon tumor model

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Abstract

AIM: To evaluate the effect of the natural diterpenoid, hypoestoxide (HE) on the growth of established colon cancer in mice.

METHODS: The CT26.WT mouse colon carcinoma cell line was grown and expanded *in vitro*. Following the expansion, BALB/c mice were inoculated s.c. with viable tumor cells. After the tumors had established and developed to about 80-90 mm³, the mice were started on chemotherapy by oral administration of HE, 5-fluorouracil (5-FU) or combination.

RESULTS: The antiangiogenic HE has previously been shown to inhibit the growth of melanoma in the B16F₁ tumor model in C57BL/6 mice. Our results demonstrate that mean volume of tumors in mice treated with oral HE as a single agent or in combination with 5-FU, were significantly smaller (> 60%) than those in vehicle control mice (471.2 mm³ vs 1542.8 mm³, $P < 0.01$). The significant reductions in tumor burden resulted in pronounced mean survival times (MST) and increased life spans (ILS) in the treated mice.

CONCLUSION: These results indicate that HE is an effective chemotherapeutic agent for colorectal cancer in mice and that HE may be used alone or in combination with 5-FU.

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Key words: Hypoestoxide; 5-Fluorouracil; Colon, Cancer; Mice

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INTRODUCTION

The clinical usefulness of chemotherapy against advanced solid tumors is limited by host toxicity and tumor resistance. Combination chemotherapy is an approach to meeting this challenge. Colorectal carcinoma is the second most common cause of cancer deaths in the United States and is responsible for the deaths of over 55 000 patients annually^[1]. 5-fluorouracil (5-FU) has long been recognized as standard chemotherapy for colorectal cancer^[2] and has been used either alone or in combination with newer chemotherapeutics such as irinotecan and oxaliplatin^[3]. Both these drugs are believed to increase efficacy but also cause more toxicity. The predominant side effects of 5-FU are diarrhea, anorexia, enteritis, hand-foot syndrome and myelosuppression. While the combination of other drugs with 5-FU has improved overall patient survival, it has also been associated with more severe side effects^[4]. Modalities that include low doses of 5-FU and combination with low doses of an effective nontoxic drug which decrease or eliminate toxicity and at the same time enhance overall efficacy, would greatly advance the treatment for colorectal cancer and other malignancies. To this end, HE is an ideal candidate.

HE is a novel natural diterpenoid with anti-tumor^[5], anti-inflammatory^[6], and anti-parasitic activities^[7]. We examined the effect of HE alone or in combination with a very low dose (25 mg/kg) of 5-FU and a low dose of HE (1 mg/kg) upon efficacy in 5-FU therapy for CT26, a mouse colon cancer model.

MATERIALS AND METHODS

Experimental animals

Female BALB/c mice were purchased from Charles River Laboratories, Wilmington, MA. The thirty-five female 6-8 wk old mice were maintained on a standard laboratory chow and under pathogen-free conditions according to institutional regulations in facilities approved by the American Association for Accreditation of Laboratory Animal Care.

Reagents

HE was prepared in our laboratory with modifications, as previously reported^[8]. 5-FU was purchased from Sigma-

Table 1 Oral administration of HE inhibits the growth of s.c. implanted CT26 colon tumor in BALB/c mice

Drug	Dose (mg/kg per day)	Tumor volume (mm ³)		ILS (%)
		(d 18) (mean \pm SE)	MST (d) (mean \pm SE)	
"Vehicle control (PBS/DMSO)"	0	1542.8 \pm 330	34.0 \pm 6.2	0
HE	1 \times 10	738.7 \pm 158	43.0 \pm 1.2	25
HE	5 \times 10	582.3 \pm 116	77.0 \pm 4.2	126
HE	50 \times 1	288.6 \pm 188	52.7 \pm 3.0	54
5-FU	25 \times 1	911.1 \pm 144	48.0 \pm 3.6	40
5-FU	100 \times 1	486.7 \pm 171	59.7 \pm 4.8	75
5-FU + HE	(5-FU) 25 \times 1 + (HE) 1 \times 10		57.7 \pm 4.0	69

PBS/DMSO: phosphate buffered saline/dimethyl sulfoxide; MST: mean survival times; ILS: increased life spans. MST: 75.0 \pm 3.8 (HE-treated mice at 5 mg/kg \times 10) *vs* Vehicle control (30.0 \pm 4.1), ILS: 150% (HE-treated mice) *vs* 0% (Vehicle control mice), $P < 0.01$.

Aldrich (St. Louis, MO). Phosphate buffered saline (PBS), Dulbecco's minimal essential medium (DMEM), and other culture media components were purchased from Irvine Scientific (Irvine, CA).

Cell line

CT26.WT mouse colon carcinoma cell line was purchased from ATCC, Manassas, VA. The cell line was grown as monolayer in DMEM culture medium containing 10% FBS and 1% L-glutamine. Full grown monolayer cultures were trypsinized for 15 min (0.25% trypsin-EDTA), harvested and passaged several times for expansion.

Experimental procedure

Following the growth and expansion of the cell line *in vitro*, trypsinized cells were harvested, washed, counted by trypan blue dye exclusion method and cell density was adjusted to 10×10^6 /mL in PBS. A suspension of 2×10^6 viable tumor cells in 0.2 mL PBS was inoculated s.c. into the left flanks of 35 mice. After tumors developed to about (80-90) \pm 10 mm³ volume, the mice were randomized into seven groups as depicted on Table 1. They were treated with varying doses of HE alone, 5-FU alone or combination of lower doses of HE (1 mg/kg) and 5-FU (25 mg/kg) *via* oral administration with a gavage needle attached to a 1.0 cc syringe.

Statistical analysis

A student *t* test was used to determine significance of difference between tumor burdens in vehicle control mice and mice treated with HE as a single agent or in combination with 5-FU. Survival data are presented in days as mean \pm SE.

RESULTS

Mice receiving varying doses of HE, 5-FU or combination experienced significant tumor growth inhibition as compared to controls. Mean % tumor growth inhibition obtained for HE was 65%; 5-FU, 55%; and HE + 5-FU, 82% relative to vehicle control. The additive effect of HE + 5-FU combination resulted in 69% ILS as compared

to 25% ILS for HE and 40% ILS for 5-FU when tested alone at each of their respective lowest doses (Table 1). Treatment was started when tumors had a mean volume of 80-90 mm³. Five female BALB/c mice were allocated to each group. Tumor volume at the start of treatment (d 0) and on d 18 after tumor implantation is shown. MST and ILS (%) were calculated for each group. MST was calculated from the period between tumor implantation and the day of death. ILS (%) was calculated using MST for each drug-treated mouse as follows:

ILS (%) =

$$\frac{[\text{MST of drug-treated mouse} - \text{MST of vehicle control}]}{\text{MST of vehicle control}} \times 100$$

This experiment was conducted twice with similar results.

DISCUSSION

HE is a novel and unique nonsteroidal antiinflammatory drug (NSAID) because it does not inhibit cyclooxygenase (COX) activity^[6]. The mechanism of action that defines NSAIDs as a class is their ability to inhibit COX activity^[9,10]. Several studies have established that numerous NSAIDs such as sulindac, aspirin, celecoxib, piroxicam and ibuprofen inhibit or prevent colorectal neoplasia in rodents and humans because of their ability to inhibit COX activity^[9-11]. HE has been shown in this report to be effective at reducing tumor burden in mice with colorectal cancer and it is thus similar to sulindac sulfide, a metabolite of sulindac sulfoxide, an NSAID which has also been shown to be effective against murine and human colorectal cancer without inhibitory effect on COX activity^[10,12]. The various mechanisms by which HE inhibits tumor growth include its ability to arrest cell cycle at G₂-M phase by interference with actin assembly, inhibit angiogenesis, vascular endothelial growth factor (VEGF)-induced cell proliferation and endothelial cell migration^[5]. All of these mechanisms have been shown to contribute to the treatment of colon cancer^[10-14]. Conversely, 5-FU is known to trigger apoptosis by depleting thymidine, partially through inhibition of thymidine synthase and partially through direct incorporation into RNA and DNA^[15,16]. Because HE lacks alkylating properties^[6], toxicity^[5], and uses several other aforementioned mechanisms, it is therefore consistent that the combination of low doses of HE with low doses of 5-FU enhances the anti-tumor responses of 5-FU.

Interestingly, consumption of the dried leaf powder of *Hypoestes rosea* (the parent plant of HE), as a dietary supplement, resulted in the elimination of existing intestinal polyps in human subjects (Nchekwube, unpublished results).

Collectively, these results indicate that HE may be a promising chemotherapeutic agent either alone or in combination with 5-FU against colorectal cancer.

COMMENTS

Background

Plants and their products have been used for medicinal purposes for thousands of years. Drugs from natural bio-resources have often been discovered on the basis of ethno-botanical information provided by herbalists living in regions of the world rich in bio-resources. Hypoestoxide is an investigational new drug isolated from the plant *Hypoestes rosea* (Acanthaceae) which is indigenous to the rain forest

regions of Nigeria. The natives have long used the *H. rosea* leaf extracts in folk medicine to treat various ailments. In this article, old world and new world medicine are brought together.

Research frontiers

The findings in this article relate well to the present state of the field in regard to the use of natural products as pharmaceutical agents. Artemisinin is an example of a natural product isolated from the Chinese plant, *Artemisia annua*. Currently, Artemisinin is used for the treatment of malaria. Interestingly, Hypoestoxide has also been found to possess anti-malarial activity. Curcumin, a polyphenolic antioxidant from a dietary spice is in clinical development for anti-cancer and anti-inflammatory activities. Vincristine is another example of a natural product (isolated from the rose periwinkle flower) which is used as a standard anti-cancer agent.

Innovations and breakthroughs

The findings are significant and novel because Hypoestoxide is an investigational new drug for which new indications are being sought. This is the first report on both the chemotherapeutic effect of Hypoestoxide on colon cancer and its additive effect with a standard chemotherapeutic agent, 5-Fluorouracil.

Applications

Future applications of the findings in this article will be in the areas of single and combination drug therapies for colorectal cancer. The findings also lend support to the use of NSAIDs such as Hypoestoxide, as anti-cancer agents. NSAIDs have long been associated with tumor chemoprevention and inhibition of the growth of established tumors.

Terminology

NSAIDs are anti-inflammatory drugs that are not steroids. Anti-angiogenesis is the process by which a drug inhibits the growth of an established tumor by shutting off the blood supply to the tumor. CT26 is an N-nitroso-N-methylurethane (NNMU)-induced, undifferentiated colon carcinoma cell line. It was cloned to generate the cell line CT26.WT (ATCC CRL-2638).

Peer review

The paper is well written. Using the simple and reliable methods, the authors studied the effect of hypoestoxide (HE) on mouse colon adenocarcinoma, and give us a definite conclusion: HE is an effective chemotherapeutic agent for colorectal cancer in mice and that HE may be used alone or in combination with 5-FU. The conclusion will help us to deeply understand HE's value in the treatment of colorectal cancer.

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Gastrectomy for patients with gastric cancer and non-uremic renal failure

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Abstract

AIM: To investigate the safety and outcome of gastrectomy for patients with gastric cancer and non-uremic renal failure (NURF).

METHODS: One hundred forty-seven patients who underwent gastrectomy for carcinoma were retrospectively divided into two groups: a group with Ccr values of ≥ 50 mL/min (Group 1; $n = 110$), and one with Ccr values of ≥ 20 to < 50 mL/min (Group 2; $n = 37$). Preoperative patient characteristics, intraoperative parameters (including operation time and blood loss), and postoperative management and complications were evaluated.

RESULTS: There were no statistically significant differences between the two groups in operation time (297.9 min vs 272.6 min, $P = 0.137$) or blood loss (435 mL vs 428 mL, $P = 0.078$). The differences in postoperative complications and hospital stay between the groups were not statistically significant. None of the patients in Group 2 required dialysis therapy, and no patients died due to gastrectomy or gastrectomy-related causes. The overall 4-year survival rates in Groups 1 and 2 were 86.6% and 81.8%, respectively ($P = 0.48$), and the corresponding 4-year disease-free survival rates for stage I, II, and III patients were 88.7% and 83.5%, respectively ($P = 0.65$).

CONCLUSION: Gastrectomy can be performed as safely in patients with NURF similar to patients with normal renal function.

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Key words: Gastric cancer; Non-uremic renal failure; Gastrectomy; Chronic kidney disease

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INTRODUCTION

Gastric cancer is the most common cancer in Japan, and the prognosis of affected patients has been improving^[1,2]. The surgical procedure for gastric cancer consists of gastrectomy, with regional and extended lymph node dissection. Although advances in surgical techniques and management have made it possible to perform gastrectomy safely, renal dysfunction remains a major risk factor for perioperative management.

Renal dysfunction is classified into non-uremic and uremic stages. Patients with non-uremic renal failure (NURF) are defined as having impaired renal function but are dependent on their own kidneys. Patients with NURF require special attention to prevent deterioration of renal function and avoid the need for dialysis therapy when undergoing gastrectomy. Recently, due to the increase of the aged population and the incidence of diabetes mellitus, patients with gastric cancer associated with NURF have been increasing^[3]. However, there have been no published reports focusing on gastrectomy in such patients. In the present study, we retrospectively evaluated the results of pre-, intra-, and postoperative management and the outcome of gastrectomy in patients with gastric cancer associated with NURF to assess the safety of gastrectomy in such patients.

MATERIALS AND METHODS

A total of 147 patients who underwent gastrectomy for carcinoma between January 2003 and December 2005 were included in this study. In all patients, renal dysfunction was evaluated by the creatinine clearance test. NURF was defined on the basis of a creatinine clearance rate (Ccr) of > 20 to < 50 mL/min according to the New York Heart Association criteria^[4,5].

The patients were divided into two groups based on the Ccr value: a group with a Ccr of > 50 mL/min (Group 1; normal renal function: $n = 110$) and a group with a Ccr of > 20 to < 50 mL/min (Group 2; NURF: $n = 37$). The characteristics of the patients are shown in Table 1.

Our treatment strategy for gastric carcinoma in patients

Table 1 Preoperative patients' characteristics

	G1	G2	P-value
Number	110	37	
Sex (male/female)	80/30	27/10	$P = 0.98 (\chi^2)$
Age	66.6 ± 9.4	73.2 ± 9.4	$P < 0.001$
BMI	22.2 ± 3.3	21.5 ± 3.6	$P = 0.24$
BUN (mg/dL)	13.5 ± 4.0	17.0 ± 4.6	$P < 0.001$
Cr (mg/dL)	0.7 ± 0.2	0.9 ± 0.2	$P < 0.001$
Ccr (mL/min)	82.5 ± 15.9	38.1 ± 8.8	$P < 0.001$

Statistical analyses were performed by Mann-Whitney *U* test, except sex. χ^2 : Chi-square test.

Table 2 Surgical procedure *n* (%)

	G1	G2	P-value
Distal gastrectomy	60 (54.5)	20 (54.1)	0.96
Total gastrectomy	44 (40)	15 (40.5)	0.95
Proximal gastrectomy	0	1 (2.7)	0.08
Remnant gastrectomy	6 (5.5)	0	0.33
Partial gastrectomy	0	1 (2.7)	0.08

Table 3 Extent of lymph nodes dissection *n* (%)

		G1	G2	P-value
Distal gastrectomy	D0	53.30	45.00	0.24
	D1	38.30	25.00	0.036
	D2	8.30	30.00	< 0.001
Total gastrectomy	D0	25.00	26.70	0.78
	D1	68.20	40.00	< 0.001
	D2	6.80	33.30	< 0.001

Extents of lymph nodes dissection (D0, D1, D2 were according to The 13th Edition, Japanese Classification of Gastric Carcinoma.

with NURF is to perform gastrectomy with regional lymph node dissection to the same extent as that for patients with normal renal function. We do not reduce the extent of lymph node dissection in patients with NURF. If patients have metastases in organs such as the liver and lung (Stage IV), removal of gastric cancer by distal gastrectomy or total gastrectomy without regional lymph node dissection is performed.

Our management strategy for patients with NURF is to maintain intraoperative and postoperative urine volume at more than 1 mL/kg per hour. To obtain this target, adequate fluid infusion and administration of diuretics are employed.

For adjuvant chemotherapy, we use oral administration of fluorouracil, and for patients whose clinical stage is more advanced than stage III, an intravenous infusion of the anti-neoplastic agents fluorouracil and cisplatin is given.

Patients' preoperative characteristics including serum blood nitrogen urea (BUN) and creatinine (Cr), surgical methods, TNM classification, surgery-related data such as operation time, blood loss, and postoperative complications, were compared between Groups 1 and 2. Postoperative increase of BUN and Cr was calculated

Table 4 TNM classification *n* (%)

	G1	G2
Stage I A	45 (40.9)	7 (18.9)
I B	20 (18.2)	8 (21.6)
Stage II	10 (9.1)	5 (13.5)
Stage III A	13 (11.8)	7 (18.9)
III B	5 (4.5)	1 (2.7)
Stage IV	17 (15.5)	9 (24.3)

Table 5 Perioperative data

	G1	G2	P-value
Operation time (min)	279 (139-643)	259 (130-521)	0.14
Operative blood loss (mL)	324 (26-2314)	250 (42-3251)	0.048 ¹
Operative urine volume (mL/kg per hour)	0.92 (0.25-7.31)	0.71 (0.17-3.03)	0.17
Postoperative complications (%)	18.2	16.2	0.79
Postoperative hospital stay (d)	15 (11-73)	15 (11-80)	0.78

¹Statistically significant.

by dividing the maximum postoperative value by the preoperative value, and multiplying by 100.

The data are expressed as mean \pm SD or median value (minimum-maximum). Statistical analyses for means and medians were performed by Mann-Whitney *U* test. Statistical analyses of surgical procedures, lymph node dissection, and TNM classification were performed using the chi-squared test. The 4-year survival and the 4-year disease-free survival rates were analyzed by the Kaplan-Meier method, and statistical analysis was performed by log-rank test. For the calculation of 4-year disease-free survival, only patients whose clinical stages were I, II, and III in Groups 1 ($n = 93$) and 2 ($n = 28$) were included. Differences at $P < 0.05$ were considered to be significant.

RESULTS

Preoperative clinical data are shown in Table 1. The mean patient age was higher in Group 2 (73.2 ± 9.4 years) than in Group 1 (66.6 ± 9.4 years). Higher Cr and BUN values were observed in Group 2 than in Group 1, and the differences were statistically significant. The Ccr value was significantly lower in Group 2 (38.1 ± 8.8 mL/min) than in Group 1 (82.5 ± 15.9 mL/min).

Table 2 and Table 3 shows details of the surgical procedure and the extent of lymph node dissection. There were 6 cases of remnant gastrectomy in Group 1 and no such cases in Group 2. Also there was one case of partial gastrectomy in Group 2 and no such case in Group 1. However, there were no significant differences in the surgical procedure between the two groups.

The details of the TNM classification are shown in Table 4. The incidence of advanced gastric cancer exceeding stage II was significantly higher in Group 2 than in Group 1 (Group 1; 40.9%, Group 2; 59.5%, $P < 0.05$).

Table 5 shows the operative data. Operation times in Groups 1 and 2 were 279 (139-643) min and 259 (130-521)

Table 6 Postoperative percentile increases in BUN and Cr

	G1	G2	P-value
BUN	19.5% (-55.6-180)	11.5% (-47.6-166.7)	$P > 0.05$
Cr	9.0% (-20.5-700)	0.0% (-20-42.9)	$P > 0.05$

Table 7 Postoperative complications

	G1	G2
Pneumonia	6	3
Pancreatitis	4	0
Anastomotic leakage	4	1
Drain infection	3	0
Catheter infection	2	1
Ileus	1	0
Others	3	3

min, respectively ($P = 0.14$), and operative blood losses were 324 (26-2314) mL and 250 (42-3251) mL, respectively ($P = 0.048$). There were no significant inter-group differences in intraoperative urine volume, occurrence of postoperative complications, or median postoperative hospital stay.

Postoperative percentage increases in BUN and Cr are shown in Table 6. There were no significant increases in BUN and Cr after surgery in either group. Furthermore, none of the patients in either group were placed on hemodialysis after gastrectomy.

Details of postoperative complications are shown in Table 7. The incidences of these complications did not differ between the two groups. No patients died as a result of the gastrectomy procedure or of gastrectomy-related causes.

The 4-year survival rates in Groups 1 and 2 were 86.6% and 81.8%, respectively ($P = 0.48$) (Figure 1A), and the 4-year disease-free survival rates were 88.7% and 83.5%, respectively ($P = 0.65$) (Figure 1B).

DISCUSSION

The critical factor to consider when performing curative gastrectomy for patients with NURF is to perform the procedure safely to prevent deterioration of renal function and avoid the need for dialysis therapy. Patients with NURF have several risk factors for major surgery. It is known that renal dysfunction is frequently associated with cardiac dysfunction, a condition known as a cardio-renal syndrome^[6]. In cardio-renal syndrome, activation of the renin-angiotensin-aldosterone system, imbalance of reactive oxygen species, and irritation of the sympathetic nervous system result in acceleration of coronary sclerosis, cardiac hypertrophy, impairment of cardiac microcirculation, and hypertension^[7]. It is also known that renal anemia is a solid risk factor for cardiovascular dysfunction (cardio-renal anemia syndrome)^[8]. Therefore, when performing major surgery in patients with NURF, careful evaluation of the cardiovascular system is essential.

Patients with NURF have impaired blood-coagulation function^[9]. Increased perioperative bleeding is the main

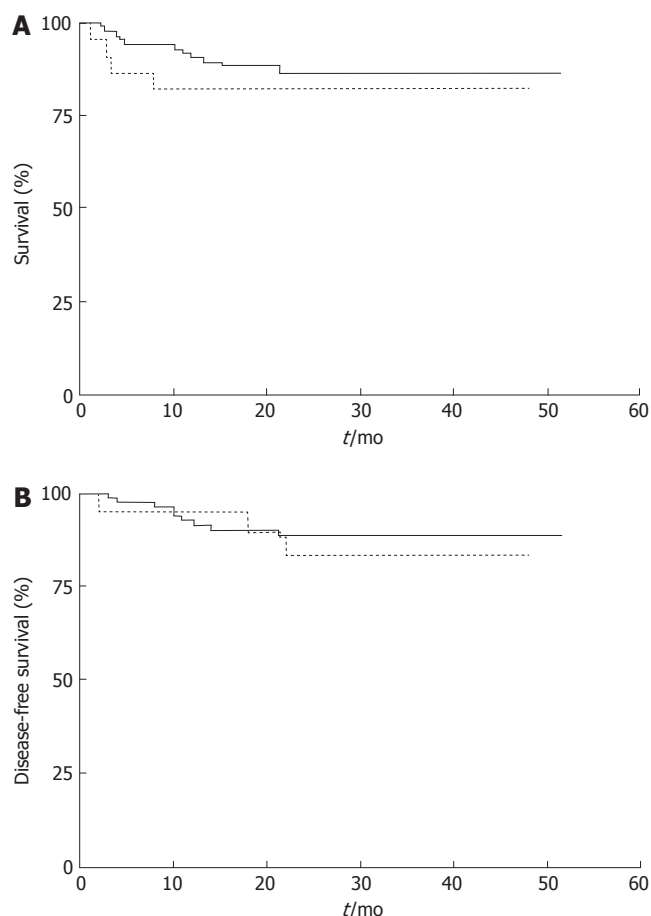


Figure 1 Outcomes of gastrectomy for carcinoma in patients with NURF. **A:** The 4-yr survival rates of patients with normal renal function (solid line) and NURF (dotted line) were 86.6% and 81.8%, respectively ($P = 0.48$); **B:** The 4-yr disease-free survival rates of patients with normal renal function (solid line) and NURF (dotted line) were 88.7% and 83.5%, respectively ($P = 0.65$).

cause for the deterioration of perioperative renal function, and often results in other postoperative complications. However, in the present study, intraoperative blood loss was significantly higher in Group 1 than in Group 2, regardless of the fact that there were no significant differences in the surgical procedures, and the more extensive lymph node dissection in Group 2 than in Group 1 (Table 2 and Table 3). Proper intraoperative hemostasis makes it possible to perform gastrectomy safely for patients with NURF.

There was no significant difference in the postoperative morbidity rates between the two groups. Postoperative complications included pneumonia, pancreatitis, anastomotic leakage, drain infection, catheter infection, ileus, and others, but none of these complications are unique to patients with NURF. The median postoperative hospital stay was 15 d in both groups, and there was no statistically significant difference between them.

Patients with renal failure are immunocompromised, and the occurrence of perioperative infection is higher than in the normal population^[10]. However, in this study, there was no significant difference in postoperative infection between the two groups. We routinely use cephalosporin-antibiotics for 3 d after surgery, and the dose of antibiotics was the same in both groups,

no cases of drug-induced renal function deterioration being observed in either of them. In terms of the TNM classification, the frequency of advanced gastric cancer above stage II was significantly higher in Group 2 than in Group 1 (Table 4). The immunocompromised state of patients with NURF may be the reason why more advanced-stage gastric cancers were present in Group 2 than in Group 1.

Previous reports have suggested that impaired innate and acquired immunity in patients with end-stage renal disease increases the incidence of cancer, the rate of recurrence after surgery, and decreases the survival rate^[11-14]. In one study, relative risks of gastric cancer in patients with end-stage renal disease in Australia, New Zealand and America were 1.2^[15]. In our study, the survival and disease-free survival rates for gastric cancer after curative surgery in patients with NURF were the same as those in patients with normal renal function. Thus, removal of gastric cancer with a sufficient normal stomach, associated with extended lymph node dissection, enables patients with NURF to achieve a satisfactory outcome.

In conclusion, with accurate preoperative evaluations, appropriate operative procedures and perioperative management, gastrectomy for carcinoma including extended lymph node dissection in patients with NURF can be performed as safely as in patients with normal renal function.

COMMENTS

Background

Renal dysfunction is classified into non-uremic and uremic stages. Patients with non-uremic renal failure (NURF) are defined as having impaired renal function but dependent on their own kidneys. Crucial issue in the surgery for the patients with NURF is to prevent deterioration of renal function and avoid the need for dialysis.

Research frontiers

We retrospectively evaluated the results of pre-, intra-, and postoperative management and the outcome of gastrectomy in patients with gastric cancer associated with NURF to assess the safety of gastrectomy in such patients.

Innovations and breakthroughs

With accurate preoperative evaluations, appropriate operative procedures and perioperative management, gastrectomy for carcinoma including extended lymph node dissection in patients with NURF can be performed as safely as in patients with normal renal function.

Applications

Malignant diseases occur likely in patients with NURF and within 2 years of the introduction of hemodialysis. Findings of the present study fascillitate the aggressive surgery in such patients.

Terminology

Non-uremic renal failure (NURF): patients having impaired renal function but dependent on their own kidneys.

Peer review

This is an unique study and as such worthy of acceptance. It is well written and presented. I think it would benefit if the authors made more of their management in maintaining urinary output both during and after the operation.

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Elucidation of the relationship of BNIP3 expression to gemcitabine chemosensitivity and prognosis

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cancer specimens with the prognosis of the patients, and found a tendency of favorable prognosis and low BNIP3 expression.

CONCLUSION: High levels of *BNIP3* expression cannot be used as one of the predicting factors for gemcitabine chemosensitivity, and some yet to be known factors will have to fill the gap for the accurate prediction of pancreatic cancer chemosensitivity to gemcitabine. However, BNIP3 expression may have an impact on prediction of prognosis of patients with pancreatic cancer.

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Key words: BNIP3; Chemosensitivity; Gemcitabine; Pancreatic cancer; Prognosis

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Abstract

AIM: To evaluate the significance of BNIP3 in the pathogenesis of pancreatic cancer, we analyzed the relationship between the expression of BNIP3 and survival rate of the patients with pancreatic cancer, or chemosensitivities in pancreatic cancer cell lines, particularly for gemcitabine, the first-line anti-tumor drug for pancreatic cancer.

METHODS: To compare the expression level of BNIP3 with the resistance to gemcitabine, eight pancreatic cancer cell lines were subjected to gemcitabine treatment and the quantitative real-time RT-PCR method was used to evaluate *BNIP3* expression. Immunohistochemical analysis was also performed using 22 pancreatic cancer specimens to study relationship between BNIP3 expression and survival rate.

RESULTS: Although no significantly positive association between *BNIP3* mRNA level and gemcitabine chemosensitivity was observed, pancreatic cancer cell lines that were sensitive to gemcitabine treatment tended to show high levels of *BNIP3* expression. The converse, an absence of *BNIP3* expression, was not correlated with gemcitabine resistance. We further compared the BNIP3 expression profiles of resected primary pancreatic

INTRODUCTION

Pancreatic adenocarcinoma is a common cancer with an extremely poor prognosis around the world because of its aggressive invasive capacity, early metastasis, resistance to existing chemotherapeutic agents and radiation therapy, and lack of specific symptoms that help in finding patients at early stages for curative operation. To improve the horrible prognosis, we need to find novel approaches to both diagnosis and treatment that are more efficient than currently available techniques.

BNIP3, the hypoxia-inducible proapoptotic gene belonging to the *BCL2* family, was originally identified as the gene encoding a protein that interacts with adenovirus E1B 19-kDa protein^[1]. The expression of *BNIP3* is increased under hypoxic conditions by a transcription factor, hypoxia-inducible factor 1 α (*HIF1* α)^[2,3], and leads to cell death by two different pathways; (1) heterodimerization with the anti-apoptotic protein *BCL2*, and (2) opening the mitochondrial permeability transition pores by direct contact with its outer membrane. Thus, *BNIP3* is considered to be a key regulator

of hypoxia-induced cell death^[4,5,6].

Hypoxia is a common phenomenon in solid tumors and has been proven to occur in pancreatic cancer, but *BNIP3* expression was shown to be decreased in pancreatic cancer compared with normal pancreas due to the hypermethylation of its promoter^[7]. On the other hand, high expression of *BNIP3* in pancreatic cancer cell lines has been reported to be associated with the chemosensitivity to gemcitabine 2', 2'-difluorodeoxycytidine (Gemzar, Eli-Lilly, Indianapolis, IN), a novel pyrimidine nucleoside analogue^[8]. It was expected that downregulation of proapoptotic *BNIP3* might contribute to chemoresistance, survival and progression of pancreatic cancer in a hypoxic environment. In the present study, we analyzed the expression of *BNIP3* in several pancreatic cancer cell lines to explore the association with chemosensitivity in pancreatic cancer.

MATERIALS AND METHODS

Pancreatic cancer cell lines and cell culture

Two human pancreatic cancer cell lines (CFPAC1 and SUIT2) were obtained from Cancer Research UK Research Services (London, UK). PK-1, PK-8, PK-9, PK-45, and PK-59 were established at Tohoku University from patients with pancreatic cancer^[9,10]. PANC-1 was obtained from Cell Resource Center for Biomedical Research, Institute for Development, Aging, and Cancer, Tohoku University (Sendai, Japan). All cells were maintained in RPMI 1640 containing 10% fetal bovine serum under an atmosphere of 5% CO₂ with humidity at 37°C.

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay

To assess cell proliferation, the MTT test was used as described previously^[11]. Cells were resuspended in fresh medium and seeded in 96-well plates at concentration of 5×10^3 cells/well. Cells were incubated for 24 h at 37°C, and then gemcitabine was added to each well at various concentrations. Each plate was incubated at 37°C for 72 h. An aliquot of 10 μ L of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (2.5 mg/mL) was added to each well, and the plate was incubated at 37°C for 2 h. The absorbance was measured at 560 nm using a microplate reader.

Quantitative real-time reverse transcription PCR

Total RNAs were extracted from the harvested cells using an RNeasy mini kit (QIAGEN, Tokyo, Japan) according to the supplier's instructions. Each purified RNA was dissolved in RNase-free water, and its concentration was measured by optical absorbance at an aliquot of 10 μ g total RNA and Super Script II Reverse Transcriptase (Invitrogen, Carlsbad, CA) by the methods described previously^[12]. The synthesized cDNA was used for a quantitative real-time PCR analysis using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) following the manufacturer's instructions. Specific primers and common probe were designed by using the Primer Express software (Applied Biosystems), and their nucleotide sequences are: 5'-tggacggagtagctcc

aagagc-3' (forward primer), 5'-agaagccctgttggtatcttg-3' (reverse primer), 5'-tctcactgtgacagtccacctcgc-3' (TaqMan probe). These primers were purchased from Nihon Gene Research Laboratories (Sendai, Japan). Expression of the β 2-microglobulin (*B2M*) gene was monitored as an internal control, and the nucleotide sequences for the primers and the probe were described previously^[13]. Amplifications were carried out in the reaction mixture in 25 μ L containing 5 μ L of cDNA samples and 12.5 μ L of 2 \times ABsolute QPCR ROX Mix (ABgene, Epsom, UK), and the final concentration of 0.2 μ mol/L of each primer pair and 0.4 μ mol/L of the probe were added in a program comprised of 2 min at 50°C, 15 min at 95°C, followed by 40 cycles consisting of 15 s at 95°C and 1 min at 60°C. The expression ratio of *BNIP3*/*B2M* was calculated and used. Each experimental reaction was performed in triplicate.

Tissue samples and immunohistochemical analysis

Pancreatic cancer specimens were obtained from 22 patients between 1995 and 2004. All the patients had undergone surgery at Tohoku University Hospital. Stages were defined according to the general rules of the Japan Pancreas Society^[14]. These patients underwent either pancreaticoduodenectomy or distal pancreatectomy with nodal dissection followed by similar chemotherapy including gemcitabine treatment. All of the patients' prognoses until 2005 were also determined. Twelve patients were male and 10 patients were female, and the mean age of operation was 64.0 years old. Specimens for immunohistochemistry were fixed in 10% formalin and embedded in paraffin for histological analysis. Research protocols for this study were approved by the Ethics Committee of the Tohoku University School of Medicine. Antigen retrieval was performed by boiling the slides in 10 mmol/L citrate buffer twice for 10 min. Peroxidase was quenched with a 3% H₂O₂ solution in 30% methanol. After an overnight incubation with mouse anti-BNIP3 antibody clone Ana 40 (Sigma-Aldrich, St. Louis, MO) at 4°C, slides were washed with PBS supplemented with 0.05% Tween-20 and exposed to the HRPO-linked anti-mouse secondary antibody for 45 min at room temperature. Color reaction was carried out by incubation for 4 min with liquid DAB+ substrate and counterstaining by Mayer's hematoxylin solution. Staining patterns were divided into three groups following the report by Erkan *et al*^[15]; punctate perinuclear staining, diffuse cytoplasmic staining, and negative/faint staining.

Statistical analysis

All experiments were done in duplicate or triplicate. A two-tailed Student's *t*-test was applied for statistical analysis of comparative data. Overall survival curves were generated according to the Kaplan-Meier method. *P* < 0.05 was considered statistically significant.

RESULTS

Efficacy of cytotoxicity induced in pancreatic cancer cell lines by gemcitabine exposure

The response of eight pancreatic cancer cell lines to gemcitabine treatment was investigated using the MTT

Table 1 Classification of the Cell Lines by IC50

	IC50 ($\mu\text{g/mL}$)	Cell lines
Sensitive group	< 0.1	PK-9, SUI2
Intermediate group	0.1-100	CFPAC1, PK-1, PK-8, PK-45
Resistant group	> 100	PK-59, PANC-1

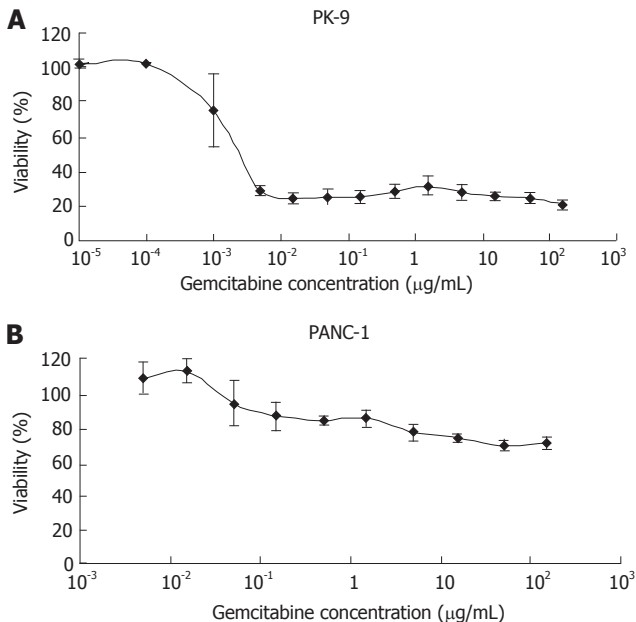


Figure 1 Response to gemcitabine in pancreatic cancer cell lines. Dose-response curves for gemcitabine in PK-9 (A) and PANC-1 (B). These are representative gemcitabine-sensitive and -resistant cell lines, respectively. Each bar represent a standard deviation (SD).

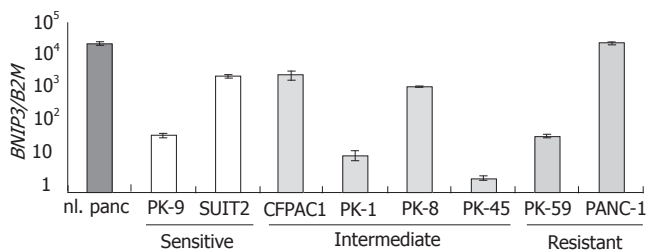


Figure 2 Relative expression levels of *BNIP3* in eight pancreatic cancer cell lines. *BNIP3* expression in pancreatic cancer cell lines as determined by quantitative real-time RT-PCR, and the expression of *B2M* was monitored as the internal control. The cell lines are arranged by IC50 for gemcitabine. No significant correlation was observed between the expression level of *BNIP3* and IC50.

assay. Representative dose-response curves are shown in Figure 1; PANC-1 is a representative resistant cell line, whereas PK-9 is a representative sensitive cell line. The pancreatic cancer cell lines were divided into three groups according to their IC50 values as shown in Table 1. Judging from the IC50 value, two cell lines (PK-9 and SUI2) showed high sensitivities to gemcitabine; less than 30% of the cells survived in the presence of 0.1 $\mu\text{g/mL}$ of gemcitabine for 72 h (Figure 1A). In contrast, PANC-1 and PK-59 showed very low sensitivity; more than 50% of the cells survived even in the presence of more than 100 $\mu\text{g/mL}$ of the drug for 72 h (Figure 1B). The remaining four cell lines (CFPAC1, PK-1, PK-8 and

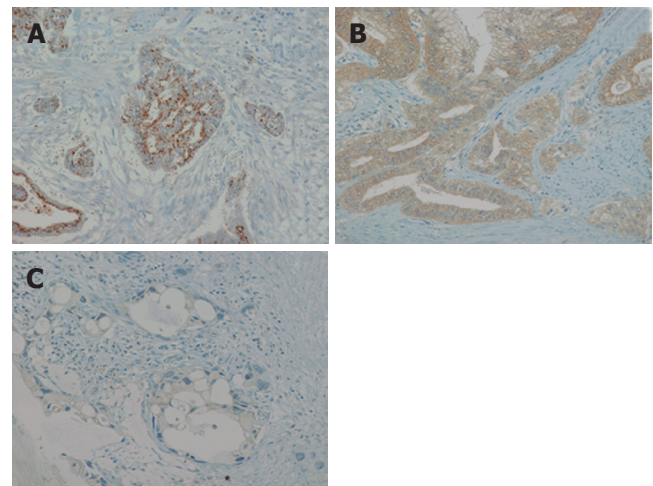


Figure 3 Immunohistochemical analysis of *BNIP3* in primary pancreatic cancer specimens. A monoclonal anti-*BNIP3* antibody (clone Ana 40) was used. A: Punctate perinuclear staining; B: Diffuse cytoplasmic staining; C: Negative/faint staining.

PK-45) showed moderate sensitivity (IC50 values 0.1-100 $\mu\text{g/mL}$) and were classified as intermediately sensitive cell lines.

***BNIP3* expression level is not associated with chemosensitivity of pancreatic cancer to gemcitabine**

The expression of *BNIP3* mRNA was examined in all the pancreatic cancer cell lines by quantitative real-time RT-PCR. Among the cell lines analyzed, *BNIP3* expression levels varied by 10^4 fold, as shown in Figure 2. Cell lines were arranged by their IC50 values from left to right. PANC-1 showed the level of *BNIP3* expression comparable to normal pancreas, but PK-45 and PK-1 showed highly suppressed levels of expression. No significant correlation was observed between the expression level of *BNIP3* and the IC50 for gemcitabine.

Next we examined the expression level of *BNIP3* before and after gemcitabine treatment to see if gemcitabine affects the *BNIP3* expression. After administration of 1 $\mu\text{g/mL}$ of gemcitabine to PANC-1, PK-45, PK-9, and SUI2, the cells were harvested on d 0, 1, 2, and 3. No significant alterations in *BNIP3* expression were observed in these cell lines (data not shown).

Expression level of BNIP3 in pancreatic cancer tissue and correlation with patient survival

To explore the question of whether the expression level of *BNIP3* affects the prognosis, we performed immunohistochemical analyses of resected tumor tissues. The staining patterns of *BNIP3* were divided into three groups^[15]. Representative examples are shown in Figure 3. Among the 22 evaluated specimens, four patients were classified as negative, 11 patients displayed diffuse cytoplasmic staining and 7 showed the punctate perinuclear pattern. The groups were comparable in terms of tumor morphology, stage, and year of treatment including usage of gemcitabine. Although significant differences were not calculated, a tendency between long survival of the patients and negative/faint *BNIP3* expression was observed (Figure 4).

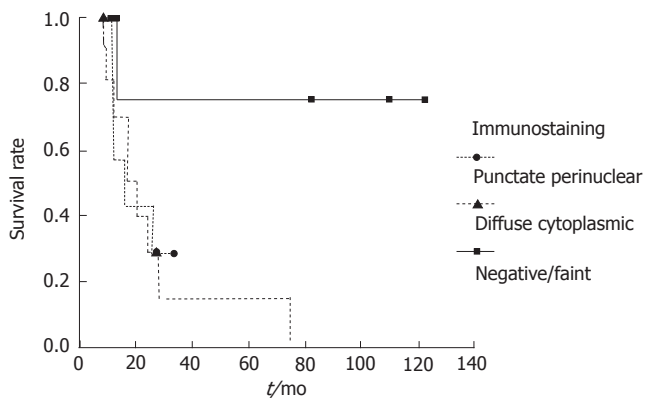


Figure 4 Kaplan-Meier analysis of the patients with pancreatic cancer by BNIP3 immunostaining patterns. The mean survival time of patients with punctate perinuclear staining group was 20 mo, diffuse cytoplasmic staining group was 26 mo, and negative/faint-staining group was 95 mo. Each group was comparable in terms of tumor stage and adjuvant therapies.

DISCUSSION

Recent studies have reported that *BNIP3* expression is silenced in pancreatic cancer by hypermethylation of its promoter and that loss of *BNIP3* expression contributes to chemoresistance and worsened prognosis^[7,8,15]. Furthermore, siRNA-mediated knockdown of *BNIP3* caused chemoresistance to gemcitabine^[8]. The information was supposed to give us some clues to finding some efficient novel methods for treatment of pancreatic cancer patients. As the first step for invention of such an efficient method for treatment, we tried to confirm these reported results by comparing *BNIP3* expression levels and gemcitabine chemosensitivity in several pancreatic cancer cell lines. However, our results did not support the previous report by Akada *et al*^[8]. The expression levels of *BNIP3* in our series of pancreatic cancer cell lines, which were basically the same as used in the previous study, did not reproduce the previous results; the chemosensitivities of pancreatic cancer cell lines to gemcitabine were quite different. Representative gemcitabine sensitive cell lines (CFPAC1 and SUIT2) and the representative resistant cell line (PK-59) were used in both studies, but PK-9, which was previously scored as moderately sensitive to gemcitabine, was actually classified as the most sensitive cell line in our study. PANC-1, which was reported also as “moderate” was the most resistant cell line. In addition, PK-8, which was not tested in the previous study, showed resistance to gemcitabine even with high expression of *BNIP3*. The cause of these discrepancies of the gemcitabine sensitivity between the two studies is unclear; the difference of the methods used in these studies, different culture media, and, probably, cell density, may have caused the discrepancy to some extent. Our immunohistochemical studies also showed results opposite to those reported by Erkan *et al*^[15]; loss of *BNIP3* expression seemed to correlate with better survival of patients.

In our present study, we could not detect any clear relationship between *BNIP3* expression and chemosensitivity that might associate with a favorable patient survival rate. *BNIP3* is a proapoptotic protein that was considered to have some effect on the chemosensitivity

and growth of the pancreatic cancer cell in itself, so it is not difficult to think that overexpression of *BNIP3* leads to cell death and that inhibition of *BNIP3* leads cells survive longer. Our present results by immunostaining supported this idea, albeit no significant difference was obtained. As the number of cases increases, a significant difference will probably be observed. However, pancreatic cancer involves very complicated molecular changes, and those *BNIP3*-positive cancer cells that appear to be weak to the cytotoxic effect and less aggressive may have other genetic and epigenetic changes which can compensate for the proapoptotic effect of *BNIP3* and eliminate the differences between *BNIP3*-positive and -negative cancer cells with regard to chemosensitivity and survival. Furthermore, chemosensitivities can be caused by factors other than those we have discussed so far, such as upregulation of the transportation system to eliminate the drugs from the cells or upregulation of metabolism. Any association between chemosensitivity and *BNIP3* expression may yet give us a clue to find a way to invent efficient methods for treatment; further studies are necessary to explain the discrepancy.

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RAPID COMMUNICATION

Dyslipidemia and *H pylori* in gastric xanthomatosis

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Abstract

AIM: To investigate the relationship among gastric xanthomatosis (GX), *H pylori*, dyslipidemia, and gastritis in Korea, a well-known *H pylori* endemic area.

METHODS: A total of 771 patients who had undergone gastroduodenoscopy by one endoscopist were included in this study. Among them, 54 patients with GX were assessed for *H pylori* infection and their endoscopic characteristics and serum lipid profiles. The findings were compared with 54 age- and sex-matched control subjects without GX.

RESULTS: The prevalence of GX was 7% (54/771) with no sex difference. GX was mainly single (64.8%) and located in the antrum (53.7%). The mean diameter was 7 ± 3 mm. Mean body mass index (BMI) of patients with GX was 23.1 ± 2.8 and no one was above 30. Compared with the controls, lipid profiles of GX group showed significantly lower HDL-cholesterol (48.8 ± 12.3 vs 62.9 ± 40.5 , $P = 0.028$) and higher LDL-cholesterol (112.9 ± 29.9 vs 95.9 ± 22.4 , $P = 0.032$). The level of total serum cholesterol, triglyceride and the existence of dyslipoproteinemia were not related to the presence of GX. However, GX showed a close relationship with endoscopically determined atrophic gastritis and histologic severity (24/53, 44.4% vs 8/54, 14.8%, $P = 0.0082$). *H pylori* infection and bile reflux gastritis were not significantly related with GX.

CONCLUSION: The prevalence of GX is 7% and it may be an increasing entity in Korea. Moreover, dyslipidemia and atrophic gastritis are found to be related to GX, but *H pylori* infection is not.

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Key words: Xanthomatosis; Dyslipidemia; *H pylori*; Gastritis

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INTRODUCTION

Xanthomatosis is a yellow tumor-like lesion of the skin or deeper structure characterized by the presence of lipid-containing histiocytes. Its presence may be a manifestation of a metabolic disturbance, such as hyperlipidemia, although it usually represents an isolated phenomenon. Many clinicians believe that yellowish plaque in the gastric mucosa is a benign lesion, and it has no clinical significance. Feyrter^[1] reported the first recorded observation of a small yellow macule in the gastric mucosa representing a fatty deposit. Since gastric xanthomatosis (GX) was first named in 1929 by Lubarsch and Borchardt^[2] as a gastric lipid island, a variety of possible causes, such as an abnormality of the lipid metabolism or inflammatory changes in the gastric mucosa, have been suggested^[3-5]. However, the detailed developmental mechanism remains unknown.

H pylori is the most important etiologic factor of peptic ulcer disease and chronic active gastritis. Chronic gastritis is thought to be involved in the gastric glandular atrophy and intestinal metaplasia sequence, which is considered a precursor of gastric cancer. Recently, Hori and Tsutsumi^[6] found *H pylori* infection on the surface of foveolar cells in 48% of biopsy samples of GX. Isomoto *et al*^[7] also reported a close relationship among *H pylori* infection, GX, and atrophic gastritis, and GX may be provoked by *H pylori* infection.

Since GX has received little attention in the Korean literatures, we undertook this study to evaluate the prevalence and the clinical significance of GX endoscopically, and to investigate the relationship among GX, *H pylori* infection and dyslipidemia.

MATERIALS AND METHODS

Patients

From August 2003 to March 2004, a total of 771 patients (398 males and 373 females) who had undergone gastroduodenoscopy by one endoscopist were included in this study. Subjects' age ranged from 17 to 88 (mean 60) years. Of these 771 patients, 54 cases (27 men and 27 women; mean age 54 and range 33-77 years) had GX. Of these 54 patients, chronic gastritis was diagnosed in 41, gastric ulcer in 4, duodenal ulcer in 4, gastric polyp in 3, gastroesophageal reflux disease (GERD) in 3, Mallory-Weiss syndrome in 1, gastric cancer in 1, congestive gastropathy in 1, and normal gastric mucosa in 2. Among them, 6 cases were

Table 1 Serum lipid profiles of gastric xanthomatosis and age- and sex-matched controls (mean \pm SD)

	GX (n = 54)	Control (n = 54)	P
Total cholesterol (mg/dL)	188.7 \pm 32.8	189.2 \pm 30.9	NS
LDL-cholesterol (mg/dL)	112.9 \pm 29.9	95.9 \pm 22.4	0.032
HDL-cholesterol (mg/dL)	48.8 \pm 12.3	62.9 \pm 40.5	0.028
TG	125.9 \pm 71.0	119 \pm 67.8	NS

NS: Not significant. TG: Triglyceride; HDL: High-density lipoprotein; LDL: low-density lipoprotein.

combined with chronic gastritis; those were gastric polyp in 2, gastric ulcer in 2, duodenal ulcer in 1 and GERD in 1.

There were no systemic signs of xanthomatosis in GX patients. Fifty-four age- and sex-matched individuals without GX served as controls. During endoscopy, six biopsy specimens had been obtained, two from GXs, two from the antrum, and two from corpus specimens. The two biopsy specimens were available from the area of the xanthomatosis and these were stained with hematoxylin and eosin (HE) for histologic examination. The one antrum and one corpus samples were used for the rapid urease test (CLO test, double chamber: Chongkeundang Co., Seoul, Korea), and stained with HE for evaluation of the activity of gastritis and *H. pylori* positivity. Anti-*H. pylori* IgG antibody was measured by enzyme-linked immunosorbent assay (ELISA) with a commercial kit (Hel-p test, Amrad Co, Melbourne, Australia) before endoscopy. Patients were classified as *H. pylori*-positive if at least two of above tests were positive or as *H. pylori*-negative if all tests were negative.

Endoscopic assessment

GX, a white-yellow nodule measuring less than 10 mm, was observed on the wall of stomach. Enterogastric or bile reflux gastritis was defined as the presence of bile-stained mucosa and a collection of bile juice on endoscopic examination.

Histologic evaluation

The histologic appearance of lipid islands was that the lamina propria was occupied by large ovoid to polygonal histiocytes having an abundant, finely vacuolated (foamy) cytoplasm staining lightly with eosin. The foam cell nuclei were regular, round to ovoid, and occupied a small portion of the cell area. No mitoses were observed. To confirm that foamy cells were present in the GX and derived from macrophages, immunohistochemical study was performed. The foamy cells were positive for the human macrophage marker (HAM-56, DAKO, Carpinteria, CA) and negative for cytokeratins (AE 1/3, Biogenix, San Roman, CA), supporting the diagnosis of xanthomatosis.

Histologic examination of the other biopsy specimens of the mucosa taken at a distance from lipid islands was also done. Biopsy specimens stained with HE and Giemsa were used to detect *H. pylori*. The histologic degree of activity (neutrophil infiltration), inflammation (mononuclear cell infiltration), glandular atrophy, and intestinal metaplasia were classified as none, mild, moderate, and severe in accordance with the updated Sydney System^[8]. Atrophy of

the gastric mucosa is defined as loss of glandular tissue. The observer should attempt to evaluate one feature at the time using a new visual analogue scale that was provided to assist in grading^[9]. Two independent observers evaluated the histologic examination without prior knowledge of the diagnosis or experimental results.

Statistical analysis

Statistical analysis was performed using the Chi-square test, Student's *t* test, and Mann-Whitney *U* test, when appropriate. A *P* value less than 0.05 was considered statistically significant. Data were expressed as mean \pm SD. SPSS 11.0 version software was used to analyze the data.

RESULTS

In this present study, the prevalence of GX was 7% (54/771). Of these 54 GX patients, 27 were men and 27 women, with mean age of 54 (range 33-77) years.

Endoscopic characteristics of GX

The mean number of GX was 1.7 ± 1.1 , and 64.8% (35/54) of cases had a single xanthoma. GX was located at the antrum in 53.7% (29/54), at the body in 35.2% (19/54), and at the fundus in 11.1% (6/54) of cases. The mean size of GX was 7 ± 3 mm, and none of the GX exceeded 10 mm in diameter.

Clinical characteristics of patients with GX

Distribution of BMI: The mean BMI of the GX group was 23.1 ± 2.8 (range, 18.0-28.2), with none over 30, which was similar to the control group (mean 24.1 ± 2.4 , range, 18.5-29.7). No correlation was found between abnormal BMI and the presence of GX.

Lipid profiles: Results of lipid profiles are shown in Table 1. When a normal HDL-cholesterol level of 30 mg/dL was selected as the lower cut-off value, 11 (20.4%) GX cases had HDL-cholesterol level lower than the cut-off value. Hypertriglyceridemia was found in 11 GX patients. However, no difference was found in the serum total cholesterol and triglyceride levels between the GX and control groups. In contrast, GX patients had lower mean HDL-cholesterol and higher mean LDL-cholesterol levels than the controls (*P* = 0.032 and *P* = 0.028, respectively).

Lipoprotein electrophoresis analysis was performed in 32 GX patients. Abnormal patterns were exhibited by only 2 (6.3%) patients, and both of them were dyslipoproteinemia type IV. No correlation was observed between the presence of dyslipoproteinemia and GX.

Underlying diseases: The underlying diseases were diabetes mellitus in 3 (5.5%), essential hypertension in 7 (12.9%), fatty liver in 3 (5.5%), chronic liver disease in 2 (3.7%), and malignancies in 6 (11.1%) GX patients. GX was not significantly correlated with any special underlying diseases.

Endoscopic findings and histologic severity

Table 2 shows the endoscopic findings of the GX patients

Table 2 Comparison of endoscopic distribution of atrophic gastritis in GX patients and controls

	Predominant distribution		
	Antrum	Corpus	Pangastritis
GX patients (24/54, 44.4%) ^b			
Mild (n = 10)	2	1	7
Moderate (n = 8)	3	0	5
Severe (n = 6)	2	0	4
Control (8/54, 14.8%)			
Mild (n = 6)	3	1	2
Moderate (n = 1)	1	0	0
Severe (n = 1)	1	0	0

The distribution and severity of atrophic change in GX patients significantly differed from that in controls (^b $P = 0.0082$).

and controls. The endoscopic distribution and severity of atrophic gastritis were determined in 24 of 54 (44.4%) GX patients as follows: mild form in 10, moderate in 8, and severe in 6. The distribution and severity of atrophic change were significantly higher in GX patients as compared with the controls in terms of both the antral distribution and the pangastritis ($P = 0.048$ and $P = 0.0082$, respectively).

Enterogastric reflux gastritis was noted in only 2 of 54 (3.7%) GX patients without other mucosal injury and in 1 of 54 (1.9%) controls. There was no significant relationship between GX and the presence of enterogastric reflux.

Prevalence of *H pylori* in GX

The positive rate of *H pylori* infection in GX patients (63%, 34/54) was similar to that of the controls (69.2%).

DISCUSSION

Gastric xanthomatosis, once considered a rare lesion, is being reported more frequently. Many reports^[1,3-5,10-12] about GX have shown its frequency of 0.018%-0.8%. Of 7816 patients who had undergone gastroduodenoscopy from January 2001 to August 2002, GX was detected in 76 (0.97%) patients, which is similar to the findings of previous studies^[11,12]. In the present study, the prevalence was found to be 7%, which is very much higher than that reported in Western countries^[12]. This finding indicates that GX is a relatively common disease entity as an increasing trend in Korea, although its etiology remains unclear. This may be associated with an increased awareness and selection bias of endoscopists; however, a large-scaled prospective study is needed in the future. In a Chinese series of 3870 patients, a moderate predominance of male over female (male:female = 3.3:1) was reported^[11], but GX showed no sex predilection in the present study.

Xanthogranulomatous gastritis is characterized by an inflammation of the gastric wall by foamy histiocytes, inflammatory cells, multinucleated giant cells, and fibrosis. The occurrence of this nodular growth in the gastric mucosa is manifested by the presence of lipid-laden macrophages containing cholesterol and natural fats in the lamina propria. The relationship between serum lipid and gastric mucosal injury due to bile reflux has been more clearly defined. After subtotal gastrectomy (Billroth II operation), GX occurred in 6.3% at 1 to 3 years after surgery,

in 35.7% at up to 15 years, and in 43.9% after 20 years^[12,13]. Isomoto *et al*^[7] reported a close relationship between atrophic gastritis or bile reflux gastritis and GX endoscopically. Several studies have shown that xanthoma is associated with gastritis, carcinoma, intestinal metaplasia of the gastric epithelium, or peptic ulcer diseases^[5,14,15]. In the present study, a close relationship was found between atrophic gastritis and GX but there was no correlation between GX and enterogastric reflux.

Endoscopically, a single GX was detected in a majority (64.8%) of GX patients, all GX were less than 10 mm in size, and most of the lesions (88.9%) were located at the antrum and body, which is in agreement with a previous study^[16]. The clinical significance of GX remains unknown and the presence of GX *per se* is probably of little clinical significance. Basic metabolic analysis to assess for hyperlipidemia or hypercholesterolemia should also be considered. Etiologically, these conditions may be associated with a primary dyslipoproteinemic state, such as diabetes, nephrosis, obesity or cholestasis. Chang *et al*^[17] reported lower mean HDL-cholesterol and higher mean triglyceride levels in GX subjects in comparison with the controls. In the present study, abnormal BMI (≥ 30) was not correlated with the presence of GX, while the lower mean HDL-cholesterol and higher mean LDL-cholesterol levels in GX subjects than controls seems statistically interesting ($P = 0.028$ and $P = 0.032$, respectively). Elevated triglyceride levels among GX patients have been occasionally mentioned in a small number of case studies^[18-20]. The present study showed insignificantly higher mean triglyceride level in GX subjects than controls. Abnormalities of lipid metabolism seem to play a role in presence of GX. The higher serum lipid content in GX patients may be partly derived from circulating lipids. Since excessive concentrations of oxygen-free radicals may result in accelerated LDL-cholesterol oxidation and the formation of GX^[7], the plasma levels of LDL-cholesterol and of oxidized LDL-cholesterol are important factors in the pathogenesis of atherosclerosis. GX cases in the present study showed no systemic signs of xanthomatosis and no significant differences were observed between GX patients and controls in the incidences of other conditions, such as diabetes, hyperlipidemia, or hypertension.

Chronic persistent infection with *H pylori* is believed to result in the development and to influence the extent of atrophic gastritis. Korea is one of the well-known *H pylori* endemic countries in the world. Some studies found a close association between *H pylori* infection and GX^[6,7]. Moreover, bacterial infection-induced foamy cell infiltration was observed in the small bowel lesions of Whipple disease^[21]. Therefore, it is possible that macrophage transformation into foamy cells may be secondary to phagocytosis caused by *H pylori* that have penetrated into the lamina propria, as suggested by Hori *et al*^[6]. In the present study, we examined *H pylori* infection by CLO test, histologic examination, or serologic test, and found that the prevalence of *H pylori* was similar in GX patients and controls, suggesting no correlation between GX and *H pylori* infection. This may be associated with high prevalence of *H pylori* infection in Korean adult population. Although the prevalence of *H pylori* infection was similar in GX patients and controls, atrophic gastritis was more frequently ob-

served in GX patients. Although atrophy of stomach was highly related to *H pylori* infection, there was no absolute correlation between atrophy and *H pylori* in some endemic area like Korea^[22]. Also atrophy could be induced by some other reasons, such as autoimmune, idiopathic, reactive, drug-associated, or other gastric irritant-induced causes.

In conclusion, although the reasons remain unclear, GX is an increasing entity in Korea. In addition, GX is associated with dyslipidemia and atrophic gastritis. However, there is no relation between *H pylori* infection or entero-gastric reflux gastritis and GX. It remains to be clarified whether dyslipidemia and atrophic gastritis observed among GX patients play any role in its etiology.

COMMENTS

Background

Xanthomatosis is yellow tumor-like lesion of the skin or deeper structure characterized by presence of lipid-containing histiocytes. This yellowish plaque in gastric mucosa (gastric xanthomatosis, GX) is a benign lesion, and it has no clinical significance. But a variety of possible causes, such as an abnormality of the lipid metabolism or inflammatory changes in the gastric mucosa, have been suggested. The aim of this study was to evaluate the prevalence and the clinical significance of GX endoscopically in Korea, and to investigate the relationship among GX, *H pylori* infection and dyslipidemia.

Innovations and breakthroughs

Although the reasons remain unclear, GX is an increasing entity in Korea. The present study showed the lower mean HDL-cholesterol and higher mean LDL-cholesterol and higher mean triglyceride levels in GX subjects than the controls. Abnormalities of lipid metabolism seem to play a role in presence of GX. However, there was no relation between *H pylori* infection or enterogastric reflux gastritis and GX. Although the prevalence of *H pylori* infection was similar in GX patients and controls, atrophic gastritis was more frequently observed in GX patients.

Applications

Even though GX is benign disease entity, it is definitely related with dyslipidemia and atrophic gastritis. If the patient has GX in endoscopic examination, it needs to evaluate the severity of atrophic gastritis and the level of lipid profile. It remains to be clarified whether dyslipidemia and atrophic gastritis observed among GX patients play any role in its etiology.

Terminology

GX was first named in 1929 by Lubarsch and Borchardt as a gastric lipid island. A variety of possible causes, such as an abnormality of the lipid metabolism or inflammatory changes in the gastric mucosa, have been suggested. The histologic appearance of GX was that the lamina propria was occupied by large ovoid to polygonal histiocytes having an abundant, finely vacuolated (foamy) cytoplasm staining lightly with eosin.

Peer review

It is an interesting and well written paper. The authors investigated the relationship among GX, *H pylori*, dyslipidemia, and gastritis in Korea. They conclude that the prevalence of GX is 7% and it may be an increasing entity in Korea. Moreover, dyslipidemia and atrophic gastritis are related to GX, but *H pylori* infection is not.

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RAPID COMMUNICATION

Prevalence of overweightedness in patients with gastro-esophageal reflux

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Abstract

AIM: To evaluate whether the prevalence of overweight and obese conditions is increased in gastro-esophageal reflux disease (GERD) patients (with 24-h pathological pH recordings) in comparison to general population.

METHODS: A total of 196 consecutive patients (103 females, age range 18-83 years) with symptoms of gastro-esophageal reflux (GER) and 24-h pathological esophageal pH-metry. Body mass index (BMI) of the patients was calculated and its distribution (%) was compared with that of the Italian general population as assessed by National Bureau of Census (ISTAT). To evaluate the association of GERD with weight categories, the binomial test was employed. $P < 0.05$ was considered statistically significant.

RESULTS: In males, overweightedness (BMI 25-25.9) was present in 43% of GERD patients *vs* 41.8% of Italian population (IP) (ns), obesity (BMI ≥ 30) in 10.9% *vs* 9.1% (ns). In females overweight was present in 34.9% of GERD patients *vs* 25.7% of IP ($P < 0.01$), obesity in 13.6% of GERD patients *vs* 9.1% of IP ($P < 0.01$). No statistically significant differences were noted in different age classes.

CONCLUSION: In comparison to the Italian general population, the prevalence of overweightedness and obesity is increased in female but not in male patients with ascertained gastro-esophageal reflux disease.

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Key words: Gastro-esophageal reflux; Obesity; Overweight; 24-h pH-metry; Body mass index

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INTRODUCTION

Increased body weight has often been considered one of the most important risk factors for gastro-esophageal reflux disease (GERD). A recent meta-analysis concluded that overweightedness and obesity are the risk factor for GERD^[1]. However, only three of the nine examined studies^[2-10] showed the relationship between obesity and GERD symptoms^[2].

Other studies have been performed only on patients with an exceedingly high body mass index (BMI), or even on patients requiring surgical^[11-15] or intragastric balloon^[16] treatment with no comparison with a normal BMI population.

Only two studies assessed the gastro-esophageal reflux (GER) by means of 24-h pH-metry but made no comparison with a reference population. Of these two studies, one was performed only on obese patients requiring intragastric balloon treatment^[16], the other was performed on 70 patients, 79% of whom had abnormal BMI, indicating a biased patient selection^[17].

Attempts to evaluate an improvement in gastro-esophageal reflux, or related symptoms following weight loss, in overweight and obese patients, have not led to a better understanding of the relationship between these two conditions since the data obtained are controversial^[16,18,19].

MATERIALS AND METHODS

A total of 196 patients (103 females, age range 18-83 years; 93 males, age range 24-74 years) referred to our GI unit for GERD symptoms (including typical and atypical symptoms), were included in this study.

In all 196 patients, the presence of pathological gastro-esophageal reflux, according to DeMeester score, was established after a 24 h pH-metry assessment [Ingold 440 M3 glass electrode, Genesi2 RAM 8Kb LEM Electronics recorder, Casalecchio di Reno (BO), Italy]. Weight and height were measured in each patient, and their BMI was calculated [weight (kg)/ height (m²)].

Patients were then assigned to one of the four categories, according to their BMI, following the 1995 World Health Organization criteria, in which distinction

between males and females is no longer considered: BMI < 18.5: underweight, BMI = 18.5-24.9: normal, BMI = 25-29.9: overweight, BMI > 30: obesity. The distribution (%) of the 196 GERD patients was compared with that of the Italian general population used, for this purpose, the data obtained in a National Bureau of Census (ISTAT) survey^[20].

To evaluate the association of GERD with weight categories, we employed the binomial test, to calculate the probabilities of the estimated proportion of GERD patients in the overweight Italian population. The expected proportion of overweight individuals, in the population, was obtained from the above-mentioned ISTAT survey, based on approximately 50000 people. This test was also performed stratifying the data according to sex and age-class. $P < 0.05$ was considered statistically significant. The association between smoking habit and presence of GERD was evaluated using the χ^2 test.

Informed consent was obtained from patients at beginning of the study.

RESULTS

Of the patients presenting with pathological gastro-esophageal reflux, 45.9% had a normal BMI which was lower than that in the general population (53.8%) while 38.8% and 12.2% overweight and obese GERD patients had a normal BMI (exceeding 33.4% and 9.1%, respectively, in the general population) (Figure 1A).

If the data on sex were considered, although the greater prevalence of BMI was still present in male GERD patients, the difference in the general population was less evident (overweight: 43% *vs* 41.8; obese: 10.8% *vs* 9.1%) (Figure 1B).

Conversely, the prevalence of overweightedness and obesity in female GERD patients was considerably greater than that in the general population (overweight: 34.9% *vs* 25.7% $P < 0.01$; obese: 13.6% *vs* 9.1% $P < 0.01$) (Figure 1C).

If the overweight and obese female patients were considered together, the total percentage reached 48.5% *vs* 35% in the general female population ($P = 0.005$).

No statistically significant differences were found in the analysis comparing different age classes. Likewise, statistical analysis failed to show any relationship between smoking habit and gastro-esophageal reflux in our patient population.

DISCUSSION

The most relevant finding of this study is that the prevalence of overweightedness and obesity was increased in female, but not in male Italian patients with ascertained gastro-esophageal reflux disease. Previous population-based studies considered GERD patients as those referring heartburn and acid regurgitation only^[1-5] and did not assess any objective pH-metric evidence of GER.

By using typical GER symptoms as entry criteria, these studies excluded subjects with atypical GER symptoms and pathological GER and, on the other hand, included subjects with functional heartburn and no GER. It is

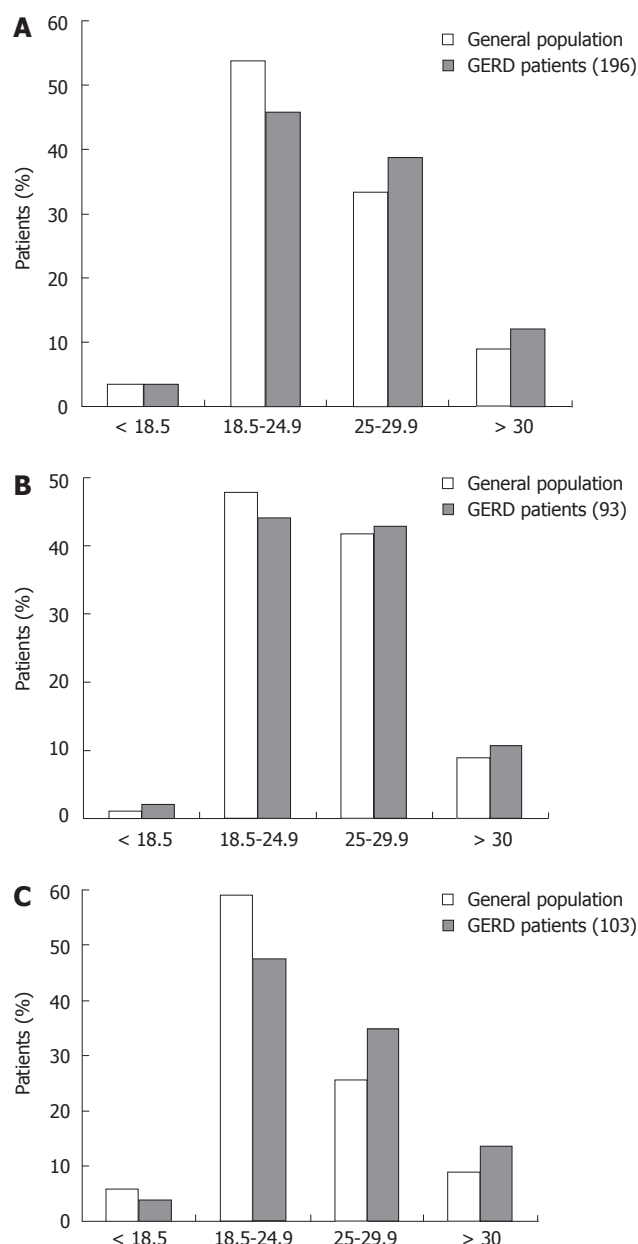


Figure 1 Distribution of BMI in general GERD patients (A), in male GERD patients (B), and in female GERD patients (C) after 24h esophageal pH-metry.

estimated that about 10% of patients complaining of heartburn have functional heartburn, namely heartburn in endoscopy-negative, pH-metry negative patients not responding to proton pump inhibitors^[21]. The prevalence of functional heartburn in patients recruited from our center is 16.5%^[22]. It is also likely that the prevalence of functional heartburn is increased in obese subjects since elevated psychosomatic score is associated with reflux symptoms^[10]. Moreover, some authors have advanced the hypothesis that esophageal mucosa is more sensitive in patients with functional heartburn and obese patients^[23].

These limitations were not encountered in the present study which assessed BMI in patients in whom pathological gastro-esophageal reflux was confirmed by 24-h pH-metry according to DeMeester score, independently of typical or atypical GER symptoms.

Almost 45% of the Italian population have an increased BMI. Although the percentage of overweight subjects is steady, if compared with data from the

ISTAT survey in 1994, the number of obese subjects has increased by 25% in the last 8 years. Elevated BMI, when accompanied with a greater intra-abdominal adipose mass, like other pathological conditions (constipation, cough and dysuria), may increase the abdominal-thoracic gradient favoring gastro-esophageal reflux episodes.

When the relationship between overweightedness or obesity and gastro-esophageal reflux is considered, it should be borne in mind that the eating habits of subjects with increased BMI values are often different from those of normal individuals, in terms of quality (particularly as lipids are concerned) and quantity of the nutrient intake. It is known that fatty foods produce a prolonged inhibitory effect on the lower esophageal sphincter (LES), particularly following intra-duodenal lipid perfusion^[24]. This inhibitory effect would appear due to a cholecystokinin-mediated action on LES^[25]. An epidemiological study revealed that overweightedness, but not excess fatty food intake, increases the risk of hospitalisation for GERD^[26]. Gastric distension following a copious meal produces a transient increase in LES relaxation which is directly correlated to the distension volume^[27] and could thus be another important factor favouring gastro-esophageal reflux. Variables such as eating habits and lifestyle were not considered in this study. The analysis of smoking failed to show any significant difference between the smoker and non-smoker groups.

Albeit, the role of LES pressure in obese patients with GERD is still controversial, and recent data^[28] show that gastro-esophageal pressure gradient but not LES pressure, plays a relevant pathogenetic role in GERD patients with elevated BMI.

All the above mechanisms secondary to overweightedness and obesity would equally affect both genders and cannot explain the association between GER and overweight and obesity in females only.

The role of sex in GERD has not been clearly defined since available data from the literature are not homogeneous. In most epidemiological studies, the prevalence of GERD is greater in males than in females among symptomatic patients^[29-31]. Some studies assessed the prevalence of sex primarily upon endoscopic esophagitis disregarding NERD patients, and found that low grade esophagitis is more frequent in females and high grade esophagitis in male patients^[32,33]. Moreover, one study showed that longer GERD duration is correlated with the male sex^[34]. It is widely known that Barrett's esophagus is far more frequent in males^[35-37].

The present study seems to indicate an association between BMI increase and gastro-esophageal reflux in female patients. Other authors have observed analogous results when studying the prevalence of male or female sex in overweight or obese patients with GERD. In fact, an association between obesity and GERD in females has been reported in some studies in which the authors are of the opinion that higher oestrogen concentration may cause more frequent transient lower esophageal sphincter relaxations^[9,38].

Since high levels of circulating free oestrogens occur in obese and overweight females due to decreased concentration of oestrogen binding globulin and a concomitant increased production of oestrone from the

fatty tissue, the hormonal effect can be indicated as a possible cause of GER. Although differences in gender fat distribution have not been considered, no data suggest a major risk of GER in males and a cardiovascular or metabolic risk of obesity in females. In addition, in this study the analysis of BMI and GER according to different age classes, failed to identify any significant difference, indicating that female postmenopausal distribution of visceral fat, similar to male one^[39], has the same but not relevant effect on GER of the premenopausal fat distribution.

However, it can be hypothesized that the hormonal effect of increased adipose tissue increases the GER risk in overweight and obese females and makes it comparable to the GER risk in males.

In conclusion, findings of the present study suggest that overweightedness and obesity are related with an increased risk of gastro-esophageal reflux in females.

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RAPID COMMUNICATION

Internal biliary fistula due to cholelithiasis: A single-centre experience

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Abstract

AIM: To discuss about the perioperative problems encountered in patients with internal biliary fistula (IBF) caused by cholelithiasis.

METHODS: In our hospital, 4130 cholecystectomies were carried out for symptomatic cholelithiasis from January 2000 to March 2004 and only 12 patients were diagnosed with IBF. The perioperative data of these 12 IBF patients were analyzed retrospectively.

RESULTS: The incidence of IBF due to cholelithiasis was nearly 0.3%. The mean age was 57 years. Most of the patients presented with non-specific complaints. Only two patients were considered to have IBF when gallstone ileus was observed during the investigations. Nine patients underwent emergency laparotomy with a pre-operative diagnosis of acute abdomen. In the remaining three patients, elective laparoscopic cholecystectomy was converted to open surgery after identification of IBF. Ten patients had cholecystoduodenal fistula and two patients had cholecystocholedochal fistula. The mean hospital stay was 13 d. Two wound infections, three bile leakages and three mortalities were observed.

CONCLUSION: Cholecystectomy has to be performed in early stage in the patients who were diagnosed as cholelithiasis to prevent the complications like IBF which is seen rarely. Suspicion of IBF should be kept in mind, especially in the case of difficult dissection during cholecystectomy and attention should be paid in order to prevent iatrogenic injuries.

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Key words: Internal biliary fistula; Cholecystoduodenal fistula; Cholelithiasis; Iatrogenic injuries

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INTRODUCTION

A biliary fistula is an abnormal passage or communication from the biliary system to an organ, cavity, or free surfaces. Fistula are classified as external (biliary-cutaneous) or internal (biliobiliary, bilioenteric, bronchobiliary)^[1]. Internal biliary fistula (IBF) is associated with chronic cholelithiasis in 90% of the cases.

Preoperative diagnosis of IBF is difficult^[2]. As the symptoms of IBF include abdominal pain, fever, nausea, vomiting, flatulence, fat intolerance, diarrhoea and weight loss, which are all non-specific and seen in most gastrointestinal pathologies, the diagnosis is often not suspected preoperatively^[3]. The diagnosis is usually made peroperatively^[4-6].

MATERIALS AND METHODS

A total of 4130 cholecystectomies were carried out for symptomatic cholelithiasis in Ankara Numune Teaching and Research Hospital, Ankara, Turkey from January 2000 to March 2004 and only 12 patients were diagnosed with IBF. The preoperative and peroperative findings as well as postoperative course of the patients with IBF were evaluated from their hospital records.

RESULTS

Pre-operative findings

Of 4130 cholecystectomies, 12 patients (5 women and 7 men) were diagnosed as IBF. The mean age of these 12 patients was 57 years and 8 of these patients were above 60 years. Five of the 12 patients were previously diagnosed cases of cholelithiasis.

Tenderness in the right hypochondrium was the most frequent physical finding presented in 10 of the 12 (83.3%) patients. One case presented with abdominal pain in the right lower quadrant, with nausea, vomiting and abdominal distension, that was preoperatively diagnosed as acute appendicitis.

Preoperative diagnosis of IBF was achieved in only two patients with gallstone ileus and based primarily on plain abdominal X-ray. The consistent findings were air-

Table 1 The characteristics of the patients

No.	Age/sex	Pre-operative diagnosis	Peri-operative findings	Operation	Hospital stay (d)	Morbidity during postoperative course
1	69/F	Gallstone ileus	Double CDF + gallstone ileus + iatrogenic sigmoid perforation	Cholecystectomy + enterolithotomy + T-tube + primary sutures to duodenal wall + Bogota bag + primary colon repair	45	Bile leakage
2	80/M	Acute abdomen, Acute appendicitis? Ileus?	Gallstones, CDF + jejunal mass + gallstone ileus	Cholecystectomy + choledochotomy + T-tube + primary sutures to duodenum + tube duodenostomy + appendectomy + jejunotomy + removal of stone	16	Myocardial infarction, death
3	60/M	Obstructive jaundice	CCDF	Partial cholecystectomy + repair of common bile duct over T-tube	20	Wound infection
4	22/M	Cholecystocholedocholithiasis	Adhesions, conversion to laparotomy, CDF, acute cholecystitis	Cholecystectomy + T tube insertion + primary sutures to duodenal wall	10	NC
5	67/F	Cholelithiasis	Adhesions, conversion to laparotomy, CDF	Cholecystectomy + primary sutures to duodenal wall	8	NC
6	54/F	Acute abdomen	CDF	Cholecystectomy + choledochotomy + T-tube insertion + primary sutures to duodenal wall	8	NC
7	71/M	Choledocholithiasis	Mirizzi's syndrome, CDF	Cholecystectomy + choledochotomy + T tube insertion + primary sutures to duodenal wall	6	NC
8	30/F	Cholelithiasis	CDF	Cholecystectomy + primary sutures to duodenal wall	4	NC
9	66/M	Cholelithiasis	Adhesions, conversion to laparotomy, CDF	Cholecystectomy + T-tube insertion + primary sutures to duodenal wall	8	Wound infection
10	38/M	Acute cholecystitis	Acute cholecystitis, CDF	Cholecystectomy + T-tube insertion + primary sutures to duodenal wall	34	ARDS, death
11	62/M	Acute abdomen, gallbladder perforation	CDF, perforation of gallbladder, pleural effusion	Cholecystectomy + T-tube insertion + primary sutures to duodenal wall + chest tube insertion ¹	28	GI bleeding, bile leakage, sepsis, death
12	70/F	Acute cholecystitis, obstructive jaundice	CCDF, acute cholecystitis	Cholecystectomy + Hepaticojejunostomy	25	Bile leakage

CDF: Cholecystoduodenal fistula; CCDF: Cholecystocholedochal fistula; NC: No complications. ¹This is the primary operation performed. Patient also underwent repeated procedures due to bile leakage.

fluid levels, distended small bowel loops and a radio-lucent stone outside the biliary tree.

Endoscopic retrograde cholangiopancreatography (ERCP) performed in two patients due to obstructive jaundice and choledocholithiasis failed to show any fistulous opening in any of them.

Among four patients diagnosed preoperatively as acute abdomen, two patients had serious coronary failure and other two had history of chronic obstructive pulmonary disease.

Nine of the 12 patients with IBF underwent emergency laparotomy with the pre-operative diagnosis of acute abdomen. In remaining three patients, elective laparoscopic cholecystectomy was converted to open surgery after identification of IBF.

Peroperative findings

In the 12 patients with IBF, peroperative findings were dense adhesion around gallbladder requiring of sharp dissections, fibrosed and contracted gallbladder, encountering difficulty in identifying cystic duct, cystic artery and common bile duct.

Intra-operative cholangiography (IOC) had to be performed only in 4 of the 12 patients in order to prevent iatrogenic injuries due to the aforementioned findings. Nevertheless, IOC was useful in identifying IBF in only one patient. The fistula tract was identified after sharp dissection in the remaining 8 patients. Regarding the types of fistulas, cholecystoduodenal fistula (CDF) and cholecystocholedochal fistula (CCDF) were found in 10

and 2 patients, respectively.

The fistula orifice in the common bile duct was usually closed by T-tube placement after exploration of the common bile duct for stone retrieval. The fistula orifice in the duodenum was primarily repaired and T-tube was inserted with the aim of biliary decompression in the patients with CDF. Cholecystectomy plus fistula repair, enterotomy and removal of the gallstone (enterolithotomy) were performed in two patients with cholecystoduodenal fistula (CDF) plus gallstone ileus. The operative details and other details are shown in Table 1.

Postoperative course

The mean (range) hospital stay was 13 (4-45) d. Two wound infections, three bile leakages and three mortalities were observed in postoperative period. Thus, the mortality rate was 25% (3/12) and morbidity rate was 42% (5/12).

One death occurred due to myocardial infarction on the 16th postoperative day after cholecystectomy + choledochotomy and T-tube insertion + repair of fistula + tube-duodenostomy + jejunotomy and removal of stone. The second death occurred due to acute respiratory distress syndrome (ARDS) on the 18th postoperative day after cholecystectomy + T-tube insertion + primary closure of the duodenum. The third death occurred following repeated procedures (abdominal lavage for intra-abdominal interloop abscess + T-tube reinsertion) due to septic complications caused by bile leakage on the 28th d after cholecystectomy + T-tube insertion + primary closure of the duodenum.

One of the two patients had bile leakage after being discharged from the hospital with T-tube and T-tube was extracted after one month later. External biliary fistula was observed in the other patient and the fistula was closed after 20 d.

DISCUSSION

Internal biliary fistula (IBF) occurs due to acute inflammation with obstruction of the cystic duct resulting in adhesions of the gallbladder to the adjacent viscus, usually to the duodenum; and repeated attacks of inflammation including gangrenous changes of the gallbladder wall and the wall of the adherent viscus, with eventual erosion and fistula formation^[1]. The common causes of IBF include cholelithiasis, peptic ulceration, malignant neoplasm (gallbladder, bile duct, duodenum, pancreas, or stomach), Crohn's disease of the duodenum and paraduodenal abscess^[1,7,8]. In this study, IBF due to cholelithiasis was evaluated.

Our data showed the IBF incidence of 0.29% (12/4130) and all fistula were secondary to cholelithiasis, which is less than the previous reports^[9,10].

Our patients with IBF usually presented with non-specific signs or symptoms of biliary fistula. Hence, the preoperative accurate diagnosis was made only in 16.6% (2/12) patients in the present study, which is in agreement with the previous study^[3]. In the present study, gallstone ileus was suspected on plain abdominal X-ray in only one patient, which was confirmed by operative findings and stone was removed by enterotomy. However, some classical findings on plain abdominal radiography include pneumobilia, intestinal obstruction, aberrantly located gallstone and change of location of a previously observed stone^[1,3]. Although ultrasonography is extremely useful in detecting a fistula, pneumobilia, impacted stones and the presence of residual cholelithiasis and/or choledocholithiasis^[11], none of the IBF in our patients was detected preoperatively by ultrasonography.

As the symptoms of IBF include abdominal pain, fever, nausea, vomiting, flatulence, fat intolerance, diarrhoea and weight loss, which are all non-specific and seen in most gastrointestinal pathologies, the diagnosis is often not suspected preoperatively^[3]. Preoperative diagnosis according to the symptoms was not possible in any of the our cases. Jaundice secondary to common bile duct obstruction was reported to be a common finding in patients with cholecystocholedochal fistula (CCDF)^[9,12]. Although two patients in our study presented with jaundice and underwent ERCP, we were unable to detect any fistulous opening.

Most studies report that the fistulas are detected incidentally or unsuspectedly during ERCP or other radiologic examinations performed for investigation of biliary or pancreatic diseases^[13]. Although ERCP failed to detect IBF in one patient in the present study, ERCP is the principal tool for diagnosis of IBF^[4]. It can easily show the fistula orifice, fistula tract, and communication with the biliary tree, as well as the etiology. ERCP has therapeutic potential by endoscopic sphincterotomy

Table 2 Data on diagnosis and management of internal biliary fistula in literature

Authors	Case number	Peri-operative findings	Procedure
Silecchia ^[18]	5	CCDF, Type II Mirizzi syndrome	NA
Carlei ^[19]	5	CDF	LC + repair
Angrisani ^[6]	34	CDF, CJF, CCF	LC + repair in 14 patients, Conversion to laparotomy in 20 patients
Moreno Ruiz ^[20]	17	14 CDF, 3 CCF, 2 CGF	LC + repair

NA: Surgical data not available; LC + repair: Laparoscopic cholecystectomy + fistula repair; CDF: Cholecystoduodenal fistula; CCDF: Cholecystocholedochal fistula; CCF: Cholecysto-colic fistula; CGF: Cholecystogastric fistula; CJF: Cholecystojejunal fistula.

and choledocholithotomy^[14]. CT is the most appropriate imaging test for further evaluation because sonographic diagnosis is often difficult^[15]. Pickhardt *et al*^[16] reported that MRCP might be useful in selected cases to confirm the diagnosis^[16].

In our study, all fistulas were diagnosed intra-operatively. Suspicion of IBF should be kept in mind, especially in cases of difficult dissection during cholecystectomy due to small, contracted, chronically inflamed and densely adherent gallbladder, and attention should be paid in order to prevent iatrogenic injuries. Moreover, intra-operative cholangiogram through the gallbladder help to identify any existing fistulous tract.

Cholecystoduodenal fistula (CDF) is the most frequent type (70%-90%) of IBF, followed by cholecystocolonic (10%), choledochoduodenal, choledochogastric, cholecystogastric, and duodeno-left hepatic duct fistulas^[1,17]. Similarly in this study, cholecystoduodenal fistula (CDF) and cholecystocholedochal fistula (CCDF) were found in 10 (83.3%) and 2 (16.6%) patients, respectively. Two of our patients with cholecystoduodenal fistula had gallstone ileus (20%).

The standard treatment of IBF is cholecystectomy and repair of the fistulous opening; however, it was performed in only two cases in our study^[2]. Common bile duct exploration, T-tube insertion, choledochoduodenostomy, tube duodenostomy, enterolithotomy and hepaticojejunostomy were also used as an additional procedure when indicated.

It was initially reported that when the IBF was diagnosed during laparoscopic cholecystectomy, it carried a higher rate of conversion to laparotomy^[6,18] (Table 2). Nowadays, as the surgeon is skilled in advanced laparoscopic procedures, such as duodenal mobilization and intracorporeal suturing and knotting, the rate of conversion is low, CDF is no longer considered a contraindication for laparoscopic treatment^[19-21]. Although the procedure was started laparoscopically in three cases in the present study, it was converted to the open due to technical difficulties.

The mortality rate of IBF was 25% (3/12) in our study, which is slightly higher than that reported in literature (i.e. 15% to 22%)^[1,9]; this might be explained as the patients underwent emergency operation for acute abdomen without enough preoperative evaluation.

In conclusion, cholecystectomy has to be performed in the early stage in the patients who were diagnosed as cholelithiasis to prevent the complications like IBF which is seen rarely. Suspicion of IBF should be kept in mind, especially in case of difficult dissection during cholecystectomy and attention should be paid in order to prevent iatrogenic injuries.

COMMENTS

Background

Internal biliary fistula (IBF) is seen rarely and it is a complication of chronic cholelithiasis. Preoperative diagnosis of IBF is difficult because of non-specific signs or symptoms. Although ultrasonography and ERCP are extremely useful in detecting a fistula, the diagnosis of IBF is usually made peroperatively.

Research frontiers

This study showed high morbidity, mortality rate and the peroperative problems encountered in patients with IBF.

Innovations and breakthroughs

Even though IBF is seen rarely, it must be remembered in the patients with chronic cholelithiasis and IBF should be diagnosed during USG and ERCP. Currently, treatment of IBF may be performed during laparoscopic cholecystectomy, but in this case the surgeon must be very skilled in advanced laparoscopic procedures.

Applications

Cholelithiasis has to be treated in early stage to prevent IBF. Suspicion of IBF should be kept in mind, especially in cases of difficult dissection during cholecystectomy due to small, contracted, chronically inflamed and densely adherent gallbladder.

Terminology

Cholecystectomy: Removing of gall bladder *via* operation. Choledocholithiasis: The position of existing one or more stones in bile ducts. Choledochotomy: Incision which is performed on ductus choledochus. Duodenostomy: Surgical fistula which is performed between duodenum and another anatomic area. Enterolithotomy: Removing of stone from intestine. Hepaticojejunostomy: Anatomises which is performed between ductus hepaticus and proximal jejunum.

Peer review

The authors investigated the occurrence of internal biliary fistula seen among the patients receiving cholecystectomy due to cholelithiasis. They concluded that laparoscopic cholecystectomy has to be performed in early stage in the patients who were diagnosed as cholelithiasis to prevent from complications like IBF which is seen rarely. IBF suspicion should be remembered during difficult dissection encountered in peroperative period and attention should be paid in order to prevent iatrogenic injuries.

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RAPID COMMUNICATION

Evidence for the role of gastric mucosa at the secretion of soluble triggering receptor expressed on myeloid cells (sTREM-1) in peptic ulcer disease

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Abstract

AIM: To investigate the role of gastric mucosa at the secretion of sTREM-1 in peptic ulcer.

METHODS: Seventy two patients were enrolled; 35 with duodenal, 21 with gastric ulcer and 16 with chronic gastritis. Patients were endoscoped and gastric juice was aspirated. Patients with duodenal and gastric ulcer underwent a second endoscopy post-treatment. Biopsies were incubated in the absence/presence of endotoxins or gastric juice. Supernatants were collected and sTREM-1 and TNF α were measured by enzyme immunoabsorbent assays. Scoring of gastritis was performed before and after treatment according to updated Sydney score.

RESULTS: Patients with duodenal and gastric ulcer and those with chronic gastritis had similar scores of gastritis. sTREM-1 was higher in supernatants of tissue samples of *H pylori*-positive than of *H pylori*-negative patients with gastric ulcer. Median (\pm SE) sTREM-1 was found increased in supernatants of patients with gastric ulcer before treatment (203.21 ± 88.91 pg/1000 cells) compared to supernatants either from the same patients post-treatment (8.23 ± 5.79 pg/1000 cells) or from patients with chronic gastritis (6.21 ± 0.71 pg/1000 cells) ($P < 0.001$ and < 0.001 , respectively). Similar differences for sTREM-1 were recorded among LPS-stimulated tissue samples of patients ($P = 0.001$). Similar differences were not found for TNF α . Positive correlations were found between sTREM-1 of supernatants from patients with

both duodenal and gastric ulcer before treatment and the degree of infiltration of neutrophils and monocytes.

CONCLUSION: sTREM-1 secreted by the gastric mucosa is an independent mechanism connected to the pathogenesis of peptic ulcer. sTREM-1 was released at the presence of *H pylori* from the inflamed gastric mucosa in the field of gastric ulcer.

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Key words: sTREM-1; Chronic gastritis; Gastric ulcer; Duodenal ulcer

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INTRODUCTION

Many aspects of the mechanisms implicated in the pathogenesis of peptic ulcer disease remain unclear^[1]. Research has been focused more on derangements of mechanisms of protection and repair of the mucosa of the stomach and duodenum^[2]. It appears that most cases of both gastric and duodenal ulcer occur in the setting of infection by *H pylori*. Evidence is mounting in support of *H pylori* as a necessary ingredient in the ulcerative process, similar to acid and pepsin. It is not known whether the bacteria or the accompanying inflammation is the most important factor in the pathophysiology of peptic ulcer disease^[3].

Triggering receptor expressed on myeloid cells (TREM)-1 is a recently discovered receptor expressed on the surface of neutrophils and monocytes. Engagement of TREM-1 has been reported to stimulate the synthesis of proinflammatory cytokines^[4]. A soluble form of TREM-1, named sTREM-1, was observed and identified at significant levels in serum samples of patients with diseases affecting the gastrointestinal tract^[5].

sTREM-1 has been found elevated in the gastric juice

of patients with peptic ulcer. Since its levels were positively correlated to the degree of infiltration of the gastric mucosa by neutrophils, leading thus to the hypothesis that sTREM-1 might be a sign of an inflammatory reaction taking place in the gastric mucosa^[6]. The latter hypothesis might be strengthened by the lack of expression of TREM-1 on cell membranes of macrophages of healthy intestinal lamina propria^[7].

Based on the latter evidence, it is questioned whether sTREM-1 might be produced from the gastric mucosa in the event of peptic ulcer disease. The current study was designed to investigate a role of gastric mucosa for the release of sTREM-1. Furthermore it was investigated whether anti-ulcerative treatment was accompanied by any change of the ability of the mucosa for the secretion of sTREM-1.

MATERIALS AND METHODS

Study group

The study was approved by the Medical and Ethics Committee (6th/11.30.05/26962) of General Hospital "Sismanoglion", Athens, Greece. A total of 72 patients, 54 men and 18 women, aged 58.92 ± 16.52 (mean \pm SD) years were enrolled; 56 patients with peptic ulcer disease and 16 patients with chronic gastritis without peptic ulcer. Admitted patients were divided into three groups based on endoscopic findings, as follows: group A: consisting of 35 patients with duodenal ulcer; group B: consisting of 21 patients with gastric ulcer; and group C, consisting of 16 patients with chronic gastritis.

Informed consent was obtained from all participants. Indications for endoscopy in these patients were abdominal pain or discomfort, epigastric pain with nausea and vomiting, and dyspepsia. All endoscopies were done by the same endoscopist. Peptic ulcer was defined as a circumscribed break in the mucosa in the duodenum (DU) or in the stomach (GU) with apparent depth covered by an exudate, as previously described^[8]. All patients with peptic ulcer disease belonged to the Forrest III score^[9]. *H. pylori* infection was defined by the presence of the bacterium both at the histopathologic findings of each biopsy and after a gastric biopsy culture at the proper growth medium^[10]. Exclusion criteria for the study were: recent upper GI bleeding, gastric carcinoma, diabetes mellitus, liver cirrhosis, acute or chronic renal failure and the ingestion of any antimicrobial or antisecretory medication for at least 15 d prior to endoscopy.

Interventions and study design

All patients were examined by upper GI endoscopy. Among patients with duodenal and gastric ulcer disease two endoscopies were performed; the first before treatment and the second 15 d after the end of the treatment. Among patients with chronic gastritis only one endoscopy was done; at each endoscopy biopsies were collected. Gastric juice was aspirated immediately after the entrance of the endoscope into the gastric lumen. At the time of endoscopy, three biopsy specimens were obtained from adjacent areas of the gastric antrum. When each biopsy

specimen was taken, the forceps were fully opened and aimed at right angles to the gastric lumen to the extent possible to obtain uniformly sized biopsies. Biopsies were obtained from endoscopically intact mucosa distant from focal lesions such as ulcers and erosions. Each biopsy was used for *in vitro* culture.

After diagnosis of peptic ulcer disease or gastritis, esomeprazole 20 mg twice daily was prescribed. It was administered for four weeks in patients with duodenal ulcers, for eight weeks in patients with gastric ulcers and for four weeks in patients with chronic gastritis. For patients with infection by *H. pylori*, clarithromycin 500 mg bid and amoxicillin 1000 mg bid for 10 d were also added. The above treatment was administered according to international guidelines^[11,12].

In brief, gastric antral mucosal biopsy tissues were weighed and cultured on a culture insert over wells containing RPMI 1640 medium with 10% heat inactivated fetal bovine serum in a 5% CO₂ incubator for 18 h^[13]. Biopsies were positioned on the insert with mucosal surfaces up. The first biopsy tissue was left unstimulated and served as control; the second was stimulated with 10 ng/mL of lipopolysaccharide of *Escherichia coli* O144:H4 (LPS), and the third with 30 μ L of gastric juice of each patient. The total volume of the added growth medium was 2.4 mL; when gastric juice was added it represented 1.25% of the total well volume. At the end of the incubation, the plates were centrifuged for ten minutes at 1400 g; then supernatants were collected from the wells and stored at -70°C, until assayed for estimation of sTREM-1 and TNF α . Results were correlated with histopathological findings.

Estimation of sTREM-1

Estimation of sTREM-1 was performed by a home-made enzyme immunoabsorbent assay in samples of supernatants. Capture antibody of sTREM-1 (R&D Inc, Minneapolis, USA) was diluted to 4000 ng/mL and distributed in a 96-well plate at a volume of 0.1 mL per well. After overnight incubation at 25°C, wells were thoroughly washed with a 0.05% solution of Tween in PBS (Merck) (pH: 7.2-7.4). Then 0.1 mL of standard concentrations of sTREM-1 (15.1-4000 pg/mL, R & D Inc) diluted with Reagent diluent (1% BSA in PBS, pH 7.2-7.4, 0.2 micron filtered) serving as a buffer or of supernatants was added in wells. After incubation for two hours, wells were washed thrice, and 0.1 mL of one 400 ng/mL dilution of sTREM-1 detection antibody (R&D Inc) was added per well. The plate was then incubated for two hours, and attached antibodies were signalled by streptavidin. Concentrations of sTREM-1 to each well were estimated by the optical density detected at 450 nm after addition of one 1:1 solution of H₂O₂: tetramethylbenzidine as a substrate (R&D Inc). sTREM-1 was expressed in pg/g of tissue. The lowest limit of detection for sTREM-1 was 3.91 pg/g of tissue. All determinations were performed in duplicate; the inter-day variation of the assay was 5.23%.

Estimation of TNF α

Tumor necrosis factor alpha (TNF α) was measured in samples of supernatants with an enzyme immunoabsorb-

Table 1 Demographic characteristics of patients enrolled in the study. Updated Sydney scores are given

Parameters	Pre treatment	Post treatment	Chronic gastritis
N of patients		56	16
Age (mean \pm SD)		60.13 \pm 17.38	57.11 \pm 15.81
Male/Female		46/10	8/8
Non smoking/smoking		16/40	6/10
Gastric ulcer	21	0	-
Duodenal ulcer	35	0	-
History of NSAID use		33/52	4/16
<i>H. pylori</i> positive/negative	41/11 ^a	7/45 ^b	11/5
Patients with evidence of gastritis	49/52 ^a	34/52 ^d	13/16
(Total updated Sydney Score > 0)			
Site of gastric inflammation			
Antrum (no of patients)	28	21	8
Corpus (no of patients)	8	6	3
Disseminated (no of patients)	13	7	5
Total updated Sydney score (mean \pm SD)	4.69 \pm 1.93 ^a	2.69 \pm 1.22 ^f	4.27 \pm 0.65
Neutrophil infiltration score (mean \pm SD)	1.77 \pm 0.59 ^a	0.81 \pm 0.32 ^f	1.73 \pm 0.73
Monocyte infiltration score (mean \pm SD)	2.15 \pm 0.68 ^a	1.05 \pm 0.61 ^f	1.73 \pm 0.38
Lymphocyte infiltration score (mean \pm SD)	0.87 \pm 0.35 ^a	0.13 \pm 0.08 ^e	0.69 \pm 0.32
Mucosal atrophy score (mean \pm SD)	0.55 \pm 0.17 ^a	0.38 \pm 0.11 ^e	0.58 \pm 0.30
Intestinal metaplasia (mean \pm SD)	0.29 \pm 0.04 ^a	0.13 \pm 0.08 ^e	0.19 \pm 0.07
Density of <i>H. pylori</i> (mean \pm SD)	1.56 \pm 0.28 ^a	0.33 \pm 0.07 ^f	1.45 \pm 0.39

^a*P* vs chronic gastritis, non significant; ^{b,d,f}*P* < 0.01, ^c*P* < 0.05, vs pre-treatment scores. ^e*P* vs pre-treatment scores; non significant.

bent assay (EIA, Amersham, London, UK). Lowest limits of detection were 6.25 pg/g of tissue. All measurements were performed in duplicate and cytokine concentrations were expressed as pg/g of tissue.

Histopathology

Formalin-fixed, paraffin-embedded tissue samples were routinely cut at 3–4 μ m and stained with haematoxylin and eosin alcian blue PAS (Periodic Acid Schiff) (at pH: 2.5) and Giemsa. Immunohistochemistry was performed for *H. pylori* detection with 1:100 dilution (Biocare Med., California, USA).

The presence of gastritis was evaluated in each biopsy sample after separate scoring for each of the following parameters: (a) disease activity as mucosal infiltration by neutrophils; (b) chronic inflammation expressed as infiltration by monocytes and lymphocytes; (c) degree of mucosal atrophy; and (d) intestinal metaplasia. Each parameter was scored from 0 to 3 (0: absent, 1: mild, 2: moderate, 3: marked). In the case of intestinal metaplasia scores indicated the following findings: 0: absence; 1: complete or type I; 2: incomplete or type II; and 3: incomplete or type III. As a consequence total Sydney score of gastritis ranged between 0 and 15, according to previously reported criteria of the updated Sydney System^[14]. The extent of gastric inflammation in the antrum, corpus or both was also recorded. The density of *H. pylori* was also evaluated semiquantitatively by the same criteria^[15]. Specimens were classified by one pathologist who was unaware of the corresponding laboratory findings.

Statistical analysis

Patients of three groups were further divided into subgroups according to the absence or presence of *H. pylori*. Concentrations of sTREM-1, and TNF α were expressed by their median \pm 95% confidence intervals (CI) or range. Updated Sydney classification scores were given by their means (\pm Standard Deviation, SD). Comparison between groups was made by Mann-Whitney *U* test with a correction according to Bonferroni; for qualitative data comparisons were performed by χ^2 test. Correlations between sTREM-1, and TNF α and the gastritis score or the density of *H. pylori* were performed according to Spearman's rank of order. Any *P* value less than 0.05 was considered as significant.

RESULTS

Patients' characteristics are given in Table 1. All patients suffering from duodenal ulcers had presence of *H. pylori* on tissue biopsy.

No differences were recorded between patients with duodenal and gastric ulcer disease before treatment and patients with chronic gastritis regarding histological parameters of gastritis according to updated Sydney score. Differences in total updated Sydney score, and several parameters of chronic gastritis before and after treatment among patients with peptic ulcer disease and patients with chronic gastritis are shown in Table 1.

Concentrations of sTREM-1 and of TNF α in supernatants of samples of gastric mucosa taken from patients with either duodenal ulcer disease or gastric ulcer disease or chronic gastritis pre-treatment are shown in Table 2. sTREM-1 was higher in supernatants of tissue samples of *H. pylori*-positive than of *H. pylori*-negative patients with gastric ulcer. That was also found when mucosa samples were stimulated by LPS. Respectively, similar differences were not found for TNF α . They were also not found for both sTREM-1 and TNF α between *H. pylori*-positive and *H. pylori*-negative patients with chronic gastritis.

In the above subgroups of patients, concentrations of sTREM-1 were higher in supernatants of gastric mucosa of *H. pylori*-positive patients with gastric ulcers than of mucosa of *H. pylori*-positive patients with duodenal ulcer after stimulation by LPS (*P* < 0.05). Concentrations of sTREM-1 were also higher in supernatants of gastric mucosa of patients with duodenal ulcer than of patients with gastritis either without or with stimulation by LPS (*P* of comparisons: < 0.01 and < 0.01, respectively). Similar differences for sTREM-1 were found between gastric ulcer and chronic gastritis for *H. pylori*-positive patients (*P* of comparisons < 0.01 and < 0.01, respectively). No changes were found when gastric juice was added in cultures. Respective differences in concentrations of TNF α were not observed.

Comparisons of concentrations of sTREM-1 and TNF α between supernatants in unstimulated, and LPS-stimulated samples of gastric mucosa in patients with duodenal and gastric ulcer pre and post treatment respectively, are shown in Table 3. In the presence of LPS, TNF α was increased in supernatants of biopsies taken from *H. pylori*

Table 2 Concentrations of soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) and tumor necrosis factor- α (TNF α) of supernatants of tissue samples taken from patients with either duodenal or gastric ulcer disease or chronic gastritis at pre-treatment

Parameters	Duodenal ulcer		Gastric ulcer		Chronic Gastritis	
	<i>H pylori</i> (+)	<i>H pylori</i> (-)	<i>H pylori</i> (+)	<i>H pylori</i> (-)	<i>H pylori</i> (+)	<i>H pylori</i> (-)
N	35	0	14	7	9	7
	sTREM-1 [median (range), pg/g of tissue]					
0	32.41 (13.32)	-	317.27 (73.98) ^a	112.41 (38.71)	6.21 (0.81) ^c	5.18 (0.77)
LPS	102.41 (40.43)	-	455.41 (98.53) ^b	214.55 (44.81)	12.67 (2.16) ^c	9.37 (1.55)
Gastric juice	17.91 (7.11)	-	150.14 (41.16) ^c	118.48 (54.93)	10.07 (2.02) ^c	7.42 (1.22)
	TNF α [median (range), pg/g of tissue]					
0	10.62 (2.07)	-	7.58 (1.75) ^c	6.40 (1.52)	8.11 (1.31) ^c	5.98 (1.45)
LPS	28.25 (3.35)	-	45.33 (13.06) ^c	21.44 (8.12)	19.07 (4.72) ^c	17.87 (2.11)
Gastric juice	18.02 (5.97)	-	10.76 (2.08) ^c	8.51 (2.05)	10.17 (3.23) ^c	9.13 (1.61)

Patients were divided as either *H pylori*-positive or *H pylori*-negative. LPS: Endotoxin of *Escherichia coli* O144:H4. ^a $P < 0.05$, ^b $P < 0.01$, *vs H pylori* negative patients. ^c P *vs H pylori* negative patients, non-significant.

Table 3 Influence of treatment on the secretion of soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) and tumor necrosis factor- α (TNF α) by the mucosa of *H pylori*-positive patients with gastric and duodenal ulcers

Parameters	<i>H pylori</i> -positive with gastric ulcer		<i>H pylori</i> -positive with duodenal ulcer	
	Pre-treatment	Post-treatment	Pre-treatment	Post-treatment
	sTREM-1 [median (range), pg/g of tissue]			
0	203.21 (88.91)	8.23 (5.79) ^b	86.82 (35.41)	3.90 (2.84) ^d
LPS	418.87 (127.56)	14.31 (7.11) ^b	144.90 (58.79)	6.13 (4.07) ^d
	TNF α [median (range), pg/g of tissue]			
0	12.67 (226.79)	9.51 (8.02) ^a	12.47 (133.45)	10.31 (20.41) ^a
LPS	31.07 (231.89)	20.12 (13.51) ^a	34.05 (150.36)	17.88 (23.11) ^a

^b $P < 0.001$, ^d $P < 0.01$, *vs* pre treatment; ^a P : non-significant, *vs* pre treatment.

Table 4 Correlations between sTREM-1 and TNF α and parameters of gastritis score in non and LPS stimulated supernatants of *H pylori* positive patients with duodenal and gastric ulcer and of patients with chronic gastritis

Parameters of Gastritis score	Supernatants of mucosa sampled from <i>H pylori</i> -positive patients with duodenal ulcer disease pre-treatment			
	Absence of LPS		Presence of LPS	
	sTREM-1	TNF α	sTREM-1	TNF α
Neutrophils infiltration	$P = \text{NS}$	$P = \text{NS}$	$P = \text{NS}$	$P = \text{NS}$
Monocytes infiltration	$P = \text{NS}$	$P < 0.01$	$P < 0.05$	$P < 0.01$
	Supernatants of mucosa sampled from <i>H pylori</i> -positive patients with gastric ulcer disease pre-treatment			
Neutrophils infiltration	$P = \text{NS}$	$P < 0.05$	$P < 0.01$	$P < 0.05$
Monocytes infiltration	$P < 0.01$	$P < 0.01$	$P < 0.01$	$P < 0.01$
	Supernatants of mucosa sampled from <i>H pylori</i> -positive patients with chronic gastritis			
Neutrophils infiltration	$P = \text{NS}$	$P < 0.05$	$P = \text{NS}$	$P < 0.05$
Monocytes infiltration	$P = \text{NS}$	$P < 0.05$	$P = \text{NS}$	$P < 0.01$

negative patients with gastric ulcer before treatment and with chronic gastritis ($P < 0.05$ and < 0.05 , respectively).

Correlations of sTREM-1 and TNF α between supernatants of patients with duodenal and gastric ulcer pre treatment and of patients with chronic gastritis when cultured in the absence/presence of LPS and parameters of gastritis score are shown in Table 4. In the absence of LPS sTREM-1 concentrations were significantly correlated with monocytes infiltration in *H pylori* positive patients with

gastric ulcer. In the presence of LPS sTREM-1 was significantly correlated with both monocytes and neutrophils infiltration in *H pylori* positive patients with gastric ulcer; respectively, significant correlations were also observed between sTREM-1 and monocytes infiltration in *H pylori* positive patients with duodenal ulcer. Respectively, both in the absence/presence of LPS TNF α concentrations were significantly correlated with monocytes and neutrophils infiltration in *H pylori* positive patients with and gastric ulcer; respectively, significant correlations were also observed between TNF α and monocytes infiltration in *H pylori* positive patients with duodenal ulcer. No significant correlation was found between any of the latter scores and sTREM-1 or TNF α of supernatants of biopsies taken from patients post-treatment.

DISCUSSION

Among patients suffering from chronic active gastritis only a minority evolves to peptic ulcer disease^[15], rendering the question what might be the underlying mechanism leading several patients with gastritis to peptic ulcer and others not. Recent data revealed that sTREM-1 was found increased in the gastric juice of patients with peptic ulcer disease compared to patients with chronic gastritis^[7]. That finding might lead to the hypothesis that sTREM-1 might constitute an independent factor leading from gastritis to peptic ulcer.

The present study applied a unique design. It is the first time, to our knowledge, in the literature where the ability of

the gastric mucosa for the release of sTREM-1 and TNF α was studied among patients with either duodenal ulcer or gastric ulcer or chronic gastritis without signs of peptic ulcer disease. Supernatants of biopsies taken from the enrolled patients were stimulated with LPS and gastric juice of patients. It is known that *H pylori* as a Gram negative bacterium secretes LPS that mediates to the gastric inflammation^[16]. As described by others, cell loss and apoptosis of gastric mucous cells was enhanced by *H pylori* LPS with less potency compared to the same effect by *E. coli* LPS. The low immunological potency of *H pylori* LPS may contribute to low-grade gastritis^[17]. In an attempt to simulate the latter process cultured biopsies were stimulated with LPS. Inflammation of the gastric mucosa was significantly reduced after treatment whereas *H pylori* was also eradicated (Table 1). Although the proper treatment was administered in patients with chronic gastritis second endoscopy was not performed; the latter was thought to be of no significance because sTREM-1 concentrations were already minimal pre treatment.

Results revealed that gastric mucosa of *H pylori* positive patients with both duodenal and gastric ulcer disease was potent to secrete sTREM-1. The potency for secretion of sTREM-1 was lost post-treatment. The release of sTREM-1 was higher by *H pylori* infected gastric mucosa than by gastric mucosa not infected by *H pylori*. The effect of *H pylori* on the release of sTREM-1 by mucosa of patients with duodenal ulcer could not be assessed since all patients with duodenal ulcer in the studied cohort were *H pylori*-positive.

Similar kinetics to sTREM-1 were not found for TNF α . TNF α was found increased in strict correlation with the degree of mucosal inflammation independently from the underlying pathogenetic status. The latter was highlighted by the fact that TNF α was increased post treatment when gastric mucosa was stimulated by LPS (Table 4).

The main assumption revealed from the presented study was that sTREM-1 was secreted by the activated inflammatory cells that infiltrate the gastric mucosa; inflammatory cells were immigrated to the inflamed gastric mucosa attracted by *H pylori* or its components. The treatment of inflamed gastric mucosa and the eradication of *H pylori* ceased the secretion of sTREM-1. It is of great importance that the latter assumption exists only in the status of gastric and duodenal ulcer disease. The release of sTREM-1 was independent from the density of mucosal inflammation at patients with no evidence of peptic ulcer. The pattern of release of sTREM-1 by the activated inflammatory cells and probably their intracellular activity should be further investigated.

The independent contribution of sTREM-1 release in the pathogenesis of gastric ulcer is further aggravated by the observation that gastric juice could not influence the activity of the inflamed mucosa per se. Stimulation of inflamed gastric mucosa with gastric juice was not lead to a significant increase to the release of sTREM-1 and TNF α (Table 4).

The present study revealed for the first time in the literature that sTREM-1 secreted by the gastric mucosa is an independent mechanism connected to the pathogenesis of gastric and duodenal ulcer. sTREM-1 was released at the

presence of *H pylori* from the inflamed gastric mucosa in the field of gastric ulcerative process. The exact pathogenetic mechanisms needs to be further clarified.

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Inhibition of angiogenesis and HCT-116 xenograft tumor growth in mice by kallistatin

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CONCLUSION: rAAV-mediated expression of KAL inhibits the growth of colon cancer by reducing angiogenesis and proliferation of tumor cells, and may provide a promising anti-angiogenesis-based approach to the treatment of metastatic colorectal cancer.

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Key words: Kallistatin; Adeno-associated virus; Angiogenesis inhibitors; Colon; Neoplasm

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Abstract

AIM: To investigate the inhibitory effect of kallistatin (KAL) on angiogenesis and HCT-116 xenograft tumor growth.

METHODS: Heterotopic tumors were induced by subcutaneous injection of 2×10^6 HCT-11 cells in mice. Seven days later, 2×10^{11} rAAV-GFP or rAAV-KAL was injected intratumorally ($n = 5$ for each group). The mice were sacrificed at d 28, by which time the tumors in the rAAV-GFP group had grown to beyond 5% of the total body weight. Tumor growth was measured by calipers in two dimensions. Tumor angiogenesis was determined with tumor microvessel density (MVD) by immunohistology. Tumor cell proliferation was assessed by Ki-67 staining.

RESULTS: Intratumor injection of rAAV-KAL inhibited tumor growth in the treatment group by 78% ($171 \pm 52 \text{ mm}^3$) at d 21 after virus infection compared to the control group ($776 \pm 241 \text{ mm}^3$). Microvessel density was significantly inhibited in tumor tissues treated with rAAV-KAL. rAAV-KAL also decreased the proportion of proliferating cells (Ki-67 positive cells) in tumors compared with the control group.

INTRODUCTION

Advanced colorectal cancer (CRC) is a critical health concern in the world; overall survival is highly dependent upon the stage of disease at diagnosis. The estimated 5-year survival rates range from 85% to 90% for patients with stage I disease to < 5% for patients with stage IV disease. Over 50% of patients with colorectal cancer presenting with metastatic or locally advanced disease experience local recurrence or develop distant metastases after potentially curative surgery. The most important treatment currently available for patients with stage IV disease is systemic chemotherapy. Although there have been recent advances in the field, with randomized trials confirming the activity of irinotecan, oxaliplatin and capecitabine, median survival remains at only 15-18 mo. There is, therefore, a critical need for more effective and better-tolerated therapies.

With the role of angiogenesis in tumor growth and progression firmly established, considerable efforts have been directed to antiangiogenic therapy as a new modality to treat human cancers. Clinical trials have provided strong evidence that antiangiogenic agents, such as bevacizumab (avastin, anti-VEGF humanized monoclonal antibody), may play a meaningful role in colorectal anticancer therapy, with an optimistic increase of 20%-30% in survival. Based upon the results of these randomized studies^[1,2], bevacizumab has now been approved by the FDA for the first-line treatment of metastatic colorectal cancer in

combination with chemotherapy.

Despite the enthusiasm and promising early results, there are still several problems to resolve in evaluating the activity of antiangiogenic agents, which are predominantly cystostatic rather than cytotoxic, and the clinical results are still disappointing according to internationally accepted RECIST criteria. Antiangiogenic gene therapy strategy holds great promise in advancing antiangiogenesis as an effective cancer therapy to be evaluated in clinical trials in the future. Several lessons can be learned from early clinical trials in antiangiogenic therapy. (1) Prolonged use of angiogenesis inhibitors is envisioned for cancer patients. Because antiangiogenic agents stabilize tumor growth but do not reduce tumor burden, constitutive expression of an antiangiogenic protein even at lower concentrations than bolus doses may be more effective than the intermittent peaks associated with repeated delivery of a recombinant protein. Preclinical experiments have shown that a constant level of these inhibitors in the circulation provides more effective anti-cancer therapy than intermittent peaks of inhibitor in mice^[3]. Therefore, in the future, antiangiogenic gene therapy may be important for protein angiogenesis inhibitors. (2) The angiogenic switch has become recognized as a critical step in tumor propagation and progression^[4]. Multiple angiogenic pathways are involved in the balance between endogenous stimulators and inhibitors. From this perspective, the body may harbor many in situ tumors, yet the tumors do not progress to lethal tumors unless there is an imbalance between a tumor's pro-angiogenic output and the body's total angiogenic defense^[5]. Gene therapy offers a strategy whereby an individual could boost their endogenous angiogenic defenses and tip the balance favorably, because multiple therapeutic genes can be engineered into one vector. (3) The production of functional proteins can be expensive, and repeated usages will not be affordable for patients. Gene therapy offers the opportunity for patients to become their own source of production, i.e., an endogenous factory for antiangiogenic protein production.

Among the identified endogenous inhibitors of angiogenesis, kallistatin (KAL) is one of the best choices because of its broad-spectrum characteristics^[6]. It is capable of inhibiting vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) mediated angiogenesis [unpublished data], as well as preventing tumor invasion *via* the activation of metalloproteinases by inhibiting tissue kallikrein activity.

Gene transfer vectors based on adeno-associated virus (AAV) are of particular interest as they are capable of inducing transgene expression in a broad range of tissues for a relatively long time without stimulation of a cell-mediated immune response. Perhaps the most important attribute of AAV vectors is their safety profile in phase I clinical trials ranging from cystic fibrosis (CF) to Parkinson's disease. The utility of AAV vectors as a gene delivery agent in cancer therapy is showing promise in preclinical studies. With the identification of different serotypes and recent progress in the improvement of AAV vectors, such as dual vectors to overcome the limited packaging capacity, self-complementary vectors to

increase the level and onset of transgene expression, and capsid modifications to mediate cell specific transduction, it will be possible in the future to design more specific and efficient therapies for use in the cancer treatment arena^[7]. Therefore, an approach whereby the KAL gene is delivered to tumors in a form enabling stable and long-term gene expression has become increasingly attractive. Our recent laboratory work revealed that KAL could be a suitable candidate for hepatocellular carcinoma (HCC) therapy [unpublished data]. In the present study, an anti-angiogenic approach by transfer of the KAL gene through an AAV vector was employed to treat colon cancer in a mouse model.

MATERIALS AND METHODS

Plasmid construction

The full-length cDNA fragments of human KAL were amplified from human liver first-stranded cDNA by PCR. Specific primers were designed from the nucleotide sequence of human KAL published in NCBI (accession number L19684), Kalli-F (5'-AAGAATTTCGAGGATGCATCTTATCGAC) and Kalli-R (5'-AAGGTACCAAGCTTCTATGGTTTCGTGGGGTC). Restriction enzyme sites (underlined) were introduced into primers for subcloning. The conditions of PCR were 45 s each at 94°C, 50°C and 68°C for 36 cycles. The PCR fragments were sequenced and subcloned into the AAV-2 vector, which has been described previously^[8,9]. The cDNA fragments of KAL were generated by PCR and were confirmed by DNA sequencing. The sequence of KAL matched that published in NCBI except for two nucleotides. The differences were at nucleotides 1145 and 1146, which resulted in a sense mutation in amino acid sequence. The residue threonine (ACG) at codon 382 was changed into a serine (AGC) residue.

To attain a constitutive and high-level expression of KAL, the KAL cDNA was inserted into the AAV vector between the inverted terminal repeats (ITRs) under the control of cytomegalovirus (CMV) enhancer/chicken β -actin promoter. A woodchuck hepatitis B virus post-transcriptional regulatory element (WPRE) was inserted into constructs immediately after the inserted genes, in order to boost transgene expression^[10].

Generation of rAAV vectors

AAV particles were generated by a three plasmid, helper-virus free packaging method^[8,15]. The viral titre was determined by real-time PCR analysis as described previously^[11].

Fifty-thousand human embryonic kidney (HEK) 293 cells were seeded into 6-well plates and were grown overnight. The medium was replaced by complete medium with reduced fetal bovine serum (FBS) (2%). A total of 5×10^9 vector genome rAAV-GFP particles were incubated with cells for 8 h. Two days later, the ability of the virus to infect and transduce the cell line was assessed by fluorescent microscopy.

Cell lines, animals and antibodies

The HEK 293 cell line and the colon adenocarcinoma

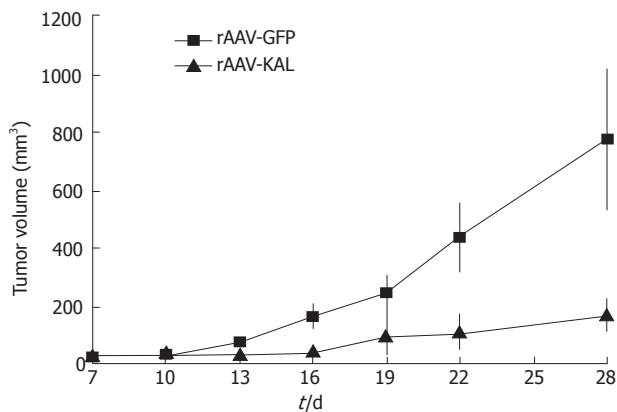


Figure 1 Tumor growth suppression curve: tumor volumes of the rAAV-KAL group versus the rAAV-GFP group on the indicated days.

cell line HCT-116 were purchased from American Type Culture Collection (ATCC). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Grand Island, NY) supplemented with 10% FBS (Gemini, Sacramento, California), 100 unit/mL penicillin and 100 µg/mL streptomycin (Invitrogen). Six- to eight-week-old male BALB/c mice were obtained from the Laboratory Animal Unit of the University of Hong Kong. All surgical procedures and care administered to the animals were approved by the University Ethics Committee and performed according to institutional guidelines. The anti-CD34 (clone MEC 14.7), anti-Ki-67 (clone B56) and anti-rat polyclonal antibodies and anti-mouse polymer conjugate were purchased from Santa Cruz (Santa Cruz, CA), Pharmingen (San Jose, CA), BD Biosciences (San Jose, CA) and Zymed (South San Francisco, CA), respectively.

Tumor model

Tumors were established by subcutaneous inoculation of 2×10^6 HCT-116 cells into the dorsal skin of mice using 25-G needles. Seven days later, 2×10^{11} rAAV-GFP or rAAV-KAL was injected intratumorally ($n = 5$ for each group). The mice were sacrificed at d 28, by which time the tumors in the AAV-GFP group had grown to beyond 5% of the total body weight.

Tumor growth was monitored for 4 wk by measuring two perpendicular diameters. Tumor volume was calculated according to the formula $0.52 \times a \times b^2$, where a and b are the largest and smallest diameters, respectively.

Evaluation of microvessel density

Microvessel density (MVD) was assessed by the method defined by Weinder and co-workers^[12] after CD34 staining. The mean value of the three hot spots was taken as the MVD, which was expressed as the absolute number of microvessels (0.7386 mm² per field).

Quantitation of Ki-67 proliferation index

Positive and negative stained cells were counted on a minimum of 10 randomly selected $\times 400$ high-power fields from representative sections of tumors. The Ki-67 proliferation index (the fraction of proliferating cells) was

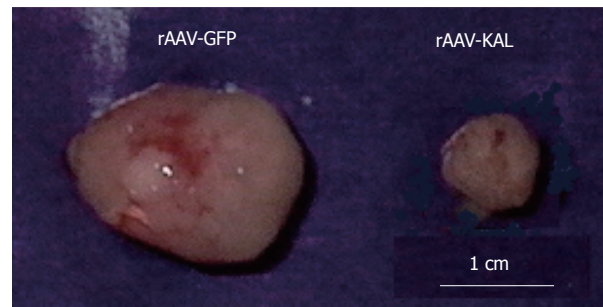


Figure 2 Representative photographs of a tumor at 21 d for mice injected with rAAV-GFP and rAAV-KAL intratumorally.

calculated from the number of Ki-67 positive cells divided by the total cell count.

Statistical analysis

Comparisons of tumor volume between groups were made with the Student's *t*-test where indicated and were considered statistically significant if the *P* value was less than 0.05.

RESULTS

KAL suppressed growth of HCT-116 tumors in vivo

Tumor formation was detected in all of the mice. Tumor growth was significantly slower in the rAAV-KAL group than in animals injected with rAAV-GFP (Figure 1). At d 21 after virus infection, tumor growth was reduced by 78% (171 ± 52 mm³) in the treatment group compared to the control group (776 ± 241 mm³, $P < 0.01$). Representative photographs of the tumor at 21 d for both groups are shown in Figure 2.

Evaluation of angiogenesis by CD34 staining

We found that delivery of KAL could significantly reduce growth of tumors, demonstrating that the treatment method was effective. Since KAL is an antiangiogenic inhibitor, in order to determine whether the suppression of tumor growth in the mice injected with rAAV-KAL was related to the antiangiogenic ability of the transgene product, the tumor blood vasculature was examined by staining for endothelial cell antigen CD34 (Figure 3). A significant reduction in microvessel density was observed in rAAV-KAL [73 ± 29 vessels/high power field (hpf), $P < 0.01$] compared with control mice (236 ± 67 vessels/hpf).

Assessment of cell proliferation by Ki-67 staining

Tumor growth retardation could also be a result of reduction in cell proliferation. To quantitatively compare the proliferation index of tumors in different groups, tumor sections were stained for expression of Ki-67. Ki-67 is strictly expressed in proliferating cells and is commonly used as a marker for cell proliferation. Treatment with rAAV-KAL decreased the proportion of proliferating cells (Ki-67 positive cells) in tumors compared with the control group. Based on the counting of 10 randomly selected microscopic fields, the proliferation index was significantly

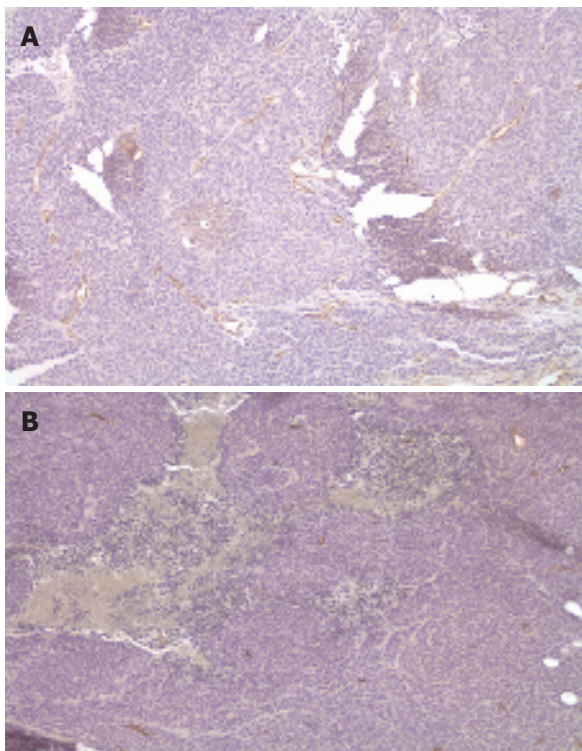


Figure 3 Evaluation of microvessel density at 21 d after intratumor injection of rAAV-GFP (A) and rAAV-KAL (B) (x200).

decreased from $63\% \pm 9\%$ in the control group to $41 \pm 6\%$ in the rAAV-KAL group ($P < 0.01$).

DISCUSSION

Our present data showed that the application of the angiogenic inhibitor KAL suppressed angiogenesis and resulted in growth retardation of colon tumors. CD34 staining of the HCT-116 tumors revealed a significant direct correlation between MVD in histological sections of cancer and size of the tumor. This finding demonstrated that continuous release of KAL in mice could successfully decrease the MVD in HCT tumors, thereby blocking angiogenesis effectively. Ki-67 protein is widely known as an appropriate and useful marker of the proliferating fraction within a given cell population. Since Ki-67 expression provides information on the proliferating status of the tumor cells, it should give good insight into the effect of treatment. The success of the current treatment method lays an important foundation, not just for colon tumor treatment, but also for anti-angiogenic gene therapy.

Miao *et al* found that human KAL significantly inhibits both VEGF and bFGF induced proliferation, migration, and adhesion of primary cultured human endothelial cells in vitro, and attenuates both VEGF and bFGF induced increases in capillary density and hemoglobin content in subcutaneously implanted Matrigel plugs *in vivo*^[6]. KAL is also a heparin binding protein. The major heparin-binding domain was identified in the region between the H helix and C2 sheet of KAL, which contains clusters of positively charged residues. KAL may act by competing with VEGF and bFGF binding to heparan-sulfate proteoglycans, a low affinity-binding site, and thus suppressing VEGF-

and bFGF-binding activity and the angiogenesis signaling cascades induced by VEGF and bFGF.

As a broad-spectrum angiogenesis inhibitor, KAL inhibits angiogenesis mediated by its heparin-binding activity, which is similar to that of endostatin^[13]. It has become clear that various growth factors and lymphokines are required to bind to two distinct classes of cell surface receptors to elicit a signal^[14]. In many ligand-receptor systems, ligands bind first to an abundant low-affinity receptor, which draws the ligand onto the cell surface and then links it to a second, high-affinity receptor that transduces the signal into cells. In addition, KAL is a specific serine proteinase inhibitor (serpin) for human tissue kallikrein. Like plasmin, tissue kallikrein may have a role in degrading extracellular matrix to promote tumor invasion. Our study results confirmed KAL's multifunction purpose for tumor inhibition.

With the role of angiogenesis in tumor growth and progression firmly established, considerable efforts have been directed to antiangiogenic therapy as a new modality to treat human cancers. There is much enthusiasm for the role that antiangiogenic agents may play in preventive therapy. Nevertheless, it is still unclear whether KAL can regress a tumor completely, even after prolonged treatment. Many leaders in the field of angiogenesis now believe that some of the most important future cancer therapies may not completely eradicate all tumor cells in an individual, but instead, may turn cancer into a chronic manageable disease^[5]. Gene therapy strategies leading to increased production of endogenous angiogenesis inhibitors would seem perfectly suited to support such an approach by tipping the balance toward a more antiangiogenic state.

Antiangiogenic approaches should be greatly encouraged, since the FDA has recently approved the angiogenesis inhibitor avastin and the SFDA approved endostatin. Because of the difficulties and high costs of manufacturing numerous endogenous inhibitors of angiogenesis, and because of the need for chronic administration of these agents, gene therapy remains an exciting strategy to circumvent these difficulties.

AAV based vectors are now being used for clinical gene transfer for cystic fibrosis, hemophilia, and Canavan's disease. Although recombinant adenoviral vectors have been utilized for a majority of both preclinical and clinical trials in cancer gene therapy, studies in animal models have suggested therapeutic benefits for tumor treatment using AAV vectors^[7]. T-cell mediated cytotoxicity to AAV vectors has not been observed even though AAV vectors can induce strong humoral immune responses. AAV can initiate long-term transgene expression and this transduction is attributed to episomal concatamer formation without integration into the host chromosome. Therefore, AAV vectors appear to be less mutagenic than other virus vectors. With new serotypes and the potential to develop targeting vectors, AAV holds great promise as a viral vector delivering therapeutic genes such as immune regulation and antiangiogenesis genes for cancer gene therapy.

In addition to AAV studies, the understanding of tumor development at the biological and molecular biological level will lead to the discovery of strong, efficient, and specific enhancers/promoters in tumor cells.

Utilization of regulatory systems will avoid the undesired side effect of systemic transgene expression delivered by AAV vectors for immune-modulation and antiangiogenesis. As better vectors are developed, combination strategies continue to evolve, and there is increased understanding of the complex role that endogenous angiogenesis inhibitors play in tumor growth. Antiangiogenic gene therapy will certainly be evaluated in future clinical trials.

COMMENTS

Background

Colon cancer is one of the most common cancers in the world, with a high propensity to metastasize. Surgical resection currently remains the only curative treatment for colon cancer. Since the majority of deaths with colon cancer result from metastatic disease, inhibition of growth and metastasis of colon cancer is expected to become an effective treatment.

Research frontiers

Antiangiogenesis strategies have been increasing and have been proven to be an attractive strategy for colon cancer therapy, as they are less toxic than conventional chemotherapy and they have a lower risk of drug resistance. Antiangiogenesis strategies can also transiently 'normalize' structure and function of tumor vasculature to make it more efficient for drug delivery and increase the efficacy of conventional therapies. Encouragingly, recent studies have demonstrated it is feasible to complete inhibition of neovascular growth in tumors by attacking multi-angiogenesis mechanisms.

Innovations and breakthroughs

There is growing evidence linking KAL to a role in the inhibition of angiogenesis. In contrast to previous reports that antiangiogenic inhibitors inhibited endothelial cells only, the results of this study clearly showed that KAL not only significantly inhibited VEGF and bFGF induced proliferation, migration, and adhesion of endothelial cells, but also suppressed the proliferation of tumor cells. The multi characteristics of KAL suggest that it is a promising candidate for a colon tumor angiogenesis inhibitor.

Applications

rAAV-mediated expression of KAL inhibits the growth of xenograft colon cancer by 78% compared with controls. Lack of toxicity may favor the clinical use of rAAV-KAL, thus demonstrating its potential in a range of clinical applications of therapy. Furthermore, rAAV-KAL may provide an effective form of therapy for other cancers in future. Elucidating the suppression of proliferation of tumor cells by KAL will provide a better understanding of the mechanism of cancer therapy.

Terminology

Tumor angiogenesis: the proliferation of a network of blood vessels that penetrates into cancerous growths, supplying nutrients and oxygen and removing waste products. Tumor angiogenesis actually starts with cancerous tumor cells releasing molecules that send signals to surrounding normal host tissue.

Peer review

This paper provides sufficient and new data of KAL's unique advantage for colon tumor treatment, and that a KAL based gene therapy has great potential therapeutic value.

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RAPID COMMUNICATION

Changes in gene-expression profiles of colon carcinoma cells induced by wild type K-ras2

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Abstract

AIM: To further elucidate the possible molecular biological activity of wild type K-ras2 gene by detecting changes in wild type K-ras2 gene-induced gene-expression profiles of colon carcinoma cells using cDNA microarray techniques.

METHODS: Total RNA was isolated from peripheral blood of health volunteers. Reverse transcription of RNA and polymerase chain reaction were used to synthesize wild type K-ras2 cDNA. K-ras2 cDNA fragment was cloned into a T easy vector and sequenced. A eukaryotic expression vector pCI-neo-K-ras2 was constructed and transfected to Caco2 cell line using the liposome method. Finally, mRNA was isolated, reverse-transcribed to cDNA from pCI-neo-K-ras2 or pCI-neo blank vector-transfected Caco cells, and analyzed by cDNA microarray assay.

RESULTS: Restriction enzyme analysis and DNA sequencing verified that the constructed expression vector was accurate. High-quality RNA was extracted and reverse transcribed to cDNA for microarray assay. Among the 135 genes, the expression was up-regulated in 24 and down-regulated in 121. All these differentially expressed genes were related to cell proliferation, differentiation, apoptosis and signal transduction.

CONCLUSION: Differentially expressed genes can be successfully screened from wild type K-ras2-transfected colon carcinoma cells using microarray techniques. The results of our study suggest that wild type K-ras2 is related to the negative regulation of cell proliferation, metabolism and transcriptional control, and provide new clues to the further elucidation of its possible biological activity.

INTRODUCTION

Carcinogenesis and progression of human colon carcinoma result from abnormal expression of many tumor-associated genes. Activation oncogenes and deactivation antioncogenes are known as one of their important mechanisms^[1,2]. Ras gene which is closely related to carcinogenesis and progression of colon carcinoma consists of three members: Hras1, Nras and K-ras2^[3,4]. It is traditionally believed that activated Ras gene plays a dominant role as an oncogene in the pathogenesis of colon carcinoma. About 30% of tumors display mutations of Ras gene members, the most frequent mutation is found in K-ras2^[5,6], and the relatively high frequency of K-ras2 mutation is observed in colon, pancreas and lung carcinomas^[7-9]. Recent studies indicate that frequent loss of wild type Ras gene occurs in human and mouse lung adenocarcinomas, thus questioning the dominant role of Ras gene in the pathogenesis of tumors^[10]. Loss of heterozygosity on chromosome 12p12-13 in K-ras2 gene has also been found in non-small-cell lung cancer^[11,12]. We have reported that frequent loss of heterozygosity occurs in this domain during carcinogenesis and progression of colon carcinoma^[13]. By observing the changes in growth curve and cell cycle of colon carcinoma cells transduced with wild type K-ras2 gene, we found that wild type K-ras2 gene could step down the growth and cell cycle of colon carcinoma cells manifested as significantly increased stage G0-G1 cells and decreased stage G2-M cells, suggesting that resting cells with proliferation activity are inhibited to advance into proliferating cell cycle^[14]. In the present study, in order to study the biological activity of wild type K-ras2 gene, we constructed a eukaryotic expression vector of wild type K-ras2 gene phenotype, screened differentially expressed genes of colon carcinoma cells transfected by wild K-ras2 gene with cDNA microarray, and detected the effect of wild K-ras2 on the gene-expression profiles

of colon carcinoma and its function *in vivo*. The results provide new clues to the exploration of the pathogenesis of colon carcinoma and the functions of K-ras2 gene.

MATERIALS AND METHODS

Cells and cDNA array

Human colon adenocarcinoma cell line Caco-2 was obtained from the ATCC. DMEM, FBS, Trizol RNA isolation kit, pCI-neo mammalian expression vector and Lipofectamine2000 were purchased from Invitrogen (Carlsbad, CA). A commercial human expression cDNA array was obtained from Shanghai Biochip Company (Shanghai, China). The array includes 8568 known genes, which can be categorized into cell division, cell signaling, cell structure, gene and protein expression, metabolism and pseudogene, *etc.*

Transfection of Caco-2 cells

Total RNA was isolated from peripheral blood of health volunteers using Trizol RNA isolation kit. Reverse transcription of RNA and polymerase chain reaction were used to synthesize the full-length sequence of wild K-ras2 cDNA. Primers containing *Mul*1 and *Sal*1 restrictions (Y1: 5'-ACCCACGCGTATGACTGAATATAAAC-3'; Y2: 5'-AACGTCGACTTACAT AATTACACACT-3') were synthesized by Shanghai Ouke Biotech Company (Shanghai, China). The PCR products were inserted into pGEM-T Easy vector (Promega) to generate pGEM-T-Ras, and positive clones were identified by blue/white color screening followed by sequencing. pGEM-T-Ras and pCI-neo eukaryotic expression vector were digested in *Mul*1 and *Sal*1 restriction enzymes and ligated using T4 DNA ligase (Promega) to produce pCI-neo-K-ras2. The recombinant pCI-neo-K-ras2 and empty pCI-neo-K-ras2 were transfected into Caco-2 cells (ATCC) using Lipofectamine2000 according to the manufacturer's instructions, and the positive clones were selected from G418 (Amresco).

cDNA microarray analysis

Total RNA was extracted from pCI-neo-K-ras2 (transfection group) and empty pCI-neo-K-ras2 (control group) using Trizol RNA isolation kit. The purity of RNA was confirmed by agarose gel electrophoresis and absorbance (A) ratio (A₂₆₀/A₂₈₀). To make cDNA probes, approximately 5 µg of total RNA was labeled with Cy3-dUTP (control) or Cy5-dUTP (transfection group) by reverse transcription. The probes were precipitated using ethanol and dissolved in 5 × SSC + 2 g/L SDS at 20°C. The microarray and probes were denatured in 95°C water bath for 5 min. Hybridization was performed at 60°C for 15-17 h. Microarray was washed with 2 × SSC + 2 g/L SDS and 1 × SSC + 2 g/L SDS for 10 min respectively and dried at room temperature. Scanning was performed with ScanArray3000 (General Scanning, Inc.). The acquired image was analyzed using ImaGene 3.0 software (BioDiscovery, Inc.). The intensities of Cy3-dUTP and Cy5-dUTP were normalized by a coefficient according to the ratio of housekeeping genes. The positively expressed

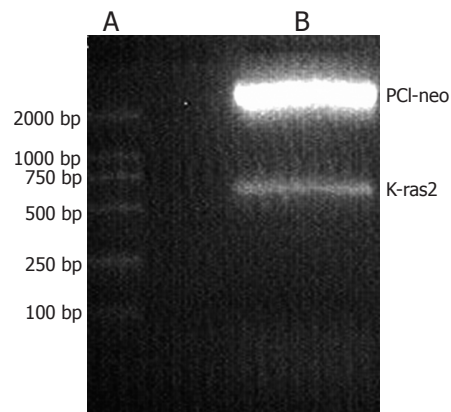


Figure 1 Restriction enzyme analysis of PCI-neo with K-ras2 genes showing a 576 bp K-ras2 gene and a 5600 bp carrier.

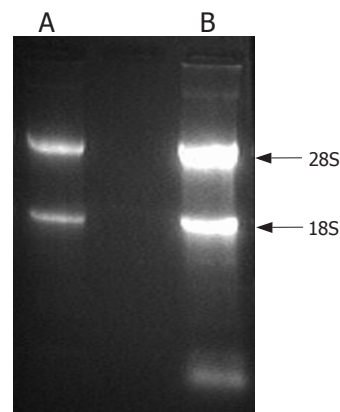


Figure 2 Electropherogram of total RNA from control (A) and transfection (B) groups.

genes were as follows: Cy5-dUTP: Cy3-dUTP signal ratio > 2.0, red fluorescent displaying up-regulated expression; Cy5-dUTP: Cy3-dUTP signal ratio < 0.5, green fluorescent displaying down-regulated expression.

Statistical analysis

Data on gene expression were analyzed by Student's *t* test using SPSS 10.0 software and *P* < 0.05 was considered statistically significant.

RESULTS

Validation of wild type K-ras2 and RNA

Restriction enzyme analysis and nucleotide sequencing of eukaryotic expression vector pCI-neo-K-ras2 showed that its sequence containing an integrity open reading frame was accurate (Figure 1). A 260/A₂₈₀ of total RNA ranging from 1.9 to 2.1 and 28S/18S of about 2 indicated that RNA was not degraded and could be used for preparation of hybridization probe (Figure 2).

Results verified by microarray hybridization system

To monitor the whole process of microarray hybridization, we set up 6 negative controls and 10 positive controls. Scanning of hybridization array and report of array detection showed that hybridization array and sample RNA were intact with good background value and well-distributed noise. The hybridization reaction system was normal and the results were reliable. To compare gene profiles between transfection and control groups, a scatter

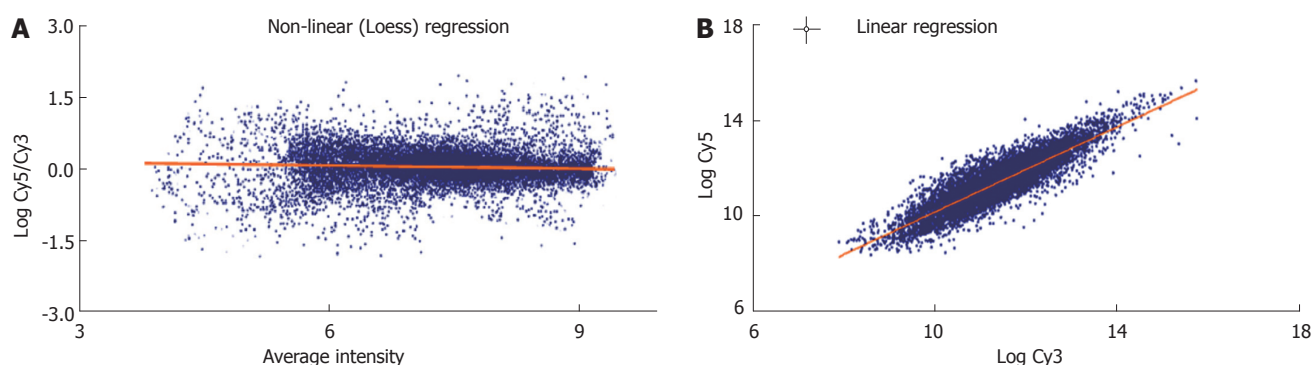


Figure 3 Scatter bar of gene-expression profiles of Caco2 cells transfected (A) and non-transfected (B) with wild-type K-ras2 gene.

Table 1 Biological function of genes with down-regulated expression

Gene	Chromosomal localization	Biological function	Cy5/Cy3
NDP	Xp11.4	Cross-cell signal transmission, signal transduction, NS development	4.509
SSX4	Xp11.23	Transcription regulation, immunoreaction	4.044
CASP1	11q23	Positive regulation of I-kappaB kinase/NF-kappaB cascade reaction, signal transduction, apoptosis	3.754
HPRT1	Xq26.1	Cytolysis, lymphocyte proliferation, purine nucleotide synthesis	3.715
TM4SF2	Xp11.4	ECM	3.554
DXS1283E	Xp22.3	Unknown	3.418
DPYS	8q22	NS development, signal transduction, nucleotide metabolism	3.386
TYRP1	9p23	Cell metabolism, melanin synthesis	3.358
CADPS	3p21.1	Calcium-regulated Exocytosis	3.238
MCF2	Xq27	Cytoskeleton	3.187
COL4A6	Xq22	ECM and ECM synthesis	3.201
F13A1	6p25.3-p24.3	Transcriptional control	3.108
HTR2C	Xq24	IP3-induced signal pathway	3.061
IL13RA1	Xq24	ECM	3.050
OGT	Xq13	Signal transduction	3.030
TOSO	1q32.1	Defence reaction, anti-apoptosis	2.994
MAB21L1	13q13	Positive regulation of cell proliferation, visual development	2.990
CYBB	Xp21.1	Cross-cell signal transmission, chemotaxis, inflammation, signal transduction	2.972
STK9/CDKL5	Xp22	Microtubule skeleton and its synthesis, histogenesis	2.946
PTPRG	3p21-p14	PTK signal pathway	2.878
ELAVL2	9p21	Transcriptional control	2.853
GLA	Xq22	Osteoclast differentiation regulation, bone resorption, cell adhesion	2.851
APXL	Xp22.3	Channel protein of sodium ion	2.839
SERPINA7	Xq22.2	ECM, transport of TH	2.833

profile was plotted for the probe signal values, showing that most genes were distributed around the regression line, and their expression in two tissue samples was similar, but a few genes had a different expression. When the difference in gene expression increased, the number of differently expressed genes decreased (Figure 3). The data were confirmed by the low hybridization signal of these genes. Cy5 fluorescein (red) and Cy3 fluorescein (green) were used to mark the probes of experimental and control groups, and the difference in color was expressed as the difference in gene expression between the two groups. Yellow indicated no expression difference. According to the experimental protocol, the expression of 24 genes with their $cy5/cy3 \geq 2$ (Table 1) was up-regulated, accounting for 17.76% of all the differentially expressed genes, and the expression of 121 genes with their $cy5/cy3 \leq 0.5$ was down-regulated, accounting for 89.63% of all the differentially expressed genes. The top 30 down-regulated genes are listed in Table 2.

Biological function classification of differentially

expressed wild type K-ras2 genes Biological function classification of differentially expressed wild type K-ras2 genes was performed based on the biological classification of genes in Affymetrix gene ontology database. Eleven subtypes were found to be closely related to carcinogenesis (Table 3).

DISCUSSION

Carcinogenesis and progression of colon cancer represent its phases from normal mucosa to atypical hyperplasia (including intestinal metaplasia) of adenoma and adenocarcinoma, involving multiple genes and factors^[15]. K-ras2 gene plays a dominant role as an oncogene in promoting carcinogenesis because of point mutation^[16,17]. In the present study, in vitro experiments demonstrated that carcinogenic agents used in the treatment of loss of heterozygosity in mice with wild type K-ras2 gene facilitated the development of cancer but not in those with normal wild phenotype K-ras2 gene. Moreover, the

Table 2 Biological function of genes with up-regulated expression

Gene	Chromosomal localization	Biological function	Cy5/Cy3
SZF1	3p21	Transcriptional control	0.181
MMP14	14q11-q12	Incision enzyme, proteolysis	0.186
WIT-1	11p13	Cell proliferation	0.189
LOC253012	7q21.3	Gas exchange, cell adhere, early nerve difference and axonogenesis	0.201
OPCML	11q25	Cell adhere, nerve identification	0.239
PLAU	10q24	Chemotaxis, proteolysis, transduction	0.258
MYC	8q24.12-q24.13	Positive-regulated cell proliferation, inhibit cell cycle, balance of iron ion, mRNA synthesis control	0.274
SNTB1	8q23-q24	Muscle contraction, skelet-matrix adhere	0.281
SERPINESL	7q21.3-q22	Ser incision enzyme inhibitor, cell component, angiogenetic regulation	0.290
C20A1	2q11-q14	PCD transport, cell component	0.294
SPTBN1	2p21	Cytoskeleton	0.298
GNAS	20q13.2-q13.3	Energy metabolism, G-protein signal pathway, signal transduction	0.302
PCOLCE	7q22	Proteolysis, cell component	0.303
ENPP2	8q24.1	Cell movement, G-protein signal pathway, phosphorylation metabolism, lytic activity p'tase activity, transcription	0.309
CDK6	7q21-q22	Proliferation, cell cycle, protein conjunct	0.315
HIST2H2BE	1q21-q23	Chromosome component and synthesis	0.323
ZNF137	19q13.4	Transcription control, ion binding	0.332
IL1B	2q14	Inflammation, proliferation, chemotaxis	0.336
IL1A	2q14	Apoptosis, proliferation, transduction, chemotaxis	0.343
MTSS1	8p22	Actin component and synthesis, cell adhere, cell movement, PTK signal pathway, muscle and NS development, internalization	0.346
ACHE	7q22	Muscle contraction, transduction	0.353
EPHB4	7q22	Proliferation, organ-formation, angiogenesis	0.368
AZGP1	7q22.1	Cell member component	0.376
PDE4DIP	1q12	Protein synthesis, actin component	0.377
PITPN	17p13.3	PHL transport	0.379
MEOX1	17q21	Growth	0.386
GNB2L1	5q35.3	Intercellular signal cascade reaction, protein localization, transduction, PKC	0.394
MERTK	2q14.1	Transduction, cross-cell signal transmission, protein phosphorylation	0.405
CUTL1	7q22.1	Transcription control	0.419
FCGR2A	1q23	Immune reaction, signal switch, defence reaction, B cell proliferation	0.428
PAX8	2q12-q14	Organ-formation, transcription control, metanephros development, mRNA synthesis	0.442

size of poorly-differentiated adenocarcinoma in mice with loss of heterozygosity was significantly larger than that of adenoma in mice with abnormal wild phenotype K-ras2 gene. It was reported that cell lines activated by wild type K-ras2 gene-transfected ras can inhibit cell growth, clone formation and tumorigenesis in nude mice, indicating that wild type K-ras2 gene may be a potential anti-oncogene^[9,18]. Changes in gene-expression of Caco2 cells induced by wild type K-ras2 gene were found in our study, showing the possible biological activity of wild type K-ras2 gene.

In our study, genes related to signal transduction, transcription control and cell differentiation were dominant, accounting for 33.33% of the total up-regulated genes. The top 30 down-regulated genes related to cell proliferation accounted for 24.79% of the total up-regulated genes. The expression of genes related to cell metabolism, cell cycle and transcription control was up-regulated. Wild type ras may inhibit cell proliferation by promoting differentiation. In fact, it has long been known that Ras proteins can induce differentiation of some cell types, such as neurons, under certain conditions^[19]. Our findings suggest that K-ras2 can negatively regulate cell proliferation, metabolism and transcription control, and inhibit the growth of colon carcinoma.

The expression of NDP is most significant. As a genetic locus, its mutation may give rise of genetic-correlated Norrie disease caused by two molecular defects in NDP gene. One is 265 C>G missense mutation in the

Table 3 Functional classification of differentially expressed genes

Type of gene	Down-regulated genes (n)	Rate of down-regulated genes (%)	Up-regulated genes (n)	Rate of up-regulated genes (%)
Metabolism-associated	18	14.88	3	12.50
Cell proliferation	30	24.79	3	12.50
Cell cycle	16	13.22	0	0
Signal transduction	18	14.88	8	33.30
Cytoskeleton	8	6.61	1	4.17
Transcription control	18	14.88	4	16.67
Cell adhere	11	9.09	1	4.17
Cell apoptosis	8	6.61	2	8.33
Cell differentiation	14	11.57	4	16.67
Immune-associated	14	11.57	2	8.33
ECM	6	4.96	1	4.17

97th codon by changing arginine into proline, the other is excision in 3'-non-translated region of the third exon^[20]. It was reported that patients with gene excision present relatively severe symptoms, whereas patients with gene mutation display relatively mild symptoms^[21]. The SSX4 gene (a member of the node point protein family) whose expression was significantly up-regulated in our study, can inhibit cell transcription, cause humor- and cell-mediated immune reaction, and may be a very valuable target for vaccine therapy of tumors^[22]. Caspase-1 encoding

apoptosis-associated thioesterase (a member of the caspase family) can lead to proteolysis and activate pro-IL-1, thus playing an important role in cell apoptosis^[23]. Its up-regulated expression in wild-type K-ras2-transduced cells may be related to apoptosis of tumor cells, suggesting that caspase-1 is one of the human p53-dependent cell modulators^[24].

The myc gene whose expression was most significantly up-regulated in our study, is closely related to tumors. It is adjusted by many factors, and can promote cell mitosis and make target cells proliferate and immortalize. This gene involving cell apoptosis is related to tumorigenesis and progression of diverse tumors^[25]. Amplification of correlated sequence of myc has been observed in diverse human tumor cell lines including cell lines of granulocytic leukemia, retinoblastoma, neuroblastoma, breast and lung cancer, as well as in human colon carcinoma cell line^[26,27]. The *MMP14* gene (MT1-MMP) is a member of the matrix metalloprotease family. Its function is modified and regulated by O-glycosylation, interaction with CD44, internalization and recycling, depending on its proper expression on the cell surface^[28]. It can invade tumors by activating MMP2 protein. It was reported that up-regulated expression of MMP2 and MT1-MMP is related to invasion of glioblastoma^[29], while the expression of MT1-MMP is related to local invasion of and metastasis to lymph nodes of oral squamous cell carcinoma^[30], supporting its function in colon carcinoma LoVo cells^[31]. The WIT-1 gene is localized in the upstream of Wilm's tumor gene sharing the same promoter. Methylation of the WIT-1 gene is related to chemotherapy-resistant tumors and acute leukemia^[32]. The EPHB4 gene whose expression was remarkably down-regulated in our study is a member of the biggest receptor tyrosine kinase (RTK) family. Its encoding protein, a receptor of ephrin-B2, promotes microvascular endothelial cell migration and/or proliferation, thus playing an important role in angiogenesis of tumors^[33]. It has been shown that EPHB4 expresses in diverse tumors such as prostate carcinoma and astrocytoma, and involves phenotype transformation post-metastasis^[34,35].

In summary, K-ras2 seems to have a dual function. On the one hand, it promotes cancer development as a gain of function oncogene. On the other hand, it inhibits cancer as a loss-of-function tumor suppressor gene. There are some interesting parallels between the Trp53 tumor suppressor gene and the unfolding story of K-ras2. Trp53 was initially described as an oncogene carrying point mutations in tumors. Later, it was found that it is in fact the wild type copy of the gene that functions as a tumor suppressor gene and is capable of reducing cell proliferation. In this case, the Trp53 mutation may, in a sense, also be considered an activating (but not necessarily gain-of-function) mutation in that it produces a dominant-negative effect over the wild type p53 protein. The two major players in human cancer have more in common than they were previously thought^[18].

only induces tumors but also inhibits tumor growth. We have reported that loss of heterozygosity on chromosome 12p12-13 K-ras2 gene occurs in colon carcinoma and wild type K-ras2 gene can effectively inhibit its growth. This study was to construct a eukaryotic expression vector of the wild type K-ras2 gene phenotype, screen differentially expressed genes of colon carcinoma cells transfected by the wild K-ras2 gene with cDNA microarray, and detect the effect of wild K-ras2 gene on the gene-expression profiles of colon carcinoma and its function *in vivo*.

Research frontiers

Based on the results of recent studies, it is hypothesized that K-ras gene plays a role both in carcinogenesis and in inhibition of cancer. It is a proto-oncogene in normal physiological conditions. However, when mutations occur, the activated K-ras2 gene changes into an oncogene and wild type K-ras2 gene becomes an anti-oncogene. This is what we want to prove in this study.

Innovations and breakthroughs

Since the inhibitory effect of wild type K-ras2 gene on tumors was reported by Zhong-Qiu Zhang, its role in suppressing long cancer has been extensively studied by foreign scholars. This is the first time to study the inhibitory effect of wild type K-ras2 gene on colon carcinoma in China.

Applications

The possible molecular pathway of K-ras2 gene in suppressing tumor cell proliferation found in this study may contribute to finding the genes closely related with colon cancer.

Peer review

This interesting paper investigated the importance of K-ras cascade at mRNA level. The major finding of this study is that wild-type K-ras results in both complex induction and more common inhibition of several genes, and may have a dual role in carcinogenesis. The new association elucidated herein may provide further insight into the carcinogenesis and may identify potentially important therapeutic targets.

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COMMENTS

Background

Studies indicate that mutation of the K-ras2 gene plays an important role in carcinogenesis and progression of tumors. It was reported that K-ras2 gene not

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RAPID COMMUNICATION

Matrix metalloproteinase-9-1562C>T polymorphism may increase the risk of lymphatic metastasis of colorectal cancer

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Abstract

AIM: To explore the role of the matrix metalloproteinase-9 (*MMP-9*) polymorphism in colorectal cancer (CRC) in a northeast Chinese population.

METHODS: Genotyping of *MMP-9*-1562C>T and 279R>Q polymorphisms was carried out on blood samples from 137 colorectal cancer patients and 199 controls using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Multivariate logistic regression models were used to calculate adjusted odds ratios (OR) and 95% confidence intervals (95% CI).

RESULTS: The distribution of *MMP-9* -1562C>T and 279R>Q genotype was not significantly associated with the risk of CRC. However, the risk of lymph node metastasis of CRC was increased in patients with the -1562T allele (OR = 2.601; 95% CI = 1.160-5.835; *P* = 0.022). The frequency of *MMP-9* 279RR + RQ genotype was higher than the QQ genotype among CRC patients younger than sixty years old (OR = 0.102; 95% CI = 0.013-0.812; *P* = 0.012).

CONCLUSION: Our results indicated that the *MMP-9*-1562C>T polymorphism affects lymph node metastasis of CRC. In addition, the *MMP-9* 279R allele may lead to a younger age of onset of colorectal cancer.

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Key words: Matrix metalloproteinase-9; Polymorphisms; Colorectal cancer; Lymphatic node metastasis

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INTRODUCTION

Matrix metalloproteinases (*MMPs*) are a family of zinc-binding proteases that process growth factors, growth factor binding proteins, and cell surface proteins^[1]. They also degrade extracellular matrix (ECM) components and thereby play a central role in tissue remodeling associated with various pathological processes, such as cancer invasion and metastasis^[2].

MMP-9 is a member of the *MMP* family that is also known as gelatinase B or type IV collagenase. (92 kDa). *MMP-9* possesses proteolytic activity against type IV collagen, a major component of the basement membrane, and has been shown to facilitate vascular smooth muscle cell migration^[3]. The expression of *MMP-9* is up-regulated in various types of human cancer, such as esophageal carcinogenesis^[4], breast cancer^[5], and gastric carcinoma^[6]. *MMP-9* expression is also significantly increased in CRC tissues^[7]. Further, over-expression of *MMP-9* represents an early event in colorectal carcinogenesis and may possibly have prognostic value^[8].

The regulation of *MMP-9* expression may be at the transcription level. Growing evidence indicates that genetic variants in the promoters of the *MMP-9* gene may result in differential expression in different individuals^[9]. A promoter variant, -1562C>T, a polymorphism due to a C to T substitution results in the loss of the binding site of a nuclear protein to this region of the *MMP-9* gene promoter^[10]. Polymorphisms in coding regions may also have altered function; a coding region polymorphism, 279R>Q, which is located in the catalytic domain, leads to substitution of arginine by glutamine.

We hypothesized with respect to the role of the two polymorphisms, speculating that they may contribute to CRC risk and metastasis. We thus conducted a case-control study to examine the relationship between *MMP-9* polymorphisms and CRC.

MATERIALS AND METHODS

Study population

The subjects for this case-control study of risk factors

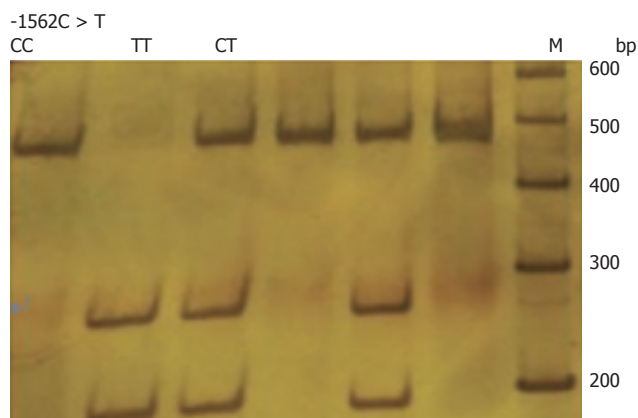


Figure 1 Genotyping of *MMP-9* -1562C>T polymorphism by PCR-RFLP.

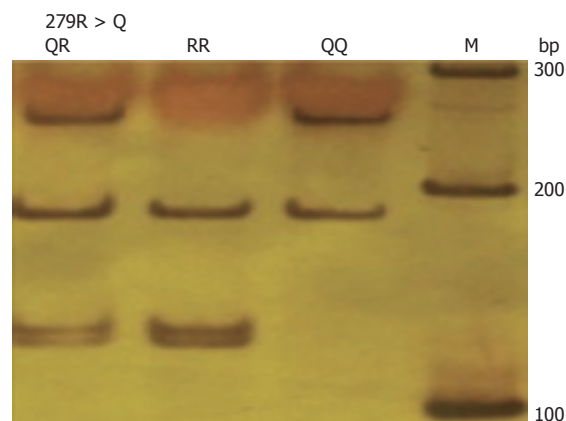


Figure 2 Genotyping of *MMP-9* 279R>Q polymorphism by PCR-RFLP.

for CRC were unrelated and from Shenyang in northern China. The trial recruited 137 CRC patients and 199 healthy control subjects. The patients were comprised of 71 men and 66 women, median age 61.29 years, with a histologically confirmed new diagnosis of CRC, made at the First Affiliated Hospital of China Medical University and Shenyang Anal Hospital, between 2005 and 2006. The CRC patients were grouped according to TNM-classification (UICC) on the basis of postoperative histopathological evaluation.

The controls, 104 men and 95 women, with a median age of 60.65 years, were randomly selected among people admitted to the same hospital during the same period. These control subjects had no history of any cancer.

All subjects gave informed consent for the study, and allowed their blood samples to be analyzed. Detailed information on risk factors including tobacco (smokers were defined as the population who intake more than one cigarette per day; all others were non-smokers), alcohol intake (drinkers were defined as the population who intake more than 50 g alcohol per day), and BMI were obtained with a baseline questionnaire. This study used the suggested WHO BMI cutoff points for Asians to assess several variables; respondents whose BMI was < 23 kg/m² were categorized as normal weight and respondents whose BMI was ≥ 23 kg/m² were categorized as overweight and obese.

DNA extraction

Five milliliters of venous blood was extracted from each subject. Genomic DNA was extracted using proteinase K digestion, followed by a salting out procedure.

Genotyping of *MMP-9* polymorphisms

MMP-9 -1562C>T genotypes were determined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay. The primers used were 5'-GGCACATAGTAGGCCCTTTAA-3'(forward) and 5'-TCACTCCTTTCTTCCTAGCCA-3'(reverse)^[11]. PCR was performed with a 20 μL volume containing 20 ng DNA template, 2.0 μL 10 × PCR buffer, 0.5 U Taq DNA polymerase, 20 pmol of each primer and 1.6 μL 2.5 mmol/L dNTP. Amplification was for 1 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 57°C, and 30 s at 72°C, and a final step at 72°C for 1 min. PCR products were digested with *Sph*I (Takara) and separated on 8%

polyacrylamide gel electrophoresis. After electrophoresis, the homozygous C allele was represented by a DNA band at 442 bp, whereas the homozygous T allele was represented by a DNA band at 264 and 178 bp, and heterozygotes at 442, 264 and 178 bp (Figure 1).

279R>Q genotypes were determined using a PCR-RFLP assay, as previously described. The primers used were 5'-GAGAGATGGGATGAACTG 3'(forward) and 5'-GTGGTGGAAATGTGGTGT-3'(reverse)^[12]. PCR products were digested with *Msp*I (Takara) and separated by electrophoresis on 8% polyacrylamide gels. The 279R allele has two restriction sites, represented by DNA bands at 187, 129 and 123 bp; the 279 Q allele has only one restriction site, represented by DNA bands at 252 and 187 bp (Figure 2).

Statistical analysis

Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated by logistic regression analyses from comparison of genotypes between CRC patients and healthy controls, using SPSS version 13.0 (SPSS, Chicago, IL, USA), adjusting for the potential confounders such as age, sex, tobacco use, alcohol use, and BMI. Association of the genotype with clinicopathological parameters was evaluated by Fisher's exact test. The χ^2 test was used to assess Hardy-Weinberg equilibrium. In all cases, $P < 0.05$ was considered statistically significant.

RESULTS

Characteristics of the study population and the association with CRC are presented in Table 1. There were no significant differences in terms of distribution for age and gender between cases and controls ($P = 0.325$ and 0.951 , respectively). However, cases tended to have a higher BMI ($P < 0.001$), and were more likely to smoke cigarettes ($P < 0.001$).

The *MMP-9* -1562C>T and 279R>Q polymorphisms genotype and allele distribution in cases and controls are shown in Table 2. The distribution of *MMP-9* 279R>Q and -1562C>T polymorphisms in cases and controls were all consistent with a Hardy-Weinberg equilibrium. In addition, there was no linkage disequilibrium between -1562C>T and 279R>Q polymorphisms ($R^2 = 0.009$, $D' = 0.376$).

The frequency of *MMP-9* -1562C>T and 279R>Q

Table 1 Characteristics of cases and controls

	Controls/cases	OR	95% CI
Sex			
Male	101/70	1	
Female	98/67	0.986	(0.638-1.524)
Age (yr)			
≤ 60	98/60	1	
> 60	101/77	1.245	(0.804-1.928)
Smoking status			
Non-smoker	147/75	1	
Smoker	52/62	2.337	(1.473-3.708) ^b
Alcohol duration (yr)			
Never	168/103	1	
1-15	16/14	1.427	(0.669-3.046)
>15	15/20	2.175	(1.066-4.437)
BMI (kg/m ²)			
18.5-22.9	101/35	1	
23-24.9	38/54	4.101	(2.329-7.220)
> 25	60/48	2.309	(1.345-3.962) ^d

^b $P < 0.001$ vs non-smoker group (Two-sided χ^2 test); ^d $P < 0.001$ vs normal BMI group (Two-sided χ^2 test).

Table 2 *MMP-9* genotypes and the risk of CRC *n* (%)

Genotypes	Controls	Cases	OR ¹ (95% CI)
C-1562T			
CC	147 (73.9)	104 (75.9)	1.00
CT	47 (23.6)	33 (24.1)	0.877 (0.527-1.462)
TT	5 (2.5)	0 (0)	
T allele	0.143	0.12	
R279Q			
QQ	16 (8.0)	12 (8.8)	1.00
RQ	85 (42.7)	58 (42.3)	0.936 (0.410-2.138)
RR	98 (49.2)	67 (48.9)	0.961 (0.424-2.177)
R allele	0.706	0.701	
C-1562T and R279Q combinations			
279 QQ and -1562CC	11 (5.5)	9 (6.6)	1.00
1-2 risk alleles ²	155 (77.9)	109 (79.6)	1.357 (0.474-3.886)
> 2 risk alleles	33 (16.6)	19 (13.9)	1.227 (0.661-2.277)

¹OR for CT/TT genotypes versus CC genotypes and adjusted for age, gender, smoking status, alcohol consumption and body mass index. ²Risk alleles were *MMP-9* 279R and -1562T.

genotypes did not differ significantly between cases and controls (-1562CT+TT vs CC: OR=0.877; 95% CI = 0.527-1.462; $P = 0.615$; 279RQ vs QQ: OR = 0.936; 95% CI = 0.410-2.138; $P = 0.875$; RR vs QQ: OR=0.961; 95% CI = 0.424-2.177; $P = 0.924$). We examined the combined effects of the two polymorphisms among cases and controls, using 279R and -1562T as risk alleles. We found that neither the genotypes containing one or two risk alleles, nor those containing more than two risk alleles were associated with increased risk of CRC.

We also estimated the association between the -1562C>T and 279R>Q genotypes and clinicopathological findings among CRC patients (Table 3). Logistic regression analysis revealed that CRC patients with the -1562CT + TT genotype showed increased risk of lymph node metastasis (CT+TT vs CC; $P = 0.022$). Age, sex, TNM classification and external membrane invasion were not correlated with the -1562 C>T genotype. There was no significant difference between the 279R>Q polymorphism and clinicopathological features. However, the frequency

Table 3 Relationship of the C-1562T and R279Q genotypes with clinicopathological features of CRC patients

Parameters	C-1562T		R279Q	
	CC	CT+TT	QQ	RQ+RR
Age (yr)				
< 60 (<i>n</i> = 60)	49	11	1	59
≥ 60 (<i>n</i> = 77)	55	22	11	66 ^c
Sex				
Male (<i>n</i> = 70)	57	13	6	64
Female (<i>n</i> = 67)	47	20	6	61
Lymph node metastasis				
N(-) (<i>n</i> = 87)	71	16	8	79
N(+)(<i>n</i> = 46)	29	17 ^a	4	42
TNM classification				
Stage I (<i>n</i> = 26)	21	5	3	23
≥ Stage II (<i>n</i> = 107)	79	28	9	98
External membrane invasion				
(+) (<i>n</i> = 102)	76	26	9	93
(-) (<i>n</i> = 31)	24	7	3	28

^a $P = 0.022$ vs CC genotype; ^c $P = 0.012$ vs QQ genotype, Fisher's exact test.

of the *MMP-9* 279RR + RQ genotype was higher than the QQ genotype among CRC patients aged < 60 years.

DISCUSSION

Increased levels of *MMP-9* have been found to be associated with CRC susceptibility^[13]. However, despite of the strong rationale for this study, our results showed that the two *MMP-9* polymorphisms did not enhance susceptibility to the development of CRC, although we did find that the *MMP-9* -1562C>T polymorphism was associated with lymph node metastasis of CRC.

Consistent with this finding is a previous report of an absence of an association between the -1562C>T polymorphism and gastric cancer susceptibility; that report also found the -1562T allele to be associated with the invasive phenotype of gastric cancer^[14]. Similarly, a Chinese study has suggested that the -1562C>T genotype distribution in CRC cancer patients and healthy controls was comparable, but an association between the -1562C allele and the invasive capability of CRC was not observed^[15]. To explain this discrepancy, two points warrant consideration. First, the difference may be due to the different populations; all our subjects were drawn from a population pool in the northern part of China, whereas their subjects were not.

Second, the association between the -1562C>T polymorphism and the risk of lymph node metastasis of CRC is consistent with the biological function of *MMP-9*. *MMP-9* is up-regulated in various human cancer types. The over-expression of *MMP-9* is positively correlated with the depth of invasion, lymphatic and venous invasion, and lymph node metastasis, such as in CRC^[7], gastric carcinoma^[16], and prostate cancer^[17]. Lymph node metastasis is considered as the most important prognostic factor of CRC^[18,19]. In addition, the 5-year survival rate of patients with CRC with lymph node metastasis is worse than that in those without lymph node metastasis^[20]. We postulated that the promoter polymorphisms could increase the risk of lymph node metastasis by affecting the expression of *MMP-9*.

The -1562 C>T polymorphism is located within an important regulatory element that appears to be a binding site for a transcription repressor protein. DNA-protein interaction is abolished by the C-to-T substitution at the polymorphic site, which results in a higher promoter activity of the T-allelic promoter^[21]. It is suggested that the *MMP-9* promoter-1562C>T polymorphism appears to regulate gene expression in an allele-specific manner.

A surprising finding was that the frequency of the *MMP-9* 279RR + RQ genotype was higher than that of the QQ genotype among patients younger than 60 years ($P < 0.05$). The results indicate that the *MMP-9* 279R allele may lead to a younger age of onset of CRC. The 279R>Q polymorphism is located in the catalytic domain of the *MMP-9* gene, and within one of the fibronectin type-II like repeats required for binding the enzyme to its substrate elastin^[22]. The polymorphism led to the substitution of a positively charged amino acid (arginine) by an uncharged amino acid (glutamine). These factors may contribute to the mechanism of our findings.

The major limitation of our study is the relatively small sample size, which could have resulted in a less precise estimation of the association between *MMP-9* polymorphisms and CRC susceptibility. Another weakness is that the study population was limited to native northern Chinese in Shenyang city, Liaoning province, and thus the results may not apply to other populations.

In conclusion, our study provides evidence of a connection between *MMP-9* -1562C>T polymorphisms and increased risk of developing CRC. Our results are consistent with a report of the multiple functions of *MMP-9* in cancer^[23], and especially with its role in tumor cell migration and invasion^[24].

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RAPID COMMUNICATION

Molecular evolution of hepatitis A virus in a human diploid cell line

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Abstract

AIM: To investigate the hotspots, direction, and the time course of evolution of hepatitis A virus in the process of consecutive cell culture passage in human KMB17 diploid cells.

METHODS: Wild type hepatitis A virus H2w was serially propagated in KMB17 cells until passage 30, and the full-length genomes of H2w and its six chosen progenies were determined by directly sequencing RT-PCR products amplified from viral genomic RNA. Alignment comparison of sequences from H2w with its six progenies and phylogenetic analysis of the whole VP1 region from H2w, progenies of H2w, and other cell culture adapted hepatitis A virus were then carried out to obtain data on the molecular evolution of hepatitis A virus in the process of consecutive passage in KMB17 cells.

RESULTS: Most of the mutations occurred by passage 5 and several hotspots related to adaptation of the virus during cell growth were observed. After that stage, few additional mutations occurred through the remaining duration of passage in KMB17 cells except for mutation in the virulence determinants, which occurred in the vicinity of passage 15. The phylogenetic analysis of the whole VP1 region suggested that the progenies of H2w evolved closely to other cell culture adapted hepatitis A virus, i.e. MBB, L-A-1, other than its progenitor H2w.

CONCLUSION: Hepatitis A virus served as a useful model for studying molecular evolution of viruses in a given environment. The information obtained in this study may provide assistance in cultivating the next generation of a seed virus for live hepatitis A vaccine production.

INTRODUCTION

The mechanism of molecular evolution is often used by epidemiologists to acquire information about the direction of an epidemic, velocity of dissemination, site of the origin of the epidemic, and even predictions concerning future epidemics^[1,2]. These methods are helpful in visualizing molecular events of viruses that have evolved in a known environment, i.e. consecutive propagation of viruses in a given cell line under given temperature and nutritional conditions. However, only a few studies had been carried out to fulfill this objective. Research of this type can provide virologists important information on the evolutionary history of viruses, as well as the virulent gene in the virus.

Our group has accumulated several years of technical expertise in the cultivation of hepatitis A virus (HAV) in the human KMB17 diploid cell line, which originated from human embryonic lung tissue^[3]. The aim of the present study was to employ HAV as a model virus to investigate the evolution of the virus in the course of consecutive cell culture passage in human KMB17 diploid cells. In the present study, we serially propagated wild type HAV H2w in KMB17 cells to obtain 30 consecutive progenies, applied cold adaptation at passage 15 to 20 to investigate the conditions resulting in mutation within virulence determinants, and acquired virus samples at the end of every 5 passages from serial progenies for genome-wide sequence analysis. The resulting 5th (H2K5), 10th (H2K10), 15th (H2K15), 20th (H2K20), 25th (H2K25), and 30th (H2K30) progenies were obtained and analyzed. We have attempted to summarize the time course of molecular events that occur in the process of consecutive passage of HAV in human KMB17 diploid cells, as well as the hotspots and the direction of the evolution of HAV in given conditions. We hope that the information obtained from this study will help explain the conditions which promote the development of mutations pertaining to viral virulence determinants, and provide help in the development of a seed virus for the production of live virus vaccine.

MATERIALS AND METHODS

Cells and viruses

The KMB17 cell line which originated from human embryonic lung^[4] is now widely used for vaccine production in China^[3]. The KMB17 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10% newborn calf serum (Invitrogen).

The H2w strain of HAV was isolated directly from feces obtained from hepatitis A patients^[5]. The KMB17 cells-adapted viruses were retrieved from cell substrate as follows: cells were first suspended in 6 mL phosphate saline buffer (pH 7.0) and treated with three cycles of freeze-thawing/sonication. The supernatant was obtained after centrifugation at 8000 r/min for 30 min and then extracted three times with chloroform. The virus in the supernatant was precipitated by the addition of polyethylene glycol 6000 to obtain a final concentration of 10% (wt/vol) and sodium chloride to give 0.4 mol/L solution^[6]. The virus pellet was collected by centrifugation at 12000 r/min for 30 min and was used directly in HAV RNA extraction.

Consecutive passage of HAV in KMB17

10⁵ 50% tissue culture infectious doses (TCID₅₀) of H2w were inoculated into 80% confluent monolayers of KMB17 cells in a 25 cm² flask (Costar). After 2 h of adsorption, the cells were washed three times and incubated at 35°C for 45 d to fulfill the first passage in the cell line^[3]. When harvested, the cells were suspended in 6 mL phosphate saline buffer (pH 7.0) and treated as described in the “cells and viruses” section above to obtain the 1st progeny virus in the supernatant. One ml of the supernatant was then inoculated into KMB17 cells to perform the 2nd passage. In this passage, the cells were kept at 35°C for 28 d. Consecutive passages from 3rd to 30th passage were carried out using the same technique as for the 2nd passage. All the passages were carried out at 35°C except for six passages from 15th to 20th, which were performed at 32°C.

Design of PCR primers

The HAV genome was divided into ten overlapping fragments, the size of about 1000 bp, and primers specific to both ends of the fragments were designed. To facilitate the process of sequencing and to ensure reproducibility of the results, we added a universal adapter from T7 promoter primer or M13 reverse sequencing primer to the routine primers used in specific amplification of HAV genome (Table 1). Our design of PCR primers allowed us to sequence PCR products with universal primers, which ensured maintaining the same reaction system for sequencing different PCR fragments, thus providing more reproducible results.

Nucleotide sequence analysis

HAV RNA was extracted from virus pellets using Trizol (Promega) according to the manufacturer's instruction. cDNA was synthesized using primers complementary to HAV nt 1567 to 1549, 3098 to 3080, 4549 to 4530, 6027 to 6008 or 3' RACE primer with AMV reverse transcriptase

Table 1 Primers used in amplifying HAV genome

Name	Sequences	Position
F1	T7 ¹ -TTTCCGGAGYCCCTCTTG	22-39
R1	M13R ² -ATCTGCCAGAGACAGGA	806-790
F2	T7-GAGGTACTCAGGGGCATTTA	690-709
R2	M13R-GGATCCAAAGCAAAAGACA	1567-1549
F3	T7-AGATTGGAGTTGCATGGATTAAC	1430-1453
R3	M13R-GGATCCTGAAACATCCA	2342-2326
F4	M13R-TTCAACAACAGTTTCTACAG	2231-2250
R4	T7-AGCTTCCTCCTCTGATCTA	3098-3080
F5	T7-GGGAGATAAGACAGATTCTACAT	2861-2883
R5	M13R-AGCTTCCACTCTTAACAAA	3813-3795
F6	M13R-GAATGCTTGGATTGTCTGG	3634-3652
R6	T7-GATCCATCCCAGTAATCTGA	4549-4530
F7	T7-ATCAATTGCATTGGCAAC	4451-4468
R7	M13R-GGATCCGCATCTAATTTAATC	5278-5258
F8	M13R-GCTGTGGGAGCTGCAGTTGG	5124-5143
R8	T7-GATCCATGGGACTCTTTCTA	6027-6008
F9	T7-GAGGAAATTCATTTCTTG	5872-5889
R9	M13R-GGATGGAGTTCAGAA	6764-6750
F10	T7-GGAGATGTGGTCTTGAT	6657-6674
R10	M13R-AGAGGGGTCTCCGGGAATTT	polyA
3'RACE	CAAGAGGGGTCTCCGGGAATTTTTTTTTTTTTT	polyA
	TTTTTT	

¹T7 indicates adapter with sequence: TAATACGACTCACTATAGGGAGA,

²M13R indicates adapter with sequence: AGCGGATAACAATTCACACAGGA.

(Promega). PCR amplifications were performed using 10 pairs of primers specific for HAV genome (Table 1) with Taq polymerase (Promega) based on the routine operation of PCR amplification. Sequences of PCR fragments were determined by directly sequencing the gel-purified PCR fragments on an ABI Prism 3730XL automated sequencer using T7 Promoter primer or M13 reverse sequencing primer. At least three reactions of the same PCR fragment were performed to ensure reliable results. The nucleotide sequence of the full-length HAV genome was obtained by splicing sequences from 10 overlapping RT-PCR fragments. Pair wise comparison of sequences from H2w and its six progenies was performed with Lasergene software package. The number of mutations in the nucleotide occurring in every 5 passages was calculated, and the mutation events as well as mutations in the deduced amino acid were summarized to provide information on hotspots and the time course of mutations of HAV during consecutive cell culture passages.

Subgenotype analysis of progeny viruses

Subgenotype analysis was performed by pair wise comparison of sequences from 168 bp VP1/2A junction at nt 3024 to nt 3191 according to the method of Robertson *et al.*^[7]. HM175 and LA were used as reference strains for IB and IA subgenotypes respectively. Divergence less than 7.5% in comparison with the reference strain was used as a criterion for distinguishing different subgenotype.

Phylogenetic assay of cell culture derivatives of H2w with other cell culture adapted HAV

Phylogenetic and molecular evolutionary analyses were conducted to evaluate the direction of evolution of HAV in consecutive passages in KMB17 cells. According to the

Table 2 Divergence of H2w and its progenies compared to reference strains in VP1/2A region (%)

	H2w	H2K5	H2K10	H2K15	K2K20	H2K25	H2K30
HM175	10.1	3.6	3.6	3.6	3.6	3.6	3.6
LA	4.8	7.7	7.7	7.7	7.7	7.7	7.7

method described by Costa-Mattioli *et al*^[8], the entire VP1 nucleotide sequences of H2w and its chosen progenies were aligned to other cell culture adapted HAV or reference strains, LA/wt^[9], GBM/wt, GBM/FRhK, GBM/HFS^[10], HM175/wt^[11], HM175/HAV7^[12], HM175/18F, HM175/24A, HM175/43C^[13], L-A-1^[14] and MBB^[15], by the CLUSTAL W program^[16]. Matrix distances for the Kimura two-parameter model were calculated from the resulting alignment output^[17] and used to plot neighbor-joining phylogenetic trees. These methods were implemented by using MEGA software package version 3.1^[18].

GenBank accession numbers

Sequences of several HAV strains were drawn from the GenBank for phylogenetic analysis. These included LA/wt (K02990), GBM/wt (X75215), GBM/FRhK (X75214), GBM/HFS (X75216), HM175/wt (M14707), HM175/HAV7 (M16632), HM175/18F (M59808), HM175/24A (M59810), HM175/43C (M59809), L-A-1 (AF314208) and MBB (M20273).

RESULTS

Nucleotide sequences of full-length HAV genomes

Our design allowed us to sequence the PCR products with a high degree of efficiency and reproducibility. All the three reactions of the same PCR fragment gave the same result, with excellent readability. The individual sequences from PCR fragments were spliced to obtain seven nearly full-length genomic sequences, which were deposited in GenBank with accession numbers: H2w (EF406357), H2K5 (EF406358), H2K10 (EF406359), H2K15 (EF406360), H2K20 (EF406361), H2K25 (EF406362) and H2K30 (EF406363).

Conversion of subgenotype during cell culture-adaptation

After pair wise comparison of H2w and its six cell culture derived progenies with HM-175 and LA, a divergence 3.6% of progenies to reference IB strain (HM175) other than 10.1% of the parent H2w was obtained. Often divergence less than 7.5% to the reference strain was a criterion for distinguishing different subgenotype, therefore, cell culture-adaptation switched the subgenotype of progenies from 1A to 1B (Table 2), which was not a common phenomenon in cell culture study of HAV conducted by other workers^[12,19].

Hotspot mutations in the course of consecutive cell passage

After alignment comparison of nearly full-length genomic sequences from H2w and its six cell culture derived progenies, we summarized hotspot mutations accumulated in the course of every 5 passages in the KMB17 cell line

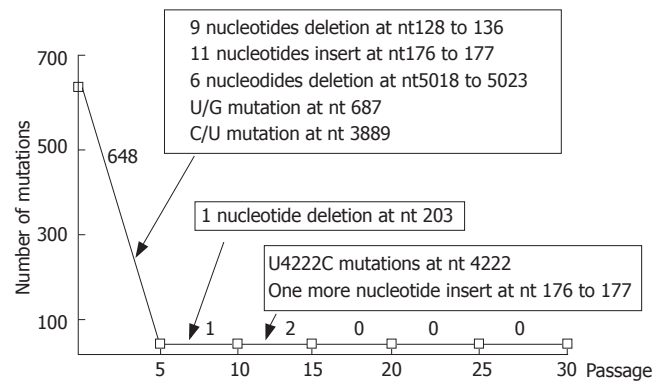


Figure 1 Summary of hotspots and time course of mutations in the process of consecutive passage of HAV in human cell line KMB17. The numbers on the curve indicate total mutations occurring at every 5 passages. Text in the boxes describes hot mutations occurring at the stage of cell culture passage.

(Figure 1). Within the first 5 passages, 648 mutations occurred throughout the genome, out of which forty were amino acid mutations. Among these 648 mutations, 9 nucleotides deletion at nt 128 to 136, 11 nucleotide insert at nt 176 to 177, 6 nucleotide deletion at nt 5018 to 5023, U/G mutation at nt 687 and C/U mutation at nt 3889, are possible hotspot mutations common to cell culture adapted HAV, or specific to HAV when adapted to grow in the KMB17 cell line^[12,19], which played crucial role in ensuring that the virus grew efficiently in human cell line KMB17. In the course of the next 5 passages, there was only one nucleotide deletion at nt 203, which was common to cell culture adapted HAV as described by other authors^[12]. A U/C mutation at nt 4222 occurred during the third 5 passages, which has been shown to be a virulence-related mutation^[20]. In the remaining consecutive passages, no new mutations were observed.

Time course of mutations pertaining to cell culture adaptation

Cell culture of HAV has been studied for many years. Several authors have reported their findings on hotspots related to changes in the HAV genome after cell-culture adaptation in different cell lines^[12,19,21-25]. Among these findings, C/U mutation at nt 3889 of 2B region^[23] was the most important in cell culture adaptation of HAV, regardless of the cell line origin. Other events, such as insertion mutation in 5' NCR and deletions at the beginning of the 3A region, have been suggested as cell line specific phenomena^[19,25]. In the present study, we found that almost all the events related to the adaptation of HAV in cell culture appeared to have been completed as early as passage 5 (Figure 1). These events included deletions near nt 131, insert at nt 176 to 177, U/G mutation at nt 687, C/U mutation at nt 3889 along with deletions near nt 5020. All the new mutations were stable during the remainder of the consecutive passage in KMB17 cells. Another mutation common in cell culture adapted HAV^[12], was deletion of 1 nucleotide at nt 203, which occurred before passage 10.

Time course of mutations within virulence determinants

It is difficult to identify mutations responsible for the

attenuation of HAV. Several studies have examined the virulence determinants of HAV through chimeric viruses constructed from wt HM175 and an attenuated derivative HAV/7^[20,26,27]. According to the studies conducted by Emerson *et al.*^[20], six nucleotides (HAV nt 3025, nt 3196, nt 4043, nt 4087, nt 4222, nt 4563) may be involved in the attenuation of HAV during cell culture adaptation. When we compared H2w and its cell culture derivatives, only nt 4222 of the six nucleotides mentioned above was found to have mutated from U to C in the long term consecutive cell culture adaptation. We thus extrapolated that in our study, nt 4222 was the main determinant for the virulence of HAV. In present study, we carefully compared H2w and its six chosen derivatives from cell culture at nt 4222, and summarized the time course of mutations related to virulence determinants of HAV. The virulence determinant did not mutate before the 10th passage in KMB17 cell line. Mutation of virulence determinant occurred between passage 11 and passage 15, and these mutations were stable throughout the remainder of the consecutive passage in KMB17 cell line (Figure 1).

Direction of evolution of HAV in prolonged cell culture passage

The complete VP1 nucleotide sequences from H2w and its six progenies were determined and aligned with those of cell culture adapted HAV strains taken from the GenBank. A phylogenetic tree was produced using Kimura two-parameter distance and the neighbor-joining method (Figure 2), which was implemented using the MEGA software package. The results showed that the six progenies of H2w were clustered more closely to MBB, a HAV adapted to human hepatocellular carcinoma cell line^[15], and L-A-1, another live attenuated HAV developed in the human diploid cells^[14], whereas the progenitor H2w clustered in another group with LA, the reference strain of HAV IA subgenotype. The situation was very different when derivative viruses from HM175 and GBM were examined. The derivatives from HM175 or GBM were clustered closely only with their progenitor virus. The phylogenetic tree suggested that progenies of H2w evolved closely to other cell culture adapted HAV rather than its progenitor virus regardless of the origin of the wild progenitor or the method used for adapting the virus.

DISCUSSION

In the present study, we used HAV as a model virus to investigate the molecular evolution of HAV in the course of prolonged consecutive passage in KMB17 cells. We obtained a good understanding of the overall time course of molecular events that occur during prolonged consecutive cell culture passage by genome-wide sequence comparison of HAV selected from serial cell culture-derived progenies. After assessing the data on the hotspot mutations at different stages of the consecutive cell culture passage, we concluded that nearly all the mutations occurred before passage 5 and these mutations remained stable throughout the remainder of the prolonged passage in KMB17 cells; several of the hotspot mutations

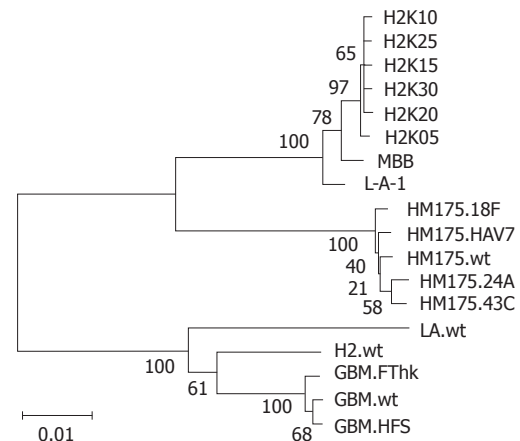


Figure 2 Phylogenetic analysis of the complete VP1 region using MEGA software package version 3.1. The numbers at nodes indicate bootstrap percentages after 1000 replications of bootstrap sampling. The bar indicates genetic distance.

played crucial roles in determining the ability of HAV to grow in cells other than hepatocytes. After this stage, no new mutations were observed except for mutations in the virulence determinants during the remainder of the consecutive passage. If the velocity of mutation is taken into account, our results were different from those described by other workers. Graff *et al.*^[19] noted that the GBM strain did not mutate its 5' non-coding region (NCR) before the 8th passage in a human cell line, and before the 20th passage in a rhesus kidney derived cell line. The main factors influencing the velocity of mutation remain unclear. Perhaps the cell substrate plays a critical role in determining the process of mutation. As the phylogenetic analysis of the whole VP1 region from H2w and its derivative progenies with other cell culture derived HAV indicates, cell culture derived progenies of H2w evolve closely to other cell culture derived HAV, i.e., MBB and L-A-1, which were successfully adapted to grow in human cells, despite the origin of the wild type progenitor or the method for adapting the virus. It is possible that the determinant factor for the direction of the evolution may be the cell substrate used, since MBB, L-A-1 and progenies of H2w were all produced in cell lines originating from human tissue.

Only a few studies have examined the time course of mutations within the virulence determinants of HAV, as well as the conditions for the occurrence of the mutations. The information obtained from this evolution study should help explain when and why the virus needed to mutate its virulence gene. In the present study, a U/C mutation at nt 4222 of 2C region with Phe/Ser conversion in amino acid occurred in H2K15, which was crucial for the attenuation of HAV, according to the identification of the virulent gene by Emerson *et al.*^[20]. Therefore, HAV may evolve its main virulence determinants in the vicinity of passage 15 in KMB17 cells. Cold adaptation was a common technique used to attenuate the virus^[28]. We therefore, hoped that this technique would mutate the virulence determinants of HAV. In our study, after the passage of virus was repeated 14 times at 35°C, we lowered the temperature from 35°C to 32°C for the following 6 passages in order to mutate the

virulent gene. The temperature was returned to the normal 35°C after completing the 20th passage so as to obtain the mutants accustomed to the higher temperature. Because U/C mutation at nt 4222 had occurred in H2K15, which was produced at lower temperature, we presumed that this mutation was temperature related. Recently, we sequenced a fragment of H2K14, the 14th progeny virus produced at the normal 35°C, and discovered that the U/C mutation at nt 4222 had already occurred (Data not shown). Therefore, cold adaptation may not influence the mutational process of virulence determinants of HAV. Perhaps, prolonged cell culture adaptation in an appropriate cell line is the real determinant for the occurrence of mutations of the virulent gene. By contrast, a study by Konduru *et al*^[29] showed that the wild type HM175 displayed no mutations during 9 passages in human hepatoma Huh7 cells.

Genetic stability is a major concern in the development of any live viral vaccine. Because of the quasispecies nature of the RNA viruses, it is believed that these viruses are heterogenous in their genome and mutations occur nonspecifically across the genome. In the case of live hepatitis A vaccine, few systematic studies have been carried out to determine the genetic stability of cell culture adapted HAV genome. In the present study, we investigated the genetic stability of cell culture adapted HAV genome after careful observation of the molecular evolution of HAV during prolonged consecutive passage in KBM17 cells. To ascertain that there would be few genetic changes during consecutive passages in KBM17 cells after completion of mutations regarding cell culture adaptation and virulence, we performed alignment analysis of genome-wide sequences from H2w and its progenies resulting from consecutive cell passage. Our results showed that no mutation occurred throughout the whole genome and none of the virulence determinants were affected by prolonged passage in KBM17 cells after the 15th passage. We concluded that the cell culture adapted genome of HAV was very stable during the course of consecutive cell culture passage in human diploid cells.

The data obtained from analyzing the molecular events during prolonged consecutive passage of HAV in KBM17 cells may provide information and technical assistance in monitoring the genetic stability of virulence determinant of seed virus used in live vaccine production, as well as the genetic stability of the live virus vaccine. Our observations may help establish an optimized design for cultivating seed virus for live vaccine production, which depends upon the knowledge of when and why the virus evolved its virulent gene, and the overall time course of mutations during consecutive passage in a given cell line. We hope that our findings are useful in breeding a seed virus for other live vaccine production, i.e., influenza, Japanese encephalitis, *etc.* In conclusion, HAV is an effective model for identifying hotspots, as well as the time course and direction of evolution of the virus in given conditions. The mechanism and possible implications of this type of evolution merits further investigation. Studies utilizing other viruses should be carried out to further evaluate this type of evolution.

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RAPID COMMUNICATION

Postoperative complications in patients with portal vein thrombosis after liver transplantation: Evaluation with Doppler ultrasonography

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Abstract

AIM: To study the postoperative complications in patients with preoperative portal vein thrombosis (PVT) undergoing liver transplantation (LT) and to evaluate the complications with Doppler ultrasonography.

METHODS: Retrospective studies were performed on 284 patients undergoing LT (286 LT) with respect to pre- and postoperative clinical data and Doppler ultrasonography. According to the presence and grade of preoperative PVT, 286 LTs were divided into three groups: complete PVT (c-PVT), partial PVT (p-PVT) and non-PVT, with 22, 30 and 234 LTs, respectively. Analyses were carried out to compare the incidence of early postoperative complications.

RESULTS: PVT, inferior vena cava (IVC) thrombosis, hepatic artery thrombosis (HAT) and biliary complications were found postoperatively. All complications were detected by routine Doppler ultrasonography and diagnoses made by ultrasound were confirmed by clinical data or/and other imaging studies. Nine out of 286 LTs had postoperative PVT. The incidence of the c-PVT group was 22.7%, which was higher than that of the p-PVT group (3.3%, $P < 0.05$) and non-PVT group (1.3%, $P < 0.005$). No difference was found between the p-PVT and non-PVT groups ($P > 0.25$). Of the 9 cases with postoperative PVT, recanalizations were achieved in 7 cases after anticoagulation under the guidance of ultrasound, 1 case received portal vein thrombectomy and 1 case died of acute infection. Ten LTs had postoperative IVC thrombosis. The c-PVT group had a higher incidence of IVC thrombosis than the non-PVT group (9.1% vs 2.6%, $P < 0.05$); no significant

difference was found between either the c-PVT and p-PVT groups (9.1% vs 6.7%, $P > 0.5$) or between the p-PVT and non-PVT groups ($P > 0.25$). Nine cases with IVC thrombosis were cured by anticoagulation under the guidance of ultrasound, and 1 case gained natural cure without any medical treatment after 2 mo. HAT was found in 2 non-PVT cases, giving a rate of 0.7% among 286 LTs. Biliary complications were seen in 12 LTs. The incidence of biliary complications in the c-PVT, p-PVT and non-PVT groups was 9.1%, 3.3% and 4.3%, respectively ($P > 0.25$ for all), among which 2 stenosis led retransplantations and others were controlled by relative therapy.

CONCLUSION: C-PVT patients tend to have a higher incidence of PVT and IVC thrombosis than non-PVT patients after LT. The incidence of postoperative complications in p-PVT patients does not differ from that of non-PVT patients. A relatively low incidence of HAT was seen in our study. Doppler ultrasonography is a convenient and efficient method for detecting posttransplant complications and plays an important role in guiding treatment.

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Key words: Portal vein thrombosis; Liver transplantation; Postoperative complications; Doppler ultrasonography

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INTRODUCTION

Portal vein thrombosis (PVT) is a common complication of chronic liver disease and malignant liver tumors. The incidence of PVT in patients undergoing liver transplantation (LT) ranges from 4.9% to 10.6%^[1-4]. In the past, technical difficulties and the potential risk of vessel reconstruction, as well as the higher rate of postoperative complications made PVT a contraindication of LT^[1,5].

However, improvement of operative management and advancement of postoperative care have meant that PVT is no longer a contraindication of LT^[6]. Only a few studies have looked in detail at the actual impact of preoperative PVT on postoperative complications. The understanding of pretransplant PVT still remains a problem. Since 1999, our center has begun to perform LT, and a portion of LT recipients have suffered from preoperative PVT. The aim of this study was to analyze the probable impact of preoperative PVT on postoperative complications, and to discuss the value of Doppler ultrasonography on monitoring the complications and guiding treatment by comparing results of LT in patients with and without PVT.

MATERIALS AND METHODS

Patients

Between February 1999 and April 2006, 302 patients received cadaveric liver transplantations. The inclusion standard was as follows: records of clinical data and ultrasonographic exams were complete. Exclusion standard was as follows: postoperative routine Doppler ultrasonography studies were not performed. Of the entire population, 284 patients (286 LT) were included in our study, with 284 primary grafts and 2 regrafts due to biliary stenosis after the primary graft. Patient group characters were as follows. There were 237 males and 47 females with an age range of 4-65 years (mean age, 43.7). The number of LTs was used as the frame of reference in our study.

Primary disease and previous surgery were as follows. There were 131 malignant liver tumors (2 regrafts), among which 8 had hepatic segment excision, 6 had splenectomy and 1 had transjugular intrahepatic portosystemic shunt (TIPS). One hundred and forty-eight cases had chronic liver disease, among which 8 had splenectomy and 3 had splenic artery embolization. Four patients had hepatic hydatidosis, 1 patient had congenital Caroli's disease, 1 patient had acute hepatic failure after liver contusion, and 1 patient had fulminant hepatitis. Orthotopic liver transplantations were performed on all patients including 284 full-size and 2 reduced-size grafts.

PVT patients: Of 286 LTs, 52 (18.2%) had preoperative PVT, with 48 males and 4 females ranging in age from 28 to 65 years (mean age, 44.3). Primary disease and previous surgery were as follows. There were 46 malignant liver tumors, with 1 liver segment excision and 3 splenectomies; 6 patients had cirrhosis, with 1 splenectomy and 2 splenic artery embolizations. PVT was confirmed by Doppler ultrasonography, contrast-enhanced CT and/or surgery as follows; there were 44 cases with tumor thrombosis and 8 with thrombus. All 52 PVT patients received full-size grafts. Portal vein (PV) reconstruction was as follows. Forty-seven patients accepted end-to-end PV anastomosis, 3 accepted porto-caval hemitransposition, 1 accepted PV to mesenteric vein anastomosis and 1 accepted PV to renal vein anastomosis.

According to the presence and grade of PVT, 286 LTs were divided into three groups. (1) Complete PVT (c-PVT) group: 22 cases, all with complete main portal vein (PV) thrombosis and 15 of them had PV branch thrombosis, 3

Table 1 Postoperative complications according to presence or absence of PVT and PVT grade

Postoperative complications	c-PVT <i>n</i> = 22 (%)	p-PVT <i>n</i> = 30 (%)	non-PVT <i>n</i> = 234 (%)
PVT	5 (22.7)	1 (3.3)	3 (1.3)
IVC thrombosis	2 (9.1)	2 (6.7)	6 (2.6)
BC	2 (9.1)	1 (3.3)	10 (4.3)
HAT	0	0	
	0	0	2 (0.9)

with superior mesenteric vein (SMV) thrombosis, 2 with splenic vein (SPV) thrombosis, and 2 with both SMV and SPV thrombosis. (2) Partial PVT (p-PVT) group: 30 cases, including 2 with partial main PV thrombosis and 28 with PV branch thrombosis. (3) Non-PVT group: 234 cases, which were the control group.

Ultrasound examination

An Agilent Sonos 4500 (HP, USA), Logiq 500 (GE, USA) and HDI 5000 (ATL, USA) were utilized in our study. The probe frequency was 2-5 MHz. Preoperative ultrasound scan included etiology of the liver, presence of PVT, and grade and extent of PVT. Postoperative ultrasound scan included size, configuration and echo of graft, the diameter of the hepatic vein, the PV and hepatic artery, anastomotic stoma, presence of thrombosis, blood flow and spectrum, presence of biliary obstruction, presence of perihepatic space effusion, and hydrothorax and ascites. Examination intervals were on the operation day and then daily or every 2 d during the first week. If any complications occurred, a scan was performed daily or according to the clinical situation.

Our study focused on early postoperative complications, which was defined as having been diagnosed within the initial hospital stay^[7].

Statistical analysis

Statistical analysis was performed with SPSS 10.0 (Chicago, IL, USA). A probability value less than 0.05 was considered significant. Differences in incidence between c-PVT, p-PVT and non-PVT groups were determined by Chi-square analysis.

RESULTS

Postoperative complications included PVT, inferior vena cava (IVC) thrombosis, hepatic artery thrombosis (HAT) and biliary complications (BC). Postoperative complications of the three groups are listed in Table 1.

PVT

Postoperative PVT was observed in 9 cases (3.1%), among which 6 were rethrombosis (11.5%). Five PVTs were found in the c-PVT group (22.7%), 1 was found in the p-PVT group (3.3%) and 3 were found in the non-PVT group (1.3%). The grades of PVT were 3 complete main PVT (Figure 1A and B), 1 partial main PVT and 5 PV branch thromboses. Nine PVTs were confirmed clinically as thrombus, appearing on 2-11 d, among which

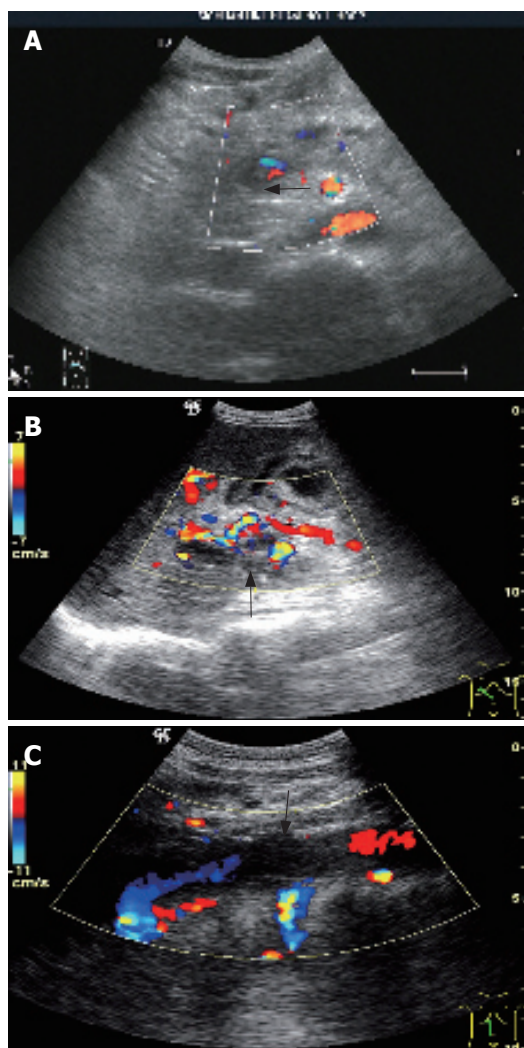


Figure 1 A 32-year-old male underwent LT due to posthepatic cirrhosis. **A:** Preoperative c-PVT; **B:** Postoperative main PV (arrow) and right branch thrombosis. Collateral circulation near PV was clearly showed also; **C:** Postoperative IVC (arrow).

recanalizations were achieved in 7 after anticoagulation, 1 received portal vein thrombectomy on the 5th d and 1 died of acute rejection on the 12th d postoperatively.

IVC thrombosis

IVC thrombosis was seen in 10 cases on 5-13 d postoperatively. All were subhepatic IVC thrombosis (Figure 1C), with 4 complete and 6 partial thromboses. Recanalizations were obtained in 9 cases after anticoagulation. One case with partial thrombosis got natural cure after 2 mo.

HAT

HAT was seen in 2 non-PVT patients on d 3 and 5. Thrombectomy was successfully performed on 2 patients. BC.

Thirteen cases suffered from BC postoperatively. Four of these were stenosis, leading to cholangioplasty in 2 cases and retransplantation in the other 2 cases. Two cases had cholangitis and were cured with drainage and anti-infective treatment. Four cases had evanescent dilation of

the intrahepatic duct. One case had biliary fistula and a repair operation was necessary. One case had secondary biliary obstruction.

The incidence of postoperative PVT in the c-PVT group was higher than in the p-PVT ($P < 0.05$) and non-PVT groups ($P < 0.005$), and there were no significant differences found between the p-PVT and non-PVT groups ($P > 0.25$). The c-PVT group had more IVC thromboses than the non-PVT group ($P < 0.05$) and did not differ from the p-PVT group ($P > 0.5$). There was no difference for IVC thromboses between the p-PVT and non-PVT groups ($P > 0.1$). Among the three groups, the incidence of BC between every two groups was not different ($P > 0.25$ for all groups).

DISCUSSION

Preoperative PVT and LT

PVT is a common preoperative complication in patients undergoing LT. In China, posthepatic cirrhosis and advanced malignant liver cancer form the majority of primary diseases in the LT population^[8], which results in an even higher incidence of PVT in LT patients. In our series, the incidence of PVT was 18.2%, which is higher than reports elsewhere^[1-4,6,16]. Gayowski *et al*^[5] also found an extremely high incidence of PVT (26%) in a predominantly male group of patients mostly with postnecrotic cirrhosis. Our study is consistent with the finding in that most of our cases were male and primary diseases were posthepatic cirrhosis and liver cancer accompanied with cirrhosis.

In past years, PVT has been taken a contraindication for LT. With improvement of surgical techniques and enhancement of posttransplant care, the outcomes of PVT patients are very close to those of non-PVT patients. The one-year survival rate of PVT patients is reported to be 81%^[6], and 1-, 2- and 4-year survival rates are not significantly different in patients with or without PVT^[5]. However, several reports confirmed that PVT had a substantial impact on increased blood loss, a higher mortality rate, a higher graft loss and more posttransplant complications^[1,5,9,10]. The mortality rate of patients with PVT and extensive splanchnic venous thrombosis undergoing LT is as high as 33%^[10]. As shown by Zhou *et al*^[11], PV tumor thrombosis is one of two major prognostic factors of HCC after LT. Therefore, strict preoperative screening and efficient postoperative examinations such as laboratory examinations, Doppler ultrasound, CT, MRI, and angiography when needed should be executed on PVT recipients^[4,12]. In addition, such surgeries should be handled by an experienced transplantation center.

Postoperative complications of PVT patients undergoing LT

PVT is a common postoperative complication of LT. Especially in children, PVT is the top transplant vascular complication^[13]. The clinical presentations include intractable ascites, variceal bleeding, shrunken livers, splenomegaly, and hepatic failure^[1-3]. It's widely

accepted that risk factors of posttransplant PVT include severe pretransplant portal hypertension, experience of treatment for portal hypertension (e.g., TIPS, portocaval shunt, splenectomy, and splenic vein embolization) and preoperative PVT^[4,14-16]. The incidence of postoperative PVT in our study was 3.1%, which is similar to 1%-3% of other reports^[2,4,17,18]. The incidence of rethrombosis in LT recipients with native PVT has been reported to range from 5% to 21%^[2,4,10,16]. A significantly increased incidence compared to the whole group of rethrombosis (11.5%) was also seen in our study, which is much higher than 1.3% of non-PVT recipients ($P < 0.005$). Although Settmacher *et al*^[16] associated splenectomy during LT with an increased incidence (10.5%) of PV complications, none of our patients endured splenectomy during LT.

In the study by Yerdel *et al*^[4], PVT was classified into four grades as follows: grade 1; $< 50\%$ PVT \pm minimal obstruction of SMV, grade 2; grade 1 but $> 50\%$ PVT, grade 3; complete PVT and proximal SMV thrombosis, and grade 4; complete PVT and entire SMV thrombosis. By Yerdel's research, postoperative complications of grade 1 did not differ from those of non-PVT, while grades 2, 3 and 4 exceeded non-PVT. We only divided the PVT patients into the p-PVT and c-PVT groups instead of classifying the recipients as in the study by Yerdel *et al* because there were too few grade 3 and 4 patients to perform statistical analysis. Our results also revealed the impact of grade and extent of PVT on postoperative complications. Retrombosis of c-PVT recipients was 22.7%, which was higher than that of p-PVT and non-PVT groups.

With regard to the probable cause of posttransplant PV complications, Settmacher *et al*^[16] took pathological changes of the vessel wall for an important reason, because all cirrhosis patients may have experienced partial or complete PVT and pathological changes of the vessel wall may increase the incidence of PV complications to 17.9%. In our study, PV branch thrombosis was predominant in the p-PVT group, which had a minute influence on PV reconstruction, while blood vessel pathological changes caused by partial thrombosis of main PV were relatively milder than c-PVT. We attribute these probable reasons to the lower incidence of postoperative PVT in p-PVT than c-PVT and similar incidences of postoperative complications between p-PVT and non-PVT groups. With regard to LT in children, postoperative PVT is much more common with the incidence ranging from 8% to 33%, which is attributed to donor/recipient portal vein diameter mismatch and application of graft veins^[7,13,19]. One of the patients with postoperative PVT in our study was a 4-year child without preoperative PVT. However, it is difficult to reach a conclusion that mismatch of donor/receptor vein diameter may lead to posttransplant PVT due to too few child cases.

IVC thrombosis

IVC thrombosis occurred in 3.5% of our series (10/286), with more in the c-PVT group than in the non-PVT group and adjacent occurrences were seen between p-PVT and non-PVT groups. Donor/recipient vessel mismatch and

recurrence of the underlying disease (e.g. Budd-Chiari syndrome) account for the majority of complications of the vena cava, while unknown causes account for the minority^[16]. At this time we can not explain the higher incidence of IVC thrombosis in c-PVT.

HAT

HAT is a common posttransplant complication, which is associated with operation techniques^[20]. In our center, vascular anastomoses are accomplished by a vascular surgeon with microsurgical techniques, which remarkably improves the quality of vascular reconstruction, especially on small vessels like the hepatic artery. Thus, posttransplant HAT in our center has been decreased to an ideal extent, which is much less than in other centers^[18,21].

BC

Biliary stenosis, biliary fistula and cholangitis account for most of the postransplant BC, for which technical failure or local ischemia are major causes^[22]. BC occurred after 13 LTs (4.5%), affecting 11 recipients in our series. No difference was found between groups with or without PVT. Biliary cirrhosis caused by biliary obstructions in two recipients led to retransplantations in the 7th and 12th mo, which suggests that BC may be the main reason for late retransplantation.

Vascular complications after LT may be more prone for placing the patient and allograft in jeopardy than BC, but they rarely necessitate retransplantations with early management^[18,23]. In our study, although 2 patients suffering from postoperative HAT endured re-exploration and thrombectomy, and 1 patient with postoperative PVT died of acute rejection, no retransplantations were caused by PVT or IVC thrombosis if early diagnosis and treatment were administered. Settmacher *et al*^[16] studied 1000 LTs and demonstrated that more than half of the patients suffering from PV complications did not require any treatment at all and treatment became necessary only when additional complications such as arterial occlusion or bile duct injuries occurred.

Doppler ultrasonography in postransplant evaluation of PVT recipients

In China, cirrhosis and unresectable liver cancer forms the majority of primary disease in LT recipients, among which PVT has a high incidence. Preoperative PVT should have the requirement for pretransplant screening and postoperative monitoring of a high quality. Since Doppler ultrasonography is rapidly available, inexpensive and provides reasonable accuracy, it is predominant in pre-, intra- and posttransplant evaluations^[4,21,24]. Accurate diagnosis of vascular abnormalities helps to determine the operation agenda^[4]. Early detection and management of postoperative complications, especially clinically unsuspected vascular complications, can satisfactorily reduce the mortality rate and avoid retransplantation^[2,21]. Conventional Doppler ultrasound provides an ideal specificity on diagnosing PVT ranging from 97% to 100%^[4,24,25]. In our series, Doppler ultrasonography detected all of the complications and

got confirmation of clinical presentations and other image examinations. In our center, if anticoagulation is essential when the posttransplant patient suffers from PVT or IVC thrombosis, a daily ultrasound examination will be performed to observe dissolution of thrombosis. Termination of treatment is thrombosis dissolution and vessel recanalization determined by ultrasound examination.

In a previous study by Hom *et al*^[25], diagnostic validity of contrast-enhanced ultrasound (CEUS) was comparable to that of angiography, and CEUS shortened study time markedly. In addition, we are currently working on CEUS on posttransplant patients and will have results when more cases are studied.

In conclusion, the incidence of PVT after LT in our study was higher in the c-PVT group than in the p-PVT and non-PVT groups. Compared to non-PVT patients, c-PVT patients are more likely to suffer from IVC thrombosis postoperatively. The incidence of BC did not differ between c-PVT, p-PVT and non-PVT groups. In our center, postoperative HAT is relatively uncommon. Doppler ultrasonography provides an accurate diagnosis of complications after LT, plays a very important role in posttransplant monitoring and is essential in guiding treatment.

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Effects of lamivudine on the function of dendritic cells derived from patients with chronic hepatitis B virus infection

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CONCLUSION: The lower expression of phenotypic molecules and impaired allogeneic mixed lymphocyte reaction function of dendritic cells derived from patients with HBV infection could be restored *in vitro* by incubation with LAM.

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Key words: Dendritic cell; Lamivudine; Chronic hepatitis B; Immune

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Abstract

AIM: To investigate if the nucleoside analogue lamivudine (LAM), a potent inhibitor of HBV replication, could restore the function of dendritic cells derived from patients with chronic hepatitis B (CHB) in an Asian population.

METHODS: Dendritic cells (DCs) derived from mononuclearcytes of patients with chronic HBV infection were cultured in the presence of IL-4, granulocyte-macrophage colony-stimulating factors (GM-CSF) and gradient concentrations of LAM (0-2 mmol/L). Cell morphology was observed under light microscopy. Cell surface molecules, including HLA-DR, CD80, CD83, and CD1 α , were analyzed with flow cytometry. The concentrations of IL-6 and IL-12 in the supernatant were assayed by ELISA. T cell proliferation was assayed by methyl thiazolyl tetrazolium (MTT).

RESULTS: The expression of CD1 α on DC treated with 0.5 mmol/L LAM (LAM-DC 0.5 mmol/L) was significantly higher than that of DC untreated with LAM (54.1 ± 4.21 vs 33.57 ± 3.14 , $P < 0.05$), and so was the expression of CD83 (20.24 ± 2.51 vs 12.83 ± 2.12 , $P < 0.05$) as well as the expression of HLA-DR (74.5 ± 5.16 vs 52.8 ± 2.51 , $P < 0.05$). Compared with control group, LAM-DC group (0.5 mmol/L) secreted significantly more IL-12 (910 ± 91.5 vs 268 ± 34.3 pg/mL, $P < 0.05$), had lower levels of IL-6 in the culture supernatant (28 ± 2.6 vs 55 ± 7.36 pg/mL, $P < 0.05$), markedly enhanced the stimulatory capacity in the allogeneic mixed leukocyte reaction (MLR) (1.87 ± 0.6 vs 1.24 ± 0.51 , $P < 0.05$).

INTRODUCTION

More than 300 million people worldwide are chronically infected with hepatitis B virus (HBV), and the majority of them are in Asia, especially in China. One of the important mechanisms resulting in the immune tolerance by chronic hepatitis B (CHB) is the impaired function of dendritic cells (DCs) derived from patients with CHB^[1]. DCs are antigen-presenting cells (APC) responsible for initiating immunity and play an important role in the induction of antiviral immune responses^[2]. Recently there has been considerable interest in using DCs as adjuvants to enhance host immunity against viral infection^[3].

Lamivudine (LAM), a nucleoside analogue, is a potent and selective inhibitor of HBV replication. LAM specifically inhibits hepadnaviral DNA polymerase by competing with the corresponding dNTPs for incorporation into nascent DNA acting as a chain terminator after incorporation^[4]. In addition to anti-HBV replication, the potential role of LAM in the regulation of immune response in HBV patients has been noticed recently. LAM treatment can restore T cell responsiveness in chronic hepatitis B^[5]. Furthermore, it has been reported that LAM could up-regulate major histocompatibility complex class II (MHC-II) expression and restore impaired allostimulatory function of mononuclear cell-derived DC in the Caucasian patients with HBV infection^[6]. In China, the influence of LAM on function of DC has not been investigated. We, therefore, HBsAg-pulsed DCs derived from the mononuclear cells

of patients with chronic HBV infection with different LAM concentrations (0-2 mmol/L) *in vitro* and observed whether LAM could enhance DCs function. We attempted to explore a new approach to combine LAM and dendritic cell-based immunotherapy for CHB infection.

MATERIALS AND METHODS

Materials

rhGM-CSF, rhIL-4, mouse anti-human HLA-DR-PE, CD80-FITC, CD1 α -FITC, CD83-PE were purchased from BioLegend. RPMI-1640 was purchased from GIBCO (USA), and fetal calf serum (FCS) from Hangzhou Sijiqing Biological Engineering, China. Ficoll-Hypaque density gradient separate solution was purchased from Tianjin Jinmai Gene Biotechnology Co., China. rhIL-6, IL-12 ELISA Kit (Peprotech) were purchased from Shanghai Shengxiong Technology Company, China. LAM was purchased from GlaxoSmithKline Company.

Subjects

Fifteen outpatients with HBeAg-positive CHB infection participated in this study. Written informed consent was obtained from all the patients and the study protocol was approved by the Ethics Committee of Zhengzhou University.

Preparation of dendritic cells

Peripheral blood mononuclear cells (PBMCs) were prepared from CHB patients. Briefly, PBMCs were washed and resuspended at 2×10^6 cells/mL. The cells were cultured in RPMI 1640 media containing 100 mL/L autologous serum, 100 U/mL penicillin, 100 μ g/mL streptomycin. After incubation for 2 h at 37°C, non-adherent cells were removed; the adherent cells were cultured with RPMI 1640 media with recombinant GM-CSF and IL-4 in a 24-well culture plate at 37°C in a humidified atmosphere containing 50 mL/L CO₂. Mononuclear cells were assigned to two groups on d 3: LAM-treated group (experimental group), and non-LAM-treated group (control group). The experimental group was further divided into subgroups according to the different concentrations (0.125, 0.25, 0.5, 1, 2 mmol/L) of LAM. Half of the medium was replaced with fresh medium every other day. DCs were harvested on the 8th d.

Analysis of DCc morphology and flow cytometry (FACS)

DCs were observed on an inverted microscope dynamically, and the phenotypes of DCs, such as CD1 α , CD80, CD83 and HLA-DR, were analyzed by FACS on the 8th day. FITC-labelled mouse anti-human CD1 α , CD80 monoclonal antibody (mAb) and phycoerythrin-conjugated mouse anti-human CD83, HLA-DR mAb were used. Staining was performed as described previously^[7].

Allogeneic mixed leukocyte reaction

To clarify whether the antigen-presenting capacity of LAM-treated DCs (LAM-DC) was different from that of non-LAM-treated DCs (non-LAM-DC), mononuclear cells were isolated from the peripheral blood of healthy

subjects. After incubation for 2 h, the non-adherent cells were collected as lymphocyte cells. DCs were collected as stimulator cells at a concentration of 1×10^5 /mL and added into a 96-well culture plate. DCs were incubated with mitomycin C (50 μ g/mL) for 30 min, and then lymphocytes were added as responder cells at a concentration of 1×10^6 /mL; each group was set to triple wells. After being cultured for 3 d, absorbance at 570 nm (A_{570}) was assayed by MTT and the stimulator index (SI) was calculated using a formula: $SI = A_{\text{experiment}} / (A_{\text{responder cells}} + A_{\text{stimulator cells}})$.

Evaluation of IL-12 and IL-6 in supernatant of DC

Levels of IL-12 and IL-6 in the supernatant of DC were determined using an enzyme-linked immunosorbent assay (ELISA) kit following the manufacturer's instructions. Samples of each group were set to triple wells.

Statistical analysis

Data were analyzed with SPSS10.0 statistical software. The significant difference between groups was determined by one-way ANOVA test. Two-sided $P < 0.05$ was considered statistically significant.

RESULTS

Morphology analyses

After being cultured for 24 h, DCs began to grow as fully differentiated swarm cells as observed under a microscope; 3 d later there was an increase in size and in numbers of DCs; 6 d later much salience on the surface of DCs was observed; many nebulous substance floating among culture media were also found on the 8th day. However, the LAM-DC at low doses had no distinct difference in morphology from control group (Figure 1A and B). There was an increase in number of grainy substance in culture media of the LAM-DC at a high dose on the 2nd day (Figure 1F), which might be cell debris resulted from the medication toxicity.

Phenotype of dendritic cells

The phenotypes of DCs were determined by FACS on the 8th day. The expressions of phenotype molecules in DCs diversified among groups treated with different concentrations of LAM. The expressions of CD1 α on LAM-DC (0.5 mmol/L) was significantly higher than those in naïve controls and other groups ($P < 0.05$), and so was the expression of CD83 ($P < 0.05$) and the expression of HLA-DR ($P < 0.05$); there was no difference in CD80 expression between LAM-DC (0.5 mmol/L) group and control group. The results demonstrated that HBsAg-pulsed DCs with LAM at certain concentration can enhance the capacities of antigen-presentation (Table 1).

Stimulatory capacity of the LAM-DC on lymphocyte proliferation

In the allogeneic mixed leukocyte reaction (MLR), the lymphocyte proliferation reflected the stimulatory function of DCs. The stimulatory capacity of the LAM-DC (0.5 mmol/L) in the allogeneic mixed leukocyte reaction

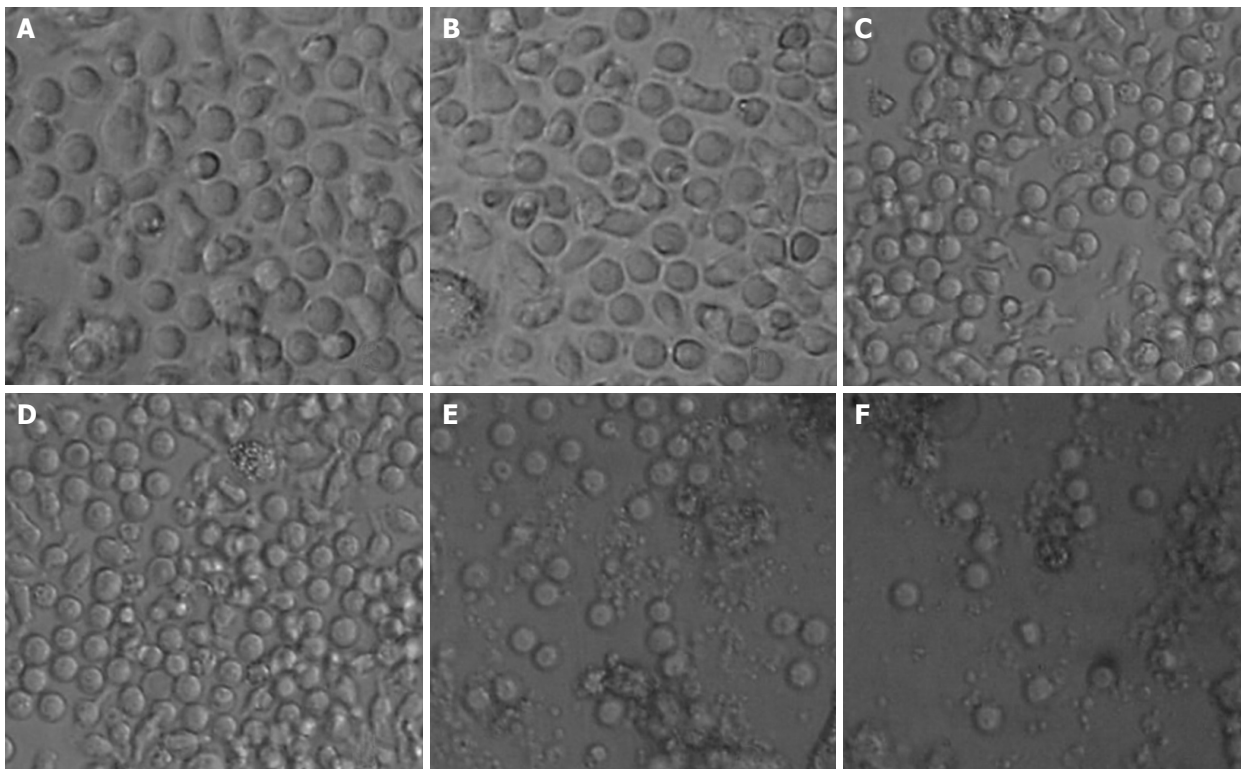


Figure 1 Morphology of DCs treated with lamivudine (LAM) at different concentrations on the 8th d (× 400). **A:** Non-LAM-treated DC; **B:** DC + LAM (0.125 mmol/L); **C:** DC + LAM (0.25 mmol/L); **D:** DC + LAM (0.5 mmol/L); **E:** DC + LAM (1 mmol/L); **F:** DC + LAM (2 mmol/L).

Table 1 The expression of DCs disposed by LAM (mean ± SD, *n* = 15)

	CD83	CD80	CD1 α	HLA-DR
DC	12.83 ± 2.12	35.82 ± 2.41	33.57 ± 3.14	52.80 ± 2.51
DC + LAM	13.40 ± 2.16	37.73 ± 3.24	40.26 ± 2.09	53.70 ± 3.27
0.125 mmol/L				
DC + LAM	18.30 ± 3.15	41.13 ± 3.61	53.10 ± 2.79	67.83 ± 4.15
0.25 mmol/L				
DC + LAM	20.24 ± 2.51 ^a	41.73 ± 4.18	54.10 ± 4.21 ^a	74.50 ± 5.16 ^a
0.5 mmol/L				
DC + LAM	12.81 ± 2.31	25.66 ± 3.05	16.55 ± 1.95	42.11 ± 3.91
1 mmol/L				
DC + LAM	11.92 ± 1.28	24.67 ± 2.62	16.54 ± 1.76	41.01 ± 3.52
2 mmol/L				

DC: Dendritic cell; LAM: Lamivudine. ^a*P* < 0.05 *vs* DC + LAM 0 mmol/L group.

(MLR) was markedly enhanced as compared with control group (*P* < 0.05). On the contrary, there was no significant difference between LAM-DC (0.125 mmol/L) and control group (Table 2).

Secretion of IL-12 and IL-6 by DCs

The concentrations of IL-12 in the supernatant of LAM-DC (0, 0.125, 0.25, 0.5, 1, 2 mmol/L) were 268 ± 34.3, 380 ± 51.2, 500 ± 89.4, 910 ± 91.5, 255 ± 73.1, 138 ± 27.9 pg/mL, respectively. The concentrations of IL-6 in the supernatant of LAM-DC (0, 0.125, 0.25, 0.5, 1, 2 mmol/L) were 55 ± 7.36, 47 ± 5.2, 32 ± 2.7, 28 ± 2.6, 69 ± 8.7, 67 ± 8.4 pg/mL, respectively. The secretion of IL-12 significantly increased in the LAM-DC (0.5 mmol/L)

Table 2 The stimulatory ability of DCs treated with LAM in allogeneic mixed leukocyte reactions (mean ± SD, *n* = 15)

	Control group	LAM (mmol/L)				
		0.125	0.25	0.5	1	2
SI	1.24 ± 0.51	1.31 ± 0.56	1.54 ± 0.45	1.87 ± 0.6 ^a	0.9 ± 0.39	0.63 ± 0.3 ^a

SI: Stimulator index; DC: Dendritic cell; LAM: Lamivudine. ^a*P* < 0.05 *vs* control group.

group in comparison with control group (*P* < 0.05). However, LAM-DC (0.5 mmol/L) group produced a lower level of IL-6 as compared with control group (*P* < 0.05) (Figure 2).

DISCUSSION

Experimental data from HBV-transgenic mice demonstrate that the immune system plays a key role in HBV clearance^[8]. After HBV infection, the body starts a series of non-specific immunology responses, including activation of natural-killing cells and secretion of interferon. However, the complete clearance of HBV relies on the activation of HBV-specific T lymphocytes^[2]. The body can produce HBV antigen-specific CTL response to kill target cells related to HBV. DCs are professional antigen-presenting cells that link innate and adaptive immunity, and are essentially involved in the initiation of primary immune responses and in the establishment of peripheral tolerance^[9,10]. The infection of CHB relates to the impairment of immunity by the mechanism resulting from the deficiency of DC function^[11]. Selective modification of DC function and promotion of HBV-

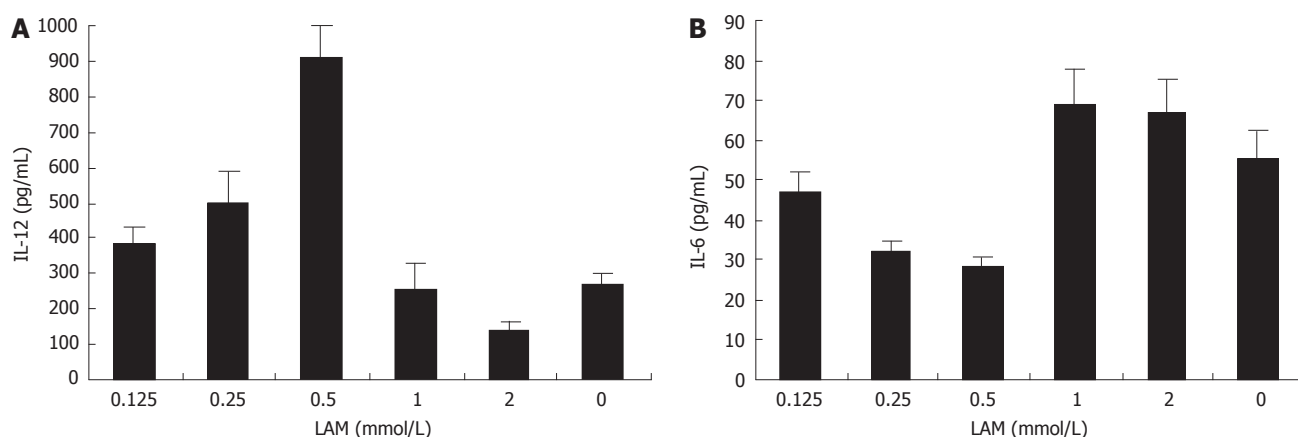


Figure 2 Modulation of cytokine secretion from DCs by LAM treatment. Bars indicate the levels of IL-12 (A) and IL-6 (B) in culture media of DCs treated with LAM at different concentrations (mean \pm SD, $n = 15$).

specific immune responses during CHB treatment have shown therapeutic significance^[12].

DCs could be assigned to two groups by their immunocompetence: mature DCs and immature DCs. Mature DCs have already encountered antigens, whereas immature DCs are able to internalize antigens, process them in the extranuclear compartment, and convert them on the cell surface in the compound of MHC-antigen peptides and present antigenic peptides to T cells. It activates the antigen-specific T cells and initiates immune response with the help of costimulatory molecules binding the corresponding T cell receptors^[13]. In the present study, we detected the expression of CD1 α , CD80, CD83 and HLA-DR on DCs. CD1 α is the specific sign of DC's function; the more CD1 α expresses on DCs, the strong function DCs have. CD83 is a mature sign of DCs; HLA-DR, one of the MHC II molecules, mostly takes part in the process of antigen presentation; CD80, one of the costimulatory molecules, promotes T cell activation *via* combining the corresponding T cell receptors^[14,15].

Beckebaum *et al*^[6] proposed that LAM could facilitate the maturation of DCs isolated from Caucasian patients with HBV infection by increasing the expression of HLA-DR and further promote DCs function. In the present study, the expression of CD1 α , CD83 and HLA-DR on DCs derived from Chinese patients with CHB infection incubated with LAM (0.5 mmol/L) *in vitro* could be enhanced; however, the increase of CD80 was not statistically different as compared with that of control group. Here we found that the lower expression of phenotypic molecules and impaired allogeneic mixed lymphocyte reaction function of DCs from patients with HBV infection could be restored *in vitro* by incubation with LAM. The present study demonstrated that LAM did promote DCs maturation and enhance DCs function.

DCs regulate Th0 cell proliferation towards Th1 cells and Th2 cells through the secretion of IL-6, IL-12 and IFN- γ ^[16]. IL-12 secreted by mature DCs regulates Th0 cells proliferation towards Th1 cells, promotes the secretion of IL-2, IFN- γ and participates in the cellular immune response. IL-6 from immature DCs drives Th0 cells to proliferate towards Th2 cells, restrains the cellular

immune and induces the immune tolerance^[17]. In the current experiment, we found that the secretion levels of IL-12, IL-6 by DCs could be affected by LAM *in vitro*. The secretion of IL-12 increased in the LAM-DC. LAM-DC produced lower levels of IL-6 as compared with non-LAM-DC. These results indicate that DCs treated with LAM at certain concentrations can promote T cell proliferation and enhance the cellular immunity.

The present data confirmed that the defect of DC function could be partially restored by LAM *in vitro* as marked by the enhanced expression of CD1 α , CD83 and HLA-DR on DCs and increased secretion of IL-12, reduced secretion of IL-6 in DCs, and enhanced mixed lymphocyte reaction ability. This indicates that, in addition to its potent anti-HBV replication role, LAM is able to modify the biological activities of DCs derived from patients with CHB infection. Therefore, LAM can be a potential candidate as an immunoregulatory therapeutic remedy used in the treatment of patients with CHB infection. Since treatment with LAM can overcome T cell hyporeactivity^[5] and restore DCs function, combination of LAM with DCs may be an effective therapeutic strategy to obtain eradication of chronic HBV infection.

COMMENTS

Background

Hepatitis B virus (HBV) infection is a global public health problem. The immune response of the host plays an important role in the pathogenesis of chronic HBV infection.

Research frontiers

One of the important reasons responsive for the immune tolerance in chronic hepatitis B (CHB) is impaired function of dendritic cells (DCs) which cannot efficiently present HBV antigens to host immune system.

Innovations and breakthroughs

DC-based therapeutic vaccine has recently been a potential approach to treat CHB. Lamivudine (LAM), a nucleoside analogue, specifically inhibits the hepadnaviral DNA polymerase.

Applications

The present study provides a new support for the application of LAM and DC-based immunotherapy in clinical practice of CHB therapy.

Peer review

This is a good paper indicating that combining LAM with DCs may be an effective therapeutic approach to obtain eradication of chronic HBV infection.

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CASE REPORT

Alcohol bingeing causes peliosis hepatitis during azathioprine therapy in Crohn's disease

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Abstract

Patients with inflammatory bowel disease have normal life expectancy and, due to modern immunosuppressive therapies, also a normal quality of life. Since mostly young people are affected, their social behaviour suits this environment. Alcohol bingeing is an increasingly disturbing factor among young people. We describe a patient with Crohn's disease, treated with azathioprine, who developed peliosis hepatitis after three episodes of alcohol bingeing. Liver toxicity was not observed previously during the course of the treatment. Azathioprine-induced peliosis hepatitis is thought to be idiosyncratic in humans. From animal studies, however, it is clear that hepatic depletion of glutathione leads to azathioprine toxicity to the sinusoidal endothelial cells. Damage of these cells causes peliosis hepatitis. Since alcohol bingeing leads to hepatic glutathione depletion, we conclude that in our patient the episodes of bingeing have reduced liver glutathione content and therefore this has increased azathioprine toxicity causing peliosis hepatitis. The problem of alcohol bingeing has not yet been addressed in IBD patients undertaking immunosuppressive therapy. This should be reviewed in future considerations regarding patients advice.

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Key words: Inflammatory bowel disease; Treatment; Side effects; Alcohol; Liver

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INTRODUCTION

Patients with inflammatory bowel disease (IBD) have normal life expectancy and, due to modern immunosuppressive therapies, also a normal quality of life^[1,2]. Since young people are often affected, their social behaviour suits this environment^[3]. Alcohol bingeing is an increasingly disturbing factor among young people^[4] and also occurs in treated IBD patients. However, there is little information about the toxicity of drugs used in IBD patients during alcohol bingeing. Azathioprine is frequently used as immunosuppressant in IBD. Adverse effects occur in 15% to 30% of patients^[5]. Hepatotoxicity is a rare but important complication of this drug. In the liver, three syndromes can be grouped: hypersensitivity, idiosyncratic cholestatic reaction, and endothelial cell injury. Liver endothelial cell injury presents with portal hypertension caused by venoocclusive disease or peliosis hepatitis^[6]. Here we describe a patient with IBD who developed peliosis hepatitis during azathioprine therapy most likely due to concomitant alcohol bingeing.

CASE REPORT

In 1996, the diagnosis of Crohn's disease was made in a 37-year-old male after a right hemicolectomy with resection of the terminal ileum. He was treated with mesalazine. In September 2005, the patient presented us with an incomplete intestinal cutaneous fistula originating from the anastomosis. He was treated with metronidazole and immunosuppression was begun with steroids (prednisolon 60 mg/d) and azathioprine (2.5 mg/kg body weight per day), mesalazine was stopped. The fistula disappeared and steroids were tapered to zero in the course of the following weeks. In April 2006, liver enzymes were within normal limits, as were white and red blood counts under azathioprine medication. At the end of July, the patient experienced an episode of ethanol bingeing at a local festival with intake of 800 g alcohol (11 g/kg body weight) over 3 d. The consumption of comparable amounts of alcohol had been uneventful in the former years. In August, he had two episodes of bingeing with intake of 20 g per day (0.3 g/kg body weight). At the end of October, he developed progressive fatigue, abdominal discomfort and pruritus. Laboratory values showed pancytopenia [WBC $2.1 \times 10^3/\mu\text{L}$ (normal range $3.6-9.6 \times 10^3/\mu\text{L}$), platelets $66 \times 10^3/\mu\text{L}$ (normal range $150-400 \times 10^3/\mu\text{L}$), erythrocytes $2.53 \times 10^6/\mu\text{L}$ (normal range $4.5-5.9 \times 10^6/\mu\text{L}$)] and elevated gammaglutamyltransferase (121 U/L, normal value < 66



Figure 1 Ultrasonography of the liver.

U/L), alkaline phosphatase (220 U/L, normal value < 129 U/L) and total bilirubin (4.03 mg/dL, normal value < 1.10 mg/dL). Transaminases were normal. Thiopurine-methyltransferase (TMA) activity was 44 nmol/g Hb × h (normal value > 20 nmol/g Hb × h). Hyaluronic acid levels were 166 ng/mL (normal value < 100 ng/mL). Ultrasound showed an enlarged liver with a macronodular surface (Figure 1) and an enlarged spleen. The diagnosis of cholestatic hepatitis with portal hypertension and related pancytopenia was made. Viral, autoimmune and hereditary causes of liver disease were serologically excluded, chronic alcohol intake was negated. Simultaneously the patient developed signs of small bowel obstruction with the presence of dilated jejunal loops. Steroid pulse therapy was administered and azathioprine was stopped. A liver biopsy was performed, which showed a sinusoidal ectasia in zone II and III without venous obliterations, minimal portal tract fibrosis without bile duct alterations (Figure 2). No cirrhotic change was noted. Thus, a diagnosis of peliosis hepatis was made. After withdrawal of azathioprine, the patient's condition improved, pancytopenia disappeared and bilirubin values returned to normal. A liver biopsy to document histological resolution was not performed.

DISCUSSION

Azathioprine-induced peliosis hepatis is thought to be idiosyncratic in humans. Possible risk factors are male gender, renal transplantation and intake of steroids^[7,8]. From our observation, we conclude that alcohol bingeing is an additional risk factor. Peliosis hepatis is a consequence of sinusoidal endothelial cell damage^[9]. This damage is demonstrated in our patient by the elevation of serum hyaluronic acid levels^[10]. *In vitro* studies with murine sinusoidal endothelial cells and hepatocytes exposed to azathioprine have suggested that the mechanism of hepatotoxicity is sinusoidal endothelial damage associated with glutathione depletion. In this model, azathioprine toxicity was selective to sinusoidal endothelial cells and hepatocellular damage was likely due to subsequent ischemia rather than direct toxicity to hepatocytes. Reducing intracellular glutathione levels in sinusoidal endothelial cells increased azathioprine toxicity. Glutathione levels in sinusoidal endothelial cells play an essential role in azathioprine detoxification^[9]. Since ethanol

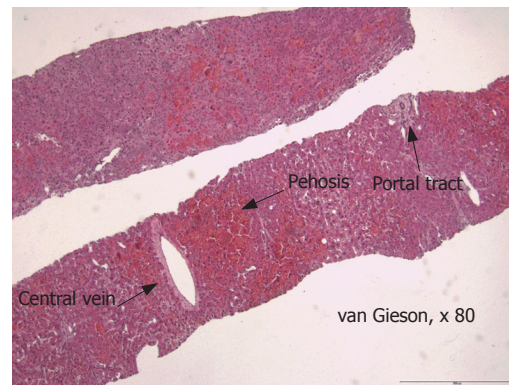


Figure 2 Peliosis hepatis with sinusoidal ectasia in Zone II and III (Elastica von Gieson stain, x 80).

bingeing depletes hepatic glutathione by 60% and therewith increases the toxicity of paracetamol to sinusoidal endothelial cells^[11], we hypothesize from our observation that the alcohol binges of the patient led to glutathione depletion in endothelial cells, thereby increasing the susceptibility to azathioprine toxicity and leading to peliosis hepatis with portal hypertension.

The observed pancytopenia was a consequence of the hypertension rather than a direct myelotoxic effect since TMA activity was normal in our patient. High TMA activity has been associated with hepatotoxicity and refractoriness on therapy^[12], while a diminished activity increases the risk of developing myelotoxicity (e.g. leucopenia or thrombocytopenia)^[13].

Of interest is also the appearance of the liver during sonography. Peliosis hepatis can be a focal, segmental or diffuse dilatation of hepatic sinusoids. According to this, imaging findings are quite variable^[14]. Cirrhotic appearance as in our patient without histological signs of fibrosis or cirrhosis has not been described so far.

The problem of alcohol bingeing has not yet been addressed in IBD patients undertaking immunosuppressive therapy. This should be reviewed in future considerations regarding patients advice.

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Watery diarrhea, hypokalemia and achlorhydria syndrome due to an adrenal pheochromocytoma

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Abstract

Watery diarrhea, hypokalemia and achlorhydria (WDHA) syndrome caused by vasoactive intestinal polypeptide (VIP) -producing tumor only rarely occurs in patients with nonpancreatic disease. A 49-year-old woman was referred for evaluation of a right adrenal tumor incidentally diagnosed by abdominal ultrasound during the investigation of chronic watery diarrhea. Laboratory findings showed hypokalemia and excessive production of VIP and catecholamines. After surgical resection of the tumor, diarrhea subsided and both electrolytes and affected hormone levels normalized. Immunohistochemical examination confirmed a diagnosis of pheochromocytoma, which contained VIP-positive ganglion-like cells. We herein present the clinical and histogenetic implications of this rare clinical entity, with literature review.

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Key words: Pheochromocytoma; Vasoactive intestinal polypeptide; Watery diarrhea, hypokalemia and achlorhydria syndrome

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INTRODUCTION

A rare syndrome of watery diarrhea associated with

hypokalemia and achlorhydria (WDHA) was first described by Verner and Morrison in 1958^[1], and has been assumed to be due to hypersecretion of vasoactive intestinal polypeptide (VIP)^[2]. In adults, this syndrome is most commonly associated with pancreatic islet cell tumors, but rarely can be caused by nonpancreatic tumors, such as bronchogenic carcinoma, medullary thyroid carcinoma, retroperitoneal histiocytoma and adrenal pheochromocytoma^[3]. In this report, we describe a woman who presented with chronic watery diarrhea and hypokalemia due to a right adrenal pheochromocytoma, which was immunohistochemically confirmed to contain immunoreactive VIP. After surgical resection of the tumor, symptoms completely disappeared and the preoperatively elevated plasma VIP level normalized.

CASE REPORT

A 49-year-old woman was referred to our hospital for evaluation of a right adrenal tumor. For several years, she had experienced protracted episodes of watery diarrhea, up to 6 times in 24 h, without blood or mucus. In the course of evaluating the diarrhea, an abdominal ultrasound was performed by her primary physician and a right adrenal tumor was incidentally discovered. She had no history of headache, palpitation, perspiration or hypertension. She also had no relevant familial history of endocrine disease. Her height was 150 cm and weight 46.8 kg. Physical examination showed blood pressure of 110/67 mmHg and regular heart rate of 70 beats/min. Laboratory tests showed hypokalemia of 3.1 mEq/L. Fasting blood sugar was 179 mg/dL and a 75-g oral glucose tolerance test (75-g OGTT) showed a pattern indicating diabetes mellitus. Results of complete blood cell count and other routine biochemical tests were within normal ranges. An abdominal computed tomography (CT) scan demonstrated a mass measuring 6 cm in diameter in the right adrenal region, but there was no apparent abnormality in the pancreas (Figure 1A). Magnetic resonance imaging (MRI) of the pituitary fossa was normal.

We then performed an endocrinological evaluation. Examination of plasma hormone levels demonstrated elevated concentrations of VIP, epinephrine, norepinephrine and dopamine (Table 1). In addition, 24-h urinary catecholamines were also highly elevated, confirming the presence of a pheochromocytoma. Plasma cortisol, gastrin, glucagon, adrenocorticotrophic hormone, prolactin, luteinizing hormone, follicle-stimulating hormone, growth

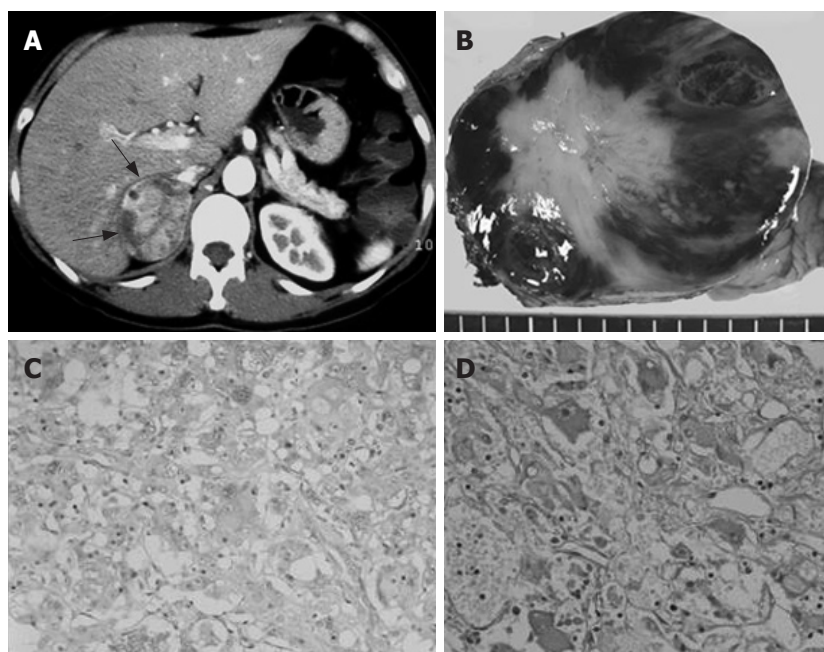


Figure 1 A: Enhanced abdominal CT scan showing a right adrenal tumor (arrows); B: Cut section of the tumor showing variegated white and red-brown areas; C: Immunohistochemical detection of chromogranin A in the tumor cells (× 200); D: Immunohistochemical detection of VIP-positive cells. Stained cells were scattered throughout the tumor tissue (× 300).

Table 1 Pre- and post-operative hormone levels

	Pre-operative	Post-operative
Plasma VIP, pg/mL (100)	225	7
Plasma catecholamines, ng/mL		
Epinephrine (0.070)	2.22	0.028
Norepinephrine (0.06-0.46)	1.81	1.53
Dopamine (0.014)	0.609	0.03
24-h urinary catecholamines, ng/d		
Epinephrine (1-29)	4890	4
Norepinephrine (26-230)	920	154
Dopamine (310-1140)	209000	474

Normal range is given in parentheses.

hormone, thyroxine, parathyroid hormone and calcitonin levels were within normal ranges.

Based on these results, the patient was diagnosed as having an adrenal pheochromocytoma with excessive catecholamine production and WDHA syndrome due to the excessive secretion of VIP, probably produced by the pheochromocytoma.

She subsequently underwent surgical resection of the tumor. At laparotomy, the tumor was found above the upper pole of the right kidney and was separated from the pancreas. Right adrenalectomy was accomplished without complications except for a transient elevation of systolic blood pressure up to 190 mmHg during manipulation of the tumor.

The patient had an uneventful postoperative course. The diarrhea soon subsided, and the laboratory data demonstrated normalization of plasma potassium level and improvement in glucose tolerance. The postoperative plasma levels of VIP and catecholamines as well as the 24-h urinary excretion of catecholamines were markedly decreased (Table 1). At follow-up 12 mo after surgery, the patient was well without any signs of recurrence.

The tumor weighed 65 g and measured 6 cm × 7 cm. It

was encased in a brownish capsule and contained a normal adrenal cortex attached to the upper part. The cut surface showed variegated white and red-brown areas, typical of a pheochromocytoma (Figure 1B). Histologically, large polygonal cells with eosinophilic granular cytoplasm were arranged in alveolar and anastomosing trabecular patterns invested by a fine fibrovascular stroma. Tumor cells showed prominent nucleoli and were strongly positive for chromogranin A on immunohistochemistry (Figure 1C). There were no ganglioneuromatous figures. We then looked for the presence of VIP-positive cells. VIP immunoreactivity was found in some of the ganglion-like cells scattered among the predominant neoplastic cell population (Figure 1D). Since pheochromocytes did not show staining for VIP, it was assumed that VIP eventually released from these scattered cells could be responsible for the WDHA syndrome presented by the patient.

DISCUSSION

The WDHA syndrome is, although rare, a well-defined clinicopathologic entity that occurs most often in association with VIP-producing tumor. VIP is a 28-amino acid peptide that stimulates intestinal water and electrolyte secretion by activating adenylate cyclase and cyclic AMP in intestinal cells^[4]. It is physiologically distributed in the digestive tract, the central and peripheral nervous systems, as well as in the adrenals^[5]. VIP also inhibits gastric acid secretion, promotes hepatic glycogenolysis and hyperglycemia, and dilates peripheral systemic blood vessels^[6]. When it is secreted in large amounts from endocrine tumors, the patient typically experiences massive secretory diarrhea, dehydration, flushing and weight loss^[6]. Characteristic laboratory abnormalities include hypokalemia, achlorhydria, hypercalcemia, hyperglycemia and metabolic acidosis^[6,7]. Unfortunately, we did not perform gastric analysis for acid secretion in our patient. However, it is now known that achlorhydria

is not necessarily part of the WDHA syndrome and that hypochlorhydria or even normal acid secretion may occur^[6,8].

To our knowledge, fifteen cases of pheochromocytoma associated with the WDHA syndrome have been reported since the first report in 1975 by Loehy *et al*^[9]. Ten cases were histologically diagnosed as pheochromocytoma^[6,7,9-16], while five cases were diagnosed as mixed pheochromocytoma-ganglioneuroma^[17-21]. In the cases of mixed pheochromocytoma-ganglioneuroma, immunoreactive VIP was confined to the ganglion cells of the ganglioneuroma component, whereas the pheochromocytes did not show staining for VIP. Eeckhout *et al*^[16] documented a pheochromocytoma in which VIP was seen only in scattered cells among pheochromocytes, as in our case. In contrast, other investigators have found VIP-positivity in the pheochromocytes themselves^[7,13,15].

The differences in these immunohistochemical findings have been partly explained by the neuronal differentiation of neoplastic cells. Both chromaffin cells and ganglion cells are derived from the neural crest and have in common a number of histochemical and biosynthetic characteristics, including amine precursor uptake and decarboxylation (APUD)^[22]. Sano *et al*^[23] suggested that tumor cells of hormone-producing neurogenic tumors can be divided into at least two cell populations: adrenergic neuron-related cells containing catecholamine and somatostatin, and cholinergic neuron-related cells containing VIP. Matsuoka *et al*^[24] demonstrated in an experimental study that some factors in glioma-conditioned medium induced the cholinergic neuronal differentiation of rat pheochromocytes. Mendelsohn *et al*^[25] immunohistochemically identified isolated VIP-containing cells that showed distinct ganglion cell differentiation in two of four pheochromocytomas without associated diarrhea. However, since pheochromocytes lacking ganglionic differentiation features may stain for VIP, it has been postulated that neoplastic transformation might elicit VIP synthesis by the tumor cells^[13]. This could be consistent with the knowledge that neoplastic growth of APUD cells may produce several polypeptides or amines, which are characteristic not only of their specific differentiated cell types but also of associated APUD cells derived from the common neuroectoderm^[6,26].

An interesting feature of this case was the absence of any hypertensive symptoms characteristic of pheochromocytoma. Most patients with pheochromocytoma complicated by WDHA syndrome remain normotensive despite high catecholamine levels. A certain proportion of patients with pheochromocytoma are indeed clinically "silent"^[27], but, in our case, it is conceivable that VIP might have masked the symptoms of catecholamine overproduction by either its vasodilator effect or subsequent induction of secretory diarrhea and dehydration.

This case is an unusual presentation of pheochromocytoma in which diarrhea was the principal clinical sign. We suggest that, in a patient with chronic diarrhea of unknown etiology, it is important to perform not only gastrointestinal investigations but also abdominal ultrasound and/or CT scan to determine if a mass is present. When an adrenal mass is discovered,

determination of catecholamines, as well as VIP is essential for a precise diagnosis even if the clinical symptoms are not characteristic of pheochromocytoma.

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Eosinophilic gastroenteritis with cytomegalovirus infection in an immunocompetent child

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Abstract

A 3-year-old boy developed transient protein-losing gastroenteropathy associated with cytomegalovirus (CMV) infection. Both IgG and IgM antibodies to CMV were positive in a serologic blood test. Upper gastrointestinal endoscopy showed multiple erosions throughout the body of the stomach, without enlarged gastric folds. Histological examination of the biopsy specimens indicated eosinophilic gastroenteritis and CMV infection. The patient had complete resolution without specific therapy for CMV in four weeks. An allergic reaction as well as CMV infection played important roles in the pathogenesis of this case.

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Key words: Eosinophilic gastroenteritis; Cytomegalovirus; Protein-losing gastroenteropathy; Allergy; Menetrier's disease

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INTRODUCTION

Eosinophilic gastroenteritis (EG) is a chronic inflammatory disorder of the gastrointestinal (GI) tract characterized by the infiltration of eosinophils. Klein *et al*^[1] proposed classification of EG based on the depth of eosinophilic infiltration; mucosal, muscular and serosal. The mucosal disease tends to present with protein-losing gastroenteropathy (PLG). As for pathogenesis, an allergic mechanism has been implicated in many cases^[2,3]. A viral etiology has also been suggested in several cases^[4]. In this

report, we present a case of PLG caused by allergy- and cytomegalovirus (CMV)-related EG in a 3-year-old boy.

CASE REPORT

A 3-year-old boy was referred to our hospital for evaluation of hypoproteinemia and edema. He had experienced allergic reactions to milk, soybeans, shrimp and pork. His initial symptoms of diarrhea and edematous swelling of the eyelids developed during one week before admission. Physical examination revealed periorbital and pretibial edema. His abdomen was slight distended. The liver and spleen were not palpable. Abdominal ultrasound detected a small amount of ascites and mild swelling of the mesenteric lymph nodes. On laboratory examination, the white blood cell count was 11 300/mm³, with 24% eosinophils. Total serum protein concentration was 3.2 g/dL, with an albumin concentration of 1.8 g/dL. Serum IgE level was within normal limits (24 IU/mL). RAST for specific IgE antibodies and skin prick test failed to reveal reactions to common food antigens. The results of a serological test for CMV were positive for IgM and IgG. Stool samples showed normal flora on culture and the absence of blood, *H pylori* antigen, ova and parasites. Enteric protein loss was confirmed by a fecal α_1 -antitrypsin clearance of 68.8 mg/d (normal, < 20 mg/d). We excluded urinary protein loss as a cause of the hypoalbuminemia. Upper GI endoscopy showed multiple erosions with abundant whitish mucus throughout the gastric body. Enlarged gastric folds were not observed. The esophagus exhibited no remarkable change and the duodenum appeared mildly reddened. A sigmoidoscopy showed mucosa with a normal vascular pattern and a mild lympho-nodular appearance. Histological examination of the gastric mucosa revealed neutrophilic and eosinophilic infiltrates with focal clustering of eosinophils in the lamina propria and regenerative epithelial changes. We also observed a small number of cells with intranuclear and intracytoplasmic inclusions that were immunohistochemically positive for CMV. *H pylori* was not detected. Duodenal and colonic biopsies showed increased numbers of eosinophils in the lamina propria. Histological diagnosis was compatible with EG.

The patient was treated with transfusions of albumin, oral famotidine, oxatamide and cromoglicic acid, but not corticosteroid or a specific therapy for CMV. There was a gradual recovery from edema with a concomitant increase in the serum protein level to 6.8 g/dL and a normalization of the eosinophil count over the next four

weeks. The endoscopic abnormalities also subsided after four weeks of hospitalization. Histological assessment of the mucosal biopsy specimens demonstrated clearing of the eosinophilic infiltration. He had no recurrences in the subsequent 36 mo.

DISCUSSION

CMV infection in the GI tract is unusual in an immunocompetent person. Primary infection with CMV is generally asymptomatic and usually remains latent for life. If host immune defenses are impaired, latent CMV may be activated and produce symptoms of overt disease. GI tract infection with CMV usually occurs in immunocompromised patients by activation of the latently infectious virus. On the other hand, there are several reports of CMV infection in normal healthy persons and the important role of CMV in the acute gastrointestinal disease has been emphasized^[5]. In our case, evidence of gastric CMV involvement was shown by histological findings of characteristic inclusion bodies and by the immunohistochemical detection of viral antigens. It was likely that mucosal disruption due to allergic mechanism facilitated the CMV infection, which then led to further injury. As well, CMV infections in the GI tract might be locally cytopathogenic, possibly allowing mucosal penetration of allergens that then stimulated the allergic reaction.

Allergy has been suggested as the cause of transient PLG in children^[6]. However, the target allergens in our patient were unclear; serum RAST and skin prick tests against common food allergens were all negative. Lin *et al*^[7] suggested that a localized IgE-mediated response could cause the gastrointestinal symptoms seen in skin-prick test-negative and serum IgE antibody-negative patients with suspected food allergy.

EG may present with a wide variety of clinical and endoscopic findings. However, the multiple erosions throughout the body of the stomach seen in our patient are unusual for EG; commonly endoscopic findings of EG are edema, erythema and erosions in the antrum of the stomach. Only one case of EG with similar endoscopic features has been reported^[8]. Additionally, the clinical course of our case was unusually benign and short for

EG; patients with EG usually have a prolonged course of relapse and remission and for control may require therapy with steroids.

Menetrier's disease is another common cause of PLG. Allergic phenomena and CMV infection have been implicated in pediatric cases^[6,9], and the course of the disease in children, unlike that in adults, is usually benign and short^[9]. EG and Menetrier's disease exhibit several common features in childhood, leading to the assumption that they may represent a continuum of gastroenteropathy associated with allergic mechanism.

Although our patient remains well, he requires long-term follow-up since the natural history of this condition remains unclear. Kristopaitis *et al*^[10] reported a case of EG in a 24-year-old female, who had experienced infantile EG, that recurred after a long period of dormancy.

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Multiple intrahepatic pseudocysts in acute pancreatitis

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Abstract

Liver pseudocysts are a very rare complication in acute pancreatitis with only a few cases previously described. The lack of experience and literature on this condition leads to difficulties in the differential diagnosis and management. We report herein a case of acute pancreatitis who developed multiple intrahepatic pseudocysts. After complete imaging evaluation, the diagnosis was still unclear and the patient was operated on. The presence of liver lesions in patients with acute pancreatitis should raise the possibility of intrahepatic pseudocysts.

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Key words: Pancreatic pseudocyst; Liver pseudocyst; Intrahepatic pseudocyst

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INTRODUCTION

Pancreatic pseudocysts are a common complication of acute pancreatitis, defined as a collection of pancreatic juice enclosed by a wall of nonepithelialized granulation tissue or fibrotic capsule^[1,2]. Most pseudocysts are located around the pancreatic gland, but they have also been described at a great distance from the pancreas; and the mediastinum to the scrotum, when the fluid dissects through tissue planes^[3,4].

A very rare location for a pseudocyst during acute pancreatitis is in the liver with only about 30 cases previously described^[5]. The lack of experience with pseudocysts located in the liver makes it difficult to establish a clear diagnosis and choose the appropriate management.

We report here a case of a patient who developed several intrahepatic lesions after an episode of acute pancreatitis. Despite a complete imaging study, the definitive diagnosis was still unclear and the patient was therefore operated on. Diagnosis and pathophysiological mechanisms were discussed herein.

CASE REPORT

A 68-year-old male with no history of hepato-biliary or pancreatic diseases or alcoholic ingestion was admitted to our hospital with abdominal pain and nausea. Physical exploration revealed moderate upper abdominal pain without tenderness or rebound and normal bowel sounds, as well as a slight conjunctival jaundice. Laboratory parameters showed 12300 white blood cells/mm³ (80% neutrophils), normal hemoglobin, 3291 IU/L serum amylase (normal value < 115 IU/L), 4003 IU/L serum lipase (normal value < 190 IU/L), 461 U/L AST (normal value < 37), 389 U/L ALT (normal value < 41) and 238 mU/mL alkaline phosphatase (normal value < 300). Abdominal ultrasound demonstrated cholelithiasis without inflammatory signs in the gallbladder wall or common bile duct dilation. The APACHE II score at admittance was 5. The patient recovered uneventfully after a few days of hospitalization, but a computed tomography (CT) scan revealed a scarcely enlarged, almost normal pancreas and an ill-defined hypodense lesion (4.5 cm × 3.5 cm) located in the left hepatic lobe of unclear etiology (Figure 1). Accordingly, liver and cholangiopancreatography magnetic resonances were carried out confirming the lesion in segment II and describing a new lesion (3 cm in diameter) located in segment III (Figure 2), and showing a normal common bile duct. The masses found were of unclear nature with a mixture of liquid and solid content. Eventually, a US-guided fine needle aspiration (FNA) cytology was performed, yielding an unspecified leukocyte infiltration without malignant cells.

Since the patient had mild acute biliary pancreatitis and cholecystectomy was indicated, we decided to proceed with the operation, despite the lack of a definitive diagnosis based on the liver lesions.

Upon operation, three soft cystic tumors were located in the left lobe of the liver with a very dense and necrotic content on exploration with intraoperative



Figure 1 CT scan showing a hypodense lesion located in the left hepatic lobe of unclear etiology.

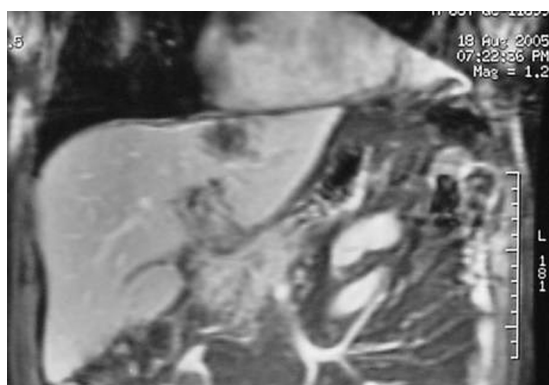


Figure 2 Magnetic resonance of the liver and bile duct showing a lesion in segments II and III and a normal common bile duct.

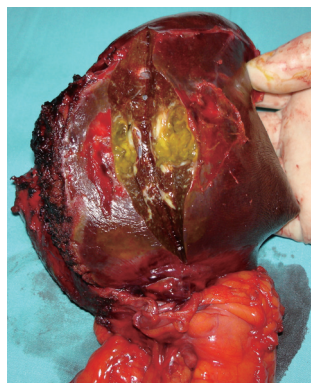


Figure 3 Left lobectomy demonstrating a lesion in segment III.

ultrasonography. There were no other lesions in the right lobe or anywhere else in the abdominal cavity. Cholecystectomy with intraoperative cholangiography was performed with the liver lesions excised by a left lobectomy (Figures 3 and 4). The pseudocysts were full of debris with a very small amount of juice. Microbiologic cultures of the content in the cysts were negative. Pathological examination confirmed the pseudocystic nature of the three subcapsular lesions by proving nonepithelialized tissue of the wall (Figure 5). There were no postoperative complications and the patient was discharged 6 d after surgery. Eight months later, the patient was completely recovered and disease free.

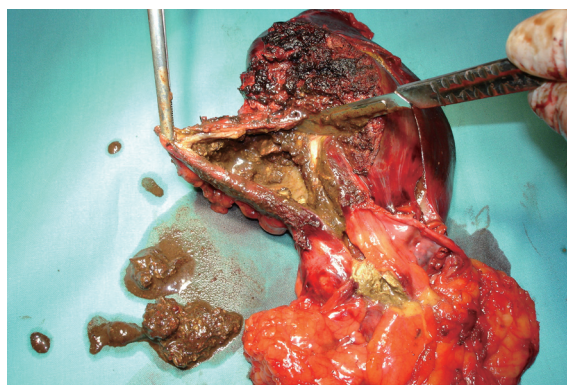


Figure 4 Cystic lesion in segment II showing the continuity with the lesser omentum and necrotic material from the pseudocyst.

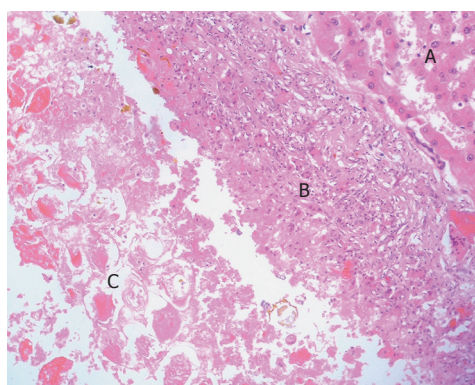


Figure 5 Microscopically the liver pseudocystic lesion is surrounded by a wall formed by macrophages and inflammatory cells (HE, x 20). A: Normal liver tissue; B: Pseudocystic wall; C: Intracystic necrotic debris.

DISCUSSION

The interest in this case presented here relies on the very rare location of the pseudocysts, the atypical evolution for the formation of a pseudocyst and the difficulties to achieve a definitive diagnosis.

So far there are only about 30 cases describing pseudocysts located in the liver, most of them are single involving the left lobe^[5]. Our case indeed involved the left lobe, but the fact that there were three pseudocysts clearly separated in the surgical specimen increases the rarity of the case^[6].

Two pathophysiological mechanisms have been proposed trying to explain the liver location of pseudocysts^[7-10]. The first suggests a release of pancreatic juice towards the lesser sac and from there towards the left lobe of the liver along the lesser omentum or gastrohepatic ligament. Nearby the liver and due to the proteolytic effect of pancreatic juice, it may dissect the liver capsule leading to formation of subcapsular collections. When another peripancreatic fluid collection or a pancreatic pseudocyst is not associated, a direct disruption or erosion on the hepatic capsule may occur. The second mechanism proposes the propagation of pancreatic juice from the head of pancreas to the porta hepatis along the hepatoduodenal ligament resulting in formation of intraparenchymal collections. The anatomic location of the case presented here seems to be better

explained by the first mechanism.

An important point of our case is the unusual evolution of liver pseudocysts. The ultrasonography performed upon admission did not detect any lesions in the liver, but the CT scan performed 6 d later vaguely identified a mass-cystic forming image. Usually a pancreatic pseudocyst requires a minimum of time, between 4 and 6 wk, to form the well-defined wall, a main characteristic of it. However, the time span between admission and identification of our patient's liver lesions was extremely short. It can be argued that the patient suffered from a previous pancreatitis and that the present episode might in fact be a relapsing pancreatitis, but this possibility should be rejected after thoroughly questioning both the patient and his family. Hence perhaps pseudocysts in the liver might not take as long to develop as in the abdominal cavity or near the pancreas. Unfortunately, this suggestion cannot be clarified from the scarce literature on liver pseudocysts.

The third point is the difficult diagnosis even with a complete radiological study and FNA. During the evaluation of this case, we were always concerned about an incidental neoplasia, although unseen in the first ultrasonography exploration, we never thought of a liver pseudocyst secondary to the inflammatory pancreatic process. In fact, the patient was sent to the operating room without a clear diagnosis of hepatic lesions, which was eventually established by the pathologist. The atypical radiological appearance of the pseudocysts full of debris may have contributed to the difficult diagnosis.

In conclusion, the presence of liver lesions in patients with acute pancreatitis should raise the possibility of intrahepatic pseudocysts, even when they are not associated with intraabdominal collections or pancreatic

pseudocysts. The atypical radiological imaging or short clinical evolution cannot rule out this complication.

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LETTERS TO THE EDITOR

Surveillance colonoscopy practice in Lynch syndrome in the Netherlands: A nationwide survey

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Abstract

Lynch syndrome, or hereditary nonpolyposis colorectal cancer (HNPCC), is the most common genetic disorder predisposing to colorectal cancer. As regular colonoscopic surveillance has been shown to reduce the incidence of colorectal cancer, this strategy is recommended worldwide. Recently, several advances in colonoscopic techniques have improved detection rates of neoplasia in Lynch syndrome. In this nationwide survey, we evaluated current surveillance colonoscopy practices for Lynch syndrome in the Netherlands and the extent to which advanced techniques have been adopted in routine clinical practice.

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Key words: Lynch syndrome; Hereditary nonpolyposis colorectal cancer; Colonoscopy; Surveillance; Chromoendoscopy

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TO THE EDITOR

Lynch syndrome, or hereditary nonpolyposis colorectal cancer (HNPCC), is caused by germline mutations in mismatch repair (MMR) genes. HNPCC is the most common hereditary disorder predisposing to colorectal cancer (CRC), accounting for up to 5% of cases. Carriers of MMR gene mutations are recommended to undergo surveillance colonoscopy once every 1-2 years starting

at the age of 20-25, a strategy which has been shown to reduce CRC mortality^[1,2]. Despite this approach, which is internationally recommended^[3-5], interval cancers can be encountered^[6]. Therefore, the identification of high-risk precursor lesions in Lynch syndrome is considered critically important. It is well known that conventional colonoscopy has a confirmed miss rate for colorectal neoplasms of up to 20%, especially small adenomas^[7]. This is of particular importance in Lynch syndrome patients since even in adenomas as small as 5-7 mm, high-grade dysplasia can be encountered^[8]. Recently, chromoendoscopy using indigo carmine was reported to increase the diagnostic yield in HNPCC patients^[9,10]. Also, several improvements involving endoscopic such as magnifying endoscopy (ME) and narrow-band imaging (NBI) techniques have recently been introduced^[11]. The role of these techniques in the clinical arena, especially in the management of Lynch syndrome patients, is still unclear. In this study, we evaluated adherence to the current guidelines for HNPCC surveillance in the Netherlands. We also surveyed the current use of new diagnostic colonoscopic techniques.

All Lynch syndrome families registered at the Netherlands Foundation for the Detection of Hereditary Tumors were reviewed. The physicians that had undertaken responsibility for these families were sent a questionnaire in which they were asked to provide data on the frequency of colonoscopic surveillance and the age at which surveillance was initiated. The current use of advanced endoscopic techniques such as chromoendoscopy, ME and NBI were also evaluated along with the physicians' expectations regarding the use of these techniques in the near future. Reimbursements for regular colonoscopic surveillance in Lynch syndrome subjects are generally authorized in the Netherlands.

Of the 446 questionnaires sent out, 228 were returned (51%). 221 out of 228 physicians (97%) adhered to the recommendation of performing surveillance colonoscopy every 1-2 years, with most physicians preferring a 2-year interval. Most physicians indicated they initiated surveillance when subjects at risk were between 25-30 years of age. The majority of physicians (196, 86%) used only conventional videocolonoscopes in their current surveillance management. The remainder of the consulted physicians regularly used advanced colonoscopic techniques, most often magnifying or high-resolution endoscopy (6%), followed by chromoendoscopy (5%) and narrow-band imaging (3%). Interestingly, 50% of the physicians expected to alter their endoscopic surveillance practices in the near future. In most cases, physicians

intended to use chromoendoscopy with indigo carmine as their standard endoscopic technique with this patient group.

In summary, there is excellent adherence to published guidelines on colonoscopic surveillance management in Lynch syndrome patients in the Netherlands. Most physicians presently use a conventional colonoscope for their regular surveillance colonoscopies. The recent introduction of new endoscopic techniques, especially chromoendoscopy, will probably be incorporated into surveillance management of this patient group in the near future. Whether Lynch syndrome patients really benefit from the introduction of these techniques, by a reduction in incidence of colorectal neoplasia, remains to be determined.

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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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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How labile is gastric infection with *H pylori*?

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Abstract

It is known that patients infected with *H pylori* can spontaneously become free from infection, and that the reverse change can occur. The time-scale of these conversions is expressed as percentages per year. Since they have been investigated in terms of serology, the changes are called sero-reversion and sero-conversion respectively. Using serological evidence to investigate these phenomena is open to the criticisms that positive serology can be present in the absence of all other evidence of infection, and that a time-lag of 6-12 mo or longer can occur between eradication of the infection and sero-reversion. Investigations using direct evidence of current infection are sparse. The few that exist suggest that some individuals can seroconvert or sero-revert within six to twelve weeks. If these findings are confirmed, it means that some patients have an ability that is variable in time to resist, or spontaneously recover from, *H pylori* infection. Evidence suggests that the deciding factor of susceptibility is the level of gastric secretion of acid.

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Key words: *H pylori*; Lability of infection; Serology; Conversion; Reversion

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EVIDENCE OF LABILITY OF *H PYLORI* INFECTION

Evidence from indirect tests: Spontaneous seroconversion and seroreversion

In papers concerned with human infections of the

gastroduodenum with *H pylori*, it is usually tacitly assumed that infection is stable, i.e., that a subject infected at any one moment will remain infected until the organism is eradicated with pharmacological agents. There is considerable evidence based on serological studies that *H pylori* infection can be more labile, with subjects undergoing spontaneous sero-reversion as well as sero-conversion. Reports from countries where the prevalence of *H pylori* infection is moderate (40%-60%) show that spontaneous cures may occur even more frequently than fresh infections, and more often in children and teenagers than in adults^[1-14]. The question is, do these figures adequately reflect the rates of the changes?

In children, of a total of 1134 children who were *H pylori* negative, 92 had converted to *H pylori*-positive in periods ranging from 9 to 14 years^[2-6]. The percentage conversion rates differed from 40 percent after 10 and 14 years to 5% or less after 2, 10 and 14 years. The same publications documented that of a total of 141 *H pylori*-positive patients 58 reverted to *H pylori* negative over the same periods. The sero-reversion rates in the five studies varied between 15% at 14 years and 80% at 10 years.

In these reports of children, there is no evidence that the length of follow-up is related to sero-conversion or sero-reversion rates. The lack of evidence of a link between the rates and the length of follow-up may be due to the (necessarily) small range of follow-up in an age group defined as children and teenagers. The salient feature of these results is that the sero-conversion rate overall was 92/1134 (8.1%), while the sero-reversion rate was 58/141 (41%). A small tendency for children to develop the infection as time passed was considerably outweighed by a five-fold tendency towards natural cure.

In adults, there is strong evidence that both sero-conversion and sero-reversion rates increase with the duration of follow-up. Eight publications^[7-14] yielded the following statistical results. Over a time-interval of 3-32 years, 94 (2.7%) of 3489 subjects sero-converted; regression analysis indicated that the number converting increased by 0.311 per cent per annum ($r^2 = 0.836$, $P = 0.0015$). The corresponding figures for sero-reversion were 109 (6.04%) of 1806 subjects; the regression values were an increased rate of reversion of 0.676 per cent per annum ($r^2 = 0.747$, $P = 0.0056$). In adults, therefore, conversion rates per annum were outweighed by a doubled rate of sero-reversion.

Comparisons between the two rates in adults and the two in children are strictly impossible because of the lack of correlation in children with length of follow-up. However, if one is prepared to accept that the yearly rates in children (in whom the average length of follow-up was

about 11 years) were, for sero-conversion $8.1/11 = 0.74\%$, for sero-reversion $41/11 = 3.73\%$, it is clear that infection status derived from antibody information in children is more labile in both directions than it is in adults.

The evidence from countries with a high prevalence of infection with *H pylori* is scanty. There are only three papers^[15-17] from Japan where, on the published evidence, the prevalence is variable (36%-87%), and only one^[15] of these papers gives data for children; and two from Brazil where the prevalence is very high (80%) - one for children^[18] and one for adults^[19]. Regression analysis to determine whether length of follow-up is related to the conversion rates is inappropriate. However, it is clear that in Japan sero-conversion rates are only slightly lower than sero-reversion rates, 5/86 (5.8%) versus 2/22 (8.1%) in children and 66/1038 (6.4%) versus 149/2103 (7.1%) in adults, whereas in Brazil the rates of sero-conversion are high 5/78 (6.41%) in less than 2 years in children, 5/46 (10.87%) in 3 years in adults, while in children there was a zero reversion rate and in adults only 1 of 173 *H pylori*-positive subjects reverted.

There seems little doubt that the sero-conversion rate rises with the overall prevalence of the infection in the population, that where the prevalence is moderate the tendency to spontaneous cure overtakes the rate of new infections, but where the prevalence is high there is practically no spontaneous cure. These conclusions depend on the assumptions that sero-positivity means the presence of infection, sero-negativity means its absence.

The time periods of the quoted studies range from 20 mo to 32 years. It is tacitly assumed by the authors that sero-reversion and sero-conversion rates represent the averages of a slow, single rate in each direction. However, it is also conceivable that during these times changes in infection status might have occurred several times in both directions.

These reports seem to assume that serological evidence of the presence or absence of antibodies to *H pylori* indicates the presence or absence of the infection. The fact is that the presence of antibodies indicates exposure to the infecting organism in the past, but does not indicate current infection. Indeed, there are reports of positive serology in the absence of other positive tests for infection^[20-23]. Moreover there is a known time lag of 6-12 mo^[24-27] or even longer between eradication of infection and reversion of serology to normal^[20-23].

Evidence from direct tests: Histology and urea breath test

Only a few reports base their opinions on direct methods such as the urea breath test (UBT) or histology. There are two reports of children showing changes either way within 3 mo^[28,29] and one reporting such changes within 6 mo in both children and adults^[30] using the urea breath test. There are two reports based on histology in adult patients, one showing 5/39 patients becoming *H pylori* negative over a ten year period^[31], and another reporting 9% of patients becoming positive and 9% becoming negative over a 6 years period^[32]. However, there is some direct evidence that infection can be even more labile than the above evidence suggests, There is one significant report

in a Master of Surgery Thesis^[33] involving adults. Some aspects of this study have been reported^[34]. Two hundred and eight patients undergoing endoscopy for dyspepsia were categorized as *H pylori*-positive or -negative, using the biopsy-rapid urease, culture and polymerase chain reaction tests. The patients received no anti-*H pylori* treatment. The first hundred of these patients to volunteer (14 duodenal ulcer, 5 gastric ulcer, 16 oesophagitis, 46 non-ulcer dyspepsia (NUD) and 19 Others) were examined between 6 and 12 wk later and re-categorized as positive or negative, using a non-invasive ¹³C-urea breath test. Of 42 patients positive for *H pylori* at endoscopy, 8 (19%) had become negative at the later breath test; and of 58 patients negative at endoscopy, 15 (26%) had become positive at the later breath test. The results suggest that *H pylori*-status in the adult can alter in both directions within a few weeks. The PCR test was done at the time of the endoscopy but, at the time of the follow-up, because it was not clinically justifiable to repeat endoscopy, the UBT was used.

It may be criticised that the results from two different tests may not be comparable. There is considerable evidence, however, that PCR and UBT vie with each other as the gold standard for *H pylori*-status, and therefore are highly unlikely to give divergent results^[24-26,35-40]. Indeed, it has been shown that PCR results can be used to determine the optimal cut-off point for the breath test results^[41], and that both tests can be used to determine not only the presence of, but also the weight of infection with, the organism^[42]. The evidence from the later breath tests can, therefore, be relied on as at least as satisfactory as that from the PCR tests at the time of endoscopy. It follows that in this study during a period of 6 to 12 wk there was a 20%-25% change of *H pylori*-status in both directions.

The possible effect of gastric pH on *H pylori* infection

One possible explanation is that the ability of *H pylori* to colonize the stomach (and gastric-type epithelium in the duodenum) is dependent on the local luminal pH. Extremes of pH in either direction kill the organism^[43,44]. The patients with peptic ulcer (whether gastric or duodenal), with reflux oesophagitis, and some of those with other lesions would have received acid-suppression agents during the period between the two examinations, and this fact might explain why patients negative at endoscopy later became positive. There is evidence that acid-suppression promotes gastritis associated with *H pylori* infection^[45]. For movements in status in the opposite direction, in patients given a clean bill of health (NUD) or those in the group with diagnoses that did not seem related to gastric hyperacidity, the later withdrawal of acid-suppressing agents given prior to the endoscopy that excluded an ulcer might be the cause of the reversion from positive to negative. It is interesting to recall that, when Marshall^[46] in 1985 swallowed a culture of *H pylori*, he took 600 mg of cimetidine 3 h before to reduce the acidity. Thereafter, stomach acidity would have returned to normal and, whilst stomach biopsies taken one week later were positive for *H pylori*, those taken at two weeks had become negative.

CONCLUSION

The above findings show that the *H pylori*-status of adults can alter in both directions in a matter of a few weeks and that the infection is much more labile than previously realised. The known time lag of 6-12 mo^[22-25] between eradication of infection and reversion of serology to normal compared with 6 wk for UBT^[22], and the unknown time lag between the inception of infection and seroconversion, are features that cast some doubt on whether serology could have demonstrated this lability.

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Should nonalcoholic fatty liver disease be regarded as a hepatic illness only?

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Abstract

The highly increasing prevalence of obesity and type 2 diabetes mellitus in the general population makes nonalcoholic fatty liver disease the most common diagnosis in every-day practices. Lifestyle changes (mainly exercise withdrawal and weight gain) have probably heightened the prevalence of nonalcoholic fatty liver disease. Mortality in patients with Nonalcoholic Fatty Liver Disease is significantly higher when compared with that of the same age-gender general population. Hepatologists claim to bear a new burden, being Nonalcoholic Fatty Liver Disease strongly linked to systemic diseases.

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Key words: Nonalcoholic fatty liver disease; Metabolic syndrome; Diabetes mellitus; Obesity; Polycystic ovary syndrome; Obstructive sleep apnea; Cardiovascular disease

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SCENARIO

Nonalcoholic fatty liver disease (NAFLD) has recently emerged as the most common cause of abnormal laboratory liver tests (LLTs) seen in patients presented to practicing hepatologists, with the overall prevalence of NAFLD in the developed world estimated to be between 20% and 30%. Changes in lifestyle have resulted in a dramatic increase in the prevalence of NAFLD. Apoptosis and insulin resistance (IR) play an important

role in the disease development and progression. Our current knowledge of the natural history of NAFLD can be summarized as follows: elevated body mass index (BMI) plays a key role; simple steatosis NAFL (Fatty liver, FL) does not generally progress to Non-Alcoholic SteatoHepatitis (NASH); patients with NASH progress, in relatively many cases, to cirrhosis; older age and advanced fibrosis are risk factors for hepatocellular carcinoma (HCC) in NASH; up to a third of patients develop liver-related morbidity or mortality. It is extremely important that physicians diagnose NASH accurately and perform appropriate treatments, because it represents an illness mirroring a systemic process, and an adjunctive cardiovascular disease (CVD) risk^[1]. A great deal of research highlight the need for surrogate serum markers for diagnosing NASH. Among them, serum cytokeratin 18 has captured certain interest^[2].

COULD NAFLD BE CONSIDERED A FURTHER EXPRESSION OF METABOLIC SYNDROME?

NAFLD and NASH are conditions gaining increasing recognition, obesity being (mainly of high grade) one of the more important risk factors. But, do other aspects of metabolic syndrome (MS) play a role? To answer this question, a prospective study was conducted in 127 consecutive obese patients (62% female, mean age 40 ± 11 years, mean BMI 42 ± 6 kg/m²) undergoing gastric bypass over a 20-month period. The report highlighted that arterial hypertension was present in 52 patients (41%) and type 2 diabetes mellitus (DM) in 18 (14%). However, NAFLD was confirmed in 80 patients (63%). Of them, 47 (37%) had FL, and 33 (26%) had NASH. Cirrhosis was found in 2 patients corresponding to 1.6% of the total population. For multivariate analysis, elevated HOMA independently predicted only NASH, (OR 4.18, 95% Confidence Interval, CI, 1.39-12.49). That NAFLD was frequently found, it is easy to deduce that the NAFLD presence coupled with obesity (visceral) and hypertension could be used as criteria to label the patients with MS^[3].

ARE PATIENTS' AGE OR LONGEVITY OF ILLNESS A CRITERION TO INFER NASH PRESENCE?

To characterize the spectrum of NAFLD in morbidly

obese adolescents, a cross-sectional study correlated in 41 adolescent subjects' (mean age, 16 years, 61% female, 83% non-Hispanic white, mean BMI 59 kg/m², undergoing bariatric surgery) liver histology with clinical features and compared data with those of the adults. The Authors wondered if NAFLD would be less severe as a result of younger age and shorter duration of obesity and which grade of portal inflammation and fibrosis would be present. Eighty-three percent had NAFLD: 24% FL, 7% isolated fibrosis with steatosis, 32% non-specific inflammation and steatosis, and 20% NASH. Twenty-nine percent had fibrosis; none had cirrhosis. Abnormal ALT was more prevalent in NASH. Mean fasting glucose was higher in NASH, but the percentage of MS was not different. The researchers concluded that NAFLD was very prevalent in morbidly obese adolescents, but severe NASH was uncommon. In contrast to morbidly obese adults, lobular inflammation, significant ballooning, and perisinusoidal fibrosis were rare, whereas portal inflammation and portal fibrosis were more prevalent, even in those who did not meet criteria for NASH. Presence of MS in morbidly obese adolescents did not distinguish NASH from FL. Conclusively; the answer is... yes it is^[4].

Could being overweight (not necessarily obese) be considered a risk factor for the NAFLD progression?

The realization that a proportion of patients with NAFLD can progress through NASH and fibrosis to full-blown cirrhosis and HCC has recently focused the attention of the liver-disease scientific community on this condition, previously considered to be benign. Although several studies have been performed on risk factors (presented in a single or combined way) and natural course of NASH, it seems that NASH is inclined to be more than a disease confined to classic boundaries. The objective of a recent study was to assess the clinical features and risk factors for NASH patients in an Iranian population. Fifty three patients (21 female, mean age 37.8 ± 11.3 years) with histologically confirmed NASH entered the study. Twenty-six patients (55.3%) were overweight, 15 (31.9%) obese, 40 (75.5%) dyslipidemic, and three patients (5.7%) were diabetic. Liver histology showed mild steatosis in 35.7%, moderate steatosis in 53.6%, and severe forms in 10.7%. In 80.2% of patients, portal inflammation was present, and 9.4% had cirrhosis. The amount of increase in LLTs bore no relationship with fibrosis, portal inflammation, and degree of steatosis. The interesting point of this research dwells with the overweight, not the obese, as having a risk factor for NASH. Obviously, a careful history was taken regarding alcohol intake^[5].

And what is the role of visceral fat?

NAFLD is increasing rapidly in the population of the Asia-Pacific region, representing a good model of study. The aim of an up-to-date research was to define the anthropometric, metabolic and histological characteristics of patients with NAFLD in these countries. Seventy-five patients with persistently raised LLTs and/or FL detected on ultrasonography (US) with exclusion of other liver disorders were prospectively enrolled (39 men, mean

age 47.0 ± 12.2 years). Fifty eight patients (77.3%) had visceral obesity, 29 (38.7%) were diabetic and 15 (20.0%) had impaired glucose tolerance (IGT). Insulin resistance was diagnosed in 62 out of 64 (96.9%) patients. FL, NASH and cirrhosis were diagnosed in three (4.3%), 59 (84.3%) and eight (11.4%) of 70 patients, respectively. The complete histological spectrum of NAFLD was seen in these patients. The majority of them were characterized by IR, central obesity and had either type 2 DM or IGT^[6].

Is isolated portal fibrosis the link between FL and NASH?

NASH is a progressive form of NAFLD that can lead to hepatic fibrosis and cirrhosis. Until now, portal fibrosis in the absence of NASH, called isolated portal fibrosis (IPF), has received less attention and has not been classified as a spectrum of NAFLD, but, when found in patients with HCV-related chronic hepatitis it is considered to be a key feature of viral infection. Liver biopsies from 195 morbidly obese subjects who underwent bariatric surgery after excluding all other causes of liver disease were analyzed. The prevalence of fatty liver (FL) only, IPF, and NASH was 30.3%, 33.3%, and 36.4%, respectively. Again, hyperglycemia was the only metabolic parameter associated with NASH (OR, 5.4; 95% CI, 2.4-12) and IPF (OR, 2.8; 95% CI, 1.2-6.5). MS was identified in 78.5% of subjects, and a significant trend for the clustering of MS criteria was observed across the spectrum of FL, IPF, and NASH^[7].

Is the exact prevalence of NASH in the severely obese known?

NAFLD has been consistently associated with every grade of obesity and IR. As previously stated, NASH is a histological entity within NAFLD that can progress to cirrhosis. The prevalence of NASH in morbidly obese patients is a crucial point of study, because they represent a well-defined population. It is unclear whether differences in insulin sensitivity exist among subjects with NASH and FL. To evaluate the prevalence and correlates of NASH and consequently liver fibrosis in this distinctive cohort of ninety-seven subjects, a recent study was employed. Thirty-six percent of subjects had NASH and 25% had fibrosis. No cirrhosis was diagnosed on histology. Markers of IR and MS, but not BMI were associated with the presence of NASH. Forty-six percent of patients suffering from NASH had normal transaminases. Subjects with NASH had more severe IR when compared to those with FL. In conclusion, NAFLD is associated with MS rather than excess adipose tissue in the severely obese^[8].

Are laboratory liver tests worth using to screen NAFLD?

A fascinating study was conducted to determine whether the current liver screening program for NAFLD has sufficient evidence to justify its continued implementation. The LLTs program to detect NAFLD was performed on 411 Japanese workers utilizing serum ALT, AST, and gammaglutamyl transpeptidase (GTP). Subjects with viral and alcohol hepatitis were excluded from the evaluation. The diagnosis of NAFLD was based on US findings. The program was evaluated by efficacy and effectiveness in comparison with the BMI. Effectiveness, based on

the efficacy determinations, was assessed by means of the positive predictive value (PPV) test performance, the disease characteristics, and the program cost. The diagnostic performances of ALT and BMI were far from excellent. The areas under the curves of the two indices were 0.69 and 0.63, respectively. The PPV ranged from 15 to 28% where the prevalence of fatty liver was 12.3%. The cost of the program was estimated at \$4 U.S. dollars per person based on the medical reimbursement fee rate. The efficacy of the liver screening program was found to be insufficient and, therefore, revealed that BMI monitoring may provide a more suitable and inexpensive alternative. The authors challenge the effectiveness of the LLTs, considering the high price of the program^[9].

What is the risk of cancer in patients with NAFLD?

The relation between NAFLD and cancer risk is poorly understood. Using the population-based National Registry of Patients, some Authors examined the incidence of cancer in 7326 patients discharged with a diagnosis of NAFLD from a Danish hospital during 1977-1993. Overall, 523 cancers were diagnosed during 47 594 person-years of follow-up, yielding a 1.7-fold increased risk (95% CI, 1.6-1.9) compared with the Danish general population. The risk of primary liver cancer was markedly elevated in patients with alcoholic liver disease (ALD) as well as NAFLD with a standardized incidence ratio of 9.5 (95% CI, 5.7-14.8) and 4.4 (95% CI, 1.2-11.4), respectively. Patients with ALD also had substantially increased risks of several types of cancer associated with alcohol and tobacco use (cancers of the lung, pharynx, larynx, esophagus, and stomach) and a moderately increased risk for cancers of the colon and breast. Among patients with NAFLD, an increased risk of some alcohol- and tobacco-related cancers was seen, and there was also an increased risk of colon and pancreas cancer^[10].

Then, what is the natural history of NAFLD in the community?

Authors sought to determine survival and liver-related morbidity among community-based NAFLD patients. Four hundred twenty patients diagnosed with NAFLD in Olmsted County, Minnesota, between 1980 and 2000 were identified using the resources of the Rochester Epidemiology Project. Overall survival was compared with the general population of the same age and sex. Overall, 53 of 420 (12.6%) patients died. Survival was lower than the expected survival for the general population ($P = 0.03$). Higher mortality was associated with age (hazard ratio per decade 2.2; 95% CI 1.7-2.7) and impaired fasting glucose (hazard ratio 2.6; 95% CI 1.3-5.2). Liver disease was the third leading cause of death (as compared with the thirteenth leading cause of death in the general population), occurring in 7 (1.7%) subjects. Twenty-one (5%) patients were diagnosed as being affected by cirrhosis, and 13 (3.1%) developed serious complications^[11].

And what about NAFLD in atopic/non-atopic children?

NAFLD in non-obese Japanese children was observed in 3.2% of non-atopic children and in 17.6% of patients

with atopic dermatitis or suffering from bronchial asthma, allergic rhinitis, in 2000. NAFLD was studied by abdominal US scans. The prevalence of NAFLD was increasing annually, and it reached 12.5% in non-atopic children, 13.1% in patients with bronchial asthma, 13.7% in patients with allergic rhinitis, or 33.9% in patients with atopic dermatitis, in 2003. Since NAFLD in childhood may be a risk factor for lifestyle-related diseases in future, care should be taken to prevent it^[12].

It would have been interesting to know how many of those subjects were on steroid therapy.

Could polyunsaturated fatty acids be used to improve an immunological disease and an apparently distant illness of liver, i.e., NAFLD?

The higher incidence of inflammatory diseases in Western countries might be related, in part, to a high consumption of saturated fatty acids and n-6 polyunsaturated fatty acids (PUFA) and an insufficient intake of n-3 fatty acids. In an intriguing study, Balb/C mice were fed for 3 wk either n-6 or n-3 PUFA-fortified diets. After inducing a contact or an atopic dermatitis, immunological parameters were analyzed by the authors to evaluate the anti-inflammatory potential of these n-3 PUFA. Accordingly, n-3 PUFA lessened innate and specific immune responses through inhibition of TH1 and TH2 responses, increase of immunomodulatory cytokines such as IL-10, and regulation of gene expression. Furthermore, reduction in edema, leukocyte infiltration, and enhancement of antioxidant defenses in the inflamed ears of mice from both models proved n-3 PUFA efficacy. Authors' data suggest that dietary fish oil-derived n-3 fatty acids could be useful in inflammatory disorders^[13].

On the other hand, is obesity a chronic low-grade inflammatory process or not? Because eicosapentaenoic acid, docosahexaenoic acid, and gamma-linolenic acid have anti-inflammatory, as well as lipid-modifying properties, the effects of supplement mixtures of these PUFA should have received much more attention by researchers in the field of NAFLD treatment.

What have two peculiar syndromes to do with NAFLD?

NAFLD and polycystic ovary syndrome (PCOS) are both associated with IR. Thus, women with PCOS may have an increased prevalence of NAFLD, including NASH. To determine the prevalence and characteristics of NASH and abnormal ALT in women with PCOS were retrospectively studied 200 women with PCOS, diagnosed with irregular menses and hyperandrogenism. Fifteen percent (29 out of 200) had AST and/or elevated ALT. Women with aminotransferase elevations had lower high-density lipoprotein (HDL), $P = 0.006$, higher triglycerides ($P = 0.024$), and higher fasting insulin ($P = 0.036$) compared with women with normal aminotransferases. Six women had NASH with fibrosis. The authors concluded that abnormal aminotransferase activity is common in women with PCOS and suggest a reflection of whether to screen PCOS women for liver disease at an earlier age than is currently recommended for the general population^[14].

Recently, obstructive sleep apnea (OSA) has been proposed as an independent risk factor for IR. The

objectives of this study were to document the prevalence of SOSA in patients with NAFLD and to determine whether prevalence rates for SOSA differ in NAFL versus NASH patients. The prevalence of OSA was similar in both biochemically ($P = 0.66$) and histologically ($P = 0.11$) defined NAFL and NASH patients. Other risk factors for NAFLD such as BMI, cholesterol and triglyceride levels, and prevalence of diabetes were also similar in the two groups. Approximately one-half of NAFLD patients, whether NAFL or NASH, have OSA^[15].

Could abnormalities in the estradiol to testosterone ratio (increased androgens and decreased estrogens) lead to insulin resistance and NASH?

Recently, the fourth case has been presented of an adult man (29 years old) affected by aromatase deficiency resulting from a novel homozygous inactivating mutation of the CYP19A1 (P450-aromatase) gene. The patient showed also a complex dysmetabolic syndrome characterized by IR, type 2 DM, acanthosis nigricans, NASH, and signs of precocious atherogenesis. The analysis of the effects induced by the successive treatment with high doses of testosterone, alendronate, and estradiol allows further insight into the roles of androgens and estrogens in several metabolic functions. High doses of testosterone treatment resulted in a worsening of IR and type 2 DM. Estrogen treatment resulted in an improvement of acanthosis nigricans, IR, and NASH, coupled with a better glycemic control and the disappearance of two carotid plaques. Data from this case provided new insights into the role of estrogens in glucose, lipid, and liver metabolism in men^[16].

CONCLUSION

For many years, research concerning acute and chronic liver diseases have been mostly confined to few areas of Medicine, i.e., virology and immunology. Vice versa, for the first time in decades, the liver comes back to have a pivotal role in Health Sciences, embracing endocrine, cardiovascular and oncological diseases, so the answer to the initial question, "Should nonalcoholic fatty liver disease be regarded as a hepatic illness only?," is... It should not!

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HFE gene in primary and secondary hepatic iron overload

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Abstract

Distinct from hereditary haemochromatosis, hepatic iron overload is a common finding in several chronic liver diseases. Many studies have investigated the prevalence, distribution and possible contributory role of excess hepatic iron in non-haemochromatotic chronic liver diseases. Indeed, some authors have proposed iron removal in liver diseases other than hereditary haemochromatosis. However, the pathogenesis of secondary iron overload remains unclear. The High Fe (*HFE*) gene has been implicated, but the reported data are controversial. In this article, we summarise current concepts regarding the cellular role of the *HFE* protein in iron homeostasis. We review the current status of the literature regarding the prevalence, hepatic distribution and possible therapeutic implications of iron overload in chronic hepatitis C, hepatitis B, alcoholic and non-alcoholic fatty liver diseases and porphyria cutanea tarda. We discuss the evidence regarding the role of *HFE* gene mutations in these liver diseases. Finally, we summarize the common and specific features of iron overload in liver diseases other than haemochromatosis.

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Key words: Hereditary haemochromatosis; Chronic liver diseases; Chronic hepatitis C; Hepatic iron overload; *HFE* gene

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INTRODUCTION

Iron is an essential element for living cells because it is

a cofactor for enzymes of the mitochondrial respiratory chain and it co-ordinates the binding of oxygen by myoglobin and haemoglobin. However, excess iron is toxic, causing increased oxidative stress; the production of reactive oxygen species is thought to be responsible for the observed oxidation of lipids, proteins and nucleic acids. Thus, iron overload can cause serious damage to organs including the liver, heart, joints and endocrine glands.

Hereditary haemochromatosis (HH) is the paradigm of heavy iron overload which can eventually lead to multiple organ failure. HH is an inherited disease of iron metabolism^[1]. Absorption of dietary iron is inappropriately high in relation to body iron stores. This leads to increased deposition of iron, predominantly in parenchymal cells of the liver, heart, joints, pancreas and other endocrine organs. Undiagnosed and untreated, iron deposition can cause hepatocellular injury, activation of hepatic stellate cells (HSCs) and increased production of collagen and other components of the extracellular matrix^[2]. The liver is the major site for storage of excess iron, which probably explains the increased risk of hepatocellular carcinoma (HCC) in HH^[3].

In non-haemochromatotic liver diseases, hepatic iron may worsen liver injury or hepatic fibrosis. This has been investigated in common chronic liver diseases (CLDs) such as chronic hepatitis C (CHC) and alcoholic and non-alcoholic fatty liver diseases. In these CLDs, the progression towards end-stage liver disease is often unpredictable; many cofactors have been proposed to explain this variability. Iron overload has been proposed as such a cofactor, but its exact role remains unclear^[4]. Iron has also been implicated in the progression of hepatitis B virus (HBV) infection^[5], but this has not been widely studied. Other conditions that may cause CLD and that have been associated with iron overload include porphyria cutanea tarda (PCT) and insulin resistance-associated hepatic iron overload (IR-HIO) syndrome^[6,7]. The IR-HIO syndrome encompasses iron overload with hyperferritinaemia and normal transferrin saturation, type 2 diabetes mellitus and non-alcoholic steatohepatitis (NASH). In these forms of secondary (acquired) iron overload, however, the hepatic iron concentration is generally lower than that seen in HH. Although major advances have been made in understanding the pathogenesis of primary iron overload in HH, the mechanism(s) whereby pre-existing CLD may lead to iron overload remain unclear. In HH, there is a strong relationship with missense mutations in the *HFE* (High Fe) gene^[8]. In non-haemochromatotic diseases, several studies have investigated the possible role of *HFE* mutations in the pathogenesis of hepatic

iron overload, with somewhat discordant results. This review aims to describe the prevalence and role of *HFE* mutations in secondary iron overload. It will discuss the effects of moderate iron excess on the natural history and its possible relevance to therapeutic approaches in CLDs other than HH.

NOMENCLATURE OF IRON OVERLOAD

Hereditary, or primary, haemochromatosis is an inherited iron storage disease. Secondary iron overload is acquired as a result of another disease. The nomenclature and classification of iron overload states is shown in Table 1. Primary haemochromatosis may be due to mutations in the *HFE* gene, or to mutations in genes other than *HFE*. The main causes of acquired iron overload are haemolytic anaemias and CLDs. Untreated HH may develop severe iron overload, whereas in secondary iron overload due to CLDs, minimal to modest iron overload is usually seen^[2].

GENETICS OF PRIMARY IRON OVERLOAD

There are two common mutations of the *HFE* gene. The first results in a change of cysteine at position 282 to tyrosine (C282Y); the second results in a change of histidine at position 63 to aspartate (H63D). Numerous studies have shown that homozygosity for C282Y is associated with typical phenotypic HH in Caucasians. C282Y homozygosity ranged from 64% of Italian haemochromatosis cases up to 100% of cases in Australia^[9-11]. A recent study of almost 100 000 North American primary care patients analysed the distribution of *HFE* mutations in a racially diverse group^[12]. It confirmed that homozygosity for C282Y is most common in whites, consistent with the hypothesis that this mutation originated in a Caucasian “founder” individual. Individuals who are compound heterozygous for C282Y and H63D may also have iron overload in the range diagnostic for haemochromatosis, although the penetrance of this genotype is lower than for C282Y homozygosity^[13-15]. The H63D mutation is variably distributed worldwide. It is more prevalent than the C282Y mutation: approximately one in five of the European population are estimated to be H63D heterozygotes^[9,16]. The C282Y/H63D compound heterozygous and H63D homozygous genotypes have mostly been associated with only biochemical evidence of mild iron overload. The clinical penetrance of these genotypes is low although there have been reports of varied phenotypic presentation^[15,17].

A third point mutation (S65C) of the *HFE* gene was also identified. It was originally considered to be a neutral polymorphism, not associated with increased transferrin saturation^[18]. However, other evidence implicated the mutation in mild iron overload^[19,20]. Other, rare mutations have been described. Two missense mutations in exon 2 of the *HFE* gene (I105T and G93R) were detected in haemochromatosis patients with atypical *HFE* genotypes, such as heterozygosity for C282Y, H63D or S65C^[21]. A splice-site *HFE* mutation (IVS3+1G/T) that prevented normal mRNA splicing was identified in a patient with classical HH who was heterozygous for the C282Y muta-

Table 1 Nomenclature for iron overload states

Primary iron overload-hereditary haemochromatosis (HH)
<i>HFE</i> -associated HH
1 C282Y homozygosity
2 C282Y/H63D compound heterozygosity
3 Other mutations
Non <i>HFE</i> -associated HH
1 Juvenile haemochromatosis
2 Tfr2-related haemochromatosis
3 Autosomal dominant haemochromatosis
Secondary iron overload-acquired
Iron-loading anaemias
1 Thalassaemia major
2 Sideroblastic anaemia
3 Chronic haemolytic anaemias
Chronic liver diseases
1 Hepatitis C
2 Alcoholic liver disease
3 Non-alcoholic steatohepatitis
4 Porphyria cutanea tarda
5 IR-HIO
6 Post-portacaval shunting
7 Transfusional and parenteral iron overload
8 Dietary iron overload
Miscellaneous
1 Iron overload in sub-Saharan Africa
2 Neonatal iron overload
3 Acaeruloplasminaemia
4 Congenital atransferritinaemia

tion. This highlighted the possibility that other rare *HFE* mutations could explain the classical HH phenotype, particularly in C282Y heterozygotes with iron overload^[22]. In this review, we will consider only the two most frequent *HFE* mutations, C282Y and H63D. The other mutations are rare, thus they are unlikely to play a major role in CLDs other than HH.

HH has been described as the most common monogenic disorder in Celtic populations. Certainly, homozygosity for the C282Y mutation occurs in about 1 in 300 people of Northern European origin, with an estimated carrier (heterozygote) frequency of 1 in 10^[8]. However, the C282Y homozygous genotype has incomplete penetrance: not all patients with this genotype show an iron overload-related phenotype. The penetrance of HH is influenced by a variety of factors. Increasing age increases the penetrance, as the body has no means of active iron excretion, so iron accumulation progresses with time. Male gender also increases the penetrance, as women may be partially protected against iron overload by iron losses incurred in childbirth and menstruation. Dietary iron content may also influence penetrance. There has been considerable debate regarding the penetrance of HH. The “biochemical penetrance” of the C282Y homozygous state is generally agreed to be high, particularly in older males, but disease-related morbidity is less frequent. A study of almost a third (3011) of the residents of Busselton, Australia showed that four (all males) of the 16 C282Y homozygotes detected had fibrosis and/or cirrhosis. If attributable to iron overload, this equates to a disease-related morbidity of 25%^[23]. In contrast, recent large studies have reported lower penetrance. Beutler and colleagues studied over

40 000 individuals attending a health appraisal clinic, where expression of haemochromatosis was investigated by questionnaire. The results for C282Y homozygotes were compared with the control group who were wild type for C282Y and H63D. The clinical penetrance of ill health or shortened lifespan in C282Y homozygotes was reported to be less than 1%^[24]. Various factors, such as the exclusion of most patients who had a previous diagnosis of haemochromatosis from the assessment of ill health, would have tended to decrease this calculated clinical penetrance of C282Y homozygosity^[25]. Despite this, a similar, low value was obtained in a survey of about 1 000 000 individuals in two health authority regions in South Wales. It was concluded that a diagnosis of haemochromatosis had been made for only 1.2% of adult C282Y homozygotes. When only male homozygotes older than 45 years were considered, the diagnosis rate rose to 2.8%^[26]. Thus, the “severity” of the definition of penetrance (disease related morbidity or clinical expression as ill health/shortened life span) is an important factor influencing the observed value. It seems that the clinical penetrance of C282Y homozygosity is low.

HFE PROTEIN AND CELLULAR IRON HOMEOSTASIS

The predicted HFE protein has sequence homology to the major histocompatibility complex (MHC) class I molecules, a family of transmembrane glycoproteins of the immune system, which present peptide antigen to T cells^[8]. Like the class I proteins, HFE is made up of three extracellular loops (α_1 , α_2 , α_3)^[27], a transmembrane domain and a short cytoplasmic C-terminal region. Two disulphide bridges stabilize the tertiary structure of the HFE protein. One of these gives rise to the α_3 loop, that is crucial for interaction with β_2 -microglobulin and for translocation to the cell surface^[28,29]. The C282Y mutation prevents formation of this disulphide bond, thereby inhibiting correct cell surface expression of HFE^[8]. The H63D mutation causes a histidine to be replaced by aspartate in the α_1 domain^[8]. H63D is generally considered to have a milder effect on body iron stores than C282Y.

The role of HFE in cellular iron homeostasis remains only partially understood. Co-immunoprecipitation experiments showed that the HFE- β_2 -microglobulin complex interacted with transferrin receptor 1 (TfR1) in human placental membrane preparations^[30] and in human embryonic kidney cells over-expressing wild type HFE^[31]. Immunohistochemical studies of human duodenum showed that TfR1 staining overlapped that of HFE in the crypt cell enterocytes, consistent with the co-trafficking of these proteins^[32] (Figure 1). These studies provided a link between HFE and the transferrin-mediated endocytosis pathway of iron uptake into cells. Under conditions of iron excess, circulating iron-saturated transferrin is understood to bind the TfR1-HFE- β_2 -microglobulin complex on the cell surface, which is internalised within an endosome. At the acidic endosome pH, iron dissociates from transferrin and enters an intracellular chelatable iron pool. The transferrin-TfR1 complex recycles to the cell

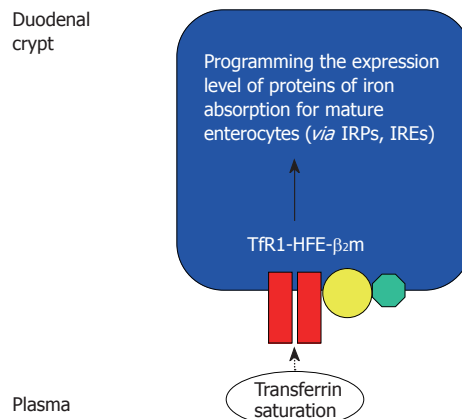


Figure 1 The duodenal “crypt cell hypothesis” of HFE function^[36]. HFE at the basolateral membrane of duodenal crypt cells co-localizes with β_2 -microglobulin and TfR1. The saturation of circulating transferrin, which reflects body iron stores, was proposed to be “sensed” by the TfR1-HFE- β_2 -microglobulin complex, through transferrin-mediated endocytosis. Wild type HFE was proposed to facilitate transferrin-mediated iron uptake. In haemochromatosis, deficiency of functional HFE would therefore decrease the iron pool within the crypt cell, despite increased body iron stores. This would increase the activity of iron responsive proteins (IRPs), leading to increased expression of genes involved in iron absorption, such as DMT1 and ferroportin. This could contribute to the iron overload seen in HH^[27,34,35]. However, recent evidence suggests that HFE may play more important roles in influencing iron metabolism in the liver.

surface, where transferrin dissociates at the pH of blood, around pH 7.4^[27,33].

However, investigations of the HFE-TfR1 interaction and its potential effect on iron metabolism in transfected cell lines gave conflicting results. Several studies indicated that overexpression of HFE may ultimately reduce iron uptake into cells *via* transferrin-mediated endocytosis^[34]. Biosensor- and radioactivity-based assays indicated that HFE competes with transferrin for binding to TfR1^[35]. Conversely, in stably transfected Chinese hamster ovary cells, HFE increased the rate of TfR1-mediated iron uptake and cellular iron concentrations, but only when co-transfected with β_2 -microglobulin^[36]. Also, in primary macrophages from haemochromatosis patients, transfection of the wild type HFE gene increased uptake of ⁵⁵Fe-transferrin and accumulation of ⁵⁵Fe in the ferritin iron pool^[37]. These latter studies are consistent with the observation that in haemochromatosis, duodenal enterocytes are paradoxically iron-deficient^[38].

Several hypotheses have been advanced to model the possible roles of HFE in the regulation of iron metabolism. It was originally proposed that the TfR1-HFE- β_2 -microglobulin complex on the basolateral surface of the duodenal crypt cell may sense the transferrin saturation (Figure 1). Where wild type HFE was present, the transferrin saturation was proposed to determine the crypt intracellular iron concentration, thereby setting the activity of iron responsive proteins 1 and 2 and ultimately the expression levels of key proteins of iron absorption in the mature enterocyte^[36]. The pathway of iron absorption in the mature enterocyte is shown in Figure 2. Duodenal cytochrome b ferric reductase (Dcytb) is expressed on the luminal surface of the enterocyte and reduces dietary ionic iron from the ferric (Fe^{3+}) to the ferrous (Fe^{2+}) state.

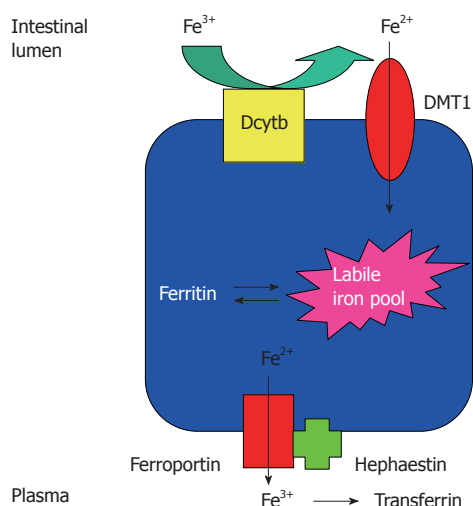


Figure 2 The pathway of iron absorption in the mature duodenal enterocyte. Dietary ferric iron is reduced to the ferrous state by ferric reductase(s), for example duodenal cytochrome b (Dcytb), which is expressed on the luminal surface of the enterocytes. Ferrous iron is taken up via DMT1 into the labile iron pool. Iron may be stored within the cell as ferritin or transferred across the basolateral membrane to the plasma by ferroportin. The exported iron is oxidized to the ferric state by hephaestin; ferric iron is then avidly bound by circulating transferrin^[39].

The ferrous iron is then taken up by the apical transporter, divalent metal transporter 1 (DMT1). Iron may be stored within the cell as ferritin or exported across the basolateral membrane by ferroportin. The exported iron is re-oxidized to the ferric state by hephaestin. Upon exiting the villus enterocyte, ferric iron can be avidly bound by circulating transferrin^[39].

The discovery of the peptide hormone hepcidin^[40-42] revived an alternative hypothesis, that the liver may be central to the regulation of iron metabolism^[43-46]. Hepatocytes act as a storage reservoir for iron, taking up dietary iron from the portal circulation and, in iron deficiency, releasing iron into the hepatic circulation. The liver is also the site of high expression of hepcidin, *TfR2* and haemojuvelin, three genes which when mutated result in HH.

Hepcidin is produced in the liver in response to dietary iron loading^[42]. Mutation of the hepcidin gene results in juvenile haemochromatosis, characterised by early onset and a rapid rate of iron loading^[47]. As in hepcidin-related haemochromatosis, inappropriately low hepcidin levels in relation to body iron stores are also seen in haemochromatosis resulting from mutation of the *HFE*, *TfR2* and haemojuvelin genes, suggesting that hepcidin may be the common pathogenic mechanism in haemochromatosis^[43,48]. Therefore, *HFE*, *TfR2* and haemojuvelin proteins may all be involved in the pathway sensing iron overload that leads to hepcidin synthesis. Experiments using cultured cells indicated that hepcidin may act by interaction with ferroportin, causing its degradation and reducing cellular iron export^[49]. The overall effect would be to “trap” iron within enterocytes, hepatocytes and macrophages. However, other studies, using parenteral injection of hepcidin into mice and analysis of iron absorption in tied off lengths of duodenum, found that hepcidin inhibited the uptake

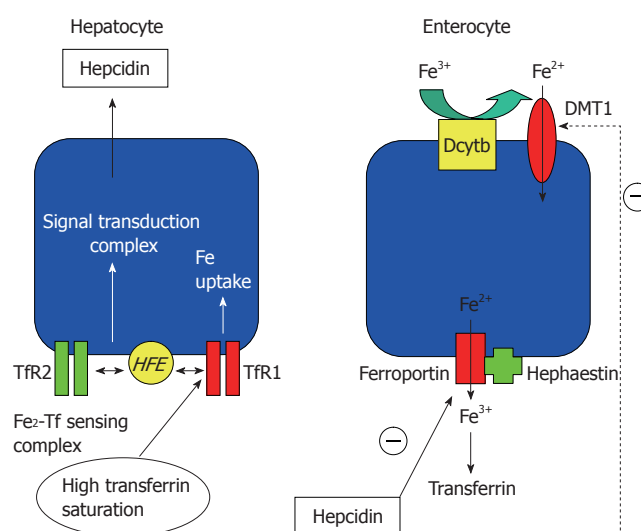


Figure 3 Current concepts regarding hepatic regulation of iron metabolism. Left panel, hepatocyte. At normal transferrin saturation, TfR1 may sequester HFE^[19]. At increased transferrin saturation, diferric transferrin competes with HFE for binding to TfR1^[55]. Freed HFE is proposed to bind TfR2; the complex conveys transferrin saturation status via a cytoplasmic signal transduction complex, leading to synthesis and secretion of hepcidin. In HH, mutations in the genes encoding HFE, TfR2, haemojuvelin or hepcidin may all disrupt this sensing system, leading to deficient hepcidin production and iron overload^[53]. Right panel, enterocyte. Circulating hepcidin may reduce iron absorption by interacting with ferroportin, causing its internalization and degradation^[49] and/or by reducing DMT1 expression^[50].

step of iron absorption. This did not require HFE. In this study, hepcidin did not influence the proportion of iron transferred into the circulation^[50]. Therefore it is not yet clear whether hepcidin acts at ferroportin in the enterocyte basolateral membrane, at DMT1 in the apical membrane, or both (Figure 3).

Because hepcidin is synthesised in hepatocytes, they have been proposed as the site of an iron sensing mechanism. TfR2 is expressed mainly in hepatocytes, haematopoietic cells and crypt cells of the duodenum, which are all also sites of HFE expression^[51,52]. Recent expression studies of intact HFE and TfR2 proteins in cultured cells showed interaction between these two proteins^[53]. It was proposed that when transferrin saturation is in the normal range, TfR1 may sequester HFE^[54]. At increased serum transferrin saturation, diferric transferrin has been shown to compete with HFE for binding to TfR1^[55]. Freed HFE is proposed to act as an iron sensor, binding TfR2 and conveying transferrin saturation status via TfR2 and a cytoplasmic signal transduction complex, leading to the synthesis of hepcidin (Figure 3). Haemojuvelin may form part of the signal transduction system. In HFE-, TfR2- or haemojuvelin-related haemochromatosis, mutations may disrupt this sensing system, leading to the observed deficiency of hepcidin; iron loading would therefore result^[53]. Thus, latest ideas propose that HFE-TfR2 interaction in hepatocytes may sense transferrin saturation, as an index of body iron status, determining production of hepcidin, which governs iron absorption^[53].

IRON AND *HFE* MUTATIONS IN CHRONIC HEPATITIS C

CHC remains a major health problem with around 200 million individuals affected worldwide^[56]. The natural course of CHC is characterised by progressive fibrosis in the inflamed liver with cirrhosis and haemodynamic changes which may be followed by end-stage complications^[57]. The progression of fibrosis in CHC is highly variable. Several factors may favour progression, including alcohol, young age at the time of infection and male gender. The role of iron in the pathogenesis of CHC has been debated. An association between iron and viral hepatitis was first described by Blumberg and colleagues^[5]. Following those observations, several studies noted elevated serum iron indices in CHC^[58-60]. Iron has been proposed as a cofactor that may both promote the progression of liver disease and reduce the response to antiviral therapy^[56,61,62]. Mechanisms proposed include production of reactive oxygen species, increased fibrogenesis through activation of HSCs and impairment of the host immune response^[63]. Several studies of CHC reported hepatic iron deposits in HSCs, which may contribute to liver damage^[64,65]. Many studies have also tried to investigate the pathogenesis of iron overload in CHC. Necroinflammation due to ongoing viral infection is considered the most important cause of iron overload. Additionally, the viral infection *per se* or the associated activation of cytokines have been proposed to modify iron metabolism in liver cells^[60,66]. A recent study showed that TfR1 expression was increased in CHC hepatic tissue irrespective of the degree of hepatic iron overload. This might, therefore, contribute to the accumulation of hepatic iron in CHC^[67]. Viral genotype may be another factor. In a large study of 242 patients, one of us analyzed the relationship between hepatitis C virus (HCV) genotypes and liver iron deposits in CHC^[68]. We found a higher prevalence of hepatic iron deposits in HCV-3 infected cases, concluding that hepatic iron deposition is HCV-genotype dependent. Indeed, the distribution of hepatic iron was mostly parenchymal in HCV-3 cases, while in non-HCV-3 patients, hepatic iron deposits were more frequently detected in the reticuloendothelial cells. These findings support the hypothesis that the expression of HCV-3 proteins in infected hepatocytes might cause specific metabolic changes resulting in enhanced oxidative stress. The non-HCV-3 cases had more severe fibrosis. Therefore their lesser hepatic iron deposits might reflect a more advanced stage of liver inflammation. A further relationship between viral genotype and iron was found in another study: Izumi and colleagues reported higher hepatic iron concentrations in HCV-1b compared with HCV-2 infected cases^[69].

A role of proteins that are involved in iron homeostasis has also been hypothesized. In a detailed histopathological study, Corengia and colleagues investigated *HFE* genotypes and hepatic iron score in hepatitis C^[70]. They found a significant relationship between *HFE* genotypes and iron deposition in hepatocytes. This suggested that some iron accumulation in CHC derives from increased iron absorption due to mutated *HFE* protein. Hecpudin has also been implicated. A study by Aoki and colleagues

evaluated the possible role of hepcidin in determining iron overload in hepatitis C^[71]. In patients with HCV, they demonstrated that hepatic hepcidin mRNA correlated with hepatic iron concentration (HIC). This suggested that iron stores regulate hepcidin expression normally; iron loading in CHC is not due to inappropriate hepcidin expression.

Serum iron indices are frequently abnormal in patients with CHC. Elevation of the serum ferritin concentration has been reported in 20%-60% of patients with chronic hepatitis C^[59,68,72,73]; stainable hepatic iron deposits were detected in 3%-38%. Furthermore, many cases of CHC, including those with elevated serum ferritin, elevated transferrin saturation, or both, showed no significant increase in HIC^[59,61,68,73-74]. In most of these studies, hepatic iron overload was generally mild to moderate and often not sufficient to be hepatotoxic *per se*^[61,75,76]. Elevated serum ferritin concentration in CHC may be explained by several associated conditions, in the absence of iron overload. Serum ferritin could be elevated as an acute phase protein because of the cytolytic necroinflammation that is common in hepatitis C. Moreover, hepatic steatosis is frequent in patients with hepatitis C^[77,78] and has been associated with raised levels of serum ferritin^[79,80]. A recent study suggested that increased serum ferritin in CHC may be mainly due to diabetes mellitus, which is commonly associated with CHC^[81]. In contrast, other observations from one of us suggested that elevated serum ferritin in CHC may be mostly multifactorial^[82].

The distribution of iron has been examined in several studies, with some discrepancies. Some studies reported iron deposits mostly in hepatocytes^[70,83,84], others in reticuloendothelial cells^[85-87] and others reported a mixed distribution^[68,88,89]. This is a notable difference with HH where, until iron loading is severe, hepatic iron deposition is almost exclusively parenchymal.

The role of *HFE* mutations as a risk factor for iron overload in CHC has been studied in different populations, with somewhat discordant results (Table 2). Piperno and colleagues studied 110 Italian patients with chronic B or C viral hepatitis; they found that all male heterozygotes for the C282Y mutation had iron overload. The H63D mutation was significantly more frequent in patients with marked hepatic siderosis than in those with mild or no siderosis and in controls^[73]. A study of 137 CHC patients from the North of England reported that patients who carried the C282Y mutation had higher serum iron indices and more frequently had stainable hepatic iron, together with more advanced fibrosis or cirrhosis, than patients without the C282Y mutation^[90]. Similar results were obtained in other studies which correlated the presence of *HFE* mutations with increased serum iron indices, hepatic iron deposits and severe hepatic fibrosis^[70,91-94]. In an Austrian study of 184 patients with CHC versus 487 controls, Kazemi-Shirazi and colleagues found that serum iron indices were increased in patients carrying *HFE* mutations. In contrast with other studies, however, there was no evidence for more hepatic siderosis or advanced fibrosis in patients with *HFE* mutations^[95]. Other studies reported no relationship of *HFE* mutations with hepatic iron deposits and severe hepatic fibrosis^[83,89]. In a Scottish study, Thorburn and colleagues prospectively investigated

Table 2 Studies of the relationship between chronic hepatitis C, iron and *HFE* mutations

Reference	Cases <i>n</i>	Ethnicity	<i>HFE</i> relationship with serum iron indices	<i>HFE</i> relationship with HI	<i>HFE</i> relationship with fibrosis
[70]	206	Italian	Yes	Yes	Yes
[83]	120	Mostly Swiss and Italian	No	No	No
[88]	242	Mostly Caucasian	No	No	No
[89]	164	Mostly Caucasian	No	No	No
[90]	137	Caucasian	Yes	Yes	Yes
[91]	135	Brazilian	Yes	Yes	Yes
[92]	119	Mostly Caucasian, non-Hispanic	Yes	Yes	Yes
[93]	316	Mostly White	Yes	Yes	Yes
[94]	401	Mostly German	Yes	Yes	Yes
[95]	184	White, non-Hispanic	Yes	No	No
[96]	273	NA	Yes	Yes (only H63D)	No
[97]	1051	Mostly White, non-Hispanic	Yes	Yes	No

NA: not available; HI: hepatic iron (histological and/or biochemical evidence).

164 consecutive patients with HCV infection. They did not find a role for *HFE* mutations in the accumulation of iron or the progression of liver disease^[89]. Overall, only a few studies have suggested an increased prevalence of *HFE* mutations in CHC patients, with respect to the general population^[90,92,95]; this observation was not confirmed in other studies^[73,91,94].

A role for the H63D mutation in the iron overload of CHC was proposed in a few of the studies. Lebray and colleagues found that the histological hepatic iron score was higher in patients who were homozygous or heterozygous for H63D; this was surprisingly associated with an increased response rate to antiviral therapy^[96]. Another study reported increased hepatic iron deposits in male patients carrying the H63D mutation^[97]. Thus, the role of the H63D mutation is unclear and, as observed in HH, is minor with respect to the C282Y mutation.

Several studies suggested that higher levels of serum ferritin or HIC were associated with a diminished likelihood of response to antiviral therapy^[84,85,97]. Data from a recent Italian multicenter study indicated that iron removal by phlebotomy improved the rate of response to interferon^[98]. The influence of *HFE* mutations on the outcome of antiviral treatment has been investigated in a few studies. Chapman and colleagues suggested that a single mutation in the *HFE* gene had no impact on the outcome of interferon treatment; this was confirmed by subsequent reports^[99-101]. However, a study of 242 patients found that presence of the C282Y mutation was positively correlated with sustained response in a multivariate analysis^[88]. The reports by Lebray *et al* and Distant *et al* suggest that *HFE* may be part of a pattern of host genes which together influence response to antiviral therapy^[88,96]. Indeed, clearance of HCV is believed to be associated with different HLA alleles; the C282Y mutation of the

HFE gene is part of an extended founder haplotype which includes the HLA region on the short arm of chromosome 6^[101]. The positive effect of the C282Y mutation may reflect linkage disequilibrium between the *HFE* mutated allele and alleles at other loci implicated in the virological response, close to the *HFE* gene on chromosome 6. Several MHC class I and II loci have been shown to be associated with a sustained virological response to interferon treatment^[102-105]. Similarly, an American study investigated the role of iron overload and *HFE* mutations in the response to antiviral therapy in over 1000 patients with advanced CHC^[97]. The authors found that *HFE* mutations correlated with histological hepatic iron score in CHC. Subjects harbouring *HFE* mutations, particularly H63D, had significantly higher likelihood of both on-treatment virological responses (at 24 and 48 wk) and sustained virological responses (24 wk after the end of lead-in therapy) to re-treatment with pegylated interferon alpha-2α plus ribavirin. Again, both the *HFE* mutation and/or associated genetic variants were considered as possible causes of the improved response to therapy.

In conclusion, elevation of serum iron indices and hepatic iron deposits are a common feature in CHC. Hepatic iron overload is generally mild to moderate and it rarely reaches the severity seen in HH. In contrast to HH, the intrahepatic iron accumulation is generally mixed, with both parenchymal and reticuloendothelial distribution. The exact mechanism of hepatic iron accumulation in hepatitis C is still not clear. The pathogenesis is likely to be multifactorial and viral and host factors have been evaluated. The viral factors suggested by several studies include necroinflammation due to viral infection, direct influence on iron homeostasis mediated by cytokines, or HCV genotypes. Among the host factors, a role for *HFE* mutations has been proposed and extensively evaluated. On the basis of current knowledge, we conclude that *HFE* mutations may have a role in the elevation of serum iron indices and hepatic iron deposition observed in CHC, but they do not fully explain the observed abnormalities of iron homeostasis. Iron may also contribute to the rate of response to antiviral therapy.

IRON AND *HFE* MUTATIONS IN CHRONIC HEPATITIS B

Chronic hepatitis B (CHB) remains a serious global health concern. Approximately 350 million people are chronically infected, and 500 000 to 1.2 million deaths per year are attributed to HBV-associated complications^[106]. Among patients with active viral replication, cirrhosis will develop in 15%-20% within five years^[107]. For patients with cirrhosis, acute exacerbation can occur and the disease may progress to end stage complications^[107]. The histopathological pathway of progressive liver disease is characterised by fibrosis leading to increasing distortion of the hepatic architecture, that is the hallmark of evolution to cirrhosis. Liver fibrosis is the result of chronic injury and plays a direct role in the pathogenesis of hepatocellular dysfunction and portal hypertension. The progression of liver fibrosis is due to many viral and host

factors. In CHC it has been proposed that iron may be a cofactor, but data for patients with CHB are more scarce. An association between iron and hepatitis B was first described by Blumberg and colleagues^[5]. They found that serum iron indices were higher in patients who developed chronic hepatitis than in those who eliminated the virus. The same team also observed that haemodialysed patients with higher serum iron indices were less likely to achieve spontaneous recovery after acute hepatitis B^[108]. Other authors, assessing the presence of hepatic iron in Kupffer cells of patients with CHB, deduced that it is derived from hepatocytes destroyed by the virus^[109,110]. Zhou and colleagues studied 40 patients with hepatocellular carcinoma, 80% of whom were hepatitis B surface antigen (HBsAg) positive. They found a significant correlation between the presence of hepatitis B core antigen (HBcAg) and iron in hepatic tissue, suggesting that iron may accumulate predominantly in the hepatocytes in which HBV replication takes place^[111]. More recently, Martinelli and colleagues evaluated the prevalence of serum iron biochemical abnormalities and iron deposits in the liver of CHB patients^[112]. They found elevated transferrin saturation in 27.1% and liver iron deposits in 48.7% of cases. Patients with liver iron deposits presented with higher scores of necroinflammatory activity and fibrosis. The authors found no relationship between *HFE* mutations and elevation of serum iron indices or liver iron.

The effect of iron on the outcome of interferon alpha therapy in patients with CHB has been investigated by very few studies. In chronic viral hepatitis, Van Thiel and colleagues reported that low hepatic iron content may predict response to interferon therapy^[61]. More recently, a Polish group investigated iron metabolism and prognostic factors in interferon therapy in children with CHB. They showed that seroconversion for hepatitis Be antigen (HBeAg) was more frequently observed in children with lower iron and ferritin values^[113].

In conclusion, only a few studies have evaluated the prevalence and the physiopathological significance of iron overload in CHB. It has been suggested that iron deposits may occur mostly in hepatocytes. It seems that patients with higher levels of serum iron indices are less likely to achieve spontaneous recovery after acute hepatitis B. No role for the *HFE* gene mutations in iron overload has been detected. Iron could influence the response to antiviral therapy, but there is not sufficient evidence to permit a definitive conclusion.

IRON AND *HFE* MUTATIONS IN ALCOHOLIC LIVER DISEASE

Alcoholic liver disease (ALD) is one of the leading causes of end-stage CLD. It is well established that only a minority of heavy drinkers, estimated at between 10 and 30%, will ever develop advanced ALD; the risk increases with cumulative alcohol intake^[114,115]. Hence, in addition to alcohol, other factors are deduced to act synergistically to enhance its hepatotoxic effects. Patients with ALD commonly have elevation of transferrin saturation and serum ferritin concentration; significant hepatic iron

Table 3 Studies of the relationship between alcoholic liver disease, iron and *HFE* mutations

Reference	Cases <i>n</i>	Ethnicity	<i>HFE</i> relationship with serum iron indices	<i>HFE</i> relationship with HI	<i>HFE</i> relationship with ALD
[120]	257	Caucasian	NA	No	No
[121]	254	Caucasian	No	Yes	No
[129]	179	White	Yes (C282Y)	NA	No
		Hispanic			
[130]	61	White, non-Hispanic	NA	NA	NA

NA: not available; HI: hepatic iron; ALD: alcoholic liver disease (histological and/or biochemical evidence).

deposition is not infrequent^[116-118]. However, most patients with ALD have normal or slightly elevated HIC, with a mixed parenchymal and reticuloendothelial pattern of distribution^[119,120]. There is growing evidence that a mild degree of iron overload is sufficient to enhance alcohol-induced liver injury. The paradigm of synergy between iron and alcohol is HH. Patients with HH and significant alcohol consumption have a higher incidence of cirrhosis and hepatocellular carcinoma than those without heavy alcohol consumption or a history of alcohol abuse^[121,122]. In ALD, stainable hepatic iron was positively correlated with fibrosis in a multivariate analysis of risk factors in 268 French alcoholic patients^[123].

There are several potential causes for hepatic iron overload in alcoholic liver disease, including increased ingestion of iron, increased intestinal iron absorption, up-regulation of hepatic TfR1, secondary anaemia due to haemolysis, hypersplenism, ineffective erythropoiesis, hypoxaemia due to intrapulmonary shunts and portosystemic shunts^[4,124,125]. Increased iron absorption could arise through three main mechanisms: an increase in the reduction of luminal iron to the ferrous state; up-regulation of DMT1 in duodenal enterocytes; upregulation of ferroportin in duodenal enterocytes. The latter mechanism may be influenced by the down-regulation by ethanol of the hepatic production of hepcidin^[126].

Several studies suggested a genetic component to disease susceptibility. Significant associations have been reported between ALD risk and polymorphisms of the genes encoding cytochrome P450 and tumour necrosis factor (TNF) α ^[126,127]. Few studies assessed the possible role of *HFE* mutations as genetic cofactors in the development of ALD (Table 3). Lauret and colleagues found a significant association between carriage of the C282Y mutation and elevation of serum iron indices, but another study did not replicate this finding^[120,128]. One study found a relationship between *HFE* mutations and hepatic iron deposits^[120] but this observation was not confirmed by others^[119]. Moreover, none of these studies found that *HFE* mutations influenced the severity of ALD^[119,120,128,129].

In conclusion, patients with ALD tend to show mild increases in hepatic and serum measures of iron status. Iron is thought to play a role in worsening the course of ALD, although the mechanisms responsible are not resolved. To date, evidence from the literature does not

suggest a role for *HFE* mutations in determining iron overload or in influencing the course of ALD.

IRON AND *HFE* MUTATIONS IN NON-ALCOHOLIC FATTY LIVER DISEASE

Non-alcoholic fatty liver disease (NAFLD) is increasingly recognized as the most prevalent liver disease, at least in the West. In the adult population of the USA, 31% of men and 16% of women were found to have NAFLD^[130]. The disease has a spectrum ranging from fatty liver alone to steatohepatitis, and progressive steatofibrosis. Although fatty liver alone is considered non progressive, up to 20% of patients with non-alcoholic steatohepatitis (NASH) may develop cirrhosis, liver failure and HCC^[131]. Many cases of cryptogenic cirrhosis may be end-stage forms of NASH^[132]. The pathogenesis of NAFLD and the reasons why some patients with fatty liver develop NASH and have progressive liver disease are not entirely understood. A “two-hit” hypothesis has been proposed, involving the accumulation of fat in the liver (“first hit”), together with a “second hit” that gives rise to increased oxidative stress. Hepatic steatosis has been recognised as the first of two hits in the pathogenesis of NASH, since the presence of oxidisable fat within the liver is enough to trigger lipid peroxidation^[133]. However, many patients with fatty liver do not progress to steatohepatitis. Potential second hits for the development of NASH include all mechanisms contributing to the development of inflammation and fibrosis. Increased expression of ethanol-inducible cytochrome P450 2E1 (CYP2E1) and an increase in the intrahepatic concentration of free fatty acids could result in oxidative stress via peroxisomal oxidation^[134]. TNF α has also been implicated, since administration of anti-TNF α antibody ameliorated liver damage in animal models^[135].

There is controversial evidence that hepatic iron may play a role in the pathogenesis of NASH. It has been proposed that iron, in relatively low concentrations, could synergize with lipid overload and induction of CYP2E1 to increase oxidative stress in hepatocytes^[136]. Elevation of serum iron indices has been found in several studies^[126,127]. In most cases hepatic siderosis was mild and HIC was only rarely elevated^[137-140]. The distribution of hepatic iron was mixed parenchymal and sinusoidal^[79]. Strong support for an important role of iron in NAFLD and NASH was recently provided by studies on iron-depletion therapy, which improved both serum aminotransferases and insulin sensitivity^[141,142].

The role of iron has been re-evaluated since the discovery of the strict pathogenetic link between NASH and insulin resistance^[143]. Iron has been proposed to contribute to the development or exacerbation of insulin resistance, which is the most important risk factor for development of NAFLD and NASH^[79,80]. Mendler and colleagues reported a new syndrome characterized by mild to moderate hepatic iron overload and features of insulin resistance; the new syndrome was named insulin resistance-hepatic iron overload (IR-HIO). In their study, liver steatosis and NASH were present in 25% and 27% of IR-HIO cases, respectively^[7,144]. A subsequent study confirmed the high prevalence of NAFLD (59.7%) in patients with

Table 4 Studies of the relationship between NAFLD-NASH, iron and *HFE* mutations

Reference	Cases <i>n</i>	Ethnicity	<i>HFE</i> relationship with serum iron indices	<i>HFE</i> relationship with HI	<i>HFE</i> relationship with fibrosis
[139]	51	Australian	Yes with transferrin saturation, no with ferritin	Yes	Yes
[142]	31	Italian	No	Yes	No
[146]	263	Italian	Yes (C282Y)	No	No
[151]	57	Caucasian	Yes	Yes	Yes
[152]	32	Mostly Caucasian	No	No	No
[153]	38	Asian	No	No	No
[154]	31	Asian Indian	No	No	No
[155]	93	Mostly Caucasian	No	Yes	No

NA: not available; HI: hepatic iron (histological and/or biochemical evidence).

insulin resistance-associated iron overload^[145]. Moreover, as in NASH, phlebotomy allows for normalization of body iron stores in IR-HIO^[146]. Indeed, IR-HIO shares some features with NASH, such as hyperferritinemia with mostly normal transferrin saturation. However, not all patients with IR-HIO have hepatic steatosis. It is likely that NASH and IR-HIO share some pathogenetic mechanisms. It has been proposed that IR-HIO may represent one end of the spectrum of NASH in which HIC is increased. Another point of view is that IR-HIO may represent a coincidental convergence of hepatic iron overload with a very common liver disorder in the general population^[128].

Genetic factors have also been proposed to play a role in the pathogenesis of NASH. Investigations of the possible contribution of *HFE* mutations in the pathogenesis of NASH have given sometimes discordant results (Table 4). George and colleagues reported increased intrahepatic iron (Perls' grade > 1) in 41% of cases; 23% of patients had HIC above the upper limit of normal^[138]. The increased HIC was attributed to the higher prevalence of homozygotes and heterozygotes for C282Y in these Australian patients with NASH versus control subjects (31% *vs* 13%, respectively). No significant difference in the prevalence of the H63D mutation was reported. A subsequent study by Bonkovsky and colleagues reported a significantly increased prevalence of H63D heterozygosity in patients versus controls (44.4% *vs* 26.4%)^[147]. Heterozygosity for C282Y was not statistically different between the two groups. Fargion and coworkers also reported an increased prevalence of *HFE* mutations in patients with respect to controls (65% *vs* 26%). Patients carrying *HFE* mutations did not present with increased serum iron indices, but showed increased HIC^[79]. In contrast to these results, other investigators have failed to observe significant associations between hepatic iron accumulation, *HFE* mutations and the severity of liver disease in patients with NASH^[139,140,80,148-151]. Angulo and colleagues found that HIC was normal in patients with NASH and abnormal serum iron indices^[139]. Another study by Chitturi and colleagues did not find any correlation

between HIC and any features of NASH, including the severity of fibrosis^[151]. The authors did not find any increase in stainable hepatic iron in the majority (90%) of liver biopsies from patients with NASH. Although they, like others, found a higher prevalence of C282Y heterozygosity in patients with NASH, no relationship with fibrotic severity was identified. Other studies did not find either significant abnormalities of serum and liver iron or an increased frequency of *HFE* mutations^[80,148,149].

Explanations for discrepant results include ascertainment bias, varied power of the studies and possible ethnic differences in the study populations. It should be underlined that elevation of serum iron indices in NASH, especially serum ferritin, may be due to insulin resistance, steatosis and inflammation, rather than to iron overload. Indeed, the discrepancies could be partially attributed to the differences between databases: the patients could be selected among subjects with hepatic iron overload or among population routinely attended a gastroenterology outpatient clinic. The association between NASH and *HFE* mutations has been described mainly in Australian and North American Caucasians^[138,147]. This hypothesis is supported by a recent study by Chitturi and colleagues that found an increased frequency of C282Y heterozygosity in NASH only in Anglo-Celtic patients^[151]. However, studies showing a positive correlation may have come from tertiary centres with an interest in iron storage disorders.

An increased prevalence of C282Y and H63D mutations has also been reported in patients with IR-HIO, but this finding was not connected with increased iron burden^[7]. A recent study of ours described four cases of H63D homozygosity associated with hyperferritinemia, macrovesicular steatosis, mild parenchymal and sinusoidal hepatic siderosis, with a granular pattern that could be related to NASH. Furthermore, three of the cases had one or more metabolic disorders which are part of the insulin resistance syndrome. The study could be consistent with proposals of a possible biological effect of the H63D mutation in IR-HIO and/or fatty liver^[152].

In conclusion, NAFLD has a heterogeneous spectrum of disease. It can progress to NASH and to end-stage liver disease, but the exact mechanism of fibrosis progression is not completely clear. A “two hits” hypothesis has been proposed and iron has been evaluated as a potential “second hit” that can cause progression of simple fatty liver to NASH. The available data are discordant about prevalence and effect of iron overload in NAFLD and NASH. Patients with coexisting NASH and hepatic iron stores undoubtedly exist, but the discordant data of the literature suggest that iron overload in NASH may be an epiphenomenon rather than have a main causative role. Insulin resistance syndrome has emerged as a key player in NAFLD and in the development of NASH. Iron may exacerbate insulin resistance; although iron overload is fairly common in NAFLD, its amount is rarely clinically significant when considered in isolation. A possible role of *HFE* mutations in NASH has been described in two studies from countries with predominantly Caucasian populations. However, this was not confirmed in subsequent studies of more heterogeneous populations. *HFE* mutations may be part of a genetic pattern

Table 5 Studies of the relationship between porphyria cutanea tarda, iron and *HFE* mutations

Reference	Cases n	Ethnicity	<i>HFE</i> relationship with serum iron indices	<i>HFE</i> relationship with HI	<i>HFE</i> relationship with fibrosis
[163]	41	Caucasian	NA	NA	NA
[164]	108	Australian	Yes	Yes	No
[165]	70	North American	NA	NA	NA
[167]	23	Brazilian	Yes	No	Yes
[168]	36	Southern France	No	NA	NA
[170]	190	German	No	No	No
[172]	68	Italian	No	NA	NA
[174]	62	German	Yes	NA	NA

NA: not available; HI: hepatic iron (histological and/or biochemical evidence).

contributing to the progression of NASH in populations of Celtic origin.

IRON AND *HFE* MUTATIONS IN PORPHYRIA CUTANEA TARDA

Porphyria cutanea tarda (PCT) is the most common of the human porphyrias. It is caused by deficient activity of hepatic uroporphyrinogen decarboxylase. Most cases of PCT are acquired; the major risk factors are CHC, alcohol abuse, iron overload and oestrogen use. The familial form of the disease is observed in 20%-25% of patients^[4]. Both sporadic and familial PCT are iron-dependent disorders. The association of PCT with iron overload has been recognized for decades. Independent of the cause of liver disease, the majority of patients with sporadic PCT have biochemical evidence of iron overload, liver siderosis and increased body iron stores. However, hepatic siderosis is generally mild or moderate, reaching the lower end of the haemochromatosis range in less than 10% of cases^[153-154]. The causes of iron overload in patients with PCT appear to be heterogeneous. Indeed, altered iron status may be secondary to cofactors such as alcohol and chronic infection with HCV, that are frequently associated with sporadic PCT^[4]. Clinical and experimental data suggest that an iron-dependent process reversibly inactivates uroporphyrinogen decarboxylase^[155]. Indeed, as initially observed by Lundvall, venesection therapy may induce remission of cutaneous lesions and an improvement of liver function tests, whereas replenishment of iron stores leads to relapse^[156,157]. Phlebotomy may also be beneficial in patients without biochemical or histological evidence of iron overload^[157]. Altered iron homeostasis, even in the absence of systemic iron overload, could reduce uroporphyrinogen decarboxylase activity^[158].

Several studies have investigated the possible association between PCT and mutations of the *HFE* gene (Table 5). Investigators from UK reported that 44% of patients with PCT *vs* 11% of the control group carried the C282Y mutation, whereas no significant difference was found for the H63D mutation^[159]. Similar results were

reported by Stuart and colleagues in Australian patients and by Bonkovsky and colleagues in patients from North America^[160,161]. A detailed study comprising 108 US patients with PCT reported a C282Y frequency of 30% compared to 6% in controls^[162]. HIC, transferrin saturation and serum ferritin were highest in PCT patients who were homozygous for the C282Y mutation. The authors also reported a high frequency of comorbidity due to factors such as alcohol and hepatitis C. They concluded that homozygosity for the C282Y mutation and HCV infection, especially with heavy alcohol consumption, are the strongest risk factors for PCT. Martinelli and colleagues reported an association between C282Y and PCT with respect to controls (17.4% *vs* 4%) in a population of Southern European ancestry^[143]. Similar results were obtained in two studies of French patients^[164,165]. In a large study of 190 sporadic PCT cases from Germany the C282Y and H63D mutations were found in 39% and 45%, respectively^[166]. C282Y was significantly more frequent in patients than controls. Serum iron, transferrin saturation, ferritin concentration, HIC and liver enzymes did not differ significantly between patients with or without *HFE* mutations. Investigators from South Africa determined the frequency of *HFE* mutations in a racially-mixed group of patients with PCT in Cape Town^[167]. They found that both the C282Y and H63D mutations were highly prevalent in South Africans of European origin. In cases of mixed or Asian origin, the H63D mutation was common but the C282Y mutation was very rare. Neither mutation was found in any African subject. They concluded that both mutations were associated with PCT, but the association was dependent on the ethnic origin of the patient. Interestingly, one study from Italy reported a strong association of PCT with the H63D mutation, which was present in half of the patients. The presence of the H63D mutation was not related to the iron status of patients. However, a subtle abnormality of iron metabolism induced by this mutation could escape detection by the standard parameters of iron status^[168]. Some major factors may account for some differences between these studies. Firstly, the C282Y mutation is more frequent in cases of Celtic ancestry, although the prevalence of this mutation appears to be lower in Southern European countries^[9,11]. Secondly, the distribution of factors predisposing for PCT also shows relevant geographical differences. In Italy and other Mediterranean countries, hepatitis C is present in 70%-90% of PCT patients while it is rare in Northern European countries, where alcohol is the prevalent aetiological agent for CLD associated with PCT^[169]. Stolzel and colleagues investigated the relationship between *HFE* gene mutations and response to chloroquine in PCT patients^[170]. Chloroquine therapy was accompanied by clinical remission and reduced urinary porphyrin excretion in 39% of patients without *HFE* mutations versus 56% of *HFE* heterozygous patients. Interestingly, all patients homozygous for the C282Y mutation had high serum iron, transferrin saturation and serum ferritin concentration, and failed to respond to chloroquine treatment.

In conclusion, PCT is frequently associated with elevated serum iron indices and sometimes with hepatic iron overload. The pathogenesis of altered iron

homeostasis in PCT is not completely understood, although iron is thought to reduce the activity of uroporphyrinogen decarboxylase. Venesection therapy may be beneficial in PCT patients. Several cofactors have been proposed to play a role in the impairment of iron homeostasis observed in PCT, such as alcohol and CHC. A role of *HFE* mutations has also been reported. The available data support a role for the C282Y mutation in many cases of PCT, especially the C282Y homozygous genotype. This is particularly true in Celtic ancestry cases, thus confirming that the importance of *HFE* mutations as modifiers of disease varies according to the ethnic group. Most studies did not detect a relationship between *HFE* mutations and serum and hepatic iron overload. This suggests that *HFE* mutations, particularly C282Y, may contribute to the pathogenesis of PCT either through immunological mechanisms that could be iron-independent, or via subtle changes in hepatic iron metabolism that may act in concert with other co-factors to inhibit the activity of uroporphyrinogen decarboxylase.

IRON AND *HFE* MUTATIONS IN HEPATOCELLULAR CARCINOMA OCCURRING IN CLDS OTHER THAN HH

HCC is a common cause of death in patients with compensated cirrhosis^[171]. European studies have reported HCC as the cause of liver-related deaths in 54%-70% of cases of compensated cirrhosis of varied aetiology and in 50% of cases with cirrhosis due to HCV^[172]. The annual incidence of HCC in patients with liver cirrhosis has been estimated at 3%-5%^[173,174]. Thus, it is important to identify patients at high risk of HCC, to increase the rate of early detection. Several risk factors for the development of HCC have been identified in Western patients with cirrhosis, including male sex, age, persistently raised serum α -fetoprotein levels, severity of cirrhosis and genetic background^[173]. A possible carcinogenic role for iron has been suggested by *in vitro* studies. Iron may promote cellular oxidative stress through the production of reactive oxygen species. These have the potential to cause lipid peroxidation as well as damage to other cellular components, including proteins and nucleic acids^[151]. *In vitro* studies showed that iron can reduce the levels of two systems that normally protect against reactive oxygen species, vitamin E and superoxide dismutases^[175-176]. Furthermore, *in vivo* studies in mammals showed that tumor growth is enhanced by iron supplementation and inhibited by iron deficiency^[175,177]. Finally, neoplastic cells highly express TfR1 and can synthesize their own transferrin^[177].

Apart from the experimental evidence, the role of iron in the step by step process that leads to HCC in CLDs other than HH is controversial. A report of 133 cases by Boige and colleagues did not find any difference in the grade of hepatic iron staining between cirrhotic patients with and without HCC^[178]. This finding was also in keeping with a previous study where no significant relationship was observed between the hepatic iron score and the occurrence of HCC in alcoholic and HCV-related

cirrhosis^[179]. A role of *HFE* mutations as part of a genetic pattern promoting carcinogenesis has been evaluated in some studies, with somewhat discordant results. In a German study, C282Y heterozygosity was significantly more common in 137 HCC cases with no history of HH versus 107 cirrhotic patients without HCC and 126 healthy controls. C282Y heterozygote HCC patients had significantly increased hepatic iron score in both HCC and non-tumorous tissue^[180]. Other studies reported that the prevalence of C282Y heterozygosity was increased above control levels in patients with HCC and alcoholic and virus-related liver disease^[129,182]. An excess of the C282Y mutation, mostly in the heterozygous genotype, has also been reported in patients with HCC developed in non cirrhotic liver. In that study, Blanc and colleagues showed that mild iron overload is frequent (54%) and that in patients with HCC in non cirrhotic liver and iron overload, C282Y mutations are frequent (36.8% of cases) and significantly increased compared to HCC in non cirrhotic liver without iron overload^[183]. In contrast with the above results, some studies did not find a relationship between HCC and mutations in the *HFE* gene. Cauza and colleagues evaluated the prevalence of *HFE* mutations in patients with HCC developed on cirrhosis of viral and alcoholic aetiology. The authors found that, except for C282Y homozygotes, *HFE* mutations did not increase the risk of HCC in patients with cirrhosis^[184]. A prospective study of 133 consecutive cirrhotic patients without HH did not find an increased prevalence of *HFE* mutations in cirrhotic patients who developed HCC compared to cases without HCC^[178]. Similarly, a study from Italy found that in patients with HCC in the absence of HH, the frequency of *HFE* mutations was not increased, compared to the controls. The authors concluded that mutations of the *HFE* gene do not play a significant role in the pathogenesis of HCC^[185].

In conclusion, iron has a clear pathogenetic role in the development of HCC in HH. The carcinogenic role of iron is deduced to be mediated through production of reactive oxygen species leading to lipid peroxidation and damage of proteins, nucleic acids and other cellular components in hepatocytes. However, similar results have not been obtained for other CLDs. The available studies about the role of *HFE* mutations in HCC in liver diseases other than HH indicate that C282Y heterozygosity may play a role in liver iron deposition and could contribute to hepatocarcinogenesis, possibly by playing a part in the immunogenetic pattern of the patient or through subtle changes in iron metabolism acting together with other cofactors.

HIGHLIGHTS

Apart from HH, a number of CLDs cause hepatic iron overload. These include hepatitis C and B, alcoholic and nonalcoholic steatohepatitis and PCT. Secondary iron overload due to CLD presents with some clinical, histological and genetic differences with respect to HH. Since the occurrence of HH is clearly related to the presence of specific genetic patterns, the role of mutations

Table 6 Secondary iron overload: common features and differences

Common features	
1	Mixed distribution of hepatic iron (parenchymal and reticuloendothelial)
2	Mild to moderate iron overload in most cases, rarely severe iron overload
3	Phlebotomy possibly improves the course of disease and response to therapy
Differences	
1	C282Y: accepted role in PCT, possible cofactor in CHC and NAFLD (especially in populations of Northern European descent)
2	H63D: unclear role in NAFLD and PCT
3	No role for <i>HFE</i> mutations in ALD or CHB

PCT: porphyria cutanea tarda; CHC: chronic hepatitis C; NAFLD: non-alcoholic fatty liver disease; ALD: alcoholic liver disease; CHB: chronic hepatitis B; CLDs: chronic liver diseases.

of the gene most commonly responsible for HH has been investigated in liver diseases other than HH. Liver diseases that cause secondary iron overload share some common features, but they also show differences (Table 6). The evidence in the literature regarding secondary iron overload in comparison to HH are consistent in showing that: (1) in HH iron overload is widespread in many organs while in secondary iron overload due to CLDs, iron is confined to the liver; (2) in HH the histological distribution of iron is mostly parenchymal while in secondary iron overload due to CLDs, iron shows mostly a mixed distribution, with both reticuloendothelial and parenchymal localization; (3) point mutations in the *HFE* gene are the most common genetic factor underlying HH; their prevalence varies according to geographic area and ethnic group. In secondary iron overload, data are controversial. When present, the penetrance of *HFE* mutations may be more influenced by cofactors, since the aetiology of CLDs is multifactorial; (4) in HH, genetics is the “primum movens” of the disease while in secondary haemochromatosis it has been proposed as a cofactor that may increase the severity of disease expression. Evidence from the literature suggests that the C282Y mutation may play a role in NASH, PCT and possibly CHC in cases of Celtic ancestry. The importance of *HFE* mutations as modifiers of disease, therefore, varies between different ethnic groups. H63D has been reported by few authors as having a role in some cases of mild iron overload in NAFLD and PCT, although any effect is generally minor, in agreement with the findings in HH. *HFE* mutations do not seem to determine iron overload or to influence the course of ALD and CHB. Iron may contribute to the rate of response to antiviral therapy in CHC and to chloroquine in PCT and a positive influence of *HFE* mutations has been suggested in CHC.

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TOPIC HIGHLIGHT

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Non-HFE haemochromatosis

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Abstract

Non-HFE hereditary haemochromatosis (HH) refers to a genetically heterogeneous group of iron overload disorders that are unlinked to mutations in the HFE gene. The four main types of non-HFE HH are caused by mutations in the *hemojuvelin*, *hepcidin*, *transferrin receptor 2* and *ferroportin* genes. Juvenile haemochromatosis is an autosomal recessive disorder and can be caused by mutations in either *hemojuvelin* or *hepcidin*. An adult onset form of HH similar to HFE-HH is caused by homozygosity for mutations in *transferrin receptor 2*. The autosomal dominant iron overload disorder ferroportin disease is caused by mutations in the iron exporter ferroportin. The clinical characteristics and molecular basis of the various types of non-HFE haemochromatosis are reviewed. The study of these disorders and the molecules involved has been invaluable in improving our understanding of the mechanisms involved in the regulation of iron metabolism.

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Key words: Haemochromatosis; Iron overload; Non-HFE; Juvenile haemochromatosis; Hemojuvelin; Hpcidin; Transferrin receptor 2; Ferroportin

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INTRODUCTION

After the identification of the *HFE* gene in 1996^[1] it became apparent that not all cases of haemochromatosis are caused by mutations in *HFE*. HFE-associated HH (HFE-HH) or type 1 HH is the most common form,

especially in populations of Northern European origin, where the C282Y mutation has a high allele frequency^[2]. Haemochromatosis that is unrelated to mutations in the *HFE* gene are collectively referred to as non-HFE haemochromatosis. Non-HFE haemochromatosis occurs in populations world wide and makes up a larger proportion of HH cases in areas where the C282Y mutation is less common, such as Southern Europe^[3] and Asia^[4]. Non-HFE HH can be further differentiated according to the gene mutated. There are four main types of non-HFE HH. The molecules mutated in all forms of HH are related in pathways involved in the regulation of iron homeostasis. Hpcidin the central regulator of iron homeostasis and hemojuvelin are mutated in juvenile or type 2 HH^[5,6]. Transferrin Receptor 2 is mutated in type 3 HH^[7] and the iron exporter ferroportin is mutated in the autosomal dominant type 4 HH or ferroportin disease^[8,9]. The genetic, clinical and laboratory features of the various types of HH are outlined in Table 1. This review will describe in detail the four main types of non-HFE HH and review the current literature in this area.

JUVENILE HAEMOCHROMATOSIS (TYPE 2)

An early onset form of juvenile haemochromatosis (JH), distinct from the typical HFE-HH has been recognised for some time^[10]. As with HFE-HH, JH or type 2 HH is an autosomal recessive disorder, and is characterised by elevated serum iron indices and iron deposition in parenchymal cells. JH usually presents before the age of 30 years and has a more rapid and severe course than HFE-HH. Unlike HFE-HH both sexes are affected equally^[10]. Cardiomyopathy and hypogonadism are more prominent features of JH, hypogonadism being the most common symptom at presentation^[11]. The rapid accumulation of iron in patients with JH can often be fatal, death usually resulting from heart failure^[11].

When the *HFE* gene was identified in 1996, it became apparent that JH was indeed a disorder genetically distinct from the typical HFE-HH. Linkage to the *HFE* gene region on chromosome 6 was ruled out, in a study utilising microsatellite markers in five Italian JH families^[12]. This was followed by a genome wide search that identified linkage to the chromosome 1q21 region in nine JH families. Subsequently a subset of families who did not have linkage to 1q21 were found to have mutations in the *hepcidin* (*HAMP*) gene on chromosome 19^[5]. This subset of JH has been termed type 2B HH and is described in more detail later. The chromosome 1 form of JH has been termed type 2A HH.

Table 1 Genetic, clinical and laboratory features of the various types of Hereditary Haemochromatosis

HH Type	Gene	Inheritance	Clinical features	Laboratory findings	Liver pathology	Functional consequences of mutations
1	<i>HFE</i>	Autosomal recessive	May include: fatigue, lethargy, arthropathy, skin pigmentation, liver damage, diabetes mellitus, endocrine dysfunction, cardiomyopathy, hypogonadotropic hypogonadism	↑ serum ferritin, ↑ transferrin saturation	Hepatocyte iron loading, fibrosis, cirrhosis	Impaired hepcidin regulation by iron, leading to increased intestinal iron absorption and release of iron from reticuloendothelial cells
2A	<i>Hemojuvelin (HJV)</i>	Autosomal recessive	As for HFE. Earlier onset (< 30 yr). Cardiomyopathy and hypogonadism more prevalent.	↑ serum ferritin, ↑ transferrin saturation	Hepatocyte iron loading, fibrosis, cirrhosis	Loss of hepcidin regulation, leading to increased intestinal iron absorption and release of iron from reticuloendothelial cells
2B	<i>Hepcidin (HAMP)</i>	Autosomal recessive	As for HFE. Earlier onset (< 30 yr). Cardiomyopathy and hypogonadism more prevalent.	↑ serum ferritin, ↑ transferrin saturation	Hepatocyte iron loading, fibrosis, cirrhosis	No/inactive hepcidin, leading to maximal iron absorption and release of iron from reticuloendothelial cells
3	<i>Transferrin Receptor 2 (TfR2)</i>	Autosomal recessive	As for HFE.	↑ serum ferritin, ↑ transferrin saturation	Hepatocyte iron loading, fibrosis, cirrhosis	Impaired hepcidin regulation by iron, leading to increased intestinal iron absorption and release of iron from reticuloendothelial cells
4	<i>Ferroportin (Fpn), SLC40A1, IREG1, MTP1</i>	Autosomal dominant	Typical presentation: as for HFE, except generally milder. May have mild anaemia and lower tolerance to venesection. Atypical: as for HFE	↑ ↑ serum ferritin, normal transferrin saturation ↑ serum ferritin, ↑ transferrin saturation	Predominant Kupffer cell iron loading, fibrosis Predominant hepatocyte iron loading, fibrosis, cirrhosis	Reduced ferroportin iron transport ability, leading to accumulation of iron in reticuloendothelial cells Loss of ferroportin regulation by hepcidin, leading to increased intestinal iron absorption and release of iron from reticuloendothelial cells

HEMOJUVELIN-ASSOCIATED HAEMOCHROMATOSIS (TYPE 2A)

The gene responsible for the chromosome 1 form of JH was identified in 2004^[6]. Fine mapping of the JH locus was performed in 12 JH families of Greek, Canadian and French origin. Sequencing of genes in this region revealed a novel gene that was mutated in all affected individuals^[6]. The gene originally named *HFE2* encodes hemojuvelin (HJV), a protein with homology to the repulsive guidance molecule (RGM) family of proteins. Six mutations were initially identified in the affected individuals either in the homozygous or compound heterozygous state. One mutation G320V was present in nine of the 12 families^[6]. Since the identification of *HJV*, numerous mutations have been identified in JH families worldwide^[6,13-24]. An Italian study identified 16 more mutations among 34 JH patients from various European backgrounds^[13]. Figure 1 illustrates the structure of hemojuvelin and position of disease-causing mutations. Most mutations are private and were detected in single families. A few have been detected in more than one population. One mutation in particular (G320V) is significantly more frequent and has been reported in JH patients in many populations^[6,13,16-20,24].

Patients with HJV-HH were shown to have low levels of urinary hepcidin^[6]. This suggested that HJV may be involved in regulating hepcidin expression in response to iron. The generation of mouse knockouts confirmed that HJV was critical for the regulation of iron homeostasis and the induction of hepcidin^[25,26]. Recent studies have suggested that HJV regulates hepcidin expression through signalling pathways involving bone morphogenic proteins (BMPs)^[27].

HEPCIDIN-ASSOCIATED HAEMOCHROMATOSIS (TYPE 2B)

While most cases of juvenile haemochromatosis have been linked to a locus on the long arm of chromosome 1 (1q21) and mutations in *HJV*, a small number of families have been described with a JH-like disorder unlinked to 1q21^[28]. This subset of patients was found to have mutations in the *hepcidin (HAMP)* gene. Two consanguineous families of Italian and Greek origin were originally reported with linkage to a region on chromosome 19, encompassing the region containing the *hepcidin* gene^[5]. Homozygosity for mutations in the *hepcidin* coding sequence was detected in both families. One family harboured a one base pair deletion (93delG), causing a frameshift and an abnormal extended protein (T31fsX180). The other family carried a single base pair substitution (166C>T) causing the replacement of an arginine with a stop codon (R56X), in the predicted cleavage site for prohormone convertases. Both of these mutations severely affect the protein sequence and would result in the absence of any mature hepcidin peptide^[5].

Hepcidin mutations remain a rare cause of JH. However, since the first report, other cases and mutations have been described^[5,29-37]. Mutations described in the *hepcidin* gene are shown in Figure 2. These include mutations affecting two of the eight highly conserved cysteine residues (C70R and C78T), important for the complex disulphide bonded structure of mature hepcidin^[33-35]. A mutation in the 5'UTR of the *hepcidin* mRNA (-25G>A) has been described in two Portuguese families^[36,37]. This mutation creates a new initiation codon upstream from the original

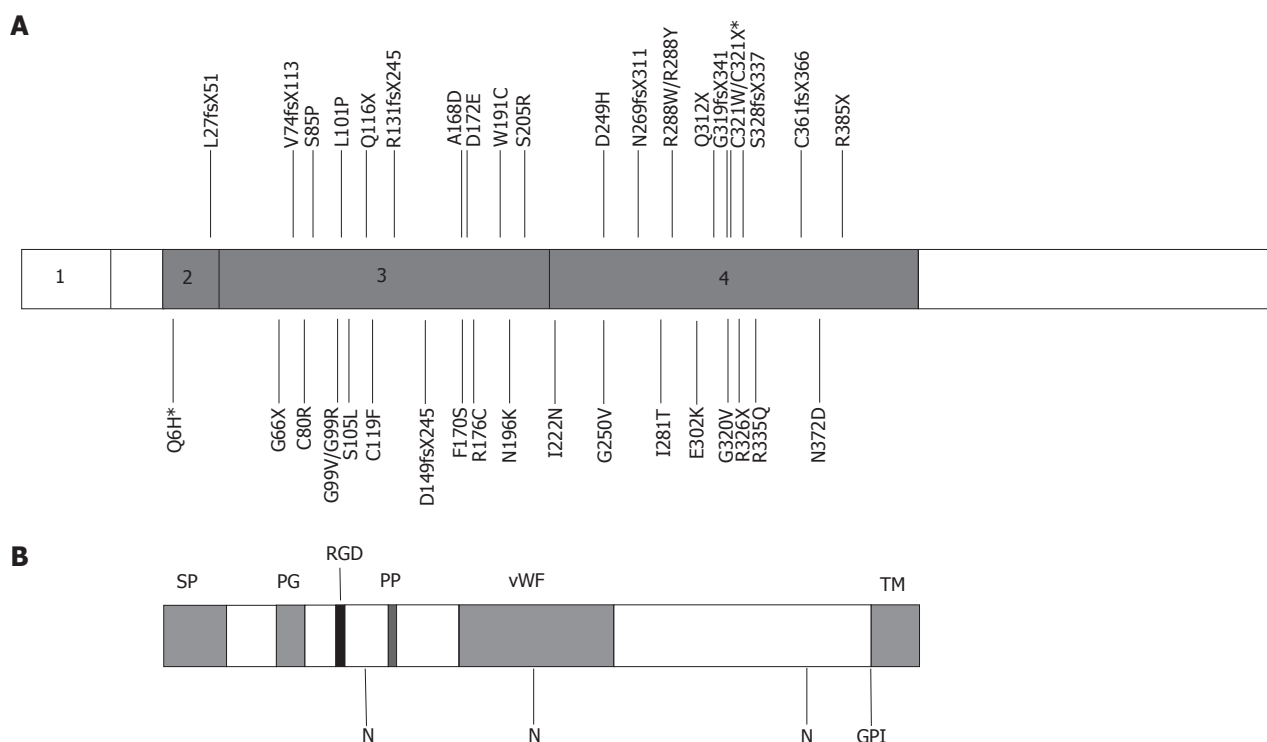


Figure 1 Structure of human hemojuvelin and positions of mutations. **A:** The exon structure of human *hemojuvelin* is shown with positions of known mutations marked^[16,13-24,30,39]. *Q6H was found associated with C321X; **B:** Predicted structure of the full length HJV protein, showing the positions of structural domains and motifs. SP, signal peptide; PG, poly-glycine sequence; RGD, RGD motif; PP, poly-proline sequence; vWF, partial von Willebrand factor type D domain; N, potential N-linked glycosylation sites; GPI, GPI-attachment site; TM, transmembrane domain, cleaved after GPI attachment.

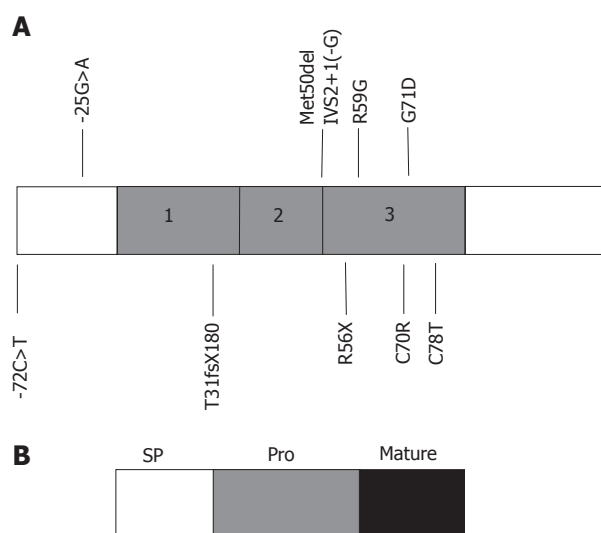


Figure 2 Structure of human *hepcidin* and positions of mutations. **A:** The exon structure of human *hepcidin* is shown with positions of known mutations marked^[5,29-37]. **B:** Predicted structure of the hepcidin peptide. SP, signal peptide; Pro, pro-region; Mature, 25 amino acid mature hepcidin peptide.

ATG. Measurement of urinary hepcidin in a patient homozygous for this mutation, suggests that steady state transcription of *hepcidin* from the original ATG codon does take place. But there is loss of upregulation of *hepcidin* transcription in response to iron^[37]. In another study it was shown *in vitro* that the out of frame upstream initiation codon was functional and prevented normal transcription from the original ATG^[38].

DIGENIC INHERITANCE AND MODIFIERS OF HFE

The remaining mutations in *hepcidin* have been detected in the heterozygous state in patients carrying *HFE* mutations. Merryweather-Clarke *et al*^[29,30] described patients with haemochromatosis who carried mutations in both *HFE* and *hepcidin*. One patient carried a four base pair deletion in *HAMP* (Met50del IVS2+1(-G)) and had a JH-like phenotype. Another family carried the G71D mutation in combination with either heterozygous or homozygous C282Y, and adult-onset iron overload. This was the first description of iron overload due to digenic inheritance of mutations in two separate genes. Two studies have detected *hepcidin* mutations in large cohorts of patients with HFE-HH. Jacolot *et al* detected *HAMP* mutations in five individuals from a cohort of 392 C282Y homozygotes and found that these were among the more iron-loaded. In addition, four of 31 subjects with iron overload, but at least one chromosome lacking an *HFE* mutation also carried a *HAMP* mutation. This supports the concept that digenic inheritance of *HFE* and *HAMP* mutations can lead to iron overload. Biasiotto *et al*^[30] also screened for *hepcidin* mutations in iron overload patients carrying the C282Y allele and detected sequence variations in some. They concluded that a novel substitution in the *hepcidin* promoter (-72C>T) may aggravate iron loading in patients with *HFE* mutations.

HJV mutations have also been detected in patients with HFE-HH. Two studies suggested that heterozygosity for *HJV* mutations may aggravate the phenotype in HFE-

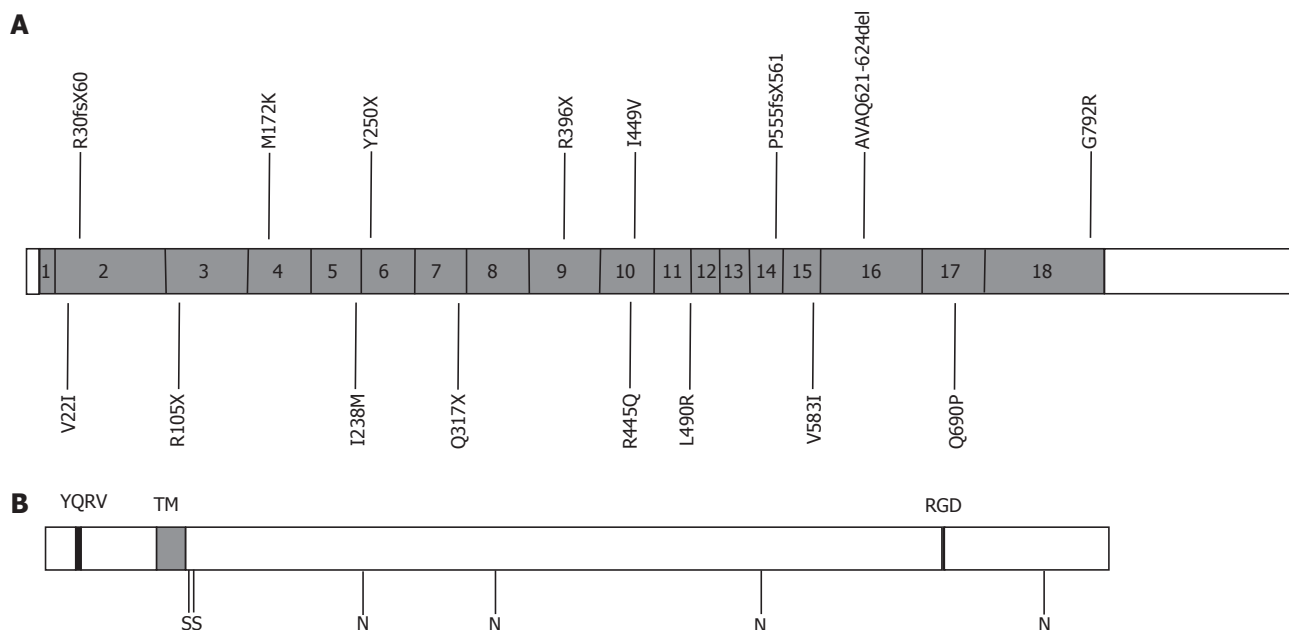


Figure 3 Structure of human transferrin receptor 2 (TfR2) and positions of mutations. **A:** The exon structure of human *TfR2* is shown with positions of known mutations marked^[7,30,40-52]. The frameshift mutations R30fsX60 and P555fsX561 are also known as E60X and V561X respectively. G792R may be associated with R396X; **B:** Predicted structure of TfR2 protein. YQRV, endocytosis signal; TM, transmembrane domain; RGD, RGD motif; S, predicted interchain disulphide bonds; N, potential N-linked glycosylation sites.

HH^[30,39]. Le Gac *et al.*^[39] reported nine of 310 C282Y homozygous patients with additional *HJV* mutations. Eight of the nine patients appeared to have a more severe phenotype, suggesting that heterozygosity for mutations in *HJV* were having a modifying effect. A similar effect of *HJV* mutations was reported by Biasiotto *et al.*^[30], the N196K mutation being associated with abnormally high iron indices in a C282Y/H63D compound heterozygote. The effect of *HJV* mutations on phenotypic expression of HFE-HH is small, and has not been detected in all studies. Lee *et al.*^[24] did not detect any *HJV* mutations in a group of 49 C282Y homozygotes. Wallace *et al.*^[7] reported a G320V heterozygous relative of a JH patient, who was also a C282Y/H63D compound heterozygote, but with normal iron indices.

TRANSFERRIN RECEPTOR 2-ASSOCIATED HAEMOCHROMATOSIS (TYPE 3)

Transferrin Receptor 2 (TfR2)-HH was first described in 2000^[7]. This was the first HH syndrome to be attributed to non-HFE mutations. Mutations in *TfR2* were first detected in six members of two Sicilian families. The defect was linked to a region on the long arm of chromosome 7 (7q22), and affected individuals were found to be homozygous for a nonsense mutation (Y250X) in *TfR2*^[7]. Affected individuals had iron overload with a similar phenotype to HFE-HH. TfR2-HH is a rare condition; however, several mutations have been reported worldwide associated with haemochromatosis^[7,30,40-52]. Mutations reported in *TfR2* are illustrated in Figure 3.

TfR2 is a homologue of the classical transferrin

receptor (TfR1)^[53], the molecule responsible for the uptake of transferrin-bound iron into cells. Unlike the ubiquitous expression of TfR1, TfR2 expression and activity is restricted almost exclusively to the liver^[54]. Rather than being involved in the uptake of transferrin bound iron, it appears that the main function of TfR2 is as a sensor of iron levels and regulator of hepcidin. In both patients and animal models with TfR2-HH hepcidin levels are low in relation to iron stores^[55-57].

The clinical features of TfR2-HH resemble that found in HFE-HH. Onset is usually in adulthood and is associated with increased serum iron indices and iron accumulation in parenchymal cells. Clinical features reported in patients with TfR2-HH include abnormal liver function, liver fibrosis, cirrhosis, arthritis, diabetes, hypogonadism, cardiomyopathy and skin pigmentation^[41,44]. All of these features are typical of HFE-HH. A direct comparison of phenotype between HFE-HH and TfR2-HH is difficult, due to the low prevalence of *TfR2* mutations and small number of reported cases. It appears, however, that TfR2-HH may have a more severe phenotype. Early onset of disease has been reported in a number of cases. Two adolescent siblings homozygous for the R105X mutation were reported with elevated transferrin saturation^[51]. However, the serum ferritin in both cases was normal and liver biopsy was not performed. Two unrelated cases, presenting at ages 3 and 16 years were reported, homozygous for the Y250X mutation^[40]. Both had raised serum iron, transferrin saturation and hepatic iron. The 16-year-old, who presented with fatigue, also had raised serum ferritin. Other cases suggestive of an earlier onset and more severe phenotype than HFE-HH have also been reported^[42, 44].

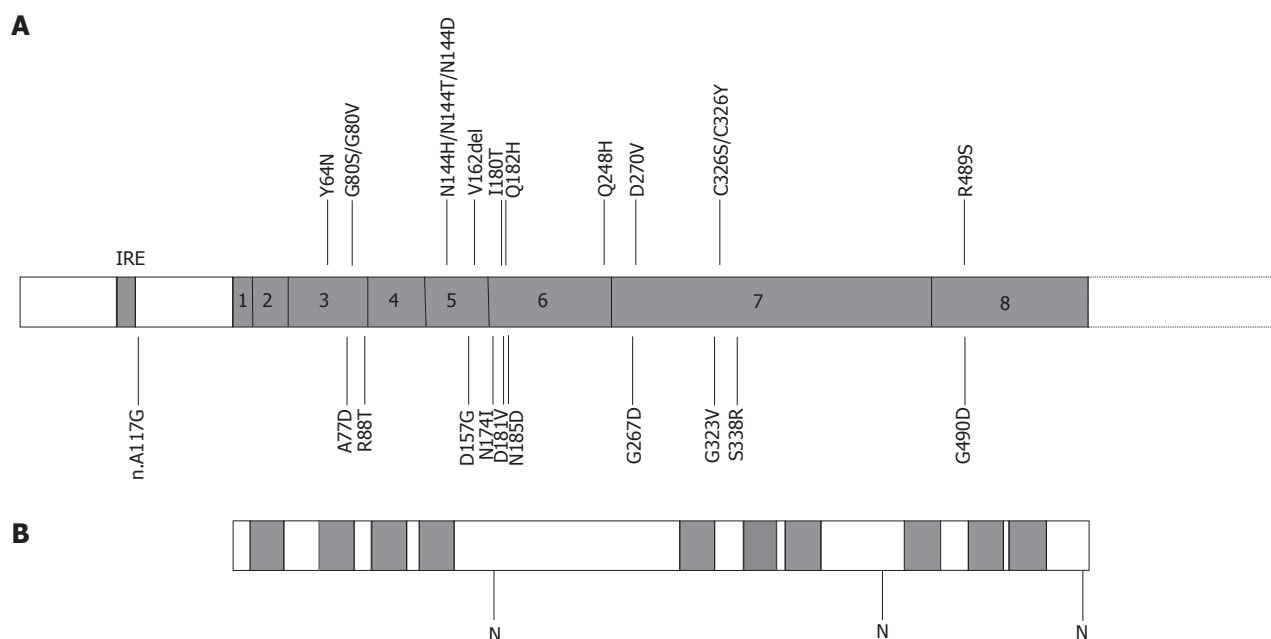


Figure 4 Structure of human *ferroportin* and positions of mutations. **A:** The exon structure of human *ferroportin* is shown with positions of known mutations marked^[8,9,60-85]. IRE, iron response element; **B:** Predicted structure of *ferroportin* protein. Predicted transmembrane domains are shaded; N, potential N-linked glycosylation sites.

Pietrangelo *et al*^[52] recently reported an unusual family with both adult and juvenile onset haemochromatosis. Two siblings presented in their early twenties with features typical of JH. These included hypogonadotropic hypogonadism, cardiomyopathy and cirrhosis. Both were found to be homozygous for a mutation Q317X in *TfR2*. In addition compound heterozygosity for *HFE*-C282Y/H63D was also present. A brother who had a less severe adult onset phenotype was homozygous for the Q317X *TfR2* mutation, but had wild type *HFE* sequence. This is the first and only report of juvenile haemochromatosis due to mutations in two genes normally associated with adult-onset haemochromatosis^[52]. This observation suggests that defects in either *HFE* or *TfR2* can be compensated to some extent by the other. Homozygosity for mutations in either one can lead to appreciable iron overload with onset normally in adulthood. The combination of mutations in both genes has an additive effect on iron loading, leading to an earlier onset and JH-like phenotype. Both *HFE* and *TfR2* are thought to regulate hepcidin expression in the liver through as yet unidentified signalling pathways. It is possible that *HFE* and *TfR2* work through either parallel or converging signalling pathways resulting in the induction of hepcidin. This would explain why the loss of one can be compensated to some extent by the other. The loss of both, however, would lead to complete loss of regulation of hepcidin by iron, as occurs in JH. Further studies will be needed to clarify the relationship between *HFE* and *TfR2*, and the signalling pathways they are involved in.

FERROPORTIN DISEASE

Ferroportin disease differs from other genetic iron overload disorders in that it is inherited in an autosomal dominant pattern. An autosomal dominant form of

haemochromatosis was first reported in 1990, in a Melanesian pedigree from the Solomon Islands^[58]. In a large 96 member pedigree, 31 of 81 members tested were affected in at least three generations. All affected individuals had raised transferrin saturation and serum ferritin levels. Liver biopsies in 19 individuals showed a pattern of iron staining consistent with *HFE*-HH, with iron present in hepatocytes and Kupffer cells. Some degree of fibrosis or cirrhosis was present in the majority of cases. This study was performed before the identification of the *HFE* gene; hence, analysis of *HFE* mutations could not be performed. However, linkage to the *HFE* locus on chromosome 6 was excluded, by HLA typing of affected and non-affected family members^[58]. Another large pedigree with multiple affected individuals, but without pathogenic mutations in the *HFE* gene was described in 1999^[59]. This Italian family consisted of 53 living members, with 15 affected across three generations. Linkage to the *HFE* region on chromosome 6 was excluded by typing of microsatellite markers.

Mutations in *ferroportin*, associated with autosomal dominant haemochromatosis were first described in 2001. An asparagine to histidine mutation (N144H) was identified in a large multi-generation family from the Netherlands^[9]. At the same time an alanine to aspartate mutation (A77D) was reported in the large Italian pedigree described previously^[8]. Since these first reports, many more *ferroportin* mutations have been described in association with autosomal dominant haemochromatosis^[60-85]. Figure 4 shows the mutations reported in the literature to date. *Ferroportin* mutations have been reported in populations throughout the world. Most of the reported *ferroportin* mutations are private and restricted to single families. Some mutations, however, are more common and have been reported in diverse populations. The most prevalent is the deletion of one of a group of three valine residues

(V162del). This mutation has been reported in families from Australia, the UK, Italy, Greece, Sri Lanka and Austria^[61-66]. It has been proposed that this mutation has occurred independently, several times, due to slippage mispairing in a repeat sequence. Other mutations reported in multiple populations include A77D (Italy and Australia)^[8,60] and Q248H^[75-79]. It is unclear whether Q248H is a mutation or polymorphism. It has been reported at high frequencies in African and African-American populations, in both controls and individuals with iron overload, where it can occur in the heterozygous or homozygous state. There is suggestive evidence that it may contribute to slightly higher serum ferritin levels^[76-79], but this effect is very small compared to other *ferroportin* mutants. There are three mutations affecting asparagine 144, suggesting that this residue is important for the functioning of ferroportin. In the original Dutch family reported by Njajou *et al*^[9], it was mutated to a histidine (N144H)^[9], in an Australian family to an aspartate (N144D)^[71] and in a Solomon Island patient to a threonine (N144T)^[67]. Whether this mutation is responsible for the autosomal dominant Solomon Island iron overload syndrome reported by Eason *et al*^[58], remains to be determined.

The phenotypic features of most cases of ferroportin disease differ significantly from that of HFE-HH. The typical features are an early elevation in serum ferritin, with normal transferrin saturation, and iron accumulation preferentially in the Kupffer cells of the liver. With increasing age, iron stores increase, and iron is seen in hepatocytes as well as Kupffer cells, and the transferrin saturation can be elevated. In these cases liver damage is minimal, with fibrosis occurring in some individuals. Venesection therapy is not always tolerated, with anaemia developing, especially in early cases when the transferrin saturation is low. It is now apparent that some cases of ferroportin disease differ from this typical pattern. A second atypical phenotype has been proposed, with features that more closely resemble HFE-HH. Atypical features include an early rise in transferrin saturation, and iron accumulation preferentially in hepatocytes, with some Kupffer cell iron apparent in some cases. Venesection therapy in these cases is usually tolerated well, but liver damage would appear to be more prevalent, with two reports of cirrhosis^[71,80].

The heterogeneity of ferroportin disease has led to the suggestion that mutational differences account for the phenotypic variation observed in patients. In general each mutation can be classified as leading to either the typical or atypical ferroportin disease phenotype. It has been proposed that particular mutations affect the function of ferroportin in different ways. The *ferroportin* gene, also referred to as *IREG1*, *MTP1*, *SLC11A3* and *SLC40A1*, encodes a multiple transmembrane domain iron transporter, highly expressed in duodenum, liver and reticuloendothelial cells. It is responsible for iron transport across the basolateral surface of enterocytes into the blood and recycling of iron in the reticuloendothelial system. Mutations such as A77D and V162del lead to the typical phenotype of reticuloendothelial iron storage, with relatively low transferrin saturation. A non-functional

ferroportin molecule would be predicted to lead to this phenotype. Heterozygosity for a non-functional mutant would be predicted to lead to haploinsufficiency for ferroportin, with only half the amount of functional ferroportin present on the surface of cells at any one time. In the reticuloendothelial system, where the vast majority of daily iron turnover occurs, this would be predicted to cause a blockage in the release of iron back into the circulation. Hence, iron would accumulate in the reticuloendothelial macrophages, and serum iron concentrations would be relatively low. These low levels of circulating iron would in turn lead to an increase in iron absorption in the duodenum, possibly involving sensors in the liver, such as HFE and Tfr2 and signalling *via* the hepcidin pathway. The turnover of iron in the reticuloendothelial system far outweighs that in the duodenum. Hence, the ferroportin required to transport iron across the basolateral surface of enterocytes would probably be sufficient to transport more iron into the body, even if a non-functional mutant was present. Over a long period of time body iron stores would increase to a point where the capacity of the reticuloendothelial cells to store iron would be reached, and iron would accumulate in parenchymal cells such as hepatocytes. This is seen in advanced typical ferroportin disease and is usually accompanied by an increase in the transferrin saturation.

It was recently reported that ferroportin expression on the cell surface can be regulated by hepcidin^[86]. It was shown that hepcidin could bind to ferroportin on the cell surface and induce its internalisation and degradation. In this way hepcidin could rapidly reduce iron absorption in the intestine and release of iron from the reticuloendothelial system, resulting in a reduction in serum iron. It has been proposed that the mutations which cause the atypical ferroportin disease phenotype affect the ability of hepcidin to internalise ferroportin. Failure to internalise would lead to a permanently “switched on” ferroportin molecule. This would lead to increased iron absorption in the duodenum and release from reticuloendothelial cells, resulting in high serum iron levels and storage of iron in parenchymal cells. Having a permanently “switched on” ferroportin molecule would effectively be the same as having hepcidin deficiency, as they both result in the same end point. This explains why the atypical form of ferroportin disease phenotypically resembles other forms of haemochromatosis, which all result from hepcidin deficiency.

CONCLUSION

There are four main genes implicated in non-HFE haemochromatosis. Mutations in these genes occur in populations world wide and account for the majority of HH cases not linked to HFE. The study of these disorders has led to a greater understanding of how the body regulates iron homeostasis. All the genes implicated in the different forms of haemochromatosis are involved in the regulation and maintenance of iron homeostasis. Hepcidin is at the centre of the iron regulatory pathway. Its expression in the liver can be regulated by the activities of HFE, Tfr2 and HJV. Hepcidin itself can regulate the activity of the iron

exporter ferroportin. Mutations in any one of these genes can disrupt the regulation of iron homeostasis and lead to iron overload. Further study of these molecules, their relationships to each other, and signalling pathways they are involved in will further illuminate our understanding of iron metabolism and its regulation.

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Iron overload and cofactors with special reference to alcohol, hepatitis C virus infection and steatosis/insulin resistance

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Abstract

There are several cofactors which affect body iron metabolism and accelerate iron overload. Alcohol and hepatic viral infections are the most typical examples for clarifying the role of cofactors in iron overload. In these conditions, iron is deposited in hepatocytes and Kupffer cells and reactive oxygen species (ROS) produced through Fenton reaction have key role to facilitate cellular uptake of transferrin-bound iron. Furthermore, hepcidin, antimicrobial peptide produced mainly in the liver is also responsible for intestinal iron absorption and reticuloendothelial iron release. In patients with ceruloplasmin deficiency, anemia and secondary iron overload in liver and neurodegeneration are reported. Furthermore, there is accumulating evidence that fatty acid accumulation without alcohol and obesity itself modifies iron overload states. Ineffective erythropoiesis is also an important factor to accelerate iron overload, which is associated with diseases such as thalassemia and myelodysplastic syndrome. When this condition persists, the dietary iron absorption is increased due to the increment of bone marrow erythropoiesis and tissue iron overload will thereafter occurs. In porphyria cutanea tarda, iron is secondarily accumulated in the liver.

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Key words: Iron overload; Cofactors; Alcohol; Chronic hepatic C; Non-alcoholic steatohepatitis; Insulin resistance; Hepatocellular carcinoma

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INTRODUCTION

In hereditary hemochromatosis, patients having HFE trait are more susceptible to iron overload when cofactors such as alcohol, hepatitis viruses, and abnormal porphyrin metabolism are present. Even in the absence of hereditary hemochromatosis, there are several conditions associated with secondary iron overload in which iron deposition is rather mild^[1]. For example, in alcoholics and patients with chronic hepatitis C, intrahepatic iron is increased and liver injury is accelerated, followed by development of fibrosis, cirrhosis and hepatocellular carcinoma (HCC). In addition, abnormal copper metabolism and several causes for iron-loaded anemia are also important cofactors which influence the background iron overload. Furthermore, there is accumulating evidences that fatty acid accumulation without alcohol and obesity itself modifies insulin resistance through iron^[2] and fibrogenesis of the liver^[3]. In this review, the role of cofactors on iron overload will be discussed in three categories such as alcohol, hepatitis C virus infection and steatosis with obesity, the most common cofactors in liver iron overload.

COFACTORS AFFECTING BODY IRON METABOLISM AND IRON OVERLOAD

There are several factors which affect body iron metabolism and accelerates iron overload. Table 1 lists cofactors and disease conditions which are known to accelerate hepatic iron accumulation independent from responsible genes for hereditary hemochromatosis. Alcoholic and hepatic viral infections are the most typical examples for clarifying the role of cofactors in iron overload. In addition, abnormal copper metabolism and several causes for iron-loaded anemia such as thalassemia and myelodysplastic syndrome are also important factors which influence the background iron overload. When this condition persists, the dietary iron absorption is increased due to the increment of bone marrow erythropoiesis^[4] and tissue iron overload will occur thereafter. These patients are usually anemic in spite of increased body iron stores (iron-

Table 1 Cofactors of iron overload

1	Alcohol (Alcoholic liver disease)
2	Infection (Hepatitis C virus infection, <i>etc</i>)
3	Obesity and insulin resistance (Nonalcoholic steatohepatitis)
4	Copper (Ceruloplasmin deficiency)
5	Porphyria (Porphyria)
6	Ineffective erythropoiesis (Thalassemia, myelodysplastic syndrome)
7	Others

loaded anemia), and require frequent blood transfusions, which further exaggerate secondary iron overload, in which conditions of **new oral iron chelators are effective**^[5]. In patients with ceruloplasmin deficiency, anemia and secondary iron overload in liver and neurodegeneration are reported^[6]. Furthermore, there are accumulating evidences that fatty acid accumulation without alcohol and obesity itself modifies iron overload states. Ineffective erythropoiesis is also an important factor to accelerate iron overload. This condition is associated with diseases such as thalassemia, aplastic anemia, and myelodysplastic syndrome. In porphyria cutanea tarda, iron is secondarily accumulated in the liver and phlebotomy and oral iron chelators are effective as well as in hemochromatosis.

ALCOHOL

Alcohol is one of the most important cofactors to modify or enhance iron accumulation in the liver. Excess intake of alcohol induces alcoholic liver diseases (ALD) such as fatty liver, fibrosis, hepatitis, and cirrhosis, in which iron overload is frequently associated^[7]. By **Perls' iron stain**, excess iron accumulation was found in hepatic tissues with ALD, but not in any normal hepatic tissues^[8]. In ALD, iron is deposited in both hepatocytes and reticuloendothelial (Kupffer) cells. In advanced cases of ALD, which is also called as "alcoholic siderosis", the reticuloendothelial iron deposition is dominant. In earlier stages of ALD such as fatty liver and fibrosis, iron deposition is mild and is preferentially present in hepatocytes **rather than in** Kupffer cells, which finding is more frequently observed in Japanese patients who have mild clinical phenotype comparing with those in US^[9].

The **reactive oxygen species (ROS) produced play** an important role in the development of ALD^[10]. The expression of 4-hydroxy-2-nonenal (HNE)-protein adducts, which is a lipid peroxidative product is **increased** in oxidized hepatocytes^[11]. Chronic alcohol ingestion in experimental animals is associated with oxidative stress as reflected by increased hepatic levels of lipid peroxidation products such as malondialdehyde and HNE, both of which have been implicated in hepatic fibrogenesis in the intragastric ethanol infusion model^[12]. Furthermore, lipid peroxidation products induce gene expression of procollagen α -1 (I) and increase collagen production by several folds in cultured hepatic stellate cell^[13]. In human ALD, there is a positive correlation between iron deposition and histological intensity of HNE-protein adduct^[14]. As shown in Figure 1, the **distribution of** HNE-protein adducts and iron granules appeared to be

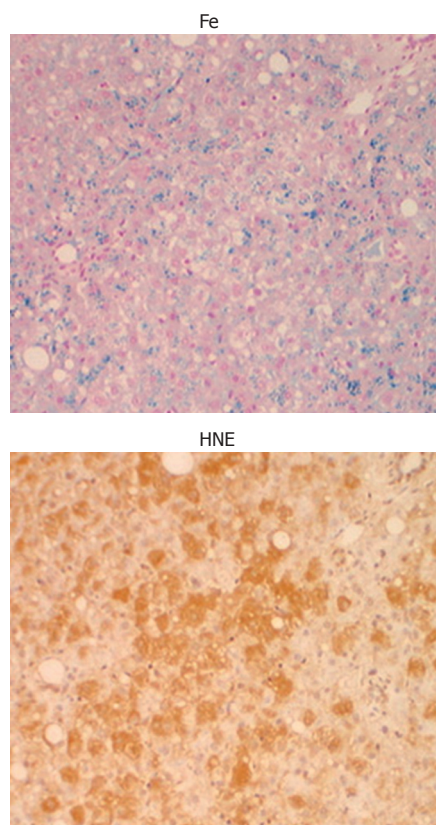


Figure 1 Iron staining and immunohistochemical staining of 4-hydroxy-2-nonenal-modified protein (HNE-protein) adducts in human alcoholic liver disease. The localization of HNE-protein adducts and iron in hepatocytes appeared to be identical (from ref. 14 with some modifications).

identical, suggesting that iron may be associated with the production of HNE-protein adduct. As hepatic iron is **visualized by Perls' reaction as an insoluble protein-bound iron** such as hemosiderin, this form of iron may be inactive for the production of ROS. But, the free iron responsible for Fenton reaction should be present close to the protein-bound iron, and may be involved in the production of HNE-protein adducts. There are two pathways to generate ROS through ethanol metabolism. Oxidation of ethanol by alcohol dehydrogenase to form acetaldehyde, which is subsequently oxidized to acetate and ultimately carbon dioxide and water. During the oxidation process of acetaldehyde involving aldehyde oxidase and xanthine oxidase, superoxide (O_2^-) is produced^[15]. In addition, cytochrome P450 is involved in the metabolism of ethanol, in which ROS are also generated in microsomes^[16]. Among ROS, hydroxy radical (OH) is most potent, which is produced via Fenton reaction in the presence of free iron and the resulted OH can easily cause **cell damage by oxidizing lipid, proteins, and nucleic acids**. In an intragastric infusion mouse model of ALD, supplementation of carbonyl iron advanced peri-venular fibrosis to bridging fibrosis and cirrhosis^[17]. **Oxidative stress arising from hepatocytes and macrophage activates** hepatic stellate cells by increasing the production of cytokines such as transforming growth factor- β (TGF β), directly or indirectly. The **dietary iron supplementation was** associated with increased NF- κ B activation^[18], and the up

regulation of NF- κ B responsive proinflammatory genes such as IL-1 β , TNF α , and MIP-1^[19].

In advanced cases of ALD, iron is accumulated more prominently in Kupffer cells than in hepatocytes, mainly due to repeated endotoxemia and hyper-cytokinemias of TNF α and IL-1 β ^[20]. These cytokines induced hepatic uptake of transferrin iron *in vitro*^[21] and *in vivo*^[22]. In mild cases of ALD, iron is preferentially stained in hepatocytes, rather than in Kupffer cells, suggesting that hepatocyte is the main site of early iron storage in the liver. However, it is not clear why iron is accumulated in liver parenchymal cells of alcoholics in such conditions. Two possibilities can be drawn: one is the increased uptake of iron in hepatocytes, and another is the increased iron absorption through hepcidin, which is a newly found antimicrobial peptide, and is a negative regulator of iron absorption and reticuloendothelial iron releases^[23]. Hepatocytes have several pathways for iron uptake. Iron in serum is usually bound to transferrin and iron-bound transferrin is taken up via transferrin receptor (TfR) with high affinity or via other unknown mechanism with greater capacity, but low affinity independent of high affinity receptor^[24]. There are two molecules of transferrin receptor: transferrin receptor 1 (TfR1) and transferrin receptor 2 (TfR2). TfR1 has a high affinity to serum transferrin and considered to be functional, while the function of TfR2 is not clear yet, even though the TfR2 gene is responsible for genetic hemochromatosis^[25]. In normal hepatocytes, TfR2 is constitutively expressed. But, TfR1 is down-regulated, suggesting that TfR1 does not contribute to the steady state hepatic iron uptake. Recently, Wallace *et al*^[26] reported that homozygous TfR2 knockout mice had no TfR2 associated with typical iron overload, and there was no upregulation of hepcidin mRNA, suggesting that TfR2 is required to iron regulated expression and is involved in a pathway to HFE and hemojuvelin. In addition, DMT1 may be involved when serum iron concentration exceeds transferrin iron binding capacity^[27]. It is noteworthy that TfR1 is regulated by cellular iron levels or oxidative stresses post-transcriptionally and it is possible that ethanol may augment TfR1 expression by producing oxidative stresses. According to immunohistochemical investigation, TfR1 expression was increased in hepatocytes in 80% of hepatic tissues with ALD, but was not detected in any normal hepatic tissues^[28]. It is noteworthy that the mean duration of abstinence of patients who demonstrated positive TfR1 expression in hepatocytes was significantly shorter than that of patients who demonstrated negative TfR1 expression.

Ethanol exposure in the presence of iron to the primary cultured-hepatocytes demonstrated an increase of TfR expression, and this augmentation was suppressed by the inhibitor of alcohol dehydrogenase, 4-methoxy pyrazole, but enhanced by a inhibitor of acetaldehyde dehydrogenase, cyanamide, suggesting that ethanol metabolite acetaldehyde itself is involved for the induction of TfR1 by ethanol^[29]. By functional uptake assay using ⁵⁹Fe-transferrin, the additional ethanol exposure increased transferrin-iron uptake into hepatocytes, while non-transferrin-bound iron (NTBI) uptake^[30] was not increased. It has been reported that TfR1 expression was

Table 2 Speculated effects of iron on HCV

1	Immunological modification (Immunological escape of HCV)
	Decrease of Th1 activity
	Impaired function of macrophage and Kupffer cells
	Decrease of innate immunity (Natural resistance macrophage protein 2)
2	Increase of liver toxicity by iron-mediated radical formation
	Reactive oxygen production through fenton reaction
	Induction of apoptosis
	Acceleration of fibrinogenesis
	DNA damage and carcinogenesis
3	Effect on cell signalling
	Decrease of interferon responsiveness by NF κ B activation
4	HCV proliferation
	Activation of translation initiation factor 3 (eIF3)
	Suppression of HCV RNA polymerase (NS5B) activity

up-regulated both transcriptionally^[31] and posttranscriptionally^[32]. This regulation is induced either by iron deficiency state or oxidative stress such as H₂O₂ and nitric oxide via iron regulatory protein, IRP^[33]. In addition to the direct cell toxicity, acetaldehyde produces free radicals^[34] and free radicals modify IRP activity^[35,36].

Body iron homeostasis is strictly regulated by a balance between the processes such as dietary iron absorption in enterocytes, iron transport by transferrin in circulation, iron utilization and storage in bone marrow and liver. The increase of intestinal iron absorption was one of the mechanisms of the hepatic iron deposition in alcoholics^[37]. In patients with hereditary hemochromatosis, serum pro-hepcidin is lower than that of normal controls, suggesting that iron absorption is increased in spite of high iron storage^[38]. It is speculated that down-regulation of hepcidin might be one of important factors for pathogenesis of iron overload in ALD^[39]. Serum pro-hepcidin concentration in ALD was significantly lower than that in healthy subjects, and pro-hepcidin/ferritin ratios in ALD were lower than healthy subjects^[40]. In the ethanol-loaded mouse model which has a mild steatotic change, the hepcidin mRNA and protein expression were significantly lower than that of control. In addition, alcohol-loading might disrupt the sensing signal of inflammatory cytokines, and then down-regulate hepcidin expression, following the increased iron absorption from small intestine. Recently, the mechanism of hepcidin downregulation by alcohol has been elucidated: a decreased hepcidin expression in mouse liver is accompanied with an increase of DMT1 and ferroportin1, and a decrease of hepcidin promoter activity and DNA-binding activity of CCAAT/enhancer-binding, protein α (C/EBP α)^[41].

HEPATITIS C VIRUS INFECTION

Hepatitis C virus infection is one of the most common disorders in liver diseases involving chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). Table 2 summarizes the effect of iron on hepatitis C virus infection. In the Third National Health and Nutrition Examination Survey, HCV infection is significantly associated with higher serum levels of ferritin and iron in the US population^[42]. The mean serum levels of

ferritin and iron were significantly higher among subjects with HCV infection than among subjects without liver disease^[43]. In addition, serum ferritin levels were directly and significantly correlated with serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ -glutamyl transpeptidase, whereas platelet counts were inversely correlated with serum ferritin. It is also found that lipid peroxidative products such as malondialdehyde are increased in hepatic tissues with CH-C^[44]. In 1994, an initial report was published that phlebotomy was effective in improving the serum ALT level in patients with CH-C^[45] and a national prospective study confirmed the results^[46]. Since then, it was reported that hepatic iron accumulation in CH-C predict a response to interferon (IFN) therapy^[47], and phlebotomy before and during IFN therapy improved virological and histological response to short-term IFN therapy evaluated at the end-of-treatment^[48]. This observation is reasonable considering the finding that oxidative stress impairs interferon alpha signal by blocking JAK-STAT pathway^[49]. The standard therapy for hepatitis C is now a combined therapy of interferon- α and ribavirin, in which patients with viral response to treatment seemed to develop higher soluble transferrin receptor levels^[50] with decline in serum iron and ferritin than non-responders, revealing intracellular reduction of iron store depending on the result of treatment including hemolytic reaction by ribavirin^[51]. This is an interesting observation that decrease of iron status may be an additional effect of the combination therapy with interferon and ribavirin. Moreover, HFE mutations are also associated with increased sustained virologic responses by antiviral long-term treatment, while it is well known that HFE mutations are associated with increased iron loading^[52]. However, some reports suggest that iron depletion was unable to trigger interferon response, so that there are conflicting data. It should be further investigated whether hepatic iron content modify the response to interferon^[53,54].

From these observations, iron and related molecules seem to be key factors in the hepatocytes to influence the disease condition of CH-C, and also development of cirrhosis and maybe hepatocellular carcinoma. Clinical data on phlebotomy on CH-C generally indicates that phlebotomy does not influence the viral load in vivo. On the other hand, in vitro study on HCV replication is controversial: iron promotes HCV translation by up-regulating expression of the translation initiation factor eIF3 by reporter assay^[55], whereas iron suppresses HCV replication by inactivating the RNA polymerase NS5B^[56].

As previously described, hepatocytes have two iron uptake systems, transferrin-mediated and nontransferrin-bound iron-mediated pathway. Transferrin and TfR1 are molecules involved in the classical pathway of cellular iron uptake, but are faintly expressed in normal hepatocytes, and is down-regulated in iron-loaded hepatic tissues with hemochromatosis. Concerning the post-transcriptional regulation of TfR1, two mechanisms are postulated through the activity change of IRP which is already mentioned. In CH-C, TfR1 expression was up-regulated and DMT1 expression was down-regulated in the condition of hepatic excess iron accumulation, suggesting that regulation of DMT1 expression is iron-

dependent, but that of TfR1 expression is iron-independent in CH-C^[57]. In patients with CH-C, serum values of inflammatory cytokines such as IL-1 β , IL-6, and TNF α have been reported to be high in comparison with those in normal controls. In addition, TfR1 was up-regulated by IL-1 β , IL-6, and TNF α in HepG2. Administration of IL-6 augments hepatic uptake of transferrin-bound iron (⁵⁹Fe), and this is mainly mediated through hepatocytes, but not through Kupffer cells. These results suggest that the up-regulation of TfR1 expression in CH-C might be caused by increase of inflammatory cytokines that proceeded from HCV infection, although there is a possibility that the components of HCV themselves may induce TfR1 expression directly or indirectly.

Like wise, the up-regulated TfR1 might act as a key molecule for hepatic excess iron accumulation in CH-C; however, there are several candidate molecules which cause this condition. For instance, each mutant of HFE, TfR2, hepcidin, hemojuvelin and ferroportin1 (also known as Ireg1 or MTP1) with substitution of amino acid causes the similar phenotype of hemochromatosis. That is, these facts indicate that at least 5 molecules are involved in the familiar hemochromatosis^[58]. In hepatocytes, TfR2 predominantly expresses in the normal condition^[59] and the disruption of TfR2 gene caused the hepatic iron overload, a phenotype of hemochromatosis, suggesting that TfR2 should also have important role in hepatic iron metabolism^[60]. This receptor might act as a sensor of iron status because hepatic TfR2 protein level was increased in iron loaded rats and was decreased in iron deficient rats. Recently, Takeo *et al*^[61] reported that in CH-C TfR2 protein expression is increased parallel with ferroportin1, although the meaning of this TfR2 elevation is still to be elucidated^[62].

In addition, there was a significant correlation of hepcidin mRNA expression in the liver with hepatic iron concentration and serum ferritin, but did not correlate with ALT, AST, HAI, or viral load. In inflammatory conditions, hepcidin is regulated transcriptionally by IL-6^[63] and IL-1 β ^[64] independent of liver iron content. It is noteworthy that, in contrast to other inflammatory states, hepcidin mRNA expression in the liver was independent of markers of inflammation in hepatitis C, suggesting that iron stores in patients with hepatitis C regulate hepcidin expression, and that iron loading in chronic hepatitis C is not due to inappropriate hepcidin expression^[65]. However, there is still a controversial result concerning the hepcidin metabolism in chronic hepatitis C that serum pro-hepcidin is down-regulated^[66]. The role of hepcidin in chronic hepatitis C seems to need further consideration.

The role of iron on the hepatocellular carcinoma (HCC) development in patients with chronic hepatitis C is another major concern. In primary hemochromatosis, iron could be involved in the development of HCC in associated with cirrhosis, suggesting a strong link between heavy iron overload and HCC development. In cases of chronic hepatitis C, it is also known that HCC are developed 20 to 30 years after the infection of hepatitis C virus through the progression of the disease from chronic hepatitis and cirrhosis. In Long-Evans Cinnamon (LEC) rat, an animal model of human Wilson

disease which spontaneously developed hepatitis and liver fibrosis, HCC is frequently developed after the rats have recovered from initial fulminant hepatitis and subsequent liver fibrosis. This is considered to relate to progressive iron accumulation in the animal^[67], and iron depletion prevents their development of hepatic cancer^[68]. Even though the iron deposition in chronic hepatitis C is mild compared with that in hemochromatosis, iron may be an independent factor on the risk of HCC. It is reported that liver fibrosis is a favorable environment of proliferation of cancer cells by releasing transforming growth factor β , and there is a strong link between liver fibrosis and liver iron deposition. In clinical trials of phlebotomy, the hepatic content of 8-OH deoxyguanosine is decreased and fibrotic score is improved. An important issue in hepatocarcinogenesis in chronic hepatitis C is the closely related sustained production of ROS during inflammation and fibrosis. Moriya *et al*^[69] reported that HCC developed in HCV core transgenic mice after the age of 16 mo, and showed high hepatic lipid peroxidation levels in old (more than 16 mo) core transgenic mice, than in control. However, the association of HCV transgenic mice, and HCC development disappeared with advanced passaging of animals, suggesting that HCC development in HCV transgenic mice cannot be simply explained by HCV infection, but requires additional cofactors. A recent study by Furutani *et al*^[70] clearly showed that hepatic iron overload induces HCC in transgenic mice expressing HCV polyprotein. Transgenic animal carrying full length polyprotein-coding region (core to NS5B, nts 342-9378) by using pAlb promoter/enhancer was fed with excess iron diet. After 6 mo feeding, the transgenic mice showed marked steatosis and increased 8 hydroxy-2'deoxyguanosine content in association with the hepatic iron accumulation. Twelve months after feeding, 45% of transgenic mice developed hepatic tumors including HCC. It is noteworthy that the steatosis does not accompany with inflammation but a remarkable ultrastructural alteration of mitochondria associated with decreased degradation activity of fatty acids.

STEATOSIS AND INSULIN RESISTANCE

Nonalcoholic steatohepatitis (NASH) is a clinical entity characterized by the development of histopathological changes in the liver that are nearly identical to those induced by excessive alcohol intake, but in the absence of alcohol abuse; the presence of macrovesicular steatosis and mixes inflammatory infiltrate associate with varying amounts of Mallory's hyaline, glycogenated nuclei, and focal hepatocyte ballooning degeneration. Clinical features of NASH include obesity, hyperlipidemia, diabetes mellitus, and hypertension. In US population, approximately 25% is obese, and at least 20% of the obese individuals have hepatic steatosis. Thus, non-alcoholic liver disease (NAFLD) is the most common cause of liver dysfunction, and it is believed that NASH becomes a cause of cryptogenic cirrhosis and hepatocellular carcinoma (HCC). In patients with homozygote of HFE-related hemochromatosis, obesity and steatosis affect liver disease progression, and will be cofactors for iron overload. There

is one study of Australia that showed that the prevalence of abnormal genotype of HFE in NASH is 31% compared to a normal prevalence of 13% in the general population, suggest that excess iron might be important. A study on North American subjects showed similar results that the prevalence of the HFE gene mutation associated with hereditary hemochromatosis are increasing in patients with NASH^[71]. In the study dealing Japanese NASH patients, who had no HFE gene mutations, a significant staining of liver iron and increased level of thioredoxin, a marker of oxidative stress in addition to the increase of serum ferritin, was observed.

As diabetes and obesity were background conditions of NAFLD, and is thought to be a initial triggering factor, insulin resistance is now considered the fundamental operative mechanism. Insulin resistance is probably the "first step" in NASH, and a close correlation between insulin resistance and iron is speculated. Even though it is not still clear whether secondary iron accumulation increases insulin resistance, or vice versa, oxidative stress may be the elusive "second" hit of possibly multiple steps in the progression of steatosis to fibrosing steatohepatitis^[72]. This may be due to the activation of stellate cells^[73].

Because hepatic iron promotes oxidative stress, it seems that iron is a contributory cofactor in NASH. This proposal is strengthened by an association with hepatic fibrosis with NASH^[74] and was confirmed by measuring serum markers of oxidative stress^[75-77]. Excess hepatic iron also occur in insulin resistance-associated iron overload (IRHIO), characterized by hyperferritinemia with normal to mild increases in transferrin saturation. There is an interesting clinical study that venesections and restricted diet are effective in patients with IRHIO^[78]. As in IRHIO, restriction of dietary calories, fat and iron improved NAFLD in addition the decrease of levels of serum aminotransferases and ferritin^[79]. It seems that the simultaneous disorder of iron and glucose and/or lipid metabolism, in most cases associated with insulin resistance, is responsible for persistent hyperferritinemia and identifies patients at risk for NASH^[80]. However, it is still unclear why iron is deposited in IRHIO and NAFLD. There is an interesting report by Bekri *et al*^[81] that there is an increase of hepcidin in adipose tissue of the severely obese but of liver, suggesting that severe obesity itself cause hypoferrremia due to the overproduction of hepcidin in the adipocytes. This finding may explain the hypoferrremia in severe obese patients, but does not show the mechanism of hepatic iron deposition in IRHIO and NASH. Further studies are needed to clarify this issue, including an increase of transferrin iron influx into hepatocytes in NAFLD.

In patients with NASH, increased transferrin saturation correlated positively with the severity of fibrosis in univariate analysis, although it became insignificant when age, obesity, diabetes, and AST/ALT ratio were controlled. A recent study showed improvement in insulin sensitivity with the use of venesection in 11 patients with NASH. Biweekly phlebotomy until serum ferritin concentration became lower than or equal to 30 ng/mL reduced mean serum ALT activity without a significant change of

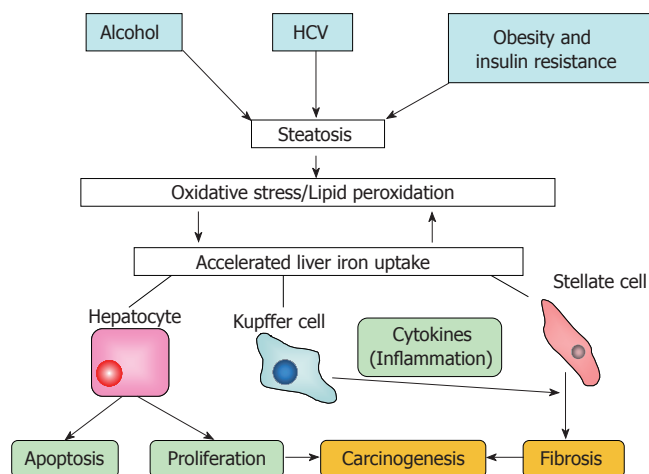


Figure 2 Postulated schema of liver damage occurred by alcohol, HCV infection, obesity and insulin resistant. A common pathway through steatosis/oxidative stress may be responsible for the development of liver fibrosis and carcinogenesis by iron.

body weight, suggesting that iron reduction therapy by phlebotomy will be one of the promising therapies for NASH^[82], although this approach cannot be implemented without extensive review.

The natural history of NASH is still unclear, but some patients follow advanced liver fibrosis progressing to cirrhosis and sometimes HCC^[83]. It is also known that diabetes increases the risk of hepatocellular carcinoma in US^[84]. Further studies are needed to clarify this issue, especially the relation between hepatocarcinogenesis from mild iron accumulation in NASH.

As shown in Figure 2, a common pathway through steatosis/oxidative stress may be present for the development of liver fibrosis and carcinogenesis by iron.

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Iron overload and immunity

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Abstract

Progress in the characterization of genes involved in the control of iron homeostasis in humans and in mice has improved the definition of iron overload and of the cells affected by it. The cell involved in iron overload with the greatest effect on immunity is the macrophage. Intriguing evidence has emerged, however, in the last 12 years indicating that parenchymal iron overload is linked to genes classically associated with the immune system. This review offers an update of the genes and proteins relevant to iron metabolism expressed in cells of the innate immune system, and addresses the question of how this system is affected in clinical situations of iron overload. The relationship between iron and the major cells of adaptive immunity, the T lymphocytes, will also be reviewed. Most studies addressing this last question in humans were performed in the clinical model of Hereditary Hemochromatosis. Data will also be reviewed demonstrating how the disruption of molecules essentially involved in adaptive immune responses result in the spontaneous development of iron overload and how they act as modifiers of iron overload.

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Key words: Iron; Iron overload; Innate immunity; Adaptive immunity

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THE TWO IMMUNITIES

The last decade has seen a growing understanding of the

numerous functions of the immune system beyond the two classical attributes of adaptive immunity: antigen specificity and memory. The key effector cell of adaptive immunity is the lymphocyte, a cell no longer known from its two principal origins in mammals, the thymus (T) and bone marrow (B), but with several subtypes characterized by different function and cytokine production profiles^[1]. “Behind” adaptive immunity lays a complex world of cells and molecules involved in less antigen-specific tasks collectively dedicated to the function of innate immunity. Two competing models have sought to explain innate immunity. One where evolutionary conserved features of pathogens are recognized by pattern recognizing receptors^[2] known generally as toll-like-receptors (TLRs). A second model, known as the danger model, is based on the assumption that products from damaged or stressed cells provide a danger signal to the host, thus evoking an innate immunity response^[3]. The effector cells of innate immunity are mostly myeloid cells and lymphocytes whose interaction with target cells does not depend on the recognition of the Major Histocompatibility Complex (MHC). A third cell, the dendritic cell (DC), was first described by Steinman and Cohn in 1973^[4]. DCs have been described most elegantly as “the nexus for translating signals from innate recognition into cells guiding adaptive immune function”^[5].

One hallmark of immune system cells, whether involved in innate or adaptive immunity, is their capacity to circulate and migrate from the blood compartment into lymphoid and non-lymphoid tissues (innate immunity cells) from non-lymphoid into lymphoid tissues (DCs) and from the blood into the lymph through the peripheral lymphoid organs (lymphocytes). The other is their role in protection from pathogen infections. Since early descriptions of immune cell functions in iron overload^[6] knowledge of the reciprocal interactions between immune cell responses, intracellular iron load and response to micro-environmental changes in iron levels has increased considerably. A decisive contribution to this understanding was the clarification of the genes involved in iron homeostasis. Reviews of the functions of such genes and their link to the reticuloendothelial system and immunity have been published recently^[7,8]. The present review will focus on the evidence that the cells of the immune system are equipped to modulate iron homeostasis through the expression of several iron related genes and proteins, and how do innate and adaptive immune cells respond in conditions of iron overload. In addition, the reader will be reminded of the evidence indicating that defective immune system models, particularly experimental models,

Table 1 Relevant genes/proteins involved in iron homeostasis and iron overload, and their related immunological functions

Genes/Proteins	Main role in iron homeostasis	Related immunological functions
Lactoferrin	Iron binding, transport and storage Iron chelator	Bactericidal and antiviral ^[13,14] . Immunoregulatory effects on Th1/Th2 cell activities ^[15]
Transferrin	Iron transporter	Present in monocyte/macrophages and T lymphocytes ^[16] Required for early T-cell differentiation ^[17]
Transferrin receptor 1	Cellular iron uptake	Iron uptake by activated lymphocytes and required for DNA synthesis and cell division of T lymphocytes ^[18]
Ferritin	Iron storage	Synthesised by macrophages and T lymphocytes ^[19,20]
Nramp1 (<i>SCLA11</i>)	Iron transfer in late phagolysosome	Resistance to infection with intracellular pathogens ^[21,22]
Nramp2/DMT-1	Iron uptake and transfer in late phagolysosome	Resistance to infection with intracellular pathogens ^[21,22]
Ferroportin 1 (IREG1)	Cellular iron exporter	TLR4 mediated downmodulation in infection ^[23]
Lipocalin	Unknown	Limits bacterial growth by sequestering the siderophore ^[24]
Regulators and modifiers of iron overload		
Hepcidin	Key regulator of iron homeostasis ^[25] . Gene disruption results in hemochromatosis ^[26] . Mutations associated with severe Juvenile Hemochromatosis (JH) ^[27]	Liver derived antimicrobial peptide ^[28] . TLR4 mediated expression in myeloid cells in response to bacterial pathogens ^[23]
Hemojuvelin	Regulation of hepcidin expression ^[29,30] . JH associated gene ^[31]	Unknown
IL-6 and IL-1	Involved in iron deprivation during infection and inflammation, through hepcidin induction and ferroportin down-modulation ^[32,33]	Derived from macrophages during infection and inflammation ^[32,33]
Heme-oxygenase 1	Required for mammalian iron reutilization. Targeted <i>Hmox1</i> mutations induce spontaneous iron overload ^[34]	High CD4:CD8 ratios with activated CD4+ cells in <i>Hmox1</i> deficient mice ^[34]
HFE	Hereditary Hemochromatosis associated gene ^[35] . HFE gene knockout produces mouse model of hereditary hemochromatosis ^[36]	Non-classical MHC-class I molecule ^[37]
β2-Microglobulin	β2m deficiency induces spontaneous iron overload and modifies the phenotype of HFE deficient mice ^[38,39]	Critical to the folding of MHC-class I molecules and selection of MHC class I molecule-associated peptides ^[40]
MHC-class I	MHC-class I deficient mice develop hepatic iron overload ^[41]	Critical molecule for viral peptide presentation ^[42]

are associated with the spontaneous development of iron overload^[9]. The mechanisms underlying what could be called the “reverse” side of this same coin, i.e. immunity and iron overload are less well understood. However, taking into account that practically all cells of the immune system express iron related genes their contribution to systemic iron homeostasis should no longer be ignored.

IRON AND INNATE IMMUNITY

As a critical element of cellular activity, iron plays a pivotal role in the fight for survival between mammalian hosts and their pathogens, each displaying a wide range of mechanisms for controlling iron acquisition and utilization. Micro-organisms have developed a large number of strategies to acquire iron from the environment and to transport the element to sites of incorporation into biologically important molecules^[10,11]. On the other side, the host has developed the capacity to modulate cellular iron metabolism, not only for its optimal utilization as a catalyst for the generation of reactive oxygen species acting as strong antimicrobial molecules, but also in order to make iron less available for the micro-organisms^[11,12]. A number of genes and proteins primary involved in iron homeostasis, namely in iron binding, transport and storage are now recognised to display related immunological functions. These are summarized in Table 1^[13-24]. In addition, it is becoming clear that the cells of the innate immune system, as part of a non-specific defense against

infection, are equipped to express genes and proteins that can modulate iron homeostasis both at the cellular and the systemic levels (see also Table 1)^[25-42]. One central player in this modulation is hepcidin, first described as a liver derived antimicrobial peptide^[28], and now well recognised as a key regulator of iron homeostasis and the anaemia of inflammation, at the interface of innate immunity and iron metabolism^[43]. Liver-derived hepcidin is strongly induced during infection and inflammation, causing intracellular iron sequestration and decreased plasma iron levels, a process mediated by the inflammatory cytokine cascade, namely macrophage derived IL-6 and IL-1^[32,33]. The mechanism underlying intracellular iron sequestration is mediated by the hepcidin-induced internalization and degradation of ferroportin, the only known iron exporter^[44]. The reduction in extracellular iron concentrations is believed to limit iron availability to invading microorganisms, thus contributing to host defense. Recently, hepcidin has been shown to be endogenously expressed by innate immune cells, i.e. macrophages and neutrophils, capable of migrating from the blood to a site of infection, constituting a newly recognised component of the local innate immune response to bacterial pathogens^[23]. Myeloid endogenous hepcidin mRNA expression in response to bacterial pathogens was shown to be dependent on the specific activation by the toll-like receptor 4 (TLR-4), the key pattern recognition receptor for LPS^[45]. This activation also produces down-regulation of the iron exporter

ferroportin. Endogenous myeloid hepcidin production is not stimulated by iron, pointing to different pathways of hepcidin activation in response to infection or iron overload^[23]. The mechanisms underlying the differential stimulatory effects of infection and iron overload on hepcidin are still not understood. Interestingly, it was recently shown that hepcidin levels in fish also respond both to iron overload and infection, demonstrating the evolutionary conservation of hepcidin's dual function^[46].

In addition to the demonstrated role of neutrophils and macrophages on hepcidin gene activation and ferroportin down-modulation, other iron genes and proteins involved in iron transport from the human phagosome into the cytosol also play critical roles in TLR-4 mediated innate immunity. The natural resistance-associated macrophage protein 1 (Nramp1) is expressed in circulating phagocytes, and is recruited from the lysosomal compartment to the phagosome membrane where it functions as an efficient antimicrobial through a mechanism of iron deprivation^[22]. Nramp1 mutations in mice are shown to cause susceptibility to infection with various intracellular pathogens including *Salmonella*, *Mycobacterium* and *Leishmania*^[21]. Mutations in the human homologue of the gene, NRAMP1, were also shown to strongly affect the susceptibility to tuberculosis^[47]. Nramp2, also known as the divalent metal transporter 1 (DMT1) is another important iron transporter in mammals^[48,49] and, as Nramp1, it is also induced by infection with intracellular pathogens, namely *Mycobacterium*^[50]. In addition, it is suggested to play an important role in recycling iron from RBC-containing phagosomes to the cytoplasm^[51]. The amount of available iron in the phagosome is also decreased by the entry in macrophages of neutrophil derived lactoferrin (Lf), another well known potent iron chelator found recently to have also a bridging role between innate and adaptive immunity. Both *in vitro* and *in vivo* studies showed that Lf is able to stimulate the proliferation and differentiation of T lymphocytes from their immature precursors into the Th1 or the Th2 phenotypes thus having an immunoregulatory effect on Th1/Th2 activities^[15].

Finally, lipocalin 2 has been recently described as a pivotal component of the innate immune system and the acute phase response^[52]. Upon infection the toll-like receptors on immune cells stimulate the transcription, translation and secretion of lipocalin 2 which then limits bacterial growth by sequestering the iron-loaded siderophore^[24].

INNATE IMMUNITY AND IRON OVERLOAD

If one or all of the above described iron related immune response genes either fail or the cells are overwhelmed by continuous iron overloading such as seen in transfusional iron overload, one consequence is the development of infection. Infections including rare microorganisms^[53] are among the major complications in patients with thalassemia, a group of common genetic disorders of hemoglobin synthesis clinically characterized by severe anemia and blood transfusion-dependent iron overload. Besides the well-known risks of blood borne viral infections associated with multiple transfusions, the

increased susceptibility of these patients to infection is known to be associated with a wide spectrum of immune abnormalities which are, at least in part, due to the effect of iron overload, including defective chemotaxis and phagocytosis by neutrophils and macrophages and decreased natural killer cell activity^[54].

In contrast with the findings in transfusional iron loading, patients with severe iron overload due to Hereditary Hemochromatosis (HH) do not show evidence of increased susceptibility to infections or iron loading of macrophages. HH is a common genetic disorder of iron overload, the majority of HH patients being homozygous for the C282Y mutation in *HFE*, a gene encoding a protein of the Major Histocompatibility Complex class I (MHC class I). The C282Y mutation disrupts the correct folding of the $\alpha 3$ domain of the protein, interfering with its interaction with $\beta 2$ -microglobulin ($\beta 2m$) and consequently abolishing the cell surface expression of the molecule^[35]. The surface expression of *HFE* was shown to have a prominent role in the regulation of iron export from macrophages^[55,56].

Monocyte/macrophage abnormalities in HH

Anomalies in monocyte/macrophage cells have been consistently described in HH patients, including low TNF- α production by peripheral blood macrophages upon stimulation with lipopolysaccharide^[57] and a significant increase in iron regulatory protein (IRP) activity in monocytes^[58], an anomaly that was corrected after phlebotomy treatment. Interestingly, subjects with a tissue iron burden similar to HH patients, but due to secondary iron overload have an IRP activity significantly decreased suggesting that the increased IRP activity in iron overload is specific to the *HFE* related hereditary form of hemochromatosis^[58]. In addition, a study that investigated the release of erythrocyte-derived iron from purified human monocytes obtained from controls and HH patients showed that although HH monocytes phagocytosed less than half the number of erythrocytes taken up by control monocytes, they released twice as much iron in the form of LMW-Fe complex than controls^[59]. More recently, increased iron content was described in macrophages from HH patients transfected with wt *HFE* when compared with HH macrophages transfected with an empty vector^[56]. These observations are consistent with previously reported observations in macrophage cell lines derived from C282Y mutated HH patients, where *HFE* was shown to lose its ability to inhibit iron release leading to a relative macrophage iron deficiency^[55]. Interestingly, *Mycobacterium tuberculosis* (*M.tb*) residing within phagosomes of macrophages from HH patients exhibit a profound defect in their ability to acquire iron from exogenous transferrin and lactoferrin relative to *M.tb*-infected macrophages from normal controls^[60]. Moreover, macrophages from HH patients failed to induce Nef-mediated iron and ferritin accumulation upon HIV-1 infection in contrast to macrophages expressing wild-type *HFE*^[61], thus suggesting that *HFE* mutated macrophages may be better equipped to protect from HIV-1 infection, compatible with the description of a long survival in a patient with AIDS

and hereditary hemochromatosis^[62]. One must therefore wonder whether the failure of macrophages from C282Y *HFE* HH patients to hold on to the iron is the expression of a putative selective advantage protecting from infection brought by the appearance and the establishment of such a mutation.

Iron and the dendritic cells

Very little work has addressed the interaction between iron loading and dendritic cell (DC) function. There is, however, recent evidence indicating that, upon endotoxin induced maturation, DCs increase significantly the expression of Tfr1 and down regulate expression of the export molecule ferroportin^[63], an observation compatible with an earlier finding of Kramer *et al*^[64] who reported that DCs generated under iron deprivation conditions were phenotypically undifferentiated and could not stimulate T cells.

IRON OVERLOAD AND ADAPTIVE IMMUNITY

The postulate that the immunological system could have a role in monitoring tissue iron toxicity, as part of its surveillance function, was first advanced in 1978, based on studies on lymphocyte traffic and positioning^[65-67]. It was implicit in that postulate that the lymphomyeloid system, and its circulating components participate in the recognition and binding of metals as a protective device against metal toxicity, and the preferential use of indispensable metals, such as iron, by bacteria or transformed cells. While a vast number of studies have clarified the reciprocal interactions between myeloid innate immunity cells and iron metabolism, fewer studies addressed this question in lymphocyte populations. Lymphocyte activation and expansion depend on the expression of transferrin receptors, required for DNA synthesis and cell division^[18] and both activated and non-activated T lymphocytes synthesize ferritin^[19,20]. Lymphocytes could, therefore, act as a “mobile” and easily “mobilizable” iron-storage compartment protecting from iron-mediated toxicity^[65]. This hypothesis motivated the study of lymphocyte function in iron overload^[68]. The influence of iron on the expansion of different T-cell subsets was demonstrated both *in vitro*^[69] and *in vivo*, namely in patients with thalassemia^[70]. Results in this clinical model, however, are difficult to interpret due to the inseparable effects of blood transfusion, splenectomy, iron chelation therapy and infection. Imbalances of the relative proportions of CD4+ and CD8+ T lymphocytes, with abnormally high CD4/CD8 ratios were later reported in HH patients^[71,72], a clinical model where iron overload is not complicated by the effects of transfusion, splenectomy or desferrioxamine. Curiously, in one experimental model of iron overload seen in mice generated with targeted *Hmox1* mutations, high CD4:CD8 ratios have been seen in the splenic cell populations of older mice aged 50 wk^[34]. These mice also exhibited numerous activated CD4+ cells. Hepatic inflammatory cell infiltrates were seen in the mice, a finding similar to that reported by Rodrigues *et al*^[73] in

aging *Hfe* deficient mice.

T lymphocyte abnormalities in HH

Abnormalities in the relative proportions of the two major T lymphocyte subpopulations have been consistently described in HH patients. Reimão and co-workers first described that patients with abnormally high CD4/CD8 ratios displayed a faster re-entry of iron into the serum transferrin pool after intensive phlebotomy treatment than patients without those abnormalities^[71]. It was shown later that the amount of iron mobilized by phlebotomy correlated significantly with the number of CD8+ T cells, but not with CD4+ T cells^[72,74]. An independent study examining patients homozygous for the C282Y mutation showed that the low percentages of CD8+ T cells seen in the peripheral blood of HH patients were associated with low numbers of the same cells in the liver and with higher levels of hepatic tissue iron^[75]. HH patients had been shown earlier to have reduced percentages of CD8+ CD28+ T cells in peripheral blood^[76]. No anomalies of CD28 expression were found in the CD4+ subset. The apparent failure of the CD8+ CD28+ T cell population to expand coincided with an expansion of CD8+ CD28- T cells in peripheral blood of HLA-A3+ but not HLA-A3-HH patients^[76]. Although the described abnormalities in lymphocyte populations were systematically found in the sub-population of CD8+ T lymphocytes, the association with total body iron stores is also reflected in the total lymphocyte counts. Low total lymphocyte counts were found associated significantly with a higher degree of iron overload in *HFE*-linked HH, but not in African iron overload^[77]. More recently, Barton *et al*^[78] also described a significant inverse relationship of total blood lymphocyte counts and severity of iron overload in hemochromatosis probands with *HFE* C282Y homozygosity. Fabio *et al*^[79] confirmed that the presence of low numbers of total lymphocyte counts and CD8+CD28+ T cells in C282Y homozygous patients was inversely related to the transferrin saturation. In addition, they found low numbers of CD4+ T and NK cells, and a major increase in IL-4 and IL-10 production in the CD3+ CD8+ T cell subset^[79]. A study of the V α / β T cell receptor (TcR) repertoire in a population of C282Y homozygous HH patients showed that the frequency of V α / β TcR expansions within the CD8+ pool in the group of HH patients was significantly higher in those with iron overload related pathology (9/16) than in patients (1/16) without pathology^[80]. In the same study it was found that control subjects heterozygous for the C282Y mutation had an absence of expansions of the V β 5.2 and V β 12 chains in the CD8+ pool, suggesting that *HFE* could have an effect in the shaping of T cell receptor repertoire.

Functional abnormalities in CD8+ T lymphocytes were also described in HH patients. The level of autophosphorylation of the CD8-associated p56lck as well as its phosphotransferase activity, as determined by phosphorylation of an exogenous substrate, was significantly reduced by two- to three-fold in HH patients relative to a control population of healthy donors^[81]. By contrast, the level of CD4-p56lck activity did not show an

overall decrease relative to controls. The decreased CD8-p56lck activity seen in patients was not corrected by iron depletion. A significantly higher percentage of HLA-DR+, but not CD45RO+ cells was also found within the peripheral CD8+ T cell subset in HH patients relative to controls^[76]. Moreover, functional studies showed that CD8+ cytotoxic T lymphocytes (CTL) from HH patients exhibited a diminished cytotoxic activity when compared with CD8+ CTL from healthy controls^[76].

The finding of a significant association of abnormally low CD8+ T lymphocytes with a more severe clinical expression of hemochromatosis in HH patients^[72,82] raises the obvious question: are if these anomalies the follow or precede the development of iron overload. The fact that those abnormalities are remarkably stable in each individual patient, that they are not corrected by phlebotomy treatment, and that they are observed in asymptomatic patients at young ages, favors the hypothesis that they are intrinsic to the genetic defect and not a consequence of the progressive iron overload. More recently it was shown that the numbers of CD8+ T lymphocytes are genetically determined, in association with other genetic determinants at the MHC class I region close to HLA and HFE^[83,84]. It is, therefore, conceivable that the inherited abnormalities in CD8+ T lymphocytes in HH are modifiers of the clinical expression of the disease as proposed by Cruz *et al.*^[82] or genetically located close to a yet unidentified modifier gene of iron metabolism.

THE REVERSE TOPIC: ADAPTIVE IMMUNITY AND IRON OVERLOAD

Genetically manipulated animal models have, therefore, become wonderful and decisive tools to address the questions of the effect on iron overload of a specific gene or protein at the systemic level. Several animal models of spontaneous iron overload were described that illustrate the influence of proteins of the adaptive immunological system on iron homeostasis, all pointing to the putative importance of the MHC class I region.

The $\beta 2m$ -microglobulin deficient ($\beta 2m^{-/-}$) mice constitute the first described model of spontaneous iron overload^[38,85,86]. These mice develop a hepatic iron overload with a distribution similar to that seen in HH liver pathology, i.e., mainly in the liver parenchyma with no evidence of iron loading in the Kupffer cells^[38,86]. These mice present severe decreased cell surface expression of the MHC-class I molecules and consequently almost no CD8+ T lymphocytes^[87,88]. Intestinal uptake of ferric iron and the subsequent transfer into the plasma is inappropriately increased in $\beta 2m^{-/-}$ mice^[86]. Upon treatment with hematopoietic cells derived from normal mice fetal liver iron overload is attenuated and shifted from the parenchymal to the Kupffer cells^[86,89]. However, TfSat and intestinal iron absorption remain high, suggesting that the primary defect of iron overload is not corrected. With the discovery of the HFE gene and the demonstration that the C282Y mutated form failed to bind to $\beta 2m$ ^[35] it was assumed that the earlier findings in $\beta 2m^{-/-}$ were due to an impaired HFE function. However, several subsequent studies showed that this was not sufficient to explain the

pathology of $\beta 2m$ deficient mice. In contrast to $Hfe^{-/-}$, the $\beta 2m^{-/-}$ mice display increased expression of the duodenal iron transporters DMT1 and ferroportin1, implicating a broader role of $\beta 2m$ in mammalian iron overload^[90]. More recently, Rodrigues *et al.*^[73] described results of a comparative study of these two models in older mice. The results confirmed that the $\beta 2m^{-/-}$ old mice present a more severe hepatic iron overload than the $Hfe^{-/-}$ counterparts. $\beta 2m^{-/-}$ old mice which also showed liver steatosis, probably as a reflection of the higher hepatic iron content causing lipid peroxidation. Earlier Levy *et al.*^[91] had reported the finding that in mice lacking both the Hfe and the $\beta 2m$ molecules, liver iron deposition is observed in greater levels than in mice lacking Hfe alone.

The $\beta 2m^{-/-}$ - $Rag1^{-/-}$ double knock out mice lack mature T and B lymphocytes as well as MHC-class I and Hfe expression. $Rag1$ is required for normal T and B lymphocyte development. $\beta 2m$ is required for correct folding and cell surface expression of MHC-class I like proteins. These mice present a more severe body iron overload than each of the single knock out models^[39]. Besides liver iron deposition in the parenchymal cells, the $\beta 2m^{-/-}$ - $Rag1^{-/-}$ also showed iron deposition in pancreas and heart. Older mice under an iron-enriched diet develop heart fibrosis, which could be prevented by treatment with normal fetal liver hematopoietic cells. To determine whether the effect of the $\beta 2m$ deficiency in the $\beta 2m^{-/-}$ - $Rag1^{-/-}$ double knock out mouse was only due to lack of Hfe expression, double knock out mice for Hfe and $Rag1$ were generated^[92]. $Hfe^{-/-}$ - $Rag1^{-/-}$ double knock out mice showed increased liver iron overload compared to each of the single knock out, or the $\beta 2m^{-/-}$ - $Rag1^{-/-}$. The distribution of the iron loading in $Hfe^{-/-}$ - $Rag1^{-/-}$ mice did not recapitulate the iron loading of the $\beta 2m^{-/-}$ - $Rag1^{-/-}$, since they did not present heart or pancreas iron loading.

The δ TCR^{-/-} mice lack the $\gamma\delta$ intraepithelial lymphocytes. Following the administration of an iron supplemented diet, these mice showed an increased liver iron accumulation in relation to control mice^[93]. In addition, δ TCR^{-/-} mice had a marked reduction of tumor necrosis factor alpha (TNF- α) production by intraepithelial lymphocytes when compared with controls suggesting a role for this cytokine in intestinal iron regulation.

Finally, mice deficient in the MHC class I molecules H2K^b and D^b have a strong reduction in CD8+ T-lymphocyte numbers^[94]. These mice present a spontaneous increase of iron content in the liver preferentially in hepatocytes with occasionally Kupffer cells iron staining^[41]. The liver iron content in this model was shown to correlate directly with the number of residual CD8+ T lymphocytes (Cardoso E and M de Sousa, unpublished observations).

The above described double knockout mouse models provide a good illustration of the modifier effect of the components of the adaptive immune system, namely a MHC class I dependent effect, on the iron overload phenotype, a conclusion also reached separately by Muckenthaler *et al.*^[90] in a study of differential gene expression in $\beta 2m$ deficient mice. Altogether the results described in animal models of hemochromatosis may help to explain why immunological anomalies

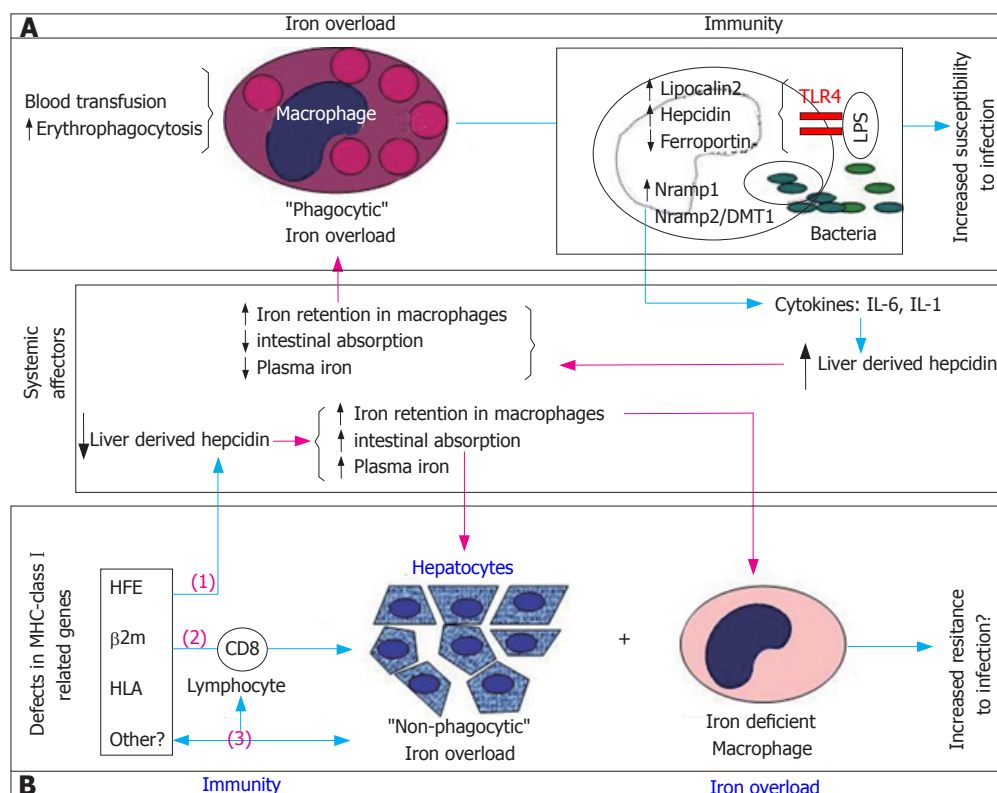


Figure 1 A model illustration of the two topics addressed in this review: how innate immunity is affected and affects “phagocytic” iron overload (Box A) and the evidence that defective immune system models, namely natural and experimental defects in MHC-class I related genes (*HFE*, β 2-microglobulin and *HLA*) are associated with the spontaneous development of “non-phagocytic” or parenchymal iron overload (Box B). **A:** Macrophages “overwhelmed” by continuous iron overloading as seen in conditions of chronic transfusion and increased erythrophagocytosis are compromised in their function of surveillance of bacterial growth resulting in increased host susceptibility to infection. As part of the natural response to bacterial infection macrophages modulate the expression of a number of genes involved in the regulation of cellular iron metabolism. By activating the cytokine cascade they induce up-regulation of liver hepcidin synthesis with further increase in macrophage iron retention and perpetuation of this cycle; **B:** A “non-phagocytic” or parenchymal iron overload is observed in hepatocytes of several defective immune system models associated with a relative iron deficiency in macrophages, recapitulating the findings observed in the human model of Hereditary Hemochromatosis. The mechanisms underlying the spontaneous development of iron overload in these models are not yet understood. Three possible (not mutually exclusive) mechanisms are represented in this figure: (1) the fact that both *Hfe* and β 2m knockout mouse models lack the liver hepcidin response to iron overload^[80,97,98] may support the notion of a liver-derived, systemically induced iron overload. The involvement of liver hepcidin in the development of iron overload in mice deficient in the MHC-class I molecules *H2K^b* and *D^b* (*HLA*) was never tested; (2) the iron overload seen in the β 2m^{-/-} *Rag1^{-/-}* and *Hfe^{-/-} Rag1^{-/-}* mouse models support the involvement of lymphocytes themselves as modifiers of the severe parenchymal iron overload phenotype seen in those mice^[39]; (3) finally, the recent demonstration of a strong link between the MHC-class I region and the genetic transmission of low CD8⁺ T lymphocyte numbers in humans and the association of total lymphocyte numbers with a more severe iron overload phenotype in patients with Hereditary Hemochromatosis^[78,82,83] opens a new possibility that another, still unidentified MHC-class I associated gene(s) may simultaneously regulate CD8⁺ T lymphocyte numbers and influence the development of parenchymal iron overload^[84].

modify the severity of iron overload in Hereditary Hemochromatosis^[82,95].

CLOSING REMARKS

The growing knowledge and availability of genetic techniques dissecting the fine components of the host response to the challenge of infection have strengthened the opportunity of revising the topic “iron and immunity”. When the main concern for this topic resided in the effects of iron deficiency on the immune response, Weinberg pioneered the opposite concern for iron overload and infection^[12]. Today it is evident that in response to infection numerous innate immunity components display metal chelating properties, including synthesis and release of lactoferrin, lipocalin and hepcidin, as discussed above. The cells involved in that response are neutrophils and macrophages. But, during evolution the macrophage, particularly the splenic macrophage, assumed the key

physiological role of recognizing senescent red blood cells and recycling the iron in hemoglobin^[96]. Exhaustion of that pool *in vivo* leads to “phagocytic” iron overload, such as seen in transfusional iron overload, with the expected consequences in the development of infection. If on the other hand the macrophage fails, it is to be expected that “parenchymal” iron overload will develop. This is the case in *HFE*-Hereditary Hemochromatosis. Therefore, iron overload must not be seen as one entity, but two separate entities with different relationships to immunity (Figure 1). Evidence was provided in this review that innate immunity is affected and affects “phagocytic” iron overload. On the reverse side, defective immune system models, namely natural and experimental defects in MHC-class I related genes (*HFE*, β 2-microglobulin and *HLA*) are associated with the spontaneous development of “non-phagocytic” or parenchymal iron overload.

The large new question looming in the horizon of this topic with which we wish to close this brief review is: does

the MHC class I region harbor, in addition to *HFE*, other gene or genes that besides regulating lymphocyte numbers also influence the development of “non-phagocytic” or parenchymal iron overload? The search for the answer to this question will probably guide many research interests for years to come.

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TOPIC HIGHLIGHT

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Molecular mechanisms involved in intestinal iron absorption

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Abstract

Iron is an essential trace metal in the human diet due to its obligate role in a number of metabolic processes. In the diet, iron is present in a number of different forms, generally described as haem (from haemoglobin and myoglobin in animal tissue) and non-haem iron (including ferric oxides and salts, ferritin and lactoferrin). This review describes the molecular mechanisms that co-ordinate the absorption of iron from the diet and its release into the circulation. While many components of the iron transport pathway have been elucidated, a number of key issues still remain to be resolved. Future work in this area will provide a clearer picture regarding the transcellular flux of iron and its regulation by dietary and humoral factors.

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Key words: Haem; Non-haem iron; DMT1; IREG1; Dcytb; Hephaestin

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INTRODUCTION

Iron is an essential trace metal for all organisms. In humans it plays numerous biochemical roles, including oxygen binding in haemoglobin and as an important catalytic centre in many enzymes, for example the

cytochromes. In normal healthy adults, some 0.5-2 mg of iron is lost each day due to blood loss and the constant exfoliation of iron-containing epithelial cells that line the gastrointestinal and urinary tracts, skin and hair. Therefore, the same amount of iron from dietary sources is required each day to replace the lost iron and maintain body iron homeostasis. Even though iron is an essential metal in human metabolism, it is highly toxic to cells and tissues if present in elevated levels. Perversely, humans do not possess the necessary machinery to rid the body of excess iron and, therefore, the absorptive process must be tightly regulated within defined physiological limits to avoid pathologies associated with both iron deficiency and overload.

Dietary iron is found in two basic forms, either as haem-found in meat and meat products-or non-haem iron-present in cereals, vegetables, pulses, beans, fruits *etc* in a number of forms ranging from simple iron oxides and salts to more complex organic chelates. Non-haem iron predominates in all diets comprising some 90%-95% of total daily iron intake. The absorption of both haem and non-haem iron takes place almost exclusively in the duodenum and the bioavailability of iron from these sources is influenced by a number of variables, e.g. the iron content of foods, the type of iron present, i.e. haem or non-haem, and other dietary constituents. Importantly, absorption is also regulated in line with metabolic demands that reflect the amount of iron stored in the body, and the requirements for red blood cell production. These humoral mechanisms are further reviewed in this volume^[1].

Despite accounting for only 5%-10% of dietary iron in western countries, haem is the most bioavailable source of iron amounting to is some 20%-30%^[2]. In contrast, the bioavailability of non-haem iron is low-only 1%-10% of the dietary load is absorbed-and is profoundly influenced by other dietary components that can enhance or inhibit non-haem iron bioavailability. The most potent enhancer is ascorbic acid (vitamin C), which acts by reducing ferric iron to the more soluble and absorbable ferrous form. Phytates found in cereal products and polyphenolic compounds found in all plant products are the most potent dietary inhibitors of non-haem iron absorption. However, it is important to note that on an equimolar basis the pro-absorptive action of dietary ascorbic acid can counteract the inhibitory effect of phytates and polyphenols^[3].

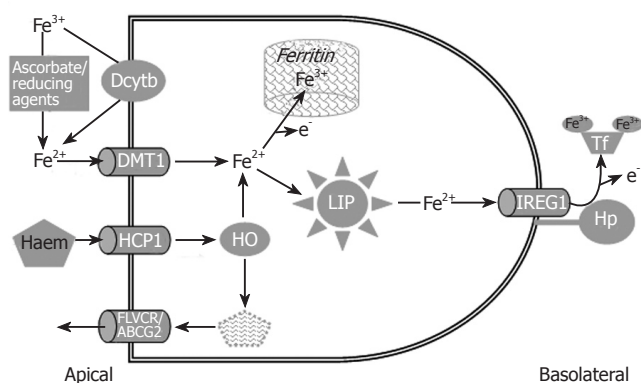


Figure 1 The cellular mechanisms involved in intestinal iron absorption. Dietary non-haem iron (mostly ferric) is reduced by the actions of the ferric reductase Dcytb and reducing agents in the diet to yield Fe^{2+} , which subsequently enters the enterocytes via DMT1. Haem is absorbed via HCP1, broken down by haem oxygenase 1 (HO) to liberate Fe^{2+} (this joins a common pool with iron from the non-haem pathway) and bilirubin (which might be removed from the cell by the efflux proteins FLVCR and ABCG2). If body iron stores are high, iron may be diverted into ferritin and lost when the cell is shed at the villus tip. Alternatively, iron passes into the labile iron pool (LIP) and is subsequently processed for efflux via IREG1 (as Fe^{2+}). The exiting iron is re-oxidized to Fe^{3+} through hephaestin (Hp) to enable loading onto transferrin (Tf).

MECHANISMS INVOLVED IN INTESTINAL IRON TRANSPORT

In recent years our understanding of the mechanisms involved in dietary iron absorption by duodenal enterocytes has increased dramatically. Both haem and non-haem iron are taken up in this proximal region of the small intestine, though their transport across the apical membrane of the enterocytes occurs through totally independent pathways (Figure 1).

Ferrous iron

The majority of dietary non-haem iron enters the gastrointestinal tract in the ferric form. However, Fe^{3+} is thought to be essentially non-bioavailable (see below) and, therefore, it must first be converted to ferrous iron prior to absorption. There are numerous dietary components capable of reducing Fe^{3+} to Fe^{2+} , including ascorbic acid^[4], and amino acids such as cysteine^[5] and histidine^[6]. It is believed that the action of these dietary reducing agents takes place in the acidic environment of the gastric lumen. Indeed the essential requirement for an acid environment in iron absorption is exemplified by the fact that achlorhydria is commonly associated with iron deficiency anaemia^[7,8]. However, ferric iron reaching the duodenal enterocytes may still be reduced by the cells endogenous reducing activity. A number of studies have demonstrated that the brush-border surface of duodenal enterocytes and cultured intestinal cells possess ferric reductase enzymic activity^[9-11]. Using a subtractive cloning strategy designed to identify intestinal genes involved in iron absorption, Dcytb (for duodenal cytochrome b), a homologue of cytochrome b_{561} , was identified as the enzyme responsible for this process^[12]. Like cytochrome b_{561} , Dcytb is a haem-containing protein with putative binding sites for ascorbate and semi-dehydroascorbate. The protein is expressed on

the brush border membrane of duodenal enterocytes, the major site for the absorption of dietary iron. Moreover, antibodies raised against Dcytb block the endogenous ferric reductase activity of the duodenal brush border membrane, providing further evidence for a functional role in dietary iron processing^[12].

Intriguingly, a recent study has reported that the targeted disruption of the *Cybrd1* gene in mice (which encodes Dcytb) does not lead to an iron deficient phenotype^[13], implying that Dcytb is not necessary for intestinal iron absorption in mice. However, an important caveat to this study is the fact that mice are able to synthesize significant quantities of ascorbic acid-unlike humans who are reliant on dietary vitamin C to satisfy their requirements-and may, therefore, have less need for a duodenal surface ferric reductase.

Following reduction either by Dcytb or dietary reducing agents, the resulting Fe^{2+} becomes a substrate for the divalent metal transporter, DMT1-also known as the divalent cation transporter, DCT1^[14], and natural resistance associated macrophage protein, Nramp2^[15]. The relatively low pH of the proximal duodenum together with the acid microclimate present at the brush border membrane^[16,17] stabilises iron in the ferrous form and provides a rich source of protons that are essential for driving iron uptake across the apical membrane of the intestinal epithelium^[14,18].

The role of this transporter in intestinal iron homeostasis has been highlighted by a number of studies. Our work^[18] and that of others^[19] have shown that antibodies to DMT1 can significantly inhibit iron absorption. Furthermore, targeted disruption of DMT1 in mice has revealed the essential role of this transporter in both intestinal iron absorption and in erythroid precursor development^[20]. In addition to these biochemical and molecular manipulations, two rodent models, the *mk/mk* mouse^[15] and the Belgrade (*b*) rat^[21], which possess a spontaneous mutation (G185R) in the DMT1 gene, exhibit defective intestinal iron uptake and microcytic anaemia. More recently a number of mutations in human DMT1 have also been identified^[22-25] which in turn lead to the development of microcytic anaemia.

The molecular identity of the functional DMT1 isoform in intestinal epithelial cells has been the subject of recent debate. At least four isoforms exist through alternate splicing in exon 16^[26] and the presence of two transcription start sites-named exon 1A and 1B^[27]. Exon 16 splicing gives rise to two variants which differ in their terminal 19-25 amino acids and their 3' untranslated sequence (UTR). Interestingly one of these variants contains an iron responsive element in its 3' UTR^[26]. The significance of this is discussed later in this review. All four isoforms can be detected at varying levels in intestinal epithelial cells^[27], but the exon 1A/IRE-containing variant has been suggested to be the major functional isoform in absorptive enterocytes.

Ferric iron

We stated earlier that Fe^{3+} is thought to be non-bioavailable. However, some workers have proposed that Fe^{3+} might be absorbed by intestinal enterocytes *via* a

mechanism that is distinct from DMT1 (reviewed in^[28]). In this model, ferric iron, which is insoluble at physiological pH, is released from the food matrix in the acidic environment of the stomach, and is chelated by mucins on the duodenal brush border surface, which maintain the iron in the ferric state. Fe³⁺ enters the enterocyte across the apical membrane via interaction with β_3 -integrin and mobilferrin (a calreticulin homologue). In the cytosol, this complex combines with flavin monooxygenase and β_2 -microglobulin to form a larger conglomerate (approximately 520 kDa) known as paraferitin, which has ferric reductase activity resulting in the conversion of the absorbed Fe³⁺ to Fe²⁺. Recent evidence suggests that the paraferitin complex may also contain DMT1^[29], which may permit the delivery of ferrous iron to intracellular organelles.

Ferritin

In animal and plant tissues, the major iron storage protein is ferritin. Most nutrition texts focus only on haem and non-haem iron, and generally ignore the possibility that ferritin may be an important nutritional source of iron. While the issue of ferritin bioavailability is still controversial, a number of studies have shown that both plant and animal ferritin sources are absorbed in humans^[30-32] with a bioavailability equivalent to that of ferrous sulphate. The ferritin iron uptake mechanism is yet to be determined. One possibility is that ferritin is broken down by protease activity in the upper gastrointestinal tract and the released iron is absorbed *via* the Dcytb/DMT1 route. However, studies have shown that ferritin is largely resistant to high temperature, low pH and protein denaturing agents^[33,34]. Therefore, it is possible that ferritin may be absorbed intact and broken down intracellularly (in the lysosomes) to liberate its iron load. In support of this latter possibility, one study has reported that iron and ferritin protein are both taken up by the intestinal Caco-2 cell line^[35]. Such a mechanism would require the presence of a ferritin receptor on the apical membrane of intestinal enterocytes. While the presence of ferritin receptors has been postulated on liver^[36] and placental^[37] plasma membranes, none has yet been identified in intestinal tissue. Taken together this evidence suggests that the molecular identity of at least one important intestinal iron transport gene may remain to be discovered.

Lactoferrin

In breast-fed infants, a major proportion of iron is bound to the human milk protein lactoferrin, an iron-binding protein capable of binding two ferric ions^[38]. Specific receptors for lactoferrin have been identified on the brush border surface of foetal enterocytes^[39] and subsequent studies have shown that these receptors mediate the uptake of lactoferrin-bound iron in intestinal epithelial cell cultures^[40]. Interestingly, a recent study looking at ontogenic changes in the expression of iron transport proteins in mouse small intestine, has suggested that the lactoferrin receptor may be the principal iron transport pathway in early life^[41]. Intriguingly, a recent human volunteer study has indicated that the nutritional importance of lactoferrin

may not be limited to infants since it is also a bioavailable source of iron (with equivalent bioavailability to ferrous sulphate) in young adult females^[42].

Haem

Non-vegetarian diets provide an additional and important source of iron in the form of haem (largely from haemoglobin and myoglobin). While haem comprises only approximately 10% of dietary iron intake, because it is more bioavailable than non-haem iron, it may contribute as much as half of the total iron absorbed from western meat-rich diets^[43]. Despite the wealth of information available on the uptake of non-haem iron, the mechanisms involved in haem iron absorption are only just beginning to emerge. Early work on intestinal absorption suggested that haem binds to the duodenal brush border membrane and is absorbed as an intact molecule^[43,44]. In support of this proposed mechanism, workers have reported the existence of haem binding proteins on pig enterocytes^[45,46] and intestinal Caco-2 cells^[47]. More recently, a number of candidate haem binding proteins have been identified in the intestinal epithelial cells including the ATP-binding cassette protein, ABCG2^[48], the feline leukaemia virus C receptor protein, FLVCR^[49] and the haem carrier protein, HCP1^[50]. Of these candidate haem transporters, ABCG2 and FLVCR mediate haem efflux and only HCP1 acts as a haem import protein. The high duodenal expression of HCP1 suggests that it may be the protein involved in haem uptake from the diet. However, the precise role of HCP1 in iron metabolism remains to be fully elucidated. This issue has been complicated by recent data indicating that HCP1 may also function as a proton-coupled folate transporter, independent from its haem transporting properties^[51].

Following absorption, haem is detectable in membrane-bound vesicles within the cytoplasm^[52,53]. Within these vesicles, it is thought that the iron contained within the protoporphyrin ring is excised by the action of haem oxygenase 1^[54] yielding ferrous iron which enters a common intracellular pool along with the iron absorbed via the non-haem transport pathways. Digestion appears to be complete within the enterocytes since a number of studies have failed to detect intact haem efflux across the basolateral membrane^[44,47]. One intriguing possibility is that the efflux proteins ABCG2 and FLVCR, also expressed in the duodenum, may act to remove bilirubin formed as a by-product of haem degradation from the enterocytes.

Intracellular storage and translocation of iron

At this stage, the absorbed iron has two fates depending on the body's requirements. If the body stores are replete, and there is no increased erythropoietic drive, a significant amount of newly absorbed iron will be stored in the enterocytes as ferritin. Because duodenal enterocytes turnover very rapidly (their lifespan is approximately 3-4 d) and the majority of enterocytes contributing to absorption lie in the mid/upper villus region, this intracellular ferritin iron is quickly lost into the intestinal lumen as the ageing cells are sloughed off at the villus tip. Interestingly, in human subjects there is a positive correlation between

dietary iron bioavailability and faecal ferritin content which supports the above mechanism^[55,56]. Indeed it is likely that this is a very important mechanism for controlling the release of iron into the circulation.

The mechanism by which iron is translocated from the apical pole of the enterocytes so that it becomes available for export across the basolateral membrane is poorly understood. A body of evidence has emerged from studies in Caco-2 cells for a vesicular transport pathway that co-ordinates the transcellular movement of iron. Central to this mechanism is apo-transferrin (apo-Tf) which when added at the basolateral surface of the Caco-2 cell monolayer stimulates transepithelial iron flux in a dose-dependent manner^[57-59]. Interestingly, in Caco-2 cells apo-Tf and Fe-Tf, once taken up from the basolateral medium, appear to be directed into distinct endosomal vesicular fractions^[59,60]. The apo-Tf containing endosomes are routed towards the apical pole of the cell where they co-localise with vesicles containing DMT1^[61]. It is proposed that the iron entering the cell along with DMT1 is transferred to apo-Tf within these endocytic vesicles, and is subsequently exocytosed into the basolateral medium as Fe-Tf. Using a combination of biochemical inhibitors to disrupt this vesicular network, it is estimated that this pathway may count for as much as 50% of the transepithelial iron flux in Caco-2 cells^[62,63].

While on the face of it, the above studies provide compelling evidence for a co-ordinated mechanism for the transcellular routing of iron, a number of caveats must be taken into consideration. (1) This model requires the expression of both DMT1 on the apical surface and transferrin receptors (TfR) on the basolateral membrane of the same enterocytes. While this requirement holds for Caco-2 cells^[64], the evidence from rat and mouse intestine suggests that TfR are predominantly expressed in the proliferating crypt and lower villus enterocytes^[65-69] while dietary iron uptake through apical membrane DMT1 occurs in the upper villus enterocytes^[70-73]. (2) Recent studies have shown that intestinal-specific inactivation of IREG1 (the basolateral iron transporter) results in anaemia confirming the essential role of this pathway in iron homeostasis^[74]. (3) Caco-2 cells, while an excellent model of the intestinal epithelium, exhibit some non-enterocyte properties including the ability to synthesize and secrete transferrin^[75-76]. Taken together, all of these studies highlight the need for further investigation into the transcellular iron transport mechanisms and their role in maintaining body iron homeostasis.

Iron export

Efflux of iron across the basolateral surface of enterocytes is achieved through the co-ordinated action of a transport protein IREG1^[77]-also known as ferroportin^[78] and MTP1^[79]-and a ferrioxidasase, hephaestin^[80]. Studies in which IREG1 was expressed in *Xenopus laevis* oocytes indicate that this is a unidirectional efflux transporter of ferrous iron^[77,78]. Interestingly, this efflux function is up-regulated in the presence of ceruloplasmin, a copper binding ferrioxidasase, plus transferrin to bind the newly liberated iron^[77]. This suggests that while ferrous iron is released through IREG1 it must be oxidised to ferric iron to

facilitate its loading onto transferrin for onward transport in the circulation. Interestingly studies with the yeast ceruloplasmin homologue, Fet3p, have highlighted the requirement for ferrioxidasase activity in iron accumulation by transferrin^[81,82].

As stated above, the use of knockout mice has elegantly demonstrated the essential role of IREG1 in basolateral iron efflux^[74]. In addition, a second genetic mutant mouse model-the sex-linked anaemia (sla) mouse-has highlighted the critical requirement for oxidation of iron leaving the enterocytes for normal iron homeostasis. The sla mouse develops a moderate to severe microcytic hypochromic anaemia^[83]. It has been shown subsequently that these mice exhibit normal uptake of iron into enterocytes^[84], but the subsequent release of iron into the circulation is diminished^[85]. As a result, iron accumulates in enterocytes, and is lost when these cells are sloughed at the villus tip^[86]. While the *in vitro* studies described earlier^[77] used the ferrioxidasase activity of ceruloplasmin to drive iron efflux, in the intestine the oxidation of iron is achieved by a ceruloplasmin homologue, hephaestin, which is also a multicopper ferrioxidasase^[87]. In the sla mouse, the hephaestin gene is defective leading to a truncated form of the protein^[80], which is mislocalised within the enterocytes^[88] and has reduced ferrioxidasase activity^[87].

REGULATION OF INTESTINAL IRON TRANSPORT

The regulation of intestinal iron absorption is complex and relies on mechanisms which sense dietary iron content as well as iron storage levels in the body and erythropoietic iron requirements (Figure 2). The iron regulatory hormone hepcidin is likely to be an important intermediate in signalling the storage and erythroid requirements and this aspect of iron homeostasis will be dealt with in an accompanying review^[1].

Basal transporter expression

In the healthy physiological state intestinal transporter expression will reflect body iron status exemplified by the circulating levels of iron bound to transferrin. Cells in the duodenal crypts of Lieberkühn express both Hfe^[89,90], the protein mutated in more than 80% of haemochromatosis patients^[91], and TfR on their basolateral surface. It is believed that Hfe binds to TfR regulating the rate at which transferrin-bound iron can enter the cell^[92,93]. One suggestion is that the cellular iron concentration established as a result of this interaction ultimately determines the basal level of expression of the proteins involved in iron absorption in the mature absorptive cells in the upper third of the villus. Importantly, in a modification to this hypothesis, we propose that in response to humoral signals, such as hepcidin, iron transport^[94] and transporter expression^[95] in mature epithelial cells, can be modified rapidly without the need to re-programme the crypt cell sensing mechanism.

The role of dietary iron

Rapid regulation of intestinal transporter expression in

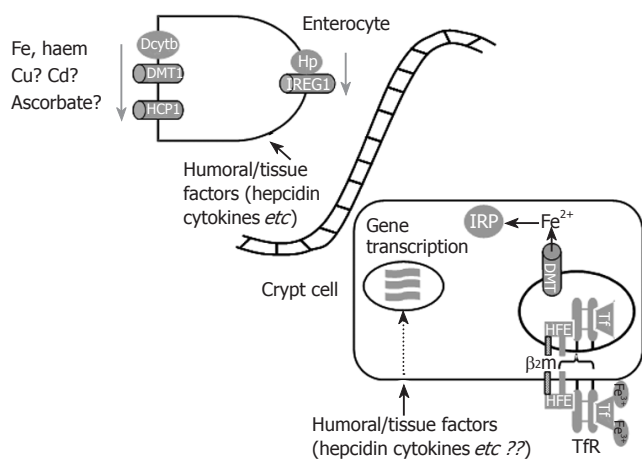


Figure 2 The regulation of intestinal iron transport. The duodenal crypt-villus axis represents a differentiation pathway that can be influenced by the dietary and humoral signals that ultimately regulate iron absorption. Immature proliferating cells in the crypt take up transferrin from the circulation via transferrin receptors (TfR). This process is governed by interactions between Hfe, β_2 microglobulin and TfR. The crypt cells are thus sensitive to the circulating levels and the iron-saturation of Tf. Iron regulatory proteins (IRP) recognise the cellular iron content and regulate iron responsive mRNAs posttranscriptionally. In addition, transcription control of iron metabolism may be exerted by a number of humoral and tissue-derived factors, such as hepcidin or cytokines interacting with their individual specific receptors. Together, these processes set the basal level of iron transporter protein expression in enterocytes as they mature and travel up the villus. Importantly, this basal level of transporter expression can be modified rapidly in mature enterocytes in response to changes in the levels of dietary factors (e.g. the iron content of the diet, other metals etc) and humoral and local tissue mediators (especially hepcidin and pro-inflammatory cytokines). This combination of basal programming in the crypts and fine tuning in villus enterocytes permits tight control of intestinal iron transport.

response to dietary factors is probably sensed by the villus enterocytes. More than half a century ago the “mucosal block” hypothesis was formulated following studies which demonstrated that a large oral dose of iron could reduce the absorption of a smaller dose administered several hours later^[96,97]. It was argued that due to the short time interval between doses, the initial dose must be having a direct effect on the mature enterocytes rather than the crypt cells. Whether such a phenomenon occurs with meaningful dietary iron levels is not clear but this may be a considerable problem with supplemental iron levels^[98]. Using the Caco-2 cell model, we have addressed the issue of whether non-haem iron can regulate iron transporter expression within a timescale and at concentrations that are consistent with digestion and absorption of a meal. We found that DMT1 (the IRE-containing isoforms) protein expression on the apical surface of these cells is decreased by iron concentrations as low as 20 $\mu\text{mol/L}$ ^[64]. The decrease in DMT1 transporter expression was rapid, occurring within 1-4 h following the exposure to iron^[99]. Further analysis revealed that DMT1 protein was internalised and targeted towards a late endosomal/lysosomal compartment. Interestingly, these iron-dependent effects were restricted to the apical uptake pathway-IREG1 protein expression was unaltered and were fully reversible (DMT1 protein levels returned to their original basal levels within 4-8 h) following the removal of iron^[99]. Our findings in this pertinent cell

culture model are consistent with those observed in rats following oral gavage with an iron bolus^[100-102] suggesting that the redistribution of DMT1 between different cellular compartments may be important physiologically for optimising iron absorption from a meal so that it matches better the body's metabolic requirements.

Iron regulatory proteins and iron responsive elements

In addition to trafficking of iron transport proteins, a number of intestinal iron metabolism genes can be regulated post-transcriptionally through interactions between cytosolic iron regulatory proteins (IRP) which bind to iron responsive elements (IRE), stem loop structures in either the 3' or 5' untranslated region (UTR) of several mRNA species, under conditions of cellular iron deficiency. TfR mRNA contains five IREs in its 3' UTR, and is stabilised following IRP binding as this blocks a target site for endonuclease activity^[103-106]. Interestingly, two isoforms of DMT1 contain a single IRE in the 3'UTR^[26]. While the DMT1 IRE can bind IRP *in vitro*^[107,108], its role in regulating DMT1 expression remains to be fully determined.

In contrast to the reported role of the 3'IRE sequences in conferring mRNA stability, the expression of mRNAs possessing 5'IREs, such as ferritin, are poorly translated with cellular iron low. But, expression is increased by high iron levels in duodenal enterocytes^[109]. This is because IRP/IRE binding blocks the association of the eukaryotic initiation factor complex to the 43S ribosomal unit^[110]. Interestingly, IREG1 mRNA contains a single IRE in the 5'UTR^[77,79]. However, the role of IRP/IRE interactions in the regulation of IREG1 transporter expression is even more controversial than its role in regulating DMT1 expression. Evidence suggests that the response to changes in iron status is tissue-specific-IREG1 expression in the liver^[79] and lung^[111] and in macrophages^[112] is up-regulated by high iron whereas in the intestine expression is elevated by iron deficiency^[77]. This may indicate that transcriptional, translational and post-translational processing of IREG1 varies between cell types^[113]. Clearly, the mechanisms involved in iron-dependent regulation of IREG1 in the intestine require further attention.

Local tissue factors

While the majority of this review has focussed on the transport pathways in the enterocytes it is important to bear in mind that the intestinal cell population is a highly heterogeneous affair. It is likely, therefore, that cross-talk between the epithelial cells and other cell types, such as macrophages, neutrophils, intraepithelial lymphocytes etc will be important in the overall regulation of intestinal iron transport. One intriguing hypothesis in this regard is the possible physiological role of the pro-inflammatory cytokine TNF α in regulating intestinal iron transport. TNF α is not only synthesized by peripheral blood monocytes and macrophages in response to inflammatory stimuli, but is also released by intraepithelial lymphocytes (IEL) that reside within the intestine in response to high iron intakes^[114]. These findings led to the formation of a hypothesis (named the piggyback-sensor model^[115])

which suggested that interaction between Hfe in the developing enterocytes with specific epitopes on the IELs was essential for regulating local TNF α production. Once released, TNF α leads to iron deposition within intestinal enterocytes *via* a TNF receptor 2-dependent mechanism^[116]. Further studies by our group^[117] and others^[118,119] have shown that TNF α has a direct influence on intestinal iron transporter expression and iron transport. These studies have opened the way for further investigations into the role of cell to cell cross-talk and the role of local tissue factors in regulating intestinal iron transport.

SUMMARY AND FUTURE DIRECTIONS

Clearly, our understanding of the molecular components of the intestinal iron transport pathway has increased exponentially in the last decade. However, there are still a number of important questions that remain unanswered: (1) How is iron shunted across the enterocytes from the apical pole to the basolateral membrane? There is some evidence for the presence of a tubulovesicular pathway. But, this work has largely been carried out in cell lines, and needs to be explored further in “normal” intestinal tissue. A role for calreticulin, a proposed component of the paraferitin pathway, remains a possibility though is still unproven. (2) What are the relative contributions of ferritin (and possibly lactoferrin) to iron nutrition? Could these iron sources be exploited for new supplemental therapies to treat iron deficiency? (3) Is there a role for cross-talk between enterocytes and other intestinal cell types in the local regulation on intestinal iron transport? If so, what are the cellular mechanisms involved? Are local tissue factors (such as TNF α) relevant physiologically in the control of iron absorption? (4) Hcpidin - how does it regulate intestinal iron transport? Is it an important physiological regulatory or is its main role in iron-related pathologies such as iron deficiency anaemia, haemochromatosis and anaemia of chronic disease?

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Liver iron transport

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Abstract

The liver plays a central role in iron metabolism. It is the major storage site for iron and also expresses a complex range of molecules which are involved in iron transport and regulation of iron homeostasis. An increasing number of genes associated with hepatic iron transport or regulation have been identified. These include transferrin receptors (TFR1 and 2), a ferrireductase (STEAP3), the transporters divalent metal transporter-1 (DMT1) and ferroportin (FPN) as well as the haemochromatosis protein, HFE and haemojuvelin (HJV), which are signalling molecules. Many of these genes also participate in iron regulatory pathways which focus on the hepatic peptide hepcidin. However, we are still only beginning to understand the complex interactions between liver iron transport and iron homeostasis. This review outlines our current knowledge of molecules of iron metabolism and their roles in iron transport and regulation of iron homeostasis.

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Key words: Liver; Iron homeostasis; Iron uptake; Iron release; Iron transporters; Hereditary haemochromatosis

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INTRODUCTION

Iron is an essential trace element for almost all forms of

life. However, under physiological conditions, the free form of iron is practically insoluble and potentially toxic. Thus, iron is always found bound to specific ligands in such a way as to render it both soluble and non-toxic. The toxicity of iron stems from its ability to redox cycle. The release of an electron from ferrous iron, if uncontrolled, may result in the formation of highly reactive oxygen species capable of oxidising lipids, proteins and DNA^[1] causing damage to the structures and processes in which they are involved. However, many catalytic and other biological processes rely on the redox properties of iron; hence, iron must be available in a form which allows it to donate and accept electrons without causing non-specific damage.

In mammals, iron is transported around the plasma bound mainly to the glycoprotein transferrin, although other forms are also present in small amounts. In normal human plasma, transferrin has a concentration of between 25 and 50 $\mu\text{mol/L}$, and is usually about one-third saturated with iron. The remaining, unoccupied, binding sites on transferrin provide a large buffering capacity in case of an acute increase in plasma iron levels, an important consideration given the toxicity of free iron. Following uptake by the tissues, iron is transferred into a cytosolic pool (the "transit pool") from where it is distributed to ferritin for storage or to iron-requiring moieties, such as haem or iron-sulphur clusters. The majority of hepatocellular iron is contained in ferritin (80%) with 2%-3% present as haem; the remainder is either bound to transferrin or present in the transit pool^[2].

The liver plays a central role in iron metabolism. It is responsible for approximately 8% of plasma iron turnover in humans^[3], most of which is mediated by hepatocytes^[4,5] and it has long been known that it is the major site for storage of iron. Histologically, iron is distributed around the periportal regions of the liver with a decreasing gradient towards the centrilobular regions. In iron overload disorders, this gradient becomes more pronounced^[6], involving mainly hepatocytes with the resident liver macrophages, Kupffer cells, leading to a much lesser extent^[7].

More recently, it has been shown that the liver expresses a complex range of molecules which regulate iron homeostasis. The liver and, more specifically, hepatocytes, also express the vast majority of genes that have been associated with hereditary iron disorders. Our understanding of these disorders as well as the normal function of the liver in iron homeostasis is, as yet, incomplete. This review focuses on iron metabolism in hepatocytes, with a specific section on the role of Kupffer

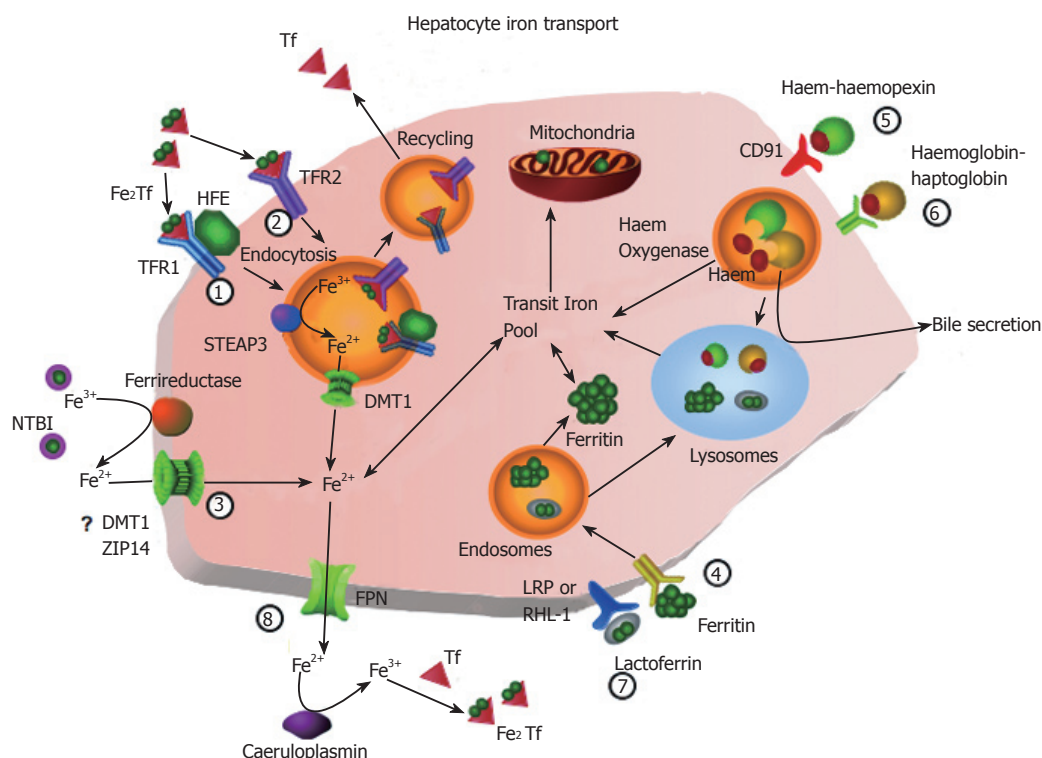


Figure 1 Hepatocyte iron transport. (1) TFR1-mediated uptake of diferric transferrin. Diferric transferrin binds to its specific receptor and is endocytosed. The endosome is acidified and Fe^{3+} is reduced by STEAP3. The iron is released and transported out of the endosome via DMT1 and apotransferrin is exocytosed. (2) TFR2-mediated uptake of transferrin. This mechanism is similar to the TFR1-specific mechanism except that transferrin binds to TFR2. (3) Uptake of NTBI. Iron is reduced and is transported into the cell via a carrier-mediated process. (4) Uptake of ferritin. Ferritin binds to its specific receptor and is endocytosed. The endosome is directed to lysosomes and the iron is transferred to the transit pool or endogenous ferritin. (5) Uptake of haem-haemopexin. The haem-haemopexin complex binds to its specific receptor CD91 and is endocytosed. Haem is removed and is degraded by haem oxygenase. (6) Uptake of haemoglobin-haptoglobin. The haemoglobin-haptoglobin complex binds to a specific receptor. Following endocytosis, the complex may be directed to the canalicular membrane for release into the bile or to the lysosomes for degradation. (7) Uptake of lactoferrin. Lactoferrin binds to LRP or RHL-1 and is endocytosed and targeted to the lysosomes for degradation. (8) Iron release. Iron is released by FPN and oxidised by caeruloplasmin and binds to apotransferrin. TFR1, transferrin receptor 1; TFR2, transferrin receptor 2; STEAP3, six-transmembrane epithelial antigen of the prostate 3; DMT1, divalent metal transporter 1; NTBI, non-transferrin bound iron; ZIP14, zinc-regulated transporter and iron-regulated transporter-like protein 14; LRP, low-density lipoprotein receptor-related protein; FPN, ferroportin.

cells, and on the molecules known to be involved in iron metabolism in the liver and their role in our current understanding of liver iron transport.

TRANSFERRIN RECEPTOR 1 (TFR1)-MEDIATED UPTAKE OF TRANSFERRIN

TFR1-mediated uptake of diferric transferrin is, perhaps, the best described process of iron uptake (Figure 1, pathway 1). Briefly, transferrin binds to TFR1 and is endocytosed^[2,5]. At the pH of the extracellular fluid, diferric transferrin is bound preferentially, the affinity of the receptor being higher for diferric transferrin than for either monoferric- or apo- transferrin^[7-10]. Following formation of the endosome, it is acidified which results in a decrease in the affinity of transferrin for iron and subsequent detachment of the metal^[11,12]. The affinity of the receptor for the (now) apotransferrin is increased by the acidic environment^[7] and apotransferrin remains bound to the receptor as the endosome returns to and fuses with the plasma membrane. At the higher extracellular pH, the affinity of the receptor for apotransferrin decreases^[7] and apotransferrin is released back into the circulation^[2]. The entire process takes between 3.8 and 15 min^[13-15].

TFR1

TFR1 expression is regulated by iron primarily by a post-transcriptional mechanism. The transcript contains five iron-responsive elements (IRE) in its 3' untranslated region (UTR) along with a number of instability elements that facilitate breakdown of the message^[16-19]. Under low-iron conditions, iron regulatory proteins (IRP) bind to the IREs, placing an inhibition on the instability elements^[18,19], increasing the half-life of the mRNA and, hence, increasing translation. When iron is abundant, IRPs do not bind IREs, resulting in a decrease of the stability of the transferrin receptor message. Two isoforms of IRP have been identified. The first (IRP1) is an iron-free form of cytosolic aconitase^[20-22]. The second (IRP2) does not exhibit any aconitase activity^[23] and appears to be the physiologically active IRP since it can respond to iron under conditions of low oxygen tension^[24], a situation which occurs in the liver *in vivo*.

TFR1 is also regulated by other mechanisms. The gene contains an hypoxia response element in its promoter region which mediates up-regulation of transcription in the presence of hypoxia-inducible factor 1^[25-27]. Transcription is also up-regulated by cytokines, such as interleukin-2, mitogens and growth factors^[28-30]. Furthermore, *TFR1*

expression is increased in proliferating cells and reduced in quiescent cells^[31-33], consistent with cellular demand for iron during periods of growth.

HFE

A mutation in HFE was the first to be shown to be causative for the iron overload disorder, haemochromatosis^[34]. HFE is a major histocompatibility complex-like protein and was originally designated HLA-H. The gene is widely expressed, with highest expression in the liver and small intestine. HFE requires the protein β 2-microglobulin for its correct localisation to the cell surface^[35].

Despite this knowledge, the normal function of the protein has been difficult to elucidate. The crystal structure of the transferrin-TFR1 complex^[36] indicates that the C-lobe of transferrin interacts with the helical domain of one of the TFR1 monomers. In contrast, the N-lobe of transferrin appears to interact partially with the helical domain, partially with the protease-like domain and, unusually, with the stalk connecting the extracellular region of TFR1 to its transmembrane region. HFE also interacts with the helical domain of TFR1^[37,38], competing with transferrin for its binding site^[39,40]. The resulting inhibition causes a reduction in transferrin-bound iron uptake in a variety of cell types^[41-44], suggesting that HFE is involved in the regulation of iron uptake by TFR1, possibly by limiting the amount of iron released from transferrin^[44]. HFE cycles with TFR1^[44], but its effect on cycling is controversial, with different groups reporting no effect^[44], a reduction in endocytosis^[45] or a reduction in exocytosis^[43].

The physiological consequences of the HFE-TFR1 interaction are difficult to ascertain given that the affinity of TFR1 for HFE is one to two orders of magnitude lower than for diferric transferrin^[46], implying that, at normal transferrin concentrations, almost no HFE would be associated with the receptor. However, these measurements were conducted on isolated proteins, so it is possible that, *in vivo*, the local environment of these proteins changes their interactions leading to a shift in the balance of competition between HFE and transferrin.

STEAP3

Following endocytosis and vesicle acidification, iron is reduced to its ferrous form prior to being transferred across the endosomal membrane. It was suggested some years ago that the transferrin receptor appeared to facilitate detachment of iron from transferrin in the endosome^[47]. More recently, it was shown that, at endosomal pH, the reduction potential of ferric iron co-ordinated by transferrin is increased when diferric transferrin is complexed to TFR1^[48], confirming the earlier observation and suggesting that reduction of iron occurs prior to release from transferrin. However, purely chemical reduction is unlikely to result in the highly efficient process of iron uptake seen in biological systems.

Despite evidence of endosomal ferrireductase activity^[49], it wasn't until recently that a candidate ferrireductase was identified^[50]. The gene *STEAP3* ("six-

transmembrane epithelial antigen of the prostate 3") is one of four genes wholly or partially deleted in the *nm1054* iron deficiency anaemia mouse. Under normal conditions, it is highly expressed in the liver, and its product is a protein which co-localises in endosomes with TFR1 and DMT1 (divalent metal transporter 1; see below). It is predicted to be a haemoprotein containing an N-terminal flavin-NADH binding domain. Most importantly, ferrireductase activity and iron uptake were lower in reticulocytes obtained from *nm1054* and *steap3* knockout mice and overexpression in HEK293T cells resulted in increased ferrireductase activity. A follow-up paper from the same group^[51] showed that the remaining three members of the Steap family (STEAP1, 2 and 4) were also ferri- and cupric- reductases. The four genes are ubiquitously expressed; however, different members are expressed more highly in some tissues than others. Foetal liver expresses all four transcripts, but adult liver expresses predominantly STEAP3 with a small amount of STEAP1^[50,51]. Like STEAP3, the other Steap proteins co-localise, at least partially, in an endosomal compartment with transferrin and TFR1.

Divalent metal transporter 1 (DMT1)

The released ferrous iron is transported from the interior of the endosome to the cytosol by DMT1 (also known as "natural resistance-associated macrophage protein 2", NRAMP2, "divalent cation transporter 1", DCT1, or "solute carrier family 11 member 2", SLC11A2). This protein is a transmembrane glycoprotein with 12 predicted transmembrane helices^[52,53] although there is no structure currently available to confirm this.

There are four known isoforms of DMT1, resulting from splice variation at the mRNA level. Alternative first exons (1 A or 1 B) give rise to the first level of variation^[54]. Secondly, each of the 5' splice variants may contain one of two 3' splice variations^[55]. The first of these contains an IRE in its 3'UTR. The second results in replacement of the final 18 codons of the open reading frame with a different sequence of 25 codons and a different 3'UTR, which, importantly, does not contain an IRE^[55]. The predominant form in the liver is the exon 1B + IRE form, although a small amount of the 1B-IRE form may also be present^[54].

Studies comparing the variants of DMT1 have indicated that the +IRE isoform is localised predominantly to the plasma membrane, exhibits slower internalisation kinetics than the -IRE isoform, and is targeted to lysosomes. In contrast, the C-terminal region of the -IRE isoform contains peptide signals which are required for efficient endocytosis and subsequent targeting to recycling endosomes^[56,57]. Thus, it is possible that the +IRE isoform is predominantly involved in iron transport across the plasma membrane whereas the -IRE isoform is involved in endosomal transport.

Evidence that DMT1 is the endosomal transporter is supported by the finding that DMT1 co-localises with TFR1^[58-60] and cycles through the endosomal compartment, appearing in acidic endosomes^[61]. DMT1 transports iron optimally at pH 5.5^[62], consistent with its presence in acidic endosomes and suggesting the energy for iron transport may be provided by a proton gradient. However, considerable transport also occurs at pH 7.4,

and a model for metal transport by DMT1 has been proposed which is consistent with symport of Fe^{2+} and H^+ from acidic endosomes and uniport of Fe^{2+} from a neutral environment^[63].

DMT1 appears to be regulated by iron levels with protein expression increased in iron loaded liver, lower in control liver, and not detected in iron deficient livers^[64]. Similar results have been obtained with the HepG2 hepatoma cell line^[65]. These findings are inconsistent with an IRE located in the 3'UTR of the transcript, which would be expected to result in a decrease in mRNA stability in iron loading with a concomitant decrease in protein expression. However, regulation of DMT1 is complex, and it is possible that the 5'UTR of the transcript or the N-terminal domain of the protein may modify the regulatory effects of the IRE in a tissue-specific manner^[54]. Additionally, regulation based around the stability of the protein cannot be ruled out.

LOW AFFINITY TRANSFERRIN UPTAKE

A second transferrin-mediated route of iron uptake (Figure 1, pathway 2) has been recognised in hepatocytes for many years^[14,66,67] and is probably responsible for the bulk of iron uptake by hepatocytes since, at the concentrations of transferrin present in the plasma, TFR1 would be saturated^[15,66,68]. The mechanism of uptake is similar to that of the TFR1-mediated pathway^[5,14,69]. After binding to the low affinity binding site, transferrin is endocytosed and iron is removed following acidification of the vesicle. Iron is sequestered away from the vesicle and apotransferrin is exocytosed^[69].

Transferrin receptor 2 (TFR2)

In 1999, Kawabata and colleagues^[70] reported the cloning of transferrin receptor 2 (TFR2), a type II transmembrane protein which shared significant sequence similarity to TFR1. It is currently the best candidate gene to code for the low-affinity binding site, with which it shares many similarities. TFR2 binds diferric transferrin specifically in a pH-dependent manner with an affinity 25-30 times lower than TFR1^[38,71]. In the liver, it is expressed predominantly in hepatocytes^[70,72,73] and mediates cellular transferrin and iron uptake^[70,73].

Regulation of TFR2 is different from regulation of TFR1. TFR2 mRNA does not contain any iron-responsive elements and cellular iron levels do not appear to change TFR2 mRNA or protein expression. Dietary and pathological iron loading do not result in decreased hepatic expression of TFR2 mRNA and neither does iron deficiency result in increased hepatic expression^[74]. Instead, TFR2 appears to be regulated at the protein level by cell cycle, with proliferating cells expressing approximately twice as many receptors as stationary cells^[75] and by the presence of diferric transferrin. Diferric transferrin causes an upregulation of receptor number and a redistribution of the protein to the cell surface in liver and hepatoma cells^[72,76]. The upregulation is caused by an increase in the half-life of the receptor conferred by its binding diferric transferrin. Removal of diferric transferrin results in a return to baseline expression^[77]. Consistent with these

findings, TFR2 protein levels were decreased with iron deficiency, and increased with iron loading in genetic models of iron overload, such as haemochromatosis, but not in the atransferrinaemic mouse which has impaired transferrin synthesis^[76]. Evidence suggests that TFR2 is a sensor of transferrin saturation and controls iron metabolism by regulating hepcidin expression^[78,79]. However, it is still not known whether changes in levels of TFR2 expression are correlated with changes in transferrin-bound iron uptake by the liver.

Given the similarities between TFR1 and TFR2, it was thought that, like TFR1, TFR2 may bind HFE. Co-localisation studies suggested an interaction in the duodenum. TFR2 was shown to co-localise with wild-type HFE in an early endosomal compartment whereas, in the presence of HFE^{C282Y}, the mutation predominantly associated with haemochromatosis type 1, TFR2 was distributed mainly to basolateral membrane^[80]. Despite this, initial *in vitro* binding studies indicated that there was no detectable interaction between soluble HFE and the soluble TFR2 ectodomain^[38]. More recently, an interaction has been demonstrated between the full-length, membrane-anchored HFE and TFR2 proteins^[81] suggesting that HFE may, indeed, be involved in TFR2-mediated iron uptake and TFR2-dependent regulation of hepcidin.

It has also been shown that TFR2, unlike TFR1, is present in lipid rafts and binding of diferric transferrin to TFR2 can activate the ERK1/2 and p38-MAPK signalling pathways^[82]. However, any connection of this to the hepcidin signalling pathway has yet to be demonstrated.

NON-TRANSFERRIN-BOUND IRON UPTAKE

The liver is one of the major sites of accumulation of iron delivered as low molecular weight chelates (Figure 1, pathway 3)^[83,84]. The pathophysiological relevance of such a process is apparent in diseases of iron overload such as hereditary haemochromatosis^[85]. The form of this low molecular weight plasma pool is likely to comprise several species; however, citrate appears to be the major component in both normal^[86,87] and haemochromatotic^[88] sera. In experimental situations, hepatocytes and their derivatives have been shown to take up iron from a variety of chelators^[89-105]. Iron taken up from these chelators has been shown to be distributed to haem and ferritin in hepatocytes^[90,101,103].

NTBI uptake by hepatocytes is linear for at least the first 15 to 60 min of incubation^[91,94,101,104]. It is also concentration dependent, with both ferrous and ferric iron, delivered as a variety of low molecular weight chelates, showing saturation kinetics^[95,97,106-108], indicating that this process is carrier-mediated. Uptake of iron as ferric citrate has been shown to be most efficient in normal rat hepatocytes at neutral pH^[92].

DMT1 as a major transporter of NTBI

NTBI uptake is increased in cells in which DMT1 mRNA and protein expression are upregulated^[109,110]. Furthermore, NTBI uptake appears to share at least one common pathway with TBI uptake since diferric transferrin has

consistently been shown to competitively inhibit uptake of NTBI^[93,102,111]. These observations, together with findings that DMT1 is active at neutral pH^[62], are consistent with DMT1 being a major transporter of NTBI in hepatocytes.

The specificity of NTBI uptake has been investigated by a number of groups and it has been generally observed that Cd, Co, Cu, Mn and Zn decrease iron uptake by normal and transformed hepatocytes^[97,104,108,109,112,113]. These observations match the range of divalent metals transported by DMT1^[53,114], adding further credence to the suggestion that DMT1 is a transporter of NTBI. Indeed, Mn and Cd appear to be transported by DMT1 with higher affinity than Fe^[114]. Although this observation is probably not relevant under normal physiological conditions, where the concentration of Fe is considerably higher than either Mn or Cd, it may become important in pathological conditions such as heavy metal poisoning in which competition for the transporter may result in a reduction in iron uptake. The alternative N and C termini conferred by the splice variants do not appear to affect the metal transport abilities^[62].

Certain inconsistencies in the data showing that some metals cause inhibition in some cell types, but not others have led to the suggestion of a family of transporters for iron and other transition metals^[101,111,115]. Several candidate transporters have been identified including calcium channels and specific transporters of other metals such as the zinc transporter, ZIP14 (zinc-regulated transporter and iron-regulated transporter-like protein 14).

ZIP14

ZIP14 (SLC39A14) is a transmembrane protein with eight predicted transmembrane helices^[112,113]. It is highly expressed in the liver, and is localised to the plasma membrane^[112]. There are two splice variants of the transcript; however, the biological functions of these two forms are yet to be determined. Originally shown to transport zinc, ZIP14 has also been shown to transport non-transferrin bound iron^[112,113,116]. But, it is not currently known whether ZIP14 is involved in hepatic iron loading. Importantly, ZIP14 has also been shown to be upregulated by interleukin-6^[112], which also upregulates hepcidin during inflammation^[117].

Calcium channels

The role of calcium channels in uptake of NTBI by the liver remains unclear. It has been suggested that L-type calcium channels are responsible for a significant component of ferrous iron uptake by cardiomyocytes, particularly under iron loaded conditions^[118,119]. However, information about any role for calcium channels in liver iron uptake is scant. Available evidence indicates that the transcripts coding for calcium channel subunits are expressed at low levels in the liver^[120], suggesting their participation in iron uptake by the liver is likely to be minor. However, levels of mRNA do not take into account any post-transcriptional modifications or functional regulation such as gating. Hence, the contribution of calcium channels to iron uptake by the liver requires further investigation.

It has also been suggested that calcium itself plays a functional role in NTBI uptake; however, this, too, needs

further clarification. Some studies have reported stimulation of NTBI uptake in cell types including hepatocytes^[99,101,104,121], whilst other studies have reported inhibition^[122] or no effect^[92]. It is possible that this spectrum of observations is due to variable chelation of calcium by the variety of chelators used to solubilise the iron^[123].

TRANSPORT OF OTHER IRON COMPLEXES

A number other forms of iron are recognised as being cleared from the circulation by the liver; however, these are likely to be mechanisms of clearance for their respective ligands rather than for uptake of iron, *per se*.

Specifically, these are ferritin, lactoferrin, the haem-haemopexin complex and the haemoglobin-haptoglobin complex. Circulating ferritin contains very small amounts of iron^[124-126] and, as such, it is not a major source of iron in the normal human. Nevertheless, the liver clears ferritin by a method involving binding to a specific ferritin receptor^[127-129] followed by endocytosis (Figure 1, pathway 4). There are several possible fates for endocytosed ferritin including catabolism of the protein in lysosomes^[130-132], excretion in the bile or inclusion in the endogenous ferritin pool^[133]. Any iron released is distributed to the mitochondria and endogenous ferritin^[130,132].

The uptake of the haem-haemopexin complex is mediated by its specific receptor, CD91 (Figure 1, pathway 5)^[134]. Following endocytosis, haem is degraded by haem oxygenase. Like transferrin, haemopexin was thought to be recycled back to the circulation^[135-137]; however, this fate has recently been questioned with evidence suggesting that it is substantially degraded in lysosomes^[134].

The haemoglobin-haptoglobin complex also binds to a high-affinity specific receptor and is endocytosed (Figure 1, pathway 6)^[138]. However, from this point, two possibilities exist for the fate of the complex. Both haemoglobin and haptoglobin may be directed to lysosomes for degradation^[139] or transported to the canalicular membrane of hepatocytes where haemoglobin is released into the bile and the receptor is recycled to the sinusoidal membrane^[140]. Clearance of the haemopexin and haptoglobin complexes by the liver is of importance in haemolytic states, especially those associated with intravascular haemolysis.

Lactoferrin is an iron-binding protein similar to transferrin which is present mainly in milk. Two lactoferrin binding sites have been reported on hepatocytes, although neither is specific for lactoferrin. The first is low-density lipoprotein receptor-related protein (LRP)^[141] and the second is the major (RHL-1) subunit of the asialoglycoprotein receptor^[142]. Lactoferrin appears to be cleared *via* receptor-mediated endocytosis regardless of its binding site (Figure 1, pathway 7)^[141,142]. Most of the internalised lactoferrin is directed to lysosomes for degradation^[143].

IRON RELEASE

Ferroportin (FPN)

The transporter, ferroportin (FPN; “solute carrier family 40 member 1”, SLC40A1; IREG1 or “metal transporter protein-1”, MTP1) was reported independently by three groups in 2000^[144-147] and appears to be the sole mediator

of iron release from hepatocytes (Figure 1, pathway 8)^[148]. Although it has not been shown directly, FPN appears to transport ferrous iron. Evidence comes from the apparent requirement of transport for ferroxidase activity. Caeruloplasmin knockout mice exhibit impaired hepatocellular and reticuloendothelial iron efflux which can be rescued by injection of caeruloplasmin^[149]. Similarly, mice with mutations in the membrane-bound ferroxidase, hephaestin, also exhibit impaired iron efflux^[150]. Further, iron efflux was stimulated in *Xenopus* oocytes over-expressing FPN in the presence of caeruloplasmin^[147]. There have been no reports to date indicating whether FPN-mediated iron transport is linked to transport of any other ion or whether there is any energy requirement for the process.

The structure and membrane topology of FPN is currently unclear with various models predicting between nine and twelve transmembrane helices^[151-153]. However, both the N- and C-termini appear to be located intracellularly^[153,154], which precludes an odd number of transmembrane segments. Similarly, the quaternary structure of FPN has been the subject of debate. Initial reports suggested that FPN was oligomeric^[155,156], but later reports cast doubt on this, suggesting a monomer^[152,157]. Recently, a comprehensive study by de Domenico *et al*^[154], demonstrated that FPN was most likely a dimer.

The quaternary structure of FPN may have important implications for regulation of iron homeostasis. Under the oligomeric model, the dominant negative phenotype of FPN-associated haemochromatosis (type 4) can be interpreted as interaction between wild-type and mutant forms of the protein interfering with its normal function^[155,156]. The alternative interpretation, haploinsufficiency, is less likely given that mice heterozygotic for a FPN knockout demonstrated a very mild phenotype and homozygotic knockout mice died *in utero*^[148]. Also, the majority of reports of human FPN-associated haemochromatosis with demonstrable iron loading involve heterozygotic point mutations which are at least partially functional^[156,158,159].

Like ferritin, FPN mRNA contains a functional IRE in its 5'-UTR^[144,147,160] indicating that translation should be augmented when iron is abundant. This has been shown to be true in HepG2 and Kupffer cells but not in the duodenum^[144,160,161] suggesting that regulation of FPN is cell-specific and one or more other regulatory mechanisms may be involved.

OTHER MOLECULES INVOLVED IN IRON HOMEOSTASIS

Hepcidin

Hepcidin is a 25 residue peptide containing four internal disulphide bonds which is produced in hepatocytes under conditions of iron sufficiency^[162-164]. It is created as a pre-pro-peptide which undergoes post-translational cleavage^[163], and its expression is regulated by inflammation and hypoxia as well as iron levels^[164,165]. It appears to be the focal point of an iron-regulatory pathway involving HFE, TFR2 and HJV, since disruption of these genes in haemochromatosis results in decreased hepcidin expression^[166-168]. Its expression is enhanced by cytokines such as interleukin-6 (IL-6)^[117].

In 2004, it was shown that FPN was a receptor for hepcidin^[169]. In HEK293 cells, the peptide was shown to bind to FPN and induce its internalisation in a dose-dependent manner. The complex was targeted to lysosomes for degradation^[169]. This is consistent with results that show increased FPN in the duodenum under conditions of iron deficiency, when hepcidin levels would be low^[144], and offers a mechanism for hepcidin-mediated anaemia of inflammation^[117,170] in which FPN levels are decreased resulting in a reduction of iron efflux to the plasma. The N-terminus of hepcidin is necessary for binding and internalisation of FPN, and the disulphide bonds appear to be necessary for its stability in the plasma^[171]. It is unclear whether hepcidin acts *in vivo* as an autocrine hormone, signalling to FPN in hepatocytes or as a paracrine hormone, signalling to FPN in Kupffer cells.

Haemojuvelin (HJV)

HJV is a protein known to play a very important role in hepatic iron homeostasis although its exact function and whether it plays a role in iron transport have yet to be ascertained. It is expressed in adult skeletal muscle, and foetal and adult liver in the periportal hepatocytes^[168,172]. Both soluble and membrane anchored forms have been demonstrated in Hep3B cells^[173]. Identified as the protein mutated in many cases of juvenile haemochromatosis^[168], human HJV shares 48% sequence identity with repulsive guidance molecules which are important in retinal development^[174]. Absence of functional HJV results in increased plasma transferrin saturation and ferritin in humans^[175,176] and studies in HJV knockout mice demonstrate decreased hepatic hepcidin expression and increased liver iron loading^[172,177]. The major function of HJV appears to be regulation of hepcidin levels, and it has been shown that HJV can bind bone morphogenic protein-2 (BMP-2), a member of the TGF- β superfamily of cytokines and activate hepcidin transcription *via* SMAD-4^[178,179]. This pathway is independent of HFE, TFR2 and IL-6^[180].

KUPFFER CELLS

Kupffer cells are the resident macrophages of the liver. Their main function in iron metabolism appears to be as a clearing house for iron from phagocytosed red blood cells^[181]. Haem breakdown is catalysed by haem oxygenase, and the products are ultimately excreted in the bile^[182]. Iron can be stored in Kupffer cells as ferritin. But, much of it is released back into the circulation^[183]. Consistent with this, Kupffer cells have been shown to strongly express both FPN transcript and protein^[184,185]; indeed, FPN is more highly expressed in Kupffer cells than hepatocytes^[144,184,186].

No functional studies on the role of FPN in iron release by Kupffer cells have been carried out. However, a number of studies have been undertaken using bone marrow-derived macrophages or macrophage cell lines. Following erythrophagocytosis or experimentally induced iron loading, the expression of many genes involved in iron metabolism, including FPN and haem oxygenase 1, are upregulated^[185,187]. In these cells, FPN is localised to intracellular vesicles, redistributing to the cell surface

following erythrophagocytosis^[188]. The upregulation of FPN results in an increase in iron release and its down-regulation results in a decrease in iron release^[189], consistent with involvement in iron recycling by Kupffer cells.

That FPN has been observed localised to intracellular vesicles in the absence of hepcidin suggests that FPN may play a role in intracellular redistribution of iron within Kupffer cells^[190] as well as in iron export. Addition of hepcidin resulted in rapid disappearance of FPN from the cell membrane, and subsequent degradation of the protein^[188], suggesting that in the absence of hepcidin, FPN may be able to cycle to intracellular compartments as necessary.

Kupffer cells also express TFR1^[191] indicating that they can obtain iron from transferrin if necessary. Interestingly, Kupffer cells also express high levels of HFE^[192]; however, they appear to be spared the level of iron loading associated with hepatocytes in HFE-associated haemochromatosis^[7]. This may indicate a difference in regulation of HFE in macrophages compared to hepatocytes or simply that iron loading of Kupffer cells is partially negated by the high level of iron exported from these cells^[183]. A recent report has suggested that GAPDH functions as a transferrin receptor in macrophages^[193]. However, the affinity of the interaction was extremely low and its importance is yet to be determined.

LIVER IRON TRANSPORT IN DISORDERS OF IRON METABOLISM

In the absence of any relevant genetic defects, an increase in plasma iron would result in an increase in transferrin saturation followed by a rise in the concentration of NTBI. Gene expression in the liver would change to sequester the iron in a non-toxic form, and signal to the duodenum to reduce iron absorption. In primary iron overload disorders, such as hereditary haemochromatosis, mutations in HFE, HJV or TFR2 result in a decrease in hepcidin production, and subsequent misregulation of iron absorption by the duodenum (as, of course, does a lack of functional hepcidin)^[194]. The mechanistic consequences of this for the liver are difficult, if not impossible, to dissect out from the resulting iron overload. This leads to the paradox of TFR2, an iron transporter which is sub-functional in type 3 haemochromatosis, resulting in hepatic iron overload^[71,195] rather than hepatic iron deficiency. This apparent paradox is probably the most telling demonstration of the liver's repertoire of iron transport and regulatory mechanisms.

Secondary iron overload is often a consequence of blood transfusions required for the treatment of certain types of anaemia such as β -thalassaemia or sideroblastic anaemia. The source of the excess iron is haem from transfused erythrocytes which are broken down in the normal way with the haem being catabolised, *inter alia*, by Kupffer cells. As with primary iron overload disorders, gene expression in secondary iron overload will change to reflect the cellular iron loading and the increase in plasma transferrin saturation and NTBI concentration despite the underlying anaemia.

Iron deficiency may be caused by a number of factors

including genetic disorders, pregnancy, an increased requirement for iron during growth, or simply by lack of dietary iron intake. The initial stage of iron deficiency corresponds to mobilisation of storage iron from the liver with decreases in hepatocyte ferritin, and an increase in iron uptake proteins such as TFR1^[196]. Plasma NTBI is generally considered to be non-existent or at very low levels in iron deficiency^[85].

CONCLUSION

Iron transport by the liver is, of necessity, tightly regulated because of the liver's myriad of transport pathways, and its role in iron homeostasis. The explosion of information in the past ten years describing many of the genes involved in liver iron transport has not only provided insight into the mechanisms involved, but also confirmed the complexities evident from the literature from previous decades. Nevertheless, much work remains to be done in piecing together this information in order to fully understand how the pathways of iron transport, the distribution of iron in the liver and the regulatory pathways interact and how they contribute to iron homeostasis.

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Liver-gut axis in the regulation of iron homeostasis

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Abstract

The human body requires about 1-2 mg of iron per day for its normal functioning, and dietary iron is the only source for this essential metal. Since humans do not possess a mechanism for the active excretion of iron, the amount of iron in the body is determined by the amount absorbed across the proximal small intestine and, consequently, intestinal iron absorption is a highly regulated process. In recent years, the liver has emerged as a central regulator of both iron absorption and iron release from other tissues. It achieves this by secreting a peptide hormone called hepcidin that acts on the small intestinal epithelium and other cells to limit iron delivery to the plasma. Hepcidin itself is regulated in response to various systemic stimuli including variations in body iron stores, the rate of erythropoiesis, inflammation and hypoxia, the same stimuli that have been known for many years to modulate iron absorption. This review will summarize recent findings on the role played by the liver and hepcidin in the regulation of body iron absorption.

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Key words: Iron; Homeostasis; Intestinal iron absorption; Liver; Hepcidin

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INTRODUCTION

Intestinal epithelial cells or enterocytes take up the predominating ferric iron from the diet through the combined action of an iron reductase (duodenal

cytochrome B or DcytB is a strong candidate) and a ferrous iron transporter known as divalent metal-ion transporter (DMT1) on the brush border membrane^[1,2]. Heme iron, on the other hand, appears to be absorbed through a separate system, and a recently identified apical membrane protein, HCP1, has emerged as a candidate heme transporter^[3]. Irrespective of the form in which iron crosses the brush border, enterocytes export iron into the circulation by the combined action of an iron reductase, hephaestin, and a basolateral membrane iron transporter, ferroportin (FPN). The newly absorbed iron is then bound to circulating transferrin which distributes it around the body to sites of utilization and storage.

The amount of iron transported across the enterocytes is ultimately influenced by body iron requirements. Thus, for example, when body iron levels are low or when erythropoietic demand is increased, iron absorption is elevated. The factors that alter iron absorption exert their effects by influencing the duodenal expression of the major iron transport molecules, particularly DMT1, Dcytb1 and ferroportin^[1,2,4]. Early kinetic studies suggested that it was the efflux of iron out of the enterocytes and into the circulation that was rate limiting for absorption^[5], and more recent molecular studies have provided support for this concept^[6,7]. In particular, this work suggested that basolateral iron transfer by ferroportin was most likely the primary regulatory step. But, how signals from distant sites modulate iron release from enterocytes has until recently remained poorly understood. The missing link in this regulatory pathway has now emerged as the liver-derived peptide hormone hepcidin, and thus we know that the liver plays a central role in the regulation of body iron homeostasis. Hepcidin regulates plasma iron levels by controlling the cell surface expression of ferroportin, and this in turn limits the efflux of iron from enterocytes, macrophages and a number of other cell types.

HEPCIDIN

Hepcidin was first discovered as an antimicrobial peptide in human blood ultrafiltrate^[8] and urine samples^[9]. The gene encoding hepcidin (*HAMP*) is very strongly expressed in the liver. But, weak expression has also been detected in heart, spinal cord, stomach, intestine and lungs^[8-10]. The first evidence linking hepcidin to iron metabolism came almost simultaneously from two groups. One group, using suppressive subtractive hybridization, demonstrated that the levels of the hepcidin transcript were greatly increased in the livers of iron loaded mice^[10]. The other group

<i>Homo sapiens</i>	D	T	H	F	P	I	C	I	F	C	C	G	C	C	H	R	S	K	C	G	M	C	C	K	T
<i>Canis familiaris</i>	D	T	H	F	P	I	C	I	F	C	C	G	C	C	K	T	P	K	C	G	L	C	C	K	T
<i>Sus scrofa</i>	D	T	H	F	P	I	C	I	F	C	C	G	C	C	R	K	A	I	C	G	M	C	C	K	T
<i>Bos taurus</i>	D	T	H	F	P	I	C	I	F	C	C	G	C	C	R	K	G	T	C	G	M	C	C	R	T
<i>Mus musculus</i>	D	T	N	F	P	I	C	I	F	C	C	K	C	C	N	N	S	Q	C	G	I	C	C	K	T
<i>Rattus norvegicus</i>	D	T	N	F	P	I	C	L	F	C	C	K	C	C	K	N	S	S	C	G	L	C	C	I	T
<i>Danio rerio</i>	Q	S	H	L	S	L	C	R	F	C	C	K	C	C	R	N	K	G	C	G	Y	C	C	K	F
<i>Salmo salar</i>	Q	I	H	L	S	L	C	G	L	C	C	N	C	C	H	N	I	G	C	G	F	C	C	K	F

Figure 1 Sequence alignment of hepcidin from various species.

inadvertently engineered a mouse strain with negligible hepcidin expression (considered in more detail below), and found that these animals accumulated high levels of body iron^[11]. *HAMP* was mapped to human chromosome 19, and it encodes an 84 amino acid pre-pro-peptide^[9,10]. Pre-pro-hepcidin is ultimately processed into several smaller peptides that consist of the 20, 22 or 25 C-terminal amino acids^[9]. The 25 amino acid peptide has eight cysteine residues forming four intramolecular disulfide bonds, and is the biologically active form of hepcidin^[9,12]. The eight cysteine residues are highly conserved among species from zebra fish to humans (Figure 1). Two-dimensional nuclear magnetic resonance (NMR) spectroscopy showed that hepcidin forms a distorted hairpin-like structure, and NMR diffusion studies demonstrated that the 25 residue peptide forms aggregates involving the first five N-terminal residues^[13]. The 20 and 22 amino acid forms, that lack the N-terminal residues, do not form aggregates and have much reduced iron regulatory capacity^[12,13]. Removal of individual disulfide bonds did not reduce the hepcidin function significantly *in vitro*. However, serial deletions of the N-terminal residues progressively reduced hepcidin activity^[12].

The first animal model describing the relationship between hepcidin and iron homeostasis came serendipitously from the knockout of an adjacent gene encoding upstream stimulatory factor 2 (USF2)^[11]. The USF2 knockout mice of this strain (Paris USF2) had very low levels of hepcidin transcript in the liver and developed multi-organ iron overload (but with relative sparing the spleen) and high transferrin saturation, a phenotype consistent with the human iron loading disease hereditary hemochromatosis^[11]. These results provided the first indication that hepcidin was a negative regulator of iron uptake from the intestine and of iron release from macrophages. Another USF2 knockout mouse strain (Houston USF2)^[14] had normal hepcidin levels, and showed no aberrations in iron metabolism^[15], indicating that the Paris USF2 knockout phenotype was due to hepcidin deficiency. Since these original studies, a specific hepcidin knockout mouse has been generated, and it also shows an iron loading phenotype^[16]. In contrast, mice over expressing hepcidin display decreased body iron levels and a microcytic hypochromic anaemia typical of severe iron deficiency^[15]. The majority of these mice die within a few hours after birth suggesting that hepcidin also inhibits placental iron transport^[15]. A similar situation has been described in humans, and patients with hepatic adenomas producing abnormally high levels of hepcidin suffer from a severe iron refractory anaemia that only resolved after resection of the tumour^[17]. As might be expected from these results, injection of synthetic hepcidin peptide into mice leads to inhibition of intestinal iron absorption and consequently hypoferrremia^[18].

Taken together, these data provide strong evidence that hepcidin is the central regulator of body iron levels.

In humans, mutations in the *HAMP* gene result in a severe form of iron loading disease that presents at early age, and is aptly named juvenile hemochromatosis (JH). *HAMP*-associated JH is inherited in an autosomal recessive manner, and two mutations have been described (93delG and C166T) that are associated with iron loading when present in the homozygous state^[13,19]. While heterozygosity for these mutations alone does not lead to iron loading, compound heterozygosity between two other *HAMP* mutations (Met50del IVS2+1(-G) and G71D) and C282Y, the most common mutation in patients with HFE-associated iron loading, has been reported to result in hemochromatosis^[20]. Thus *HAMP* mutations may act as modifiers of the HFE-associated hemochromatosis phenotype.

Clearly hepcidin plays a major role in the regulation of intestinal iron absorption. But, how does it exert its effects? Soon after the link between hepcidin and iron was recognised, a close inverse correlation between *HAMP* expression and iron absorption and the expression of duodenal iron transporter transcripts was described^[21]. It was suspected that hepcidin interacted with a receptor on the basolateral surface of the enterocytes, thereby activating one or more signal transduction pathways that ultimately led to changes in the expression of the iron transport genes. The truth turned out to be elegantly simple. Hepcidin acts by directly binding to the sole basolateral iron export molecule, ferroportin, and causing its internalisation and subsequent degradation^[22]. Thus ferroportin is the hepcidin “receptor”. This loss of ferroportin on the cell surface reduces iron export from the cells leading to intracellular iron accumulation. As ferroportin is responsible for iron export from both enterocytes and macrophages, loss of this protein will result in reduced supply of iron to the plasma and, hence, will cause hypoferrremia and, ultimately, anaemia. Consistent with this mechanism is the observation that mice lacking hepcidin show decreased iron in the spleen, an organ rich in macrophages, in the face of increased hepatic iron^[15]. Similarly, J774 mouse macrophages treated with hepcidin peptide showed decreased levels of ferroportin and reduced the efflux of iron^[23]. Hepcidin likely acts on iron export from other cell types, such as hepatocytes, in a similar fashion, and this can explain its key role in regulating iron traffic into and around the body.

Since hepcidin interacts with FPN, it might be expected that mutations in FPN that alter this interaction could essentially mimic hepcidin deficiency. This has been found to be the case. Two classes of FPN mutations have been identified in human subjects and both lead to iron loading.

However, there are subtle differences in the phenotypes, with one being consistent with reduced iron transport by the protein and, the other consistent with impaired interaction with hepcidin^[24].

SYSTEMIC FACTORS THAT REGULATE HEPCIDIN

Consistent with its role as a central regulator of body iron metabolism, hepcidin levels are modulated by the same factors that alter iron homeostasis. Changes in body iron stores, the rate of erythropoiesis, inflammation and hypoxia all influence iron absorption and iron release from macrophages and these are the major systemic factors that regulate *HAMP* mRNA levels in the liver.

Hepcidin levels are increased in response to oral and parenteral iron loading and decreased under iron deficient conditions^[10]. This inverse relationship is seen with chronic changes in body iron status. But, it can also occur quite quickly, and *HAMP* mRNA levels in the liver can decrease within days of transferring rats from a control to iron deficient diet^[21]. The regulation of hepcidin by body iron levels acts as a feedback mechanism to allow sufficient iron to enter the plasma when demand is high, but to limit iron intake/release in times of iron sufficiency. How hepcidin responds to changes in body iron levels is incompletely understood. Since hepcidin expression is largely restricted to the liver, it is highly likely that the hepatocyte is the site of action of the regulatory stimulus. But, whether hepatocyte iron levels per se play a primary role or whether an external signal is involved is unclear. This will be considered in more detail in the following section.

Interestingly, *in vitro* loading of hepatocyte cell lines and primary hepatocytes with iron decreases *HAMP* mRNA expression, the opposite effect to that seen *in vivo*^[25]. Why this is the case has proved difficult to resolve. One possibility is that the iron supplied to the cultured cells is of a different form to that presented to the liver *in vivo*. This may be the case, but the *in vitro* reduction in hepcidin expression is seen when both iron salts and transferrin-bound iron is presented to the cells. A second possibility is that during the isolation and culture procedure liver-derived cells lose some factor that is critical for their normal physiological response to iron. Since the same effect is seen on freshly isolated primary hepatocytes this appears unlikely. But, it remains possible. Potentially the most satisfying explanation for the observations is that the response of the liver to iron *in vivo* requires the interaction of two or more types of cells, and that this interaction is lost after the cells are isolated. The liver macrophages or Kupffer cells are strong candidates for cells that might interact with hepatocytes to regulate iron homeostasis. But, several studies have now shown that when animals are depleted of macrophages (including Kupffer cells) their livers respond normally by increasing hepcidin expression in response to iron^[26,27]. Thus it appears that macrophages are not required for hepcidin regulation in the liver in response to iron *in vivo*. A final possibility is that reduced expression of hepcidin is the normal physiological response of hepcidin to iron, and that the *in vivo* situation is complicated by a range of interacting stimuli that

influence expression of the *HAMP* gene. Further work is required to resolve this issue.

The largest single sink for iron in the body is haemoglobin in the red blood cells, and consequently iron demand is closely linked to the rate of erythropoiesis. Thus when erythropoiesis is stimulated, following phenylhydrazine-induced hemolysis for example, hepcidin expression is suppressed to allow increased iron flow into the plasma and consequently to the developing red cells^[15,28]. This hepcidin response is observed only in the presence of erythropoiesis as suppression of erythropoiesis by irradiation or by post-transfusion polycythemia leads to increased hepcidin levels^[29,30]. The regulation of hepcidin mRNA levels by erythropoiesis is independent of direct erythropoietin effects^[30], and is likely to reflect several stimuli. The iron requirement of the erythroid marrow is certainly a major factor. But, hypoxia too is also likely to be important as reduced haemoglobin may reduce oxygen delivery to the tissues. The response of hepcidin to hypoxia is considered in more detail below. In a recent study by Ganz and colleagues, it was concluded that in addition to iron requirements and hypoxia, there is an erythropoiesis-specific factor that affects hepcidin expression^[30]. However, this factor has yet to be characterized.

Another situation where body iron homeostasis is perturbed is during inflammation or infection. Under these circumstances, iron absorption declines and iron is sequestered in macrophages, with the consequence that the plasma iron level is decreased (hypoferrremia). With chronic inflammation or infection, anemia may result, and this condition is often called the anemia of chronic disease^[31]. Consistent with the reduction in plasma iron is the demonstration that inflammatory stimuli positively regulate hepcidin levels^[32-35]. Increased hepcidin means decreased iron entry into the plasma. That hepcidin is responsible for the hypoferrremia accompanying inflammation has been shown by studies with *Hamp* null mice. These animals mount a standard inflammatory response to a stimulus such as bacterial lipopolysaccharide (LPS), but the expected hypoferrremia does not occur^[32].

One of the major mediators of the inflammatory response is the cytokine IL-6. IL-6 infusion in humans or administration to experimental animals leads to an increase in hepcidin production and decrease in serum iron levels within a few hours^[36]. A time course analysis in human subjects injected with LPS revealed a strong temporal correlation between increases in serum IL-6 and urinary hepcidin, and the decrease in serum iron^[34]. Similarly, IL-6, other pro-inflammatory cytokines like IL-1 α and IL-1 β and LPS stimulate hepcidin in primary hepatocytes and hepatoma cell lines^[36,37].

Many inflammatory processes have a systemic component, and these are able to influence hepcidin expression in hepatocytes. However, there is increasing evidence that hepcidin may also be relevant in the local, extra-hepatic setting. For example, in an *in vivo* murine granulomatous pouch model of infection the host animals responded to bacterial infection by upregulating hepcidin at the local level, presumably to limit availability of iron to the pathogens in the immediate vicinity^[35]. The

cells responsible for the local production of hepcidin are unknown, but may be infiltrating macrophages and neutrophils. Indeed hepcidin expression has been detected in myeloid cells in response to systemic infection^[38]. Hepcidin production also has been demonstrated in adipose tissue. But, again the responsible cell type is not known^[39].

At the whole body level, hypoxia is usually associated with a reduced amount of circulating hemoglobin, and the body's response to this deficiency is to stimulate erythropoiesis. This in turn requires an increased iron supply. It thus comes as no surprise that hepcidin levels drop 2-4 d after animals are placed in a hypoxic chamber^[32], and that luminal iron uptake is increased in the small intestine under the same conditions^[40]. Some of the *in vivo* effects of hypoxia cannot be attributed to the direct repression of hepcidin expression by low oxygen as increased erythropoiesis may reduce hepcidin levels by other mechanisms^[30]. But, the demonstration that hypoxia down regulates hepcidin mRNA levels in human hepatoma cell lines^[32] suggests that the *HAMP* gene itself may be regulated by hypoxia. In addition, hypoxia could also trigger a stress response in cells and animals, and *HAMP* is a known stress response gene^[33].

The factors that regulate hepcidin levels described above vary in their relative strength, and in certain situations, where more than one stimulus is present, one may predominate. A good example of this is in β -thalassemia. In both mice and humans with this disorder, hepcidin levels are initially low despite increased levels of storage iron, and thus the erythropoietic stimulus is predominating^[41-43]. However, as the disease advances the effect of the increasing iron stores become relatively stronger and hepcidin levels increase. A similar situation is found in hypotransferrinaemic mice^[17] and in iron loaded animals treated with PHZ to induce anaemia and erythropoiesis^[32] where the erythropoietic stimulus predominates.

THE MOLECULAR BASIS OF HEPCIDIN REGULATION

While the major physiological factors that alter hepcidin expression have been identified, how these stimuli signal the liver to alter hepcidin expression, and how the changes in expression are brought about is complex and only partially understood. Important clues in dissecting these regulatory pathways have come from the analysis of human iron loading disorders and their equivalent murine models. Patients with mutations in the *HFE*, transferrin receptor 2 (*TfR2*), hemojuvelin (*HJV*) or *HAMP* genes all show a histological pattern of iron deposition that is similar and consistent with elevated iron absorption. Furthermore, *HFE*, *TfR2* and *HAMP* all show their highest expression in the liver and *HJV* shows strong expression in this organ. These similarities suggested that the proteins these genes encode may form part of the same regulatory pathway. This has now proved to be the case.

Classical, adult onset hereditary hemochromatosis (HH) results from mutations in the *HFE* gene, and both human

patients and mouse strains with a disrupted *HFE* gene show periportal iron deposition with relative sparing of the Kupffer cells^[44,45]. *HFE* is expressed on the cell surface as a complex with β_2 -microglobulin (β_2m) and mouse models of β_2m deficiency show a similar iron loading phenotype^[46-48]. Since hepcidin expression is increased with iron loading, it was expected that disruption of the *HFE* gene would lead to enhanced hepcidin levels. When this was investigated, however, the opposite was seen. Human patients with *HFE*-associated hemochromatosis showed significantly lower levels of liver hepcidin expression than control subjects^[49]. In *Hfe* knockout mice, hepcidin levels were similar to those of wild-type animals. But, the level remained low even when the knockout animals were fed a high iron diet^[50]. Taken together, these observations show that *HFE* is an upstream regulator of hepcidin, and not a downstream target as was previously believed^[49]. Proof of this was provided by Nicolas and colleagues who showed that mice over expressing hepcidin, but null at the *Hfe* locus, did not accumulate excess iron^[51]. Despite low hepcidin levels in patients with HH, hepcidin levels still increase as the body iron load increases indicating that hepcidin regulation is not completely disrupted when *HFE* is mutated^[49,52]. This is consistent with the milder phenotype of patients with *HFE*-associated HH compared to those with mutations in the *HAMP* gene^[24].

If *HFE* acts as an upstream regulator of hepcidin, how does it respond to changes in body iron demand? The answer to this question is not clear. But, the level of circulating diferric transferrin has emerged as a potential regulator^[53,54]. *HFE* is able to bind to TfR1, the major cell-surface diferric transferrin binding protein^[55,56], and *HFE* and transferrin are able to compete for TfR1 binding. Such competition could modulate the amount of *HFE* that is not bound to TfR1 that in turn could transduce a signal to alter hepcidin expression. It is also possible that *HFE* interferes with the cellular uptake of transferrin-bound iron, and that this in turn affects *HAMP* expression^[57]. Support for diferric transferrin as a signal to alter *HAMP* expression has come from the demonstration that another transferrin binding protein, TfR2, also is involved in hepcidin regulation.

Transferrin receptor 2 encodes a protein that shares 45% identity with TfR1^[58]. However, in contrast to its widely expressed homolog, TfR2 expression is restricted to the liver, spleen, brain and heart, with highest expression in the liver^[58]. Mutations in TfR2 lead to iron overload in human patients^[59] with a clinical picture similar to classical *HFE*-associated hereditary hemochromatosis^[60]. The same phenotype is seen in TfR2 knockout and mutant mice^[61,62]. As in *HFE*-associated iron loading, hepcidin expression is decreased in the liver of TfR2 mutant mice^[62,63], and patients with TfR2-related hemochromatosis also show decreased urinary hepcidin levels^[64]. The severity of TfR2-related hemochromatosis is much less than that associated with mutations in *HAMP* or *HJV*, but is similar to *HFE*-associated hemochromatosis.

Like *HFE*, TfR2 appears to be an upstream regulator of hepcidin. But, how it exerts its effects is unknown. It has been reported that, unlike TfR1, TfR2 does not bind to *HFE*, and shows 25 times less affinity for transferrin compared to TfR1^[65] making it unlikely that TfR2 makes a

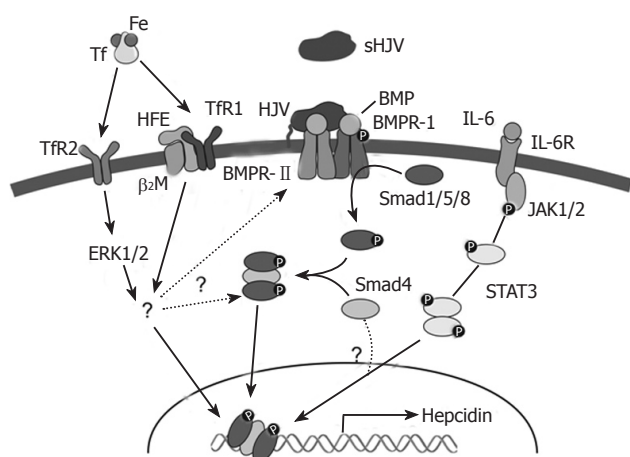


Figure 2 Signalling pathways for *HAMP* regulation.

major contribution to cellular iron uptake. Indeed, in *Tfr2* knockout mice, the liver accumulates iron very efficiently indicating that *Tfr2* is not essential for hepatic iron uptake. In contrast, a recent study has shown that when *Tfr2* and *HFE* are over expressed together in the same cells they can be co-immunoprecipitated^[66]. Whether this interaction is physiologically relevant or represents an overexpression artefact remains to be determined. Interestingly, two independent studies have demonstrated that *Tfr2* protein levels are upregulated by diferric transferrin, but do not respond to apotransferrin or non-transferrin bound iron^[67,68]. This effect of diferric transferrin is not observed at the transcript level, and appears to be post-translational^[68]. Recently, it has been reported that the interaction between diferric transferrin and *Tfr2* activates ERK1/ERK2 and p38 MAP kinase, but only when *Tfr2* is present on the lipid rafts of the exosomes^[69]. However, the precise mechanisms and signalling pathway of *Tfr2*-hepcidin axis are not yet understood and remain to be further explored.

The third important protein known to be involved in the regulation of hepcidin is hemojuvelin (HJV, RGMc). HJV, is a member of the repulsive guidance molecule (RGM) family, and shares some common features with RGMa and RGMb, including a C-terminal glycosylphosphatidylinositol-linked membrane anchor (GPI-anchor), N-terminal signal sequence, proteolytic cleavage site and partial von Willebrand factor type D domain^[70,71]. Mutations in the *HJV* gene cause severe iron overload, and lead to juvenile hemochromatosis due to greatly decreased hepcidin levels in human patients^[72] and mice^[73,74]. Moreover, knocking down HJV with siRNA in Hep3B cells leads to a decrease in hepcidin levels^[74]. Interestingly, HJV expression is strongest in heart and skeletal muscle, but also shows moderate expression in the liver^[72], where *HAMP* is most strongly expressed. Treating primary hepatocytes with soluble HJV led to a decrease in hepcidin levels suggesting that a binding competition exists between soluble and cell-associated hemojuvelin^[75]. In addition, increasing iron concentrations led to a decrease in soluble HJV (sHJV) in cells over expressing HJV^[75], indicating that iron could regulate HJV at the post-transcriptional level.

HJV acts as a co-receptor for the bone morphogenetic proteins (BMPs), in a similar fashion to other molecules of the Rgm family, and HJV mutants have impaired BMP signalling^[76]. BMPs represent a subfamily of transforming growth factor-beta (TGF- β) ligands that signal by binding to and bringing together type I and type II BMP receptors on the cell surface, and then propagating the signal through phosphorylation of the Smad proteins^[77] (Figure 2). BMPs phosphorylate receptor-regulated Smads 1, 5 and 8 that in turn form heteromers with the co-mediator Smad 4. The activated complex then translocates to the nucleus and, in combination with other factors, regulates target genes such as *HAMP*^[77]. Cells transfected with the co-receptor HJV or treated with the ligand BMP-2 showed increased levels of hepcidin, and BMP-2 induction was enhanced in the presence of HJV^[76]. Other BMPs, BMP-4 and BMP-9, have been shown to have a similar effect on hepcidin expression independent of *HFE* and *Tfr2* status^[78]. This suggests that HJV acts *via* a *HFE*/*Tfr2* independent pathway to alter *HAMP* levels. Further insight into this aspect of hepcidin regulation comes from the studies of the liver-specific Smad 4 knockout mouse. These animals showed markedly decreased levels of hepcidin, increased duodenal transporters and iron overload^[79]. Overexpression of Smad 4 led to transcriptional activation of *HAMP* due to epigenetic modification of histone H3 protein^[79]. Smad 4-deficient hepatocytes showed no increase in hepcidin levels upon treatment with BMP, iron, TGF- β or IL-6^[79]. This response to BMP is expected as Smad 4 acts downstream in the signalling pathway^[79]. However, the result with IL-6 is interesting as it indicates that the IL-6 pathway and the BMP-Smad pathway converge at some point. Overall, these data indicate that the BMP/SMAD pathway plays an important role in the regulation of *HAMP* gene expression. Whether this is the major pathway operating or other pathways are dominant remains to be determined, as the relationship between *HFE*, *Tfr2* and HJV (Figure 2).

Pro-inflammatory molecules like LPS and FCA positively regulate hepcidin expression by inducing the expression of cytokines such as IL-6. This induction of hepcidin by pro-inflammatory cytokines appears to be independent of *HFE*, β_2m and *Tfr2*^[80,81], although one study provided evidence that these proteins may play some role^[82]. The differences in these studies could be due to the difference in the timing and dosage of the treatments. IL-6 alters *HAMP* transcription through the classical JAK-STAT pathway^[83,84]. This pathway ultimately leads to activation of STAT3 that binds to an element in the proximal 100 bases of the *HAMP* promoter. The interesting observation that the IL-6 dependent stimulation of the *HAMP* gene is abrogated in Smad 4 knockout mice indicates that Smad 4 is involved in the signalling process as well. This has yet to be investigated in detail, as have the mechanisms by which other pro-inflammatory cytokines stimulate *HAMP* expression.

Although it is widely considered that the *HAMP* gene is regulated predominantly at the level of transcription, relatively few promoter analyses have been carried out. As noted above, STAT3 is known to bind to the promoter, and several other transcription factors have been

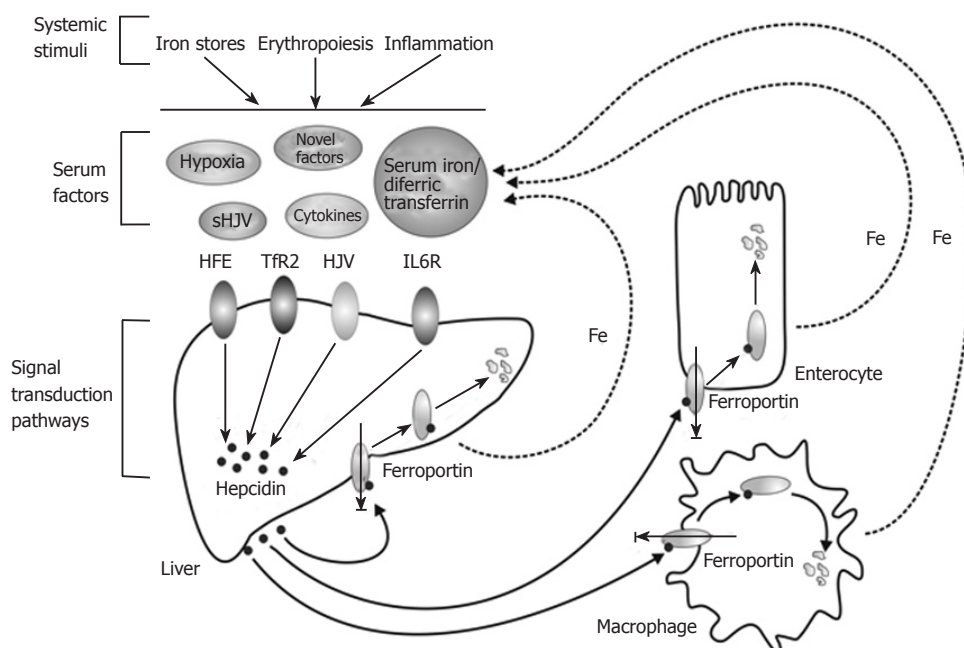


Figure 3 A model for the liver-dependent regulation of iron homeostasis.

identified. C/EBP α , a liver-enriched transcription factor, has been shown to bind the *HAMP* promoter 230-250 bp upstream of the transcription start site^[85], and may drive basal transcriptional activity of the gene. Supporting such a role, C/EBP α knockout mice showed decreased hepcidin levels and increased iron staining in their livers^[85]. In another study, the role of the basic helix-loop-helix leucine zipper (bHLH-ZIP) family of transcriptional regulators, and notably USF2, in *HAMP* transcription was studied in order to investigate the reasons behind the lack of hepcidin expression in the USF2 knock out mouse. It was demonstrated using site-directed mutagenesis, chromatin immunoprecipitation assays and mobility shift assays that USF1/USF2 and c-Myc/Max bind to E-boxes in the hepcidin promoter, and regulate its transcription^[86]. Because some genes with E-boxes show rhythmicity, it has been proposed that hepcidin might also be under pulsatile or rhythmic transcriptional control. However, the signals and cellular pathways that lead to the activation of these proteins in the context of hepcidin regulation remain to be resolved.

A MODEL FOR THE LIVER-DEPENDENT REGULATION OF IRON HOMEOSTASIS

The discussion above has highlighted various factors that regulate hepcidin, and the current knowledge about the molecular mechanisms behind their effects. We previously proposed a model to explain the regulation of hepcidin in a physiological context^[53], and present an updated version of the model here (Figure 3). In this model, signals that alter body iron homeostasis (iron stores, erythropoiesis, inflammation, hypoxia) act on the hepatocytes in the liver to modulate *HAMP* gene expression. Some of these stimuli may act directly on the liver cells (e.g. hypoxia), while others may act indirectly e.g. iron stores may act through changing the levels of diferric transferrin in the circulation. How the signals alter *HAMP* expression at the

molecular level is incompletely understood. HFE, TfR2 and HJV are clearly involved in the process and, in the case of the latter; signalling through the BMP/SMAD pathway is a likely mode of action. Pro-inflammatory cytokines such as IL-6 act through the JAK/STAT pathway to regulate *HAMP* transcription. Hepcidin secreted by the liver acts on mature enterocytes in the proximal small intestine to reduce iron export into the circulation. Thus, high hepcidin means reduced iron absorption and vice versa. Hepcidin also acts on macrophages, hepatocytes, and likely other cells in the body to regulate their release of iron, so it plays a universal role in iron homeostasis.

Hepcidin levels show a positive correlation with transferrin saturation that is independent of the liver iron content^[21] indicating that the levels of iron bound to transferrin may be a major signal that regulates the expression of hepcidin in the liver. Diferric transferrin is essential for iron delivery to the tissues making it suitable as an indicator of plasma iron status. Transferrin saturation thus decreases with iron deficiency and increases with iron loading. Diferric transferrin competes with HFE for binding to TfR1, and their binding sites overlap. But, it has a higher affinity for its principal receptor than HFE^[87], and thus out-competes HFE for binding to TfR1. This leaves HFE “free” on the cell surface to initiate a signal to stimulate hepcidin expression. Similarly, diferric transferrin binds to TfR2, although with lower affinity, and sends a signal via the proposed ERK1/2 and MAPK pathways^[69]. The signal transduction pathways driven by HFE and TfR2 that lead to the regulation of hepcidin expression are not yet clear. HFE and TfR2 may form a stable complex with each other as has been proposed^[66] or may interact with HJV/BMPRs to propagate the signalling *via* the Smad1, 5, 8/Smad4 pathway. But, the precise details have yet to be elucidated.

In iron deficiency, TfR1 and transferrin levels increase, and transferrin saturation drops. The balance shifts towards monoferric transferrin that has lower binding affinity for TfR1 compared to diferric transferrin. This

results in increased binding of HFE to TfR1, decreased signalling, and consequently lower hepcidin production. Similarly, as the plasma iron level falls, TfR1 binds residual diferric transferrin more efficiently than TfR2. This down regulates the TfR2-ERK signalling pathway, and thus hepcidin synthesis. When body iron levels are high the opposite pattern is seen, and hepcidin expression is increased. Stimulated erythropoiesis is another major stimulus for iron absorption, and at least one mechanism by which it might exert its effects on reducing hepcidin expression is *via* reduced transferrin saturation.

CONCLUSION

Recent developments in understanding the molecular mechanisms of iron homeostasis have greatly enhanced our knowledge of iron absorption in the gut. The most important advance in this area has been the recognition that the liver-derived peptide hepcidin responds to variations in body iron demand, and acts on the proximal small intestine to regulate iron efflux into the plasma. This shows that the liver plays a central role in the regulation of body iron homeostasis. Various systemic stimuli including iron stores, the rate of erythropoiesis, and oxygen levels regulate hepcidin expression, and consequently iron absorption. But, how systemic signals are received by the liver, and how these signals are transduced into changes in *HAMP* gene expression are incompletely understood. Strong evidence now suggests that signalling through the BMP/Smad pathway plays a major role in regulating hepcidin. But, how universal this pathway is has yet to be resolved.

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TOPIC HIGHLIGHT

Nathan Subramaniam, PhD, Series Editor

Role of iron in hepatic fibrosis: One piece in the puzzle

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Abstract

Iron is an essential element involved in various biological pathways. When present in excess within the cell, iron can be toxic due to its ability to catalyse the formation of damaging radicals, which promote cellular injury and cell death. Within the liver, iron related oxidative stress can lead to fibrosis and ultimately to cirrhosis. Here we review the role of excessive iron in the pathologies associated with various chronic diseases of the liver. We also describe the molecular mechanism by which iron contributes to the development of hepatic fibrosis.

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Key words: Iron; Fibrosis; Oxidative stress; Hepatic stellate cell; Haemochromatosis; Hepatitis C; Non-alcoholic fatty liver disease; Alcoholic liver disease

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INTRODUCTION

Iron, created by stellar nucleosynthesis, is the most abundant element on Earth in terms of mass, making up 35% of total planetary mass. Iron, in its ferrous (Fe^{2+}) and ferric (Fe^{3+}) forms, is critical to all life forms from the simplest filamentous algae through to the most complex multicellular organisms. Iron is an essential element mainly present within the cells in association with haemoprotein (haemoglobin, myoglobin) or within an iron-sulphur cluster of various metalloproteins (e.g., aconitase and

Rieske proteins of the respiratory chain)^[1]. Iron is involved in the redox-driven processes of oxygen transport, electron transport and various enzymatic reactions such as DNA synthesis, transcriptional regulation, catalysis as well as nitric oxide (NO) and oxygen sensing^[1]. Mammals do not have any major physiological pathway for iron excretion. Therefore, iron uptake and storage is closely regulated so as to avoid deficiency, and excess. In humans, iron deficiency is manifested as anaemia and with increasing severity can eventually result in cardiac failure. Iron overload, especially at sites of storage, enhances oxidative stress ultimately leading to lipid, nucleic acid and protein peroxidation. Within the liver, which is the major site of iron storage, enhanced oxidative stress can lead to fibrosis, cirrhosis, hepatocellular carcinoma (HCC) and death.

In this review, we outline the hepatic disease states where iron is an important factor in disease progression. We also discuss the role of iron in promoting liver fibrosis and those cells and mechanisms most important in the underlying wound healing/fibrotic processes.

HEPATIC FIBROSIS

Hepatic fibrosis is promoted by various pathogenic, mechanical and toxic insults to the liver, and is part of a physiological wound healing response. If the injurious stimuli are chronic, the degree of fibrosis worsens, leading to cirrhosis, and eventually to hepatic failure and death. The interactions between various resident hepatic cell populations and immune cells that lead to the establishment of fibrosis are complex, and not yet fully understood. However, some profibrogenic pathways and clinical outcomes are common to several disease states, and these will be briefly outlined below.

In the normal liver, a balance is struck between extracellular matrix (ECM) deposition and degradation, a process that is tightly regulated by matrix metalloproteinases (MMP) and their specific inhibitors (TIMPs)^[2]. Fibrosis is associated with major quantitative and qualitative changes in the ECM. These changes are mainly due to an increase in the expression of TIMP-1^[3] and an increase in the expression of various ECM components, which include fibrillary collagens I and III, collagen IV, fibronectin, elastin and laminin^[4].

The hepatic stellate cell (HSC) resides within the space of Disse, and is responsible for the majority of ECM deposition in the normal and fibrotic liver^[5,6]. In the normal liver the primary role of the HSC is the regulation of

vitamin A homeostasis and storage^[6]. In times of chronic injury, the HSC transdifferentiates into a myofibroblastic cell exhibiting contractile, proliferative, inflammatory and fibrogenic properties. Transdifferentiation occurs in response to soluble fibrogenic and proliferative factors released mainly by Kupffer cells (KC), (PDGF, TGF β 1) or by damaged hepatocytes (IGF-1, TNF α , EGF)^[2]. Once transdifferentiated, the HSC expresses a number of myogenic markers (including α smooth muscle actin (α SMA), c-myc and myocyte enhancer factor-2) that allows them to be readily identified by immunohistochemical techniques^[4]. In addition, HSC also express a number of neuroendocrine proteins (e.g., glial fibrillary acidic protein or GFAP, synaptophysin and nestin^[4]) and receptors for various neurotransmitters^[7]. Once activated, HSC also release TGF β 1 and PDGF-BB, thereby ensuring a self-sustained phenotypic change^[8,9]. Fibrosis was once thought to be irreversible; however, a growing body of evidence now suggests that once the underlying source of liver injury is treated, fibrosis, and even cirrhosis, to some extent, are reversible. Fibrosis resolution is known to be accompanied by HSC apoptosis, and to some degree of reversion of HSC to their original phenotype^[10].

IRON AND DISEASES OF THE LIVER

Iron is proposed to play a role in promoting hepatic fibrosis in a number of different chronic liver disease states. The evidence supporting or contradicting those propositions is outlined in the following section.

Haemochromatosis

Haemochromatosis is a term used to describe excessive iron loading of the liver leading ultimately to cirrhosis and frequently to HCC^[11]. The knowledge regarding the underlying causes of haemochromatosis is gradually expanding, and the term has now been subdivided according to the specific genetic mutation involved. Iron loading of the liver is a life long process in patients with haemochromatosis, occurring without any apparent overt inflammation or elevation of serum liver enzymes such as alanine aminotransferase (ALT) and aspartate aminotransferase (AST). The symptoms associated with hereditary haemochromatosis (HH) include; hepatomegaly, malaise, insulin resistance and arthritis, to name but a few. When hepatic iron reaches the threshold concentration of 60 μ mol/g dry weight, HSC begin to exhibit the early stages of cellular activation (namely α SMA expression), a key event in the initiation of hepatic fibrosis^[12]. Evidence also suggests that there is a hepatic iron concentration threshold (about 250 μ mol/g dry weight) beyond which cirrhosis can develop in patients with HH^[13,14]. Initial iron loading of the liver occurs in hepatocytes located in Rapoport zone 1, progressively extending to hepatocytes in zones 2 and 3. In the later stages of the disease, KC also accumulate iron and it is the co-loading of both hepatocytes and KC that is, believed to allow fibrosis to become established^[15]. Patients with well established hepatic iron loading are also at a significantly greater risk of developing HCC^[11]. Phlebotomy is widely used to reduce iron burden

in patients with haemochromatosis and, is also an effective way to reverse hepatic fibrosis^[14,16], although regression of cirrhosis remains controversial^[14,16].

Transition of the iron-loaded liver from non-fibrotic to fibrotic, and then onwards to cirrhotic is not always a clear-cut process, and there is growing evidence to suggest that other factors, such as excessive alcohol consumption^[17], viral hepatitis^[18] and steatosis^[19] among others, have an important role in that transition. In fact, iron overload has been shown to be important in the pathology of other liver disease such as non-alcoholic fatty liver disease (NAFLD), alcoholic liver disease (ALD) and chronic hepatitis C.

Non-alcoholic fatty liver disease

Increase in caloric intake and an evermore sedentary lifestyle have led to a surge in the prevalence of people being overweight or obese. Current estimates suggest that as much as 60% of the over 18 age group in Australia will be overweight or obese by the year 2010^[20]. Obesity impacts the liver in the form of NAFLD, which is now recognised as the most common form of liver disease. NAFLD begins with steatosis (fat accumulation within the liver) and can remain in this state without any apparent associated pathology. In its more advanced form NAFLD is often referred to as non-alcoholic steatohepatitis (NASH). It is believed that in order for steatosis to advance to NASH a secondary liver insult is also required often termed the "second hit"^[21]. Iron is among several co-factors known to increase hepatic oxidative stress, and constitutes a "second hit" agent that is postulated to contribute to the progression of steatosis to NASH.

Several studies have highlighted mutations (C282Y and H63D) in the HFE gene that positively correlate with the presence of NAFLD/NASH^[19,22-24]. These studies suggest that while combined NASH/NAFLD and HFE mutation have a negative effect on disease severity as highlighted by serum ALT concentrations and fibrosis grade, hepatic iron concentration is not always elevated. Interestingly Mendler and colleagues also noted that patients with an elevated iron burden almost always displayed insulin resistance irrespective of liver damage^[24]. Insulin resistance is known to play a central role in the accumulation of triglycerides within the hepatocytes, and in the initiation of the inflammatory cascade ultimately leading to cirrhosis^[25]. The correlation between elevated hepatic iron and disease severity in patients with NAFLD/NASH is not always consistent. Several studies have failed to find any evidence of increased hepatic iron levels in patients with NAFLD/NASH, with histological severity being linked to the presence of ballooning hepatocyte degeneration, Mallory hyaline, older age, obesity and the presence of diabetes mellitus^[26-28]. The role of iron in the pathologies associated with NAFLD is, however, further supported by several studies that demonstrate the beneficial effects of iron depletion on serum ALT concentrations and insulin response in patients with NAFLD^[29]. How and why steatosis and iron combined lead to exacerbated fibrosis is possibly linked to the oxidative stress that both can exert.

Alcoholic liver disease

ALD is caused by high risk alcohol consumption over a number of years. Of those engaging in high risk alcohol consumption, only approximately 30% go on to develop liver cirrhosis^[21]. This would suggest that other factors influence disease severity and progression in patients with ALD. Iron is one such factor known to affect the pathogenesis of ALD. In pre-cirrhotic ALD, approximately 29% of patients demonstrate an elevated hepatic iron concentration^[30]. As the disease progresses to cirrhosis, as many as 57% of patients have iron overload ($> 25 \mu\text{mol/g}$)^[31]. It is also apparent that iron is an independent risk factor for the development of fibrosis^[32] in ALD, and higher iron concentrations also correlate with reduced survival^[33]. Alcohol has been postulated to enhance iron uptake by a number of different mechanisms, which have recently been reviewed by Brittenham^[34]. In addition, recent work by Bridle and colleagues suggest that ethanol may perturb IL-6-mediated hepcidin expression resulting in enhanced absorption of iron and hemosiderosis^[35]. This is important when one considers that mammals lack an effective mechanism by which excess iron can be eliminated from the body^[34].

Hepatitis C

Viral hepatitis encompasses a range of different entities from hepatitis A through E, which are caused by distinct viruses. Of these, hepatitis B and C are perhaps the most significant in terms of patient morbidity and mortality. Hepatitis B virus (HBV) is a member of the *Hepadnaviridae* family. Estimates suggest that between 3% and 6% of the world's population are infected with HBV, with up to one third having been previously exposed^[36]. Hepatitis C virus (HCV) is a member of the *Flaviviridae* family and is thought to infect somewhere between 150 and 200 million people worldwide. Both types of viral hepatitis contribute to the development of cirrhosis and HCC. As is common with many forms of liver disease, the severity and rate at which fibrosis progresses are influenced by a great number of external factors and in viral hepatitis these include male gender, alcohol consumption, iron status and age at infection^[37].

The association between elevated serum iron and hepatitis B was first described by Blumberg *et al* in 1981^[38]. Some years later, a link was also found between serum iron, ferritin, transferrin saturation levels and HCV infection^[39,40]. However, these results did not correlate with an increased hepatic iron concentration. Where hepatic iron was found to be elevated in association with HCV infection (in 5% of cases), it was seldom to levels deemed hepatotoxic^[39]. In HCV-infected patients, iron is deposited in hepatocytes, sinusoidal cells, and portal mesenchymal cells, with the degree of portal mesenchymal iron deposition correlating with both hepatic inflammation and fibrosis^[41]. Martinelli and colleagues demonstrated a significant correlation between liver iron scores and the number of GFAP and αSMA -positive HSC in patients with HCV. These αSMA -positive or "activated HSC" were located primarily in Rappaport zones 1 and 3^[42]. Similar findings were also reported by Rigamonti and colleagues,

who also described a correlation between hepatic iron concentration and HSC activation. They suggested that, in patients with HCV infection, iron was important in both HSC activation and fibrosis progression^[43]. Besides being directly correlated with inflammation and fibrosis, hepatic iron has also been linked to a lack of response to IFN α in chronic hepatitis C patients^[44,45]. One mechanism by which this may occur was suggested by Di Bona and colleagues, who found that oxidative stress prevented IFN α induced phosphorylation of STAT-1 & 2 and subsequent upregulation of the antiviral proteins MxA and interferon regulatory factor 9, thereby impairing the antiviral action of IFN α ^[46].

The link between iron and fibrosis in chronic hepatitis C patients has, however, been challenged by Guyader and colleagues who looked at other confounding factors also known to influence both iron overload and fibrosis^[47]. In their studies, liver iron was elevated in only 17% of patients, and correlated with age, male sex, and alcohol intake. They found no association between liver iron and fibrosis after adjusting for confounding variables, and suggested that iron should be considered more as a surrogate marker for disease severity rather than as a fibrogenic factor^[47]. Iron is still further implicated in chronic hepatitis C progression by a number of groups investigating the effects of iron depletion on HCV-associated fibrosis. Kaito and colleagues demonstrated that phlebotomy treatment of HCV-infected patients alone was enough to reduce markers of liver damage (AST and ALT values), lipid peroxidation and oxidative stress^[48]. In addition, patients treated by maintenance phlebotomy (keeping patients in a state of near iron deficiency with a serum ferritin level of 10 ng/mL) showed less inflammation and suppressed fibrosis progression^[49]. Phlebotomy has also been used to increase the therapeutic effect of IFN α treatment of chronic hepatitis C by a number of groups^[50,51]. How and why HCV should interfere with iron homeostasis is open to conjecture. However, there is some evidence to suggest that HCV infection may influence the expression of the iron homeostasis peptide, hepcidin. Nagashima and colleagues suggested that a failure in the regulation of serum prohepcidin levels (mediated by HCV) leads to elevated serum ferritin concentrations, and subsequently to progression of liver injury by iron overload in chronic hepatitis C patients^[52]. Conversely, Aoki and colleagues found hepcidin mRNA expression in the liver did not correlate with AST, ALT levels, or viral load and no differences in hepcidin mRNA were found based on viral genotype or the presence of fibrosis^[53].

MOLECULAR MECHANISMS OF IRON-INDUCED FIBROSIS

This section outlines the mechanisms by which iron is able to cause liver damage and the subsequent pathways activated that lead to hepatic fibrosis.

Role of iron in oxidative stress

In hepatocytes and KC, iron catalyses the production

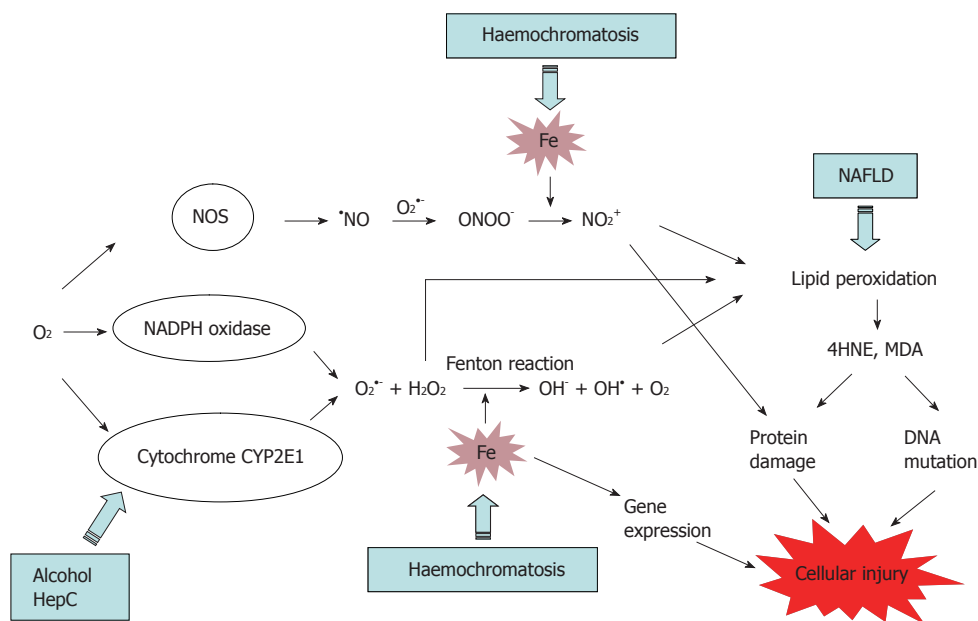


Figure 1 The involvement of iron in oxidative stress and its cytotoxic consequences. Iron catalyses the production of the reactive molecules OH^\bullet (via the Fenton reaction) and NO_2^+ , which promote lipid peroxidation and protein damage leading to cellular injury. NOS, nitric oxide synthase; NO, nitric oxide; ONOO^- , peroxynitrite; $\text{O}_2^{\bullet-}$, superoxide radical; OH^\bullet , hydroxyl radical; H_2O_2 , hydrogen peroxide; NO_2^+ , nitronium anion; Fe, iron; NAFLD, non-alcoholic fatty liver disease.

of hydroxyl radical (OH^\bullet) from reactive oxygen species (ROS), superoxide ($\text{O}_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) *via* a chemical reaction known as the Fenton reaction (Figure 1). ROS are the by-products of aerobic respiration reactions by cytochrome P450 (CYP) 2E1, and are also produced by the membrane-bound NADPH oxidase complex^[54]. In addition, Fe catalyses the formation of NO_2^+ from peroxynitrite (ONOO^-). ONOO^- is formed when the cellular level of $\text{O}_2^{\bullet-}$ is elevated as this later reacts with nitric oxide (NO), produced by the constitutive and inducible nitric oxide synthase (NOS)^[55,56] (Figure 1). Interestingly, nitric oxide has also a protective effect against oxidative stress, as it can inhibit lipid peroxidation and the generation of OH^\bullet by reacting with Fe^{3+} ^[56,57]. NO_2^+ and OH^\bullet induce oxidative deterioration of biomolecules (lipid, protein and DNA), leading to tissue injury and cell death. In addition, iron can modulate gene expression in the cells leading to an alteration of cell function^[58,59].

Alteration of essential biomolecules: NO_2^+ and OH^\bullet catalyse lipid peroxidation which is the process whereby electrons are transferred from the lipids in cell membranes to the free radical, resulting in cell damage. This process proceeds by a free radical chain reaction mechanism. It affects polyunsaturated fatty acids, as they contain multiple double bonds, between which lie methylene $-\text{CH}_2-$ groups that are especially susceptible to peroxidation. Lipid decomposition leads to the generation of thiobarbituric acid (TBA)-reactants and breakdown by-products 4-hydroxynonenal (4HNE) and malondialdehyde (MDA), which are used as marker of lipid peroxidation.

Lipid peroxidation affects the plasma membrane of the cell, but also increases the membrane fragility of a number of different cell organelles, such as lysosomes, which store excess iron, mitochondria and endoplasmic reticulum, leading to impaired cell function^[2]. Lipid peroxidation of the mitochondrial membrane can lead to an increase in their permeability resulting in loss of the electrochemical gradient and release of the proapoptotic cytochrome C.

This phenomenon is called mitochondria permeability transition. Damaged mitochondria generate yet more ROS that further enhance cell damage and activate proapoptotic signals^[60].

Another consequence of lipid peroxidation is the damage of DNA and proteins, as lipid peroxidation products such as 4HNE and MDA can react with DNA bases^[61] and the ϵ - NH_2 group of lysine and histidine residues^[62]. The presence of acetaldehyde, resulting from ethanol oxidation, increases the binding of MDA and its own binding to proteins in a synergistic manner, generating new hybrid adducts called MDA-acetaldehyde adducts^[63]. These adducts may play a role in the development and progression of liver fibrosis as they have been shown to stimulate the secretion of several cytokines and chemokines by liver endothelial cells (TNF α , MCP-1, MIP-2, fibronectin) and HSC (MCP-1, MIP-2, uPA)^[64-66].

Gene expression modulation: Iron or iron-induced oxidative stress have also been found to activate cell signaling cascades triggering apoptosis and necrosis pathway *via* NF- κ B and AP-1 pathways respectively^[60]. NF- κ B promotes the synthesis and release of cytotoxic, proinflammatory and fibrogenic factors such as TNF α , IL-6 and MIP-1 that alter KC and hepatocyte function, and trigger HSC activation^[58,59]. In HSC, AP-1 transcription factors are involved in the regulation of procollagen (I)^[56]. In addition, AP-1 and NF- κ B-dependent gene products modulate hepatocyte death induced by oxidative stress^[67].

Regulation of intracellular levels of ROS: In the normal liver, hepatocytes are able to remove or neutralize oxidative molecules *via* enzymatic and non-enzymatic antioxidant processes, thereby maintaining a safe cellular level of ROS. For example glutathione (GSH) is a tripeptide that neutralizes free radicals and ROS directly *via* a chemical reaction or *via* enzymatic reactions involving glutathione-reductase or glutathione-peroxidase^[68]. Antioxidant agents such as vitamin A, C and E are also able to impair

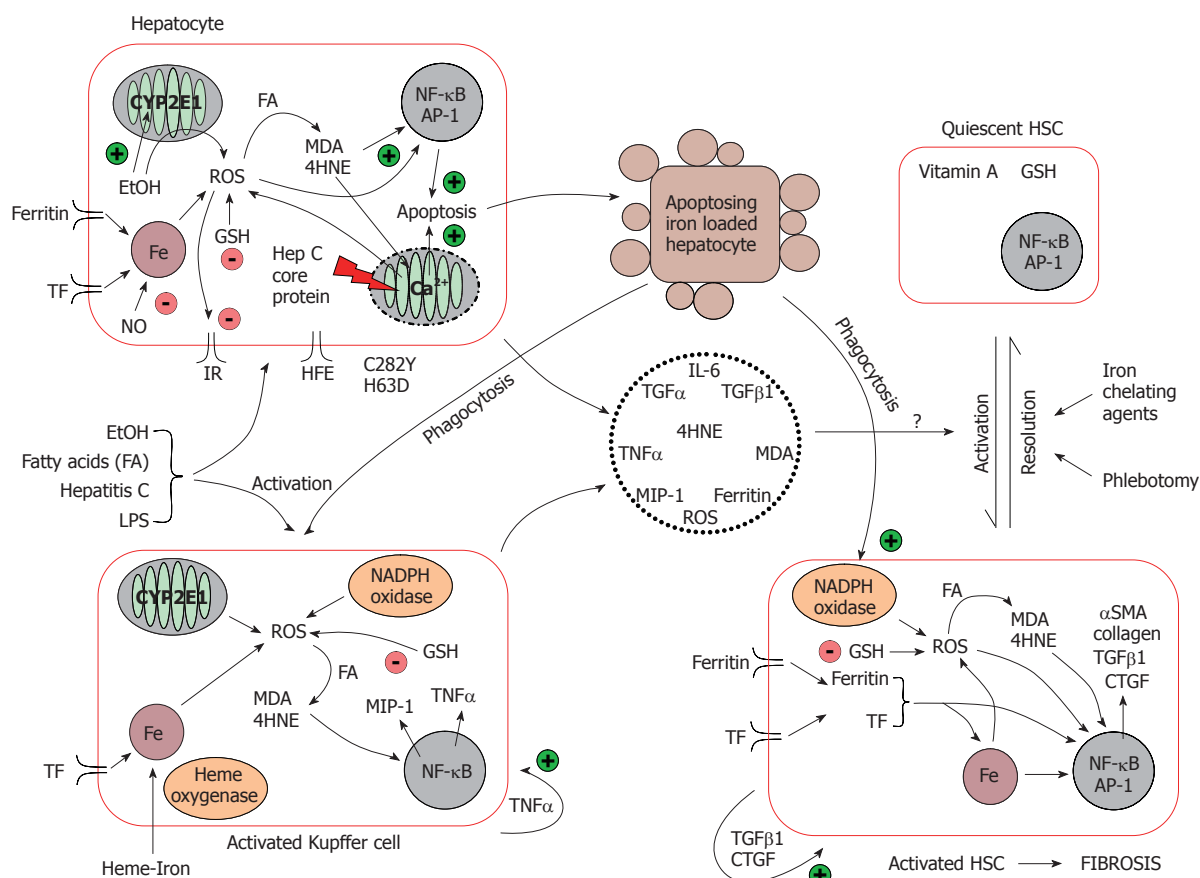


Figure 2 Mechanisms of iron-induced liver injury and fibrosis. Iron catalyses the formation of several reactive oxygen species in hepatocytes. Under normal circumstances, hepatocytes are able to effectively cope with oxidant stress. When the liver is subjected to a secondary insult that enhances hepatic oxidant stress, hepatic fibrosis begins to develop. Increased oxidative stress and other pathological modes of action of HCV, ethanol and steatosis, lead to mitochondrial dysfunction and hepatocyte apoptosis. Kupffer cell activation is achieved by phagocytosis of apoptosing hepatocytes in conjunction with the direct effects of iron, HCV infection, ethanol and steatosis on Kupffer cells. The concomitant hepatocyte damage/apoptosis and Kupffer cell activation is able to drive and maintain hepatic stellate cell activation leading to fibrosis and ultimately cirrhosis if left unchecked. Fe, iron; TF, transferrin; ROS, reactive oxygen species; FA, fatty acids; IR, insulin receptor; GSH, glutathione; TNF α , tumour necrosis factor α ; NO, nitric oxide; IL-6, interleukin 6; TGF β 1, transforming growth factor β 1; CTGF, connective tissue growth factor; α SMA, α smooth muscle actin; MDA, malondialdehyde; 4HNE, 4-hydroxynonenal; MIP-1, macrophage inflammatory protein-1; TGF α , transforming growth factor α ; NF- κ B, nuclear factor κ B; AP-1, activating protein-1.

lipid peroxidation by breaking the chain reaction. Other antioxidants can act as inhibitors of CYP2E1, such as diallylsulfide^[56]. However, when cellular defences are overwhelmed, there is an accumulation of ROS, which trigger cellular damage and apoptosis and, when this occurs in the liver, it can lead to fibrosis.

Iron driven oxidative stress promotes the development of hepatic fibrosis: Iron from the diet is absorbed into the enterocyte, and is either stored bound to ferritin or exported out into the plasma coupled to transferrin. In the liver, iron is taken up mainly by hepatocytes, and secondarily by KC. Hepatic iron uptake is mediated via HFE and the transferrin receptor, and iron is stored within the storage protein, ferritin^[69]. Macrophages phagocytose senescent blood cells, and recycle the iron back to plasma. In iron-loading disorders, although hepatocytes remain the dominant site of iron deposition, KC can also store iron^[70]. As mentioned earlier, the interplay between hepatocytes, KC and HSC and other extracellular proteins in the liver is important in the progression of hepatic fibrosis, and can be influenced by iron. Iron, as a key player in the oxidative reaction, may contribute directly and indirectly to HSC activation although the precise pathways remain

unclear (Figure 2).

Indirect activation of HSC: Hepatocytes and KC are the main cells where iron-induced oxidative damage occurred as described earlier, i.e. lipid peroxidation and activation of proapoptotic/necrotic signalling. It is proposed that HSC activation occurs as a result of soluble factors (TGF- α / β 1, TNF α , MIP-1, IL-6) produced by injured hepatocytes or KC following oxidative stress^[71-73]. Indeed, the gene expression of two markers of HSC activation, collagen type I and α SMA, is increased in studies where HSC are incubated with conditioned medium from iron loaded hepatocytes^[72,74]. Collagen upregulation was also observed when HSC were co-cultured with hepatocytes expressing CYP2E1, which is the principal cytochrome involved in the oxidation of ethanol when the concentration of ethanol is elevated^[74]. This effect was even more pronounced in the presence of iron, and was prevented by several antioxidants suggesting a oxidation mediated upregulation of these profibrogenic genes^[74].

KC have been shown to play a role in HSC activation. When KC and HSC are co-cultured, HSC proliferation is increased along with HSC expression of α SMA and collagen type I, when compared to the culture of HSC

alone^[73]. Friedman and Arthur demonstrated that KC conditioned medium activated HSC and stimulated HSC proliferation^[75]. After exposure to several stimuli such as LPS, ethanol, fatty acid, HCV infection or the phagocytosis of injured hepatocytes, KC become activated and produced ROS *via* PKC dependant activation of NADPH oxidase and *via* the CYP1E2^[55,59,76,77]. Deugnier and colleagues observed KC containing phagocytosed hepatocellular debris in hemochromatosis liver tissue^[78]. Tsukamoto and colleagues demonstrated that haeme-derived iron primed hepatic macrophages for NF- κ B activation, and enhanced the expression of the pro-inflammatory genes TNF α and MIP-1^[79]. TNF α is known to play a key role in promoting KC activation^[80], and also prevents the HSC from undergoing apoptosis^[81] thereby promoting fibrosis.

In addition to the production of profibrogenic and proinflammatory cytokines that activate HSC, KC release iron-loaded tissue ferritin that may interact with the ferritin receptor on activated HSC^[82]. Furthermore, hepatocyte and KC-generated ROS can be released from the cell, and enhance the perpetuation of HSC activation^[83]. In contrast, the role of the toxic by-products of lipid peroxidation such as MDA and 4HNE has been studied by Olynyk and others who showed that these compounds did not directly activate HSC^[84,85].

Direct activation of HSC: Activated HSC express a specific receptor for H-ferritin^[86] which appears to regulate the expression of α SMA^[82]. Ruddell and colleagues have preliminary evidence which suggest that ferritin upregulates genes involved in HSC activation via a PKC ζ dependent pathway^[87]. In addition, activated HSC are also known to express a transferrin receptor which can enhance the expression of α SMA and collagen type I within these cells^[88]. The role of free iron in the induction of HSC activation has been studied by Gardi and colleagues who demonstrated that iron can stimulate type I collagen gene expression but this was not mediated by lipid peroxidation^[89]. This is supported by other studies which rule out the involvement of intracellular lipid peroxidation in the activation of HSC^[84,85,90].

However, in other studies, intracellular oxidative stress has been shown to induce collagen expression in HSC, and that HSC lipid peroxidation can be triggered by hepatocytes^[91,92]. A recent study by Zhan and colleagues supports the role of lipid peroxidation in HSC in the upregulation of procollagen expression^[93]. They showed that HSC are able to phagocytose apoptotic bodies (i.e. injured hepatocytes), which triggers the activation of the stellate cells NADPH oxidase and its production of superoxide within and outside the cell. They also showed that the upregulation of procollagen I following the phagocytosis of apoptotic bodies was NADPH oxidase-dependent.

In conclusion, iron may have a direct effect on the activation of HSC or *via* the modulation of intracellular oxidative stress. However, this may be a minor role in the activation of the HSC when you consider that hepatocytes are the main cells that take up iron, and that quiescent HSC do not express the transferrin or ferritin

receptors^[86,88]. The inherent ability of iron to catalyse the production ROS in hepatocytes and KC appears likely the main mechanism by which it is able to influence hepatic disease progression. Iron, in conjunction with other factors, may overwhelm the liver in terms of its innate ability to cope with oxidative stress thereby causing disease.

Iron and other oxidation-related molecules

As described in Figure 1, iron is a key player in oxidative reactions that produced toxic ROS. However, the cellular level of other molecules, such as H₂O₂ and unsaturated lipids, also influence the degree of oxidation that occurs in the cells and thereby the degree of fibrosis. Indeed, the role of iron along with alcohol, steatosis and HCV in exacerbating liver injury has been the subject of fairly intensive study. The effects of feeding rats a diet supplemented with iron and alcohol was found to enhance hepatic localisation of MDA and 4HNE as well as increase serum levels of ALT and AST to two-fold that of rats fed ethanol alone^[94]. Both liver tissues and HSC isolated from rats fed ethanol and iron were also found to express elevated levels of procollagen α_1 (I) and TGF β 1 mRNA compared to those fed ethanol alone^[94]. Kato and colleagues also suggested TGF α derived from alcohol exposed hepatocytes may also contribute to hepatic fibrosis in ALD^[95]. Ethanol is known to upregulate the expression of CYP2E1^[74] which would exacerbate the potential oxidative stress exerted by iron in hepatocytes due to the catalysis of more ROS. In the same manner, accumulation of fatty acid within the cells will increase oxidative damage due to the enhanced peroxidation of lipid.

HCV alone has been shown to increase ROS generation and also lipid peroxidation in hepatocytes^[96]. It does this *via* the core protein, which is able to alter mitochondrial Ca²⁺ uptake, and also it induces endoplasmic reticulum (ER) stress and enhances ER to mitochondria Ca²⁺ transfer^[97]. Iron overload is also able to induce mitochondrial dysfunction (as visualised by ultrastructural alterations), and in mice over expressing the HCV core polyprotein, iron increases the risk of those mice going on to develop HCC^[98].

CONCLUSION

Iron plays an integral part in the progression of hepatic fibrosis, and it does this via its ability to catalyse the formation of highly reactive and damaging ROS. The damage done by ROS includes lipid peroxidation, protein and DNA modification leading ultimately to apoptosis and necrosis. However, the literature also indicates that co-factors such as steatosis, ethanol and HCV infection contribute to iron-induced hepatic injury. Antioxidant therapies have been tested in clinical trials with limited success with regards to the prevention and reversal of liver fibrosis. Iron depletion therapies have also been used with varying degrees of success to treat all four diseases of the liver outlined in this review. Continued investigation into the molecular mechanisms of iron toxicity, and how it leads to hepatic fibrosis may give a better understanding of the role of iron as a cofactor in the

progression of liver disease leading ultimately to novel anti-fibrotic therapies.

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Pathology of hepatic iron overload

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Abstract

Although progress in imaging and genetics allow for a noninvasive diagnosis of most cases of genetic iron overload, liver pathology remains often useful (1) to assess prognosis by grading fibrosis and seeking for associated lesions and (2) to guide the etiological diagnosis, especially when no molecular marker is available. Then, the type of liver siderosis (parenchymal, mesenchymal or mixed) and its distribution throughout the lobule and the liver are useful means for suggesting its etiology: HLA-linked hemochromatosis gene (HFE) hemochromatosis or other rare genetic hemochromatosis, nonhemochromatotic genetic iron overload (ferroportin disease, aceruloplasminemia), or iron overload secondary to excessive iron supply, inflammatory syndrome, noncirrhotic chronic liver diseases including dysmetabolic iron overload syndrome, cirrhosis, and blood disorders.

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Key words: Iron; Liver; Biopsy; Hemochromatosis; Ferroportin; HLA-linked hemochromatosis gene; Hfe; Metabolic syndrome

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INTRODUCTION

Progress in molecular genetics and in liver imaging have allowed for the noninvasive diagnosis of most cases of inherited disorders of iron metabolism. However, liver pathology remains often useful to assess associated lesions—especially fibrosis—in patients with HLA linked hemochromatosis gene (HFE) and, mainly, nonHFE hemochromato-

sis, and to guide the etiological diagnosis of iron overload in the absence of a molecular marker. Moreover, because iron is more and more considered as a putative (co) factor of morbidity in patients with chronic liver diseases of various causes, iron excess must be recognized, indicated, qualified, quantified and interpreted even when it seems to be contingent upon a well established hepatic disorder.

ASSERTION OF IRON OVERLOAD

In the normal liver, iron is present at a concentration lower than 20 $\mu\text{mol/g}$ of dry weight^[1]. But, it is not histologically visible. Iron deposits are usually difficult to identify on usual stains unless they are abundant. Therefore, every liver biopsy should be routinely stained using not only hematoxylin-eosin-safron and connective stains, but also iron stain. Because it is easier and more reproducible than the Tirmann-Schmeltzer's method, Perl's staining is the most widely used, despite, it identifies Fe^{2+} only^[2].

DESCRIPTION OF IRON OVERLOAD^[2,3]

Distribution

The cellular distribution of iron deposits within hepatocytes, sinusoidal and portal macrophages, sinusoidal and portal endothelial cells, and biliary cells must be precisely described according to lobular areas in order to differentiate the following types of liver siderosis.

Parenchymal iron overload: It is related to intestinal iron hyperabsorption. Then, because it comes to the liver through the portal vein, iron deposits within hepatocytes as fine granules at the biliary pole of cells, and is distributed throughout the lobule according to a decreasing gradient from periportal to centrolobular areas. Mesenchymal iron deposits may be found at a latter stage when the amount of hepatocytic iron is high and responsible for sideronecrosis.

Mesenchymal iron overload: It corresponds to iron deposition within Kupffer cells and/or portal macrophages. Iron loaded cells are either isolated or grouped together without any lobular systematization. When associated, hepatocytic iron deposits are rough, sparse and usually located within cells close to iron loaded macrophages.

Mixed iron overload: It presents with the histological characteristics of the previous two types and corresponds usually to complex conditions or to massive iron loading.

Table 1 Histological grading of iron storage^[2]

Grade	Ease of observation and magnification required
0	Granules absent or barely discernible at × 400
1	Granules barely discernible at × 250 and easily confirmed at × 250
2	Discrete granules resolved at × 100
3	Discrete granules resolved at × 25
4	Masses visible at × 10, or naked eye

Table 2 Histological grades of iron storage from Deugnier Y and Turlin B^[3-12]

Hepatocytic iron	0, 3, 6, 9 or 12	HIS
	According granules size	0-36
	In each Rappaport area	
Sinusoidal iron	0, 1, 2, 3 or 4	SIS
	According granules size	0-12
	In each Rappaport area	
Portal iron	0, 1, 2, 3 or 4	PIS
	According to % of iron overloaded macrophages, biliary cells, and vascular walls	0-12
Total iron score		0-60

HIS: hepatocytic iron score; SIS: sinusoidal iron score; PIS: portal iron score.

It is also important to precise whether iron distribution throughout the liver is homogeneous or not, i.e. whether lobules (or, in case of cirrhosis, nodules) are equally iron loaded or not^[4,5].

Quantification

Biochemical hepatic iron concentration: Irrespective of the method used (colorimetry or atomic absorption), the biochemical determination of hepatic iron concentration (HIC) is considered as the reference method for quantifying iron in the liver^[1,6]. In normal subjects, HIC ranges from 10 to 36 μmol/g of dry weight. Iron excess is considered as mild up to 150, moderate between 150 and 300, and important above 300. Cases with HIC greater than 1000 are exceptionnal. Results obtained from fresh tissue and from deparaffinized blocks are equivalent^[7]. Therefore, determination of HIC on deparaffinized tissue should be the rule because it allows for histological control. This is especially relevant when iron distribution is heterogeneous as in the cirrhotic liver^[4,5].

Histological semiquantitative assessment: Several scoring systems have been proposed. The Scheuer's scoring system, either in its original presentation^[8] or modified according to Rowe *et al*^[9] or to Searle *et al*^[2] is widely used because it is simple (Table 1). However, it was not satisfactorily validated. The system proposed by the authors (Table 2) was well validated in both hemochromatotic^[10,11] and nonhemochromatotic iron overload disorders^[12]. But, it remains mainly used for research purposes due to its relative complexity.

ETIOLOGICAL DIAGNOSIS

Both the type of iron overload and associated hepatic

Table 3 Main causes of hepatic iron overload according to the histological type of siderosis and associated lesions

Parenchymal iron overload
With normal liver
Early genetic hemochromatosis
Nontransfused dysmyelopoiesis
Hereditary aceruloplasminemia
With cirrhosis
Iron overload secondary to cirrhosis
Mixed iron overload
With normal liver
Insulin resistance syndrome
Ferroportin disease
Transfused dysmyelopoiesis
Oral or parenteral iron supplementation
With steatosis or steatohepatitis
Insulin resistance syndrome
Chronic alcoholism
With chronic hepatitis
Hepatitis C or B
Wilson's disease
With intrahepatocytic inclusions
Porphyria cutanea tarda
With cirrhosis
Late genetic hemochromatosis
Mesenchymal iron overload
With normal liver
Inflammatory syndrome
Repeated transfusions

lesions may guide towards the right etiology (Table 3).

Genetic iron overload

Genetic hemochromatosis: Genetic hemochromatosis^[3] corresponds to 4 disorders transmitted as autosomal recessive traits, two with late onset (adult type: HFE hemochromatosis and iron overload related to mutation on the receptor transferrin 2 gene) and two with early onset (juvenile type related to mutations on the hemojuvelin or the hepcidin gene). From a histological point of view, these disorders are very similar because of a common pathophysiology consisting in an impairment of hepcidin production whose degree modulates the severity of iron burden^[13].

In early GH, iron remains located within hepatocytes, at the biliary pole of cells. It is distributed according to a decreasing gradient from periportal to centrilobular areas. This results in a typical parenchymal iron overload pattern.

With the passage of time, hepatocytic iron load increases, and then periportal sideronecrosis occurs. Sideronecrosis is responsible for macrophage activation, which leads to both development of fibrosis, and redistribution of iron towards nonparenchymal cells. In the absence of other cause of chronic liver disease, cirrhosis develops when hepatic iron concentration exceeds 400 μmol/g. GH related cirrhosis consists of large fibrous septa resembling biliary cirrhosis, while preserving the vascular architecture of the liver for a long time. This likely explains why portal hypertension and hepatic failure are rare features in GH patients. According to series, 25% to 50% of GH patients are still diagnosed at the cirrhotic stage.

Liver cancer is a frequent complication in GH^[10]. Most cases are hepatocellular carcinomas (HCC) developed in

a cirrhotic liver. However, some cases of HCC have been reported in GH patients with no cirrhosis, and frequency of cholangiocarcinoma reports is increasing^[14]. Two types of preneoplastic lesions have been reported and should be sought for at histological examination: (1) Iron-free-foci^[15] consist of sublobular nodular clusters of hepatocytes devoid of iron or with a low iron content within an otherwise iron-overloaded liver. Most often, they exhibit a proliferative pattern with either large or small cell dysplasia in 50% of cases. More than half the patients with IFF on their initial liver biopsy will develop. (2) Von Meyenburg complexes have also been reported as abnormally numerous in the surrounding liver of patients with GH complicated with cholangiocarcinoma^[14].

Nonhemochromatotic genetic iron overload

The ferroportin disease: The ferroportin disease^[16] is a dominant hereditary iron overload disorder characterized by phenotypic variability. In most cases, iron deposits are found within macrophages (mesenchymal type) with no significant fibrosis. This corresponds to the classical asymptomatic form with elevated hyperferritinemia contrasting with normal or mildly increased transferrin saturation. Rarely, iron is predominantly located within parenchymal cells, and the histological picture is similar to that of genetic hemochromatosis with, in some cases, either severe fibrosis or cirrhosis. Then, transferrin saturation is usually markedly elevated.

Hereditary aceruloplasminemia: Hereditary aceruloplasminemia^[17,18] is an exceptional disease transmitted as a recessive trait. Under the lens, iron is found predominantly in parenchymal cells. No case of liver cirrhosis has been described even in the most iron overloaded cases.

African iron overload : African iron overload^[19] is a rare disorder characterized by a mixed hepatic iron overload frequently complicated with cirrhosis. It is related to excessive iron intake, and likely underlain by nonHFE genetic factors.

Excessive iron supply

When brought parenterally: (i.e. through multiple transfusions), iron is initially localized within Kupffer cells and portal macrophages. With time, it is usually redistributed towards surrounding parenchymal cells, which results in a mixed and heterogenous pattern.

In case of excessive chronic iron intake: mixed hepatic iron overload may develop as reported in elite road cyclists^[20].

Inflammatory syndrome

It is a frequent cause of mesenchymal hepatic siderosis related to a defect of iron release from Kupffer cells due to increased production of hepcidin^[13]. Iron deposits are usually sparse and distributed throughout the lobule.

Noncirrhotic chronic liver diseases.

Dysmetabolic iron overload syndrome (DIOS): Dios

is a frequent condition corresponding to the association of an unexplained hepatic iron overload with usually normal transferrin saturation and features of metabolic syndrome^[21,22]. The histological pattern of DIOS is mixed, both mesenchymal (throughout the entire lobule) and parenchymal (predominating in periportal area)^[23]. Iron excess is usually mild and averages 100 $\mu\text{mol/g}$. However, in 30% of cases, the hepatic iron concentration to age ratio exceeds 2, a threshold previously considered as highly suggestive of GH before the discovery of the HFE gene. Either steatosis or steatohepatitis is present in 50% of cases. Bridging fibrosis or cirrhosis is found in 12% of cases. Whether iron may be involved in the development of fibrosis in DIOS patients, and its removal may be beneficial remains debated.

Alcoholic liver disease: Mixed and mild hepatic siderosis is found in 5% to 20% of chronic alcoholics, even in the absence of cirrhosis. A direct effect of alcohol on hepcidin production could be involved^[24].

Chronic hepatitis: Hepatic iron deposition is found in 35% to 56% of patients with chronic hepatitis. This was especially demonstrated in patients with chronic hepatitis C. The histological pattern is usually mesenchymal with frequent iron deposits in endothelial cells. Iron excess has been shown to be correlated with necrotico-inflammatory changes, and to decrease after interferon therapy. Moreover, iron removal before or at the time of interferon therapy could result in histological improvement, even in nonresponders^[25].

Wilson disease: Mixed iron overload is frequently found in the liver of patients with Wilson disease. Its mechanism is likely multifactorial, and involves low serum ceruloplasmin levels, hemolysis, necrotico-inflammatory changes and cirrhosis.

Cirrhosis: Significant liver siderosis is found in 35 to 78% of patients with end-stage cirrhosis, irrespective of the cause of cirrhosis^[4,5]. Iron deposits are mainly located within hepatocytes, predominate in remaining periportal areas, and, in some cases, can mimic GH. However, liver siderosis secondary to cirrhosis is distributed very heterogeneously from a nodule to another and absent from fibrous septa, biliary cells and vascular walls. This usually allows for correct diagnosis, and points to the need, in case of cirrhosis, for interpreting hepatic iron concentration according to histological findings. It is likely that nontransferrin bound iron (NTBI) plays a key role in the development of iron overload in cirrhosis^[26]. Indeed, in severe cirrhosis, serum transferrin levels are low due to hepatic failure which results in increased saturation of transferrin and, then, in the appearance of NTBI, a special form of iron that is avidly taken up by hepatocytes.

Hepatocellular carcinoma: Parenchymal or mixed iron overload is frequently present in the nontumorous part of the liver of patients with hepatocellular carcinoma (HCC), whether they have cirrhosis or not^[27]. This supports the

(co)carcinogenic role of iron suggested by numerous experimental and epidemiological studies^[28].

Blood disorders

Porphyria cutanea tarda: Mixed, heterogeneous and mild hepatic siderosis is encountered in 60% to 70% of patients with porphyria cutanea tarda (PCT). Searching for intrahepatocytic inclusions when faced with mixed iron overload is suitable, because the clinical diagnosis of PCT may be missed.

Dyserythroipoiesis: In well compensated dyserythropoietic syndromes, intestinal iron absorption is increased secondary to impairment in iron incorporation into red cell precursors. With time, severe hepatic iron overload resembling GH may develop, even in the absence of blood transfusions. Once transfusions are required, iron deposits in both parenchymal and mesenchymal cells resulting in a mixed pattern.

PLACE OF LIVER BIOPSY IN THE MANAGEMENT OF IRON OVERLOAD DISORDERS

Recent progress in both imaging and genetics have resulted in reducing the role of liver biopsy in the diagnosis of hepatic iron overload.

Positive diagnosis: MRI allows for a specific and sensitive detection and reliable quantification of excessive hepatic iron content when comprised between twice the upper limit of normal and 300 $\mu\text{mol/g}$ dry weight^[29]. Then, liver biopsy is no longer necessary to ascertain iron overload.

Aetiological diagnosis: MRI also allows for classifying iron excess as parenchymal, mesenchymal or mixed according to organ iron deposition (liver and/or spleen and/or spine)^[30]. Then it provides guidance for the aetiological diagnosis process, especially with respect to the first choice of genotyping tests. This was recently well illustrated by Pietrangelo *et al*^[31] in patients with ferroportin mutations. Moreover, in these patients, MRI contributed to establish phenotypic/genotypic correlations, and to understand the pathophysiology of the disease by demonstrating, beside the classical mixed pattern of iron accumulation, a non-classical parenchymal pattern related to the N144H ferroportin mutation^[31].

Disease severity: Once the positive and aetiological diagnosis of hepatic iron overload has been made, it is mandatory to assess the degree of hepatic damage, that is to determine whether severe fibrosis has developed or not. This will remain the major goal of liver biopsy as long as noninvasive tests for fibrosis-including biochemical markers, and elastometry-are not validated in patients with iron overload syndromes.

Currently, indication of liver biopsy can be discussed according to the phenotypic presentation of iron excess.

In case of hemochromatotic phenotype (= increased transferrin saturation with parenchymal iron deposition),

performing liver biopsy depends on HFE genotyping:

In C282Y homozygotes, liver biopsy is no longer necessary for diagnosis, but remains suitable with respect to prognosis. Guyader *et al*^[32] demonstrated that, when the liver was not clinically enlarged AND serum ferritin level was lower than 1000 ng/mL AND serum AST level was normal, there was never significant liver fibrosis (i.e. grade 3 or 4 fibrosis according to the METAVIR scoring system). On the contrary, when one, two or all these conditions were not met, there was a significant risk of fibrosis calculated as $1/(1 + \exp[-(-6.7620 + 3.2934 \text{AST}_{(\text{iu/l})} + 0.0013 \text{ferritin}_{(\text{ng/ml})} + 2.5317 \text{hepatomegaly}_{(0:1})])$. Accuracy of Guyader's algorithm was further validated in Canadian patients^[32]. Since then, other equations of prediction of (non)fibrosis have been proposed^[33,34] based upon age and serum ferritin level or serum ferritin level, serum AST level and platelets count. But, either they were not further correctly validated or they were not as simple for clinical use as Guyader's algorithm. Then, currently there is a global consensus to perform liver biopsy for fibrosis evaluation in C282Y homozygotes with either increased liver size, serum ferritin level higher than 1000 ng/mL or abnormal serum AST levels except when the diagnosis of cirrhosis is clinically obvious or when the predictive equation gives a risk close to 100%. Recently, Powell *et al*^[35] showed that obesity-related steatosis may have a role as a cofactor in liver injury-especially fibrosis-in C282Y homozygotes. This has clinically important implications, but does not modify indications of liver biopsy in these patients.

In a C282Y-H63D compound heterozygote presenting with mild increase in transferrin saturation-usually comprised between 45% and 60% and in serum ferritin-usually < 500 ng/mL and with no biochemical abnormalities and clinical liver symptoms, it can be reasonably assumed that the HFE genotype is responsible for the abnormalities in iron metabolism^[36], and that the patient is free of risk of fibrosis. Then, liver biopsy is not necessary.

In all other cases, the diagnostic procedure must be conducted irrespective of HFE genotype. Indeed, C282Y and H63D heterozygosity as well as H63D homozygosity not only are frequent (up to 1/3 of subjects in European general populations), but do not result, in a given subject, in clinically relevant perturbances of iron metabolism even if large genotyping studies^[36-39] have shown that some of them induced slight but significant increase in serum ferritin and/or transferrin saturation. Then, liver biopsy remains suitable to search for an additional cause of either iron overload or chronic liver disease. The most frequent finding is heterogeneous parenchymal iron overload complicating alcoholic or viral liver cirrhosis^[4,5,40]. Much more rarely, liver biopsy discovers marked iron overload suggesting an associated mutation on another gene involved in iron metabolism. In that case, the precise description of iron deposition, and associated lesions may participate in defining the choice of diagnostic molecular tests: mesenchymal or mixed iron deposition with no significant fibrosis is suggestive of ferroportin disease (which sometimes presents with TS > 60%) while parenchymal iron overload suggests the diagnostic

of juvenile haemochromatosis in a young adult with usually severe fibrosis (mutation on the hemojuvelin or hepcidin gene) and that of transferrin receptor 2-related haemochromatosis in an adult with or without fibrosis.

In the absence of hemochromatotic phenotype (= low, normal or slightly elevated transferrin saturation), the question is whether increased serum ferritin levels are related to iron overload or not. MRI can replace liver biopsy to answer this question, and histological examination of the liver can be restricted to patients with significant iron deposition at MRI (i.e. hepatic iron concentration > 100 µmol/g dry weight) and/or elevated serum transaminase levels and/or abnormal noninvasive predictive tests of fibrosis^[41]. In such a situation, the most frequent finding is mild and mixed iron overload with either metabolic or alcoholic steatohepatitis or chronic hepatitis C or porphyria cutanea tarda. Much more rarely, histological examination reveals marked iron overload with no significant fibrosis corresponding to ferroportin disease (mesenchymal type-normal or slightly increased transferrin saturation) or to hereditary aceruloplasminemia (parenchymal type-low transferrin saturation).

Due to the widespread use of genotyping, of MRI and of noninvasive predictive markers of hepatic fibrosis, liver biopsy is less and less performed for diagnostic and prognostic purposes in C282Y homozygous patients. Conversely, it remains often necessary in other patients in order to guide the etiological diagnosis of hepatic iron overload by describing and semi-quantifying iron excess and by assessing associated lesions.

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Inhibition of histone deacetylase for the treatment of biliary tract cancer: A new effective pharmacological approach

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significant *in vivo* activity and potentiated the efficacy of gemcitabine. Therefore, further clinical evaluation of this new drug for the treatment of biliary tract cancer is recommended.

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Key words: Histone deacetylase inhibitor; Biliary tract cancer; Cholangiocarcinoma; NVP-LAQ824; NVP-LBH589

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Abstract

AIM: To investigate *in vitro* and *in vivo* therapeutic effects of histone deacetylase inhibitors NVP-LAQ824 and NVP-LBH589 on biliary tract cancer.

METHODS: Cell growth inhibition by NVP-LAQ824 and NVP-LBH589 was studied *in vitro* in 7 human biliary tract cancer cell lines by MTT assay. In addition, the anti-tumoral effect of NVP-LBH589 was studied in a chimeric mouse model. Anti-tumoral drug mechanism was assessed by immunoblotting for acH4 and p21^{WAF-1/CIP-1}, PARP assay, cell cycle analysis, TUNEL assay, and immunohistochemistry for MIB-1.

RESULTS: *In vitro* treatment with both compounds significantly suppressed the growth of all cancer cell lines [mean IC₅₀ (3 d) 0.11 and 0.05 µmol/L, respectively], and was associated with hyperacetylation of nucleosomal histone H4, increased expression of p21^{WAF-1/CIP-1}, induction of apoptosis (PARP cleavage), and cell cycle arrest at G2/M checkpoint. After 28 d, NVP-LBH589 significantly reduced tumor mass by 66% (bile duct cancer) and 87% (gallbladder cancer) *in vivo* in comparison to placebo, and potentiated the efficacy of gemcitabine. Further analysis of the tumor specimens revealed increased apoptosis by TUNEL assay and reduced cell proliferation (MIB-1).

CONCLUSION: Our findings suggest that NVP-LBH589 and NVP-LAQ824 are active against human biliary tract cancer *in vitro*. In addition, NVP-LBH589 demonstrated

INTRODUCTION

Interactions between histones and DNA are regulated by the acetylation status of histones, which in eukaryotic cells, plays a pivotal role in chromatin remodeling and in the regulation of gene expression: hyperacetylation determines transcription activation while hypoacetylation transcription repression. The balance between two classes of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs), can affect the acetylation status of histones. Altered HAT or HDAC activity has been identified in several cancers^[1]. HDACs have been found to be associated with aberrant transcription factors, and can mediate the function of oncogenic translocation products in specific forms of leukemia and lymphoma. They are divided into three classes: class I (HDAC 1-3, 8, 11) is generally nuclear; class II (HDAC 4-7, 9, 10) is generally tissue-dependent for the expression, and can shuttle between the cytoplasm and the nucleus; and class III requires nicotinamide adenine dinucleotide (NAD) as cofactor with substrate; they were discovered recently and are poorly characterized^[1]. To date, several structural unrelated classes of HDAC-inhibitors (HDACIs) demonstrating anti-tumor activity both *in vitro* and *in vivo* in animal models have been identified. These classes include carboxylic acids such as phenylbutyrate (PB), phenylacetate (PA), sodium butyrate (SB), AN-9 (Pivanex) and valproic acid; cyclic tetrapeptides such as trapoxin A; cyclic peptides such as depsipeptide or FK-228 and apicidine; benzamides such as MS27-275 and CI-994

(*N*-acetyldinaline); ketones such as trifluoromethyl ketone and α -ketomides; hydroxamic acids such as trichostatin A (TSA), suberoylanilide hydroxamic acid (vorinostat, SAHA), azelaic bis-hydroxamic acid (ABHA), scriptaid, oxamflatin, pyroxamide, *m*-carboxycinnamic acid bis-hydroxamide (CBHA), and the recently developed NVP-LAQ824, NVP-LBH589, and PXD101^[2]. Multiple phase I, II clinical trials are either completed or currently ongoing with several HDACIs, either as single agent or in combination with conventional chemotherapy, or biologicals. Clinical studies published so far have shown that HDACIs can be administered safely in humans and that treatment of some cancers with such agents seems to be beneficial^[3]. NVP-LAQ824 and NVP-LBH589 are a new chemical entity belonging to a structurally novel class of cinnamic hydroxamic acid compounds^[4-6] which are currently in phase I clinical evaluation in advanced refractory solid tumors and hematologic malignancies^[7-12]. However, little is known about their potential efficacy in biliary tract cancer, a rare tumor with a grim prognosis and up to now only limited treatment options. Therefore, the objectives of the current study were to investigate the efficacy of *in vitro* and *in vivo* treatment with the two novel pan-HDACIs NVP-LAQ824 and NVP-LBH589 and to evaluate the combination with gemcitabine.

MATERIALS AND METHODS

Materials

Seven biliary tract cancer cell lines - five extra-hepatic bile duct cancer cell lines (EGI-1, TFK-1, CC-SW-1, CC-LP-1, and SK-ChA-1)^[13-17] and two gallbladder cancer cell lines (Mz-ChA-1, Mz-ChA-2)^[16] were examined. All cell lines were cultured with appropriate media and incubated at 37°C in a humidified atmosphere containing 50-100 mL/L CO₂ in air, and the media were changed every three days. The HDACIs NVP-LAQ824 and NVP-LBH589 were provided by Novartis (Basel, Switzerland) and dissolved in dimethyl sulfoxide (DMSO) (10 mmol/L stock). Hoechst dye, sodium butyrate and monoclonal (mc) β -actin antibody were purchased from Sigma (Sigma-Aldrich Chemie GmbH Munich, Germany), mc p21^{WAF-1/Cip-1}, and polyclonal (pc) cleaved-poly(ADP-ribose) polymerase (PARP) antibodies from Cell Signaling (Cell Signaling Technology, Beverly, USA), mc acH4 antibody from Upstate (Upstate Biotechnology, Lake Placid, USA), mc MIB-1 antibody from Dako (Glostrup, Denmark), and gemcitabine [diluted in 50 g/L dextrose in water (D5W) and 50 mL/L DMSO] and etoposide (dissolved in normal saline to 10 mmol/L stock) from our hospital pharmacy. Six to eight-week-old female athymic NMRI nude mice were supplied from Taconic (Taconic Europe, Ry, Denmark) and held under pathogen-free conditions. Humane care was administered, and study protocols complied with the Institutional Guidelines.

Inhibition of cell growth detected by MTT assay

Cytotoxic effects of both drugs were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldridge Chemie GmbH Munich, Germany) assay. About 1.5×10^3 cells were seeded in

triplicate in 96-well plates (100 μ L/well) and allowed to attach overnight. The medium was then replaced with medium (100 μ L) containing the designated drug or vehicle control (50 mL/L DMSO in D5W), followed by an incubation for 3 or 6 d. For the 6-d experiment, medium was changed after 3 d. Three hours before the end of the incubation period, 10 μ L of phosphate-buffered solution (PBS) containing 5 g/L MTT was added to each well. Following this, the medium was removed. The precipitate was then resuspended in 100 μ L of lysis buffer (DMSO, 100 g/L SDS). The absorbance was measured on a plate reader at 590 nm and a reference wavelength of 630 nm. Each experiment was performed in triplicate.

Immunoblotting

Cell culture monolayers were washed twice with ice-cold PBS, and lysed with RIPA-buffer containing Tris-HCl (50 mmol/L, pH 7.4), NP-40 (10 g/L), sodium-desoxycholate (2.5 g/L), NaCl (150 mmol/L), EDTA (1 mmol/L), sodium-orthovanadate (1 mmol/L), and one tablet of complete mini-EDTA-free protease inhibitor cocktail (Boehringer, Mannheim, Germany) (in 10 mL buffer). Histones for anti-acH4 immunoblotting were isolated by acid extraction [cells were lysed in ice-cold lysis buffer (HEPES 10 mmol/L; pH 7.9), MgCl₂ (1.5 mmol/L), KCl (10 mmol/L), DTT (0.5 mmol/L), PMSF (1.5 mmol/L), and additional protease inhibitor]. One molar HCl was added to a final concentration of 0.2 mol/L, followed by incubation on ice for 30 min, and centrifugation at 13000 *g* for 10 min. The supernatant was kept and dialysed against 200 mL of 0.2 mol/L acidic acid twice for 1 h and against 200 mL of H₂O overnight. Proteins were quantified by Bradford protein assay (Bio-Rad, Munich, Germany) and stored at -80°C, and 50 μ g of cell or tissue lysates were separated on SDS-polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Freiburg, Germany). Membranes were then incubated in blocking solution [50 g/L dry milk in 10 mmol/L Tris-HCl, 140 mmol/L NaCl, 1 g/L Tween-20 (TBS-T)], followed by incubation with the primary antibody at 4°C overnight (50 g/L BSA in TBS-T). The membranes were then washed in TBS-T, and incubated with horseradish peroxidase (HRPO)-conjugated secondary antibodies for 1 h at room temperature. Antibody detection was performed with an enhanced chemoluminescence reaction (SuperSignal West Dura, Pierce, Rockford, USA).

Cell cycle analysis

Cells (2×10^5) were seeded in T-25 flasks, treated with various concentrations of NVP-LAQ824 or NVP-LBH589 or vehicle control (50 mL/L DMSO in D5W) for 72 h, washed with PBS, trypsinized, centrifuged, and fixed in 750 mL/L ice-cold ethanol/phosphate-buffered saline containing 10 g/L EDTA. DNA was labelled with 100 mL/L propidium iodide. Cells were sorted by FACScan analysis, and cell cycle profiles were determined using ModFitLT V2.0 software (Becton Dickinson, San Diego, USA). Each experiment was performed in triplicate.

Animal studies

Tumors were induced by injecting 5×10^6 Mz-ChA-2 or

EGI-1 cells in 200 μ L of PBS subcutaneously into the flank region of NMRI nude mice. Treatment was started when an average tumor volume of 150 mm³ was reached (usually after 2 wk). The verum groups intraperitoneally received either NVP-LBH589 (40 mg/kg, 5 \times weekly) or gemcitabine (5 mg/kg, 2 \times weekly) or a combination of both (NVP-LBH589 at 20 mg/kg, 5 \times weekly plus gemcitabine at 5 mg/kg, 2 \times weekly), whereas the control group received placebo (carrier solution 50 mL/L DMSO in D5W) only. Treatment was continued for 28 consecutive days, tumors were daily measured with a Vernier caliper and tumor volumes were calculated using the formula: tumor volume = $0.5 \times L \times W^2$, where *L* represents the length and *W* the width of the tumor. When treatment was finished, animals were sacrificed and tumors were excised and weighed.

TUNEL POD test

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (*In Situ* Cell Death Detection Kit, POD) was used to detect apoptosis in paraffin sections from mouse tumor tissue. TUNEL test was carried out following the manufacturer's instructions (Roche, Penzberg, Germany) as previously described^[18]. Apoptotic cells (red) were counted under a light microscope after fluorescence signal conversion using antibody with conjugated peroxidase and the substrate for peroxidase (DAB, Roche, Penzberg, Germany). The number of positive cells was counted by an experienced pathologist in a total of 8 high-power fields (HPFs) and expressed as mean percentage of total cells in these fields of the tumor. Necrotic tumor cells were excluded from cell count.

Immunohistochemical staining

For MIB-1 staining, we used paraffin sections following a protocol that has been described elsewhere^[19]. The number of positive cells was counted in a total of 4 HPFs and expressed as mean percentage of total cells in these fields of the tumor.

Statistical analysis

Statistical calculations were performed using SPSS version 10.0 (SPSS Inc., Chicago, USA). Numeric data were presented as mean \pm SD or SEM. Inter-group comparisons were performed using Student's *t* test. *P* < 0.05 was considered statistically significant.

RESULTS

Inhibition of cellular growth by NVP-LAQ824 and NVP-LBH589

After 3 d of incubation, all tested cell lines were sensitive to NVP-LAQ824 [mean IC₅₀ (3 d) = 0.11 ± 0.06 μ mol/L] and even more to NVP-LBH589 [mean IC₅₀ (3 d) = 0.05 ± 0.05 μ mol/L]. There was no significant difference in response between the group of extra-hepatic bile duct cancer cell lines [mean IC_{50/LAQ824} (3 d) = 0.10 ± 0.07 μ mol/L and mean IC_{50/LBH589} (3 d) = 0.05 ± 0.06 μ mol/L] and the group of the two gallbladder cancer cell lines [mean IC_{50/LAQ824} (3 d) = 0.12 ± 0.03 μ mol/L and mean IC_{50/LBH589} (3 d) = 0.06 ± 0.05 μ mol/L].

Inhibition of cell growth was more pronounced if incubation time was extended to 6 d, with a mean IC₅₀ value of 0.05 ± 0.02 μ mol/L for NVP-LAQ824 and 0.02 ± 0.01 μ mol/L for NVP-LBH589. Once again, there was equal response between the group of extra-hepatic bile duct cancer cell lines [mean IC_{50/LAQ824} (6 d) = 0.05 ± 0.02 μ mol/L and mean IC_{50/LBH589} (6 d) = 0.01 ± 0.01 μ mol/L] and the group of the two gallbladder cancer cell lines [mean IC_{50/LAQ824} (6 d) = 0.06 ± 0.03 μ mol/L and mean IC_{50/LBH589} (6 d) = 0.02 ± 0.02 μ mol/L] (Figure 1 and Table 1). In addition, DMSO, the solvent of NVP-LAQ824 and NVP-LBH589, alone had no influence on cell growth (data not shown).

Mechanism of drug action

Treatment of cell lines EGI-1 and Mz-ChA-2 with 0.1 μ mol/L NVP-LAQ824 or 0.1 μ mol/L NVP-LBH589 for 24 h resulted in acetylation of histone H4 (Figure 2A). Protein extract from HELA cells that were treated with 5 mmol/L sodium butyrate served as positive control. The same treatment caused an induction of p21^{WAF-1/CIP-1} expression (Figure 2B). Cell lysate from HEK 293 cells served as positive control. A dose increase to 0.2 μ mol/L NVP-LAQ824 or 0.2 μ mol/L NVP-LBH589 corresponded with an increase of p21^{WAF-1/CIP-1} levels. Histone H4 acetylation was higher in treated Mz-ChA-2 than EGI-1 cells, whereas p21^{WAF-1/CIP-1} expression was higher in treated EGI-1 cells. Immunoblotting for cleaved-PARP was positive when cells were treated with 0.1 μ mol/L NVP-LAQ824 or 0.1 μ mol/L NVP-LBH589 for 24 h (Figure 2C, Lanes 2 and 5). PARP cleavage was more pronounced when the concentration of NVP-LAQ824 or NVP-LBH589 was increased to 0.2 μ mol/L (Figure 2C, Lanes 3 and 6). Lysate from untreated NIH-3T3 cells (Figure 2C, Lane 7) and from cells that were treated with 25 μ mol/L etoposide for 5 h [Figure 2C, Lane 8 (lane *)] served as negative control. Lysate from NIH-3T3 cells that were treated with 10 μ mol/L etoposide for 24 h [Figure 2C, Lane 9 (lane **)] served as positive control. Positive and negative controls were selected according to the recommendations of the manufacturers of antibodies. Staining with β -actin antibody confirmed equal protein loading in all immunoblots.

Treatment of cell lines EGI-1 and Mz-ChA-2 with 25 nmol/L NVP-LAQ824 or 25 nmol/L NVP-LBH589 for 72 h resulted in G2/M phase arrest. This arrest was more pronounced if the dose of NVP-LAQ824 or NVP-LBH589 was increased to 50 nmol/L. For both concentrations, the effect of NVP-LBH589 was stronger than the effect of NVP-LAQ824 (Table 2). Further increase of the NVP-LAQ824 and NVP-LBH589 concentration to 100 nmol/L and 200 nmol/L led to no further significant changes in G2/M phase arrest (data not shown). Sub-G1-peak percentage was not analyzed.

Inhibition of tumor cell growth by NVP-LBH589 \pm gemcitabine in nude mice

Tumors were induced in nude mice by subcutaneous injection of Mz-ChA-2 and EGI-1 cell lines. These cell lines were selected because they had shown the best growth capability in nude mice in our previous studies^[20,21].

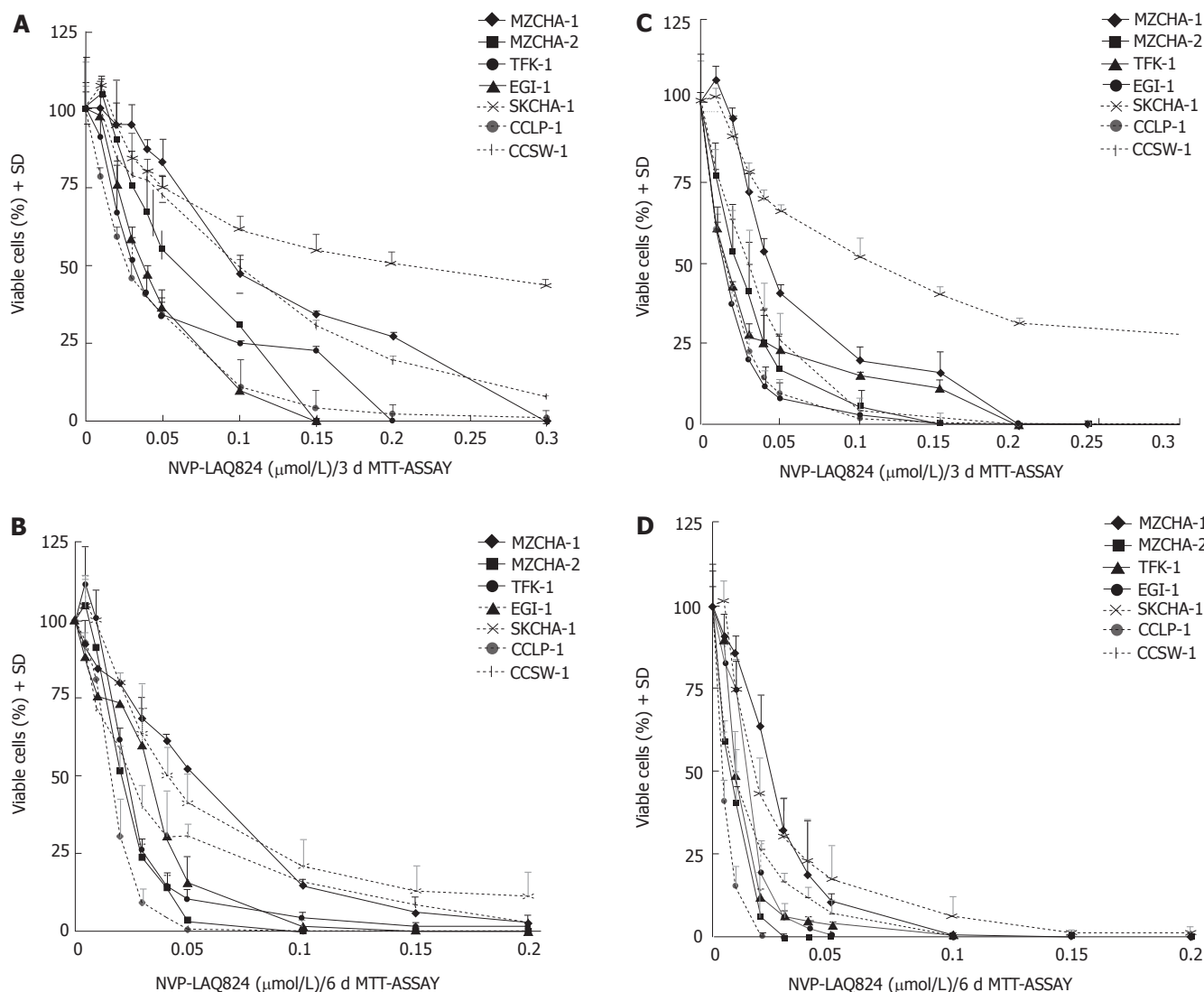


Figure 1 *In vitro* treatment of biliary tract cancer cell lines with NVP-LAQ824 and NVP-LBH589. **A:** 3-d incubation with NVP-LAQ824 ($n = 3$); **B:** 6-d incubation with NVP-LAQ824 ($n = 3$); **C:** 3-d incubation with NVP-LBH589 ($n = 3$); **D:** 6-d incubation with NVP-LBH589 ($n = 3$).

Table 1 Inhibition of cell growth by NVP-LAQ824 and NVP-LBH589

Cell line	IC ₅₀ (μmol/L)			
	NVP-LAQ824		NVP-LBH589	
	3 d	6 d	3 d	6 d
TFK-1	0.06	0.05	0.01	0.01
EGI-1	0.07	0.05	0.01	0.01
CC-LP-1	0.05	0.02	0.01	0.01
CC-SW-1	0.13	0.05	0.04	0.01
Sk-ChA-1	0.21	0.08	0.15	0.04
Mz-ChA-1	0.14	0.07	0.09	0.04
Mz-ChA-2	0.10	0.04	0.02	0.01

Since NVP-LBH589 seemed to have a higher *in vitro* activity than NVP-LAQ824 in these two cell lines (Table 1), only NVP-LBH589 was investigated *in vivo* in order to save costs. Treatment of mice consisted of intraperitoneal injections with NVP-LBH589, gemcitabine, NVP-LBH589 plus gemcitabine (COMBO) or placebo (50 mL/L DMSO in D5W). Starting at d 19, respectively at d 26, of the

experiment, EGI-1 cell tumors showed a significantly reduced volume in comparison to control after treatment with NVP-LBH589 or COMBO ($n = 7$ for each group; $P < 0.05$). The statistically significant difference was maintained until the end of the experiment. In contrast to that, treatment of mice with gemcitabine alone did not result in any significant reduction of tumor growth compared to control (Figure 3A). Treatment of Mz-ChA-2 tumors with NVP-LBH589, COMBO, or gemcitabine resulted in a significantly reduced volume in comparison to control at d 5, 5, and 8 ($P < 0.01$), respectively ($n = 7$ for each group; Figure 3B). The statistically significant difference was maintained until the end of the experiment. Regarding tumor latency (representing the time for the tumor to increase to 150% of the initial volume when treatment was initiated), the extrapolated data were as follows for EGI-1: placebo d 6, LBH d 26, COMBO d 12, and gemcitabine d 4. The data for Mz-ChA-2 were: placebo d 3, LBH d 14, COMBO "not reached", and gemcitabine d 7. At the end of the experiment at d 29, tumor mass in EGI-1 cells-bearing mice was significantly diminished as compared to placebo after

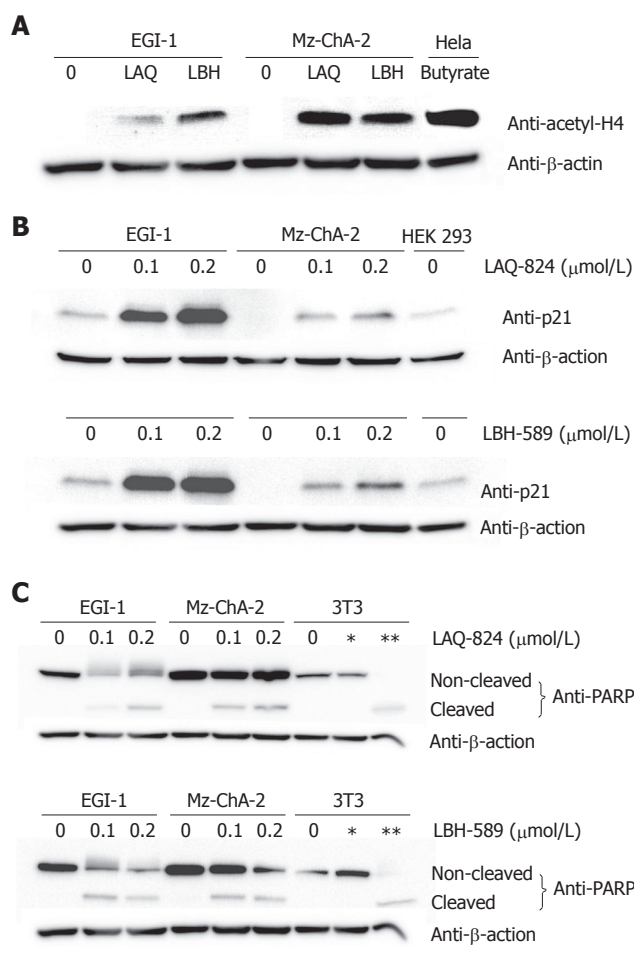


Figure 2 Mechanism of drug action after *in vitro* treatment with NVP-LAQ824 and NVP-LBH589 for 24 h. Lane *: NIH-3T3 cells treated with 25 $\mu\text{mol/L}$ etoposide for 5 h (negative control); lane **: NIH-3T3 cells treated with 10 $\mu\text{mol/L}$ etoposide for 24 h (positive control). **A**: acetylation of histone H4; **B**: p21^{WAF-1/CIP-1} expression; **C**: PARP cleavage.

Table 2 Cell cycle analysis (mean \pm SD, %)

	MZ-CHA-2			EGI-1		
	G0/G1	S	G2/M	G0/G1	S	G2/M
CTRL	38.3 \pm 3	44.7 \pm 3	17.0 \pm 3	58.7 \pm 5	29.2 \pm 2	12.1 \pm 1
LAQ824 (25 nmol/L)	37.0 \pm 1	42.8 \pm 2	20.1 \pm 2	50.1 \pm 7	31.9 \pm 4	18.1 \pm 3
LAQ824 (50 nmol/L)	36.9 \pm 2	38.7 \pm 5	24.4 \pm 4	41.2 \pm 2	34.8 \pm 2	24.1 \pm 4
LBH589 (25 nmol/L)	35.5 \pm 1	27.6 \pm 3	36.9 \pm 2	38.8 \pm 5	33.8 \pm 3	27.4 \pm 4
LBH589 (50 nmol/L)	38.8 \pm 2	9.0 \pm 3	52.3 \pm 2	46.0 \pm 1	15.1 \pm 2	38.9 \pm 1

treatment with NVP-LBH589 (-66%) or COMBO (63%) ($P < 0.05$; Figure 3C). In contrast to that, treatment of mice with gemcitabine alone (-6%) did not result in any significant reduction of tumor mass as compared to control (Figure 3C). For Mz-ChA-2 cells-bearing mice, tumor mass was significantly diminished after treatment with either NVP-LBH589 (-87%), COMBO (96%), or gemcitabine (-74%), respectively ($P < 0.01$; Figure 3C). For both cell lines, EGI-1 and Mz-ChA-2, the combination of NVP-LBH589 and gemcitabine was more effective in tumor mass reduction in comparison to gemcitabine alone ($P < 0.05$ and $P < 0.01$, respectively). Moreover, for tumor

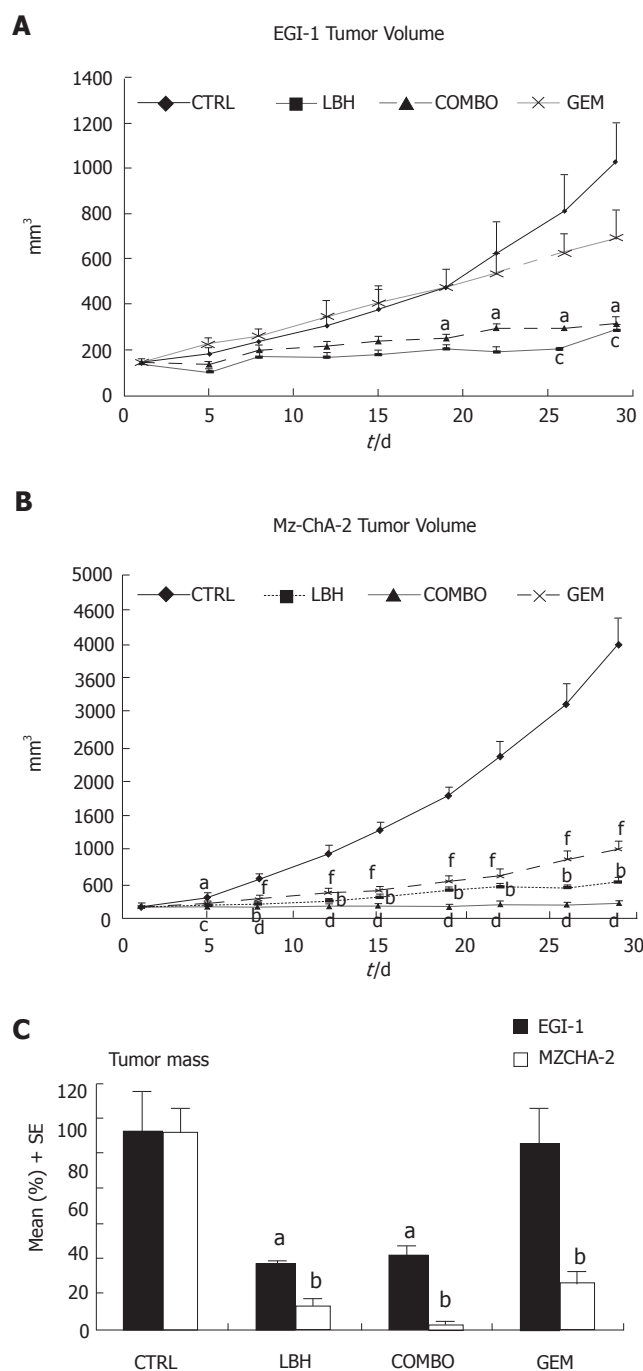


Figure 3 *In vivo* treatment with NVP-LBH589 \pm gemcitabine in chimeric mice. **A**: Effect on tumor volume of EGI-1 cells ($^aP < 0.05$, NVP-LBH589 vs control; $^cP < 0.05$, COMBO vs control); **B**: Effect on tumor volume of Mz-ChA-2 cells ($^aP < 0.05$, $^bP < 0.01$, NVP-LBH589 vs control; $^cP < 0.05$, $^dP < 0.01$, COMBO vs control; $^eP < 0.01$, gemcitabine vs control); **C**: Effect on tumor mass ($^aP < 0.05$, NVP-LBH589 or COMBO vs control; $^bP < 0.01$, NVP-LBH589 or COMBO or gemcitabine vs control).

cell line Mz-ChA-2, COMBO reduced tumor mass at a higher degree than NVP-LBH589 alone ($P < 0.05$), although NVP-LBH589 was administered at half dose in the COMBO group. Concerning side effects of the different drugs used in our experiments with EGI-1 and Mz-ChA-2 cells tumor-bearing mice, NVP-LBH589 alone (-17% and -19%, respectively) and COMBO (-7% and -10%, respectively) induced weight loss in the animals at d 29 of therapy as compared to the initial body weight, whereas

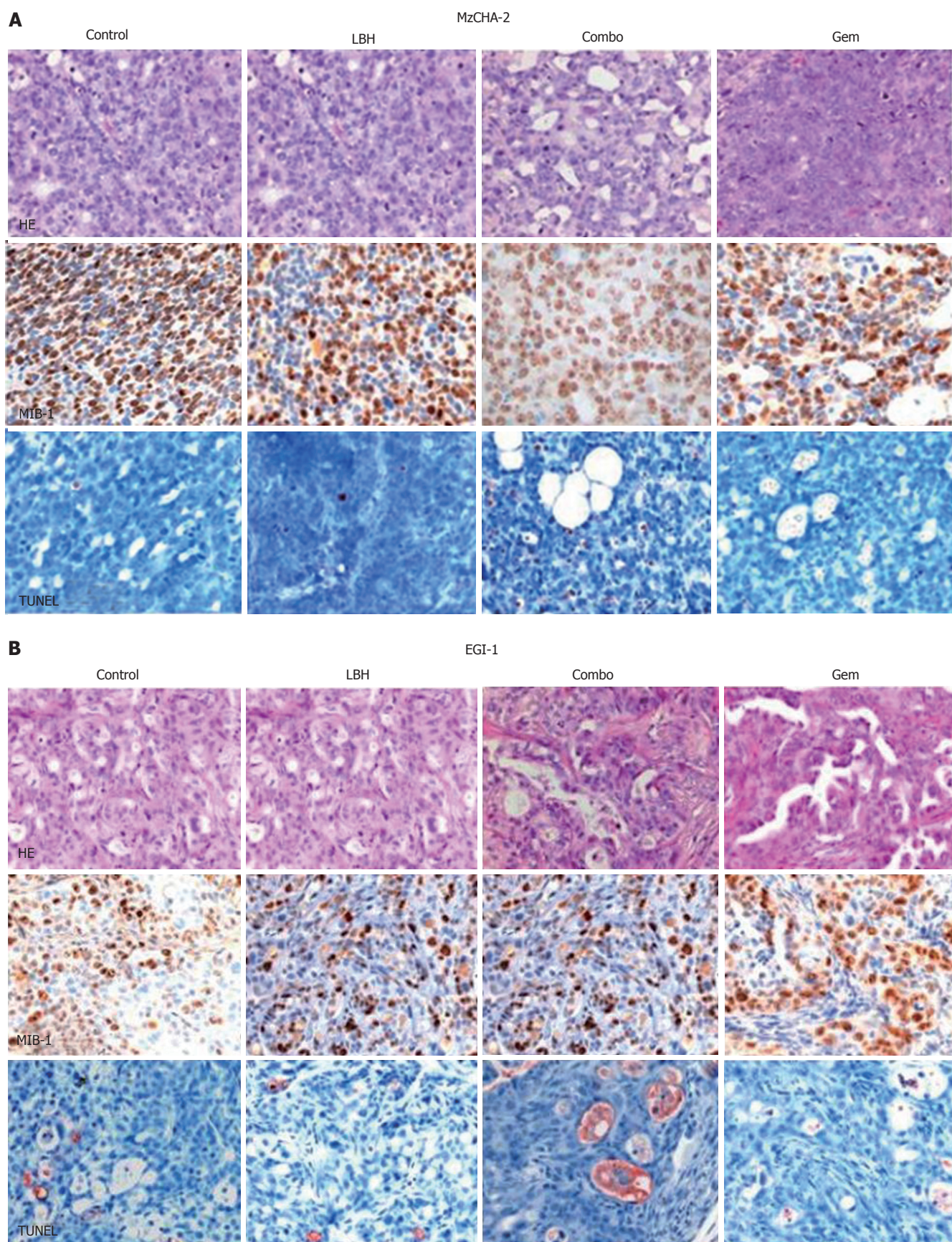


Figure 4 Hematoxylin-eosin (HE), MIB-1 (proliferation marker) and TUNEL (apoptosis marker) staining of mouse tumors (SABC, $\times 40$). **A:** cell line Mz-ChA-2; **B:** cell line EGI-1.

gemcitabine alone did not. One animal died at d 12 (EGI-1 experiment) in the COMBO group, and 2 animals died at d 22 and d 26 (Mz-ChA-2 experiment) in the NVP-

LBH589 group from significant weight loss. These three mice were replaced by back-up mice in order to guarantee equal numbers in the different groups ($n = 7$) for the

Table 3 MIB-1- and TUNEL-staining of mouse tumor specimens (mean, %)

	MZ-CHA-2		EGI-1	
	MIB-1	Apoptosis	MIB-1	Apoptosis
CTRL	85	0.1	38	4.3
GEM	77	2.2	29	6.5
LBH	62	2.9	26	5.9
COMBO	67	7.8	17	9.0

comparisons of tumor volume and tumor mass at the end of the experiment. Finally, NVP-LBH589 alone at a dose of 40 mg/kg (5 × weekly) caused diarrhea and exsiccation in the animals starting at d 16 of the experiment. We performed no further microscopic evaluation for organ damage in these animals. However, there was no macroscopic tissue damage.

In order to assess the anti-tumoral drug mechanism, paraffin sections of mouse tumors were stained with hematoxylin-eosin (HE), MIB-1 (proliferation marker) and TUNEL (apoptosis marker) (Figure 4). Treatment with NVP-LBH589, gemcitabine, and COMBO reduced proliferation (reduced MIB-1 staining) and induced apoptosis (increased TUNEL staining) (Figure 4, Table 3).

DISCUSSION

Cholangiocarcinoma (CC) is the second most common primary hepatic tumor, with increasing incidence and a high mortality^[22-24]. Tumors may occur anywhere along the biliary ductal system, and are arbitrarily defined anatomically as intra-hepatic or extra-hepatic CC as well as adenocarcinoma of the gallbladder^[25,26]. Unfortunately, the vast majority of patients with CC typically seek treatment with advanced disease, and often these patients are deemed poor candidates for curative surgery. Moreover, conventional chemotherapy and radiation therapy have not been shown to be effective in prolonging long-term survival, and although photodynamic therapy combined with stenting has been reported to be effective as palliative treatment, it is not curative^[27-30]. Thus, there is a need to develop novel therapeutic strategies for CC based on exploiting selected molecular targets that would have an impact on clinical outcome^[31,32].

One possible approach may be the use of HDACIs, perhaps in combination with conventional chemotherapy or other so called biologicals. Therefore, in our current study, we investigated the two novel cinnamic hydroxamic acid compounds NVP-LAQ824 and NVP-LBH589 for *in vitro* and *in vivo* treatment of CC. Cell-growth inhibition by NVP-LAQ824 and NVP-LBH589 was studied *in vitro* in 7 human biliary tract cancer cell lines by MTT assay. Treatment with the both compounds significantly suppressed the growth of all cancer cell lines after 3 and 6 d with a mean IC₅₀ (3d) of 0.11 and 0.05 μmol/L, and with a mean IC₅₀ (6d) of 0.05 and 0.01 μmol/L, respectively. In previous *in vitro* studies, NVP-LAQ824 exhibited a potent anti-proliferative activity against the HCT116 colon carcinoma cell line (IC₅₀ = 0.01 μmol/L), as well as against H1299 (IC₅₀ = 0.15 μmol/L) and A549 non-small cell

lung carcinoma cells (IC₅₀ = 0.15 μmol/L), DU145 (IC₅₀ = 0.018 μmol/L), PC3 prostate cancer cell lines (IC₅₀ = 0.023 μmol/L), Cal27 (IC₅₀ = 0.04 μmol/L), SCC25 (IC₅₀ = 0.095 μmol/L), SCC9 (IC₅₀ = 0.245 μmol/L), FaDu (IC₅₀ = 0.340 μmol/L) head and neck squamous carcinoma cells, and MDA-MB-435 (IC₅₀ = 0.039 μmol/L) and BT-474 (0.03 μmol/L) human breast adenocarcinoma cells after 72 h of exposure^[6,33,34]. The *in vitro* effects of NVP-LAQ824 on hematologic malignancies have been examined in several human cell lines, including AML (HL60, K562), lymphoma (Namalwa, DHL10), and multiple myeloma (IM9, 8226). Death of more than 90% of all cells was seen in all cell lines following 48 h of drug incubation, with exposures as low as 0.1 μmol/L^[35-37]. NVP-LBH589, the second investigated compound, was even more effective *in vitro* for the treatment of human chronic myeloid leukemia blast crisis K562 and LAMA-84, multiple myeloma, and acute leukemia MV4-11 cells^[5,38-40].

The *in vitro* anti-tumoral drug mechanism in our study was assessed by immunoblotting for acH4 and p21^{WAF-1/CIP-1}, PARP assay, and cell cycle analysis. Treatment with both compounds was associated with hyperacetylation of nucleosomal histone H4, increased expression of p21^{WAF-1/CIP-1}, induction of PARP cleavage, and cell cycle arrest at G2/M checkpoint. Hyperacetylation of nucleosomal histones H3 and H4 has been previously reported as the primary mechanism of action of NVP-LAQ824 and NVP-LBH589 for the treatment of HCT116, A549, MDA-MB-231, BT-474, MCF-7 (human breast adenocarcinoma), HL-60, K562, MV4-11, and multiple myeloma cells^[5,6,34,35,41]. Inactivation of tumor suppressor p14/mdm-2/p53/p21^{WAF-1/CIP-1} signaling pathway is a phenomenon that occurs frequently in CC, mostly due to p53 mutations or by up-regulation of mdm-2, an inhibitor of p53^[42]. P21^{WAF-1/CIP-1} binds to the cell division kinase (CDK) 4:cyclin D complex and prevents it from phosphorylating Rb. Therefore, the release of the bound E2F transcription factor that regulates genes encoding for proteins critical to entrance into the S-phase of the cell cycle is prevented^[25]. Up-regulation of p21^{WAF-1/CIP-1} induced by NVP-LAQ824 and NVP-LBH589 has been described for BT-474, MCF-7, MDA-MB-468 (human breast adenocarcinoma), human umbilical vein endothelial cells (HUVEC), LNCaP (prostate cancer), K562, LAMA-84, MV4-11 and multiple myeloma cells^[5,34-36,38,43,44]. P21^{WAF-1/CIP-1} is likely to be transcriptionally activated by a p53-independent mechanism, since it has been shown for NVP-LAQ824 to activate the p21^{WAF-1/CIP-1} promoter^[6]. Other commonly induced tumor suppressor genes include p53, p57 and the CDK inhibitor p27^{kip1}^[34,36,38,40].

Another mechanism of *in vitro* drug action found in our study was induction of apoptosis as shown by the increase of PARP cleavage, a process mediated by active effector caspase-3. This phenomenon, induced by NVP-LAQ824 and NVP-LBH589, has been described previously for several other cell lines^[5,34-36,39,43]. Furthermore, transcriptional up-regulation of pro-apoptotic genes such as *FAS*, *TRAIL*, *BID* or *BAX* and down-regulation of anti-apoptotic genes such as *BCL-XL* or *FLIP*, although not examined in our study, may explain apoptotic cell death induced by HDACIs, besides priming of malignant cells

for innate immune effector mechanisms^[36,45-49]. Whereas a number of different mechanisms were described for HDACi-induced cell death, including the death receptor-activated caspase cascade, usually the intrinsic apoptotic pathway, mediated by mitochondrial membrane disruption, is commonly activated, which has also been shown for NVP-LAQ824 and NVP-LBH589^[38,50]. Among several genes repressed by HDACis are cyclin D1 and thymidylate synthase. The result is a G1 or G2-M cell cycle arrest and differentiation, common mechanisms of the anti-proliferative effect of HDACis. Further potential growth inhibitory mechanisms include induction of other cell cycle regulatory genes such as GADD45 α and β and up-regulation of transforming growth factor β (TGF- β) receptor signaling, leading to repression of c-myc and cell cycle arrest. Several studies, including ours, investigating NVP-LAQ824 and NVP-LBH589, could demonstrate G2-M cell cycle arrest, induced by those compounds^[6,34,44], others detected G1-arrest^[5,35,36].

Encouraged by our *in vitro* results, we decided to test the most effective drug NVP-LBH589 *in vivo* in comparison to placebo using the chimeric mouse model. At d 29 of the experiment, NVP-LBH589 significantly reduced tumor mass in comparison to placebo and potentiated the efficacy of gemcitabine. The NVP-LBH589 dose of 40 mg/kg (5 d/wk) was selected according to a study testing different intravenous doses of NVP-LAQ824 between 5 and 100 mg/kg (5 d/wk) in a similar chimeric mouse model using the human colon cancer cell line HCT116^[6]. *In vivo* data for NVP-LBH589 were only available after completion of our study, using human prostate carcinoma cell PC-3 xenografts, being able to show tumor reduction at a dose of 10 mg/kg per day^[44]. Surprisingly, weight loss of the animals, as observed in our study, was not previously reported for NVP-LAQ824^[6]. Therefore, further studies are required to examine animals microscopically for organ damage. Unfortunately, this was not done in our experiments. Since we already experienced this phenomenon in a small pilot study with 3 animals (data not shown), we decided to have the dose of NVP-LBH589 for the combination therapy with gemcitabine. The latter drug was selected since it is a commonly used drug for CC monotherapy in the palliative setting^[30]. Even with this dose reduction of NVP-LBH589, our results do strongly support an additive effect which, however, requires further investigation. In human breast cancer cell lines SKBR-3 and BT-474, NVP-LAQ824 also enhanced taxotere-, epirubicin B-, and gemcitabine-induced apoptosis *in vitro*^[34]. For head and neck squamous carcinoma cells, the combination of NVP-LAQ824 with gemcitabine was more effective *in vitro* than a combination with docetaxel, paclitaxel, or cisplatin, especially when used in the sequence of the cytotoxic agent first for 24 h, followed by 48 h of NVP-LAQ824^[33]. There might be also a synergistic *in vitro* effect of a combination of NVP-LAQ824 and VEGFR-inhibitor PTK787/ZK222584 as demonstrated for PC3 and MDA-MB-435 cells^[41]. Similar results were observed *in vitro* for a combination of NVP-LBH589 and proteasome inhibitor bortezomib^[39], NVP-LBH589 and tyrosine kinase inhibitor AMN107^[40], and NVP-LBH589 and heat-shock protein 90 (hsp90) inhibitor

17-allyl-amino-demethoxy geldanamycin (17-AAG)^[5].

Anti-tumoral drug mechanism in our *in vivo* model was assessed by TUNEL assay, and immunohistochemistry for MIB-1, which revealed increased apoptosis (TUNEL) and reduced cell proliferation (MIB-1), confirming our *in vitro* data. Surprisingly, the calculated numbers were much smaller than expected from the *in vitro* experiments.

Finally, it should be mentioned that other mechanisms of anti-tumor activity of HDACis include anti-angiogenic properties through alteration of VEGF-, HIF-1 α -, Ang-2/Tie-2-, and survivin-signaling, down-regulation of endothelial nitric oxide synthase (eNOS), suppression of tumor invasion through negative regulation of matrix metalloproteinases expression, and depletion of several oncoproteins that are normally stabilized by binding to the hsp90 multi-chaperone complexes, including bcr-abl, ErbB2, Raf-1, or AKT (HDACis may induce acetylation of hsp90 *via* inhibition of HDAC 6, thus inhibiting chaperone activity, resulting in polyubiquitination and proteasomal degradation of the hsp90 client proteins), which was not further investigated in our study^[44,51].

In conclusion, our findings suggest that NVP-LBH589 and NVP-LAQ824 are active against human biliary tract cancer *in vitro*. In addition, NVP-LBH589 demonstrated significant *in vivo* activity and potentiated the efficacy of gemcitabine. Therefore, further clinical evaluation of NVP-LBH589 alone or its combination with gemcitabine for the treatment of biliary tract cancer is recommended.

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COMMENTS

Background

Carcinoma of the biliary tree are rare tumors of the gastrointestinal tract with worldwide rising incidence for intrahepatic cholangiocarcinoma during the last several years. At present, complete resection is the only potentially curative therapy. But, most of the patients present with already advanced disease. In the palliative setting, systemic chemotherapy (chemoradiation) has not been clearly proven to prolong survival significantly.

Research frontiers

Receptor tyrosine kinase (TKs), cyclooxygenase-2 (COX-2), mammalian target of rapamycin (m-TOR), and histone deacetylase (HDAC) inhibitors are currently under preclinical and clinical evaluation as new treatment options.

Innovations and breakthroughs

In 2006, the results of a two-stage phase I and a phase II study suggested a therapeutic benefit for EGFR blockade with erlotinib and combined erbB-1/erbB-2 signaling inhibition with GW572016 (Lapatinib) in patients with biliary cancer. In 2007, a phase II study was presented examining the combination of gemcitabine, oxaliplatin and angiogenesis inhibitor bevacizumab (GEMOX-B) in unresectable or metastatic biliary tract cancer/gallbladder cancer. Early evidence of antitumor activity was seen.

Applications

The aim of our study was to investigate the *in vitro* and *in vivo* treatment with histone deacetylase inhibitors NVP-LAQ824 and NVP-LBH589 in biliary tract cancer. Our findings suggested that NVP-LBH589 > NVP-LAQ824 are active

against human biliary tract cancer *in vitro*. In addition, NVP-LBH589 demonstrated significant *in vivo* activity and potentiated the efficacy of gemcitabine.

Terminology

Histones (positively charged proteins) are the major components of chromatin. Histone acetylation and deacetylation modulate chromosome structure and regulate gene transcription. Two families of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs), activate and repress gene expression, respectively. Aberrant HAT or HDAC activity is associated with various epithelial and hematologic cancers. HDACs may play an important role in human oncogenesis through HDAC-mediated gene silencing and interaction of HDACs with proteins involved in tumorigenesis. HDAC inhibition could potentially restore normal processes in transformed cells without affecting normal cells.

Peer review

This manuscript is original, interesting and well-written. It deserves to be published.

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Increasing dietary fiber intake in terms of kiwifruit improves constipation in Chinese patients

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Abstract

AIM: To investigate if increased dietary fiber, in terms of kiwifruit, is effective in Chinese constipated patients.

METHODS: 33 constipated patients and 20 healthy volunteers were recruited for a 4-wk treatment of kiwi fruit twice daily. Response during wk 1-4 was defined as an increase in complete spontaneous bowel motion (CSBM) ≥ 1 /wk. Secondary efficacy included response during wk 1-4, individual symptoms and scores of bowel habits and constipation. Responses were compared with the baseline run-in period. Colonic transit time and anorectal manometry were performed before and after treatment.

RESULTS: Responder rate was 54.5% in the constipated group. The mean CSBM increased after treatment (2.2 ± 2.6 vs 4.4 ± 4.6 , $P = 0.013$). There was also improvement in the scores for bothersomeness of constipation ($P = 0.02$), and satisfaction of bowel habit ($P = 0.001$), and decreased in days of laxative used ($P = 0.003$). There was also improvement in transit time ($P = 0.003$) and rectal sensation ($P < 0.05$). However, there was no change in the bowel symptoms or anorectal physiology in the healthy subjects.

CONCLUSION: Increasing dietary fiber intake is effective in relieving chronic constipation in Chinese population.

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Key words: Constipation; Dietary fiber; Kiwifruit; Chinese

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INTRODUCTION

Functional constipation is a common problem in clinical practice. In the Western population, the prevalence was reported to be as high as 24% in elderly persons and more commonly among women^[1]. We observed a prevalence of 14% of constipation in the Hong Kong population^[2].

In mild cases of functional constipation, it has been suggested that the treatment includes general measures like increased intake of water and dietary fiber, and use of simple laxatives. A diet with enough fiber (20-35 g each day) helps form a soft, bulky stool. Sufficient dietary fiber is also needed to promote normality in bowel movement frequency over the long term^[3,4]. Colonic transit has been shown to be related to stool weight and dietary fiber intake^[5,6]. On the other hand, there are only a few studies that looked for dietary fiber intake by patients with chronic constipation^[7-9]. In addition, in a trial with a proprietary fiber product, less than a half of the patients with self-defined constipation responded^[10]. Also, anorectal physiology was not assessed in most of these studies.

Numerous anecdotal reports have suggested that kiwifruit has laxative effects, and suggested it could be an acceptable dietary supplement, especially for the elderly who often experience constipation. It has been reported that the dietary fiber in kiwifruit is about 3.4 g/100 g, thus being a good source of dietary fiber. Rush *et al*^[11] has demonstrated the laxative effect of kiwifruit in the elderly subjects.

In the current study, we aimed at assessing if increasing dietary fiber intake could improve functional constipation. We chose kiwifruit to be the source of dietary fiber in the study. In addition to constipation symptoms, we also assessed if there was any improvement in anorectal physiology after fiber intake.

MATERIALS AND METHODS

Protocol

This is a single center, age and sex-matched, case control study performed from 31 December, 2006 to 31 March, 2007, consisting of a 2-wk baseline assessment and 4-wk treatment period. Both patients and controls were given two Zespri Kiwi fruit (1 in the morning after breakfast and 1 in the evening after dinner) throughout the four-week treatment period. The kiwifruit were of the green, 'Hayward' variety, and were supplied at an optimum ripeness for consumption. The kiwi fruit was dispatched in two batches, the first on d 0 and the second at the begin-

ning of wk 3 to ensure the freshness of the fruit. All the participants were seen by the investigator on d 0 and at the end of wk 4. Bisacodyl was given as rescue medicine and patients were instructed to take it if no bowel motion for ≥ 4 d. The subjects were asked to fill in the diary during the 2-wk baseline period, and throughout the treatment period. Anorectal physiology tests including both colonic transit and anorectal manometry were performed for both the constipated patients and healthy controls before and after the treatment with kiwi fruit. The study was performed in accordance with the Declaration of Helsinki regarding informed patient consent and institutional review board approval.

Patients and controls

Thirty-five age ≥ 18 years constipated subjects were recruited for the study. They were recruited from the Gastroenterology outpatient clinic of Department of Medicine, Queen Mary hospital, Hong Kong, after assessment by a gastroenterologist, and satisfied the following criteria: constipation for 6 mo or more, with an average of less than three complete spontaneous bowel motion (CSBM) per week; and at least one of the following occurring for more than 25% of the time: straining, passage of lumpy or hard stools, and sensation of incomplete evacuation. Complete spontaneous bowel motion referred to the feeling that defecation led to complete passage of stool rather than partial or incomplete evacuation of stool without relief of symptoms. In addition, these patients also satisfied the Chinese constipation questionnaire^[12]. Patients with a previous history of constipation predominant irritable bowel syndrome using the Rome II criteria were excluded from the present study. Additional exclusion criteria were inability to understand Chinese, constipation due to secondary causes, history of malignancy and significant systemic disease. Pregnant or breast feeding women were also excluded. Patients who failed to complete the diary or if the constipation was not confirmed by diary were excluded.

Twenty age and sex matched volunteers were recruited by posters distributed at the university campus and community. All the volunteers were screened and then assessed by the same gastroenterologist. All the subjects will be asked to maintain normal diet pattern and activity during study period.

Assessment

Assessment of constipation symptoms was done in a diary format. Patients recorded their constipation symptoms in a diary throughout the 2-wk baseline and 4-wk treatment period. On a daily basis, they recorded the symptoms of CSBM, straining score (using a 3-point score: no straining/acceptable straining/too much straining), the 7-point Bristol stool scale form (range from 1, separate hard lumps, to 7, watery with no solid pieces)^[13], and the intake of Bisacodyl as rescue medicine. Final assessment included the patients' satisfaction with bowel habits over the past week (using a 5-point ordinal scale, 0 = very satisfied, 4 = not at all satisfied), and the bothersomeness of constipation (using a 5-point ordinal scale, 0 = not at all, 4 = a very great deal).

Assessment of bowel transit time

Normal and slow transit constipation were confirmed by X-ray and colonic motility studies performed in all patients. Colonic transit time was assessed through the use of radiopaque markers, as modified from the method described by Metcalf *et al*^[14] (14). In brief, 4 sets of distinctive radiopaque markers of different shapes and size (circle on d1, semi-cylinder on d 2, dot on d 3 and cylinder on d 4) were ingested by the volunteers on 4 consecutive days. X-ray of the abdomen was taken on d 5 to assess the mouth to anal transit and segmental colon transit. Transit in the right, left, and rectosigmoid colon was calculated by adding all markers seen in these regions on d 5. Slow total colonic transit was defined as > 67 h, the mean transit plus 2 standard deviations averaged from published studies.

Anorectal manometry

The manometry catheter (Zinetics Manometric Catheter, Medtronic) had a latex balloon on its tip that could be distended with air *via* a handheld syringe, and it had 8 perfusion ports spaced 0.5 cm apart beginning 2 cm below the balloon to measure pressures. The catheter was perfused with degassed water at a rate of 0.5 mL/min by a low-compliance pump (Densleeve Manometric infusion pump-16 channel E4500). The outer diameter of the catheter was 4.5 mm. Pressures were recorded and displayed using a model (Polygraph Medtronic Functional testing Software 2.05). Pressure recordings were analyzed manually.

With the patient in the left lateral position, the manometry catheter was lubricated and inserted into the rectum. It was then pulled back in 1-cm steps, and pressures were recorded at each position while the patient was instructed to relax. The peak pressure (averaged across all 8 perfusion ports) defined anal canal resting pressure. The second perfusion port was then positioned in the high-pressure zone of the anal canal, and the rectal balloon was distended with varying volumes of air (10, 20, 30, 40, 50 mL) to determine the smallest volume of distention that elicited a rectoanal inhibitory reflex (RAIR, defined as the reflex decrease in anal canal pressure that is elicited by rectal distention). Next, the rectal balloon was inflated in 20-mL steps up to 200 mL to assess the threshold for the first sensation, sensation of urge to defecate and the maximum tolerable volume. A phosphate enema was administered approximately 30 min before the anorectal manometry and balloon defecation tests.

Statistical analysis

Primary efficacy variable: The primary efficacy variable was the responder rate for CSBM during the first 4 wk of treatment. Patients with a mean increase of CSBM ≥ 1 /wk compared with the last 14 d of baseline were defined as responders, provided that they had completed at least 7 d of treatment.

Secondary efficacy variables: These included the change from baseline in scores for individual constipated symptoms (stool form, straining scores, bothersomeness of constipation, and satisfaction of bowel habit). Days of laxatives used and percentage of patients needed laxatives were assessed.

Table 1 Demographic data and constipation symptoms of the constipated patients and healthy subjects

	Treatment group (<i>n</i> = 33)	Control group (healthy subjects) (<i>n</i> = 22)	<i>P</i> value
Sex (female)	72.70%	80%	0.74
Female to male ratio	2.67:1	04:01	
Ethnicity (Chinese)	100%	100%	
Age (yr \pm SD)	49.9 \pm 12	50.8 \pm 14	0.82
Duration of constipation (yr \pm SD)	20.3 \pm 15	-	
Complete spontaneous bowel motion per week (mean \pm SD)	1.9 \pm 1	-	
Passage of hard stool	72.70%	-	
Incomplete evacuation	84.80%	-	
Straining	90.90%	-	

Tertiary efficacy variables: These included any improvement in anorectal physiology, which included the colonic transit time, simulated defecation pressure and also the rectal sensation.

Statistical analysis was performed using SPSS (SPSS, Chicago, IL, USA) statistical software. Demographics for patients and controls were summarized by calculating means (SD) (or median (range)) for continuous variables (for example, age and severity score) and proportions for categorical variables (for example, sex). Comparisons were performed with the Student's *t* test for continuous variables, and with Chi square test for categorical data. Paired *t* test was used to assess the bowel habit and anorectal physiology parameters before and after treatment within the same group. All statistical tests were two-sided, and *p* value of less than 0.05 was considered to indicate a statistically significant difference.

RESULTS

Demographic data

There were 35 patients screened, but only 33 patients were enrolled into the study. Two of them refused to participate. The demographic data, baseline constipation symptoms of both the patients and controls are summarized in Table 1. There was no difference in the demographic data as they were purposely matched. The anorectal physiology was markedly different between the constipated patients and healthy controls. These include prolonged colonic transit time and impaired rectal sensation.

Primary efficacy

The mean CSBM per week before and after treatment in patients was 2.2 ± 2.6 *vs* 4.4 ± 4.6 (*P* = 0.013). The responder rate, ie mean increase of CSBM ≥ 1 /wk, was 54.5% (18 patients). Whereas in the controls, the mean CSBM per week before and after treatment was 6.5 ± 1.6 *vs* 7.1 ± 2.2 (*P* = 0.31). There was no difference in the responder rate between those with slow and normal transit (42.9% *vs* 63.2% , *P* = 0.25).

Secondary efficacy

The scores for stool form, straining, bothersomeness of constipation, satisfaction of bowel habit, and use of

laxatives before and after treatment are summarized in Table 2. The scores for bothersomeness of constipation and satisfaction of bowel habit were significantly lower after treatment. The number of days of laxatives used, and the percentage of patients needed laxatives as rescue therapy was lower after treatment with Kiwi fruit (Table 2). There was no significant change in bowel habits or satisfaction in the control group after treatment.

Tertiary efficacy

The parameters of anorectal physiology before and after treatment are summarized in Table 3. There was marked decrease in total colonic transit time, especially in the sigmoid-rectal segment, in the constipated patients after treatment. In addition, improvement in rectal sensation, in terms of the first sensation, urge sensation and the maximum tolerable volume and the rectoanal inhibitory reflex, was seen in the constipated patients after treatment. On the contrary, there was almost no significant change in the anorectal physiology parameters in the healthy subjects after treatment.

Safety and side effects

There were no side effects reported in both the patient and control groups. None of them, including the healthy subjects, reported diarrhea.

DISCUSSION

The current study demonstrated the efficacy of increasing dietary fiber in Chinese constipated patients, with significant improvement in complete spontaneous bowel motion, decrease laxatives used, decrease bothersomeness of constipation and improved satisfaction of bowel habit. More importantly, anorectal physiology was shown to improve significantly after treatment.

It has always been suggested that increasing dietary fiber might be helpful in patients with mild constipation. However, there have been few studies done on this area, and few studies assessed the improvement objectively using the criteria of CSBM, as well as anorectal physiology.

It is interesting to note that in this study, improvement was seen not only in the constipation symptoms, but also in terms of colonic transit time and rectal sensation in the constipated patients. It has been reported that gas production from fiber metabolism may limit acceptance, which is particularly true for bran^[15] and other insoluble fibers. Despite most of the fibers in kiwifruit are insoluble fiber, none of the patients or controls reported bloating or gas, or intolerance. Voderholzer *et al*^[10] reported in their study on proprietary fiber in self-defined constipation, only 20% of slow transit patients profited from fiber, whereas more than 80% of patients without identifiable cause of their complaints had a partial or complete improvement. We observed in this study that the responder rate did not differ in both groups of patients. This is probably the first study reporting an improvement in rectal sensation: the first sensation, urge and maximal tolerable volume in constipated patients after increasing fiber intake. Despite the change in anorectal physiology parameters in constipated patients, there was no significant change observed in

Table 2 Secondary efficacy variables before and after the kiwi fruit treatment in the constipated patients and healthy subjects (mean \pm SD)

	Constipated patients			Healthy subjects		
	Before treatment	After treatment	P value	Before treatment	After treatment	P value
Bristol stool scale	3.1 \pm 1.9	3.3 \pm 1.2	0.58	4.0 \pm 0.9	4.2 \pm 0.8	0.94
Straining	1.9 \pm 0.5	1.8 \pm 0.5	0.88	0.3 \pm 0.5	0.4 \pm 0.5	0.97
Bothersomeness of constipation	2.6 \pm 0.9	2.0 \pm 1.1	0.02	0.2 \pm 0.5	0.2 \pm 0.4	1.0
Satisfaction of bowel habit	2.7 \pm 0.9	1.6 \pm 1	0.001	0.3 \pm 0.5	0.2 \pm 0.5	0.97
Number of days taking laxatives (rescue therapy)/wk	2.2 \pm 2.5	0.8 \pm 1.5	0.003	0	0	-
Percentage of patients taking laxatives	60.60%	30.30%	0.013	0	0	-

Table 3 Anorectal physiology parameters before and after the kiwi fruit treatment in the constipated patients and healthy subjects

	Constipated patients			Healthy subjects		
	Before treatment	After treatment	P value	Before treatment	After treatment	P value
Transit time in right segment (h)	11.6 \pm 12	12.6 \pm 12	0.77	4.2 \pm 7	4.1 \pm 6	0.55
Transit time in left segment (h)	23.3 \pm 16	19.6 \pm 13	0.23	6.0 \pm 9	6.7 \pm 8	0.97
Transit time in sigmoid-rectal segment (h)	19.5 \pm 16	7.6 \pm 7	< 0.0001	7.2 \pm 11	3.4 \pm 5	0.045
Total colonic transit time (h)	54.5 \pm 29	39.6 \pm 22	0.003	16.8 \pm 23	14.1 \pm 14	0.23
Rectal pressure (mmHg)	26.3 \pm 18	30.4 \pm 19	0.3	23.6 \pm 19	27.2 \pm 18	0.54
Upper anal canal pressure (mmHg)	51.9 \pm 18	48.9 \pm 29	0.67	49.1 \pm 20	58.4 \pm 30	0.33
Lower anal canal pressure (mmHg)	63.9 \pm 22	67.9 \pm 25	0.44	58.4 \pm 23	74.4 \pm 27	0.025
Simulated Defecation-Push Maneuver (rectum) (mmHg)	65.8 \pm 45	90.8 \pm 55	0.019	72.2 \pm 50	88.2 \pm 50	0.31
Simulated Defecation-Push Maneuver (upper anal canal) (mmHg)	98.6 \pm 43	108.6 \pm 43	0.5	96.1 \pm 46	118.1 \pm 66	0.29
Simulated Defecation-Push maneuver (lower anal canal) (mmHg)	101.3 \pm 47	101.1 \pm 56	1.0	77.8 \pm 53	90.3 \pm 64	0.43
Rectoanal inhibitory reflex (R.A.I.R.) (mL)	17.2 \pm 7	11.3 \pm 4	0.003	12.1 \pm 4	12.2 \pm 4	1.0
First sensation (mL)	39.0 \pm 11	33.1 \pm 9	0.03	30.5 \pm 9	29.4 \pm 6	0.61
Constant sensation/urge (mL)	68.7 \pm 20	60.3 \pm 20	0.049	52.7 \pm 13	50.0 \pm 6	0.46
Maximum tolerable volume (mL)	99.6 \pm 35	85.1 \pm 21	0.031	83.2 \pm 14	78.9 \pm 14	0.07

normal healthy subjects. The reason for the difference in anorectal physiology response in the two groups is still unknown. However, Muller-Lissner in their meta-analysis did show that the improvement in transit time in constipated patients was greater than in healthy subjects^[16].

The current study aimed at assessing the effect of increasing fiber intake in constipated subjects. However, other nutrients that are present in the kiwifruit may also contribute partly to the laxative effect. The whole fruit (minus skin) was consumed, making it difficult to isolate the mechanism. One of the novel compounds in kiwifruit that has been suggested to interact in laxation is actinidin, a proteolytic enzyme belonging to the class of thiol-proteases.

It is important to note that double blinding was impossible in this study or other studies involving dietary fiber. However, the symptoms were compared to those at the baseline, and that patients themselves were their own controls. The additional age and sex matched control group was recruited to assess if there is any side effects or change in anorectal physiology in healthy subjects. The current study thus showed that dietary fiber in terms of kiwifruit is effective in Chinese patients with functional constipation, and with improvement in anorectal physiology. Further studies on the fiber intake in both constipated patients and healthy controls are warranted. In addition, studies may be needed to dissect the individual nutrient in kiwifruit, other than fiber, that may contribute to the laxative effect.

COMMENTS

Background

Dietary fiber has been suggested to improve functional constipation. However, are only a few studies that looked for dietary fiber intake by people with chronic constipation. In addition, in a trial with proprietary fiber product, less than a half of the patients with self-defined constipation responded. Also, anorectal physiology was not assessed in most of these studies.

Research frontiers

Therefore, we investigate if increased dietary fiber, in terms of kiwifruit, is effective in Chinese constipated patients. The anorectal physiology before and after the introduction of kiwifruit was assessed.

Innovations and breakthroughs

We found that responder rate to kiwi fruit was 54.5% in the constipated group. They have improvement in complete spontaneous bowel motion per week, symptom scores for constipation, as well as in colonic transit time.

Applications

Increase dietary fiber may improve functional constipation.

Peer review

This article is novel and provides an insight in the pathophysiology of constipation.

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RAPID COMMUNICATION

Distribution of solitary lymph nodes in primary gastric cancer: A retrospective study and clinical implications

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Abstract

AIM: To investigate the distribution pathway of metastatic lymph nodes in gastric carcinoma as a foundation for rational lymphadenectomy.

METHODS: We investigated 173 cases with solitary or single station metastatic lymph nodes (LN) from among 2476 gastric carcinoma patients. The location of metastatic LN, histological type and growth patterns were analyzed retrospectively.

RESULTS: Of 88 solitary node metastases cases, 65 were limited to perigastric nodes (N₁), while 23 showed skipping metastasis. Among 8 tumors in the upper third stomach, 3 involved right paracardial LN (station number: No.1), and one in the greater curvature was found in No.1. In the 28 middle third stomach tumors, 10 were found in LN of the lesser curvature (No.3) and 6 in LN of the left gastric artery (No.7); 5 of the 20 cases on the lesser curvature spread to No.7, while 2 of the 8 on the greater curvature metastasized to LN of the spleen hilum (No.10). Of 52 lower third stomach tumors, 13 involved in No.3 and 19 were detected in inferior pyloric LN (No.6); 9 of the 29 cases along the lesser curvature were involved in No.6.

CONCLUSION: Transversal and skipping metastases of sentinel lymph nodes (SLN) are notable, and rational lymphadenectomy should, therefore, be performed.

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Key words: Gastric cancer; Metastatic lymph node, Lymph node dissection; Rational lymphadenectomy; Sentinel lymph node

INTRODUCTION

Lymphatic metastasis is the most important factor for prognosis of gastric carcinoma. To avoid missing positive lymph nodes, surgeons have performed an extensive radical lymphadenectomy for gastric cancer, a method which is also used when early tumors are present. As a result, patients that did not have lymph node metastasis have had to undergo operations and face potentially avoidable risks^[1,2].

As the SLN concept gains acceptance, onco-surgery researchers are optimistic that the concept may serve as a breakthrough management tool to be used in gastric cancer; however, the concept is still considered to be at an investigative stage^[3]. We studied the distribution pathway of solitary or positive lymph nodes limited to a single station to provide a foundation for undertaking rational lymphadenectomy for gastric carcinoma.

MATERIALS AND METHODS

Patients

One hundred and seventy-three patients were selected from the 2476 patients with gastric cancer for whom radical operations were performed at the first affiliated hospital of the China Medical University between 1980 and 2003. The criteria used for inclusion was: (1) D2 lymph node dissections had been performed^[4]; (2) there were greater than 15 lymph nodes, and the resected specimens had been analyzed pathologically^[5]; (3) patients with pT4 and M1 stage were excluded^[5]; (4) patients' medical records were complete. Among the 173 cases, 88 had solitary lymph metastasis and 85 involved a single station lymph node. Sixty-four of the 88 patients were male and 24 female. The average age of the patients in this group was 57.6 ± 7.2 years (range 30-80). With respect to tumor location, the tumor was found in the upper third stomach area (U) in 8 cases, in the middle third (M) in 28, and in the lower third (L) in 52. Amongst the 85 patients with

Table 1 Distribution of metastatic lymph nodes (cases)

	L tumors			M tumors			U tumors		
	SN ¹	SSN ²	P value	SN ¹	SSN ²	P value	SN ¹	SSN ²	P value
No.1	2	0	- ³	2	2	NS	3	3	NS
No.2	1	0	-	0	0	-	1	5	NS
No.3	13	11	NS ⁴	10	1	NS	1	6	NS
No.4	4	9	NS	5	1	NS	0	2	NS
No.5	4	3	NS	1	1	-	0	0	-
No.6	19	21	NS	1	2	NS	0	3	NS
No.7	4	5	NS	6	3	NS	1	1	-
No.8	3	1	-	1	0	-	1	0	-
No.9	1	0	-	0	1	-	0	1	-
No.10	0	0	-	2	0	-	0	1	-
No.11	1	0	-	0	0	-	0	1	-
No.12	0	0	-	0	1	-	0	0	-
TOTAL	52	50	-	28	12	-	8	23	-

No.2, left paracardial node; No.4, greater curvature node; No.5, superior pyloric node; No.8, common hepatic node; No.9, celiac artery node; No.11, splenic artery node; No.12, hepatoduodenal node. ¹Solitary metastatic node; ²Single station metastatic node; ³Differences of the frequency distributions between the two groups were not determined; ⁴Not significant.

single station node metastasis, 60 were male and 25 female. The average age of the patients in this group was 58.2 ± 8.3 years (range 32-76). In respect of tumor location; the tumor was in the U in 23 cases, in the M in 12, and in the L stomach areas in 50.

Reference standard

The location of the tumor, the classification of the lymph node and histological type were by the Japanese Classification of Gastric Carcinoma^[6]. For classification, the symbol "No." indicates lymph node station number and "N" indicates the lymph node group. The histological types included differentiated and undifferentiated. An "adjacent metastasis" was defined as when lymph node metastasis is limited to the tumor side of N₁; "transversal metastasis" is limited to the region of N₁ opposite the tumor; and a "skipping metastasis" indicates a lymph node metastasized outside of N₁.

Histological growth patterns included massive, nest, and diffuse types^[7].

Statistical methods

All data were analyzed using SPSS13.0 statistics software. The differences of the frequency distributions between the two groups of lymph nodes were determined by a χ^2 -test or by Fisher's exact test. A χ^2 -test was adopted in the analysis of a single factor and a P value of less than 0.05 was considered statistically significant.

RESULTS

The distribution of metastatic nodes

Among the 88 patients with a solitary metastatic lymph node, in 65 (73.9%) the lymph nodes involved were within N₁, and 23 (26.1%) were over N₁. In 8 cases the tumor was in the U location, amongst which 6 (75%) were observed to be in N₁ and 2 (25%) in N₂. In 28 cases the tumor was in the M region, amongst which 19 (67.9%) were involved

Table 2 Distribution of solitary metastatic lymph nodes according to transversal zoning (cases)

	L tumors			M tumors		U tumors	
	A ¹	B ²	C ³	A ¹	B ²	A ¹	B ²
No.1	0	2	0	1	1	1	2
No.2	0	0	1	0	0	0	1
No.3	3	9	1	0	10	0	1
No.4	2	2	0	3	2	0	1
No.5	1	2	1	0	1	0	0
No.6	7	9	3	1	0	0	0
No.7	1	2	1	1	5	0	1
No.8	0	3	0	0	1	0	1
No.9	1	0	0	0	0	0	0
No.10	0	0	0	2	0	0	0
No.11	0	0	1	0	0	0	0

¹Tumors located at the greater curvature; ²Tumors located at the lesser curvature; ³Tumors extended in a circle of the stomach.

in N₁ and 9 (32.1%) in N₂. Among the 52 patients with L region cancers, 40 were found with solitary metastatic nodes in N₁, 10 (19.2%) with nodes in N₂ and 2 (3.9%) in N₃ (Table 1).

Comparisons were also made between cases with a solitary metastasis lymph node and single station nodes. No statistically significant difference was found with respect to the distribution of metastatic lymph nodes in the U, M and L regions using a χ^2 -test or Fisher's exact test (Table 1).

Adjacent metastasis

In 7 of the 8 cases with a U region tumor, the tumor located at the side of the lesser curvature region. Amongst them, metastatic lymph nodes in 2 (28.6%) were detected within No.1. Among the 28 cases with a tumor in the M area, in 20 the tumor was observed in the lesser curvature region and in 8 in the greater curvature region. Metastatic lymph nodes in 10 of the 20 cases were found within No. 3, while 3 of the 8 were within No. 4.

Fifty-two cases had tumors in the L stomach area. Amongst these the tumors in 29 located at the lesser curvature region, 15 at the greater curvature side and 8 extended in a circle. Metastatic lymph nodes in 9 of the 29 cases were found within No.3, in 7 of the 15 within No.6, and in 3 of the 8 within No. 6 (Table 2 and Figure 1).

Transversal metastasis

There was just one case with a tumor in the U area, and solitary metastatic lymph nodes were found within No.1. Twenty-nine patients had tumors at the lesser curvature side in the L area, and 9 (31%) of them were found to have metastatic lymph nodes within No.6. Of the 15 cases with tumors at the greater curvature side, in the L stomach area, 3 (20%) were involved in No.3 (Table 2 and Figure 1).

Skipping metastasis

Among the 8 cases with tumors in the U area of the lesser curvature region, one solitary metastatic lymph node (14.3% of total) was observed within No.7, and another node (14.3%) was found within No.8. In the M area, 5 (25%) of the 20 patients who had tumors at the lesser curvature

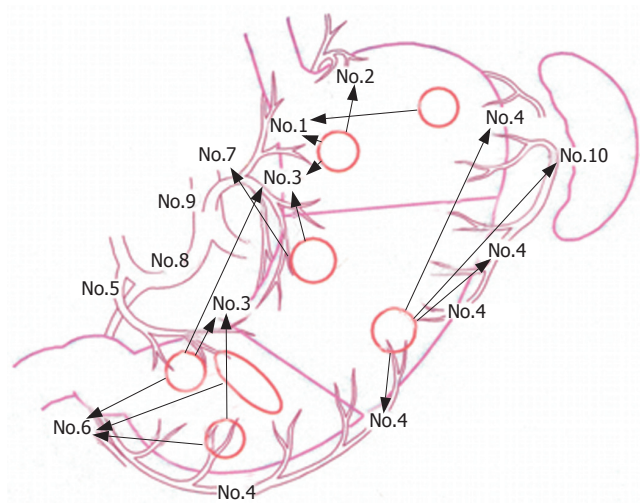


Figure 1 The metastatic pathway of SLN. Tumors are identified by circles and tumors extend in a circle in the lower third stomach.

Table 3 Histological type and skipping metastasis (cases)

	Group N ₁	Group > N ₁	P value
L tumors			0.192
Differentiated	22	4	
Undifferentiated	18	8	
M tumors			0.299
Differentiated	3	3	
Undifferentiated	16	6	
U tumors			1.000
Differentiated	3	1	
Undifferentiated	3	1	
Total			0.489
Differentiated	28	8	
Undifferentiated	37	15	

side had metastatic lymph nodes within No.7, while a metastatic node was detected within No.10 in 2 (25%) of the 8 patients with a tumor in the greater curvature side. Among the 29 cases with a tumor in area L, the number of lymph node metastases involved in No.1, No.7 and No.8 were 2 (6.9%), 2 (6.9%) and 3 (10.3%), respectively (Table 2 and Figure 1).

Relationships among histological type, growth pattern, and distribution pattern of solitary lymph nodes

Comparing the 65 patients with metastasis within N₁ and the 23 with a skipping metastasis, no statistical significance was found between histological type and the growth patterns of the two groups (Tables 3 and 4). Also, there was no significant difference between the adjacent metastasis group (39) and the transversal metastasis group (20) (Tables 5 and 6).

DISCUSSION

The distribution pattern of solitary metastatic nodes

There is little clinical literature available on the lymphatic routes of the stomach^[8-10]. After analyzing 51 cases, Kosaka *et al*^[11] reported that 44 gastric carcinomas with solitary lymph node metastasis were involved within N₁. Ichikura

Table 4 Histological growth pattern and skipping metastasis (cases)

	Group N ₁	Group > N ₁	P values
L tumors			0.087
Massive	8	3	
Nest	21	3	
Diffuse	11	6	
M tumors			0.456
Massive	2	2	
Nest	8	2	
Diffuse	9	5	
U tumors			0.645
Massive	1	1	
Nest	2	0	
Diffuse	3	1	
TOTAL			0.051
Massive	11	6	
Nest	31	5	
Diffuse	23	12	

Table 5 Histological type and transversal metastasis (cases)

	TN ¹	AN ²	P values
L tumors			0.793
Differentiated	8	11	
Undifferentiated	7	8	
M tumors			0.426
Differentiated	0	3	
Undifferentiated	3	13	
U tumors			0.114
Differentiated	0	3	
Undifferentiated	2	1	
Total			0.793
Differentiated	8	17	
Undifferentiated	12	22	

¹Transversal metastatic nodes; ²Adjacent metastatic nodes.

et al^[12] analyzed 69 cases with solitary lymph node metastasis, and found that in 6 of the 28 cases that had a lesion along the lesser curvature region, lymph node metastasis was observed along the greater curvature region. Kikuchi^[13] reported that skipping metastasis occurred in 14% of their patients with a solitary lymph node.

We investigated 173 cases with a solitary or single station metastatic lymph node from 2476 gastric cancer patients. First, 74.2% of the solitary metastasis lymph nodes were limited to within N₁. The lesion found in each region had one or more adjacent lymph node stations where a metastasis was more frequently found. These lymph node stations were relatively certain for the lesion in each region. Second, the frequency of transversal lymph node metastases, referring to the lesion in each region, was also relatively high. For example, with reference to a lesion in the L region, 31% of patients with a primary tumor on the lesser curvature area involved No.6, while 20% with a lesion on the greater curvature involved No.3. Third, the frequency of skipping metastases in our study was 25.8%. The frequencies of metastases in N₂ in regions U, M and L were 25%, 32.1% and 19.2%, respectively. We noted that the frequency of skipping metastasis in region M was 25%, that is, 25% of the lesions at the lesser curvature side were

Table 6 Histological growth pattern and transversal metastasis (cases)

	TN ¹	AN ²	P values
L tumors			0.513
Massive	6	2	
Nest	5	11	
Diffuse	4	6	
M tumors			0.453
Massive	0	2	
Nest	2	6	
Diffuse	1	8	
U tumors			0.687
Massive	1	0	
Nest	1	1	
Diffuse	1	2	
TOTAL			0.863
Massive	7	4	
Nest	8	18	
Diffuse	6	16	

¹Transversal metastatic nodes; ²Adjacent metastatic nodes.

detected in No.7 and No.8, and 25% of the lesions at the greater curvature were found in No.10.

From the above results, it is apparent that the distribution patterns of solitary nodes in gastric carcinoma are basically adjacent metastases; however, transversal and skipping metastasis were also found. The histological type and growth patterns did not influence the distribution of solitary metastatic nodes.

The first possible nodes of metastasis along the route of lymphatic drainage from the primary lesion should be SLN^[14]. However, because of the multidirectional and complicated lymphatic flow from the GI tract, when using methods that inject dyes or radioactive tracers, there is likely to be some bias in the description of the distribution pathway of SLN in gastric carcinoma^[15-17]. By analyzing the location of solitary metastatic lymph nodes, the distribution pathway of SLN in gastric carcinoma can be accurately assessed.

Reasons for skipping metastasis

After analyzing the clinical records of 51 patients with solitary lymph node metastasis, Kosaka *et al.*^[11] reported 7 cases of lymph node metastases in N₂-N₃ without being in N₁. Kosaka *et al.*^[11] suggested that the following could have a role in skipping metastasis: (1) occult metastases may remain unseen in a routine histopathological examination; (2) there may be a great number of lymphatic routes in the minor omentum; and (3) there may have been only a few perigastric nodes in those cases.

In this study, transversal and skipping metastasis were found to be notable. However, to date the reasons for the occurrence of skipping metastasis remain poorly understood. Chen *et al.*^[18] studied the dynamic role of stomach lymphatic flow from 138 infant corpses using 20% Prussian Blue Chloroform Solution as lymphatic dye. They reported that in 40 of 41 cases in which the drainage pointed at the greater curvature of the corpus gastricum, the lymphatic channel flowed directly to No.10. Therefore, with reference to lesions in a certain region, the so-called “skipping metastasis” of a SLN may be the first lymph

node in the lymphatic drainage system. In cases where metastasis first occurs in N₂ or N₃, the function of N₂ or N₃ is considered to be the same as N₁^[14].

Clinical implications

In the past 23 years, 2476 patients with gastric cancer were treated at our hospital. Among them, for the 728 patients without metastatic lymph nodes an extended D1 dissection was also performed, and as a result some cases had complications connected with the dissection. A great achievement of gastric surgeons in the last century, one that deserves unequivocal respect, has been to establish radical surgery with extensive lymph node dissection for gastric cancer. However, we now need to proceed to another stage by improving post-operative function and quality of life after gastric cancer surgery without impairing long-term outcomes^[19,20]. The concept of a “minimally invasive, curative, safe operation” has gradually gained acceptance^[21,22]. Here, we have attempted to discover the distribution pathway of SLN in order to provide clinical data for rational lymphadenectomy.

Based on the study we suggest that: (1) for patients with U area cancer at the lesser curvature region, No.7 and No.8 should be treated in the same way as N₁; (2) for patients with cancer in the M area in the lesser curvature region, No.7 should be treated in the same way as N₁; (3) for patients with cancer in the M area at the greater curvature region, No.10 should be inspected more carefully, although No.10 can be regarded as the same as N₃ for an M area cancer^[6]; further, if No.10 is questionable, a resection of the spleen should be undertaken^[4]; and 4) for patients with cancer in the L area at the lesser curvature region, No.1, No.7 and No.8 should be inspected more carefully.

COMMENTS

Background

The first possible nodes of metastasis along the route of lymphatic drainage from the primary lesion should be a sentinel lymph node (SLN). If a SLN is negative, patients can be considered to be without lymph node metastasis, and should not have to endure possible operations and face avoidable risks. The SLN concept is gaining greater acceptance, and thus clinicians and researchers can be optimistic that it may serve as a breakthrough management tool for use in gastric cancer.

Research frontiers

Many clinicians and researchers are presently undertaking studies of the distribution pathway of SLN in gastric carcinoma. However, because of the multidirectional and complicated lymphatic flow from the GI tract, there is likely to be some bias in the description of the distribution pathway of SLN in gastric carcinoma when using methods that inject dyes or radioactive tracers. The first possible sites of metastasis along the route of lymphatic drainage from the primary lesion are known as SLN. Therefore, a solitary metastatic lymph node could be regarded as SLN in gastric carcinoma. Some onco-surgery scholars have attempted to assess the distribution pathway of SLN in gastric carcinoma by analyzing the location of solitary metastatic lymph nodes.

Innovations and breakthroughs

Based on a large sample study, we described the distribution pathway of solitary metastatic lymph nodes and put forward concrete suggestions for lymph node dissection in gastric cancer.

Applications

We attempted to discover the distribution pathway of solitary metastatic lymph nodes in gastric cancer. Our results provide clinical data for rational lymphadenectomy and for experimental study of SLN.

Terminology

Minimally invasive surgery (MIS) is well recognized; however, rational lymphadenectomy operations are more focused on in this study. MIS is more about treatment of early gastric cancer, while rational lymphadenectomy is more about advanced gastric cancer.

Peer review

This manuscript presents a good overview of the distribution of lymph node metastasis in gastric cancer and should be of interest to GI researchers and clinicians.

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Gastric adenocarcinoma with features of endodermal sinus tumor

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Abstract

Extragenadal germ cell tumors are rare. The most common sites for EGGCTs are in midline locations such as the mediastinum, retroperitoneum and pineal gland. These tumors rarely present in the stomach. We describe here a case where a middle aged man presented with typical symptoms of gastric cancer. After extensive workup, which included blood work, CT abdomen scan, upper endoscopy, and endoscopic ultrasound, the patient was diagnosed with gastric cancer. However, due to very high blood levels of alpha-fetoprotein, the specimen was sent for special histochemical staining, which demonstrated that the tumor had features of both adenocarcinoma and endodermal sinus tumor. This is a very aggressive tumor with a very poor prognosis.

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Key words: Extragonadal germ cell tumor; Endodermal sinus tumor; Seminoma; Non-seminoma; Adenocarcinoma; Mediastinum; Retroperitoneum; Metastasis; Alpha fetoprotein

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INTRODUCTION

Extragenadal germ cell tumors are rare, accounting for only 1% to 4% of all germ cell tumors. Ninety-five percent of all testicular tumors are germ cell tumors. These tumors originate in the sperm forming cells in the testicles (the male gonads) or egg producing cells in the ovary (female gonads). On rare occasions, however, germ cell tumors

develop elsewhere in the body without any evidence of cancer in the testes. When this happens, they are referred to as extragonadal germ cell tumors (EGGCTs) (from the Testicular Cancer Resource Center)^[1].

At approximately 4 to 6 wk of embryonic development, the germ cells migrate into the embryo where they populate the developing testes or ovaries. If these cells miss their destination, they are likely to come to rest in one of a number of midline sites in the body. Extragonadal tumors arise when these cells become cancerous.

EGGCTs can be either benign or malignant and the malignant tumors can be either seminoma or non-seminoma. Extragonadal tumors are much more common in females. Malignant extragonadal tumors are much more common in men.

Extragenadal tumors can arise anywhere in the body. But, the majority are found in 3 common sites: anterior mediastinum, the retroperitoneum, and pineal gland in the brain. EGGCTs are aggressive and are usually seen in young adults. The treatment and prognosis of the disease depends on a variety of factors including the type of cancer, the tumor location, and the size of the tumor.

CASE REPORT

A 67-year-old Hispanic male presented to our institution with a complaint of epigastric pain radiating to the right upper quadrant, 8/10 in intensity, and it worsened with eating for 2 to 3 mo. The patient also complained of a weight loss of 10 pounds over the past month and vomiting for 2 d. He denied melena, hematochezia, and hematemesis. Past medical history was only significant for hyperlipidemia. The patient smoked 1 pack of cigarettes for 40 years, but denied use of alcohol. Family history was only significant for diabetes and hypertension. On physical examination, the patient felt a slight pain on deep palpation in the epigastrium with voluntary guarding. The remainder of his physical examination, including genital examination, was normal. Initial blood work obtained in the emergency room showed iron deficiency anemia, and elevated AST, ALT, and alkaline phosphatase. Tumor markers were sent and his serum alpha-fetoprotein was very high (4178; normal 0-1.8). Beta-HCG was normal. A CT scan of the abdomen showed gastric wall thickening and several 2 cm lymph nodes in mesenteric fat inferior to the stomach and liver extensively replaced with metastatic disease (Figure 1). An upper endoscopy, and endoscopic ultrasound were obtained (Figures 2 and 3). Endoscopy showed a large 8-10 cm exophytic-ulcerating lesion along the greater

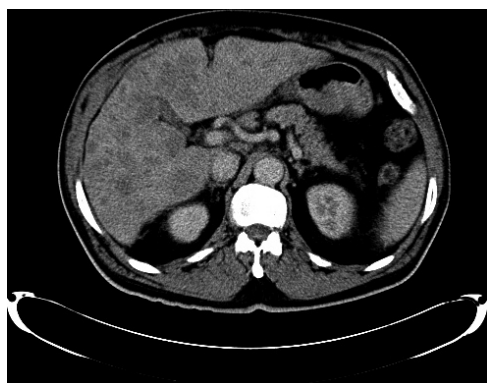


Figure 1: CT scan of the abdomen shows gastric wall thickening, several lymph nodes, and liver extensively replaced with metastatic disease.

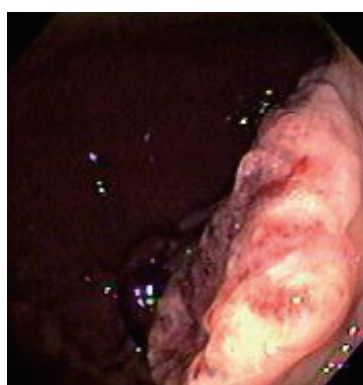


Figure 2: Upper Endoscopy shows 8-10 cm exophytic-ulcerating lesion along the greater curvature, extending from mid body to antrum

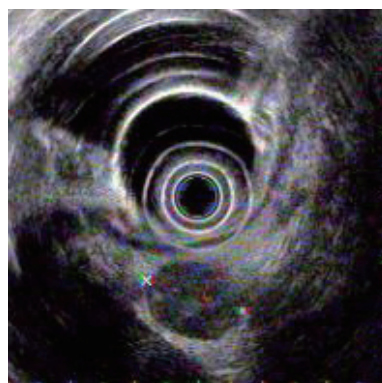


Figure 3: EUS showing a mass extending through all layers of the stomach.

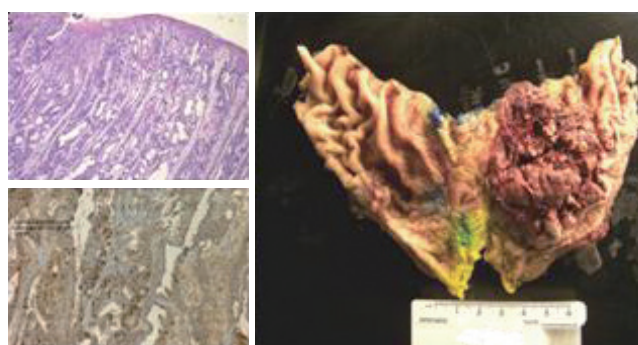


Figure 4: Immunohistochemical stains for alpha-fetoprotein strongly reactive with tumor cells.

curvature, extending from mid body to the antrum. A tissue biopsy was obtained. EUS demonstrated a mass extending through all layers of the stomach. Tissue biopsy obtained *via* EGD demonstrated invasive adenocarcinoma, which was poorly differentiated, and there were no *H Pylori* organisms. In view of the patient's very high serum alpha fetoprotein, a mixed component of extragonadal germ cell and adenocarcinoma was suspected, and the biopsy specimen was sent for special immunohistochemical staining (Figure 4). The immunohistochemical stains for alpha-fetoprotein strongly reacted with the tumor cells. Synaptophysin was focally positive and chromogranin was negative. These findings support the diagnosis of gastric adenocarcinoma, which was moderate to poorly differentiated, with features of endodermal sinus tumor and endocrine type cells.

The patient underwent exploratory laparotomy with partial gastric resection. The patient also received platinum based chemotherapy with Platinol and Taxol. He had a very eventful 2 mo of hospital course with multiple infections, upper gastrointestinal bleeding and eventual respiratory failure and death.

DISCUSSION

Extragonadal germ cell tumors are rare, representing 1% to 4% of all germ cell tumors. Seminomas account for 30% to 40% of these tumors, and non-seminomas account for 60% to 70%. The most common site of EGGCTs is the mediastinum (50%-70%) followed by the retro

peritoneum (30%-40%), the pineal gland (5%), and the sacrococcygeal area (less than 5%). In children, benign and malignant EGGCTs occur equally in males and females. In adults, only benign EGGCTs (teratomas) occur at equal frequency in both sexes; more than 90% of malignant EGGCTs occur in males^[2].

Symptoms vary depending on the site and the size of the tumor. A complete physical examination is required. Workup of EGGCTs begins with lab studies with the tumor markers alpha-fetoprotein and beta-HCG. These tumor markers provide diagnostic, staging and prognostic information. Pure seminomas and pure choriocarcinomas do not produce AFP. A CT scan of the chest, abdomen, and pelvis should be obtained. Treatment consists of surgery and chemotherapy. A classification system has been developed by the International Germ Cell Collaborative Group (IGCCG). This system categorizes tumors on the basis of histologic type, localization of metastases, and initial levels of serum AFP, beta-HCG, and LDH. Our patient who had very high levels of AFP and liver metastases expired within two months of the diagnosis^[3].

As mentioned earlier, these tumors generally present at an early age. However, few cases of EGGCTs in the elderly have been described in the literature^[4]. A case of yolk sac tumor with adenocarcinoma has been described in a 56-year-old female^[5]. On even rarer occasions, a gastric adenocarcinoma can present with a combination of these tumors. A case with such a tumor has been described in a Korean pathology journal where gastric adenocarcinoma was mixed with gastric choriocarcinoma and endodermal sinus tumor^[5].

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LETTERS TO THE EDITOR

Can *Campylobacter jejuni* play a role in development of celiac disease? A hypothesis

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Abstract

Celiac disease (CD) is an enteropathy with malabsorptive condition in which an allergic reaction to the cereal grain-protein (gluten) causes small intestine mucosal injury. CD is a multifactorial disorder in which both genetic and environmental factors contribute to the disease development. Mechanisms have been described to explain the pathology of CD. T cells specific for multiple gluten peptides are found in virtually all patients. Generation of such a broad T cell response may be a prerequisite for disease development. CD is associated with multiple extraintestinal presentations, including neurological deficits. Recent studies have shown a significant correlation between anti-ganglioside antibodies and neurological disorders in patients with underlying CD. Gangliosides are glycosphingolipids which are abundant in nervous system and in other tissues including gastrointestinal tract. It is not known what triggers the release of anti-ganglioside antibodies in people with gluten sensitivity. But, the mechanism is likely to involve the intestinal immune system response to ingested gliadin, a component of wheat gluten. Studies showed that mechanisms different from gluten exposure may be implicated in antibody formation, and other environmental factors may also exist. In addition, considering the fact that genetic predisposition dysregulating mucosal immune responses in the presence of certain environmental triggers like gastrointestinal infections may be strong etiological factors for developing chronic intestinal inflammation including CD, the hypothesis raised in our mind that antiganglioside antibody formation in CD may play a role not only in development of neurological complications in celiac patients, but also in development of CD itself. As presence of *Campylobacter jejuni* in other diseases with antigangliosides antibody formation has been established, we propose the possible role of *Campylobacter jejuni* in development of CD in association

with other genetic and environmental factors by the mechanism that molecular mimicry of gangliosides-like epitopes common to both lipo-polysaccharide coats of certain strains of *Campylobacter jejuni* and gangliosides in cell structure of gastrointestinal mucosa may cause an autoimmune response and consequently lead to atrophy and degeneration of mucosa possibly by apoptosis.

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Key words: Celiac disease; Gangliosides; *Campylobacter jejuni*; Molecular mimicry

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TO THE EDITOR

Celiac disease (CD) is an enteropathy with malabsorptive condition in which an allergic reaction to the cereal grain-protein (gluten) causes small intestine mucosal injury. CD is a multifactorial disorder in which both genetic and environmental factors contribute to the disease development^[1]. Most of CD patients carry human leukocyte antigens (HLA)-DQ2 or HLA-DQ8. But, this genetic predisposition cannot fully explain the pathogenesis of CD, as CD just develops in minority of HLA-DQ2 and HLA-DQ8 positive individuals^[2]. Several mechanisms have been described to explain the pathology of CD. Gluten proteins have several unique factors that contribute to their immunogenic properties. They are extremely rich in amino acid proline and glutamine. Due to high proline content, gluten is highly resistant to proteolytic degradation within the gastrointestinal tract. Moreover, high glutamine content makes gluten a good substrate for tissue transglutaminase (Ttg), which can convert glutamine into negatively charged glutamic acid. Such modified gluten peptides can bind to HLA-DQ8 and subsequently cause T cell response. T cells specific for multiple gluten peptides are found in virtually all patients. Generation of such a broad T cell response may be a prerequisite for disease development^[3]. CD is associated with multiple extraintestinal presentations, including bone disease, endocrine disorders and neurological deficits^[4].

Neurological disorders and CD

Limited neurological disorders are recognized in association with CD. But, their spectrum becomes wider as complications of prediagnosed CD and/or as an initial manifestation of CD. Neurological disorders include cerebellar ataxia, peripheral neuropathy, epilepsy, dementia, migraine, encephalopathy and Guillain-Barre-like syndrome. Vitamin deficiency due to malabsorption was a first described as the etiology of neurological manifestations. But, it could not explain neurological disorders in patients with normal level of vitamins or in individuals with vitamin deficiency, but without neurological syndromes^[5]. Recent studies have shown a significant correlation between anti-ganglioside antibodies, and neurological disorders in patients with underlying CD. Gangliosides are glycosphingolipids which are abundant in the nervous system and in other tissues including gastrointestinal tract^[6]. It is not known what triggers the release of anti-ganglioside antibodies in people with gluten sensitivity. But, the mechanism is likely to involve the intestinal immune system response to ingested gliadin, a component of wheat gluten. Two mechanisms have been postulated for the release of anti-ganglioside antibodies: one is the presence of ganglioside-like epitopes in gliadin and the other is the potential for complex formation between gliadin and GM1 ganglioside. One study evaluated the feasibility of these two mechanisms, and found that certain gliadin spices are glycosylated. But, they do not appear to carry GM1-like carbohydrate moieties^[7]. In contrast, *in vivo* formation of gliadin-GM1 complexes is probably feasible, since abundant GM1 is found in gut epithelial cells^[7].

It was reported that antibody titer is reversed in some patients after gluten-free diet, whereas it increases in other patents after such a diet^[8], suggesting that mechanisms different from gluten exposure may be implicated in antibody formation, and other environmental factors may exist.

Hypothesis

The above findings, and the fact that a genetic predisposition dysregulates mucosal immune responses in the presence of certain environmental factors such as gastrointestinal infections are strong etiological factors for development of chronic intestinal inflammation including CD (We can define the hypothesis in our mind that anti-ganglioside antibody formation in CD may play a role not only in developing neurological complications of celiac patients, but also in developing CD itself).

Among disorders associated with anti-ganglioside antibody formation, we focused on an autoimmune disorder with some neurological presentations like CD, and Guillain-Barre syndrome (GBS). In GBS a preceding infection may trigger an autoimmune response through

molecular mimicry in which the host generates an immune response to an infectious organism which shares ganglioside-like epitope with the host's peripheral nervous system. Among bacterial organisms which have a role in development of GBS, *Campylobacter jejuni* has been best studied, showing that about 25% of patients with GBS have a recent *Campylobacter jejuni* infection. Now, it is well established that lipo-oligosaccharide located in the wall of *Campylobacter jejuni* cross-reacts with ganglioside in axonal membrane of neurons.

We proposed a possible role of *Campylobacter jejuni* in development of CD in association with other genetic and environmental factors by the mechanism that molecular mimicry of gangliosides-like epitopes common to both lipo-polysaccharide coats of certain strains of *Campylobacter jejuni* and gangliosides in cell structure of gastrointestinal mucosa may cause an autoimmune response, and consequently lead to atrophy and degeneration of mucosa damage possibly by apoptosis in a manner similar to nerve tissue injury in GBS. The proposed mechanism can also explain the presence of neurological manifestations of CD.

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www.cag-acg.org/cddw/cddw2007.htm

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Meeting BSG Annual Meeting
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- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A* 2006; In press

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- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

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- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325]

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- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900]

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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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Transient elastography for the assessment of chronic liver disease: Ready for the clinic?

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Abstract

Transient elastography is a recently developed non-invasive technique for the assessment of hepatic fibrosis. The technique has been subject to rigorous evaluation in a number of studies in patients with chronic liver disease of varying aetiology. Transient elastography has been compared with histological assessment of percutaneous liver biopsy, with high sensitivity and specificity for the diagnosis of cirrhosis, and has also been used to assess pre-cirrhotic disease. However, the cut-off values between different histological stages vary substantially in different studies, patient groups and aetiology of liver disease. More recent studies have examined the possible place of transient elastography in clinical practice, including risk stratification for the development of complications of cirrhosis. This review describes the technique of transient elastography and discusses the interpretation of recent studies, emphasizing its applicability in the clinical setting.

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Key words: FibroScan; Transient elastography; Liver stiffness measurement; Hepatic fibrosis; Hepatitis

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INTRODUCTION

The management and prognosis of chronic liver disease is strongly influenced by its severity. While percutaneous liver biopsy remains the gold standard, there is increasing awareness, not only of the associated morbidity and mortality of the procedure, but also its diagnostic limitations. There is considerable sampling variability, and inter- and intra-observer variation in the assessment of liver pathology. Antifibrotic therapies are in development, but it has been stated that “the lack of robust markers of fibrosis represents the single greatest factor limiting both the validation of progression or regression of fibrosis and the testing of antifibrotic therapies”^[1].

A number of approaches to non-invasive assessment of chronic liver disease have been developed. Serum markers, and serum panel markers for the assessment of chronic liver disease, such as the APRI (AST to platelet ratio index) score, Enhanced (European) Liver Fibrosis (ELFTM) test and FibroTest, have been proposed, and are the subject of several comprehensive reviews^[2-4]. Investigations based on imaging modalities, including ultrasound and magnetic resonance, are liver-specific and provide structural information related to the liver^[5]. Microbubble contrast-enhanced ultrasound to obtain hepatic vein transit times (HVTT) and phosphorus-31 magnetic resonance spectroscopy (³¹P MRS) have been shown to delineate cirrhotic and pre-cirrhotic disease stages, but require considerable operator skill and access to the relevant technology^[6-10]. Other MR techniques, such as diffusion-weighted imaging (DWI) and ultrashort echotime (UTE) have shown promise, but require further development^[11,12]. Moreover, these techniques require assessment in larger subject groups in the setting of multi-centre trials.

Liver stiffness measurement using transient elastography (TE) (FibroScan[®]) is a recently developed technique designed for the assessment of liver fibrosis, and has been extensively evaluated in several recent studies. The aim of this article is to review the current data on the use of transient elastography in clinical practice and to make recommendations for future research. A Medline search using the terms “FibroScan” and “transient elastography” was conducted. The proceedings of the 41st annual meeting of the European Association for the Study of the Liver 2006, and the 57th annual meeting of the American Association for the Study of Liver Diseases 2006 were also searched for relevant articles.

THE BASIS OF TRANSIENT ELASTOGRAPHY

Transient elastography allows liver stiffness measurement (LSM) which enables the assessment of liver disease severity, using a 1-dimensional ultrasound transducer and receiver mounted on the same axis as a vibrator, producing a low-frequency pulse or shear wave. When the probe tip is placed perpendicularly against the skin between the ribs overlying the liver and triggered, the rate of progression of the shear wave is measured.

The speed of propagation depends on the elasticity or stiffness of the tissue under examination and is measured by a series of ultrasonic pulses, which detect the transient local deformations in the liver tissue as the shear wave progresses. The elasticity of the liver is derived from the velocity of the wave approximating to the Young's modulus, E , according to the equation: $E = 3\rho V_s^2$, where V_s is the shear velocity and ρ is the mass density, assumed to be close to that of water. The deformation of tissue is plotted as a function of time and depth to create a two-dimensional "elastogram". The slope of the elastogram represents the speed of propagation and thus the liver stiffness, expressed in kPa^[13].

This technique is simple to learn, can be performed quickly by a single operator, and provides an objective measure of liver stiffness. TE has been employed in a number of clinical paradigms over the last few years, however, there is at present no consensus on its indications for use, interpretation and applicability.

ANALYSIS AND EVALUATION

Most studies carried out to evaluate the performance of transient elastography compare LSM to the histological assessment of liver biopsy. Of these, the METAVIR scoring system which is widely used in clinical practice has been employed in the majority of studies^[14]. Fibrosis is staged semi-quantitatively on a five-point scale from F0 to F4 (F0, no fibrosis; F1, portal fibrosis without septa; F2, portal fibrosis and few septa; F3 numerous septa without cirrhosis; F4, cirrhosis). Divisions of clinical significance are considered to be between F1 and F2 (from minimal to significant fibrosis) and between F3 and F4 (from fibrosis to cirrhosis). The data obtained is commonly represented by boxplots with liver stiffness (or log₁₀ liver stiffness) on the y axis and fibrosis stage on the x axis, representing the median, interquartile range and range of values for individuals at each fibrosis stage. Despite an apparently high diagnostic accuracy, there is substantial overlap between groups, especially in the pre-cirrhotic stage of liver disease. This has clinical implications since for a given liver stiffness measurement, the patient's true fibrosis score may vary from F0-1 to F4^[15].

A major criticism of liver biopsy and an important stimulus to the development of non-invasive techniques is the small portion of liver that is assessed. The specimen obtained by standard liver biopsy techniques represents just 1/50 000 of the liver, and typically only 16% of such biopsies exceed the optimal length of 25 mm required

for adequate histological assessment^[16]. This results in significant sampling variability since hepatic fibrosis, inflammation and steatosis may have a patchy spatial distribution within the liver. Added to this drawback is the presence of inter- and intra- observer variability in histological assessment, making this "gold-standard" considerably flawed. Thus, non-invasive measures such as TE are judged by liver biopsy which may be a flawed standard. Clearly, the results obtained should be regarded as a probability of correctly predicting liver fibrosis and interpreted in the context of clinical, epidemiological and biochemical data, and moreover should be correlated prospectively with robust clinical outcome measures.

ASSESSMENT OF DIAGNOSTIC ACCURACY

While the sensitivity, specificity, positive and negative predictive values describe the performance of a test with respect to a gold-standard, diagnostic accuracy provides a measure of the overall performance of a test. Diagnostic accuracy of transient elastography with respect to histology has been measured in clinical studies using the area under the receiver operator characteristic (ROC) curve, a convenient non-parametric method for the assessment of diagnostic tests, compared to a gold-standard. The sensitivity is plotted against 1- specificity for all possible cut-off values between two states. For example, this could be expressed as: positive or negative; cirrhosis or no cirrhosis; insignificant or significant fibrosis. A measure of diagnostic accuracy of the test may be derived, whereby an area near 1 represents high diagnostic accuracy. ROCs may be used to select cut-off values appropriate to different scenarios. Cut-off values may then be selected for a given situation according to the required sensitivity or specificity of a given value to distinguish the two states. This allows cut-off values to be chosen to answer clinically relevant questions. For example, to rule out cirrhosis effectively in a group of patients, a cut-off value with a specificity of 95% may be used, indicating that there is a 95% probability that patients below the cut-off value will not have cirrhosis. Alternatively, an optimum cut-off may be calculated, where the cut-off is chosen at the point where the sum of sensitivity and specificity is maximal, although this is affected by the shape of the ROC curve and therefore may vary between studies. A more inclusive measure would be obtained by using a cut-off associated with high sensitivity but lower specificity.

The largest study published to date demonstrates that different cut-off values for the diagnosis of cirrhosis exist, depending on the sensitivity and specificity required for the decision and for different aetiologies, such as chronic hepatitis B, chronic hepatitis C and alcohol-related and non-alcoholic related fatty liver disease (Table 1)^[17].

TRANSIENT ELASTOGRAPHY FOR STAGING OF HEPATIC FIBROSIS

Initial clinical studies using TE investigated the ability of

Table 1 Liver stiffness cut-off values for the diagnosis of cirrhosis according to the primary cause of liver disease

	Optimum cutoff (kPa)		
	Hepatitis C (<i>n</i> = 298)	Hepatitis B (<i>n</i> = 122)	Alcohol or NASH (<i>n</i> = 122)
Sensitivity 95%	10.0	6.0	13.2
Max. sum of sensitivity and specificity	10.4	10.3	21.5
Best diagnostic accuracy	20.2	16.9	21.5
Specificity 95%	14.1	14.3	27.7

NASH: Non-alcoholic steatohepatitis. Data obtained from Ganne-Carrie *et al* 2006^[17].

the technique to assess hepatic fibrosis when compared to the gold standard of liver biopsy. This was assessed by Sandrin and colleagues in a “proof-of-principle” study. They demonstrated a graduated increase in liver stiffness with increasing hepatic fibrosis^[13]. Chronic hepatitis C was chosen as the paradigm for many studies as the patients are numerous and the natural history of the disease and histological classification systems have been well described^[14,18]. Other workers have investigated the ability of TE to assess fibrosis compared to liver biopsy in several disease paradigms, as summarised in Table 2^[13,15,17,19-25].

Hepatic fibrosis is a complex and multistep process. Therefore, a precise description of disease severity may require assessment of more than one aspect of the disorder. TE measures liver stiffness which is thought to be due largely to the extent of fibrosis. Indeed digital image analysis demonstrates a correlation between the fibrotic area and liver stiffness^[26]. Yet, the extent of fibrosis does not provide the complete picture. Histological scoring systems are not linear and in addition to the extent, they describe the pattern of deposition of fibrous tissue^[18,14]. The effect of collagen cross-linkage on liver stiffness, associated with more severe disease, has not been clearly established. More recently, a strong relationship between liver stiffness and the hepatic venous pressure gradient (HVPG) has been described, demonstrating an association with portal hypertension^[27]. The relative contribution of fibrosis, inflammation and haemodynamic changes have yet to be determined.

A recent well-conducted study by Fraquelli and colleagues looked specifically at the reproducibility of LSM in 195 patients with liver disease of mixed aetiology (predominantly HCV)^[24]. The results obtained by two different operators working under highly regulated conditions, showed a very high degree of inter-observer agreement, with an intraclass correlation coefficient (ICC) of 0.98, representing an estimated 98% of variability due to patient characteristics, as opposed to observer variability. This agreement was substantially reduced when groups such as overweight patients, those with histological or ultrasound evidence of hepatic steatosis and especially those with mild disease (ICC 0.6 for METAVIR F0-1 *vs* 0.99 for F ≥ 2) were assessed. There were stringent inclusion criteria such as inclusion of only those patients in whom a success rate of > 65% was achieved and where the

interquartile range of the readings was less than 30% of the median. Additionally, 76% of liver biopsy specimens exceeded 20 mm in length, thus minimising sampling error. Despite the high reproducibility of LSM, there was substantial overlap in the findings between adjacent stages of hepatic fibrosis, which the authors acknowledged would limit the diagnostic accuracy of TE, particularly in intermediate fibrosis stages.

Disease activity or necro-inflammation are not directly assessed by TE, although LSM has been shown to increase with increasing necroinflammatory scores at histology^[24], and in biochemically-assessed flares of hepatitis and cirrhosis^[28]. Steatosis is a cause as well as a consequence of chronic liver disease^[29], and its effect on liver stiffness is believed to be minimal based on multivariate analyses in studies investigating other endpoints^[15,30]. However, studies assessing the severity of liver stiffness stratified by the degree of steatosis are awaited. The development of serum panel markers of hepatic fibrosis demonstrates recognition of the fact that multiple diagnostic measures are required. Castera and colleagues compared TE with FibroTest, an indirect serum panel marker, and the AST to platelet ratio index (APRI) in a cohort of patients with chronic hepatitis C, and found equivalent results with these techniques, but noted that a combination of TE and the serum panel marker provided the greatest diagnostic accuracy^[19]. On this basis, an algorithm was proposed whereby liver biopsy can be avoided in most patients with chronic hepatitis C when the tests are in agreement^[19]. Other studies have demonstrated a similar diagnostic accuracy of TE in the context of hepatitis C virus (HCV) and human immunodeficiency virus (HIV) coinfecting patients^[23], and also in the presence of biliary fibrosis in patients with primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC)^[20,23].

CIRRHOSIS AND ITS COMPLICATIONS

In view of the wide range of LSM for any single fibrosis stage, attention has turned to the use of FibroScan in the diagnosis of cirrhosis and of its complications such as varices, risk of variceal bleeding, hepatocellular carcinoma and ascites. Foucher and colleagues examined prospectively a cohort of patients with liver disease of varying aetiology^[25]. In addition to establishing the cut-off values for fibrosis of varying severity as assessed by liver biopsy, they calculated the cut-off values below which there was a 90% chance that complications such as varices (stage 2/3) (27.5 kPa), Child-Pugh score of B/C (37.5 kPa), ascites (49.1 kPa), and oesophageal bleeding (62.7 kPa) were absent. Although the number of subjects with each complication was small (between 14 and 42 cases), these cut-off values may serve to identify patients with cirrhosis at risk of such complications. However, history of ascites, hepatocellular carcinoma, and variceal bleeding may be readily obtained by direct questioning of the patient, therefore for these observations to be clinically useful, they need to be borne out in prospective studies on the development of these complications over time. Kazemi and colleagues correlated endoscopic evidence of oesophageal varices with LSM in a cohort of patients with

Table 2 Results of studies in which liver stiffness was compared with histological fibrosis stage (METAVIR system) to establish diagnostic accuracy and cut-off values

Author, yr	Patient group	Number of subjects	Cut-off for $F \geq 2$ (kPa)	AUROC $F \geq 2$	Cut-off for $F = 4$ (kPa)	AUROC $F = 4$
Fraquelli M <i>et al</i> 2007 ^[24]	Mixed (HCV)	195 (155)	7.9 (72%; 84%)	0.86	11.9 (91%; 89%)	0.90
Ganne-Carrie N <i>et al</i> 2006 ^[17]	Mixed (HCV)	1007 (298)			14.6 (79%; 95%)	0.92
De Ledinghen V <i>et al</i> 2006 ^[23]	HCV/HIV co-infected	72	4.5 (93%; 17%)	0.72	11.8 (100%; 93%)	0.97
Gomez-Dominguez E <i>et al</i> 2006 ^[22]	Mixed (HCV)	94 (62)	4 (94%; 33%)	0.74	16 (89%; 96%)	0.94
Carrión J <i>et al</i> 2006 ^[21]	HCV post transplant	169	8.5 (90%; 81%)	0.90	12.5 (100%; 87%)	0.98
Corpechot C <i>et al</i> 2006 ^[20]	PBC/PSC	101	7.3 (84%; 87%)	0.92	17.3 (93%; 95%)	0.96
Foucher J <i>et al</i> 2006 ^[25]	Mixed	354	7.2 (64%; 85%)	0.80	17.6 (77%; 97%)	0.96
Castera L <i>et al</i> 2005 ^[31]	HCV	183	7.1 (67%; 89%)	0.83	12.5 (87%; 91%)	0.95
Ziol M <i>et al</i> 2005 ^[15]	HCV	251	8.8 (56%; 91%)	0.79	14.6 (86%; 96%)	0.97
Sandrin <i>et al</i> 2003 ^[13]	HCV	106		0.88		0.99

Cut-off values were those proposed by the authors. If more than one cut-off value was available, the value set for optimum diagnostic accuracy (i.e. cut-off at which sensitivity (se) + specificity (sp) is maximal) was used. Cut-off values are followed by the relevant sensitivity and specificity in parenthesis. HCV: Hepatitis C virus; HIV: Human immunodeficiency virus; PSC: Primary sclerosing cholangitis; PBC: Primary biliary cirrhosis.

compensated cirrhosis, and observed that a cut-off value of 19 kPa predicted varices (stage II / III) with a sensitivity of 91% and specificity of 60%^[31]. These findings suggest that endoscopic surveillance can be avoided in up to 60% patients with cirrhosis.

TOWARDS THE CLINIC

Ganne-Carrie and coworkers have published the largest series to date, but have concentrated on separation of cirrhotic from precirrhotic disease^[17]. These authors highlighted the fact that the cut-off values vary on the basis of the aetiology of liver disease as well as the level of sensitivity and specificity required for the issue under question (Table 1). There were substantial differences in the cut-off values for the prediction of cirrhosis with 95% specificity depending on the number of patients studied (775 *vs* 1007). It remains to be seen whether the cut-off values vary depending on the population of interest, for example in affluent societies with high level of co-existent type 2 diabetes and hepatic steatosis, compared to a group with few risk factors for steatosis.

Of particular interest is the question whether liver stiffness measurement affects clinical decision making. For example, a non-invasive technique, such as TE, is particularly desirable in patients with bleeding disorders, such as haemophilia. Two studies have addressed this issue. Masaki and colleagues correlated LSM with ultrasound assessment of liver disease and several serum markers in haemophiliacs coinfecting with HCV and HIV, although the findings were not linked to a clear standard or to clinical outcome measures and decisions^[32]. Posthouwer and colleagues studied a cohort of patients with haemophilia and chronic hepatitis C in whom percutaneous liver biopsy was contraindicated^[33]. Cut-off values from a previous study were applied^[19] and validated in a separate cohort of patients without a bleeding disorder. This study demonstrated the pragmatic use of TE in a specific scenario with acknowledgement that there will be inaccurate assessment in a proportion of patients. In such a scenario, a false positive test may result in a patient with mild disease receiving antiviral therapy while a patient

with a false negative test, may have severe disease and not receive treatment. It can be argued that the latter group may have a detrimental outcome, and therefore a cut-off with a higher sensitivity is indicated.

An important indication for non-invasive staging of hepatitis C-related liver disease is to determine whether antiviral treatment is appropriate in a particular patient^[15]. However, on the basis of recent studies in patients with mild hepatitis C, current UK treatment guidelines suggest that treatment is both clinically effective and cost-effective in histologically mild, moderate and severe stages of pre-cirrhotic disease^[34-36]. Increasingly, patients with functionally-compensated cirrhosis are being treated, although the likelihood of a good response to treatment is smaller in this group. Since treatment is not contraindicated in any histological group of patients with hepatitis C, the role of such non-invasive technologies should be questioned. It is possible that the evaluation of liver stiffness may provide risk assessment on the basis of which further investigations should be planned. Such a scheme may allow a period of “watchful waiting” prior to a decision on starting treatment, in addition to providing reassurance to patients with mild disease. Another area of interest is the patient’s response to treatment. Preliminary data indicates that LS decreases in patients with hepatitis C after treatment with pegylated interferon and ribavirin and that the decrease is greater in virologic responders compared to non-responders^[37]. This alteration may reflect biochemical changes associated with disease activity, as opposed to changes in fibrosis *per se*, and the long term outcome data in a large cohort of patients is awaited.

Nahon and colleagues addressed the issue of how LSM may affect clinical assessment by inviting four physicians to predict the fibrosis stage on the basis of routine history, physical examination and biochemical tests^[38]. Liver stiffness measurement was performed at the time of liver biopsy. The physicians were allowed to modify their estimate of disease severity in the light of the LSM findings, which were then compared with the biopsy results. LSM did not significantly enhance the physicians’ prediction of pre-cirrhotic disease staging compared to the assessment based on routine investigations. In the

prediction of cirrhosis, the addition of LSM improved the diagnostic accuracy by about 10% in results obtained by 3 out of the 4 physicians.

The utility of TE in routine clinical assessment of precirrhotic disease appears questionable in the light of data currently available. Routine abdominal ultrasound is carried out in nearly all patients with chronic liver disease to assess structural abnormalities, and to look for cholestasis, portal hypertension and hepatocellular carcinoma. The presence of findings such as increased heterogeneity, irregular liver outline or nodularity, caudate lobe hypertrophy, increased spleen size, and portal vein Doppler blood flow measurement all provide evidence of cirrhosis, with high specificity, but relatively low sensitivity^[39,40]. It is possible that routine ultrasound enhances the physician's assessment of whether a patient has cirrhosis, while in addition providing the additional information described above. However, it should be noted that in the study by Nahon and colleagues, of the 15 patients misclassified as cirrhosis after LSM by the senior physicians, 3 had features of cirrhosis on ultrasound or endoscopy^[38]. It is not clear how many of the patients correctly classified on the basis of LSM would have also been correctly classified had routine ultrasound information been provided. Therefore, before LSM is recommended for routine clinical use, the existing technologies should be compared by employing analogous methods of analysis.

CONCLUSION

Assessment of TE using FibroScan is a novel technique that has been evaluated in a number of well-conducted studies. It is a safe, acceptable and quick technique that provides an objective and reproducible measure of liver stiffness. LSM correlates with histological fibrosis score, but the cut-off values vary depending on the study referred to, the aetiology of the disease and the sensitivity and specificity required. LSM provides high diagnostic accuracy for the detection of cirrhosis, making it a potentially useful tool for population-based screening for cirrhosis in areas of high prevalence. However, the delineation of precirrhotic stages is less clear, although LSM compares well with serological markers of fibrosis such as the APRI score and FibroTest^[19]. LSM has a number of drawbacks which include that it is technically challenging in obese individuals, where it is associated with reduced success rate; it is not possible to perform in the presence of significant ascites; and the effect of marked steatosis has not been addressed.

Recent studies have begun to address the likely place of LSM in routine clinical practice and the impact it may have on physicians' assessment of disease severity. However, several important questions remain to be resolved including: what are the relative contributions of fibrosis and haemodynamic alterations on LSM? How does steatosis affect LSM? Which cut-off values should be used for which indication? Does LSM substantially add to the information already obtained by routine clinical assessment, abdominal ultrasound and simple blood tests such as the APRI score? Is LSM sufficiently sensitive to detect changes in fibrosis over the long term; in terms of both disease progression and response to treatment?

While such information is awaited, LSM is being performed increasingly worldwide as a result of increased awareness of the technique. The following recommendations are made for consideration when employing the technique: (1) As different cut-off values exist for different diseases, the diagnosis should always be obtained prior to interpretation of the LSM. (2) The clinical question should be defined in order that a cut-off value is used incorporating the appropriate sensitivity and specificity. (3) Criteria in terms of success rates and minimum number of readings should be defined so that assumptions are not made on the basis of inadequate data. (4) Comparison should be made with liver biopsy, if available, in order to provide continual validation. (5) The process of continuous audit should be instituted, with particular emphasis on how such measurements influence decision-making.

THE FUTURE OF IMAGING BIOMARKERS

Fibrosis and fibrogenesis is a complex multistep process. It would be surprising if a single biomarker was able to provide complete evaluation of the disease. FibroScan is an innovative and user-friendly technology but, despite strong academic and commercial promotion, its limitations have been described in a several well-conducted studies. Assessment of precirrhotic disease and the longitudinal assessment of change in fibrosis have not been fully evaluated. A comprehensive, non-invasive assessment of chronic liver disease will be very helpful for baseline assessment of disease and to evaluate the impact of new antifibrotic therapies. Serum panel markers and imaging techniques including ultrasound and magnetic resonance modalities need to be investigated longitudinally in a number of disease states in order to develop and identify the most effective combination of tests, of which TE with FibroScan may be one. The challenge is to develop and validate such a protocol, and to correlate the results with clinically meaningful outcome measures.

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EDITORIAL

Stimulating erythropoiesis in inflammatory bowel disease associated anemia

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Abstract

Anemia is a frequent complication in patients with inflammatory bowel disease (IBD), and is associated with decreased quality of life and increased rate of hospitalization. The primary therapeutic targets of IBD-associated anemia are iron deficiency and anemia of chronic disease. An important prognostic parameter of the success or failure of therapy is the outcome of the underlying disease. Iron deficiency should be appropriately managed with iron supplementation. However, the use of oral iron therapy is limited by several problems, the most important being gastrointestinal side effects leading occasionally to disease relapse and poor iron absorption. Intravenous iron preparations are more reliable, with iron sucrose demonstrating the best efficacy and tolerability. Treatment with erythropoietin or darbepoetin has been proven to be effective in patients with anemia, who fail to respond to intravenous iron. Patients with ongoing inflammation have anemia of chronic disease and may require combination therapy comprising of intravenous iron sucrose and erythropoietin. After initiating treatment, careful monitoring of hemoglobin levels and iron parameters is needed in order to avoid recurrence of anemia. In conclusion, anemia in the setting of IBD should be aggressively diagnosed, investigated, and treated. Future studies should define the optimal dose and schedule of intravenous iron supplementation and appropriate erythropoietin therapy in these patients.

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Key words: Anemia; Crohn's disease; Erythropoiesis; Erythropoietin; Iron; Ulcerative colitis

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INTRODUCTION

Anemia is a frequent and serious complication in patients with inflammatory bowel disease (IBD), with reported prevalence varying from 6% to 74%^[1,2]. Anemia is associated with a decrease in the quality of life and increased rate of hospitalization^[3,4]. In the past, it was common to ascribe anemia as an unavoidable accompaniment of IBD, but recently, correction of anemia is being emphasized as a specific therapeutic objective in these patients^[5,6]. Both iron deficiency and anemia of chronic disease contribute to the development of anemia in IBD^[3]. The therapeutic targets in the treatment of IBD patients with anemia are iron deficiency and the mechanisms underlying anemia of chronic disease^[3].

Anemia is defined as "a reduction in the number of circulating RBCs, hemoglobin concentration, or the volume of packed red cells (hematocrit) in the blood"^[7]. The World Health Organization (WHO) specifies the laboratory definition of anemia as hemoglobin below 120 g/L (non-pregnant females), 110 g/L (pregnant women) and 130 g/L (men). A hemoglobin level below 100 g/L is commonly considered as severe anemia and should be aggressively treated. However, in clinical practice, the timing of therapeutic intervention is based not only on the hemoglobin level but also on the relative degree of hemoglobin reduction, the underlying comorbidities, and the presence or absence of symptoms.

Anemia is an important manifestation of IBD and patients with anemia have increased disease severity and reduced quality of life, indicating the need for effective treatment. The underlying cause of anemia in IBD is either iron deficiency, resulting from intestinal bleeding due to mucosal inflammation and ulceration, or the so-called anemia of chronic disease, resulting from inhibition or suppression of erythropoiesis and dysfunction of iron transport, mediated by inflammatory cytokines^[3,4]. Thus, the pathogenesis of IBD-associated anemia is complex and represents an example of combined iron deficiency anemia (IDA) and anemia of chronic diseases (ACD). Cobalamin or folate deficiency, drug induced anemia, and other causes of anemia such as haemolysis are seen less frequently.

Most patients with IBD-associated anemia respond to iron therapy. Oral iron supplementation appears to be effective for short periods but drug intolerance leads to discontinuation of therapy in up to 21% of patients^[8]. Moreover, the use of oral iron is associated with several limitations^[3]. By contrast, intravenous administration of iron sucrose has been found to be an effective treatment in these patients^[9,10]. Erythropoietin (EPO) administration

is associated with beneficial results in patients who are refractory to oral iron^[3]. The combination of iron sucrose and EPO has been proposed as the most efficacious treatment in IBD-associated anemia^[6,11]. The aim of the present review is to transfer our knowledge, especially the strategies for stimulating erythropoiesis, into the current clinical practice of treatment of anemia in IBD.

ANEMIA IN IBD

Iron deficiency (ID) is the most common cause of anemia in IBD. Iron, which is present in all mammalian cells, is of pivotal importance not only for oxygen transport and storage but also for many non-hematological functions. In normal subjects, the daily iron loss is 1-2 mg which needs to be replaced from the diet. The causes of iron deficiency are reduced intake, either from dietary deficiency or malabsorption, or increased losses. Chronic intestinal bleeding in IBD may exceed the quantity of iron that is absorbed from the diet, resulting in a negative iron balance^[12]. Such an imbalance occurs frequently in IBD, leading to anemia.

When the rate of iron supply to the developing erythroblast is reduced as a result of ID, red cell haemoglobinisation is impaired. The red cells emerging from the bone marrow are microcytic and hypochromic. The increase in EPO response secondary to a drop in haemoglobin levels stimulates erythropoiesis, creating an even greater demand for iron, which is unmet. As a result, there is a high degree of ineffective erythropoiesis^[13]. The most appropriate definition of iron deficiency is bone marrow proliferation in response to intravenous iron supplementation, an approach that has not been studied in IBD. Moreover, in addition to a possible iron deficiency in anemia of chronic disease, functional iron deficiency occurs because of intense erythropoiesis^[14,15] during therapy with erythropoietic agents, with a decrease in transferrin saturation and serum ferritin. Furthermore, during erythropoietin therapy, the absorption of iron increases by as much as 5 fold^[16].

After iron deficiency, anemia of chronic disease is the next important cause of anemia in these patients. There is general correlation between disease activity and the severity of anemia. Abnormal iron metabolism and improper use of iron stores are the typical features of anemia of chronic disease, characterized by low circulating iron concentration in the presence of ample reticuloendothelial iron stores. Thus, anemia of chronic disease can be easily diagnosed by the presence of hypoferraemia and increased serum levels of ferritin.

Because of differences in the therapeutic response, it is important to distinguish anemia of chronic disease from iron deficiency. Iron parameters need to be checked regularly. When both ID and ACD are present, many of the laboratory measures of iron status become unreliable. Circulating iron concentrations are subnormal in both situations. Measurement of serum ferritin reflects the level of iron in the body stores. When serum ferritin is less than 15 µg/L, iron stores are definitely depleted. When ferritin is less than 30 µg/L there is a strong suggestion of iron deficiency, whereas, a value > 200 µg/L indicates

that iron deficiency is unlikely. Moreover, diagnosis of iron deficiency in the setting of IBD is difficult and no single laboratory test is reliable. Undoubtedly, low serum ferritin levels are indicative of iron deficiency. However, in the presence of inflammation, ferritin levels may increase despite iron deficiency. Furthermore, in IBD patients the diagnostic criteria for iron deficiency depend on the level of inflammation, thus in quiescent disease a serum ferritin < 30 µg/L or TfS < 15% indicates iron deficiency, whereas, in active disease a value < 100 µg/L suggests iron deficiency. A combination of true iron deficiency and anemia of chronic disease is possible when the ferritin level is between 30 and 100 g/L. If necessary, a bone marrow aspirate can be examined, which will show absence of macrophage iron in iron-deficient subjects. The ratio of soluble transferrin receptor/log ferritin has also been proposed as a marker for differentiating ID from ACD^[17]. A ratio above 2 is suggestive of iron deficiency, whereas a ratio of less than 1 indicates anemia of chronic disease.

IRON TREATMENT

Since iron deficiency is the most prevalent cause of IBD-associated anemia, iron supplementation is the most relevant therapeutic intervention.

Oral iron preparations

Treatment of anemia in IBD with oral iron is limited by poor absorption, drug intolerance, and induction of oxidative stress at the site of bowel inflammation^[3]. Moreover, there is evidence that oral iron may increase the disease activity in IBD. Oral iron supplements commonly contain iron in the form of ferrous salts (ferrous sulphate, ferrous gluconate, and ferrous fumarate). All ferrous compounds are oxidized in the gut lumen or within the mucosa with the release of activated hydroxyl radicals, which act on the gut wall and produce a range of gastrointestinal symptoms such as nausea, bloating, diarrhea and upper abdominal pain. Absorption of iron appears to be reduced in IBD. The acute phase protein hepcidin may play a central role in this process as it is over expressed in the liver leading to reduced iron uptake by the duodenum^[18]. Moreover, oral iron enhances intestinal inflammation as well as colon carcinogenesis in animal models of colitis^[19,20].

Intravenous iron preparations

Patients with IBD and anemia respond well to intravenous iron therapy with an increase in hemoglobin levels^[9]. Administration of iron directly into the circulation requires formulations that prevent cellular toxicity of iron salts^[21]. Three different products are available: Iron dextran, iron gluconate and iron sucrose. The stability of the dextran complex allows administration of large doses at a single setting. The molecule however may cause dextran-induced anaphylactic reactions. On the other hand, iron gluconate may cause the so-called transient capillary leak syndrome. Non-transferrin bound free ionic iron may induce acute endothelial cell injury, with the development of symptoms such as nausea, hypotension, tachycardia, dyspnoea, and oedema of the hands and feet. Iron sucrose is safer

than iron dextran and is well tolerated even by patients who have previously reacted adversely to iron dextran^[22]. Single doses of up to 300 mg of iron sucrose are safe^[23]. The maximal recommended dose is 600 mg/wk^[24]. If the infusion speed is too rapid, or the single total iron dose is too high, non-transferrin bound free iron may cause transient hypotension, tachycardia and dyspnoea, as described with iron gluconate.

Recently, a new intravenous iron preparation, ferric carboxymaltose (FeCarb) has been introduced. FeCarb can be administered intravenously in single or multiple doses of up to 1000 mg within 15 min. A recent report suggests that administration of FeCarb in IBD-associated anemia provides faster hemoglobin response, a greater increase in iron stores and better patient tolerance^[25]. Intravenous iron therapy for IBD-associated anemia has been recommended since the 1970s^[26], but clinical trials were only performed in the early 1990s^[6]. During the last several years, experience in the use of intravenous iron sucrose in IBD has evolved greatly. Gasche *et al* demonstrated the efficacy of such form of iron supplementation^[6,27,28]. Moreover, intravenous iron sucrose has a good safety profile in IBD-associated anemia, with a 65%-75% response rate in 4-8 wk, associated with improvement in the quality of life^[6]. In IBD patients, high levels of serum transferrin, soluble transferrin receptor, and serum EPO predict the response to intravenous iron supplementation, while low levels indicate the need for concomitant EPO therapy^[9]. Two comparative trials (oral *vs* intravenous iron) have recently been published^[29,30], indicating better tolerability of iv iron sucrose therapy.

The current management of IBD-associated anemia with iron sucrose is based on infusing the total iron dose by multiple small infusions, since a single dose should not exceed 500 mg (or 7 mg/kg body weight). Depending on the body weight, the usual dose for single infusions varies from 200 mg to 300 mg of iron sucrose in 100 mL sodium chloride. The total iron infusion may take between 4 to 10 wk. After this period, about 25% patients will fail to show a significant hemoglobin increase, defined as an increase in hemoglobin concentration of ≥ 20 g/L at the end of the wk 4.

One limitation of intravenous iron in patients not receiving EPO therapy is that much of the administered iron is transported into the reticuloendothelial system as storage iron, where it is less readily available for erythropoiesis.

Safety issues

Intravenous iron therapy has been carefully analyzed for risks and adverse events. Iron is an essential nutrient for proliferating microorganisms, and the sequestration of iron from microorganisms into the reticuloendothelial system is a potentially effective defense strategy against pathogens^[31]. In addition, iron therapy in the setting of long-term immune activation promotes the formation of highly toxic hydroxyl radicals that can cause tissue damage and endothelial dysfunction, with increased risk of acute cardiovascular events^[31-33]. Thus, intravenous iron therapy may predispose patients to infections and unfavorable

coronary outcomes.

Iron therapy is currently not recommended for patients with anemia of chronic disease who have high or normal ferritin levels (above 100 g/L), because of the risk of possible adverse outcome.

ERYTHROPOIETIN AND ANEMIA IN IBD

Erythropoietin, a glycoprotein hormone secreted by the kidney, is the primary growth factor regulating erythropoiesis^[34]. Erythropoiesis must maintain a steady state level of circulating RBCs and in addition respond to acute challenges. The bone marrow is a highly dynamic organ that produces two to three million red cells every second. These red cells contain hemoglobin and are replaced after 75-150 d^[3]. Any imbalance in the rates of red-cell loss and production due to inadequate release of EPO, absence of co-factors required for red-cell formation (in particular iron), or an impaired ability of the erythroid progenitor cells to respond to EPO, results in anemia^[35]. Erythropoiesis is controlled by the hypoxia sensing mechanism in the kidney which responds by modulating the output of EPO^[36].

EPO acts on committed erythroid progenitor cells in the bone marrow to regulate their proliferation, to promote their differentiation and to maintain their viability as they differentiate. Thus, EPO is the major regulator of erythropoiesis^[37]. Under normal physiological conditions, EPO expression is inversely related to tissue oxygenation and hemoglobin levels, and there is a semilogarithmic relationship between the EPO response (log) and the degree of anemia (linear)^[38]. Moreover, the concentration of EPO that is normally between 5 to 29 U/L, can increase 100-fold in the presence of severe anemia. However, in some patients, EPO concentrations fail to increase despite significant anemia^[39].

Measurement of serum EPO levels is useful only in anemic patients with hemoglobin levels less than 100 g/L, since EPO levels at higher hemoglobin concentrations remain well within the normal range^[40]. Serum EPO levels that are inappropriately low for the degree of anemia, indicates blunted EPO response, and is encountered in anemia of chronic disease^[41].

Higher serum EPO levels have been reported in IBD patients compared to the normal population^[39,42,43]. The EPO levels increase with the degree of anemia^[27]. Interestingly, it has been shown that in IBD-associated anemia, EPO production is inadequate in relation to the degree of anemia^[5,9]. This finding may be of help when considering EPO therapy^[9].

Although anemia is a frequent complication of many diseases, its clinical relevance and the importance of its correction have long been neglected. Anemia was found to have a marked effect not only on the quality of life, but also on various physiological functions^[44-46]. Before the mid-1980s there was no effective therapeutic means of stimulating erythropoiesis. The clinical use of EPO began in 1986 with treatment of patients with chronic kidney disease^[47,48]. Since then, indications for the use of EPO therapy to boost erythropoiesis have broadened considerably.

ERYTHROPOIETIC AGENTS

Three erythropoietic agents are currently available in routine clinical practice: epoetin alfa, epoetin beta, and darbepoetin alfa, which differ in terms of their pharmacologic modifications, receptor-binding affinity, and serum half-life, thus allowing for alternative dosing and scheduling strategies^[49].

Recombinant Human Erythropoietin

Epoetin- α and Epoetin- β are the two available brands of recombinant human erythropoietin (rHu EPO). In 1977, small amounts of human EPO were obtained from the urine of patients with aplastic anemia^[50]. Based on limited peptide sequence information of this purified material, the gene of human EPO was isolated and cloned in 1983^[51], and the use of genetic engineering techniques allowed large-scale production of recombinant human EPO in a suitable mammalian cell line. The clinical success of this product has resulted in the use of epoetin in millions of anemic patients.

Like the endogenous hormone, epoetin binds to the dimerised EPO receptor on the surface of erythroid progenitor cells, inducing a conformational change in the receptor. This change induces phosphorylation of tyrosine residues by JAK-2 kinase on several intracellular molecules, including STAT-5, which is the major signal transducer and activator of transcription, causing gene activation in the cell nucleus^[52]. Activation of these pathways stimulates proliferation and inhibits apoptosis of the erythroid progenitor cells^[53].

Epoetin is highly effective in stimulating erythropoiesis^[5,6,54,55]. In addition there is increasing experimental evidence that EPO has a range of non-erythropoietic, pleiotropic effects, including tissue protection of the nervous system, myocardium, kidneys, intestines and the joints^[56].

Darbepoetin alfa

Despite the undoubted therapeutic efficacy of epoetin, a major limitation of this treatment is that it has to be administered parenterally two or three times a week. Much effort has therefore been directed at producing longer-acting EPO analogues that would retain their biological activity, but require less frequent dosing. The first to be synthesized was darbepoetin (DPO) alfa^[57]. DPO alfa has five N-linked glycosylation chains compared with the endogenous and recombinant EPO, both of which have three. All three molecules have, additionally, a single O-linked glycosylation chain^[58]. The molecular weight of DPO alfa is 37.1 kDa, compared with 30.4 kDa for EPO, and its elimination half-life in humans after an intravenous injection is about three-fold longer (25.3 h *vs* 8.5 h for epoetin alfa)^[59]. This allows less frequent dosing, with most patients receiving injections once weekly or once every 2-3-4 wk^[60].

Continuous erythropoietin receptor activator

Continuous EPO receptor activator (CERA) is the another erythropoietic agent, that has recently completed phase III of its clinical development program^[61,62]. CERA was developed by the integration of a single 30 kDa

polymer chain into the EPO molecule, thus, increasing the molecular weight to twice that of epoetin to about 60 kDa, and considerably increasing the elimination half-life in humans to about 130 h. The hypothesis being tested in phase III clinical studies is whether CERA can be administered safely and effectively every 3-4 wk. The preliminary data suggests that this is perhaps the case^[63,64].

Next generation erythropoietic analogues

Synthetic erythropoiesis protein, EPO fusion protein and EPO-mimetic peptides are the next generation of erythropoietic agents that exploit recent advances in drug development for stimulating erythropoiesis. These agents stimulate erythropoiesis through the activation of EPO receptors^[65-67].

ROLE OF ERYTHROPOIETIN IN IBD-ASSOCIATED ANEMIA

Hemoglobin increase

Several studies have examined the efficacy and the safety of EPO in the treatment of refractory anemia in patients with IBD^[5,6,11,68,69]. Horina *et al* first tested tEPO therapy in three patients with a long-standing history of IBD and refractory chronic anemia (Hb < 100 gr/L, plasma EPO < 100 U/L). A marked increase in hemoglobin values was noted in all three patients^[68]. Gasche *et al*^[27] reported that after 5 wk of treatment with intravenous iron alone or in combination with EPO, all anemic patients had a marked increase in hemoglobin levels. However, the mean increase in EPO-treated patients was higher compared to patients receiving iron therapy alone (50 g/L *vs* 20 gr/L respectively). Subsequently, recombinant human EPO was found to be effective in a controlled trial of patients with anemia refractory to oral iron supplementation^[5]. In this double-blind study, the superiority of combination therapy (iron + rHEPO) over iron alone was again demonstrated. In another placebo-controlled study in a patient with Crohn's disease-associated anemia that was refractory to oral iron, intravenous iron, and EPO resulted in greater and quicker hemoglobin response compared with intravenous iron alone^[6]. Concomitant EPO therapy was associated with a more rapid incline in hemoglobin levels. EPO treatment has also been shown to be safe and effective in children with iron refractory IBD-associated anemia^[11].

In a recent study^[70], Darbepoetin-alpha (DPO) was tested in patients with IBD-associated refractory anemia. This study, the first to assess DPO alfa in IBD-associated refractory anemia, demonstrated that the administration of DPO in combination with intravenous iron sucrose raised hemoglobin levels. Furthermore, the longer half-life of DPO compared to EPO allowed less frequent administration and was more convenient to the patients. Thus, all the trials have demonstrated a significant beneficial effect of erythropoietin agents in this patient population.

Improvement in quality of life

Most studies have shown that successful treatment of anemia with EPO, is accompanied with improvement in

the energy and activity level and the overall quality of life^[71]. Surprisingly, in the hemoglobin range of 80-140 g/L, the largest improvement in quality of life occurred when hemoglobin levels increased from 110 to 130 g/L^[72]. Alterations in the quality of life were also examined in anemic patients with Crohn's disease treated with iron sucrose and EPO^[6]. The sense of well being, and improvement in mood, physical ability, and social activity accounted for most of the improvement in quality of life. Moreover, avoidance of blood transfusions enhanced the improvement in quality of life^[15]. Thus, the quality of life is significantly improved in patients with IBD-associated anemia with erythropoietin therapy.

MECHANISMS OF ACTION

The therapeutic effect of EPO involves counteracting the antiproliferative effect of cytokines, along with stimulation of iron uptake and heme biosynthesis in erythroid progenitor cells. Accordingly, a poor response to treatment with erythropoietic agents is associated with increased levels of proinflammatory cytokines, and poor iron availability.

There is little data on the possible effects of therapy with erythropoietic agents and the correction of anemia, on the course of the underlying disease, particularly since epoetin can exert additional biologic effects, including interference with the signal transduction cascade of cytokines. Treatment of rats with EPO was found to reduce the degree of colitis caused by DNBS suggesting that EPO may be useful in IBD treatment^[73]. Furthermore, EPO may enhance the healing of colonic anastomosis after colonic surgery by increasing the number of fibroblasts and accelerating the maturation of new blood vessels^[74].

PREDICTIVE FACTORS OF RESPONSE TO ERYTHROPOIETIC THERAPY

The response (erythropoiesis) to a dose of EPO is not related to the patient's gender or age, suggesting that patient-specific factors such the underlying chronic disease, iron-restricted erythropoiesis, and other factors that normally result in a wide distribution of hemoglobin responses, account for the variability in erythropoietin response to EPO in different individuals^[15].

There are no baseline indices of response to erythropoietin that can be used in routine clinical practice. If functional iron deficiency and vitamin deficiency are excluded, a low serum EPO level appears to be the only established predictive factor of some importance^[75]. In IBD patients, high levels of serum transferrin, soluble transferrin receptor, and serum EPO predict the response to intravenous iron supplementation, while low levels indicate a need for concomitant EPO therapy^[9].

Further studies are needed to investigate the value of hepcidin, C-reactive protein and other measures as predictive factors. The iron-regulatory hormone hepcidin is of particular interest as it is believed to be the primary factor in anemia of chronic disease. Cytokine-mediated induction of hepcidin in inflammatory or infectious

conditions^[76] decreases duodenal absorption of iron and induces iron retention by macrophages. Likewise, CRP is also increased in inflammatory conditions, and high CRP levels are associated with reduced Hb levels and resistance to EPO treatment. Therefore, pre-treatment levels of hepcidin and CRP may provide important information on the response to erythropoietic proteins in IBD patients.

INDICATIONS FOR ERYTHROPOIETIC THERAPY IN IBD-ASSOCIATED ANEMIA

It is difficult to determine as to which patient with IBD-associated refractory anemia will require combination therapy with erythropoietic agents in view of the absence of any long-term data. However, EPO should be reserved for symptomatic patients who may otherwise require blood transfusions, who have not responded to intravenous iron, and in whom aggressive management of IBD (including immunosuppressive therapy) has not suppressed the mucosal inflammation. EPO is an adjunct and not an alternative to appropriate treatment of IBD^[77]. Several issues remain to be resolved including the use of EPO for prevention of anemia, the target hemoglobin levels, and the immunologic role of EPO in the setting of chronic bowel inflammation.

SIDE EFFECTS AND CONCERNS ABOUT EPO THERAPY

Erythropoietic therapy is highly effective in stimulating erythropoiesis and has an excellent safety profile. Apart from the rare induction of antibodies, all adverse effects of epoetin appear to be directly related to its pharmacodynamic properties i.e., the increase in red-cell number. In patients with kidney disease, such an increase may lead to a moderate rise in the blood pressure. In addition, there is a risk of thromboembolic complications with higher hemoglobin levels. Clinical trials in patients with cancer^[78] and chronic kidney disease^[79], designed to assess the effect of increasing the haemoglobin concentration to the normal range, compared with a subnormal target, have suggested no overall benefit and even the risk of potential harm. Moreover, it has recently been observed that "on the basis of available data, the maintenance of haemoglobin concentrations above 130 g/L appears to be unsafe in patients with chronic renal failure"^[80].

The success of epoetin has been clouded by the discovery in a few patients of the development of neutralizing antibodies against recombinant proteins that cross-react with native EPO, and cause pure red-cell aplasia^[81]. This problem is likely caused by the use of a new buffer (polysorbate 80), which replaced human serum albumin, and which induces the release of organic compounds with adjuvant properties from the rubber stoppers of prefilled syringes^[82].

Another issue of concern is the presence of EPO receptors on several malignant cell lines, including mammary, ovarian, uterine, prostate, hepatocellular, and renal carcinomas, as well as on the myeloid cell lines^[83]. There are contradictory reports concerning the effect of

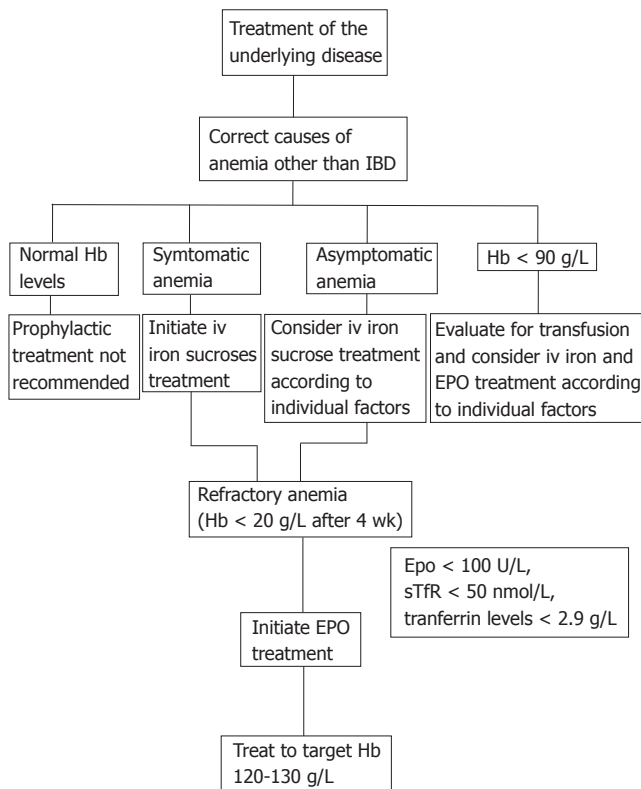


Figure 1 IBD-associated anemia. Suggested treatment algorithm for stimulating erythropoiesis in patients with IBD-associated anemia. Hb: Hemoglobin; iv: Intravenous; EPO: Erythropoietin.

epoetin on such cells. Although the use of EPO caused tumor regression in a murine model of myeloma^[84], administration to EPO-receptor-expressing human renal carcinoma cells *in vitro* stimulated their proliferation^[85]. Large amounts of EPO receptors were found in 90% of biopsy specimens of human breast carcinoma^[86]. The production of EPO receptors by cancer cells appears to be regulated by hypoxia, and in clinical cancer specimens the highest levels of EPO receptors were associated with neoangiogenesis, tumor hypoxia, and presence of infiltrating tumors. One potential adverse effect may be the induction of neoangiogenesis, since EPO increases inflammation and ischemia-induced neovascularization by enhancing the mobilization of endothelial progenitor cells^[87]. In a study in nude mice, implantation of EPO-receptor expressing cell lines and subsequent inhibition of EPO-receptor signaling resulted in inhibition of angiogenesis and destruction of the tumor mass^[83].

The effects of erythropoiesis-stimulating agents are still not fully understood. Not only do they increase haemoglobin concentration, but they may also act *via* alternative dose-dependent pathways that are harmful. Careful studies on the potential harmful effects of EPO therapy in patients with different forms of anemia of chronic disease remain to be carried out.

STRATEGIES FOR STIMULATING ERYTHROPOIESIS IN IBD

Anemia is a common complication of IBD and poses a

serious therapeutic challenge. The optimal management of IBD-associated anemia is to promote an increase in hemoglobin levels accompanied with an improvement in the quality of life. Therapy is targeted at the mechanisms underlying anemia of chronic disease and iron deficiency. The most important approach is to treat the underlying disease (Figure 1). In previous studies assessing EPO in anemic IBD patients, current or recent immunosuppressive therapy was a specific exclusion criterion^[5,6], suggesting that the patients may not be receiving optimal anti-inflammatory therapy. Moreover, as the IBD disease activity correlates with the degree of anemia, treatment of IBD should lower its incidence. Thus, as effective therapy (including immunosuppressants and anti TNF- α) induces mucosal healing^[88,89], one would expect a lower incidence of anemia.

Additionally, causes of anemia other than IBD (nutritional defects, bleeding, haemolysis, chronic kidney disease, cancer, metabolic disorders and cardiovascular disease) should be evaluated and treated. Asymptomatic anemia warrants correction, especially in patients older than 65 years of age, those with additional risk factors (such as coronary artery disease, pulmonary disease, and chronic kidney disease), or a combination of these factors.

A stepwise approach for stimulating erythropoiesis should be followed in patients with IBD. First, iron deficiency which is the most common cause of anemia should be corrected. Recent studies have demonstrated the efficacy and the safety of intravenous iron supplementation with iron sucrose. About two-thirds of patients respond to this treatment within 4-10 wk. The remaining subjects who fail to improve likely have anemia of chronic disease. Gasche *et al* suggest that serum levels of EPO, soluble transferrin receptor, and transferrin predict a positive response to iron infusion, and may identify those patients who will benefit from early treatment with EPO^[9].

EPO administration has been shown to be of benefit in patients who do not respond to iron supplementation. Several studies have demonstrated the efficacy and the safety of EPO in IBD patients with refractory anemia. EPO treatment results in a marked increase in hemoglobin values and improvement in quality of life. However, there is controversy as to whether EPO is cost-effective in treating anemia in IBD. Many physicians have employed erythropoietic agents which are not currently approved by the FDA. Thus, clinicians will have to make treatment decisions based on limited data currently available. These agents are effective in increasing hemoglobin level, and improving the quality of life for the duration they are used. However, the length of treatment, optimal dose and target hemoglobin level remain to be established. The disadvantage of these agents is that they add another drug to the patient's treatment regimen, thereby increasing the costs, inconvenience, and potential side effects. Several studies in patients with renal failure and cancer indicate that the target hemoglobin level with EPO should be 120-130 g/L. However, a normal haematocrit value may not be optimal.

IBD-associated anemia can also be managed with blood transfusions which are widely used for rapid and effective intervention. Transfusions are particularly helpful in the context of severe anemia (Hb < 90 g/L)

and life-threatening anemia (Hb < 70 g/L). However, the optimal hemoglobin threshold for red-cell transfusion in IBD patients is unknown. Recent evidence^[90,91] does not support the unrestricted use of blood transfusions, because of the risks associated with this procedure such as iron overload and sensitization of the immune system. Thus, the timing of blood transfusion in IBD-associated anemia must take into consideration not only the hemoglobin level but also the relative degree of hemoglobin decrease, any underlying comorbidities and the presence of anemia-related symptoms. It is also important that the use of blood transfusions should be followed by treatment with iron supplementation, with or without EPO.

In our clinical practice, most patients with IBD-associated anemia respond well to intravenous iron alone. It is important that the bowel inflammation is treated adequately and that sufficient iron is given. In anemic patients with active IBD, we usually start treatment with corticosteroids, in order to alleviate the clinical symptoms, followed by intravenous iron sucrose to replenish the iron stores. This strategy helps to stimulate impaired erythropoiesis and thus reduce the need for concomitant EPO injections. However, patients with ongoing inflammation have anemia of chronic disease and may require combination therapy with intravenous iron sucrose and erythropoietic agents. It has been suggested that about 25% of patients with IBD-associated anemia require combined treatment with iron sucrose and EPO^[8]. Recently, we examined the use of DPO to determine whether it is effective in refractory anemia in IBD^[70]. The administration of DPO in combination with intravenous iron sucrose was effective in these patients. Careful monitoring of hemoglobin levels and iron parameters is required to avoid recurrence of anemia in IBD patients. Additional clinical trials are warranted to establish the optimal dose and schedule of intravenous iron supplementation, and erythropoietic therapy in IBD-associated anemia.

CONCLUSION

Anemia is a frequent complication of IBD. Patients with anemia usually have greater disease severity and lower quality of life, demanding aggressive diagnosis and treatment. Iron deficiency is managed most reliably by intravenous preparations. Iron sucrose demonstrates the best efficacy and tolerability. Difficulties arise in patients with refractory anemia, who do not respond to intravenous iron. EPO administration has proven to be effective in these patients. EPO has additional beneficial effects on hemoglobin concentration. However, EPO is expensive and not without potential side effects. It is difficult to determine as to which patient with refractory anemia will require combination therapy with erythropoietic agents. The responsible use of medical resources, as well as the absence of data on long-term safety of EPO in patients with IBD, suggests that iron sucrose should be considered as the first-line therapy in IBD-associated anemia. Therefore, EPO has a secondary role, in patients who do not respond to intravenous iron alone. The long-term outcome of alleviating anemia depends on whether the bowel inflammation can be adequately treated.

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Robert Thimme, MD, Professor, Series Editor

Immunity to hepatitis C virus infection: Update 2007

From the editor

This 8-part topic review series, "Hepatitis C virus: virology, immunology and pathogenesis", was designed to provide expert opinion and new insights for the Journal's readership.

Hepatitis C virus (HCV) is a small single-stranded RNA virus that belongs to the *Flaviviridae* virus family. More than 120 million people are chronically HCV infected. The factors that determine the outcome and natural course of HCV infection are not completely understood. However, it is generally accepted that next to virological factors innate and adaptive immune responses play an important role in both, control of HCV infection and as disease pathogenesis (Figure 1).

In recent years, significant progress has been made in understanding basic mechanisms of viral replication, innate and adaptive immune responses, such as CD4⁺ and CD8⁺ T cells as well as neutralizing antibodies, and the possible role of regulatory T cells in orchestrating the adaptive immune response. However, the central question why HCV is able to evade the innate and adaptive immune response and to establish viral persistence in the majority of acutely infected patients is still unknown. In this review series, recent advances

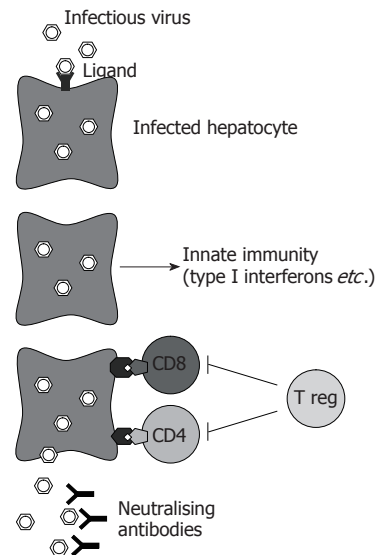


Figure 1 After HCV infection of hepatocytes, innate immune responses are induced. In addition, virally infected cells present antigens to CD4⁺ and CD8⁺ T cells. Both T cell subtypes are regulated by Tregs. Secreted virus can be bound by neutralizing antibodies. All parts of these interactions will be discussed in the following review series.

in HCV virology and immunology will be highlighted. Clearly, a better understanding of the dynamic host-virus interactions during HCV infection is crucial for the development of new prophylactic and therapeutic vaccines.

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TOPIC HIGHLIGHT

Robert Timme, MD, Professor, Series Editor

Sequence diversity of hepatitis C virus: Implications for immune control and therapy

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Abstract

With approximately 3% of the world's population (170 million people) infected with the hepatitis C virus (HCV), the WHO has declared HCV a global health problem. Upon acute infection about 50%-80% of subjects develop chronic hepatitis with viral persistence being at risk to develop liver cirrhosis and hepatocellular carcinoma. One characteristic of HCV is its enormous sequence diversity, which represents a significant hurdle to the development of both effective vaccines as well as to novel therapeutic interventions. Due to a polymerase that lacks a proofreading function HCV presents with a high rate of evolution, which enables rapid adaptation to a new environment including an activated immune system upon acute infection. Similarly, novel drugs designed to specifically inhibit viral proteins will face the potential problem of rapid selection of drug resistance mutations. This review focuses on the sequence diversity of HCV, the driving forces of evolution and the impact on immune control and treatment response. An important feature of any therapeutic or prophylactic intervention will be an efficient attack of a structurally or functionally important region in the viral protein. The understanding of the driving forces, but also the limits of viral evolution, will be fundamental for the design of novel therapies.

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Key words: Hepatitis C Virus; Evolution; Escape; Drug resistance; Selection

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INTRODUCTION

With approximately 3% of the world's population (170 million people) infected with the hepatitis C virus (HCV), the WHO has declared HCV a global health problem^[1]. Upon acute infection about 50%-80% of subjects develop chronic hepatitis with viral persistence being at risk to develop liver cirrhosis and hepatocellular carcinoma. The current standard treatment for chronic HCV infection is based on a combination of pegylated interferon alpha and ribavirin^[2,3]. However, this combination is successful in only 50% of all subjects with response rates substantially influenced by the infecting viral genotype. Moreover, the side effects and costs of treatment further limit the use in many regions. Alternative treatment approaches including immunotherapeutic interventions are, therefore, highly warranted. One characteristic of HCV is its enormous sequence diversity, which represents a significant hurdle to the development of both effective vaccines as well as to novel therapeutic interventions. This review focuses on the sequence diversity of HCV, the driving forces of evolution and the impact on immune and control and treatment response.

GENOME STRUCTURE

The HCV genome is an RNA molecule of approximately 9600 nucleotides structured in a coding region that contains one large open reading frame and flanked by non-translated regions at the 5' and 3' ends. The polyprotein is cleaved into structural (core, envelope 1 and 2) and non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) with one additional small protein at the junction between the structural and non-structural elements (p7 protein)^[4]. Until the recent development of an *in vitro* replication system that produces infectious viral particles^[5-7] many of the protein functions have been studied in sub-genomic replication systems or with purified protein after expression.

The 5' NTR forms a highly structured RNA element that contains an internal ribosomal entry site (IRES) that allows interaction with the 40S ribosomal subunit and initiation of cap-independent translation of the viral RNA^[8]. The polyprotein is translated as one large reading frame and subsequently cleaved by host-cellular proteases and virally encoded proteases into the individual proteins^[9]. The structural proteins core, envelope 1 and envelope 2 are cleaved

by host-cellular proteases. Processing of these proteins is believed to take place in a membrane associated complex at the endoplasmic reticulum by signal peptidases^[10]. The core protein forms the viral capsid, binds to the viral RNA and interacts with envelope proteins to form viral particles. Different receptors have been suggested for the interaction of viral particles with the hepatocyte that mediates HCV entry including CD81, scavenger receptor class-B type-I (SR-BI), low-density lipoprotein receptor (LDL), mannose binding lectins (L-SIGN and DC-SIGN) and glycosaminoglycans^[11]. E2 binds with high affinity to the extracellular loop of CD81, a tetraspanin that is expressed on different cell types including hepatocytes^[12]. Cell entry of the virus is CD81 and SR-BI dependent, suggesting that these molecules serve as receptors or co-receptors for infection^[13-16]. However, this interaction does not explain the hepatotropism as CD81 and SR-BI are not exclusively expressed on hepatocytes. Moreover, it has been shown that CD81 and SR-BI are necessary for cell entry of viral particles, but are not sufficient^[16,17]. Some cell lines that express both proteins do not support viral entry. Recently a new candidate has been suggested that may close this gap. Claudin-1, a tight junction component that is highly expressed in the liver, was recently identified as a key factor in the late entry process^[18].

These structural components of HCV are flanked by the non-structural proteins NS2 to NS5B. The function of one additional protein (p7) between these elements remains to be elucidated. It has been suggested that p7 forms an ion channel in planar lipid bilayers^[19,20]. However, it is still unclear whether it is a virion component. NS2 contains an autoprotease, which cleaves the junction between NS2 and NS3^[4]. NS3 is a multifunctional protein with a N-terminal protease domain and a C-terminal RNA helicase/NTPase domain. The NS3 protease cleaves the remaining non-structural proteins with NS4A as a cofactor for this activity. The NS3 RNA helicase/NTPase unwinds RNA and DNA^[21]; however, its role during viral replication is unclear. The integral membrane protein NS4B is sufficient to induce membranous web formation and has been proposed to serve as a scaffold for replication complex assembly. The role of NS5A is again unclear. Numerous protein-protein interactions have been suggested including a role in silencing the host's innate immune response and determining responsiveness to interferon alpha^[22]. Some of these interactions are discussed later. NS5B encodes the viral RNA-dependent RNA polymerase. In the viral replication cycle the positive-strand RNA genome serves as a template to make a negative-strand intermediate, which then again serves as a template to produce multiple nascent genomes. Recently, one additional protein resulting from frameshifted translation of the core protein has been identified (alternative reading frame protein ARFP)^[23]. However, the function of this protein is unknown.

GENOTYPES

Phylogenetic analysis of HCV genomes revealed that sequences fall into different clusters. This observation led to a classification of HCV into different genotypes and a standardized nomenclature was proposed in a consensus

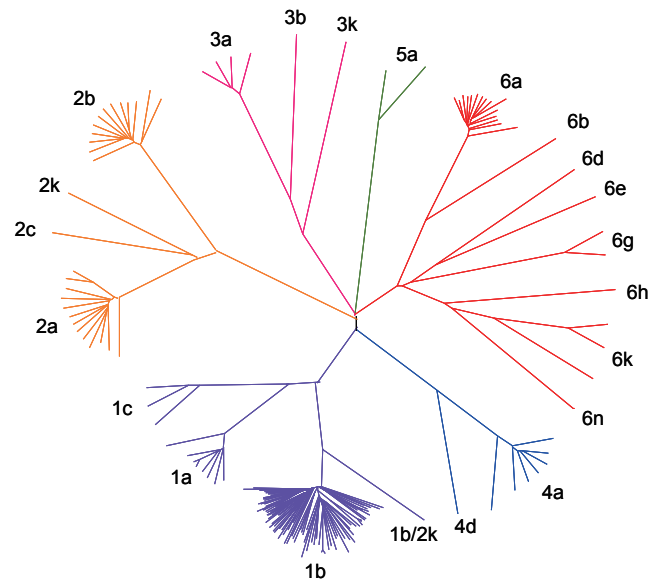


Figure 1 Published HCV Full Genomes. 169 full length HCV sequences available from the Los Alamos National Laboratory (LANL) HCV Sequence Database are illustrated in a phylogenetic tree. HCV sequences fall into six different clusters (genotype 1-6) and are further classified into subtypes. The sequence from one recombinant virus (genotype 1b/2k) is included.

paper in 1994^[24]. The global distribution of HCV genotypes is regionally specific. The predominant genotype in most areas is genotype 1. However, some areas are almost exclusively infected with other genotypes. For example, the predominant genotype in Egypt is genotype 4 and the HCV epidemic in this country could be linked to parenteral treatment of schistosomiasis in the 1950s^[25,26]. In some regions in Africa genotype 2 is more frequent^[27]. In some areas of Asia, however, genotypes 3 and 6 are predominant^[28-30]. Despite substantial sequence variation all genotypes share the same structure of linear genes of nearly identical size. The genotype specific variation of the different genes is remarkably consistent and has enabled many of the currently recognized variants of HCV to be provisionally classified based on partial sequences from subgenomic regions such as core/E1 and NS5B. The original nomenclature was recently updated further standardizing the nomenclature of existing variants^[30]. Based on phylogenetic analysis a classification into 6 major genotypes was proposed and criteria for the designation of new HCV variants were formulated. These proposals provide an HCV nomenclature scheme for the three major public HCV sequence databases (Europe^[31], USA^[32] and Japan: <http://s2as02.genes.nig.ac.jp/>) and eliminate inconsistencies of the current classification procedures. HCV genotypes differ from each other by 31%-33% on the nucleotide level^[33]. The genotypes are further divided into multiple epidemiologically distinct subtypes differing by 20%-25% from one other^[33]. A phylogenetic tree depicting all published complete HCV genomes is presented in Figure 1. For many of the HCV subtypes, particularly for the less frequent ones, complete genome sequences are not available. The lack of sequence data for rare genotypes is profound prompting a sequencing initiative supported by the NIH to improve the HCV sequence databases^[32].

SEQUENCE VARIATION

As a member of the flaviviridae, the virally encoded RNA polymerase of HCV lacks a proof reading function. Replication of this positive-stranded RNA genome is, therefore, characterized by ongoing error rates between 1 in 10000 and 1 in 100000 bp copied, which are typically found for RNA polymerases^[34]. Together with a high turnover rate of estimated 10^{12} virions per day^[35], theoretically every possible mutation in every single position of the genome will be generated in one infected host every day. This high error rate is reflected in the generation of a heterogeneous, but closely related swarm of viruses within the same host referred to as quasispecies. The quasispecies nature of HCV can be best illustrated by sequence analysis of a short, but highly polymorphic region in envelope 2 designated as the hypervariable region 1 (HVR 1). Analysis of clonal sequences reveals that sequences of the viral population from the same subject are highly variable, but still phylogenetically closely related. In public databases normally the consensus sequence, as the most predominant residue at any given position within the quasispecies population, is presented. The quasispecies nature of HCV may have important consequences during a transmission event. Depending on the transmission route the number of transmitted viral RNA copies can be limited and may not represent the true complexity of the sequence diversity of the donor. This bottleneck phenomenon has been described for sexual transmission of HCV^[36] and in the chimpanzee model^[37]. However, the bottleneck could also be interpreted as selection of the optimal strain in the new host during the earliest infection events. Different aspects about the nature of the observed HCV sequence evolution have been published. The next section gives an overview of the different mechanisms.

DRIVING FORCES OF EVOLUTION

Genetic drift

Longitudinal analysis of isolates from subjects with chronic HCV infection calculated a mutation rate on the order of $1.5\text{--}2.0 \times 10^{-3}$ nucleotide substitutions per site per genome per year^[38,39]. In the model of neutral evolution mutations are selectively neutral^[40]. The spread of these neutral mutations is mainly influenced by stochastic factors and is called genetic drift. As a consequence of this stochastic process even disadvantageous mutations can reach fixation when the virus circulates through a sufficiently small population. In turn, advantageous mutations are also affected by genetic drift when they are rare and are occasionally lost from the population. Several studies have described the rapid sequence drift of HCV over time^[33]. The model of neutral evolution and the presumption that such diversification should occur at a constant rate over time provide a framework to estimate times of spread of HCV in specific transmission networks^[41,42]. For example, a recent analysis of viral sequences obtained from an HCV and HIV outbreak in children at the Al-Fateh hospital in Libya, utilizing this molecular clock, demonstrated that the origin of the outbreak predated the year of 1998^[43]. Noteworthy, this analysis excluded the possibility that the

source virus was transmitted by foreign medical staff as suggested by local authorities.

Sequence drift has been suggested in a few studies as the major driving force of HCV evolution. An analysis by Allain *et al* suggested a dominant role in the evolution of envelope 2^[44]. They analyzed clonal sequences of six different transmission pairs years after the transmission event. In this study the ratio of non-synonymous to synonymous mutations in the analyzed region did not support the hypothesis of positive or negative selection. The author's, therefore, concluded that neutral evolution is a major component of the observed sequence diversity. Moreover, the authors did not find a correlation between the strength of the antibody response and the rate of evolution in these patients. However, in this study the strength of the humoral immune response was determined in serum collected years after the transmission event utilizing different HVR 1 peptides corresponding to autologous and heterologous sequences. Therefore, the true antibody response against the virus present during the acute phase of infection might have been underestimated. Another shortcoming of this study is that the inoculum sequence at the time of transmission was unknown and only a single time point years later was available making conclusions about the true evolutionary rate and kinetics difficult.

Positive and negative selection

In contrast to being neutral mutations may also be selected. Many mutations are probably disadvantageous or even deleterious for the virus and these variants are eliminated in a negative selection process. However, some mutations may not have an impact on replication capacity and a few may even be beneficial and confer a replication advantage. Variants harbouring these beneficial mutations will out compete for others in a dynamic process of continuous positive selection. Similar to other highly variable pathogens a complex process of continuous selection has been proposed for HCV^[45]. Theoretically, infections with persistent viruses such as HIV and HCV have time to evolve within the same host before transmission to the next host and may adapt to the specific environment in an individual. The evolution of HCV may, therefore, be substantially influenced by host factors mediating selection pressure on the virus. Even though the consensus sequence may be close to the maximum of viral replication capacity at any one time, the existence of a large and diverse viral population allows rapid, adaptive changes in response to changes in the replication environment. Many variants that are beneficial in a new environment may already be present in a low frequency in the quasispecies population and subsequently out competes the existing dominant sequence. The impact of the quasispecies complexity on the clinical outcome can be profound. Farci *et al* analyzed sequences covering HVR1 obtained during the acute phase of infection from subjects who spontaneously resolved viremia and subjects who continued to chronic infection^[46]. Spontaneous resolution of viremia was predicted by a decrease in quasispecies complexity during the first weeks of infection.

In turn patients with viral persistence had increasing viral diversity suggesting a fast adaptation process to the new environment. A similar effect of quasispecies diversity on the outcome of treatment is discussed^[47,48]. In recent years many studies have been published that aim to characterize the driving forces of this selection process.

Antibodies

The most variable region in the HCV genome is a short fragment spanning 27 amino acids of envelope 2 and is, therefore, designated the hypervariable region 1 (HVR 1). There is strong evidence that the profound sequence diversity in this region is the result of immune pressure by virus specific antibodies. Importantly, there is a close association between the observed sequence diversity in this region and the appearance of HCV specific antibodies in the sera of subjects with acute infection^[49,50]. Patients suffering from common variable immunodeficiency (CVID) who present with hypogammaglobulinemia are not able to produce high titres of HCV specific antibodies and, therefore, are not able to mount humoral immune selection pressure. Analysis of sequences of HVR1 revealed that patients with hypogammaglobulinemia had significantly less amino acid substitutions in this region over time as compared to controls^[51]. In a similar analysis, the rate of non-synonymous and synonymous mutations was compared between core and envelope in patients with and without CVID^[52]. The rate of synonymous or silent mutations was similar in the core and envelope protein. In patients without CVID, as expected, the rate of non-synonymous mutations was much higher in envelope as compared to core, a protein that is known to be highly conserved. However, this high rate of non-synonymous mutations in envelope was not observed in patients with CVID suggesting that evolution is triggered by the presence of anti-HCV antibodies. In the chimpanzee model, it was demonstrated that a high turn-over rate is not sufficient to explain HVR1 sequence diversity. Only minor sequence variation was observed in this region upon serial infection with passage of an infectious HCV clone between 8 different animals^[53]. Noteworthy, samples for the subsequent infection of the next animal were taken during the acute phase before antibodies became detectable. Again, this study indicates that this region in the envelope remains stable in the absence of antibodies despite high level viremia that was present in all animals during the acute phase of infection. Taken together, all these studies suggest that without immune selection pressure only minor sequence changes occur in HVR1.

The lack of an *in vitro* culture system has hampered direct evaluation of these putative escape mechanisms in the envelope protein. Recently, more elegant tools for this type of analysis became available by pseudotyping retroviral particles with HCV glycoproteins (HCVpp)^[54-56]. Utilizing this technique, the impact of neutralizing antibodies on the evolution of HVR1 was demonstrated in a study by von Hahn and co-workers^[57]. Here longitudinal samples were obtained over a time period of 26 years from patient H who was infected in 1977 with genotype 1a. Sera were analyzed for the presence of neutralizing

antibodies against the autologous isolate present at the time of sampling. A neutralizing antibody response could be detected as early as 8 wk after infection against the inoculum strain. Interestingly, the antibodies present in a given sample continuously failed to neutralize HCV pseudoparticles bearing the autologous sequence from the same time point. Longitudinal analyses demonstrate continuous escape from emerging antibodies over the time of infection demonstrating humoral immune pressure as the major driving force for the observed sequence diversity in HVR1.

CD8 T cells

Mutational escape from CD8 T cells targeting viral proteins has been well documented for highly variable pathogens such as HIV and SIV. Similarly, in the chimpanzee model of HCV infection selection of mutations in CD8 epitopes that inhibit recognition by specific T cells has been described by Weiner and co-workers^[58]. In a follow-up analysis the majority of targeted CD8 epitopes in chimpanzees infected with HCV evolved over time and an important role for mutational escape as a contributor for viral persistence has been suggested^[59]. However, acute infection is rarely detected in humans due to lack of specific symptoms making the design of similar longitudinal studies difficult. First evidence for selection pressure by CD8 T cells was obtained from sequence analyses of patients with chronic infection^[60]. Here the T cell response against previously defined CD8 epitopes was determined. In some cases the autologous viral sequence present in the patient differed from the described prototype sequence of the epitope and was not targeted by specific T cell lines derived from that patient. The study included a case where sequence evolution was observed in a follow-up sample suggesting that CD8 escape also plays a role during chronic HCV infection. More recently, several longitudinal studies on patients with acute HCV infection have been published providing compelling evidence for CD8 escape in humans^[61-64]. Probably the most comprehensive analysis was done by Cox *et al.*^[62]. They prospectively followed subjects with ongoing intravenous drug use and high risk behaviour for evidence of acute HCV infection. Using this approach they were able to identify eight patients with acute HCV infection. Samples from these patients were obtained during acute infection (at the time of diagnosis) and after 6 mo. Utilizing comprehensive techniques with overlapping peptides spanning the entire HCV polyprotein the breadth of the immune response was determined. At the same time, sequence evolution between the first and second sample obtained 6 mo later was analyzed. Seventeen of 25 targeted epitopes evolved over time consistent with selection of escape mutations. Of note, the single subject without selection of escape mutations cleared viremia spontaneously. In turn, 50% of the observed sequence changes outside the envelope were associated with a detectable CD8 response. In line with these findings Ray *et al.* analyzed sequences from a single source outbreak infected with HCV genotype 1b and observed reproducible selection of mutations in previously

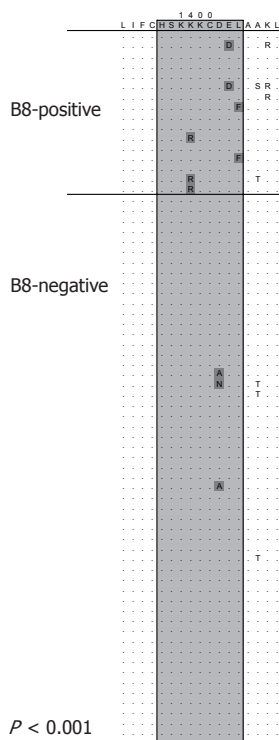


Figure 2 HLA class I-associated sequence polymorphisms in a CD8 epitope. Viral sequences are aligned to a consensus sequence and sorted into sequences derived from HLA B8-positive and HLA B8-negative subjects. Boxed is the region of a described HLA B8-restricted CD8 epitope. Differences from the consensus sequence are significantly more frequent in HLA-B8 positive subjects ($P < 0.001$). This association was reported in [68].

described CD8 epitopes in subjects expressing the restricting HLA-allele^[65]. The observation of widespread escape in individuals during acute HCV infection prompted efforts to determine whether adaptation to HLA class I-restricted selection pressure also occurs at the population level. Moore *et al* analyzed sequences spanning the reverse transcriptase protein of HIV-1 in a large HLA-diverse cohort^[66]. This study revealed accumulation of viral sequence polymorphisms at different sites of the protein in patients sharing the same HLA-allele. Many of these sequence polymorphisms were located inside previously described CD8 epitopes that are restricted by the associated HLA-class I allele. This study demonstrated that MHC class I-associated selection pressure has a major impact on the evolution of HIV-1. A similar analysis was done by the same group in a cohort chronically infected with HCV^[67]. Here, sequences spanning parts of the NS3 protein were analyzed and again a number of associations between sequence polymorphisms and particular HLA-alleles were identified. This analysis was extended to all non-structural proteins in a cohort of 70 subjects with chronic HCV genotype 1a infection with similar results^[68]. An example of an HLA class I-associated sequence polymorphism is illustrated in Figure 2. Viral sequences from all 70 subjects are aligned to a majority consensus sequence and sorted into sequences derived from HLA B8-positive and HLA B8-negative subjects. Boxed is the region of a described HLA B8-restricted CD8 epitope. Differences from the consensus sequence are significantly more frequent in HLA-B8 positive subjects compared to HLA B8-negative subjects indicating that there is reproducible selection pressure on this region in HLA B8-positive subjects. This study included a phylogenetic analysis approach for the detection of HLA-associated sequence polymorphisms highlighting the potential for

false positive detection of such associations by pure statistical approaches. However, these studies suggest that the same evolutionary forces act on HCV and HIV-1 and that selection pressure by virus specific CD8 T cells is an important driver of viral evolution.

CD4 T cells

Spontaneous resolution of viremia after acute infection with HCV has been associated with the emergence of a broad and functionally intact T cell response. There is convincing evidence that HCV specific CD4 T cells are important for viral control in the early phase. However, little is known about mutational escape in targeted CD4 T cell epitopes during HCV infection. In theory, a similar selection process as observed for CD8 T cells could be present. A quasispecies that is mutated inside an immunodominant CD4 epitope may have a selection advantage in an individual with acute HCV infection and could out compete others. Eckels *et al* found evidence for selection of mutations in CD4 epitopes in NS3^[69,70]. Analysis of a large number of clonal sequences revealed a high degree of polymorphisms in regions targeted by CD4 T cells and the ratio of synonymous versus non-synonymous mutations was consistent with positive selection. However, none of the observed variants became the dominant sequence at a second time point 16 mo later. More recently, two studies have described mutational escape from CD4 responses. The first report is part of a vaccination study in the chimpanzee model^[71]. One animal was vaccinated with DNA followed by recombinant vaccinia virus in a prime/boost strategy with HCV NS3 and NS5A/B and subsequently infected with a genotype 1a isolate. After primary infection and transient control of viremia the animal developed chronic infection. Longitudinal sequence analysis of the NS3 and NS5A/B region revealed two non-synonymous mutations. Both of them were located inside regions targeted by CD4 T cells. Additional experiments with synthetic peptides showed that the mutated sequence was not recognized by specific T cells from this animal. A second study focusing on the evolution of the envelope protein in one subject similarly identified amino acid substitutions inside targeted CD4 epitopes consistent with escape^[57]. These findings suggest that CD4 T cells may select mutations during HCV infection; however, the overall extent of CD4 escape as a contributor to the evolution of HCV remains to be clarified.

Innate immunity

Infection with HCV initiates a cascade of events within the infected cell with the goal to generate an antiviral state. The first line of defence builds the innate immune system that is triggered by engagement of pathogen-associated molecular patterns (PAMPs) to specific PAMP receptors^[72]. In case of HCV toll-like receptor 3 (TLR3) and retinoic-acid-inducible gene I (RIG-I)-receptor recognize dsRNA resulting in activation of multiple cellular factors that mediate transcription and secretion of interferon alpha and beta. Engagement of these type I interferons with their cellular receptor activates a series of interferon stimulated genes (ISG) with the goal to initiate an antiviral state with-

in the infected cell. The hepatitis C virus has developed strategies to evade this first line of host immune defence. Recently it was demonstrated that the NS3/4A protease is able to specifically cleave Cardif (CARD adaptor inducing interferon beta) and TRIF (Toll-interleukin-1 receptor domain-containing adaptor-inducing beta interferon)^[73-75]. Both proteins are involved in the activation of interferon regulatory factors (IRFs) and their inactivation, therefore, interferes with the production of interferon alpha and beta. Gale *et al.*^[76] demonstrated *in vitro* that NS5A is able to inhibit protein kinase R (PKR). PKR is activated by interferon alpha and involved in the inhibition of viral RNA translation. Its inhibition, therefore, represents a functional antagonism to the interferon alpha response. Interactions between virus and cellular host factors are sequence specific and negative selection of mutations undermining these immune evasion strategies seems reasonable. However, the underlying mechanisms of the innate immune response are highly conserved in all humans and less dependent on the genetic background of the individual. Therefore, the direction of selection pressure does not change upon transmission to the next host, which makes the observation of positive selection unlikely. Moreover, the interactions between virus and the host's innate immune response take place during the earliest phase of infection making it even more difficult to directly show that viral escape variants are selected.

Drugs

The current standard treatment regimen for patients with chronic hepatitis C is a combination of pegylated interferon alpha with ribavirin^[2,3]. Interestingly, the response rate to this treatment regimen is dependent on the infecting genotype suggesting that sequence differences between genotypes influence the susceptibility to these drugs. Patients infected with genotype 2 and 3 usually show a much faster decline in viral load after initiation of therapy associated with higher sustained response rates. The determinants of this differential responsiveness of different genotypes are poorly understood. Interferon alpha predominantly modulates the immune system^[77]. Engagement of its specific receptor turns on a cascade of IFN-stimulated genes (ISGs) resulting in a non-pathogen specific antiviral state. Different HCV sequences seem to have different capabilities to interfere with this anti-viral strategy. The response rates to treatment dramatically differ not only between different genotypes, but also between isolates of the same subtype. Comparison of sequence isolates that have been successfully treated with isolates that did not respond has put a 40 amino acid stretch of the HCV NS5A protein into the spotlight^[78]. The degree of sequence variation in this region has been associated with treatment outcome and has therefore been designated as the interferon-sensitivity determining region (ISDR). Subsequently, conflicting results have been published in similar studies; however, a meta-analysis supported the impact of this region on treatment outcome^[79]. A correlate of this observation may be the reported inhibitory action of HCV NS5A on PKR^[76]. For this interaction the ISDR and an additional 26 C-terminal amino acid stretch of NS5A are crucial. Therefore, selection of viral variants

during treatment that successfully enhance this interaction seems reasonable. However, neither was selection of mutations observed in the presence of this antiviral drug *in vitro*^[80] nor has selection of variants during treatment in longitudinal studies formally been shown. It remains, therefore, unclear how interferon alpha contributes to evolution.

The exact antiviral mechanisms of ribavirin are even less well established. Several mechanisms have been suggested including inhibition of the HCV polymerase and early chain termination during the replication process. Higher mutation rates in the presence of ribavirin have been reported potentially resulting in an 'error catastrophe'. Two recent studies analyzed the mutation rate in the presence and absence of ribavirin in patients receiving treatment. Hofmann *et al.* analyzed the NS3 and NS5B gene in 14 subjects receiving either ribavirin monotherapy or in combination with interferon alpha^[81]. Based on a comparison of clonal sequences in the quasispecies population before and after initiation of therapy they concluded that the mutation rate of HCV is higher in the presence of ribavirin. These results were reproducible in cell culture with HCV replicon bearing hepatoma cell lines. Even though the overall effect was weak, a dose dependency could be demonstrated and the inactive L-enantiomer did not show the same effect. In a similar analysis by Lutchman *et al.* the mutation rate for NS5B was calculated based on analysis of bulk and clonal sequences of the NS5B gene in 18 subjects receiving ribavirin and 13 subjects receiving placebo^[82]. A significant increase of the mutation rate in the presence of ribavirin was observed after 4 wk of treatment; however, there was no significant difference between the mutation rates after 24 wk compared to the placebo group. The authors of this latter study conclude that ribavirin unlikely acts through an increase of the mutational error rate resulting in an error catastrophe. One study demonstrated selection of a Phe to Tyr mutation in position 415 of the HCV NS5B protein in the presence of ribavirin^[83]. This mutation was associated with a less susceptible phenotype for this drug when tested in the replicon model *in vitro*. This mutation was also observed in 5 out of 16 subjects infected with genotype 1a in the study by Lutchman *et al.* However, it was not reproducible in HCV genotype 1b. It is, therefore, still unclear if specific mutations are selected in the presence of ribavirin.

Future treatment strategies will include small molecules as inhibitors of virus specific protein functions. Drugs like protease and polymerase inhibitors are very successful for the treatment of HIV. However, selection of variants that are resistant to these antiviral compounds is a major challenge in the management of patients infected with HIV. In recent years many compounds have been tested as inhibitors of the HCV protease and polymerase. Some are now available in early clinical trials. The first compound that was tested in humans was the protease inhibitor BILN 2061. In patients infected with genotype 1 the viral load was dramatically decreased after only 2 d of treatment^[84]. However, the drug was designed to inhibit the HCV protease from a genotype 1 isolate with high affinity. As expected, the efficacy was, therefore, much

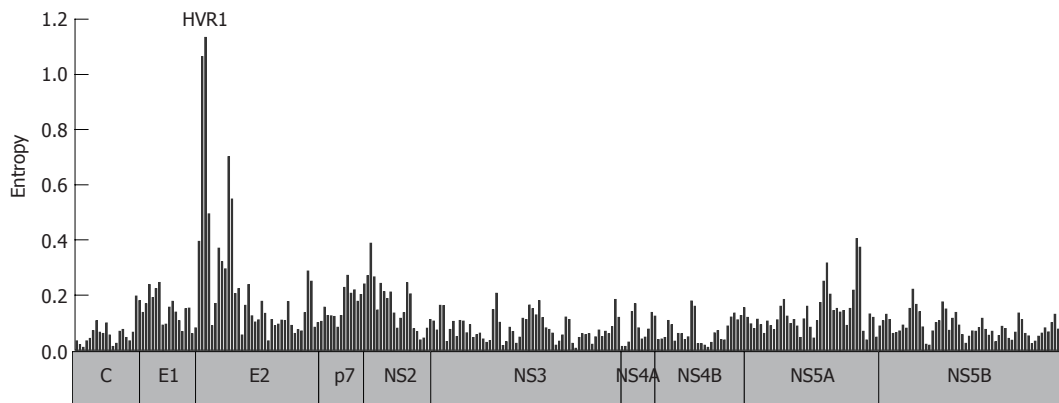


Figure 3 Entropy across the HCV polyprotein. 100 HCV genotype 1b sequences were retrieved from the Los Alamos National Laboratory (LANL) HCV Sequence Database. The entropy score was calculated for 300 windows of 20 residues overlapping by 10 residues utilizing the algorithm implemented in the database.

lower in subjects infected with HCV genotype 2 or 3^[85]. Due to observed toxic effects of this drug in high dosage in animal models further clinical trials were stopped. Other small compounds have meanwhile reached early phases of clinical testing (reviewed in^[86]) including protease inhibitors (VX-950 and SCH 503034) and polymerase inhibitors (NM283 and HCV-796). Along with their proof of excellent efficacy *in vitro* and *in vivo* several reports have been published describing resistance mutations^[87,88]. A recent study by Sarrazin *et al* analyzed clonal sequences from subjects treated with the protease inhibitor VX-950 in a clinical trial^[89]. In this analysis mutations associated with phenotypic resistance were rapidly selected during treatment and the number of resistant clones in each patient correlated well with the virologic response to the drug. The mutations were reproducibly located in only a few positions in the HCV protease gene. Interestingly, the number of resistant clones decreased after cessation of therapy indicating that some mutations are associated with fitness costs and revert back to wild type in the absence of the drug. Future studies will show if combinations of different drugs such as polymerase and protease inhibitors are beneficial to decrease the risk of resistance mutations similar to HIV.

Constraints on sequence diversity-purifying selection

Analysis of available HCV full genome sequences from public databases shows that the degree of sequence variation varies both between different proteins, but also between regions of the same protein (Figure 3). Some regions are highly conserved even across different HCV genotypes. Many of these highly conserved regions represent functionally important motifs in the viral protein in which substantial sequence variation is not tolerated. Viral evolution is clearly limited by structural constraints forcing the virus into a state in which it is able to functionally exist. Many mutations that occur during the replication process are deleterious or disadvantageous to the fitness of the virus and are, therefore, negatively selected. In contrast, as highlighted in this review multiple forces of the immune system or in some cases drugs exact positive selection pressure away from the consensus sequence in the individual. Selection of variants is, therefore, a trade-off between host pressure and functional needs. Purifying selection describes the driving force towards a sequence with optimal replication capacity

in the absence of outside pressure on the virus. Reversion of resistance mutations that have been selected in the presence of antiviral drugs back to the consensus sequence have been first described in the influenza model^[90]. For HIV, reversion of drug resistance mutations is well documented and the concept of a salvage therapy was based on this observation. In this concept treatment is re-initiated after interruption and genotypic reversion of drug resistance. However, the clinical benefit is controversial and with a wide range of antiviral drugs now available for the treatment of HIV this strategy received less attention.

In HCV reversion was first described for an escape mutation that has been selected by virus-specific CTLs^[64]. In this study a virus harbouring an escape mutation in an HLA-B8 restricted epitope in NS3 was transmitted to a host who is HLA-B8 negative and who, therefore, was not able to mount the same T cell response. In the new host the virus continued to evolve back to the prototype sequence and the variant disappeared. Similarly, Ray *et al* analyzed viral sequences from a single source outbreak years after the transmission event^[65]. Interestingly, the source of the virus again had an escape mutation in the same HLA-B8 restricted epitope in NS3. Years later the mutation has reverted back to the prototype sequence in most recipients. Of note, the mutation was stable in subjects who are HLA-B8 positive and who are theoretically able to target this region. Similar to the reversion of CTL escape mutations Sarrazin *et al*^[89] describe in their study on drug resistance to the protease inhibitor VX-960 reversion back to the wild type sequence after treatment was discontinued. These studies illustrate the main selecting forces of HCV evolution. On one side, there is positive selection pressure mainly by the immune system but also in the presence of antiviral drugs. These selection forces are not constant and vary in different hosts and different environments largely depending on the host's genetic background. On the other side, there is negative selection pressure, which presses the virus into a state of optimal replication capacity. This force is more or less constant, but largely depends on the pre-existing sequence configuration such as the genotype or presence of compensatory mutations.

CONCLUSION

The inherent sequence diversity of HCV represents a

major challenge for any treatment. HCV has the ability to rapidly adapt to a hostile environment in the acutely infected individual with an activated immune system at work. An important feature of any therapeutic or prophylactic intervention will be the efficient attack of a structurally or functionally important region in the viral protein. Ideally the attack is targeted against a region that does not tolerate substantial sequence variation in order to limit the ability to evade. Alternatively, a combination of different attacks at different sites may decrease the risk of efficient mutational escape. Recent studies in HIV have suggested that selection of escape mutations may actually be linked to control of viral replication^[91]. Indeed, many of the described CTL escape mutations particularly in the presence of protective HLA-alleles such as HLA-B27 and -B57 as well as some drug resistance mutations in HIV are associated with dramatic fitness costs for the virus. For HCV, little is known about the impact of escape and drug resistance mutations on replication capacity even though their documented reversion in the absence of the drug or immune pressure suggests similar fitness costs. With the recent development of culture systems that include the complete replication cycle we now have the tools at hand to address important questions on the interaction between different selecting forces acting on HCV. The understanding of this interaction is fundamental for the design of novel therapies.

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TOPIC HIGHLIGHT

Robert Thimme, MD, Professor, Series Editor

Interaction of hepatitis C virus with the type I interferon system

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Abstract

Hepatitis C virus (HCV) needs to tightly manipulate host defences in order to establish infection. The innate immune response slows down viral replication by activating cytokines such as the type I interferons (IFN- α / β), which trigger the synthesis of antiviral proteins and modulate the adaptive immune system. HCV has therefore developed a number of countermeasures to stay ahead of the IFN system. Here, I will attempt to summarize the current state of research regarding IFN responses against HCV and the viral escape strategies. Particular emphasis will be put on the newly discovered mechanisms HCV employs to avoid the induction of IFN in infected cells.

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Key words: Hepatitis C virus; Innate immunity; Interferon system; Escape mechanisms

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INTRODUCTION

The type I interferon system which mainly involves IFN- α and - β is a powerful and universal intracellular defence system against viruses. Knockout mice which are unresponsive to IFN- α / β due to targeted deletions in the type I IFN receptor quickly succumb to viral infections although they have a regular adaptive immune system^[1,2].

Likewise, humans with genetic defects in STAT-1, which is involved in the signaling cascade of the IFN system, die of viral disease at an early age^[3].

INTERFERON INDUCTION

All nucleated cells of the mammalian body are able to synthesize and secrete type I IFNs in response to virus infection. Secreted IFNs are then recognized by neighboring cells and cause them to express potent antiviral proteins^[4,5]. As a result, virus multiplication is slowed down or even stopped, and the organism buys time for the establishment of an adaptive immune response.

Type I IFNs are classified according to their amino acid sequence and comprise a large number (at least 13) of IFN- α subtypes and a single IFN- β ^[6], as well as some additional family members^[7,8]. Expression patterns, i.e. which IFNs will be synthesized at which time point, mostly depend on the particular cell type.

Fibroblasts secrete mainly IFN- β as an initial response to infection but switch to IFN- α during the subsequent amplification phase of the IFN response^[9]. By contrast, dendritic cells, which play an important role in immunosurveillance, directly secrete high levels of IFN- α subtypes^[10,11].

Induction of IFN- β gene expression in fibroblasts occurs by the intracellular, so-called "classic pathway" (Figure 1). In infected cells, a signaling chain is activated by viral RNA molecules which are generated during genome transcription and replication^[12]. Two intracellular RNA helicases, RIG-I^[13] and MDA5^[14], act as sentinels for viral RNA^[15-17]. Then, a recently discovered adaptor protein binds to RIG-I and MDA5 and mediates the signal to downstream factors. It is called either Cardif for "CARD adaptor inducing IFN- β "^[18], IPS-1 for "interferon- β -promoter stimulator 1"^[19], MAVS for "mitochondrial antiviral signaling" molecule^[20], or VISA for "virus-induced signaling adaptor"^[21]. Cardif/IPS-1/MAVS/VISA activates two I κ B kinase (IKK)-related kinases, IKK ϵ and TANK-binding kinase-1 (TBK-1), which phosphorylate the transcription factor IRF-3^[22,23]. IRF-3 is a member of the IFN regulatory factor (IRF) family and plays a central role in the activation of the IFN- β promoter^[24]. Phosphorylated IRF-3 homo-dimerizes and moves into the nucleus where it recruits the transcriptional coactivators p300 and CREB-binding protein (CBP) to

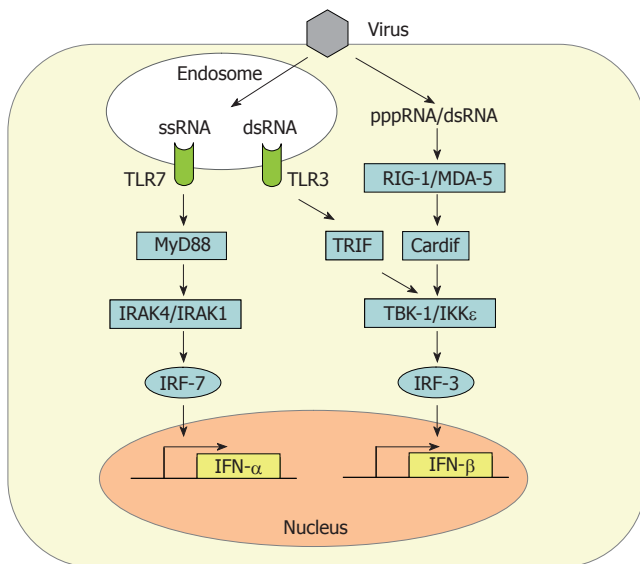


Figure 1 Type I IFN gene expression. Detection of viral ssRNA and dsRNA leads to transactivation of IFN- α and IFN- β promoters by IRF-7 and IRF-3. IRF-3 is phosphorylated by the kinases IKK ϵ and TBK-1 which in turn are activated by the intracellular RNA-sensor proteins RIG-I and MDA5. RIG-I preferentially senses 5'triphosphorylated ssRNAs (pppRNA) whereas MDA-5 recognizes dsRNA. Cardif (also termed IPS-1/MAVS/VISA) serves as an adaptor protein connecting RNA sensing and IRF-3 phosphorylation. A second dsRNA signaling pathway involves endosomal TLR-3 and the adaptor protein TRIF which also activates IKK ϵ and TBK-1. The endosomal ssRNA receptor TLR7 utilizes the adaptor protein MyD88 to stimulate IFN- α synthesis via the kinases IRAK4 and IRAK1 and the transcription factor IRF-7.

initiate IFN- β mRNA synthesis^[24,25]. This first-wave IFN triggers expression of a related factor, IRF-7, which in fibroblasts is only present in low amounts^[26]. IRF-7 can be activated the same way as IRF-3^[27-29], leading to a positive-feedback loop that initiates the synthesis of several IFN- α subtypes as the second-wave IFNs^[9,30]. In addition, NF- κ B and AP-1 are recruited in a dsRNA-dependent way^[31,32]. Together these transcription factors strongly upregulate IFN- β gene expression.

Until very recently, it was assumed that the main trigger of intracellular cytokine induction by all viruses is double-stranded RNA (dsRNA) which supposedly forms as a by-product of genome replication. However, we have recently found that some viruses do not produce substantial amounts of dsRNA^[33]. Instead, ssRNA containing a 5' triphosphate group is much more potent than dsRNA in activating RIG-I-dependent IFN induction^[34-36].

Among the cells of the lymphatic system, myeloid dendritic cells (mDCs)^[11] and, most prominently, plasmacytoid dendritic cells (pDCs)^[10] are the main IFN producers. In addition to the classical, intracellular pathway of IFN induction described above, pDCs sense the presence of viruses by the extracytoplasmic toll-like receptors (TLRs)^[37-39]. It is thought that TLRs serve as sensors for viral infection of phagocytosed cells^[40]. Human pDCs mostly express TLR7 and TLR9 which recognize viral single-stranded(ss) RNA and dsDNA, respectively^[41], whereas mDCs express TLR3 which responds to dsRNA^[42]. Upon activation, TLRs signal through different intracellular adaptor molecules such as

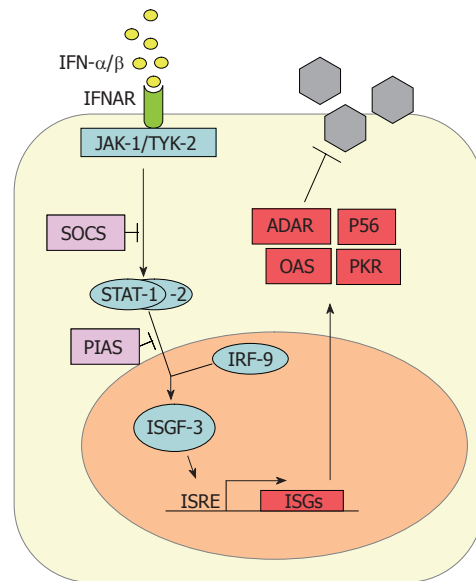


Figure 2 Cellular response to IFNs. Newly synthesized IFN- α/β binds to its cognate receptor (IFNAR) and activates the expression of numerous IFN-stimulated genes (ISGs) via the JAK/STAT pathway. ADAR, P56, OAS and PKR are IFN-stimulated gene products with antiviral properties against HCV. The SOCS and PIAS proteins negatively regulate the IFN-induced signaling pathway at different stages.

MyD88 (TLR7 and 9) or TRIF (TLR3) to induce IFN transcription^[41]. Interestingly, DCs already contain high levels of IRF-7^[43,44], thus explaining their ability to rapidly produce high amounts of alpha-IFNs. Furthermore, TLR7 and TLR9 are retained in the endosomes of pDCs to allow prolonged IFN induction signaling^[45].

INTERFERON SIGNALING

IFN- α/β subtypes all bind to and activate a common type I IFN receptor. It consists of two subunits (IFNAR-1 and IFNAR-2) and is present on virtually all host cells^[5,6]. Binding of IFN- α/β leads to heterodimerization of the IFNAR subunits and to conformational changes in the intracellular parts of the receptor which activate the so-called JAK-STAT signaling pathway (Figure 2). The signal transducer and activator of transcription (STAT) proteins are latent cytoplasmic transcription factors which become phosphorylated by the Janus kinase (JAK) family members JAK-1 and TYK-2^[46]. Phosphorylated STAT-1 and STAT-2 recruit a third factor, IRF-9 (also called p48), to form a complex known as IFN stimulated gene factor 3 (ISGF-3). The ISGF-3 heterotrimer translocates to the nucleus and binds to IFN-stimulated response elements (ISRE) in the promoter regions of IFN-stimulated genes (ISGs), thereby inducing their transcription.

Several specialized proteins serve as negative regulators and inhibitors of the JAK-STAT pathway. For example, the suppressor of cytokine signaling (SOCS) proteins specifically prevent STAT activation by binding to activated cytokine receptors, inhibiting the activity of JAKs, and targeting bound signaling proteins for proteasomal degradation^[47]. Also, the protein inhibitor of activated STAT (PIAS) family members function as small

ubiquitin-like modifier (SUMO) E3 ligases and inhibit the transcriptional activity of STATs^[48].

INTERFERON EFFECTOR PROTEINS WITH ANTIVIRAL ACTIVITY AGAINST HCV

IFN- α combined with ribavirin is the standard treatment for HCV infection, and its effect can be potentiated by co-administration of IFN- γ ^[49,50]. IFN- α/β activates the expression of more than 300 IFN-stimulated genes (ISGs) which have antiviral, antiproliferative, and immunomodulatory functions^[51,52]. IFN-induced proteins include enzymes, transcription factors, cell surface glycoproteins, cytokines, chemokines and a large number of factors that need to be further characterized. Up to now, only a few antiviral proteins have been characterized in detail. Type I IFNs are known to be effective against HCV replicon systems^[53,54], and several IFN-induced proteins have documented anti-HCV activity, namely protein kinase R (PKR)^[55], the RNA-specific adenosine deaminase 1 (ADAR 1)^[56], the 2'-5' oligoadenylate synthetases (2-5 OAS) / RNaseL system^[57], and P56^[58].

PKR, ADAR1, and 2-5 OAS are constitutively expressed in normal cells in a latent, inactive form. Basal mRNA levels are upregulated by IFN- α/β and these enzymes need to be activated by viral dsRNA. PKR is a serine-threonine kinase that phosphorylates the α subunit of the eukaryotic translation initiation factor eIF2^[59]. As a consequence, translation of cellular and viral mRNAs is blocked. ADAR 1 catalyzes the deamination of adenosine on target dsRNAs to yield inosine. As a result the secondary structure is destabilized due to a change from an AU base pair to the less stable IU base pair and mutations accumulate within the viral genome^[5]. The 2-5 OAS catalyzes the synthesis of short 2'-5' oligoadenylates that activate the latent endoribonuclease RNaseL^[60]. RNaseL, in turn, then degrades both viral and cellular RNAs, leading to viral inhibition^[61]. P56 binds the eukaryotic initiation factor 3e (eIF3e) subunit of the eukaryotic translation initiation factor eIF3. It functions as an inhibitor of translation initiation at the level of eIF3 ternary complex formation and is likely to suppress viral RNA translation^[62,63].

INTERACTION WITH INNATE IMMUNE RESPONSES

Several recent studies have clarified that the RNA of HCV is a potent trigger of IFN induction, leading to the establishment of an antiviral state. Therefore, in order to establish infection and to persist in the human host, HCV has been forced to evolve efficient counterstrategies. Intracellular IFN induction by HCV appears to be mostly mediated by RIG-I binding to viral RNA^[64]. Extracellularly, no specific TLR has been identified yet, but by deduction from data on related flaviviruses, TLR3 and TLR7 would be the most obvious candidates. The dsRNA-binding TLR3 was shown to be activated by West Nile virus^[65], and the ssRNA-binding TLR7 is activated by Dengue virus^[66]. Moreover, TLR7 can elicit HCV immunity, and a synthetic

TLR7 agonist reduced HCV mRNA and protein levels in HuH-7 hepatocytes^[67]. It is important to note that TLR7 is expressed in hepatocytes of normal as well as HCV-infected people^[67]. Thus, TLR7 may indeed play a role during natural infection.

On the other hand, HCV is capable of disturbing the IFN response at multiple levels^[68,69]. With respect to IFN induction, it was recently discovered that the NS3/4A protease specifically cleaves Cardif^[418] as well as TRIF^[70,71]. Since both these adaptor proteins are important for IFN induction via the classical intracellular pathway (Cardif) and the TLR3-driven endosomal pathway (TRIF), NS3/4A is the key factor of HCV to disturb IRF-3 activation^[72] which would otherwise result in IFN gene transcription. In addition, NS3 directly interacts with TBK1 to inhibit its association with IRF-3 and its activation^[73].

With respect to the IFN response, it was shown that expression of the full-length virus genome or the core protein suppresses IFN signal transduction^[74,75]. Most likely, this is due to an up-regulation of protein phosphatase 2A by ER stress^[76], resulting in association of STAT1 with its inhibitor PIAS1^[77]. Moreover, for the core protein it was shown that it interferes with the JAK/STAT pathway^[78] and is able to activate the JAK-STAT signaling inhibitor SOCS-3^[79], further contributing to the HCV-induced block of IFN signaling.

HCV also directly counteracts the antiviral IFN response. The NS5A protein, which confers a multitude of functions in virus replication^[80], also plays a key role in escape from the antiviral action of IFN. A stretch of 40 amino acids on NS5A, termed the IFN sensitivity region (ISDR), was correlated with responsiveness to IFN therapy^[81-83]. Moreover, NS5A was shown to directly bind to and repress PKR, and this interaction involved the ISDR^[84]. However, other groups did not find a connection between viral IFN susceptibility and a particular ISDR sequence^[85-87], and PKR activity was not affected by expression of the HCV genome^[88] or NS5A^[89], although NS5A clearly reduced the antiviral effects of IFN^[89]. A possible solution for this discrepancy could be that ISDR sequence variations affect the efficiency of HCV replication^[90,91]. Thus, the correlation between particular ISDR sequences and IFN sensitivity could be caused by differences in HCV replication strength. In addition, NS5A induces IL-8 (also termed CXCL-8), a chemokine which inhibits the antiviral actions of IFN^[92]. Elevated IL-8 levels were indeed detected in the sera of IFN non-responders^[93]. Moreover, in cell culture CXCL-8 protein levels are positively associated with chronic HCV replication and CXCL-8 removal inhibits HCV replication^[94]. Interestingly, CXCL-8 cannot only be induced by NS5A, but also by the HCV RNA-sensitive RIG-I pathway^[95].

NS5A also interferes with the 2-5 OAS/RNaseL pathway by binding to 2-5 OAS^[96]. Furthermore, the HCV genome sequences of IFN-resistant strains have fewer RNase L recognition sites than those of more IFN-sensitive ones^[97], thus allowing escape from nucleolytic cleavage^[97]. PKR activity is also modified by the internal ribosome entry site (IRES) of HCV^[98] and the E2 protein^[99].

The multiple countermeasures of HCV to avoid a

fully-fledged IFN response appear to be quite efficient, since 85% of the HCV-infected patients develop a chronic infection, and up to 60% of those patients do not respond to IFN therapy or experience a relapse when therapy is stopped^[100]. Our rapidly increasing knowledge about HCV immune escape will certainly lead to a significant improvement in both prevention and therapy for hepatitis C.

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TOPIC HIGHLIGHT

Robert Thimme, MD, Professor, Series Editor

Neutralizing antibodies in hepatitis C virus infection

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Abstract

Hepatitis C virus (HCV) is a major cause of hepatitis world-wide. The majority of infected individuals develop chronic hepatitis which can then progress to liver cirrhosis and hepatocellular carcinoma. Spontaneous viral clearance occurs in about 20%-30% of acutely infected individuals and results in resolution of infection without sequelae. Both viral and host factors appear to play an important role for resolution of acute infection. A large body of evidence suggests that a strong, multispecific and long-lasting cellular immune response appears to be important for control of viral infection in acute hepatitis C. Due to the lack of convenient neutralization assays, the impact of neutralizing responses for control of viral infection had been less defined. In recent years, the development of robust tissue culture model systems for HCV entry and infection has finally allowed study of antibody-mediated neutralization and to gain further insights into viral targets of host neutralizing responses. In addition, detailed analysis of antibody-mediated neutralization in individual patients as well as cohorts with well defined viral isolates has enabled the study of neutralizing responses in the course of HCV infection and characterization of the impact of neutralizing antibodies

for control of viral infection. This review will summarize recent progress in the understanding of the molecular mechanisms of antibody-mediated neutralization and its impact for HCV pathogenesis.

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Key words: Hepatitis C virus; Virus-host cell interaction; Viral entry; Neutralizing antibodies

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INTRODUCTION

With an estimated 170 million infected individuals, hepatitis C virus (HCV) has a major impact on public health^[1]. HCV is a major cause of hepatitis world-wide. The majority of infected individuals develop chronic hepatitis which can then progress to liver cirrhosis and hepatocellular carcinoma. Treatment options for chronic HCV infection are limited and a vaccine to prevent HCV infection is not available.

HCV is a small enveloped positive-strand RNA virus that belongs to the genus *Hepacivirus* of the *Flaviviridae* family. This virus exhibits high genetic heterogeneity and has been classified into six genotypes and several subtypes. The HCV genome encodes a single precursor polypeptide of about 3000 amino acids that is cleaved co- and post-translationally by host and viral proteases into functional structural and non-structural proteins. The virion is composed of three different structural proteins: The core protein forming the viral nucleocapsid and two envelope glycoproteins, E1 and E2.

In vivo, HCV infects only humans and chimpanzees^[2]. Each individual is infected with a mixture of distinct but closely related HCV genomes, termed quaspecies. The liver is the primary target organ of HCV, and the hepatocyte is its primary target cell. Replication of the HCV genome has been demonstrated *in vivo* and *in vitro* in liver hepatocytes, and hematopoietic cells including dendritic cells and B lymphocytes^[3,4]. HCV establishes persistent infection in the majority of infected individuals

despite the fact that it is recognized and targeted by the host's immune system^[5].

Viral proteins are recognized as non-self by the host's immune system and induce the production of antibodies. During the natural course of infection, a large number of antibodies targeting epitopes of both structural and non-structural proteins are produced. The vast majority of antibodies induced have no antiviral activity, either because they are elicited by intracellular, degraded or incompletely processed proteins released from dying cells or because they are directed against epitopes that do not play any role in the virus entry process^[6,7]. A small proportion of antibodies termed "neutralizing antibodies" are able to target exposed epitopes of the viral structural proteins and neutralize the infectious virus by preventing or controlling viral infection. This review will summarize the current knowledge about host neutralizing responses in HCV infection. It starts with a brief description of the current model systems allowing the study of neutralizing responses, followed by viral targets of neutralizing antibodies. Finally, neutralizing responses in the course of HCV infection and the impact of neutralizing antibodies for HCV pathogenesis are discussed.

MODEL SYSTEMS FOR THE STUDY OF ANTIBODY-MEDIATED VIRUS NEUTRALIZATION

For many years, studies of host neutralizing responses against HCV had been hampered by the lack of a convenient tissue culture system for HCV entry and infection. In recent years, several *in vitro* models have been developed to study defined aspects of HCV host cell interaction and antibody-mediated virus neutralization: These include recombinant HCV envelope glycoproteins^[8,9], HCV-like particles^[10], HCV pseudotyped particles^[11-13], and, more recently, cell-culture derived infectious HCV^[14-16]. Recombinant HCV envelope glycoproteins have been successfully used as a surrogate model to study virus-host cell interaction leading to the identification of putative HCV receptor candidates including CD81^[8], scavenger receptor class B type I (SR-BI)^[9] and heparan sulfate^[17] as well as antibodies inhibiting cellular binding of envelope glycoproteins^[18]. HCV-like particles (HCV-LP) generated by self-assembly of the HCV structural proteins in insect cells have been shown to exhibit morphologic, biophysical, and antigenic properties similar to putative virions isolated from HCV-infected patients^[10]. In contrast to individually expressed envelope glycoproteins E1 and E2, E1/E2 heterodimers of HCV-LPs are presumably presented in a native, virion-like conformation. HCV-LPs have been shown to bind and enter human hepatoma cells as well as primary hepatocytes and dendritic cells in a receptor-mediated manner, therefore representing a useful model system for the study of HCV-host cell interaction including the characterization of antibodies interfering with cellular binding of particles^[17,19-25].

Retroviral HCV pseudotyped particles (HCVpp) represent a convenient and elegant approach to study

viral entry and antibody-mediated neutralization^[11,12]. Infectious HCVpp consist of unmodified HCV envelope glycoproteins E1 and E2 assembled onto retroviral or lentiviral core particles^[11,12]. HCVpp are produced by transfecting cells with expression vectors encoding the full-length E1/E2 polypeptide, retroviral or lentiviral core proteins, and a packaging-competent retro- or lentiviral genome carrying a marker gene. The presence of a green fluorescent protein or luciferase reporter gene packaged within these HCVpp allows reliable and fast determination of infectivity mediated by the envelope glycoproteins. HCVpp are infectious for certain cell lines of hepatocyte origin, principally Huh-7 cells, as well as for human primary hepatocytes^[11,12]. This system has been extremely useful in identifying neutralizing antibodies as well as characterization of the molecular mechanisms of antibody-mediated neutralization^[12,25-30]. Vesicular stomatitis viruses (VSV)/HCV pseudotypes expressing HCV E1 or E2 chimeric proteins containing transmembrane and cytoplasmic domains of the VSV G glycoprotein have been developed as another HCV pseudotype model system to study HCV entry and antibody-mediated neutralization^[13,31]. VSV/HCV pseudotypes infect human hepatoma cell lines and sera from HCV-infected chimpanzees or humans neutralize the pseudotype virus infectivity^[13,32]. In contrast to retroviral HCV pseudotypes demonstrating strong tropism for liver-derived cell lines, VSV/HCV pseudotypes are generated in relatively lower titer and can infect a broad range of mammalian cell lines, including cell lines not derived from the liver.

Most recently, several laboratories succeeded in establishing the efficient production of infectious HCV particles using a unique clone derived from a viral isolate of a Japanese patient with fulminant hepatitis C (JFH-1)^[14-16]. Successful infection of naïve Huh-7 and Huh-7-derived hepatoma cells with cell-culture derived HCV (HCVcc) was demonstrated by detection of viral proteins and a highly reproducible time-dependent increase of viral RNA in infected cells^[14-16]. Virus production in Huh-7 cells was dependent on an active viral polymerase and expression of a functional viral envelope containing the HCV envelope glycoproteins E1 and E2^[14-16]. Inoculation of naïve chimpanzees with JFH-1 or chimeric J6/JFH-1-derived HCV particles synthesized *in vitro* resulted in viral infection *in vivo* demonstrating the biological significance of this model system^[14,33]. The ability to generate infectious HCVcc of different genotypes – such as the development of chimeric HCVcc or HCVcc derived from HCV genotype prototype 1a strain H77 certainly improves the scope of the cell culture system for HCV infection^[34,35]. Infection of HCVcc has been shown to be efficiently neutralized by anti-HCV antibodies derived from human sera^[14] as well as polyclonal anti-envelope antibodies^[34].

The chimpanzee remains the only natural occurring animal model for the study of HCV infection *in vivo*. The clinical course of infection is usually milder in chimpanzees than in humans. However, these animals have provided unique opportunities to study adaptive immune responses to HCV^[36]. Using the chimpanzee model, antibodies with neutralizing properties have first been described^[37,38]. These antibodies were directed against epitopes in the envelope

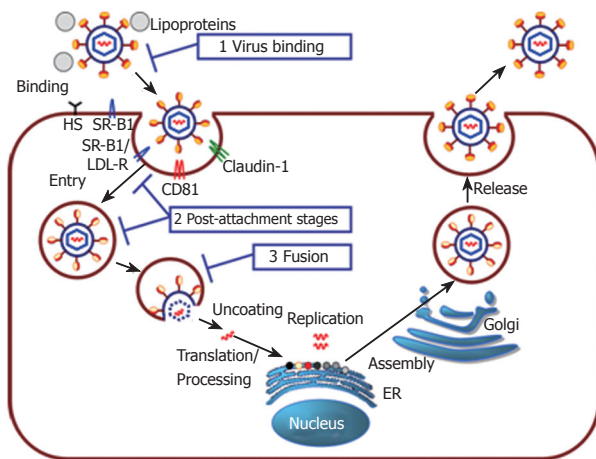


Figure 1 Potential targets for neutralizing antibodies within the HCV life cycle. A model of HCV infection with potential targets for virus neutralizing antibodies is shown. Antibodies can potentially interfere with the viral life cycle at different steps. ER: Endoplasmic reticulum; HS: Heparan sulfate; LDL-R: LDL receptor; SR-BI: Scavenger receptor class B type I.

glycoprotein E2 hypervariable region 1 (HVR-1) of HCV and appeared to be isolate-specific. The chimpanzee model has also been used to study protective immunity against re-exposure. Vaccination studies^[39] and passive immunization with rabbit anti-sera^[38] have shown some protection but infection of chimpanzees with HCV does not provide complete protective immunity against re-infection with homologous or heterologous virus^[40-44].

TARGETS OF HOST NEUTRALIZING RESPONSES

In recent years, rapid progress has been made in the understanding of the molecular mechanisms of HCV life cycle (Figure 1). Attachment of the virus to the target cell is mediated through binding of HCV envelope glycoproteins to binding factors present on the host cell surface, such as the glycosaminoglycan heparan sulfate^[17,25]. Binding and entry of HCV is believed to be a multistep process involving several attachment and entry factors, such as CD81, SR-BI and claudin-1^[45-47]. HCV is most certainly internalized in a clathrin-dependent manner and HCV genome delivery into the host cell cytosol prior to HCV replication is pH dependent^[48-51]. In analogy to other viral infections, antibodies neutralizing HCV may render virions non-infectious by interfering with different steps of the viral life cycle^[52,53]. Binding of the antibodies to the virus may directly block attachment of the virus with the host cell and thus inhibit dissemination of infection. Neutralizing antibodies may also interfere with post-binding steps such as interaction of the virus with host entry factors. If endocytosis is an obligate replicative step, internalization of the virus into the host cell by endocytosis may also cause neutralization. Neutralization of viruses by antibodies may also take place during fusion at the cell surface or in endosomes: Neutralizing antibodies may directly interfere with the fusogenic protein, hinder conformational changes necessary for the fusion process or simply obstruct contact between cellular

Table 1 HCV epitopes targeted by antibodies interfering with cellular HCV envelope glycoprotein binding, viral entry or infection

Envelope glycoprotein	Epitope (Amino acids)	Potential function	Model system
E1	192-226	Membrane fusion	HCV-LP binding ^[85]
	197-207		HCV-LP binding ^[24]
	270-284		HCVpp entry ^[63]
	313-332		HCV-LP binding ^[85]
E2	HVR-1	SR-BI/heparan sulfate binding	Chimpanzee ^[38]
	HVR-1		E2 binding, HCVpp entry ^[18,27]
	396-407		HCVpp entry ^[12]
	408-422		HCV-LP binding ^[22]
	412-419	Membrane fusion	HCVpp entry ^[55]
	412-423		HCVpp entry, HCVcc infection ^[12,57]
	416-430		HCVpp entry ^[63]
	432-443		HCVpp entry ^[12]
	436-447		HCVpp entry ^[12]
	474-494	CD81 binding	E2 binding, HCVpp entry ^[56,86]
	522-551	CD81 binding	E2 binding, HCVpp entry ^[56,86]
	600-620	Membrane fusion	HCVpp entry ^[63]
	640-653		HCV-LP binding ^[24]
	644-655		HCVpp entry ^[12]
	CD		E2 binding, HCVpp entry ^[58-61]

Envelope glycoprotein epitope (position amino acid within the HCV polypeptide) and its potential function with the viral entry process is shown. The experimental system for antibody-mediated inhibition of viral binding, entry or infection is listed together with the respective reference. CD: Conformation-dependent; E1 and E2: Envelope glycoproteins E1 and E2; HCV-LP: HCV-like particles; HVR-1: Hypervariable region-1; HCVpp: HCV pseudotyped particles; HCVcc: Cell-culture derived HCV; SR-BI: Scavenger receptor class B type I.

and viral membranes. In addition, neutralizing antibodies may also interfere with viral uncoating or the first steps necessary for viral replication (Figure 1). The identification and characterization of antibodies targeting distinct steps of viral entry is thus an important strategy for the understanding of the molecular mechanisms of antibody-mediated neutralization.

Using the above described model systems, it could be demonstrated that envelope glycoproteins E1 and E2 are critical for host cell entry and thus represent important targets for virus neutralization. Monoclonal or polyclonal antibodies targeting both linear and conformational epitopes of envelope glycoprotein E2 have been shown to inhibit cellular binding of HCV-LP binding, entry of HCVpp and infection of HCVcc (Table 1)^[10-12,14-16,19,20]. Several viral epitopes targeted by neutralizing antibodies have already been identified: epitopes of the E2 HVR-1 region (aa 384-410)^[12,18,27], two epitopes adjacent to the N-terminal HVR-1 region (aa 408-422 and aa 412-419)^[22,54,55], the E2 CD81 binding region (aa 474-494 and aa 522-551)^[12,54,56,57] and conformational epitopes within glycoprotein E2^[58-61]. These epitopes may represent potential candidate targets for antibodies in passive immunoprophylaxis. Indeed, two studies have demonstrated that monoclonal antibodies directed against

conformational epitopes^[60] or epitope aa 412-423 exhibited broad cross-neutralizing activity among all major genotypes of HCVpp entry^[54] as well as HCVcc infectivity^[62]. Most recently, at least three epitopes (aa 270-284, 416-430, 600-620) playing a role in membrane fusion processes have been identified in the envelope glycoproteins E1 and E2^[63]. Since one epitope (aa 416-430) has been shown to represent a target for monoclonal antibodies efficiently neutralizing HCV infection^[54], it is conceivable that membrane fusion may represent another target for anti-HCV antibodies with neutralizing properties.

IMPACT OF VIRUS NEUTRALIZING ANTIBODIES FOR PATHOGENESIS OF INFECTION

HCV RNA is detectable already one week following infection. Despite the rapid onset of viral replication, there is a delay in the appearance of HCV-specific T-lymphocytes and HCV-specific antibodies which only appear several weeks after infection. Patients who spontaneously clear HCV infection have been described to mount a vigorous multi-epitope-specific CD4 and CD8 T-cell response^[64,65]. Antibody-mediated neutralization occurs during HCV infection *in vivo* but the role of antibodies for the control of HCV infection has been difficult to study. Antibody-mediated neutralization has been suggested by study of patients undergoing liver transplantation for HCV- and hepatitis B virus (HBV)-related liver cirrhosis. Infusion of anti-HBs hyperimmune globulin containing anti-HCV appeared to reduce HCV infection in the transplanted liver^[66]. In addition, HCV-infected patients with primary antibody deficiencies have been reported to have accelerated rates of disease progression^[67,68]. Moreover, passive protection against HCV has been demonstrated in a cohort of patients that had been administered immunoglobulin preparations derived from HCV RNA-positive plasma but containing HCV-neutralizing antibodies^[69]. However, in the majority of patients, HCV infection is established despite the induction of an humoral immune response that targets various epitopes of the HCV envelope glycoproteins^[22,26,28,70,71].

Until recently, functional studies analyzing the neutralizing antibody response during acute and chronic HCV infection using HCV model systems demonstrated a lack of neutralizing antibodies in the majority of patients with acute HCV infection^[22,26,70,72]. These studies were limited by the fact that the viral surrogate ligand was derived from a different isolate than the virus present in the infected patient thus precluding the detection of isolate-specific antibodies. Most recently, studies using well defined nosocomial or single-source HCV outbreaks with a defined inoculum enabled to study the role of isolate-specific neutralizing antibodies for control of HCV infection in humans. Using the HCVpp model system, two studies have demonstrated that neutralizing antibodies are induced in the early phase of infection by patients who subsequently clear the virus^[29] or control viral infection^[73]. In a well characterized single-source outbreak of hepatitis C, viral clearance was associated with

a rapid induction of neutralizing antibodies in the early phase of infection. In contrast, chronic HCV infection was characterized by absent or low-titer neutralizing antibodies in the early phase of infection^[29]. In addition, patients with resolution of infection were shown to exhibit a broader cross-neutralizing activity of antibodies in the early phase of infection. An impaired ability to cross-neutralize viral variants rapidly emerging during acute infection may thus contribute to viral evasion from neutralizing responses in persistent HCV infection^[29]. These results suggest that a strong early broad neutralizing antibody response may contribute to control of HCV in the acute phase of infection and assist cellular immune responses in viral clearance. This conclusion is further supported by recent findings for HIV demonstrating that neutralizing antibodies act in concert with antiviral cellular responses for control of HIV infection^[74-76]. Furthermore, experimental data obtained in animal models have demonstrated that immune control of poorly cytopathic viruses, such as lymphocytic choriomeningitis virus (LCMV) or simian immunodeficiency virus requires a collaboration of both the cellular and humoral arms of the immune system^[77]. Indeed, gene-targeted mice expressing the immunoglobulin heavy chain of virus-neutralizing antibodies exhibit an accelerated LCMV clearance^[75]. Applying these findings to HCV infection- another prototype of persistent-prone non-cytopathic viruses- it is conceivable, that both cellular^[5,64,65,78,79] and neutralizing responses^[29,73] may contribute to control of HCV infection during the very early phase of viral infection.

Patients who do not clear the virus develop high-titer and even cross-neutralizing antibodies during the chronic phase of infection^[14,22,26,29,70,80]. Paradoxically, these antibodies are not able to control HCV infection. Viral escape from antibody-mediated neutralization in these patients may occur on several levels: (1) HCV exists as a quasispecies with distinct viral variants in infected individuals changing constantly over time and his variability has been shown to represent a mechanism of escape from antibody-mediated neutralization in the chimpanzee model^[26]; (2) the interplay of HCV glycoproteins with high-density lipoprotein and SR-BI has been shown to mediate protection from neutralizing antibodies present in sera of acute and chronic HCV-infected patients^[27,74]; (3) as shown for other viruses such as human immunodeficiency virus (HIV), escape from neutralizing antibodies may occur through a combination of different mechanisms, for instance point mutations, insertions/deletions or changes in glycosylation patterns of the viral envelope^[30,81] or conformational masking of receptor binding sites following envelope-antibody interaction^[82] preventing neutralizing antibody binding^[83].

Most recently, it has been shown for a chronic HCV patient who has been meticulously followed-up for 30 years that HCV continuously escapes the host's immune system by repeated mutational changes resulting in loss of recognition of the HCV envelope glycoproteins by antibodies^[80]. In fact, neutralization of heterologous strains does not reflect neutralization of the viral variants present in the patient's serum at the time of sampling^[80]. These data suggest that the neutralizing antibody response of

the host lags behind the rapidly evolving HCV envelope glycoprotein sequences of the quasispecies population. The fact that envelope glycoprotein sequences and neutralizing antibody specificity change over time suggest that neutralizing antibodies exert selective pressure on HCV evolution. In line with this hypothesis, it has been shown that HCV quasispecies complexity is associated with the inability to clear HCV infection and development of chronic disease^[84].

CONCLUSION

The development of robust tissue culture model systems for HCV infection within recent years has finally allowed for the study of antibody-mediated neutralization in HCV infection. Rapid progress has since then been made in determining the kinetic and targets of host neutralizing responses in the course of HCV infection. The novel model systems and patient cohorts with well defined viral isolates will now allow the identification of the molecular mechanisms of antibody-mediated neutralization as well as mechanisms of viral escape from host neutralizing responses. The elucidation of these mechanisms will be crucial for the understanding of HCV pathogenesis as well as the development of novel preventive and therapeutic strategies for control of HCV infection.

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CD4+ T cell responses in hepatitis C virus infection

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Abstract

Hepatitis C virus (HCV) infection is a major cause of liver damage, with virus-induced end-stage disease such as liver cirrhosis and hepatocellular carcinoma resulting in a high rate of morbidity and mortality worldwide. Evidence that CD4+ T cell responses to HCV play an important role in the outcome of acute infection has been shown in several studies. However, the mechanisms behind viral persistence and the failure of CD4+ T cell responses to contain virus are poorly understood. During chronic HCV infection, HCV-specific CD4+ T cell responses are relatively weak or absent whereas in resolved infection these responses are vigorous and multispecific. Persons with a T-helper type I profile, which promotes cellular effector mechanisms are thought to be more likely to experience viral clearance, but the overall role of these cells in the immunopathogenesis of chronic liver disease is not known. To define this, much more data is required on the function and specificity of virus-specific CD4+ T cells, especially in the early phases of acute disease and in the liver during chronic infection. The role and possible mechanisms of action of CD4+ T cell responses in determining the outcome of acute and chronic HCV infection will be discussed in this review.

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Key words: Hepatitis C virus; CD4 T cells; HLA class II; Immune responses; Cytokines; Interleukin 2; Proliferation; Escape; Exhaustion

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INTRODUCTION

Hepatitis C virus (HCV) infection is a major cause of liver damage, with virus-induced end-stage disease such as liver cirrhosis and hepatocellular carcinoma resulting in a high rate of morbidity and mortality worldwide. Since the discovery of the virus, considerable evidence has emerged that CD4+ T cell responses to HCV play a key role in the outcome of infection. However many questions remain and these will be discussed in this review.

Cellular immune responses, involving both CD8+ cytotoxic T lymphocytes (CTL) and CD4+ T-helper cells, play an essential role in the control of HCV infection, as they do in other persistent viral diseases. Whereas CTLs are traditionally thought to be the main effector cells that eliminate HCV-infected cells^[1], it is clear that HCV-specific CD4+ T cells also play a critical role. These cells can potentially act in multiple ways and are central to the initiation and maintenance of adaptive immunity. Two likely major roles are in providing help for CD8+ T cells by cytokine production and activation of antigen-presenting cells, but there are multiple other mechanisms of action including direct antiviral effects, a role in B cell maturation, and regulatory functions.

A very clear example of the overall importance of HCV-specific CD4+ T cell responses *in vivo* is an experiment where the antibody-mediated depletion of CD4+ T cells before re-infection of two immune chimpanzees was performed. Such depletion resulted in persistent, low-level viraemia despite functional intra-hepatic memory CD8+ T cell responses^[2]. In this experiment incomplete control of HCV replication by memory CD8+ T cells in the absence of adequate CD4+ T cell help was associated with emergence of viral escape mutations in Class I MHC-restricted epitopes and failure to resolve HCV infection^[2]. This experiment is important in that it shows that CD4+ T cells are necessary for resolution of HCV infection. However, in this case the exact function of the CD4+ T cells was not elucidated. The fact that CD8+ T cell responses were maintained and were functional (inducing viral escape) suggests that their role is not purely in providing support for CD8+ T cell responses.

Overall, most data on CD4+ T cells comes from experiments in infected persons, particularly those

comparing chronic and resolved infection. During chronic HCV infection, HCV-specific CD4⁺ T cell responses are typically described as weak or absent whereas in resolved infection these responses are generally vigorous and multispecific. However, much of the data is derived from cross sectional studies and also based on analysis of one function-proliferation *in vitro*. There is no doubt that CD4⁺ T cell responses in resolved infection and in persistent infection look very different, but how they diverge and what the true functionality of the populations are is not yet clear. In our view, given their central importance in adaptive immunity, understanding why CD4⁺ T cell responses may fail in acute infection is the key question in HCV pathogenesis. Current data on the function and role of virus-specific CD4⁺ T-helper cells in acute and chronic HCV infection will be reviewed here and models discussed.

CD4⁺ T CELL RESPONSES IN ACUTE HCV INFECTION

Clearly, the best place to study the CD4⁺ T cell response against HCV is at the site of infection, but CD4⁺ T cell responses in the liver of acute HCV infection in humans have not been characterized to date. In chimpanzees, liver CD4⁺ T cells have been expanded with anti-CD3 and IL-2 and then tested for proliferation in response to HCV proteins^[3]. In those who failed to control the virus, no CD4⁺ T cell responses were identified whereas in those chimps who transiently or permanently controlled the virus, strong proliferative CD4⁺ T cell responses were detectable. This experiment is limited by the need to expand the cells first *in vitro*, which might exclude certain cell populations, especially at the peak of infection. What is interesting is not only the dichotomy between those animals that cleared virus and those that did not, but also the “transient” group. This group does mount an early CD4⁺ response against HCV, but it is not sustained. The clinical phenomenon of transient control is common in human infection, with “yo-yo” patterns often described, and will be discussed further below.

All human studies in acute disease have been performed on blood. In these, overall, acute resolving HCV infection has been associated with a sustained response by HCV-specific CD4⁺ T cells. In one representative study patients who failed to clear the virus were divided into two groups^[4]. Group 1 was unable to mount an HCV-specific CD4⁺ T cell response and developed chronic HCV. In Group 2, HCV RNA was cleared initially and was associated with strong HCV-specific CD4⁺ T cell responses. However, these responses diminished just before a rebound of viraemia that resulted in chronic infection. Therefore, a vigorous anti-viral CD4⁺ T cell response (as measured by proliferation assays) in the early and late phase of acute HCV seems necessary to achieve long-term viral control (CD4⁺/Th1 response). However, it is not sufficient (as in the chimpanzees), since those who mount such a response do not necessarily go on to clear virus.

Although what the Gerlach paper described is especially clear cut in relation to the “yo-yo” pattern, other groups

have also reported that the vigour of the T cell response during the early stages of infection may be a critical determinant of disease resolution and control of infection^[5]. That persistent infection can develop despite the presence of acute-phase HCV-specific CD4⁺ T cell responses has been shown in a study of healthcare workers exposed to needle-stick injuries^[6]: although two individuals had strong HCV-specific CD4⁺ T cell proliferative responses in the acute phase with significant decreases in HCV RNA initially, they subsequently became chronically infected. This was an important study since the individuals were available for analysis shortly after infection-before clinical symptoms arose. Since responses are sometimes transient, it may be that in studies where patients are only analysed after they present clinically with acute infection, early T cell responses have been missed.

Such analyses have traditionally relied on proliferation. Using alternative techniques, it has been shown that evolution of the infection to chronicity can be associated with HCV-specific CD4⁺ T cells which survive initially despite failing to proliferate or produce IFN- γ : these diminish eventually as infection persists^[7]. A study of a single patient using a novel approach with Class II MHC peptide complexes (“tetramers”) revealed the loss of functionality of cells before the final loss of detection of such populations^[8]. The failure of such responses in this patient occurred before the re-emergence of virus after transient control. This is important since it suggests that not all the failure of CD4⁺ T cell responses may occur as a consequence of prolonged viremia.

Overall, these studies suggest that a range of HCV-specific CD4⁺ T cell responses in the acute HCV phase can exist in blood and liver. In a subgroup of individuals, it may be that CD4⁺ T cell responses are not mounted initially, or are not detectable at the time of presentation. These individuals are very likely to develop chronic infection^[9]. In another group, strong responses are detected and sustained, which is typically associated with resolution of infection. An important third group (probably the majority of patients) do mount a CD4⁺ T cell response initially, but loss of such responses is associated with progression to chronicity. What the differences are in these initial responses to HCV and why certain key CD4⁺ T cell responses are not sustained remain important questions in understanding HCV persistence. To understand this further it is necessary to consider the exact targets of the CD4⁺ T cell response.

TARGETS OF THE CD4⁺ T CELL RESPONSES IN HCV INFECTION

A more effective immune response against HCV could result from targeting more epitopes, mounting larger responses or targeting a key region. There is some evidence for all three. Overall, permanent resolution of infection has been related to both the breadth (number of CD4 epitopes targeted) and the magnitude of HCV-specific CD4⁺ T cell responses. While no one target has been identified as the key determinant, a number of candidates have been proposed.

Table 1 Displays the most commonly described CD4+ restricted epitopes

Amino acid position	HCV protein	Amino acid sequence	HLA-restriction	Reference
aa 21-40	Core	DVKFPGGGQIVGGVYLLPRR	DRB1*1101,DQB1*0301	Day, 2002
aa 31-45	Core	VGGVYLLPRR GPRLG	DRB1*1101	Godkin, 2001
aa 141-155	Core	GAPLGGAARA LAHGV	DRB1*1101	Godkin, 2001
aa 393-410	E2	GFATQRLTSLFALGPSQK	DRB1*1101	Frasca, 1999
aa 1241-1260	NS3	PAAYAAQGYKVLVLPNSVAA	DRB1*15, DRB1*0301 +	Day, 2002
aa 1248-1261	NS3	GYKVLVLPNSVAAT	DR4, DRB1*1101	Wertheimer, 2003
aa 1248-1267	NS3	GYKVLVLPNSVAATLGFAY	DQB1*0301	Lamonaca, 1999
aa 1251-1259	NS3	VLVLPNSVA	DRB1*1101,DRB1*1201, DRB1*0401, DRB1*1302	Day, 2002
aa 1384-1401	NS3	VIKGGRHILFCHSKKCD	DRB1*15	Eckels, 1999
aa 1411-1426	NS3	GINAVAYYRGLDVSVI	DRB1*15	Gerlach, 2005
aa 1531-1550	NS3	TPAETTTRLRAYMNTPLPV	DRB1*0701	Day, 2002
aa 1539-1554	NS3	LRAYMNTPLPVCQDH	DRB1*15	Gerlach, 2005
aa 1581-1600	NS3	ENLPYLVAAYQATVCARAQAP	DRB1*1001	Day, 2002
aa 1686-1705	NS4a	VVLSGKPAIIPDREVLREF	DRB1*0301	Harcourt, 2003
aa 1746-1765	NS4b	IAPAVQITNWQKLETFWAKHM	DRB1*16 or DRB3*0202	Harcourt, 2003
aa 1767-1786	NS4b	NFISGIQYLAGLSTLPGNPA	DRB1*1104	Carlos, 2004
aa 1771-1790	NS4b	GIQYLAGLSTLPGNPAIASL	DRB1*0404	Day, 2002
aa 1806-1818	NS4b	TLLFNILGGWVAA	DRB1*0101	Gerlach, 2005
aa 1907-1926	NS4b	GPGEAGAVOWMNRIFAASRG	DRB1*1104,DQB1*0501	Lamonaca, 1999
aa 2268-2282	NS5a	VSVPAEILRK SRRFA	DRB1*1101	Godkin, 2001
aa 2571-2590	NS5b	KGGRKPARLIVFPDLGVRVC	DRB1*0404,DRB1*0407	Day, 2002
aa 2841-2860	NS5b	ARMILMTHFFSVLIARDQLE	DRB1*1101	Day, 2002
aa 2941-2955	NS5b	CGKYLFNWAV RTKLK	DRB1*1101	Godkin, 2001
aa 2941-2960	NS5b	CGKYLFNWAVRTKLKLTPIA	DRB1*1101	Day, 2002

The NS3 protein has been shown to be one dominant target of CD4+ T cell responses in humans clearing HCV infection^[10]. Some epitopes in NS3 have been identified in both humans and chimps with resolved infection (see below)^[11-14]. Numerous studies indicate that CD4+ T cells targeting most HCV proteins, including non-structural (NS) proteins, are the norm in self-limited infections^[3-6,10-11,15-17]. However, some caution is needed in interpreting the exact targeting as the genomic variation between different HCV genotypes is substantial and relatively non-conserved epitopes (as might occur in envelope genes) may not always be picked up in such screens.

The number of CD4+ T cell epitopes recognised during acute HCV infection has been estimated by characterising memory CD4+ T cell populations in blood after permanent resolution of the virus: one study showed at least four, and up to 14 epitopes from the core, NS3, NS4 and NS5 proteins were recognised by CD4+ T cells in patients several months or even years after loss of HCV RNA^[11]. A recent study identified 13 CD4+ T cell epitopes within the NS3-NS4 region that were recognised by $\geq 30\%$ of patients with acute or resolved HCV^[18]. Of these, eight peptides were also recognised recurrently from different donors by specific CD4+ T cell clones in independent cloning procedures. Multispecific CD4+ T cell responses were also detectable in blood of individuals during acute HCV infection acquired by needlestick injury^[6] or IV drug use^[15]. Importantly, in some patients whose infection became chronic after the acute phase infection, responses were similar to those who spontaneously cleared the virus after acute infection^[6]. The only difference (as above) was that these responses in individuals with chronic infection were not sustained.

A recent study identified a single epitope restricted

by HLA DR1 (a common HLA molecule) in NS4, which appears to be very commonly targeted in infected individuals^[18] (Table 1). New analysis using Class II tetramers revealed that in acute infection, all DR1+ donors made a response to this peptide^[19]. This highly reproducible response is somewhat unusual, but may reflect the fact that this peptide is extremely conserved. Although the responses at presentation were indistinguishable between those who went on to resolve infection or not, early loss of tetramer+ cells was seen in those with persistent infection. The loss of such cells was not associated with mutation within the epitope and populations of such cells were not found localised in the liver only. Interestingly, when compared to controls in long term chronic infection, some very low level tetramer+ populations could be identified in the absence of proliferative responses. This suggests that specific responses were not deleted entirely, but persist at very low levels and with only limited function.

Table 1 HCV T helper epitopes mapped within a region of 21 amino acids or less. The protein, the sequence and HLA restriction elements of the T helper epitopes are provided.

ASSOCIATION BETWEEN HLA CLASS II ALLELES AND INFECTION OUTCOME

It is not yet clear whether responses to specific epitopes are clearly related to outcome. However, the T cell response to peptides derived from the virus is directed by the MHC genotype, and this could have a major influence on the quality of T cell responses. Importantly, some MHC Class II alleles in humans have been associated with persistence or resolution of HCV infection. For example, HLA-DRB1*0701 has been shown to be associated with

persistence in patients who were homogeneous in terms of gender, source of infection (genotype 1b) and ethnicity^[20]. In contrast, a number of other studies of more mixed populations have shown a strong association between other HLA Class II alleles and viral control. These alleles are HLA-DRB1*0101, HLA-DRB1*1101, and HLA-DQB1*0301^[21-23].

Although specific HLA alleles have been defined as protective, the link between the possession of a specific allele and the presentation of a specific peptide or set of peptides has not yet been made. HLA-DRB1*1101 and HLA-DQB1*0301 (these genes are in close linkage disequilibrium) have been associated with a sustained CD4⁺ T cell response in resolved HCV infection; these responses were stronger than in non-DQB1*0301+ controls^[24]. However, our understanding of this association still remains incomplete. Some of these HLA Class II restricted peptides have been identified through epitope prediction programs^[25], and the full repertoire of naturally presented peptides is not completely defined. A recent study showed that viral variation might play a role in determining the dominance of epitopes seen within a population. Here, an HLA DR11-restricted epitope (NS3 aa1248-1261) that is highly conserved within viral genotypes was found not to be the immunodominant response, despite being the most commonly recognised epitope for this HLA allele^[26]. Epitopes in viral regions that can tolerate amino acid substitutions may thus appear to be less dominant, even if they are important. This is because their capacity for increased variability means that they may not occur in the prototype viral peptide sequences often used for immunological study, or might only be represented in a fraction of the patients studied.

CD4⁺ T CELL QUALITY AND ASSOCIATION WITH ACUTE OUTCOME

It has been suggested in studies of acute disease that viral clearance is more likely to occur when HCV specific responses of patients display a Th1 profile (IFN- γ and IL-2). Those with a more typical Th2 profile (IL-4 and IL-10) were more likely to become chronic^[27] suggesting that the Th1 phenotype generates more protective immune responses in HCV. In that study, CD4⁺ T-cell proliferation and cytokine secretion in response to a panel of recombinant HCV antigens were assayed in 17 patients with acute HCV. All six patients with self-limited disease had a significant CD4⁺ T-cell proliferation to C22, E1, C100, C200, and NS5, running parallel with the antigen-stimulated secretion of IL-2 and IFN- γ , but not with IL-4 and IL-10, indicating predominant Th1 responses. Among the remaining 11 patients who developed chronicity, several cases showed specific CD4⁺ T cell responses, but their antigen-stimulated IL-2 and IFN- γ production were significantly lower than those of cases with recovery. Importantly, IL-4 and IL-10 (Th2 responses) were detectable in the group who developed chronicity. The data suggested that activation of Th2 responses in acute hepatitis C patients might play a role in the development

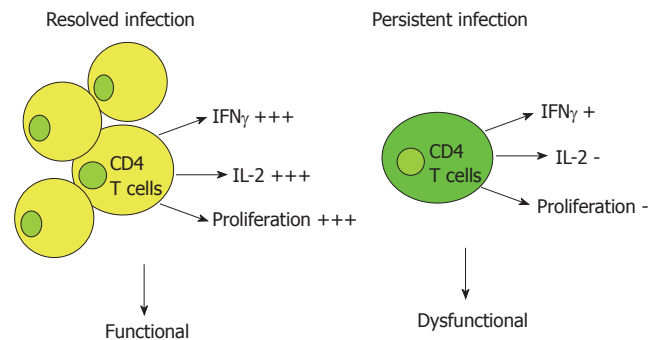


Figure 1 Cytokine secretion patterns in persistent versus resolved HCV infection.

of chronicity, but much more work needs to be done in this area, and why such responses might differ at the start of the disease needs some explanation.

HCV SPECIFIC RESPONSES IN CHRONIC INFECTION

As discussed above, once chronic HCV infection is established, cellular immune responses are rarely or barely detectable using current technology. Several groups have aimed to analyse these HCV-specific CD4⁺ T cell responses during chronic HCV infection using proliferation assays, cytokine assays and, more recently Class II tetramers.

Analysis of HCV-specific CD4⁺ T cell responses in chronic HCV infection using ELISpot or ICS showed responses at low frequency in blood and only targeted a limited number of epitopes^[4,7,11,15-17,28]. In an expanded analysis of responses to recombinant HCV proteins in persons with resolved infection, an average of 10 epitopes was targeted, whereas in persons with chronic viraemia never more than one epitope was targeted^[11].

The question of whether HCV-specific CD4⁺ T cell responses persist in chronic infection but lack function in terms of proliferation and cytokine production (IFN- γ) has been addressed by staining for expression of the IL-2 receptor α -chain (CD25), which is an early marker of activation in a study that showed that a small proportion of CD4⁺ T cells from the blood of chronically infected individuals did upregulate CD25 after stimulation with recombinant HCV proteins, but lacked the capability for proliferation and IFN- γ production^[7]. However, the use of the marker CD25, which is present normally on a fraction of CD4⁺ T cells including Tregs, limits the usefulness of this assay.

It has generally been assumed that loss of proliferation equates to loss of the specific T cell populations. Low levels of IL-2 secretion have been shown in independent studies in humans and mice to be accompanied by a loss of proliferative capacity both *in vitro* and *in vivo*^[29-32]. In HCV infection, recent data suggest that, in the presence of viraemia, HCV-specific CD4⁺ T cell populations do exist but lack proliferative capacity^[33]. This status is associated with the production of IFN- γ upon antigen stimulation, but little or no IL-2 is expressed^[34] (Figure 1).

The best approach for detecting functionally impaired

CD4⁺ T cells in chronic HCV infection is the use of MHC Class II tetramers. Using this technique, a correlation has been shown between the clinical outcome and the presence of circulating CD4⁺ T cells directed against the virus^[35]. Here, with the use of 3 HCV HLA Class II tetramers, HCV-specific CD4⁺ T cells could be detected in subjects who spontaneously resolved HCV viraemia, but not in those with chronic HCV infection, suggesting that HCV-specific CD4⁺ T cell frequencies are very low in PBMC. A further application of this technology for the analysis of intrahepatic CD4⁺ T cells could shed important light on their differentiation state and functionality.

The expansion of HCV-specific CD4⁺ T cell lines by repeated stimulation with recombinant antigens indicated that antigen-specific populations do persist^[36-38]. A note of caution should be injected here in the analysis of sustained CD4⁺ T cell responses in chronically infected patients. In many cases, these may represent a historical response to a previous viral genotype which is no longer circulating in the patient. In one case described, the response was directed against genotype 1 even though the patient carried genotype 3, with a historical genotype 1 infection^[39]. Since the peptide in the genotype 3 sequence was substantially different and not recognised by host CD4⁺ T cells, this indicates that the detected response is effectively a memory response after removal of the original virus. Since superinfection is relatively common, this issue may be a substantial one for both CD4⁺ and CD8⁺ T cells^[40].

The role of IL-10 as Th2 anti-inflammatory cytokine has been demonstrated *in vivo* in humans chronically infected with HCV^[41]. Here, individuals with advanced fibrosis were treated three times a week with IL-10 for 12 mo. Administration of IL-10 resulted in a decreased number of IFN- γ -secreting HCV-specific CD4⁺ and CD8⁺ T cells. At the same time ALT levels as a marker of inflammation were reduced, indicating the role of IL-10 as anti-inflammatory cytokine. However, with the loss of specific CD4⁺ and CD8⁺ T cells, HCV RNA levels were increased, suggesting that these same cells are responsible for viral control^[41].

One hypothesis why in chronic HCV the Th2 type may occur is that dendritic cells from patients with chronic HCV infection have defective function, possibly due to inhibition of IL-12^[42,43]. The latter cytokine is required for the induction of Th1 type cells. This dendritic cell dysfunction might result in biased T cell polarisation which could favor, for example, a Th2-type response. However, to what extent this is a major factor in pathogenesis is still controversial and the findings are not uniformly reproducible.

Overall, failure of CD4⁺ T cells is a key factor in HCV persistence and clearly in chronic disease there are relatively few functional CD4⁺ T cells to find, by whatever method. To some extent this appears to be due to loss/deletion of antigen-specific cells. On the other hand there is some evidence that a change in function also occurs in persistent infection, although whether this is cause or effect requires a great deal more study. It should be noted that although CD4⁺ T cell responses are regarded as weak in chronic HCV mono-infection, they are even weaker in HCV/HIV co-infection^[44,45]. Since co-infection is associated with an

increase of HCV load of about 0.5-1 log, this data suggests that in chronic mono-infection the remaining CD4⁺ T cell response is still playing a significant role.

MODELS FOR FAILURE OF CD4⁺ T CELL RESPONSES

If failure of the CD4⁺ T cell response against HCV is associated with virus persistence, what mechanisms could account for this? Here we outline three major contenders, escape, exhaustion and regulation.

Escape through mutation

Numerous studies in both animal and human models have documented immune escape from virus-specific CTL responses by viral mutations in CTL epitopes that lead to loss of immune control and viral persistence^[46-52]. Less information is currently available about the potential for immune escape from viral CD4⁺ T cell epitopes, although limited studies in chronic HIV and HCV infection have identified multiple autologous virus variants for specific CD4⁺ T cell epitopes^[53-56]. Peptides corresponding to viral variants were synthesised and tested in *in vitro* assays, and the majority of variants failed to stimulate proliferation or cytokine production by CD4⁺ T cells^[54-56]. That viral variants may play a role in HCV persistence has also been shown previously in a study with four HLA-DRB1*15 patients chronically infected with HCV^[57]. Here, naturally occurring single amino acid substitutions in the DRB1*15-restricted Th1 epitope (aa 358-375) in the NS3 protein failed to stimulate proliferation. This was also accompanied by a shift in cytokine secretion patterns from one characteristic of a Th1 anti-viral response to a Th2 form. These data suggest that viral immune escape from specific CD4⁺ T cell responses is possible, but clear data showing the evolution of CD4⁺ T cell escape mutants in response to T cell selection pressure are still needed.

A recent study analyzed the effects of an induced T-cell response in three immunized chimpanzees, targeting nonstructural proteins in the absence of neutralizing antibodies^[58]. The immunized animals were challenged with clonal HCV, which had the same sequence as the antigens used for immunization. Persistent control of the virus was observed in two animals, whereas in the third animal viral control was transient, followed by a resurgence concomitant with the emergence of new dominant viral populations bearing single amino acid changes in the NS3 and NS5A regions. These mutations resulted in a loss of CD4 T-cell recognition and subsequent to viral resurgence and immune escape a large fraction of NS3-specific T cells became impaired in their ability to secrete IFN- γ and proliferate.

Exhaustion

In addition to escape from virus-specific T cell responses, escape from neutralising antibody (nAb) responses is thought to be one potential mechanism leading to the persistence of some viruses with knock on effects on CD4⁺ T cells^[53,59,60]. Recent data generated in an LCMV model have provided additional insight into the relationship between

CD4⁺ T cells and immune escape from nAb responses. CD8^{-/-} mice were infected with the WE strain of LCMV to establish a long-term infection with high levels of virus production that is transiently controlled by nAbs^[60]. However, the lack of CD8⁺ CTL responses and consequently high viraemia in this model leads to escape from polyclonal nAb responses. Associated with this is the rapid induction of CD4⁺ T cell unresponsiveness^[61]. Although the molecular mechanism of CD4⁺ T cell unresponsiveness is not clear from this study, it has been postulated that the high antigenic load in this model system may have resulted in activation of all virus-specific CD4⁺ T cells, leading to exhaustion and activation-induced cell death as has been described for CTLs^[62,63]. Importantly, in the absence of LCMV-specific CD4⁺ T cells, these mice failed to generate new effective humoral responses against emerging neutralisation-escape mutants and the viral infection persisted^[61]. These data provide further evidence for the importance of interactions between the cellular and humoral immune responses for efficient control of viral infections. A similar phenomenon of exhaustion of CD4⁺ T cells could easily arise through any mechanism, which leads to long-term viremia, including escape from interferon or mutation in epitopes recognised by CD8⁺ T cells.

Regulation

Recent years have seen a revival of interest in the role of regulatory T cells—notably the CD4⁺ CD25⁺ FoxP3⁺ subset^[64]. It is plausible that in HCV infection excessive regulation is involved in the suppression of HCV specific T-cell responses. Recently, CD4⁺ CD25⁺ regulatory T-cell activity has been shown to be present in patients with chronic HCV infection, which may contribute to weak HCV-specific T-cell responses and viral persistence^[65-69]. An important question that derives from these studies is to what extent the Treg activity seen in persistent infection relates to the activity of antigen-specific cells. Treg cells may arise in the thymus (natural Tregs) but additionally virus-specific cells, which are repetitively stimulated with antigen over time, may develop regulatory characteristics. This activity may be promoted by the action of dendritic cell subsets modulated by persistent viremia. Such Treg cells might serve to downregulate both CD4⁺ and CD8⁺ T cell responses in persistent infection, particularly within the inflamed liver. A recent study revealed that approximately one in two to one in three CD4⁺ T cells in the liver of chronically infected patients are FoxP3⁺, a remarkably dominant potential Treg population^[70]. This could have a major effect on the maintenance and growth of antigen-specific CD4⁺ and CD8⁺ T cells.

CONCLUSION

HCV specific CD4⁺ T cells hold a pivotal role in disease pathogenesis. There is a consensus that there are real differences between the responses seen in resolved infection vs persistent infection, although to what extent these are cause or consequence is not clear yet. The differences include not only number, but also function, including cytokine secretion, such as the key mediator IL-2. A number of pieces of evidence point to the fact that a robust CD4⁺

T cell response is associated with a good outcome from acute infection and there is no doubt that such responses should be elicited in a vaccine. To what extent it matters which epitopes are targeted or not is not yet clear, but most observers argue that breadth is important, especially given the huge genomic variation in HCV, and that numbers are important. For those lucky enough to inherit protective HLA types, a vaccine may simply augment an already favourable response and sustain adaptive responses from CD8⁺ and B cells. For the rest, perhaps a pool of CD4⁺ T cell responses which are primed in a normal uninfected individual, and which can expand rapidly during acute infection may be sufficient to tip the balance in favour of host clearance.

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Host and viral factors contributing to CD8+ T cell failure in hepatitis C virus infection

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Abstract

Virus-specific CD8+ T cells are thought to be the major anti-viral effector cells in hepatitis C virus (HCV) infection. Indeed, viral clearance is associated with vigorous CD8+ T cell responses targeting multiple epitopes. In the chronic phase of infection, HCV-specific CD8+ T cell responses are usually weak, narrowly focused and display often functional defects regarding cytotoxicity, cytokine production, and proliferative capacity. In the last few years, different mechanisms which might contribute to the failure of HCV-specific CD8+ T cells in chronic infection have been identified, including insufficient CD4+ help, deficient CD8+ T cell differentiation, viral escape mutations, suppression by viral factors, inhibitory cytokines, inhibitory ligands, and regulatory T cells. In addition, host genetic factors such as the host's human leukocyte antigen (HLA) background may play an important role in the efficiency of the HCV-specific CD8+ T cell response and thus outcome of infection. The growing understanding of the mechanisms contributing to T cell failure and persistence of HCV infection will contribute to the development of successful immunotherapeutical and -prophylactical strategies.

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Key words: Hepatitis C virus; CD8+ T cells; T cell failure; Viral escape; Programmed death 1; Regulatory T cells; T cell maturation; Human leukocyte antigen

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INTRODUCTION

The host immune response to pathogens involves various components of the immune system, including innate, humoral, and cellular immunity, the latter consisting of CD4+ and CD8+ T cells. All components of the immune response might have distinct roles in the outcome and pathogenesis of HCV infection and will be discussed in separate reviews in this issue of *WJG*. In this review, we will focus on the CD8+ T cell response to HCV infection. CD8+ T cells recognize viral antigen presented by HLA class I molecules on professional antigen presenting cells (CD8+ T cell priming) and on infected target cells (e.g. hepatocytes). Their antiviral activity includes cytotoxicity as well as the secretion of antiviral cytokines such as interferon-gamma (IFN- γ). In the following, successful virus-specific CD8+ T cell responses associated with viral clearance as well as ineffective CD8+ T cell responses present in persistent HCV infection will be described. The main focus of this review, however, is the multiple mechanisms that contribute to CD8+ T cell failure and viral persistence.

CD8+ T CELL RESPONSE IN ACUTE HCV INFECTION

During acute resolving HCV infection, vigorous virus-specific CD8+ T cell responses that target multiple epitopes can be detected approximately 4-8 wk after infection, and their emergence is temporally associated with the onset of liver disease^[1-4] (Figure 1A). However, the virus-specific CD8+ T cells are not able to secrete antiviral cytokines such as IFN- γ in this early phase of infection, a status referred to as 'stunned phenotype'^[2,3]. In a later phase of infection, virus-specific CD8+ T cells regain their ability to secrete antiviral cytokines, and this is temporally associated with a rapid decline of viremia and finally viral clearance. Knowledge about the intrahepatic virus-specific CD8+ T cell response during acute HCV infection was obtained from experimentally infected chimpanzees. Responses accumulate in the liver 8-14 wk after infection and coincide with liver disease as well as viral clearance^[5,6]. After resolution of infection, virus-specific CD4+ and

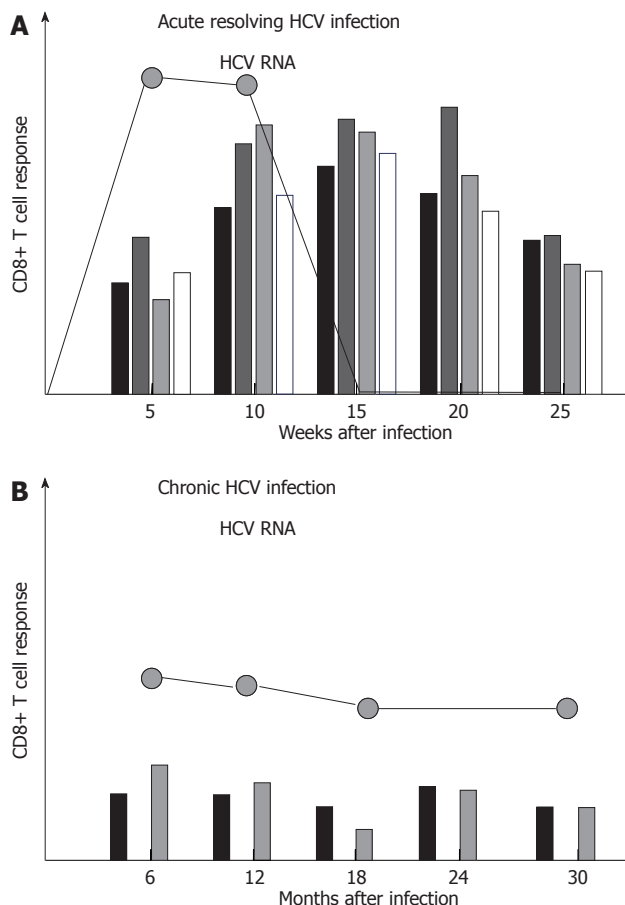


Figure 1 A: Virus-specific CD8⁺ T cell responses are strong, multi-specific, and sustained in acute resolving HCV infection; B: Virus-specific CD8⁺ T cell responses are weak and narrowly focused in chronic HCV infection.

CD8⁺ T cell responses persist for decades and can even outlast humoral responses^[7]. Virus-specific CD8⁺ T cells also play a role in mediating protective immunity. Indeed, evidence for protective immunity comes from both epidemiological studies as well as experimental studies^[8]. Chimpanzees re-challenged by HCV showed a shorter period and lower level of viremia than naïve animals^[9-11]. Sterilizing immunity against HCV, however, may not exist, since multiple episodes of heterologous or homologous re-infection have been observed in both, humans and chimpanzees.

In contrast to acute resolving HCV infection, the CD8⁺ T cell response in acute persisting HCV infection has been less defined. Previous reports comparing the CD8⁺ T cell response in acute resolving versus acute persisting HCV infection in chimpanzees and men found significantly weaker and more narrowly focused virus-specific CD8⁺ T cell responses in those subjects developing persistent infection^[1,2,5,6]. More recent studies, however, could not confirm this finding^[4,12,13]. For example, Cox *et al* performed a prospective longitudinal study in young iv drug users and analyzed the T cell response in 4 individuals with resolution of acute HCV infection and 15 individuals who progressed to chronic infection. Although all 4 individuals with resolving infection mounted virus-specific CD8⁺ T cell responses and those 4 individuals who lacked CD8⁺ T cell responses developed chronic infection, the

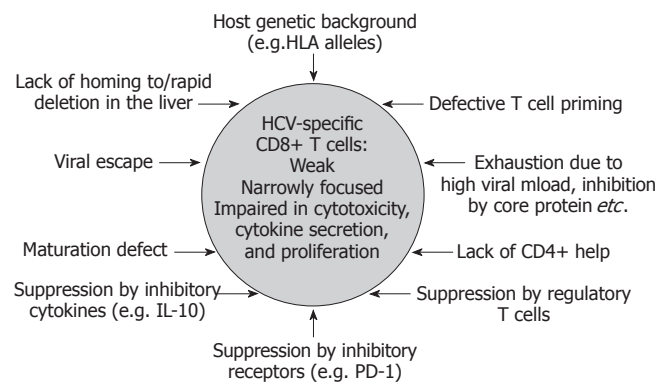


Figure 2 Possible Mechanisms of CD8⁺ T cell failure in persistent HCV infection.

CD8⁺ T cell response did not differ significantly between resolvers and persistently infected individuals^[4]. Urbani *et al* studied 6 patients with acute resolving and 11 patients with acute persisting HCV infection and found an association between strong and multispecific CD4⁺, but not CD8⁺ T cells with viral clearance. However, patients developing chronic infection displayed prolonged CD8⁺ T cell dysfunctions and maturational defects^[12]. This discordant role of CD4⁺ and CD8⁺ T cells was confirmed by Kaplan *et al*, albeit their analysis was limited to two HLA-A2 restricted CD8⁺ T cell epitopes^[13].

In sum, acute resolving HCV infection is associated with strong, broadly directed and sustained CD8⁺ T cell responses, while a universal picture of the CD8⁺ T cell response in acute persistent HCV infection has not yet been defined.

CD8 + T CELL RESPONSE IN CHRONIC HCV INFECTION

In contrast to acute resolving infection, CD8⁺ T cell responses are usually weak or even absent in chronic HCV infection, targeting only few epitopes^[14-22] (Figure 1B). In this context, it is important to point out that at least in some chronically infected patients, the CD8⁺ T cell response targets several epitopes^[17,18,22]. Importantly, however, these HCV-specific CD8⁺ T cells display functional impairments, including reduced cytotoxicity, reduced secretion of antiviral cytokines such as IFN- γ , and a reduced proliferative capacity^[23-25]. In addition, many CD8⁺ T cell responses do not target a present antigen, but rather a historical antigen due to viral escape (see below). Different mechanisms which might be involved in the failure of the HCV-specific CD8⁺ T cell response in persistent infection will be discussed in the following (Figure 2).

MECHANISMS OF CD8 + T CELL FAILURE

Primary failure and exhaustion

As discussed above, some patients with chronic HCV infection lack strong and multispecific CD8⁺ T cell responses, however, it is difficult to distinguish if virus-specific CD8⁺ T cell responses were not primed initially (primary CD8⁺ T cell failure) or responses were primed,

but vanished quickly (CD8+ T cell exhaustion). Results obtained from the early phase of acute HCV infection in chimpanzees and in health care workers infected through needle stick exposure support the hypothesis that CD8+ T cells are not primed at least in some patients with acute persisting HCV infection^[2,5,6]. In a prospective longitudinal study of young iv drug users, however, CD8+ T cell exhaustion was indeed demonstrated for at least one targeted epitope in each subject developing chronic infection^[4].

An impaired priming of HCV-specific CD8+ T cells might be mediated by numeric and functional impairments of antigen-presenting cells, e.g. macrophages and dendritic cells; however, this topic remains controversial^[26-35].

CD8+ T cell exhaustion might be explained by general as well as HCV-specific mechanisms. Of note, it has been demonstrated in the lymphocytic choriomeningitis virus (LCMV) mouse model that high viral loads lead to an unresponsive state of virus-specific CD8+ T cells, downregulation of T cell receptors, and finally physical deletion of virus-specific CD8+ T cells^[36-39]. Recent data indicate that the inhibitory receptor PD-1 might be involved in this process (see below), and it has been postulated that the detrimental effect of high viral load may not only apply in LCMV infection, but in different viral infections including HCV infection. Regarding HCV-specific mechanisms of CD8+ T cell exhaustion, the core protein has been reported to impair CD8+ T cell activation, e.g. through interaction with membrane-bound complement receptor gC1qR^[40-42].

Lack of CD4+ help

While CD8+ T cells are considered the major effector cells against viral pathogens, the successful elimination of HCV might be highly dependent on sufficient CD4+ T cell help. Indeed, it has been demonstrated in the LCMV mouse model that CD4+ T cell help is needed to sustain cytotoxic CD8+ T cell responses during chronic viral infections^[43]. In chronic HCV infection, however, CD4+ T cell responses are very weak or even absent and functionally impaired, e.g. secrete low amounts of IL-2^[44,45]. Findings in the chimpanzee model support the central role of CD4+ help in CD8+ T cell mediated viral clearance. When CD4+ T cells were depleted by neutralizing antibodies prior to viral re-challenge, HCV viremia was prolonged, CD8+ escape variants were selected and HCV finally persisted^[46]. Consistent with this concept, HCV-specific CD8+ T cell responses were seen almost exclusively in the face of a strong CD4+ T cell response in a study of acutely HCV infected patients^[1]. A recent study demonstrated that the outcome of acute HCV infection was associated with efficient virus-specific CD4+ T cell responses. In this study, however, HCV-specific CD8+ T cell responses were induced irrespective of virological outcome or HCV-specific CD4+ T cell responses^[13].

Suppression by regulatory T cells

In the last few years, the concept of regulatory T cells has undergone a comeback and different types of regulatory T cells have been characterized in different clinical settings. In HCV infection, the role of CD4+CD25+

Foxp3+ regulatory T cells as well as IL-10 producing CD8+ T cells has been defined. In chronically HCV infected patients, CD4+CD25+ T cells have been found in a higher frequency compared to individuals with resolved HCV infection or healthy controls^[47-49]. These regulatory T cells suppress the proliferation as well as interferon-gamma secretion of virus-specific CD8+ T cells *in vitro*. The suppression by CD4+CD25+ T cells was cell-cell contact dependent^[47,48], it was independent of suppressive cytokines such as IL-10 and TGF- β in some but not all studies^[47,48,50]. Interestingly, the suppression was not restricted to HCV-specific CD8+ T cells, but also included CD8+ T cells specific for other viruses, such as EBV or influenza^[47,50]. However, specificity *in vivo* might be mediated by the enrichment of CD4+CD25+ T cells in the liver^[51]. While CD4+CD25+ T cells might limit immunopathology in the chronic phase of HCV infection^[52], it has been suggested that they may facilitate viral persistence in the acute phase of infection. However, studies in larger cohorts of patients with acute HCV infection have not yet been reported. The induction of CD4+CD25+ regulatory T cells is still little characterized, however, they could be induced by certain HCV peptides from peripheral blood mononuclear cells (PBMCs) from HCV-infected, but not healthy individuals *in vitro*^[53].

Another type of regulatory T cells in HCV infection is virus-specific regulatory CD8+ T cells that express high levels of IL-10. These regulatory T cells have been detected in the liver of HCV-infected individuals; they could be expanded upon stimulation with HCV epitope peptides and their suppression of virus-specific CD8+ effector T cells could be blocked by neutralizing IL-10 antibodies^[54]. This virus-specific regulatory T cell population might have an important role in the prevention of liver damage during chronic HCV infection^[55].

The spectrum of regulatory T cells involved in HCV infection may further expand, since we recently described the induction of regulatory CD8+ T cells from the PBMC of HCV-infected patients which also expressed high levels of FoxP3 and CD25^[56]. A comprehensive review on the different types of regulatory T cells in HCV and HBV infection by Billerbeck *et al* is also included in this issue of *WJG*.

Inhibitory receptors: PD-1

The inhibitory receptor PD-1 ("programmed cell death 1") has been demonstrated to be a strong marker for exhausted virus-specific CD8+ T cells in the LCMV mouse model. The antibody-mediated blockade of the interaction between PD-1 and its ligand PD-L1 led to the restoration of cytokine secretion, proliferation, and cytotoxicity by the exhausted virus-specific CD8+ T cells and a substantial reduction in viral load^[57]. Similar roles of PD-1 have been shown in human chronic viral infections^[58] such as HIV^[59], HBV^[60,61], and HCV. In the acute phase of HCV infection, similar to LCMV infection, PD-1 is up-regulated on HCV-specific CD8+ T cells independent of outcome. However, in individuals with resolving infection PD-1 expression decreases soon, while in patients with a chronic course of infection, HCV-specific CD8+ T cells remain PD-1 positive^[62]. This finding is in parallel with

the “stunned” phenotype of HCV-specific CD8⁺ T cells in the early acute phase of infection, which is restored in resolving infection but remains in persisting infection^[2,3,24]. In chronic HCV infection, HCV-specific CD8⁺ T cells in the peripheral blood^[63] as well as liver^[64] have been shown to express high levels of PD-1. Blockade of PD-1/ PD-L1 interaction by antibodies restored cytokine production and proliferation of the exhausted CD8⁺ T cells from acute and chronic infection *in vitro*.

It is important to note, however, that the antibody-mediated blockade of the PD-1/PD-L1 pathway in chronically LCMV-infected mice did not result in viral clearance although a significant reduction of viral load was achieved. Even more importantly, PD-L1-/- mice died due to immunopathologic damage after infection with a LCMV strain usually establishing persistent infection^[57]. These findings indicate that a subtle balance in the blockade of the PD-1/PD-L1 pathway must be granted before it can be applied in the clinics.

Inhibitory cytokines: IL-10

Two recent reports on the role of IL-10 in the dysfunction of virus-specific T cells and viral persistence gained much attention in the field. These reports showed that in mice with persistent LCMV infection, IL-10 was highly up-regulated early in infection, which was associated with the dysfunction of virus-specific CD4⁺ and CD8⁺ T cells. The blockade of the IL-10/IL-10 receptor (IL-10R) pathway by a genetic approach or by an anti-IL-10R antibody early in infection, however, led to the restoration of T cell function and to clearance of infection^[65-67].

Although these reports definitely point towards an important general mechanism of T cell dysfunction, a role of IL-10 in HCV infection has been postulated before, and IL-10 therapy has even been tested in clinical trials in HCV infected patients. Indeed, many reports showed an association of IL-10 polymorphisms and outcome, disease progression, or response to antiviral therapy of HCV infection^[68-74], while other studies failed to confirm these data^[75-79]. Clinical trials with administration of recombinant IL-10 to patients with chronic HCV infection who had failed antiviral therapy with interferon-alpha led to a decrease in transaminases and histological disease progression; however, viral titers strongly increased in some IL-10 treated patients^[80,81]. This indicates that IL-10 might not only mediate viral dysfunction and thus facilitate viral persistence in acute infection, but may also reduce immunopathology in the chronic phase of infection. In this context, it is important to point out, however, that IL-10/IL-10R blockade in the LCMV mouse model did not result in severe immunopathology^[65,66].

The exact mechanism of HCV-induced up-regulation of IL-10 remains elusive. Some groups have reported induction of IL-10 production by monocytes^[82] or natural killer (NK) cells^[83] through core^[74,84], non-structural protein 3^[84], or 4^[82]. More intriguingly, HCV-specific CD8⁺ T cells with regulatory properties which produce IL-10 have been described in the liver of chronically HCV infected patients^[54]. These IL-10 producing intrahepatic CD8⁺ T cells were associated with mild inflammation and low progression of fibrosis in liver histology^[55], once more sug-

gesting that IL-10 may protect from immunopathology in chronic HCV infection. Blockade of the IL-10 pathway by anti-IL10R antibodies *in vitro* led to increased HCV-specific CD4⁺ T cell responses^[85]. In addition, antiviral therapy led to reduced production of IL-10 by virus-specific T cells in patients with chronic HCV infection^[86]. A direct inhibition of the IL-10 pathway, however, needs further careful evaluation in additional animal models before it can be transferred to men.

Viral escape

HCV is a RNA virus with an enormous replication rate (approximately 10¹² virions per day) with a RNA-dependent RNA polymerase that lacks a proofreading function. Therefore, multiple viral variants, called quasispecies, circulate in a single individual. It has been suggested that the selection of viral variants escaping from CD8⁺ T cell responses might facilitate the persistence of HCV infection. Indeed, the first evidence for viral escape in HCV infection came from chronically infected patients^[87] and experimentally infected chimpanzees^[88,89]. Chronically infected patients harbored variant viral sequences in targeted epitopes which were non-immunogenic and not cross-reactive with the prototype peptides. These viral escape mutations remained fixed over a follow-up time of up to four years, indicating that escape mutations occur early in infection^[87]. In the chimpanzee model, it could further be demonstrated that viral escape mutations occurred during the first 16 wk of infection and were associated with a chronic course of infection^[89].

Important additional information came from studies in acutely infected patients^[90-92] as well as population-based approaches^[90,93]. In these studies, viral escape from CD8⁺ T cell responses was demonstrated in patients developing persistent infection^[90-92], but not in individuals with resolving infection^[91,92]. Interestingly, many mutations outside of targeted CD8⁺ T cell epitopes represented conversion to consensus^[91], and the transmission of an HLA-B8 associated escape mutation to an HLA-B8 negative subject resulted in rapid reversion of the mutation^[90]. These results were supported by a study in a well-defined cohort of Irish women accidentally infected with HCV from a single source more than 20 years ago. In this unique cohort, amino acid substitutions in known epitopes were directed away from consensus in women having the HLA allele associated with that epitope, and toward consensus in those lacking the allele^[93]. These findings are in agreement with the concept of viral fitness cost, indicating that viral escape mutations are often associated with a reduced replicative capacity of the virus^[94]. In the absence of the T cell pressure, e.g. upon transmission to an individual negative for the restricting HLA allele, the virus reverts to consensus and thus regains its full replicative capacity. This phenomenon has been analyzed in more detail in the background of an immunodominant HLA-A2 restricted epitope, identifying that certain amino acid residue substitutions abolish HLA binding without strongly influencing viral replication, while some substitutions lead to a strong reduction of viral fitness^[95]. Importantly, there might be some CD8⁺ T cell epitopes which are not affected by viral escape

due to high functional constraints. For example, we have recently identified an HLA-A26 restricted epitope located at the NS5A/5B cleavage site which was targeted in all studied HLA-A26+ patients (3/3) with acute HCV infection and a significant number of patients with chronic HCV infection (3/15). However, the epitope sequence was highly conserved in HLA-A26 positive and negative patients, indicating that viral escape did not occur in this functionally constrained region^[96].

Based on the finding that immunodominant CD8+ T cell epitopes leave their footprint in viral sequences in chronic HCV infection^[90], viral genome sequencing studies were performed in order to identify footprints of additional potential CD8+ T cell epitopes^[97,98]. In addition to previously defined epitopes, these studies identified HLA allele dependent polymorphisms and thus candidate CD8+ T cell epitopes. Importantly, the strongest association with any HLA allele in the study by Timm *et al* was found for HLA-B27 in a region that was shown to contain an immunodominant HLA-B27 restricted CD8+ T cell epitope by an independent study in another patient cohort^[99].

There are different molecular mechanisms by which a certain mutation escapes from the CD8+ T cell response. Especially those mutations located at the HLA binding anchors, usually P2 and the C-terminal amino acid, lead to the interruption of the peptide binding to the HLA molecule. Mutations in the center of the epitope, in contrast, are more likely to interfere with T cell receptor (TCR) recognition^[95]. Mutations in the flanking region, however, prevent proteasomal epitope processing^[90,100,101].

The determinants of viral escape are less understood. In the chimpanzee model of HCV, it has been shown that upon depletion of CD4+ T cells in the acute phase of infection viral escape from the CD8+ T cell response occurs and is associated with a persistent course of infection^[46]. This finding has led to the hypothesis that viral escape is caused by insufficient CD4+ help. Other studies indicate that a limited T cell receptor (TCR) diversity might be responsible for viral escape^[102]. Of note, viral escape does not occur in the context of dysfunctional CD8+ T cell responses^[103]. The strong association between HLA-B27 and viral escape within an immunodominant HLA-B27 restricted epitope as well as the suggestion that escape variant epitopes might preferentially be restricted by HLA-B alleles indicates that the restricting HLA allele background also plays an important role in determining viral escape^[97-99].

Lack of homing to the liver

Experimentally HCV infected chimpanzees which progressed to viral persistence without temporary viral control lacked virus-specific CD8+ T cell responses in the liver despite of detectable responses in the peripheral blood^[2]. This finding led to the tempting hypothesis that the failure of the virus-specific CD8+ T cell response might be caused by an insufficient homing to the primary location of infection, the liver. However, in chronically HCV infected patients virus-specific CD8+ T cells are detectable and even enriched in the liver^[22,25,104-108]. In a comprehensive study comparing the overall breadth and

vigor of CD8+ T cell responses in the peripheral blood and liver of chronically HCV infected patients, we found that virus-specific CD8+ T cell responses were strongly enriched in the liver. Many responses were only detectable in the liver; however, few responses were limited to the peripheral blood (Neumann-Haefelin *et al*, unpublished results). Therefore, it is possible that a defective homing of HCV-specific CD8+ T cells or their rapid deletion in the liver also contributes to T cell failure and viral persistence in a subset of patients.

ROLE OF THE HOST HLA CLASS I BACKGROUND

CD8+ T cells recognize antigens presented by human leukocyte antigen (HLA) class I molecules. It has, therefore, been suggested that different HLA class I alleles are associated with differential outcomes of HCV infection, e.g. viral clearance versus persistence^[109]. Analysis of the role of HLA alleles in viral infections are hindered by multiple factors, including the wide polymorphism of HLA alleles, their association with other genetic characteristics e.g. in certain racial backgrounds (founder effect)^[110], and the variability of viral strains (genotypes, quasispecies *etc.*). However, an Irish cohort of women accidentally infected with HCV (genotype 1b) from a single source more than 20 years ago, represents a homogeneous group in which the role of HLA alleles in the outcome of HCV infection could be studied^[111]. Importantly, the HLA class I alleles A3, B27 and Cw*01 were significantly associated with viral clearance, while B8 was associated with viral persistence. Interestingly, the strongest protective effect was observed for HLA-B27: 80% (12/15) of B27 positive women were able to clear the infection spontaneously, while only a minority developed chronic infection. We recently identified an immunodominant HLA-B27 restricted HCV-specific CD8+ T cell epitope, which was targeted in the vast majority (5/6) of B27 positive Irish women who had cleared the infection^[99]. Of note, such a clear dominance of a single epitope-specific CD8+ T cell response has not been described for any other HLA allele in HCV infection. In chronically infected patients, still a remarkable percentage of patients (3/8) recognized the epitope. However, most B27 positive chronically infected patients had evidence of viral escape within the otherwise conserved viral region containing this epitope. Thus, a single immunodominant HLA-B27 restricted CD8+ T cell epitopes might mediate both, clearance of HCV infection in the majority of B27 positive individuals, and viral evolution associated with viral persistence in a minority of individuals.

Strikingly, a very similar frequency of viral escape variation was demonstrated within an immunodominant HLA-B8 restricted CD8+ T cell epitopes^[90]. This indicates that in the background of both, a protective HLA allele (B27) as well as a detrimental HLA allele (B8) the principle mechanisms of CD8+ T cell failure might be the same. More precise details such as viral fitness cost associated with the respective escape variation^[94,95], T cell receptor (TCR) diversity^[102] or heterologous immunity^[112,113]

may play an additional critical role in the definition of a protective HLA allele.

Two other population studies in more heterogeneous cohorts showed an association between HLA-B57 and HCV clearance in Caucasian as well as African Americans and West Africans^[114,115]. Interestingly, HLA-B27 and HLA-B57 have also been shown to be protective in HIV infection, being strongly associated with low viral titers, low CD4+ T cell decline, and long-term non-progression of the disease^[116]. Thus, a picture emerges that the same HLA alleles may confer protection in different clinical infections, indicating that similar mechanisms of viral control and disease progression apply in these infections. A better understanding of the host-virus interactions leading to different clinical outcomes of HCV infection will be important not only to understand the mechanisms of viral clearance and persistence, but also for the development of new antiviral vaccine strategies.

CONCLUSION

CD8+ T cells are generally thought to be the major effectors in viral infections; however, multiple host and viral mechanisms contribute to the failure of antiviral CD8+ T cell responses and viral persistence in the majority of HCV infected patients. In the last few years compelling progress has been achieved in the understanding of these mechanisms (compare with^[117]). These findings are not only important for the development of successful immunoprophylactic approaches, but may also be more directly adopted for immunotherapeutic interventions.

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TOPIC HIGHLIGHT

Robert Thimme, MD, Professor, Series Editor

Memory CD8+ T cell differentiation in viral infection: A cell for all seasons

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Abstract

Chronic viral infections such as hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV) are major global health problems affecting more than 500 million people worldwide. Virus-specific CD8+ T cells play an important role in the course and outcome of these viral infections and it is hypothesized that altered or impaired differentiation of virus-specific CD8+ T cells contributes to the development of persistence and/or disease progression. A deeper understanding of the mechanisms responsible for functional differentiation of CD8+ T cells is essential for the generation of successful therapies aiming to strengthen the adaptive component of the immune system.

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Key words: Viral infection; Hepatitis C virus; Memory T cell phenotype; Differentiation

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INTRODUCTION

The adaptive immune response is characterized by the

ability to respond specifically and quickly to antigens that the host has encountered previously. Virus-specific CD8+ T cells critical in this response can be divided into naïve, effector and memory CD8+ T cells. In the strictest sense, the memory response should be maintained in the absence of antigen, poised to respond quickly, specifically, and with sufficient amplitude to protect the host from repeated infection by a previously encountered pathogen^[1,2]. The ability to survive in the absence of antigen differentiates memory T cells from effector cells that exist at the peak of the immune response, while antigen is present. However, in the context of viral infection, differentiation from effector T cells into memory cells may differ depending on the nature of the pathogen.

Many viral infections are acutely cleared by the immune response, whereas others result in persistent infection and are associated with altered differentiation of host T cells. For example, antigen-specific CD8+ T cells isolated from persons with resolved infections such as influenza (Flu) or respiratory syncytial virus (RSV) clearly represent functional memory. With “latent” infections such as Epstein-Barr virus (EBV) or cytomegalovirus (CMV) where low level of virus may still be intermittently present, a strict definition of memory may not be appropriate for these antigen-specific CD8+ T cells^[2]. In fact, van Leeuwen *et al*^[3] proposed to classify these cells as “resting vigilant effector cells” due to their ability to continuously control the latent virus. Currently, neither the frequency nor mechanism of re-encounter with antigen after resolved, primary EBV or CMV infection is well understood. For chronic viral infections such as HIV, hepatitis B virus (HBV) and hepatitis C virus (HCV), isolated antigen-specific CD8+ T cells also may not represent true memory T cells, but rather effector-type cells, or perhaps a population comprising effector and memory cells.

The criteria used to define the differentiation of virus-specific T cells are complicated. In mouse models of viral infection, time after experimental infection is often used to delineate effector T cells from memory T cells. For example, after experimental LCMV infection and clearance, d 40+ has been set as a time at which stable memory CD8+ T cells can be isolated, with many memory qualities being acquired between d 8 and 21 post infection^[4,5]. In addition to time-after-infection, a number of surface antigen markers have been used to differentiate effector CD8+ T cells from memory CD8+ T cells, and to differentiate subsets of memory cells that possess varied levels of differentiation and function. Activation markers

such as CD38 and HLA-DR are often used to identify effector cells which up-regulate these markers during an immune response and which are generally not expressed on quiescent memory cells. Memory cells that re-encounter antigen re-express these activation markers^[6]. Surface expression of the IL-7 receptor- α (CD127) is also used to differentiate effector from memory cells. Naïve and memory cells require an ability to survive and proliferate in the absence of antigen, a process called “homeostatic proliferation”. Cytokines, such as IL-7, and signaling through CD127 are critical in this process^[7]. Naïve cells exposed to antigen decrease expression of CD127 on their cell surface (effector phenotype). Once antigen is cleared, CD127 is re-expressed on memory cells and enables their maintenance. This re-expression characterizes them as memory cells. However, CD127 expression may not be a fool-proof marker of memory, since some subsets of CD8+ T cells identified as memory CD8+ T cells express only low levels of CD127, particularly in latent/chronic viral infections. Whether these cells represent true memory in the strict sense of the definition, or rather represent a population of effectors amidst memory cells is not yet fully elucidated. Finally, immediate cytolytic activity may be the best way to differentiate effector from memory T cells, though some memory cell types also possess immediate cytolytic activity albeit at lower levels^[8].

Improved understanding of functional memory CD8+ T cell development and the identification of unique phenotypic markers of memory CD8+ T cells could be helpful in vaccine development for viral infections where adaptive immune responses play an important role in control and/or clearance (HIV, HBV, HCV). In theory, if efficient memory T cell responses could be induced by vaccination, protective immunity could be achieved. Additionally, further enlightenment into the transition from effector CD8+ T cell into memory CD8+ T cell could aid in the discovery and use of immune modulating therapies that might heighten the response to vaccination. As an example, adoptive transfer studies in mice of antigen-specific CD8+ CD127+ T cells taken during the effector phase of the immune response to LCMV infection have identified CD127 as a marker of CD8+ T cells able to control virus upon re-infection^[5,9]. Therefore, it can be hypothesized that vaccine induction of CD127 expression on CD8+ T cells may be beneficial and should be a goal of any effective vaccine for chronic viral infections. In this review, we will summarize current knowledge of antiviral CD8+ T cell differentiation with a focus on persistent infections such as HCV.

MODELS OF MEMORY CD8+ T CELL DIFFERENTIATION

Several models have been proposed to describe the differentiation of CD8+ T cells from naïve cells to memory cells^[10,11]. In the “linear” or “progressive” model, naïve cells undergo an effector T cell phase prior to developing into memory cells, and all memory T cells are direct descendents of effector cells. This model posits that memory T cells do not develop until antigen is

markedly reduced or eliminated. In the “divergent model”, a stimulated naïve T cell gives rise to either an effector or memory T cell. In this model, naïve T cells can directly give rise to memory T cells without going first through an effector phase. The “decreasing-potential” model accounts for scenarios where antigen persists after primary infection and posits that naïve T cells differentiate into effector cells first. If antigen is cleared early after infection, functional memory T cells develop. If antigen persists, the function of the effectors is sequentially impaired and memory CD8+ T cell development is compromised. Eventually, persistent antigen leads to a non-functional effector cell and eventual cell death by apoptosis. As in the linear model, functional memory cells do not develop until antigen is cleared.

Subsets within the memory CD8+ T cell compartment have been segregated based on markers other than CD127. Sallusto *et al.*^[12] utilized the expression of the lymph node homing receptor, CCR7, and a transmembrane phosphatase involved in T cell signaling, CD45RA, to distinguish central memory (CCR7+ CD45RA-) and effector memory (CCR7-CD45RA- and CCR7-CD45RA+) CD8+ T cell populations^[12]. Central memory cells were characterized by rapid proliferation after antigenic stimulation, while effector memory cells were more capable of immediate effector functions^[12]. In theory, central memory cells are most capable of surveying lymph nodes and responding to antigen with enhanced proliferative capacity, while effector memory cells are more capable of migrating into tissues and exacting immediate effector functions. Appay *et al.*^[13] have proposed another model of CD8+ T cell differentiation during chronic or persistent infection. Studying antigen-specific CD8+ T cells during several different viral infections, they hypothesized that there is a progressive memory differentiation based on differential expression of CD27 and CD28 co-stimulatory molecules^[13]. They defined early (CD27+, CD28+), intermediate (CD27+, CD28-) and late memory CD8+ T cell subsets of virus-specific cells^[13]. The early subset had the greatest proliferative capacity while the intermediate and late subsets had progressively greater cytotoxic potential^[13]. Furthermore, the late subset also expressed CD57, a marker of replicative senescence^[13].

Recently, Romero *et al.*^[14] combined the phenotypic markers used by Sallusto *et al.*^[12] and Appay *et al.*^[13] to further dissect the memory CD8+ T cell pool. They identified four subsets within the effector memory (CD45RA-, CCR7-) pool based on differential staining of CD27 and CD28. Interestingly, the different subgroups differed not only phenotypically, but showed a progressive reduction in telomere length coinciding with a progressive increase in cytotoxic molecules (granzyme B, perforin). Their model lends support to the idea that there is a progressive up-regulation of cytolytic activity and a stepwise loss of CCR7, CD28 and CD27 during the differentiation process. They showed that CD8+ T cells specific for a resolved infection (Flu) consisted of both a central memory (CCR7+, CD45RA-, CD27+ and CD28+) population and a sub-population of effector memory cells termed EM1 (CCR7-, CD45RA-, CD27+ and CD28+).

They hypothesized that this effector memory population, which has only a low expression of the lymphocyte homing receptor, CCR7, confers memory functions and provides surveillance in peripheral tissues^[14].

ACUTE VIRAL INFECTION

Effector phase

In the classic understanding of an adaptive T cell immune response, there is an initial massive expansion of antigen-specific T cells, followed by a period of marked contraction as the pathogen is cleared. This period of expansion and contraction can be referred to as the “acute” or “effector” phase of the immune response. Exceptions to this paradigm likely exist following chronic or latent infections, for example, in CMV infection of humans, where the contraction phase may be more limited^[3]. During this process of expansion and contraction, functional memory cells are formed and persist to protect the host from future infection. Upon infection of mice with LCMV, rare naïve T cells specific for cognate antigen increase exponentially within secondary lymphoid tissues^[15,16]. The responding T cells in this “clonal burst” clear the infection *via* dissemination to non-lymphoid tissues (common sites of infection), the secretion of anti-microbial cytokines such as interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α), and direct lysis of infected host cells^[17-19]. Interestingly, no antigenic stimulation is needed after the initial clonal stimulation, meaning that daughter-cell expansion and differentiation into a memory population occurs in an antigen-independent manner^[20-22]. However, mediation of effector functions, such as cytokine production and killing, is dependent upon contact with antigen bearing targets. In situations where virus is successfully eliminated, > 90% of effector T-cells undergo apoptosis (contraction), and a small population of CD127+ surviving cells undergo further differentiation from an activated phenotype to a resting phenotype to generate a long-lived memory pool that is ready to respond more rapidly upon secondary infection^[5,10]. This pool is maintained in the absence of antigen, and is characterized by IL-7 and IL-15-dependent homeostatic proliferation resulting in relatively constant numbers of CD8+ T cells^[23-25]. During the progression from naïve to effector to memory cells, the homing potential of these cells changes. Upon differentiation to effectors, CD8+ T cells down-regulate lymphoid homing molecules such as CD62L and CCR7, and begin to migrate to nonlymphoid effector sites. Eventually, these lymphoid homing molecules are gradually up-regulated, giving cells the ability to home to lymphoid tissues. This dichotomy of homing potential has prompted researchers to further define memory cells into effector memory or central memory subsets, with the latter having lymphoid homing potential^[26,27].

Much of what we know about memory T cell differentiation is from murine models of infection since identifying humans in the acute phase of viral infection is often difficult, and time from acquisition of infection is rarely precisely known (exceptions described below for accidental infection). Additionally, for some viral infections the acute phase of infection may be relatively

short in duration, while for other infections the “acute” phase may last for weeks to months (HCV). Nevertheless, studying the phenotype of antigen-specific CD8+ T cells during different human acute viral infections has shown that there is a remarkable similarity in terms of surface marker expression and function. During acute infectious mononucleosis, EBV-specific CD8+ T cells show massive expansion (up to 44% of total CD8+ T cells in peripheral blood), and the majority express the activation markers, HLA-DR and CD38, and the memory marker CD45RO (also CD45RA low), but have down-regulated the lymphocyte homing molecule CD62L^[28]. CD28 expression on EBV-specific cells has been shown to range from 9% to 86% depending on the donor, with CD57 expression ranging from 2% to 37%. Appay *et al*^[13] have also shown that during acute infection, some EBV-specific CD8+ T cells express the proliferation marker Ki67. Additionally, these cells were prone to apoptosis since they expressed minimal levels of the anti-apoptotic factor Bcl-2^[13].

In an individual with acute CMV infection, Carmichael *et al*^[29] showed that at the peak of clinical symptoms (3 wk after symptom onset), 80% of CMV-specific CD8+ T cells were CD45RO high, CD28 negative, and CCR7 negative. Studying primary CMV infection after kidney transplantation in humans, Gamadia *et al*^[30] showed that CMV-specific CD8+ T cells evaluated at wk 31 after first positive CMV PCR, were nearly all CD27 positive, but with mixed expression of CD28 (54%). Nearly all expressed CD45RO (minimal CD45RA), and most were CCR7 negative (91%)^[30]. They expressed significant levels of Ki67 (78%), granzyme B (93%) and perforin (100%)^[30]. Few of these acute CMV-specific CD8+ T cells expressed CD127 (1%)^[30].

Other viruses known to cause more persistent infections including HIV, HBV, and HCV, still show characteristic effector T cell development in the early stages after acute infection. For example, in primary HIV infection, nearly all HIV-specific CD8+ T cells expressed CD38 and approximately 11%-41% were Ki67 positive^[13,31]. There was minimal Bcl-2 expression, which correlated inversely with CD38 expression^[13]. Very early after HIV infection, the majority of HIV-specific CD8+ T cells also expressed CD27 and approximately 40% expressed CD28^[31].

Evaluation of 5 persons with acute HBV infection showed that the majority of HBV-specific CD8+ T cells were HLA-DR positive (92%-98%) and CD45RO positive (95%-100%), and most were CCR7 and CD45RA negative, again consistent with an effector phenotype^[32]. The majority expressed CD27, and similar to HIV, 40%-50% expressed CD28^[32]. The frequency of CD127 expression on these antigen-specific cells was also very low^[33].

The phenotype of HCV-specific CD8+ T cells from the peripheral blood of patients evaluated during the acute phase of HCV infection also showed the characteristic expression patterns of effector T cells^[13,34]. Studying 9 patients with acute HCV infection, Lechner *et al*^[34] demonstrated that during acute infection, the activation marker CD38 was up-regulated on HCV specific CD8+ lymphocytes from all patients irrespective of their clinical outcome. By wk 20 after the acute phase, there was a loss

of expression of this activation marker on HCV-specific CD8+ T cells^[34]. Similarly, HLA class II was elevated early during infection and decreased over time^[34]. Studying five health care workers exposed to HCV via accidental needle stick, Thimme *et al.*^[35] demonstrated that HCV-specific CD8+ T cells detectable from a patient with spontaneous viral clearance expressed CD38 on wk 8 and 10 after infection, but by wk 12 and thereafter they were CD38 negative^[35]. CD38 expression correlated with hepatitis, as measured by ALT level^[35]. Interestingly, these activated cells were unable to produce IFN- γ when stimulated by cognate peptide *in vitro*, and the appearance of HCV-specific, IFN- γ producing CD8+ T cells coincided with the disappearance of CD38 expression^[35]. Lechner *et al.*^[36] studied acute infection in one patient, and noted that in addition to increased expression of CD38 and HLA class II on HCV-specific CD8+ T cells early during infection, CCR5 expression was also maximal during the first 20 wk. During the acute phase, CD127 expression was minimal on HCV-specific CD8+ T cells^[37,38].

In summary, for the majority of these viral infections, during the acute phase, there is an increase in expression on antigen-specific CD8+ T cells of activation and proliferation markers, and a decrease in lymph node homing molecules and CD127 expression. Cytolytic molecules are increased but anti-apoptotic factors are decreased. In general, CD27 expression remains present and there are intermediate levels of CD28 expression.

VIRAL PERSISTENCE OR CLEARANCE

Memory phase

While acute models of infection in mice have provided much phenotypic and functional insight into memory CD8+ T cell generation, chronic models of infection have shed light on the inadequacies of cellular responses, and how the resulting persistence of antigen load can affect the differentiation and function of these antigen-specific cells. Indeed, the course of memory CD8+ T cell differentiation during chronic infections can vary greatly from that which is seen in acute infections, including unique tissue distribution of antigen-specific T cells, dominance of T cell populations that normally have subdominant specificities, and even gradual exhaustion or deletion of entire T cell populations from the repertoire^[25]. The hallmark of differentiation in chronic infection is a stepwise loss of T cell effector functions that becomes more severe as time progresses, as opposed to the gain of effector functions that is seen in acute infections^[39]. This “exhaustion” can be broken into several categories, corresponding to the severity of impairment of effector function and proliferative potential. Initial antigen stimulation leads to CD8+ T cells that are functionally competent in that they can produce IFN- γ , TNF- α , IL-2, are cytolytic, and have robust proliferative capacity. However, if virus persists, these cells become partially exhausted, losing their ability to lyse target cells and produce IL-2 first, followed by decreased TNF- α production^[40,41]. Interestingly, cells that are partially exhausted may still have the ability to proliferate and produce IFN- γ , albeit with reduced efficiency. As antigen persists, cells may become fully

exhausted, completely losing both effector functions and the ability to proliferate^[39,41,42]. It has also been shown in chronic LCMV infection that deletion of antigen-specific CD8+ T cells can occur if antigen load is both extremely high and persistent^[41,43]. Additionally, CD4+ T cells play an important role in the chronic exhaustion of CD8+ T cell responses, both throughout infection and in the priming of cells during the acute phase of infection. Unlike antigen load, CD4+ T cell help is directly related to the functionality of the CD8+ T cell effector response: the absence of this help leads to a more rapid and severe progression to the exhausted phenotype^[25].

Unlike the similarities in the range of phenotype of antigen-specific cells seen during the acute phase of different viral infections in humans, the phenotype of antigen-specific cells isolated during different latent/chronic viral infections is more diverse. In the chronic phase of HCV infection, Lechner *et al.*^[36] were unable to detect the activation markers CD38 or HLA class II expression on any HCV-specific CD8+ T cells. This is different from HIV infection, where during chronic infection a proportion of HIV-specific CD8+ T cells in the blood expressed CD38 and HLA-DR^[44]. Appay *et al.*^[13] compared HIV, CMV, EBV and HCV-antigen specific CD8+ T cells taken from blood during the latent/chronic stage of infection. Though the majority of antigen-specific CD8+ T cells for these viral infections had all lost evidence for activation (minimal CD38 expression) and proliferation (minimal Ki67 expression), and had up-regulated the survival factor, Bcl-2, the expression of CD27 and CD28 differed^[13]. The majority of HCV-specific CD8+ T cells expressed both CD27 (90%) and CD28 (90%), while EBV-specific CD8+ T cells had comparable levels of CD27 expression but lower CD28 expression (60%). HIV-specific CD8+ T cells had relatively high levels of CD27 expression (80%), but very low levels of CD28 expression (10%). Finally, the majority of CMV-specific CD8+ T cells had relatively low expression of both CD27 (30%) and CD28 (20%). This comparison of differentiation in the chronic/latent phase of multiple infections prompted the authors to label EBV- and HCV-specific CD8+ T cells “early”, HIV-specific CD8+ T cells “intermediate” and CMV-specific CD8+ T cells as “late” differentiated. CCR7 expression was low on HIV-, CMV- and EBV-specific CD8+ T cells^[13], whereas others have shown that peripheral HCV-specific CD8+ T cells are largely CCR7+^[45,46]. Others have shown that EBV-specific CD8+ T cells may be better represented as a mixture of effector memory and central memory cells (or early and late differentiation states) as delineated by differential staining of CCR7 and CD45RA^[47].

For cleared viral infections such as influenza and RSV, antigen-specific CD8+ T cells resembled these HCV and EBV-specific cells in that they were mostly CD27+ and CD28+^[48,49]. RSV-specific CD8+ T cells were mostly CCR7 negative (92%)^[49] in contrast with influenza, where a greater frequency of specific CD8+ T cells were CCR7 positive^[49]. The range of CCR7 expression on both RSV and Flu, however, were broad among different patients in this study (0%-71% for RSV and 0%-57% for Flu)^[49].

Similar to the mouse, CD127 expression appears to be

Table 1 Phenotype of antigen-specific CD8⁺ T cells found in blood and tissue during different viral infections

Virus	Peripheral blood phenotype	Tissue	Tissue phenotype
Flu	CD27 ⁺ CD28 ⁺ CCR7 ⁺ CD127 ⁺	Lung	CD27 ⁻ CD28 ⁻
RSV	CD27 ⁺ CD28 ⁺ CCR7 ⁻ CD127 ⁺	Lung	CD27 ⁻ CD28 ⁻
EBV	CD27 ⁺ CD28 ⁺ CCR7 ⁻ CD127 ⁻	Tonsil	↑ CD38 ↑ CCR7 ↑ CD127
CMV	CD27 ⁻ CD28 ⁻ CCR7 ⁻ CD127 ⁻ CD45RA ⁺	Tonsil	↑ CD127
HIV	CD27 ⁺ CD28 ⁻ CCR7 ⁻ CD127 ⁻	Rectum	CCR7 ⁻ CD127 ⁻ ↓ perforin
HCV	CD27 ⁺ CD28 ⁺ CCR7 ⁺ CD127 ⁺	Liver	↑ CD69 CCR7 ⁻ CD127 ⁻
HBV	CCR7 ⁺ CD127 ⁺	Liver	↑ HLA-DR

Arrows indicate increase or decrease relative to expression in blood.

associated with memory phenotype and viral clearance of some infections in humans. CD8⁺ T cells isolated from persons with resolved viral infections such as influenza or RSV expressed high levels of CD127^[50]. Similarly, longitudinal analysis of 6 patients with acute resolving HBV infection showed that after viral clearance, CD127 expression increased markedly and correlated with the loss of CD38 and PD-1 expression, acquisition of CCR7 expression and enhanced proliferative capacity^[33]. In contrast, for latent infections (EBV, CMV) and persistent viral infections (HIV) low levels of CD127 have been noted on virus-specific CD8⁺ T cells^[50-52]. However, in EBV infection CD127 expression was higher on cells specific for latent epitopes compared with lytic epitopes^[47]. When we evaluated CD127 expression on peripheral HCV-specific CD8⁺ T cells from patients with chronic HCV infection, we were surprised to find that in the majority of patients, nearly all expressed high CD127 expression despite the high level of antigen present^[53]. This phenotype is reminiscent of resolved infection such as influenza. However, Bengsch *et al*^[37] identified two subsets of patients with chronic HCV: One with HCV-specific CD8⁺ T cells predominantly expressing low levels of CD127 and the other expressing higher levels of CD127. Interestingly, the CD127 low group also had higher level of CD38⁺ frequencies and lower level of CCR7 expression hinting that in this group, re-activation of these cells may have induced the down-regulation of CD127.

Table 1 summarizes the phenotype of antigen-specific cells from the peripheral blood of persons with resolved, latent and chronic viral infections. There is substantial heterogeneity between the different viral infections, as noted in the table.

MECHANISMS OF VARIED MEMORY DIFFERENTIATION IN PERSISTENT OR LATENT INFECTION

Currently, the explanation for the variation in differentiation phenotype seen in the setting of different viral infections is not completely understood, but a number

of hypotheses exist. Clonal expansion, effector functions and memory formation require three signals during the immune response: antigen (signal 1), co-stimulation (signal 2) and cytokine (signal 3)^[54]. Alterations in these signals could contribute to alterations in CD8⁺ T cell phenotype and function. Optimal expansion and function of naïve CD8⁺ T cells required antigen and co-stimulation to be present for approximately 36 h and cytokine (IL-12) present from about h 12 to h 60^[54,55]. These signals launch a complex program of proliferation and differentiation. Given this “autopilot” response^[56], the quality and context of the original signal may have a critical impact on subsequent T cell differentiation^[57]. Differing numbers of naïve precursor cells, antigen loads, cytokine milieu and primary location of infection (lymph node, gut, lung, liver) seen with the different viral infections would offer additional possible explanations for the diversity of these CD8⁺ T cells. In line with this hypothesis, Marzo *et al*^[58] have shown that initial precursor frequency is critical in determining effector and central memory CD8⁺ T cell differentiation. Increasing the input number of antigen-specific CD8⁺ T cells during the primary immune response resulted in increasingly larger populations of central memory cells. Furthermore, effector memory CD8⁺ T cells generated from high or low numbers were fundamentally different, in that cells generated from low initial naïve T cell precursor frequency were unable to interconvert and re-express CD62L^[58].

Even after establishment of persistent viral infection, initial events in naïve T cell proliferation and differentiation likely continue to play a critical role in the varied CD8⁺ T cell differentiation phenotypes that are seen in different viral infections. Vezys *et al*^[59] have recently shown that during persistent viral infection, there is a continuous recruitment of naïve T cells that contributes to the heterogeneity of antiviral CD8⁺ T cells. In their model, antigen-specific memory T cells were not maintained in the presence of antigen without replenishment from thymic emigrants. By induction of a partial hematopoietic chimerism in persistently infected mice using busulfan and congenic bone marrow, they showed that there were variations in the expression of CD27, CD62L, CD127 and bcl-2 between cell populations primed at different times^[59], and that heterogeneity in the memory population was related to this. Their study certainly complicates the current models of T cell differentiation described above, and highlights the dynamic nature of chronic viral infections, even despite relatively stable levels of viral load measured in the peripheral blood of patients with chronic HBV, HCV or HIV.

After initial viral infection and programming of naïve CD8⁺ T cells, downstream events may also impact on CD8⁺ T cell differentiation. Wherry *et al*^[60] have recently shown that in the setting of chronic infection, it is viral antigen and extensive division of virus-specific CD8⁺ T cells that maintains cell numbers, in marked contrast to the slow turnover seen during homeostatic proliferation of memory T cells from cleared viral infection. These cells, in the context of persistent infection, would be expected to display a different phenotype from those isolated from cleared infection, given differences in cell turnover. After

naïve CD8+ T cells are activated and become effector cells, they shortly lose the ability to produce IL-2 upon re-encounter with antigen and co-stimulation, a condition referred to as activation induced non-responsiveness (AINR)^[54]. IL-2 (provided by CD4+ T cells) is able to reverse this state. Co-stimulation via molecules other than CD28, such as OX40 (CD134), and 4-1BB (CD137) may also be critical in providing a stimulation for continued expansion once AINR develops^[54]. Interestingly, we have shown in chronic HCV infection that the co-inhibitory molecule, PD-1, is highly expressed on HCV-specific CD8+ T cells and on total CD8+ T cells at the site of infection in the liver^[53]. It is possible, that a lack of adequate co-stimulation and/or vigorous co-inhibitory signals prevent reversion of AINR and explains the exhaustion and CD8+ T cell deletion seen during this chronic viral infection. In addition to loss of IL-2 production, chronic antigen stimulation eventually also leads to characteristic progressive loss of TNF- α and finally IFN- γ production by cytotoxic CD8+ T cells^[39,57,61]. Since cytokines alone have been shown to induce T cell differentiation^[62] a lack of available cytokine during persistent infection may also explain altered differentiation patterns seen with different viral infections. In a transgenic mouse model, HBV-specific CD8+ T cells are rapidly induced to produce IFN- γ when they enter the liver, but are then rapidly suppressed despite continued antigen^[63]. Suppression of cytokine production was mediated by the co-inhibitor, PD-1, since blockade of PD-1 led to a delay in the suppression of IFN- γ producing cells^[63].

The potency of the pathogen and the antigen load may also influence memory differentiation. In a mouse model of infection, reducing the stimulation of CD8+ T cells by using an attenuated pathogen led to primarily a central memory subset, while infection with a more virulent pathogen led to effector cell development^[64]. Tussey *et al*^[65] compared the phenotype of HIV-specific CD8+ T cells in the setting of viral control (using anti-retroviral medication) and in the setting of uncontrolled viremia. They showed that the phenotype of these antigen-specific cells differed based on the level of viremia, and hypothesized that the level of antigen burden determined the differentiation state. Similarly, Papagno *et al*^[66] showed that in HIV infection excessive levels of antigen stimulation as determined by level of HIV disease progression lead to a progressive differentiation toward a state of replicative senescence. Given these studies, the importance of the quantity and duration of persistent antigen would seem to be very important factors leading to varied differentiation programs. However, in chronic HCV infection, antigen burden is large, with viral loads often on the order of 10^6 - 10^7 , yet as previously described, differentiation of HCV-specific CD8+ T cells is more limited and these cells have been termed “early”. Viral escape is one possibility for the lack of progressive differentiation, yet when we sequenced HCV isolated from the peripheral blood at the epitope specific for the tetramer used to identify these “early” differentiated HCV-specific CD8+ T cells, no mutations were seen^[53]. Another possibility to explain variation in differentiation phenotype is differences in innate signals among different infections,

and differences in these signals occurring at early and late time-points during persistent infection^[67]. This could be particularly relevant for HCV infection, since it has been shown that the NS3-4A serine protease of HCV degrades the adapter molecule, Cardif^[68], and thereby interferes with the RIG-I mediated process of innate recognition of dsRNA. The “early” differentiated phenotype of HCV-specific CD8+ T cells during chronic infection could be a reflection of this impairment in innate signaling. Pulendran *et al*^[67] has also speculated that during the early stages of an immune response, highly stimulatory DC subsets might deliver strong TCR signals favoring effector T cell differentiation, while at later stages, a milder form of T cell stimulation by less stimulatory DC subsets could favor the development of central memory T cells.

Location, location, location

Finally, a perhaps somewhat overlooked cause for differences in memory phenotype in different viral infections may be related to the anatomic location of the different viral infections, and to discrepancies between the active site of infection (liver, lung, *etc.*) and the site from which cells were obtained for study. Mice infected with vesicular stomatitis virus (VSV) developed memory cells with functional differences in cytolytic activity based on their location in either tissue (lung, liver, small intestine) or secondary lymphoid organs, highlighting the importance of anatomic location in type of isolated memory cell subset^[69]. Masopust *et al*^[70] have also shown that virus specific intraepithelial lymphocytes in the gut resemble neither central nor effector memory CD8+ T cells isolated from spleen or blood by almost all properties examined, including effector function, differentiation, homing receptors and cell cycle. In fact, memory CD8+ T cells changed phenotype upon change of location^[70].

As noted above, we were surprised to find a phenotype of HCV-specific CD8+ T cells that resembled the phenotype of a resolved infection (Flu) in terms of high expression of CD127, CCR7, CD28 and CD27. However, analysis of antigen-specific cells at the site of active infection, the liver, revealed that nearly all of the antigen-specific CD8+ T cells displayed a different phenotype, with low CD127 expression^[37,53]. Similarly, Accapezzato *et al*^[71] have shown that HCV-specific CD8+ T cells isolated from liver express markedly lower CCR7 expression in comparison with peripheral blood. HCV-specific CD8+ T cells in the liver also displayed an activated phenotype with elevation of expression of the early activation marker CD69^[72]. We hypothesize that a lack of exposure to antigen by the peripheral CD8+ T cells enabled up-regulation of CD127 and CD62L and memory formation, since exposure to cognate peptide *in vitro* induced a down-regulation of CD127 on these same cells. The effector-like cells isolated from the liver were likely actively involved in the immune response occurring at the site of infection. Similar to our findings with chronic HCV infection, peripheral blood HBV-specific CD8+ T cells expressed high levels of CD127 during chronic HBV infection despite high levels of antigen load^[73]. Analysis of HBV-specific CD8+ T cells in the liver of patients with resolving HBV infection showed that a greater frequency

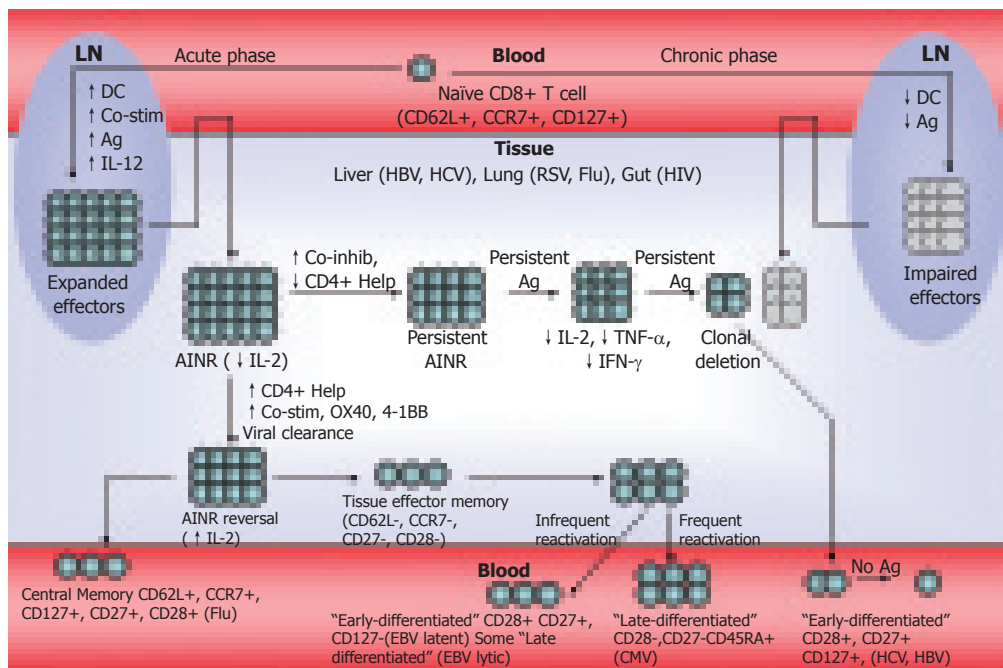


Figure 1 Tissue-specific model of antigen-specific memory CD8+ T cell differentiation.

were activated, as demonstrated by HLA-DR expression, in comparison with the peripheral blood^[74]. It will be interesting to further analyze the phenotype of these HBV-specific cells at the site of infection.

In addition to the liver, human memory CD8+ T cells at other locations are clearly influenced by the anatomic site where they reside. De Bree *et al*^[75] have compared Flu and RSV-specific CD8+ T cells in the lungs and peripheral blood. They found that the lung contained markedly higher frequencies of Flu- and RSV-specific cells compared with the peripheral blood^[75]. A substantial percentage of these lung residing antigen-specific CD8+ T cells had progressed to a relatively late differentiation phenotype with low expression of CD28 and CD27^[75]. EBV-specific CD8+ T cells isolated from the tonsils of long-term carriers were more likely to express the activation marker CD38 and CD103, an integrin induced by epithelium-derived cytokine TGF- β ^[76]. Both EBV (lytic)- and CMV-specific CD8+ T cells in the tonsils were shown to have increased CD127 expression in comparison with peripheral blood^[47]. In HIV infection, rectal HIV-specific CD8+ T cells expressed a similar effector memory phenotype as in the peripheral blood (CCR7-), but expressed minimal perforin, unlike in the peripheral blood where as many as 23% of Gag-specific CD8+ T cells expressed perforin^[77]. Table 1 summarizes the phenotype differences of viral-specific CD8+ T cells between peripheral blood and tissue (liver, lung, tonsil, gut). Clearly, there is much to be learned about memory T cell differentiation by further evaluation of T cells residing in tissues other than peripheral blood.

A MODEL OF TISSUE DEPENDENT MEMORY DIFFERENTIATION

So, how might the variation in differentiation phenotypes of antigen-specific CD8+ T cells from different viral infections and from different anatomic locations look? A

schematic is shown in Figure 1. During the acute phase of infection, naïve CD8+ T cells surveying the lymph node (LN) may encounter antigen presented by activated dendritic cells (\uparrow DC) in the context of high antigen concentration (\uparrow Ag), significant co-stimulatory signal (\uparrow Co-stim), and cytokine (IL-12 or type I interferon) (\uparrow IL-12). This leads to an expansion of competent effectors that have decreased lymph node homing receptors (CD62L, CCR7) and migrate to sites of infection (e.g. liver, lung, gut). These cells lose the ability to produce IL-2 (activation induced non-responsiveness, AINR)^[54]. With proper CD4+ T cell help (\uparrow CD4+ Help) or other co-stimulatory signals, such as via 4-1BB (\uparrow Co-stim, OX40, 4-1BB), these cells maintain function and succeed in clearing virus. One population of cells forms central memory cells (CD62L+, CCR7+, CD127+, CD45RA-) that have up-regulated lymph node homing molecules and are easily detected in the peripheral blood. A second population of effector memory cells is maintained in the tissue (perhaps by homeostatic mechanisms) (CD62L-, CCR7-, CD27-, CD28-). For the latent viruses, EBV and CMV, we hypothesize that differences in the frequency and/or location of re-activation offers an explanation for the "early" vs "late" phenotype. Perhaps CMV reactivation or even low-level persistence occurs at peripheral sites, and sampling of antigen-specific CD8+ T cells from the peripheral blood reflects this (late differentiation). EBV reactivation may be less frequent (and not persistent) and, therefore, a phenotype similar to a resolved infection such as Flu or RSV is sometimes seen (though CD127 expression is diminished). EBV reactivation may also lead to a population of more differentiated cells specific for the lytic epitope. If AINR cannot be reversed, as might be the case with a lack of CD4+ T cell help (\downarrow CD4+ Help) or via enhanced co-inhibitory signals (\downarrow Co-inhib), there is a progressive loss of function of virus-specific CD8+ T cells (\downarrow IL-2, \downarrow TNF- α , \downarrow IFN- γ) eventually leading to clonal deletion. For HCV and HBV infections,

perhaps at the site of infection in the liver, where antigen is present and concentrated, persistent antigen maintains a population of effector-like cells. As Wherry *et al.*^[78] have described in chronic LCMV infection, this cell population would not be maintained without antigen present. These effector cells would be expected to express low levels of CD127 and lymph node homing molecules. Additionally, naïve cells encounter antigen during the chronic phase; however in this setting, DCs are less stimulatory (\downarrow DC) and antigen is lower (\downarrow Ag) than what is seen during acute infection. The resulting impaired effectors contribute to the pool of antigen-specific cells. In the periphery, HCV- and HBV-specific CD8⁺ T cells are not maintained by persistent antigen, but rather proceed to form functional memory T cells and are maintained via homeostatic signals. We and others have found that peripheral blood HCV-specific CD8⁺ T cells expressing CD127 have a capacity for proliferation upon ex vivo antigen encounter similar to other functional memory T cells. For HIV infection (and persistent LCMV infection), where antigen is located in the periphery, these cells are maintained only by the presence of antigen and thereby, display the phenotype of an effector-like cell in the periphery.

CONCLUSION

The phenotype of antigen-specific CD8⁺ T cells persisting during different viral infections is quite varied. This variation may be related to a number of factors including level of antigen persistence, strength of antigen presenting cell interactions, balance of co-stimulatory/co-inhibitory signals, and the influence of the anatomic location of infection. The requirement of continued antigen in the maintenance of virus-specific CD8⁺ T cells during chronic viral infection and continued recruitment of naïve CD8⁺ T cells into the population of antigen-specific cells highlight the dynamic nature of these infections and the cells responding to them. Improved understanding of the relative contribution of each of these factors in the formation of functional memory cells may aid in the development of virus-specific treatments to enhance the immune response to infection or vaccines.

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TOPIC HIGHLIGHT

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Regulatory T cells in viral hepatitis

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Abstract

The pathogenesis and outcome of viral infections are significantly influenced by the host immune response. The immune system is able to eliminate many viruses in the acute phase of infection. However, some viruses, like hepatitis C virus (HCV) and hepatitis B virus (HBV), can evade the host immune responses and establish a persistent infection. HCV and HBV persistence is caused by various mechanisms, like subversion of innate immune responses by viral factors, the emergence of T cell escape mutations, or T cell dysfunction and suppression. Recently, it has become evident that regulatory T cells may contribute to the pathogenesis and outcome of viral infections by suppressing antiviral immune responses. Indeed, the control of HCV and HBV specific immune responses mediated by regulatory T cells may be one mechanism that favors viral persistence, but it may also prevent the host from overwhelming T cell activity and liver damage. This review will focus on the role of regulatory T cells in viral hepatitis.

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Key words: Regulatory T cells; Viral hepatitis; Immuno-regulation

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INTRODUCTION

An infection with HCV or HBV activates the immune system to defend the host with a broad range of innate and adaptive immune responses. Macrophages, natural killer cells and neutrophils are an important part of the innate immune response that produces inflammatory and antiviral cytokines. Activated dendritic cells induce the differentiation of naïve T cells into virus specific CD4+

and CD8+ T cells for adaptive immunity. CD4+ T cells Type 2 induce B cells to produce antiviral antibodies; CD4+ T cells Type 1 cells secrete IFN- γ and activate the massive proliferation of cytotoxic CD8+ cells that destroy infected cells or secrete proinflammatory cytokines^[1-3].

These complex molecular and cellular mechanisms help to control and, in the best case, to eliminate the virus in the acute phase of infection. However, in the majority of HCV and a significant amount of HBV infections the immune system fails to eliminate the virus and viral persistence is established. Although essential for successful virus elimination, the virus specific T cell responses of the host may also cause tissue damage and autoimmune reactions in the liver, especially in the setting of chronic viral infection. Therefore, many regulatory mechanisms of the immune system control the virus specific immune responses in order to prevent massive tissue damage or autoimmune disease.

Over the last few years, it has become evident that regulatory T cells (T_{reg} cells) may play an important role in the suppression of virus specific immune responses^[4-6]. Indeed, several studies suggest a role of diverse populations of T_{reg} cells in the natural course of HCV and HBV infections. In this review, we will summarize the current knowledge about the role of regulatory T cells in HCV and HBV infection.

REGULATORY T CELL SUBSETS

A regulatory phenotype of a T cell population was first described for a CD4+ T cell subset that constitutively expresses the interleukin 2 receptor α -chain (CD25). In 1995 Sakaguchi *et al* showed that the transfer of lymphocytes depleted of CD4+CD25+ T cells into athymic mice caused the development of various autoimmune diseases in the recipient mice. Interestingly, the reconstitution with CD4+CD25+ T cells prevented autoimmune reactions in these mice, indicating a function of this T cell subpopulation in the control of self tolerance^[7]. In the last decade numerous studies in mice and men showed that diverse T cell populations exhibit regulatory capacity and play an important role in the suppression of immune responses to self as well as foreign antigens^[8]. Regulatory T cells are divided into a natural CD4+CD25+ T_{reg} cell population and diverse populations of induced or adaptive T_{reg} cells^[9]. Natural T_{reg} cells develop in the thymus under strong TCR engagement with self peptides and play an important role in the maintenance of self-tolerance and immune homeostasis.

About 5%-10% of CD4⁺ T cells in mice and humans are natural T_{reg} cells^[10]. Natural CD4⁺CD25⁺ T_{reg} cells constitutively express cytotoxic T-lymphocyte antigen 4 (CTLA4), glucocorticoid-induced TNF receptor family related protein (GITR)^[11,12] and the forkhead family transcription factor FoxP3^[13,14]. Of note, mutations in the gene *foxP3* cause the absence of natural T_{reg} cells and a loss of self tolerance. Indeed, it has been shown that FoxP3 expression is the essential factor for the induction of the natural T_{reg} cell population. FoxP3 is the best marker for the identification of natural T_{reg} cells in mice and men, thus far^[15]. However, it has recently been shown that FoxP3 may also be transiently induced in activated human T cells^[16,17].

In contrast to natural T_{reg} cells, induced T_{reg} cells develop from non regulatory T cells in the periphery and not in the thymus. Diverse populations of induced regulatory T cells have been identified, thus far. Among those, CD4⁺ cells secreting IL-10 or TGF- β , named TR1- or TH3 cells, as well as CD4⁺CD25⁺FoxP3⁺ T cells and CD8⁺ T cells with various phenotypes have been shown to have a regulatory capacity^[8,18]. The extrathymically conversion of non-regulatory T cells into regulatory T cells requires special immunological conditions. In many respects, the cytokine TGF- β and a distinct mode of antigen exposure have been shown to play an important role in the development of induced T_{reg} cells^[8,19]. Indeed, several studies in both mice and humans demonstrated that naïve and memory CD4⁺ T cells can be converted into CD4⁺CD25⁺FoxP3⁺ regulatory T cells in the presence of TGF- β ^[20-23]. In addition, it has been shown in several mouse models that a specific way of antigen presentation, e.g. continuous exposure to low dose antigen or to a systemic peripheral antigen, can lead to the expansion of induced CD4⁺CD25⁺FoxP3⁺ regulatory T cells^[24-26].

Overall, these results and the phenotypical diversity of induced T_{reg} cell subpopulations indicate that several different mechanisms of T_{reg} cell development may exist in the periphery that still need to be defined for each subpopulation of induced T_{reg} cells. Furthermore, lineage relationship and functional overlap of induced T_{reg} cells and natural T_{reg} cells still need to be characterized in detail.

FUNCTIONAL CHARACTERISTICS OF REGULATORY T CELLS

The major function of natural and induced T_{reg} cells is the suppression of immune responses to self or foreign antigens. Indeed, numerous studies in mice and humans showed that regulatory T cells suppress the proliferation, cytokine-production (IFN- γ , IL-2) and cytolytic activity of naïve and antigen specific CD4⁺ and CD8⁺ cells. In addition, T_{reg} cells are able to suppress the functions of antigen presenting cells and B cells^[27]. T_{reg} cells may mediate their suppressive activity either through the secretion of anti-inflammatory cytokines like IL-10 or TGF- β , direct killing of the target cells or distinct cell-cell contact dependent mechanisms^[27]. The surface molecules CTLA4 and GITR have been suggested to play a role in direct cell-cell contact mediated suppression. CTLA-4

expressed on T_{reg} cells may bind to CD80/CD86 expressed on antigen presenting cells to activate the IDO (indoleamine 2,3-dioxygenase) dependent generation of tryptophan. A decrease of free tryptophan reduces T cell activation^[28,29]. The transfer of cAMP from T_{reg} to effector cells via gap junction contact has recently been proposed to be another possible mode of cell-cell contact mediated suppression. cAMP inhibits the proliferation and IL-2 secretion of T cells^[30]. In addition, T_{reg} cells have also been shown to generate extracellular adenosine that suppresses T cells responses^[31]. However, the mechanisms and the antigen-specificity of T_{reg} cell mediated immunosuppression are still largely unknown.

In addition to the ability to control immune responses, at least those T_{reg} cell populations that express FoxP3 share other functional characteristics that distinguish them from effector T cells. Indeed, the expression of the transcription factor FoxP3 significantly influences the phenotype and function of T_{reg} cells^[32]. FoxP3⁺ T cells show different TCR signaling patterns from effector T cells, do not proliferate well, when cultured *in vitro* and do not produce IL-2 or other inflammatory cytokines^[32]. Recently, genome wide analysis of FoxP3 target genes in mouse natural T_{reg} cells revealed that FoxP3 regulates those genes involved into TCR signaling pathways and cytokine-production as well as genes encoding for T_{reg} cell associated surface molecules like CD25 or GITR^[33,34]. In conclusion, the functional as well as the phenotypical differences between T_{reg} cells and effector T cells are largely dependent on the expression of FoxP3 in regulatory T cells.

REGULATORY T CELLS IN VIRAL INFECTION

Many viruses, like HCV and HBV, are able to evade the host immune response and to establish chronic infection. Efficient virus specific T cell responses are critical to eliminate the virus in the acute phase of infection. Importantly however, viruses have evolved strategies of immune evasion to subvert innate and adaptive immune responses and to facilitate viral persistence^[35].

Growing evidence suggests that regulatory T cells may play an important role in the suppression of antiviral T cell responses in the acute and chronic phase of infection. Indeed, the virus specific induction of regulatory T cells may have two very different consequences: first, it may help the virus to establish viral persistence and second, it may be an important process that occurs to prevent excessive immunopathological damage^[4,5].

First evidence that regulatory T cells may play a role in viral infection was forthcoming from a study by Souvas *et al* who showed that the depletion of CD4⁺CD25⁺ T cells in mice infected with herpes simplex virus (HSV) enhanced virus specific CD8⁺ T cell activity in the acute phase of infection as well as after viral clearance. Furthermore, HSV infection appeared to have a direct effect on natural T_{reg} cells, since regulatory T cells from HSV infected mice showed an increased suppressive capacity towards HSV specific and unspecific CD8⁺ T cell responses^[36]. While these findings indicate a detrimental role of natural T_{reg} in the host-virus immune balance, another study with HSV infected mice proposed a

protective role of natural T_{reg} cells in viral infection. Mice suffering from blinding keratitis, caused by HSV infection, showed much more severe lesions in the eyes when depleted of natural CD4+CD25+ regulatory T cells^[37].

Regulatory T cells have also been shown to play a role in chronic retroviral infections, indicated by studies with mice infected with Friend virus (FV). CD8+ T cells are critical for clearance of FV in the acute phase of infection, while insufficient CD8+ T cell effector functions are associated with viral persistence. A study by Dittmer *et al* showed that immunosuppression and CD8+ T cell impairment in chronic FV infection is a result of induced T_{reg} cell activity. Indeed, adoptive transfer experiments revealed that IL-10 secreting CD4+ cells from persistently infected mice suppressed antigen specific IFN- γ secretion of CD8+ cells^[38]. Furthermore, a kinetic analysis of T cell responses in acute FV infection demonstrated that the onset CD8+ T cell dysfunction as early as 2 wk after infection was associated with the expansion of induced T_{reg} cells^[39].

Taken together, these *in vivo* results from different mouse models suggest the important role of regulatory T cells in the suppression of virus specific T cell responses. In the following, recent human studies indicating the role of regulatory T cells in viral hepatitis will be discussed in more detail.

REGULATORY T CELLS IN HCV INFECTION

About 170 million people worldwide suffer from chronic hepatitis C virus infection. HCV is able to persist in up to 70% of those infected and may cause chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. HCV is a positive-stranded RNA virus that belongs to the Flaviviruses^[1].

HCV clearance is associated with a vigorous HCV specific CD4+ and CD8+ T cell response in the acute phase of infection. In contrast, viral persistence is associated with a weak and dysfunctional virus specific T cell response^[40]. Several possible mechanisms of T cell failure and HCV immune evasion have been proposed, like T cell deletion, T cell dysfunction and the emergence of viral escape mutations^[2,41]. Recently, the possible role of different regulatory T cell populations in HCV persistence has also been suggested.

Indeed, several groups have shown a higher frequency of CD4+CD25+ regulatory T cells in the blood of chronically HCV infected persons versus recovered or healthy persons^[42-45] and the presence of CD4+FoxP3+ T cells in the liver of chronically HCV infected patients^[45,46]. However, whether these cells are natural T_{reg} cells that originate from the thymus or whether these cells are induced T_{regs} that developed from conventional CD4+ T cells upon HCV antigen contact in the periphery, remains to be determined. Of note, CD4+CD25+ regulatory T cells from chronically HCV infected patients are capable of suppressing HCV specific CD8 T cell and CD4+ T cell proliferation as well as CD8+ T cell IFN- γ secretion in a dose-dependent manner that requires direct cell-cell contact. However, CD4+CD25+ T_{reg} cells of infected subjects did not only suppress HCV specific T

cell proliferation, but also Influenza-, CMV-, and EBV specific T cell responses, suggesting an antigen unspecific inhibition of CD8+ T cells^[42,45].

The role of immunosuppressive cytokines, such as IL-10 and TGF- β , in the CD4+CD25+ T_{reg} cells dependent suppression remains controversial. Some studies reported that T_{reg} cells secrete IL-10 and TGF- β after HCV antigen stimulation and that TGF- β neutralization reverses T_{reg} cell mediated suppression of virus specific T cell responses^[43,47], while others did not observe this effect^[42,45].

It is important to note that CD4+CD25+ T_{reg} cells obtained from chronically HCV infected patients had an increased suppressive activity against HCV specific CD8+ T cells compared to natural T_{reg} cells isolated from subjects that had recovered from acute HCV infection, suggesting that chronic HCV infection leads to the expansion and activation of CD4+CD25+ T_{reg} cells. However the suppressive effect observed in patients who successfully cleared the virus was still significant^[42]. Furthermore, a recent study with HCV infected chimpanzees, the only animal model for HCV infection, showed that the frequency of CD4+CD25+FoxP3+ T_{reg} cells and the suppressive capacity of those cells against virus specific T cell responses were as high in HCV recovered chimpanzees as in persistently HCV infected chimpanzees^[48]. These results suggest that CD4+CD25+ T_{reg} cells do not only suppress virus specific T cell responses in chronic infection but may also control memory T cells after virus recovery. Of note, further analysis of CD4+CD25+ T_{reg} cells in chimpanzees revealed that T_{reg} cells from chronically HCV infected and recovered chimpanzees displayed fewer T cell receptor excision cycles and a better proliferative capacity *in vitro* compared to T_{reg} cells from HCV naïve chimpanzees. These data may indicate HCV specific proliferation of T_{reg} cells in HCV infected chimpanzees.

First evidence that T_{reg} cells may be induced by HCV antigens was provided by a study that examined the CD4+ T cell response to the HCV core protein. Interestingly, HCV specific IL-10 secreting T cells were detected in the blood of chronic HCV infected persons^[49]. These regulatory Tr1 cells recognized the same epitopes on the core protein as IFN- γ producing Th1 cells. However, HCV specific IL-10 secretion is not limited to the CD4+ T cell subset, since IL-10 producing HCV specific CD8+ T cells were identified that suppressed IFN- γ secretion of CD8+ T cells targeting the same epitope as the IL-10 secreting cells^[50]. Of note, both cell subsets were predominantly present in the liver, suggesting a compartmentalization of effector and regulatory T cells to the site of infection. In addition, another study also reported the accumulation of HCV specific IL-10 producing CD8+ T cells in the liver of chronically infected patients. *In situ* staining of liver biopsies revealed that IL-10+ HCV specific CD8+ T cells are located in liver areas with low hepatocellular apoptosis and low liver fibrosis, further supporting a potential role of these cells in the prevention of liver damage^[51]. However, these studies did not determine the FoxP3 expression of these CD8+ T cells.

The conclusion, that regulatory CD8+ T cells may play an important role in chronic HCV infection is further underlined by the observation that HCV specific CD8+

Table 1 Regulatory T cell subsets in HCV and HBV infection

Regulatory T cell phenotype	Virus	Compartment	Suppression	Cytokines
CD4+CD25+ (FoxP3+) ^[42-46,48,54,56,58,61,62]	HCV	Blood/Liver	Cell-cell contact dependent suppression of antigen specific and unspecific T cell proliferation and cytokine production	In part IL-10/ TGF-β
CD4+ (T _H 1 cells) ^[49]	HCV	Blood		IL-10
CD8+ (FoxP3+) ^[50-52]	HCV	Blood/Liver	Suppression of antigen specific T cell proliferation and INF-γ secretion	IL-10/TGF-β
CD8+FoxP3+ CTLA-4+GITR ⁺ ^[53]	HCV	Blood	Cell-cell contact suppression of antigen unspecific T cell proliferation	IFN-γ

CD25⁺FoxP3⁺ T cells from the blood of chronically infected patients suppress HCV specific T cell responses *via* TGF-β secretion. Of note, the blockade of TGF-β markedly enhanced the HCV specific IFN-γ secretion by CD4⁺ and CD8⁺ T cells^[52].

A population of HCV specific FoxP3⁺CD8⁺ T cells that suppressed CD4⁺ and CD8⁺ T cell proliferation in a cell-cell contact dependent manner has also been described^[53]. Indeed, these cells expanded simultaneously with FoxP3⁺CD8⁺ effector T cells after *in vitro* HCV specific peptide stimulation of peripheral blood mononuclear cells (PBMC) from chronically HCV infected patients. These results suggest that stimulation with a defined viral antigen leads to the expansion of two distinct CD8⁺ T cell populations: FoxP3⁺ effector as well as FoxP3⁺ regulatory T cells. FoxP3 expression on virus-specific CD8⁺ T cells *ex vivo* has not been shown, however. Of note, *in vitro* stimulation of PBMC from chronically HCV infected patients with HCV specific antigens also resulted in an expansion of HCV specific CD4⁺CD25⁺FoxP3⁺ regulatory T cells^[54]. However, at least for the CD8⁺ T cell compartment this is not a HCV specific effect since the expansion of virus specific FoxP3⁺ regulatory CD8⁺ T cells after *in vitro* peptide stimulation was also detected in influenza specific CD8⁺ T cells.

In summary, all these studies suggest a role of different regulatory T cell populations in the pathogenesis of HCV infection (Table 1). The elevated frequency of CD4⁺CD25⁺ regulatory T cells in the blood of chronically HCV infected patients, the ability of these cells to suppress HCV specific T cell responses, the accumulation of FoxP3⁺ T cells in the liver as well as the existence of different HCV specific regulatory T cell populations, strongly indicate that HCV infection induces virus-specific regulatory T cells that may contribute to viral persistence by suppressing HCV specific T cell responses. The presence of T_{reg} cells, especially in the liver, may also protect the host from tissue damage. In this context, it is interesting to note, that one study showed that HCV infected patients with normal alanine transaminase (ALT) levels have an increased frequency of HCV specific TGF-β secreting CD4⁺CD25⁺ regulatory T cells combined with decreased liver inflammation compared to patients with elevated ALT levels^[47].

REGULATORY T CELLS IN HBV INFECTION

Hepatitis B virus is a hepatotropic DNA virus infecting

about 300 million people worldwide. About 5%-10% of acutely infected patients develop a persistent HBV infection that is associated with T cell hyporesponsiveness and dysfunctions^[55]. Thus, T_{reg} cells may also play a role in HBV infection. Indeed, one study reported a high frequency of CD4⁺ CD25⁺ T_{reg} cells in the blood of chronically HBV infected subjects and an increase of HBV specific T cell proliferation after depletion of CD4⁺CD25⁺ T cells^[56]. Although these data suggest a potential role of T_{reg} cells in mediating T cell dysfunction during chronic HBV infection, another study reported discrepant results^[57]. In fact, neither a higher frequency nor an elevated suppressive capacity of CD4⁺CD25⁺ T_{reg} cells isolated from the blood of chronically infected patients compared to CD4⁺CD25⁺ T_{reg} cells from persons recovered from HBV infection, were observed in this study^[57].

However, recent studies shed more light on these controversial results about the role of T_{reg} cells in HBV infection. Indeed, a broad analysis of the frequency and function of CD4⁺CD25⁺ T_{reg} cells in the blood and liver of patients with acute HBV, chronic HBV, chronic severe HBV and healthy controls revealed that only patients with a chronic severe HBV infection showed a significant higher level of CD4⁺CD25⁺ T_{reg} cells in the blood compared to patients with mild course of chronic HCV and acute HCV infection^[58]. A significant accumulation of CD4⁺CD25⁺FoxP3⁺ T_{reg} cells in the liver was found in patients with chronic HBV and chronic severe HBV infection^[58,59]. A positive correlation between HBeAg level, HBV DNA level and the frequency of CD4⁺CD25⁺ T_{reg} cells in the blood of chronically infected patients further supports the role of T_{reg} cells in HBV infection^[58-60]. Finally, two studies showed the presence of HBeAg-specific CD4⁺CD25⁺FoxP3⁺ T_{reg} cells in chronically HBV infected patients^[61,62]. Interestingly, HBeAg specific CD4⁺CD25⁺ T_{reg} cells declined in the blood of patients during acute exacerbation of hepatitis in the immunoactive phase in chronic HBV infection while the HBeAg specific CD8⁺ T cell frequency increased at the same time. These data indicate that HBV specific T_{reg} cells may suppress HBV specific CD8⁺ T cells responses during flares in chronic HBV infection and may thus contribute to protection from severe hepatitis^[61].

The analysis of T_{reg} cells during acute HBV infection showed that T_{reg} cells may also play a role in this state of the infection. Indeed, the frequency of CD4⁺CD25⁺ T_{reg} cells was normal in the early acute phase of infection, increased during the convalescent phase and decreased

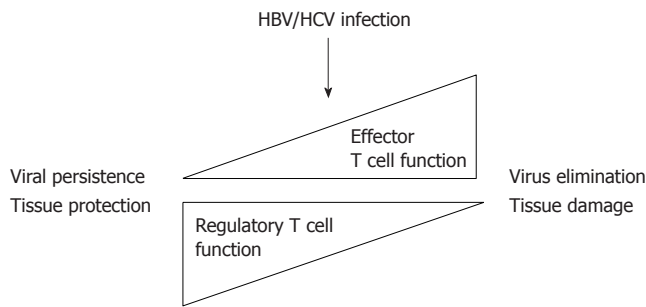


Figure 1 Regulatory T cells may contribute to viral persistence but they may also prevent tissue damage.

again to normal after virus resolution^[58]. Of note, CD4+CD25+ T_{reg} cells isolated from the blood of acutely HBV infected patients had an increased suppressive activity against HBV specific T cell responses compared to antigen unspecific T cell responses, suggesting the generation of HBV antigen specific T_{reg} cells during acute HBV infection^[58]. In summary, as in HCV infection, recent studies suggest that regulatory T cells do also play a role in HBV infection (Table 1).

FUTURE PERSPECTIVES

Taken together, there is strong evidence that different regulatory T cell populations mediate virus-specific T cell suppression in HCV and HBV infection. This immune suppression may contribute to viral persistence, but also to protection from overwhelming liver damage (Figure 1). Although, the studies discussed in this review give important first insights into the role of regulatory T cells in viral hepatitis, they also raise many questions. In fact, HCV infection in particular seems to induce a striking number of distinct HCV specific regulatory T cell populations. However, the lineage relationship between these T_{reg} cell populations as well as their relationship with HCV specific effector T cells remains elusive. It is unclear whether the CD4+CD25+ T_{reg} cells analyzed in HCV infected patients natural T_{reg} cells derive from the thymus develop from CD4+ T cells upon HCV antigen encounter in the periphery. The latter is most likely for HCV specific regulatory CD8+ T cells. However, if HCV specific T_{reg} cells are induced in the periphery, do they develop from naïve T cells as distinct induced CD4+ or CD8+ regulatory T cell lineages or do they generate from HCV specific effector memory T cells in a certain immunological context of the virus infection?

Other important questions that need to be addressed are the place and time point of regulatory T cell induction in HCV and HBV infection, as well as the mode and antigen specificity of T_{reg} cell mediated suppression in both viral infections. In addition, the presence and action of regulatory T cells in the liver, the site of virus replication and chronic inflammation should be analyzed in more detail. Furthermore, it will be important to determine if virus specific regulatory T cells do only play a role in the progression of a chronic infection or if they are already activated during the acute phase of infection and contribute to the development of viral persistence, as has

already been indicated in the case of HBV infection.

Of note, T_{reg} cells may serve as a potential target for therapeutic interventions. The depletion of regulatory T cells during an acute viral infection may have the therapeutic potential to prevent viral persistence. Furthermore, a manipulation of regulatory T cells may also improve vaccine efficiency. Studies with mouse models already suggest that depletion of CD4+ CD25+ T cells can help to resolve an infection^[36] or to enhance the effect of a virus DNA vaccination^[63,64]. However, a depletion of regulatory T cells in the setting of a chronic viral infection could also lead to massive tissue damage, if virus specific CD8+ T cells are no longer suppressed. Therefore, an application of these approaches in humans requires a more detailed knowledge about the exact interplay of regulatory T cells, viruses and virus specific immune responses. Otherwise, the overall function of regulatory T cells in the maintenance of immune homeostasis and self tolerance could be dangerously disturbed.

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Hepatitis C virus infection and apoptosis

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Abstract

Apoptosis is central for the control and elimination of viral infections. In chronic hepatitis C virus (HCV) infection, enhanced hepatocyte apoptosis and upregulation of the death inducing ligands CD95/Fas, TRAIL and TNF α occur. Nevertheless, HCV infection persists in the majority of patients. The impact of apoptosis in chronic HCV infection is not well understood. It may be harmful by triggering liver fibrosis, or essential in interferon (IFN) induced HCV elimination. For virtually all HCV proteins, pro- and anti-apoptotic effects have been described, especially for the core and NS5A protein. To date, it is not known which HCV protein affects apoptosis *in vivo* and whether the infectious virions act pro- or anti-apoptotic. With the availability of an infectious tissue culture system, we now can address pathophysiologically relevant issues. This review focuses on the effect of HCV infection and different HCV proteins on apoptosis and of the corresponding signaling cascades.

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Key words: Hepatitis C; Spoptosis; TRAIL; CD95/Fas; TNF α ; Perforin

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INTRODUCTION

Hepatitis C virus (HCV) infection persists in approximately. Eighty percent of patients and is a leading cause of liver

cirrhosis and hepatocellular carcinoma^[1-4]. Worldwide, about 300 million individuals are HCV infected. The only antiviral treatment available to date with PEG-INF and ribavirin does not eliminate HCV infection in a large proportion of patients, especially in HCV genotype 1 infection, and, at the same time, has multiple severe side effects. With the availability of an infectious tissue culture system, we now can address pathophysiologically relevant issues for new treatment options^[1-3]. HCV belongs to the flaviviridae. It has an enveloped, positive strand RNA genome of 9.6 kb length containing one open reading frame translated into a single polypeptide. Posttranslational cleavage yields 4 structural (E1, E2, core, p7 (probably) and 6 nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B). Six different genotypes (1 [a, b, c], 2 [a, b, c], 3 [a, b], 4a, 5a, 6a) and 52 subtypes have been described. Due to the lack of proofreading function of the RNA-dependent RNA-polymerase (NS5B), HCV has a high mutation rate and exists as genetically heterogeneous quasispecies in individual patients^[5-7]. The different genotypes differ genetically from one another by at least 30%, and the different subtypes within a genotype by more than 20%. This genetic heterogeneity makes it difficult to compare apoptotic pathways obtained with different HCV genotypes. In general, apoptosis is central to viral clearance. In HCV-infected liver, however, despite enhanced hepatocyte apoptosis, viral persistence is observed.

APOPTOSIS IN HCV-INFECTED LIVER

Immune cell deficiency

The immune response to viral infections includes different components of the innate and the acquired immune system. They induce apoptosis as a host defense against viral infections. The innate immune system as the first line of defense directly activates inflammatory cells, such as macrophages (e.g., granulocytes, Kupffer cells in the liver) and natural killer (NK) cells which may directly cause death of the infected cells. On the other hand, viral RNA or proteins can bind to intracellular molecules that modulate or directly induce cell death^[8]. In this immune cell-independent, virus-induced apoptosis of the host cell protein kinase R (PKR)^[9,10] and the cytoplasmic RNA helicase RIG-I^[11] play central roles. RIG-I activates Cardif, a cytosolic protein that localizes to the mitochondrial membrane where it acts pro-apoptotic^[12,13]. PKR is also activated by interferons

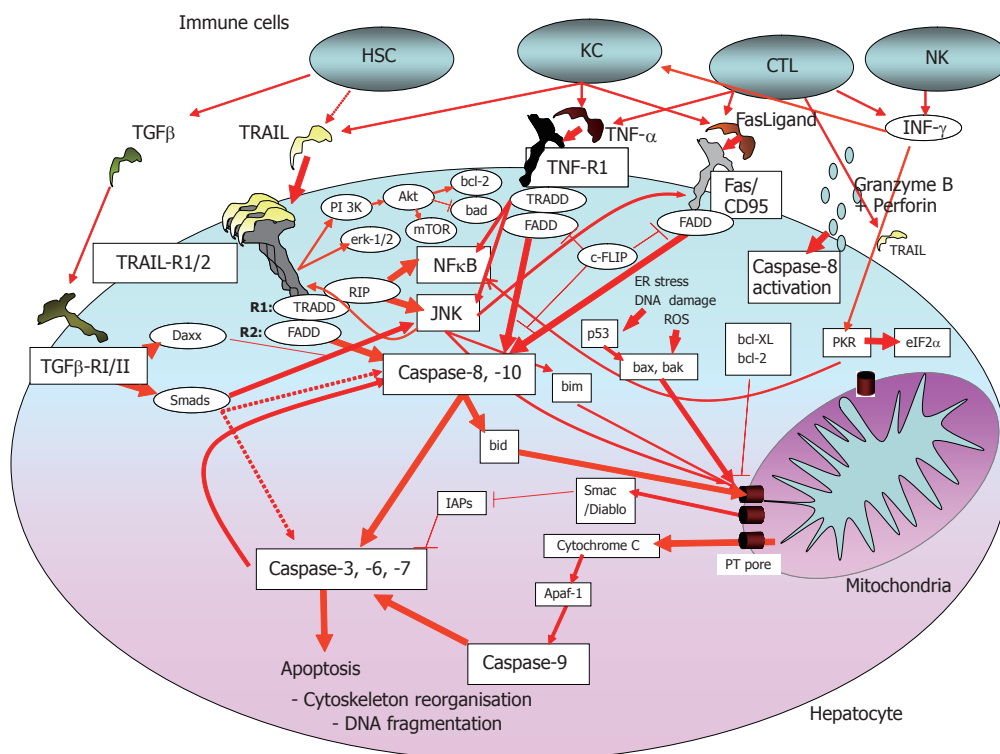


Figure 1 Signal transduction pathway of apoptosis in hepatocytes. Immune cells induce apoptosis in hepatocytes by death receptor ligands (TRAIL, TNF α , CD95Ligand, TGF- β) and granzyme B/perforin. Ligand-induced apoptosis activates caspase-8, whereas intrinsic apoptosis occurs via the mitochondrial permeability transition (PT) pore and activation of caspase-9. Caspase-9 and -8 activation converge in activation of the effector caspases-3, -6 and -7, resulting in irreversible apoptosis induction. HSC: Hepatic stellate cells; KC: Kupffer cells; CTL: Cytotoxic T-lymphocytes; NK: Natural killer cells.

(Figure 1) and acts *via* the downstream transcription factor eIF-2 α ^[14,15]. In HCV infection, the activated innate immune system alone is obviously insufficient to eliminate the virus^[16]. The acquired immune system consists of the humoral (antibody-secreting B-lymphocytes/plasma cells) and the cellular immune system (CD4+- and CD8+-T-lymphocytes). This system is essential for the clearance of most viral infections and depends on complex intercellular interactions and the recognition of viral antigens presented by specific cells (e.g., dendritic cells). CD4+-T-lymphocytes activate CD8+-T-lymphocytes, cytotoxic T lymphocytes (CTLs), macrophages and B-lymphocytes^[16]. The antigen-primed CD8+-T-lymphocytes/CTLs directly kill infected cells *via* direct cell-cell-contact, and release of cytotoxic and/or antiviral cytokines (e.g., IFN γ , TNF α), whereas IFN γ and IFN α are also able to eliminate the virus without killing the host cell^[17,18]. In chronic HCV infection, the acquired immune system is, among others, impaired by T cell failure, dysfunction and exhaustion^[19]. This failure includes CD4+- as well as CD8+-T-lymphocytes.

ENHANCED HEPATOCYTE APOPTOSIS IN HCV INFECTION *IN VIVO*

Most of the cytotoxic effects mentioned above occur *via* programmed cell death, with activation of the intracellular suicide program through specific signals. Because chronic viral infection may reflect a failure of the immune system, specific apoptosis induction may not occur. In chronic HCV infection, however, enhanced hepatocyte apoptosis has been described, independent from the HCV genotype^[20]. Apoptosis varies between 0.54%^[20] and 20.00% of hepatocytes^[21], depending on the methods used. Typical pathomorphological features

of apoptosis (e.g., nuclear fragmentation, cell shrinkage) may be seen only in a minority of hepatocytes. The close physical proximity of apoptotic hepatocytes and infiltrating lymphocytes suggests an immune cell-mediated apoptosis^[20,22]. Apoptosis correlates with liver pathology^[20,21] and may contribute to fibrogenesis^[23]. Due to the difficulty to identify HCV infected hepatocytes, it is unknown whether apoptotic hepatocytes are indeed HCV infected. The number of HCV infected hepatocytes is in the range between 1% and 10%^[24]. Therefore, we actually do not know whether apoptosis is indeed related to HCV clearance. In an animal model of cholestasis, inhibition of hepatocyte apoptosis reduced fibrogenesis^[25] and excessive apoptosis lead to fulminant hepatitis^[26,27]. Therefore, anti-apoptotic therapy to prevent HCV-related liver damage has been suggested^[28,29]. By contrast, in a chimeric mouse-human model, pro-apoptotic gene therapy with proapoptotic Bid, engineered to contain a specific cleavage site for NS3/NS4A protease, results in a considerable decline of HCV RNA in serum^[30]. The relation between PEG-IFN/ribavirin-induced viral clearance and apoptosis of infected hepatocytes is largely unknown. INFs induce apoptosis in hepatoma cells, activate pro-apoptotic PKR^[10] and upregulate death receptor ligands. However, anti-apoptotic effects have also been described^[17,31-33].

LIGAND-INDUCED HEPATOCYTE APOPTOSIS IN HCV INFECTION

Hepatocytes most likely represent so-called type-II cells, for which external activation of the death signaling pathway often is insufficient to induce apoptosis. Here, apoptosis requires in addition amplification by the mitochondrial pathway (intrinsic apoptosis pathway). The

latter is affected by oxidative stress, DNA damage, and viral proteins (Figure 1).

Targeted apoptosis induction *via* CTLs and macrophages largely occurs *via* the ligands and receptors of the TNF α family: TNF α /TNF-receptor 1, CD95/CD95Ligand and TRAIL/Trail receptor-1 and -2, respectively (Figure 1). Ligand binding induces the formation of a death-inducing signaling complex, resulting in the activation of caspase-8 (caspases are the proteases involved in the apoptosis signaling cascade^[34]). Active caspase-8 can trigger two signaling pathways. The first pathway involves cleavage of bid, followed by mitochondria-dependent activation of caspase-9 *via* cytochrome C release and apaf-1^[35] (Figure 1). Mitochondria-dependent apoptosis is amplified by pro-apoptotic bax, bad, bak and others, while molecules like bcl-2 or bcl-XL act anti-apoptotic. These proteins converge at the mitochondrial permeability transition (PT) pore that regulates release of apoptotic regulatory proteins, e.g., procaspase-9, cytochrome C, apoptosis inducing factor (AIF) or Smac/Diablo^[36-38]. The second pathway involves caspase-8 activation that may bypass mitochondria resulting in the direct activation of effector caspases (caspase-3, -6, -7). Cellular inhibitors of apoptosis (IAPs, survivin, c-FLIP) are able to block caspase activation and apoptosis^[39] (Figure 1).

Growth-factor activated MAP-kinases Erk-1/2 and PKB/Akt inhibit apoptosis directly (e.g., through inactivation of pro-apoptotic bad) or *via* upregulation of anti-apoptotic proteins (e.g., bcl-2). By contrast, sustained stress activation of c-jun kinase (JNK) enhances death ligand-induced apoptosis *via* bim activation and consecutive mitochondrial apoptosis or *via* enhanced death-receptor membrane trafficking^[40-42]. Most death ligands, especially TNF α and TRAIL, activate NF κ B, which has anti-apoptotic effects in hepatocytes by upregulation of anti-apoptotic proteins, e.g., c-FLIP and bcl-XL^[43].

Death receptor ligands may be secreted by immune cells (e.g., macrophages) or may be membrane-bound. The latter form induces apoptosis more efficiently^[44]. In the normal liver, INF γ -activated Kupffer cells can kill neighbouring cells *via* TRAIL and CD95Ligand^[37,44]. By contrast, in injured liver, activated hepatic stellate cells release TGF- β that may induce apoptosis of hepatocytes^[45,46]. While TGF- β 1 expression is increased in the HCV-infected liver^[22], the impact of TGF- β on hepatocyte apoptosis in HCV-infected patients remains elusive. Apart from apoptosis induction, TGF- β is a key molecule in the pathogenesis of liver fibrosis^[47].

Hepatocytes undergo apoptosis in response to CD95Ligand and TNF α , whereas TRAIL presumably only induces apoptosis in infected or malignantly transformed hepatocytes/hepatoma cells, but not in normal liver cells. For all three death ligands, in chronic HCV infection, upregulation has been described^[20,48-51]. Further, HCV-specific CTL clones induced CD95Ligand-, TNF α - and perforin-dependent hepatocyte apoptosis^[52,53]. In HCV-infected liver, CD8+ T cells express CD95Ligand^[49] and TRAIL^[54] (Fischer, Blum Schmitt-Gräff *et al.*, unpublished data). Interestingly, CD95Ligand-induced apoptosis did not depend on HCV infection/antigen presentation, because bystander killing of non-HCV infected hepatocytes was

observed. TRAIL-induced apoptosis seems especially important in viral defense. Adenoviral-infected murine and human hepatocytes are sensitized to TRAIL-induced apoptosis, while CD95Ligand-induced cell death is not affected^[50,55]. In TRAIL knock-out mice resolution of pulmonary influenza infection is TRAIL-dependent^[56], and CMV infected colon epithelial cells or skin fibroblasts become sensitive to TRAIL-induced apoptosis^[57]. Further, in mice infected with encephalomyocarditis virus, blocking of TRAIL resulted in higher viral titers and early death^[58]. In concanamycin- and listeria-induced hepatitis, liver cell apoptosis is TRAIL-dependent^[59]. PEG-INF/ribavirin therapy of patients with chronic HCV infection results in a rapid and sustained TRAIL elevation, suggesting a role of TRAIL in viral clearance^[60]. Similar observations have been made for soluble CD95Ligand^[61,33]. Therefore, TRAIL-induced apoptosis may play a major role in HCV defense and elimination.

Another mechanism of apoptosis involves the release of granzyme B and perforin by CTLs^[62,63]. Exocytosed perforins form transmembrane channels in the target cell that allow the entry of granzyme B. Similar to death-ligand induced apoptosis, granzyme B-mediated apoptosis largely depends on caspase activation and the sensitivity of the target cell. Hepatocytes seem resistant to granzyme B mediated cell death, and CTL killing of infected hepatocytes is perforin/granzyme B- independent^[29,64]. Therefore, a contribution of this apoptosis mechanism in patients with viral hepatitis is very unlikely.

MODIFIED HEPATOCYTE APOPTOSIS *IN VITRO*

Viral proteins interfere with the cellular apoptotic signaling pathway and block key cellular elements of the host cell. Until recently, the lack of an infectious HCV tissue culture system did not allow to study the impact of HCV infection on hepatocyte apoptosis. Overall, the data regarding the role of different HCV proteins are controversial and ascribe to a given viral protein pro- and anti-apoptotic effects, depending on the experimental system used. Since in most models viral proteins are overexpressed by non-viral promoters, for virtually all HCV proteins a pro-apoptotic effect has been described. Apart from the unphysiological expression of viral proteins, these models further lack the balance of intracellular viral expression of the different HCV proteins and their interactions. Especially in HCV infection, intracellular viral protein expression is very low.

Further, HCV is genetically highly variable and exists as quasispecies in a given patient. Different pro- and anti-apoptotic effects of the HCV core protein from an individual patient have been described^[65], suggesting special properties of different quasispecies proteins. These protein differences may explain in part the different effects of viral proteins on apoptosis. Studies of the contribution of genotypes or quasispecies to the effects on apoptosis are largely missing. Further, experiments designed to study the impact of HCV infection on hepatocyte apoptosis must also consider the interactions between the different

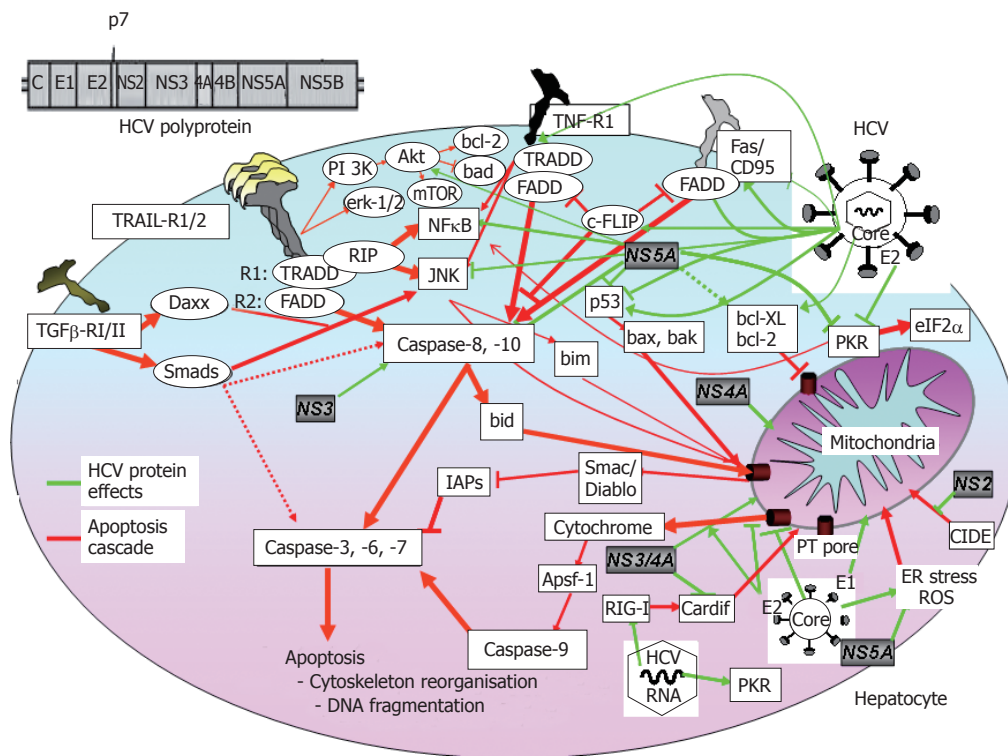


Figure 2 Interference of HCV proteins with the apoptosis cascade. Pro- and anti-apoptotic effects of HCV proteins converge at the mitochondria (e.g., NS2, NS3/4A, NS5A, E2, core), partly indirectly via p53 (NS5A, core) and activation of PKB/Akt, c-Jun kinase JNK (core) or NFκB (NS5A). HCV interacts directly with death receptors (core), the corresponding death receptor domains (FADD) and caspase-8 (NS5A). HCV double-strand RNA-activated protein-kinase R (PKR) induced signaling via RIG-I (retinoic acid inducible gene-I) and Cardif is directly (E2, NS5A) and indirectly (NS3/4A) disturbed.

HCV proteins. Therefore, only models based on the complete and infectious virus may reflect to some extent the *in vivo* situation.

HCV core protein

The structural HCV core protein makes up the virion nucleocapsid^[1,5,66]. The core protein has been shown to affect various cellular signaling pathways^[67] and to activate different promoters, e.g., c-myc, c-fos^[68-70]. It has further been shown to have pro- and anti-apoptotic effects in death ligand-mediated hepatocyte apoptosis. Core-dependent inhibition of TNF-α^[71] and CD95Ligand-induced apoptosis^[72] has been described in a hepatoma cell line. In other models, overexpressed HCV core protein did not prevent CD95Ligand-induced apoptosis in hepatoma cells^[73] or transgenic mice expressing HCV core protein, E1, E2 and NS2, respectively. HCV core protein inhibits CD95Ligand-mediated apoptosis by prevention of cytochrome C release from mitochondria and consecutive activation of caspase-9, -3 and -7^[74]. Direct physical and pro-apoptotic interaction of the core protein with the cytoplasmatic domains of CD95, TNF-R1^[75] and lymphotoxin-β^[76] receptors have been reported. Further, direct binding to the downstream death domain of FADD and c-FLIP^[77] has been shown to result in anti-apoptotic effects. Recently, inhibition of the TGF-β-pathway by direct interaction of the core protein with the DNA-binding domain of Smad3, important apoptosis mediators of TGF-β-receptor-I/II, has been demonstrated^[65].

Several studies demonstrated binding of the HCV core protein to p53, either inhibiting or activating p53^[69,78-80] with consecutive anti- or pro-apoptotic effects. In some studies apoptosis was inhibited in hepatoma through core-dependent phosphorylation and activation of STAT3 that induces the anti-apoptotic bcl-XL^[81,82]. Other studies

showed core-induced apoptosis through mitochondrial cytochrome C release and indirect activation of bax^[83,84]. TRAIL-induced apoptosis in hepatoma cells seems enhanced by core-dependent bid-cleavage^[83]. Mitochondrial functions are altered by core-induced oxidative stress, making cells more susceptible to apoptosis^[85]. Machida *et al*^[86] showed HCV-dependent production of reactive oxygen species (ROS), lowering of the mitochondrial transmembrane potential and consecutive caspase-independent cell death.

Taken together, it remains unclear whether HCV core protein inhibits or induces death receptor-mediated apoptosis of hepatocytes (Figure 2).

HCV envelope proteins E1 and E2

HCV proteins E1 and E2 are envelope proteins, that mediate viral binding and entry^[7,87]. In a transgenic mouse model expressing HCV proteins, CD95Ligand-mediated hepatocyte apoptosis is inhibited by E1, E2, NS2 and core, respectively. The activation of mitochondrial apoptosis (intrinsic pathway) is involved, because release of cytochrome C and caspase-9, but not caspase-8 activation are inhibited. To date, the contribution of the individual HCV proteins was not investigated^[74]. In E1-expressing hepatoma cells, apoptosis depends on the presence of the C-terminal transmembrane domain of E1, presumably altering membrane permeability of E1^[88,89].

Inhibition of TRAIL-induced apoptosis in hepatoma cells by E2, presumably through inhibition of mitochondrial cytochrome C release has been demonstrated^[90], while E1 had no effect and core did not counteract the anti-apoptotic effect of E2. Comparable results were obtained in core-E1-E2 transfected hepatoma cells or transgenic mice. In both models, core-E1-E2 induced less apoptosis than core-transfected

cells/transgenic mice and controls, respectively^[91]. By contrast, E2 induces mitochondria-related and caspase-dependent apoptosis in the same hepatoma cell line^[92]. These controversial data may reflect the use of different promoters that overexpress E2, while at the same time, the HCV genotype or the individual sequence of E2 have not been considered. Therefore, it still remains unclear whether HCV E1 has apoptosis-modulating activity *in vivo*, and whether HCV E2 acts anti- or pro-apoptotic (Figure 2).

HCV nonstructural proteins

The non-structural HCV proteins NS2 and NS3 are the two viral proteases required for posttranslational cleavage of non-structural proteins. NS2 is a transmembrane protein localized in the endoplasmic reticulum (ER) that directly binds and inhibits CIDE-B-induced apoptosis (cell death-inducing DFF45 (DNA-fragmentation-factor)-like effector^[93]). CIDE-B-induced apoptosis is assumed to occur *via* the mitochondrial pathway^[94,95]. Its role in hepatocyte apoptosis and viral hepatitis remain to be determined, however.

NS3 has a helicase and NTPase activity that are involved in RNA replication^[7]. Importantly, NS3 prevents viral RNA-induced pro-apoptotic RIG-I effects by specific cleavage of downstream Cardif, a protein that translocates to the mitochondrial membrane when activated^[13]. The precise role of Cardif in hepatocyte apoptosis and viral hepatitis is unknown, however. In contrast, NS3 induces caspase-8 dependent apoptosis in hepatocytes^[96] and in dendritic cells^[97]; the underlying mechanism remains unknown.

HCV NS4A is a cofactor that binds to NS3. NS4A alone and complexed with NS3 is localized in mitochondria and induces the release of cytochrome C and caspase-8 independent apoptosis^[98]. NS4B is an integral ER membrane protein that may play a role in anchoring the replication complex^[6,7]. A role in the apoptotic signaling pathway has not yet been described.

The function of NS5A is not yet well defined. NS5A interferes with the response to IFN and seems to play an important role in viral replication^[5,7]. NS5A has sequence homologies with bcl-2 and binds to FKBP38, thereby augmenting the anti-apoptotic effect of bcl-2^[99] and inhibiting the pro-apoptotic action of bax in hepatoma cells^[100]. Anti-apoptotic effects of NS5A are further mediated by cytoplasmatic sequestering of p53^[101], activation of PI3-kinase-Akt/PKB survival pathway^[102], activation of STAT3 with enhanced expression of bcl-XL and p21^[103] and activation of NFκB^[104]. By contrast, the direct inhibition of pro-apoptotic bin1, a tumor suppressor protein with a SH3 domain, has been described in hepatoma cells^[105], and a direct NS5A-induced apoptosis has also been shown^[97,106]. NS5B is the viral RNA-dependent RNA polymerase^[5-7]. There are no studies demonstrating a role of NS5B in apoptosis of hepatocytes/hepatoma cells, while a pro-apoptotic effect of NS5B has been demonstrated in dendritic cells^[97].

In conclusion, similar to HCV structural proteins, the effect of non-structural proteins on hepatocyte apoptosis *in vivo* remains unclear.

CONCLUSION

The role of apoptosis in HCV infection is not well defined. Kinetics and extent of hepatocyte apoptosis as well as the pro- and anti-apoptotic mechanisms involved remain unclear. It remains further unclear whether enhanced hepatocyte apoptosis in HCV infection is related to viral clearance, and whether it has a therapeutic benefit.

Most experimental models have fundamental shortcomings and there are no data from primary hepatocytes, tissue cultures or animal models. The majority of the data were obtained with different tumor cell lines that may in themselves be inhomogeneous. Different HCV genotypes and quasispecies may induce different effects, and most studies employ nonphysiologically overexpressed viral proteins. In HCV infected patients, by comparison, only very low quantities of HCV proteins are detectable, and the balanced expression of these proteins may be essential. Therefore, the results obtained to date have to be interpreted with great caution. The now available infectious tissue culture systems^[1-3] as well as future *in vivo* model systems may give answers to these questions, may better reflect the *in vivo* situation and may help to clarify the interference of HCV with apoptotic pathways and its role in the pathogenesis of HCV infection and clearance.

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Effects and mechanisms of electroacupuncture at PC6 on frequency of transient lower esophageal sphincter relaxation in cats

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Abstract

AIM: To investigate the effects of electroacupuncture (EA) at neiguan (PC6) on gastric distention-induced transient lower esophageal sphincter relaxations (TLESRs) and discuss the mechanisms of this treatment.

METHODS: Protocol I: Twelve healthy cats underwent gastric distention for 60 min on the first day. Electrical acupoint stimulation was applied at the neiguan or a sham point on the hip in randomized order before gastric distention, on the third day and fifth day. Those cats that underwent EA at neiguan on the fifth day were named "Neiguan Group" and the cats that underwent EA at a sham acupoint on the fifth day were named "Sham Group" (control group). During the experiment the frequency of TLESRs and lower esophageal sphincter (LES) pressure were observed by a perfused sleeve assembly. Plasma levels of gastrin (GAS) and motilin (MTL) were determined by radioimmunoassay. Nitrite/nitrate concentration in plasma and tissues were measured by Griess reagent. The nuclei in the brain stem were observed by immunohistochemistry method of c-Fos and NADPH-d dyeing. Protocol II: Thirty six healthy cats were divided into 6 groups randomly. We gave saline (2 mL iv. control group), phaclofen (5 mg/kg iv. GABA-B antagonist), cholecystokinin octapeptide (CCK-8) (1 µg/kg per hour iv.), L-Arginine (200 mg/kg iv.), naloxone (2.5 µmol/kg iv.) and tacrine (5.6 mg/kg ip. cholinesterase inhibitor) respectively before EA at Neiguan and gastric distention. And the frequencies of TLESRs in experimental groups were compared with the control group.

RESULTS: Protocol I: Not only the frequency of gastric distention-induced TLESR in 60 min but also the rate of common cavity during TLESRs were significantly

decreased by EA at neiguan compared to that of sham acupoint stimulation. C-Fos immunoreactivity and NOS reactivity in the solitarius (NTS) and dorsal motor nucleus of the vagus (DMV) were significantly decreased by EA at neiguan compared to that of the sham group. However, the positive nuclei of C-Fos and NOS in reticular formation of the medulla (RFM) were increased by EA at neiguan. Protocol II: The inhibited effect of EA at neiguan on TLESRs frequency was completely restored by pretreatment with CCK (23.5/h vs 4.5/h, $P < 0.05$), L-arginine (17.5/h vs 4.5/h, $P < 0.05$) and naloxone (12/h vs 4.5/h, $P < 0.05$). On the contrary, phaclofen (6/h vs 4.5/h, $P > 0.05$) and tacrine (9.5/h vs 4.5/h, $P > 0.05$) did not influence it.

CONCLUSION: Electric acupoint stimulation at Neiguan significantly inhibits the frequency of TLESR and the rate of common cavity during TLESR in cats. This effect appears to act on the brain stem, and may be mediated through nitric oxide (NO), CCK-A receptor and mu-opioid receptors. But the GABAB receptor and acetylcholine may not be involved in it.

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Key words: Electroacupuncture; Transient lower esophageal sphincter relaxation; C-Fos; Nitric oxide; Cat

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INTRODUCTION

Transient lower esophageal sphincter relaxations (TLESRs) is the most important mechanism of gastroesophageal reflux (GER) either in the patients of gastroesophageal reflux disease (GERD)^[1] or in normal subjects^[2]. Distention of the proximal stomach is a major stimulus for triggering TLESRs^[3]. The stimulus passes through vago-vagal reflex and is integrated in the brain stem. There is ongoing interest in developing drugs that can decrease GER by interfering with TLESRs, including

GABA-B receptor agonist^[4], Cholecystokinin-A receptor antagonist^[5], nitric oxide synthase (NOS) inhibitor^[5,6], morphine^[7] and atropine (act through central cholinergic blockade)^[8-10]. The aim of our study is to explore new approaches (EA at Neiguan acupoint) to decrease the rate of TLESRs during gastric distention, and discuss the mechanisms of this treatment.

Acupuncture has been used to treat functional gastrointestinal disorders in eastern countries for centuries. It can modulate visceral sensation as well as function through stimulation at selected acupoints along the meridians (channels of acupoints)^[11]. EA at zusanli (ST-36) can increase the basal LES pressure^[12]. Transcutaneous electric nerve stimulation (TENS) at Hukou acupoint increased the degree of LES relaxation in volunteers^[13] and reduced basal LES pressure in patients with achalasia^[14,15]. Previous studies have suggested that TENS at neiguan may inhibit the rate of TLESRs triggered by gastric distention^[16] and reduce the perception to gastric distention^[17] in human beings. But the precise mechanisms for this phenomenon have not been extensively investigated and are not fully understood.

MATERIALS AND METHODS

Materials

Protocol I was performed on 12 adult cats weighing 3.7 ± 0.2 kg (M/F: 8/4), and 36 adult cats weighing 3.6 ± 0.5 kg (M/F: 25/11) were studied for protocol II. Cats were provided by the Animal Center of the First Hospital of Peking University. They were kept in individual cages in a controlled environment with a temperature of 22-26°C, 12/12-h light/dark cycles, and fed with standard cat diet. The animals were deprived of food 10 h before each experiment. All procedures were approved by the Committee for Animal Care and Usage for Research and Education of the Peking University. Anesthesia was initially induced with katamine (30 mg/kg im). Supplementary doses of katamine (15 mg/kg ip) were given whenever necessary to maintain an appropriate depth of anesthesia, as assessed they remained motionless yet still had cornea reflex. They were euthanized with pentobarbital sodium (0.5 mL/kg ip) at the end of the protocol.

Phaclofen, CCK octapeptide and tacrine were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA). Naloxone was provided from Beijing Shuanghe Chemical Company. L-arginine was offered by Beijing Dingguo Chemical Company.

The GAS Radioimmunoassay kit was purchased from the China Institute of Atomic Energy, Beijing, China. The MTL Radioimmunoassay kit was purchased from the Neurobiological Technique Center of the Second Military Medical University, Shanghai, China. The Griess reagent for testing nitrite/nitrate concentration was purchased from Promega Corporation, USA. The reduced form nicotinamide adenine dinucleotide phosphate (NADPH) of and NBT (nitro blue tetrazolium) were obtained from Biomol Corporation, London, UK. The antibody of C-Fos was obtained from Santa Cruz Biotechnology, California, USA.

Recording methods

The manometry catheter (outer diameter 0.5 cm) consisted of a multilumen silicone tube with five side holes located at 9, 6, 3, 0 and -6 cm from the upper margin of the 6 cm-long Dent sleeve sensor (Dentsleeve, Belair, Australia). The catheter was continuously perfused with distilled water by a low compliance pneumohydraulic capillary infusion system (UPS-2020, Holland) at a rate of 0.2 mL/min. The external pressures transducers were connected via an analog/digital converter to a personal computer system. The data were displayed continuously on a monitor and stored on the personal computer system (MMS B.V. the Netherlands).

After anesthesia the cat was set in a supine position. A manometry catheter was placed through the mouth into the esophagus and positioned so that the sleeve sensor straddled the LES to register LES pressure. The distal side hole was used as a reference point for intragastric pressure. And the upper LES side holes were used to measure esophageal body pressure.

A mylohyoid electromyography (MMS B.V. the Netherlands) was used to record swallowing^[11,18]. The pinhead electrode was inserted in the mylohyoid muscle, and the reference electrode was fixed to the interscapular region of the back.

Electroacupuncture

Two acupuncture needles of 0.22 mm in diameter (Suzhou global acupuncture instrument Co. Ltd, Suzhou, China) were inserted perpendicularly at the bilateral Neiguan acupoint (PC6, located 1.5-2.0 cm above the wrist between the ligaments of the flexor carpi radialis and the palmaris longus^[19]) overlying the median nerve to a depth of 5 mm. An electrical stimulator (Model LH202H Hans, Beijing Huawei Medical Instrument Co. Ltd, Beijing, China) provided current to the needles. Wave patterns were sparse with dense pulse intervals ranging from 2 to 100 Hz (2/100 Hz), with constant amplitude and current flow (3-4 mA). The duration was 60 min. Correct positioning was confirmed by observing slight repetitive paw flexion during stimulation^[20].

Control stimulation on a sham acupoint was conducted at the hip, a point away from the traditional meridians and dermatomes.

Gastric distention

Air was insufflated (at a rate of 15 mL/s) into the stomach through a 2.0-mm diameter tube intubated through mouth to stomach. It's depth equal to 5 cm plus the esophageal body length. 30 mL air into the stomach every 6 min amount to 300 mL were insufflated in the 1 h period of gastric distention.

Study protocol I : Effects of EA on LES motor function

Twelve adult cats were divided into Neiguan group and Sham group randomly. Each cat was studied for three sessions, and an interval of each session is 2 d (Figure 1). In the first session, the basal rate of TLESRs triggered by gastric distention was observed for one hour. In session 2 and session 3, the influence of EA at neiguan or sham

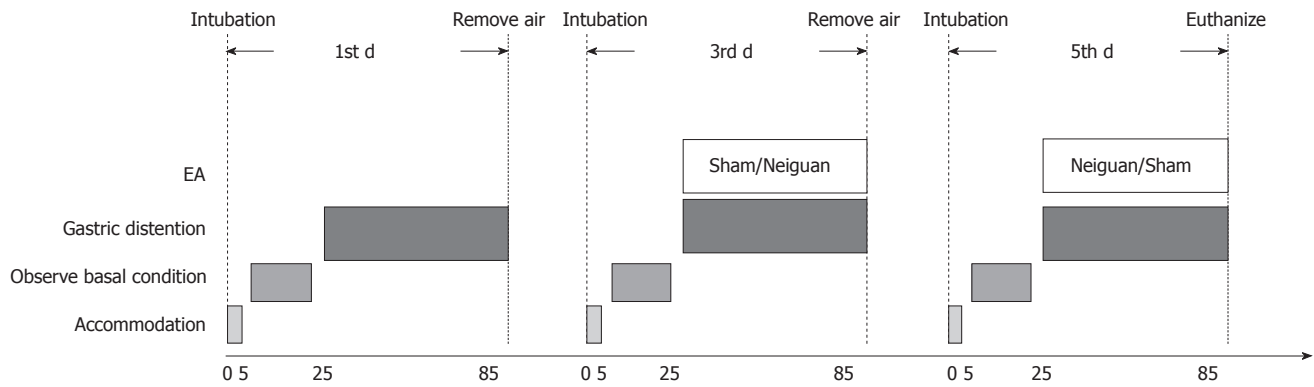


Figure 1 The flow chart of protocol I.

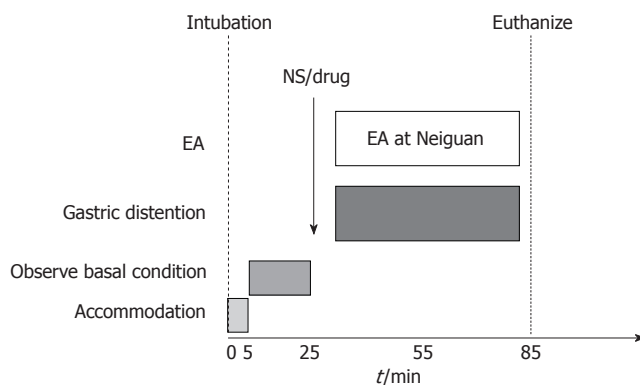


Figure 2 The process of protocol II.

acupoint to the rate of gastric distention induced TLESRs was observed in a random order.

After 5min of accommodation, the basal conditions were observed for 20 min (including basal rate of TLESRs and basal LES pressure). And then the gastric distention was applied in session 1. In session 2 and 3, gastric distention and electroacupuncture were applied at the same time. The remaining air was removed at the end of each session. At the end of session 3, the cats were euthanized and the blood, gastric fundus and brain stem were obtained for further research.

Study protocol I : Effects of drugs (phaclofen, CCK octapeptide, L-arginine, naloxone and tacrine) on inhibition of TLESRs by electric acupoint stimulation

Thirty six healthy cats were divided into 6 groups randomly. We gave normal saline(NS) (2 mL iv.), GABA-B antagonist phaclofen^[21] (5 mg/kg iv.), CCK octapeptide^[5] (1 µg/kg per hour iv.), L-arginine^[5] (200 mg/kg iv.), naloxone^[22] (2.5 µmol/kg iv.) and tacrine^[23] (5.6 mg/kg ip. cholinesterase inhibitor in central nerve system) respectively before EA at Neiguan and gastric distention, and observed the frequency of TLESR and LES pressure (Figure 2). At the end of this study, the cats were euthanized and the blood, gastric fundus and brain stem were obtained for further research.

Data analysis

TLESRs were defined according to established methods^[9].

Basal LES pressure was measured at the end of expiration relative to gastric pressure. The LES pressure during gastric distention was measured for 1 min every 6 min, and an overall mean for each period of the study was calculated. Common cavities were defined as abrupt simultaneous and sustained rises of basal esophageal pressure to intragastric pressure in at least the two lower esophageal body manometry recording sites^[24]. Common cavities are considered as markers of gas or liquid reflux from the stomach into the esophagus.

Assay of gastrointestinal hormones and nitrite concentration

When the cats were euthanized, 15 mL venous blood was collected into a test tube containing 400 µL of 10% EDTA-Na₂ (an anticoagulant) and 200 µL of trasylol. The blood samples were centrifuged at 4°C at 3500 r/min for 20 min. The serum was separated and stored at -70°C before analysis. The tissue of gastric fundus is about 2 cm × 2 cm. After weighting, tissues were added to 1 mL of 1 mol/L acetic acid and mixed evenly in a homogenizer to obtain a homogenate and were refrigerated at 4°C for 100 min. The extracted homogenates were added to 1 mL of 1 mmol/L NaOH and centrifuged at 3500 r/min 4°C for 20 min. The supernatant fluid was collected and stored at -70°C.

Gastrin (GAS) and motilin (MTL) levels were measured with commercial radioimmunoassay kits. Nitrite/nitrate levels were tested by Griess reagent.

c-Fos immunohistochemistry and NADPH-d histochemistry

After 60 min of gastric distention on the fifth day, the animal was transcardially perfused with 9 g/L saline followed by 40 g/L paraformaldehyde in 0.1 mol/L phosphate buffer saline (PBS, pH 7.3). The brain stem was removed and postfixed in the same fixative overnight and cryoprotected by immersion in 200 g/L sucrose for 72 h.

Coronal sections (40 µm) of the brain were cut in a cryostat. Every fourth section was used to reveal c-Fos immunoreactivity and the second set of sections was used to reveal NADPH-diaphorase (NADPH-d) staining.

The first set of sections were placed into a 50 g/L goat serum for 30 min at room temperature (RT), and incubated overnight at RT in primary antibody c-Fos (1:200). After washing for 15 min with PBST, the sections were incubated in biotinylated anti-rabbit IgG

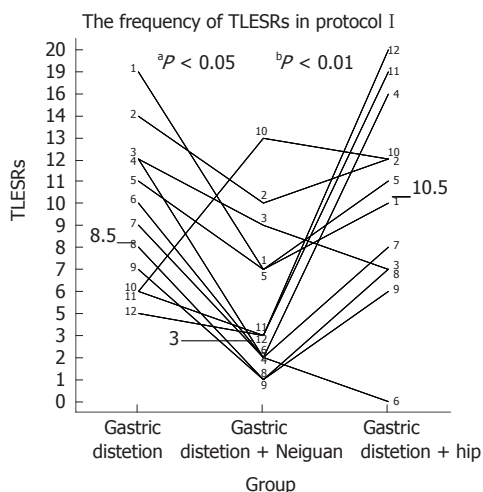


Figure 3 EA at neiguan decreased the rate of TLESRs induced by gastric distention, compared with EA at sham acupoint ($^aP < 0.01$) and baseline distention ($^bP < 0.05$). Each number represents one cat.

(Zymed, South San Francisco, Canada) diluted 1:300 in PBST at RT for 2 h, and then incubated in peroxidase-conjugated streptavidin (1:300 dilution, Zymed) for 2 h at RT. The immunoreactivity was visualized by incubating with 0.05 mol/L Tris-HCl buffer containing 0.1 g/L 3, 3'-diaminobenzidine, and 0.3 mL/L H_2O_2 for 10-20 min at RT. The stained sections were mounted on APES-coated glass slides, dehydrated and coverslipped.

The second set of sections were incubated at 37°C for 2 h in a solution containing 1 mmol/L NADPH, 0.5 mmol/L NBT, Tris-HCl 50 mmol/L, and Triton X-100 2 g/L. After a rinse in PBST, sections were mounted on APES-coated glass slides, dehydrated and coverslipped.

The distribution of c-Fos and NADPH-d positive cells was detected under a microscope (Olympus, Tokyo, Japan), and the cells were counted on LEICA Q550CW system (Leica Microsystems Imaging Solutions Ltd, Wetzlar, Germany), 10 sections for NTS/DMV, 8 sections for RFM. The average number of c-Fos or NADPH-d positive neurons per section for each cat was calculated, respectively.

Statistical analysis

The number of TLESRs was compared using Wilcoxon signedrank test and expressed as median (interquartile range). Basal LES pressure and intragastric pressure were presented as means \pm SD and were compared using repeated-measures. The rate of common cavity is compared using paired sample χ^2 . Nitrate concentrations, plasma hormone levels and the average number of c-Fos or NADPH-d positive nucleus of respective brain areas per section were expressed as mean \pm SD and was compared using independent t .

SPSS 11.0 was used for statistical analysis, and $P < 0.05$ was considered statistically significant.

RESULTS

Study protocol I: Effects of EA on LES motor function

Transient LES relaxations: The frequency of TLESRs

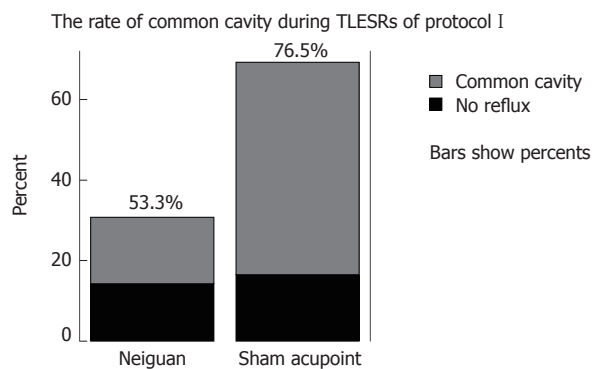


Figure 4 EA at Neiguan decreased the rate of common cavity during TLESRs compared with EA at sham acupoint ($P < 0.05$).

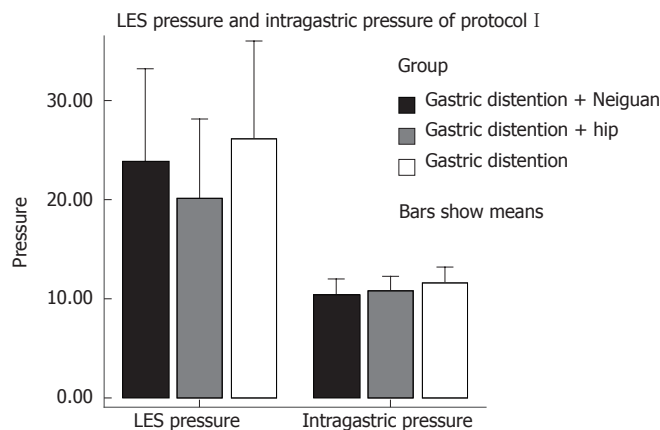


Figure 5 Electric stimulation at Neiguan didn't influence the LES pressure and intragastric pressure.

during acupoint stimulation at neiguan [3 per hour (range, 1-13)] was significantly lower than that during both the baseline period without any stimulation [8.5 per hour (range, 5-19), $P < 0.05$] and the period of sham stimulation at the hip [10.5 per hour (range, 0-20), $P < 0.01$] (Figure 3).

Common cavities during TLESRs: During EA at neiguan, a total of 60 TLESRs induced by gastric distention were observed in one hour, from it 32 were associated with common cavity, and its rate was 53.3%. During EA at sham acupoint, there are 136 gastric distention induced TLESRs in all, and 104 were associated with common cavity. The rate was 76.5%. Between-group comparisons showed that the rate of common cavity during EA at Neiguan was significantly lower than that occurring during EA at Sham acupoint ($P < 0.05$) (Figure 4).

LES pressure: EA at neiguan (PC6) had no effect on LES pressure. Overall mean LES pressure during electrical acupoint stimulation at neiguan (34.33 ± 18.16 mmHg) was similar to that during stimulation at the sham acupoint (30.97 ± 15.72 mmHg, $P > 0.05$) and during the baseline period without any acupoint stimulation (37.74 ± 18.69 mmHg, $P > 0.05$) (Figure 5).

Intragastric pressure: EA at neiguan (PC6) had no effect on gastric pressure during gastric distention. Overall

Table 1 Nitrite concentration in plasma and gastric fundus of protocol I (mean \pm SD)

Group	Serum (mmol/mL)	Gastric fundus (mmol/mg)
Neiguan	0.85 \pm 0.62	3.50 \pm 0.87
Sham	0.66 \pm 0.59	4.18 \pm 1.09

No significant difference was found between the two groups.

Table 2 Plasma GAS and MTL in each group of protocol I (mean \pm SD)

Group	GAS (pg/mL)	MTL (pg/mL)
Neiguan group	79.43 \pm 28.84	82.23 \pm 43.79
Sham group	156.30 \pm 72.53	103.6 \pm 68.16

GAS: gastrin; MTL: motilin. No significant difference was found between the two groups.

Table 3 c-Fos and NADPH-d staining in CNS

group	C-Fos		NOS	
	NTS and DMV	RFM	NTS and DMV	RFM
Neiguan group	21.9 \pm 6.9 ^a	96.6 \pm 16.5 ^a	23.6 \pm 4.6 ^a	75.6 \pm 17.0 ^a
Sham group	45.3 \pm 10.1	61.0 \pm 13.8	43.9 \pm 11.0	30.6 \pm 8.5

NADPH: nicotinamide adenine dinucleotide phosphate; CNS: central nerve system; NOS: nitric oxide synthase; NTS: nucleus tractus solitarius; DMV: dorsal motor nucleus of vagus; RFM: reticular formation of medulla. ^a $P < 0.05$ vs Sham group.

mean gastric pressure during EA at neiguan (10.37 ± 3.17 mmHg) was similar to that during stimulation at the sham acupoint (10.81 ± 2.89 mmHg, $P > 0.05$) and during the baseline period without any acupoint stimulation (11.58 ± 3.16 mmHg, $P > 0.05$) (Figure 5).

Nitrite concentration: EA at neiguan (PC6) did not influence the nitrite levels in plasma and gastric fundus tissues compared with Sham group ($P > 0.05$) (Table 1).

Gastrin (GAS) and motilin (MTL) levels: EA at neiguan (PC6) did not influence the GAS and MTL levels in plasma (Table 2).

C-Fos and NADPH-d staining in the central nerve system (CNS)

The c-Fos positive cell nuclei of activated cells showed the characteristic dark brown staining of oxidized DAB. Compared with the Sham group, electroacupuncture at neiguan significantly inhibited the number of C-Fos-labeled neurons in nucleus tractus solitarius/dorsal motor nucleus of vagus (NTS/DMV) (Figure 6A and B). However, it stimulated a significantly greater number of C-Fos positive nuclei in areas of reticular formation of the medulla (RFM) (Figure 7A and B).

NADPH-d activity was visualized as a vibrant blue color within perikarya, dendrites and axons. Electroacupuncture at neiguan significantly decreased the number of

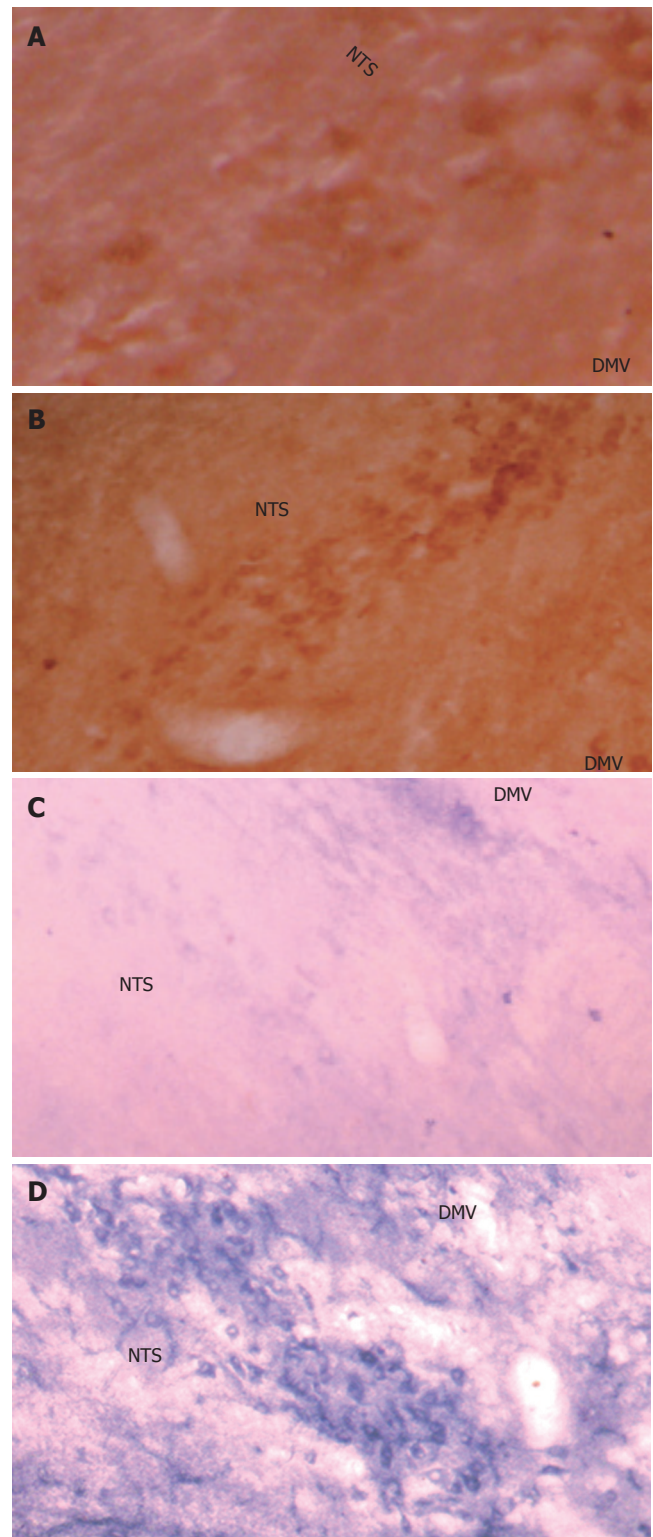


Figure 6 Photomicrographs showing C-Fos and NOS positive neurons in nucleus tractus solitarius/dorsal motor nucleus of vagus (NTS/DMV). A: C-Fos immunohistochemistry of neiguan group; B: C-Fos immunohistochemistry of sham acupoint group; C: NADPH-d histochemistry of neiguan group; D: NADPH-d histochemistry of sham acupoint group ($\times 10$).

NADPH-d stained cells in NTS/DMV (Figure 6C and D), but increased the number of positive cells in RFM (Figure 7C and D).

The number of positive nuclei in each group is listed in Table 3.

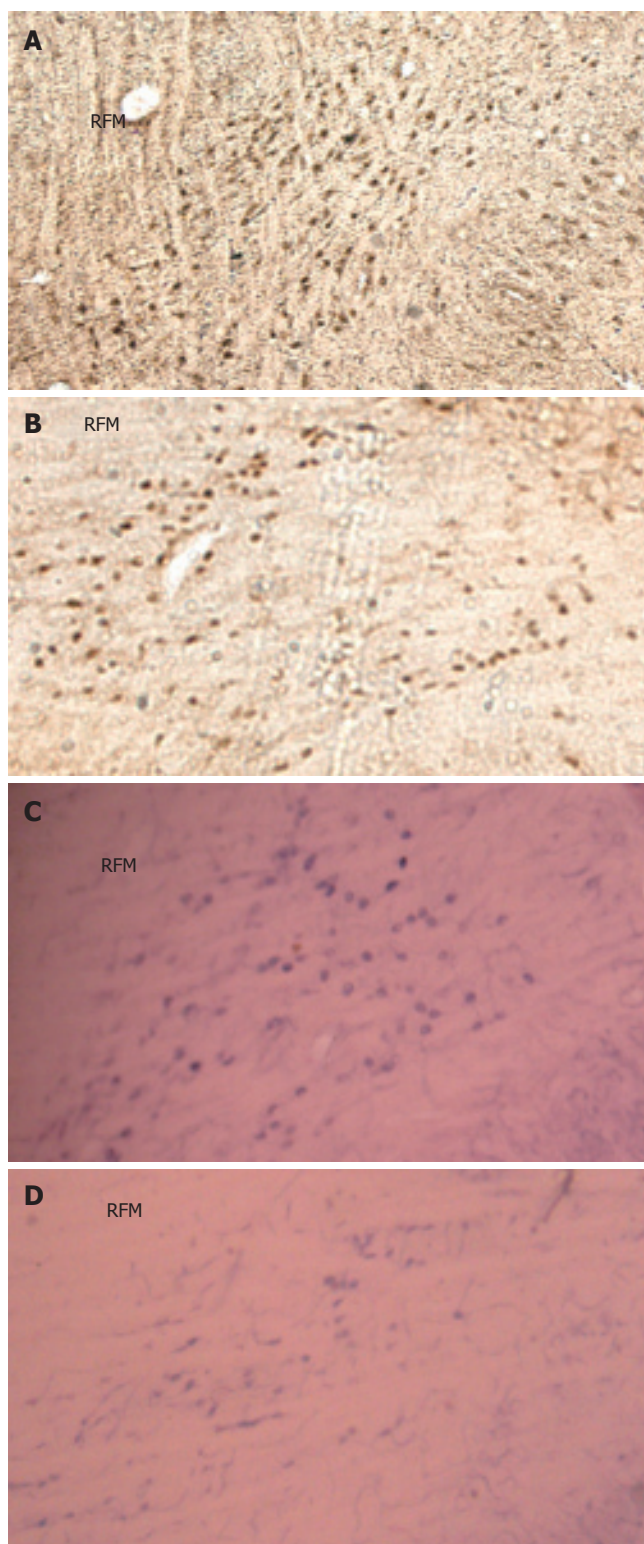


Figure 7 Photomicrographs showing C-Fos and NOS positive neurons in reticular formation of medulla (RFM). **A:** C-Fos immunohistochemistry of neiguan group; **B:** C-Fos immunohistochemistry of sham acupoint group; **C:** NADPH-d histochemistry of neiguan group; **D:** NADPH-d histochemistry of sham acupoint group ($\times 10$).

Study Protocol II : Effects of Drugs (phaclofen, CCK octapeptide, L-arginine, naloxone and tacrine) on inhibition of TLESRs by Electric Acupoint Stimulation

Transient LES relaxations: After saline infusion, the frequency of TLESRs during acupoint stimulation at neiguan [4.5 per hour (range, 1-11)] was still significantly

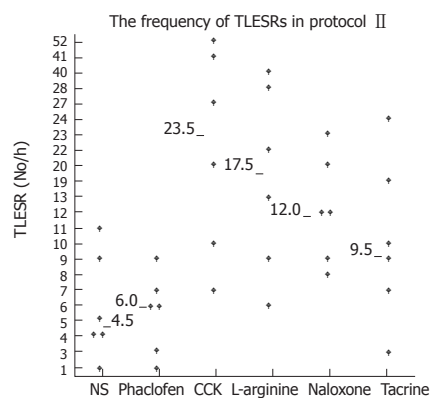


Figure 8 CCK, L-arginine and naloxone significantly increase the frequency of TLESRs compared with control group ($^{\circ}P < 0.05$). But phaclofen and tacrine did not change the frequency of TLESRs compared with control group ($P > 0.05$). It was indicated that the inhibited effects of EA at neiguan were completely restored by pretreatment with CCK-A, L-arginine and naloxone. But it was not influenced by phaclofen and tacrine.

lower than that during sham stimulation at the hip in protocol I [10.5 per hour (range, 0-20), $P < 0.05$].

Infusion of Phaclofen and tacrine did not influence the rate of TLESRs during acupoint stimulation at neiguan. They were [6 per hour (range 1-9) *vs* 4.5 per hours (range 1-11), $P > 0.05$] and [9.5 per hour (range 3-24) *vs* 4.5 per hour (range, 1-11), $P > 0.05$] respectively. However, during infusion of CCK octapeptide, L-arginine and naloxone, the inhibited effect of EA at neiguan had been completely restored. The rate of TLESRs were CCK [23.5 per hour (range 7-52) *vs* 4.5 per hours (range 1-11), $P < 0.05$], L-arginine [17.5 per hour (range 6-40) *vs* 4.5 per hours (range 1-11), $P < 0.05$] and naloxone [12 per hour (range 8-23) *vs* 4.5 per hours (range 1-11), $P < 0.05$] respectively (Figure 8).

DISCUSSION

Acupuncture has been used to treat functional gastrointestinal disorders in the eastern countries for centuries. A large amount of clinical evidence supports the effectiveness of acupuncture for treating functional disorders of the gastrointestinal tract, and the most commonly used acupoints in treating gastrointestinal symptoms are Neiguan (PC6) and Zusanli (ST-36). In the present study, we found electric acupoint stimulation at the neiguan acupoint resulted in a significant reduction of the rate of TLESRs induced by gastric distention. This result was consistent with previous investigations on human beings [12]. Furthermore, the present work also demonstrated the action site and neurotransmitters of this effect.

Speculation on the action site of electroacupuncture at neiguan

We postulate that the site at which electric acupuncture stimulation acts to inhibit the occurrence of TLESRs may be as follows: First, it may increase the proximal gastric motility or increase the gastric fundus tone, so that to decrease the volume of gastric fundus, and inhibit

the stretch receptors localized in the gastric fundus (As we know, the stretch receptors were the major receptor in triggering TLESRs^[25,26]) and then reduce the sensory input from gastric distention. Second, it may inhibit the integration of TLESRs in some area of the brain stem, such as NTS (nucleus tractus solitarius) and DMV (dorsomotor nucleus of the vagus nerve). Third, electric acupoint stimulation at Neiguan may exert its action primarily on the efferent motor pathway.

With present research, electric stimulation at Neiguan did not change the intragastric pressure and nitrite levels in the tissue of gastric fundus compared with EA at sham acupoint. Nitric oxide is well accepted as an inhibitory neurotransmitter in the gastrointestinal tract, and may exert a tonic inhibition on the proximal stomach^[27]. So the result of our study suggested that EA at neiguan cannot change the tone of gastric fundus. And it is consistent with previous research^[12,28].

In the current research, electric stimulation at neiguan had no effect on the residual LES pressure, so it is unlikely that it exerts action primarily on the efferent motor pathway.

The dorsal vagal complex (DVC) comprising nucleus tractus solitarius (NTS) and dorsomotor nucleus of the vagus nerve (DMV) is the center of the integration of TLESRs^[9]. The brain stem dyeing shows that when compared with sham group EA at neiguan significantly decreases the C-Fos and NOS positive nucleus in NTS/DMV. However, it stimulated a significantly greater number of C-Fos and NOS positive nucleus in areas of reticular formation of the medulla (RFM). And RFM may be one of the acupuncture action sites^[29].

Consequently, the action site of EA at neiguan may be localized within the brain stem. It may increase NOS in the nucleus of RFM, so that it inhibits NOS in NTS/DMV, and then decreases the frequency of TLESRs.

Speculation on the neurotransmitter of electroacupuncture at neiguan

In the second part of this research, the inhibited effect of EA at neiguan on TLESR's rate was completely restored by pretreatment with CCK, L-arginine and naloxone. On the contrary, phaclofen and tacrine did not influence it. So this effect appeared to be mediated through nitric oxide (NO), CCK-A receptor and mu-opioid receptors. But the GABAB receptor and acetylcholine may not be involved in it.

In our study, electric stimulation at Neiguan also did not influence the gastrin and motilin levels in plasma. It suggested that the inhibited effects of electric stimulation were not through these two neuropeptides.

The variance between previous research

Endogenous opioid peptides (EOPs) are considered as major candidates for a role in acupuncture action because numerous investigations have clearly demonstrated that electroacupuncture effect is antagonized by the opioid receptor antagonist naloxone^[30]. And in our research, the naloxone can reverse the inhibited effect of electroacupuncture on TLESRs. In contrast to our results in cats, Zou *et al.*^[12] found the inhibited effect of

acupoint stimulation was not inhibited by naloxone. In that study, the frequency of electrical stimulation is 100 Hz. However, our wave patterns were sparse and dense pulse intervals ranging from 2 Hz to 100 Hz (2/100 Hz). Previous studies have demonstrated that low-frequency (2 Hz) electroacupuncture analgesia (EAA) is induced by the activation of μ - and δ -opioid receptors *via* the release of enkephalin, β -endorphin, and endomorphin; and high-frequency (100 Hz) EAA is caused by activation of κ opioid receptors *via* release of dynorphin^[31]. So it may be the reason of the variance between our findings and Zou's research.

In conclusion, electric acupoint stimulation at the Neiguan result in a significant reduction of the rate of TLESRs induced by gastric distention. This effect appears to act at the brain stem, and may be mediated through NO, CCK-A receptor and mu-opioid receptor.

COMMENTS

Background

Gastroesophageal reflux disease (GERD) is a disorder characterized by an increased exposure of the esophagus to the intragastric contents. Recent studies have suggested that transient lower esophageal sphincter relaxation is the main mechanism underlying gastroesophageal reflux. It involves a prolonged relaxation of the lower esophageal sphincter, mediated by a vago-vagal neural pathway, synapsing in the brainstem. Acupuncture has been used to treat functional gastrointestinal disorders in the eastern countries for centuries. It can modulate visceral sensation as well as function through stimulation at selected acupoints along the meridians (channels of acupoints).

Research frontiers

Transient lower esophageal sphincter relaxation (TLESR) is the most important mechanism of gastroesophageal reflux (GER) in the patients of GERD. So it had become an important target in dealing with gastroesophageal reflux disease.

Innovations and breakthroughs

Although there have one article indicated that electroacupuncture at neiguan (PC6) may decrease the frequency of TLESRs, our research is the first paper to observe the mechanisms carefully.

Applications

Our research observed the relationship between electroacupuncture at neiguan (PC6) and TLESRs. And there may be a significant clinical impact in the future.

Terminology

TLESR: It is a spontaneous relaxation of LES without swallow induced. Its definition included: (1) absence of swallowing for 4 s before to 2 s after the onset of LES relaxation. (2) relaxation rate of $> \text{or} = 1 \text{ mmHg/s}$. (3) time from onset to complete relaxation of $< \text{or} = 10 \text{ s}$. (4) nadir pressure of $< \text{or} = 2 \text{ mmHg}$. Exception: a markedly prolonged LES relaxation $> \text{or} = 10 \text{ s}$, and nadir pressure $< \text{or} = 2 \text{ mmHg}$ can also be classified as TLESR, irrespective of the relation with swallow; Common Cavity: It was defined as abrupt simultaneous and sustained rises of basal esophageal pressure to intragastric pressure in at least the two lower esophageal body manometry recording sites.

Peer review

This manuscript it is well written and the experimental design is sound. In addition, there may be a significant clinical impact if these results will be confirmed in the future.

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Impaired contractility and remodeling of the upper gastrointestinal tract in diabetes mellitus type-1

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Abstract

AIM: To investigate that both the neuronal function of the contractile system and structural apparatus of the gastrointestinal tract are affected in patients with longstanding diabetes and autonomic neuropathy.

METHODS: The evoked esophageal and duodenal contractile activity to standardized bag distension was assessed using a specialized ultrasound-based probe. Twelve type-1 diabetic patients with autonomic neuropathy and severe gastrointestinal symptoms and 12 healthy controls were studied. The geometry and biomechanical parameters (strain, tension/stress, and stiffness) were assessed.

RESULTS: The diabetic patients had increased frequency of distension-induced contractions (6.0 ± 0.6 vs 3.3 ± 0.5 , $P < 0.001$). This increased reactivity was correlated with the duration of the disease ($P = 0.009$). Impaired coordination of the contractile activity in diabetic patients was demonstrated as imbalance between the time required to evoke the first contraction at the distension site and proximal to it (1.5 ± 0.6 vs 0.5 ± 0.1 , $P = 0.03$). The esophageal wall and especially the mucosa-submucosa layer had increased thickness in the patients ($P < 0.001$), and the longitudinal and radial compressive stretch was less in diabetics ($P <$

0.001). The esophageal and duodenal wall stiffness and circumferential deformation induced by the distensions were not affected in the patients (all $P > 0.14$).

CONCLUSION: The impaired contractile activity with an imbalance in the distension-induced contractions likely reflects neuronal abnormalities due to autonomic neuropathy. However, structural changes and remodeling of the gastrointestinal tract are also evident and may add to the neuronal changes. This may contribute to the pathophysiology of diabetic gut dysfunction and impact on future management of diabetic patients with gastrointestinal symptoms.

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Key words: Diabetes; Autonomic Neuropathy; Biomechanics; Contractility; Ultrasound; Esophagus; Duodenum; Deformation; Stress

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INTRODUCTION

Gastrointestinal (GI) symptoms (nausea, vomiting, bloating, abdominal pain, diarrhea, etc.) are frequent in patients with diabetes mellitus^[1-4]. The symptoms are often severe and substantially compromise quality of life. Abnormal GI function in diabetic patients has been demonstrated with methods such as manometry, scintigraphy, radiography, and breath tests. For example, the esophagus is characterized by dysmotility with fewer contractions having decreased amplitudes and abnormal wave forms^[5]. The pathogenesis of the GI symptoms in diabetes is complex in nature, multi-factorial and not well-understood^[5]. Dysmotility and delayed emptying of the stomach have been demonstrated, and in the small and large intestine dysmotility, delayed transit, and bacterial overgrowth have been observed^[5]. The GI dysfunction and symptoms may be caused by autonomic neuropathy being one of the most prevalent complications affecting

up to 40% of patients with long-standing diabetes^[6,7], and several clinical studies have demonstrated neuropathy of the autonomic nervous system (especially vagal but also sympathetic), as well of enteric nerves^[8]. Impaired visceral sensory function, glycemic control and psychological factors may also be contributing factors^[9-13]. Finally, mechanical factors may also contribute to the symptoms. Hence, studies in animals with experimental diabetes have shown structural remodeling and protein cross-linking in the GI wall layers compared to control animals^[14-18]. Structural remodeling caused by diabetes in animals is known to cause changes in the biomechanical properties, resulting in increase of both stiffness and thickness of the GI wall^[19-22].

The impact of the structural changes in the human GI wall on the function and on biomechanical properties has not been studied in detail due to inaccessibility of the organs and lack of suitable methodology. Such studies are needed since it is still generally assumed that the contractile (and structural) apparatus of the GI tract is normal, and that the disordered function and abnormal contractile activity predominantly reflects neuronal abnormalities^[23,24]. Better ways of studying the GI tract may impact on the future management of diabetic patients with GI symptoms. Cross-sectional ultrasound imaging have recently been developed to study the biomechanical properties of the GI tract during distension in animals^[25,26] and humans^[27-29]. The deformation pattern, the radial distributions of strain, stress and stiffness, and the distension-induced sensation have been assessed in the human esophagus^[30]. The technique has also been applied to assess the biomechanical properties of the human duodenum^[31]. Ultrasound has no known short or long term hazards and provides excellent soft tissue imaging with a good temporal and spatial resolution and is a valuable tool for studying GI function *in vivo*.

The hypotheses in the present study were that (1) the biomechanical properties of the esophageal and duodenal wall were changed due to diabetes-induced tissue remodeling and that (2) the contractile activity of the esophagus and duodenum was affected by the neuronal dysfunction related to diabetic autonomic neuropathy. Hence, the aims were to apply the new ultrasound based testing approach to (1) assess the distension-induced contractile activity in the human upper gut in healthy controls and in patients affected by gastrointestinal dysfunction due to diabetic autonomic neuropathy, and (2) to look into the mechanism of the findings by assessing the GI remodeling including the wall thickness and distension-induced deformation patterns.

MATERIALS AND METHODS

Study subjects

Data were obtained from 15 diabetic patients recruited at the Department of Endocrinology M, Aarhus University Hospital (13 males, 2 females, mean age 43 years, range 25-62 years) and 12 healthy controls (7 males, 5 females, mean age 37 years, range 29-50 years) recruited among the hospital staff and at the university. The local Ethical Committee approved the study protocol (VN

2003/120mch) which also conforms to the Declaration of Helsinki. Oral and written informed consent was obtained from all subjects.

All of the 12 patients who completed the study (Table 1) had type 1 diabetes lasting 12 to 46 years (average 23 years) and all suffered from debilitating symptomatic diabetic autonomic neuropathy (shown by a minimum of two symptoms from different organ systems) and verified by abnormal cardiovascular reflexes (heart rate variability and blood pressure changes during deep breathing and going from lying to standing). All suffered from peripheral neuropathy as demonstrated by absent or diminished patellar reflexes and abnormal biotensimetry values. The patients underwent examinations that were justified by their symptoms to exclude any organic diseases affecting the GI tract. Clinical data from the 12 patients who completed the study are presented below and in Table 1. All patients had severe GI symptoms: nausea (12 of 12), vomiting (10 of 12), abdominal pain (4 of 12), diarrhea (9 of 12), and constipation (2 of 12). Five of the 12 patients were taking medication known to affect gastrointestinal function (erythromycin, metoclopramide and proton pump inhibitors) while the rest were not treated because of previous insufficient response to various drugs. Four patients suffered from neuropathic pain and were treated with analgesics (oxycodone, gabapentin, pregabalin and paracetamol). None of the patients had prior abdominal surgery or suffered from psychiatric diseases or had any suspicion of psychological abnormalities. The control subjects did not take medications, had no prior abdominal surgery and did not suffer from any GI symptoms or pain-related diseases. They all had normal physical examination and blood tests.

Experimental probe design

The probe consisted of a 120 cm catheter (Ditens A/S, Aalborg, Denmark) with a 6.2 mm outer diameter and eight lumens of different sizes (Figure 1 top)^[30]. A 50 μ m thick polyurethane bag (Ditens A/S, Aalborg, Denmark) was attached to the catheter with 5 cm between the attachment points and with its centre positioned corresponding to the crystal of the ultrasound probe. The bag could be inflated to a maximum diameter of 50 mm (cross-sectional area (CSA) of 2000 mm²) with a constant bag length without stretching the bag wall. The size of the bag was chosen on the basis of previous studies of the duodenum where the CSA never exceeded 2000 mm² when the bag was inflated to the point where moderate pain was reported^[32].

The largest lumen in the probe contained a 20 MHz endoscopic 360 degrees ultrasound probe (UM-3R, Olympus Corporation, Tokyo, Japan). The signal from the endoscopic ultrasound unit (EU-M30, Olympus Corporation, Tokyo, Japan) was directly captured and stored digitally (AVI MPEG4 format) by frame grabber software (Studio 8, Pinnacle Systems Inc., CA, USA) for later analysis. Another large lumen was for infusion and withdrawal of fluid to the bag. The lumen was connected to a roller pump (Type 110, Ole Dich, Hvidovre, Denmark) for inflation and deflation of the bag with 37°C sterile water at a constant rate^[30,33].

Two small lumens (< 1 mm in diameter) were used for

Table 1 Clinical data describing the 12 type-1 diabetic patients that completed the study

Patient No.	1	2	3	4	5	6	7	8	9	10	11	12
Gender	M	M	F	M	M	M	M	M	M	M	M	F
Age (yr)	30	47	25	32	40	46	42	39	41	62	53	33
Height (cm)	176	180	165	176	169	182	180	185	168	174	175	170
Weight (kg)	64	86	65	60	90	78	75	85	66	79	95	61
Diabetes duration (yr)	18	37	16	12	32	27	32	14	13	46	20	14
Autonomic neuropathy	S,T	S,T	S,T	S,T	S,T	S	S,T	S	S	S	S	S,T
Peripheral neuropathy	S,T	S,T	S	S,T	S,T	S	S,T	S	S	S	S	S,T
Neuropathic pain	-	+	-	-	-	-	+	+	-	-	-	+
Retinopathy	+	+	+	+	+	+	+	+	+	+	+	+
Nephropathy	-	+	+	-	+	+	+	-	+	+	+	+
Bladder paresis	-	-	-	-	+	-	-	-	-	-	-	+
Gastroparesis ¹	+	+	+	+	+	-	-	-	-	+	-	+
Sexual dysfunction	-	-	-	-	-	-	-	-	+	-	-	+
HbA _{1c} (%)	11.1	8.6	14.1	10.5	7.7	10.7	9.2	9.3	10.4	10.2	9.2	7.3
Creatinine (mmol/L)	75	101	111	63	73	87	123	78	96	108	118	52
Nausea	+	+	+	+	+	+	+	+	+	+	+	+
Vomiting	+	+	+	+	+	-	+	+	+	+	+	-
Abdominal pain	+	-	+	-	+	-	-	-	-	+	-	-
Diarrhea	+	-	+	+	+	+	+	+	+	-	-	+
Constipation	-	-	-	-	+	-	-	+	-	-	-	-
Gastroscopy	N	N	N	N	N	N	N	N	N	N	N	N
Colonoscopy	ND	ND	N	ND	N	ND	ND	N	N	ND	ND	N
Small bowel radiology	ND	ND	N	N	N	N	ND	ND	N	N	ND	N
Breath test ²	N	ND	N	N	N	N	ND	ND	ND	N	ND	N
Insulin treatment	inj	pump	inj	inj	inj	inj	inj	inj	inj	inj	inj	pump
GI medication	-	-	+	-	-	-	+	-	+	+	-	+
Analgetics	-	+	-	-	-	-	+	+	-	-	-	+
Smoking	+	-	+	+	-	+	-	+	-	+	-	-

M: male, and F: female. S: verified by classic symptoms, and T = verified by tests. “-”: not present, and “+”: present. N: normal examination, and ND: not done. “inj”: injection by insulin pen. “pump”: injection by pump system. ¹Assessed by scintigraphy. ²To exclude bacterial overgrowth. Normal range of HbA_{1c} is < 6% and Creatinine < 125 mmol/L (M) or < 115 mmol/L (F).

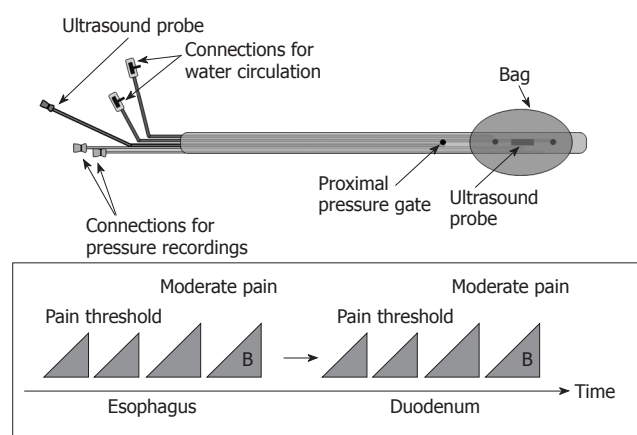


Figure 1 The probe design allows bag distension of the esophagus and duodenum together with cross-sectional ultrasound imaging and recording of the bag and proximal pressures (top). The distension protocol is shown at the bottom. The first distensions to the pain threshold were preconditioning stimuli also used for learning. They were followed by a distension (15 mL/min in the esophagus and 25 mL/min in the duodenum) to the perception of moderate pain. Finally, a bolus of 20 mg of butylscopolamine (B) was given intravenously in order to diminish distension-evoked smooth muscle contractions and a final distension to the perception of moderate pain was done.

recording of pressures inside the bag and 6 cm proximal to the center of the bag. The channels were continuously perfused at a rate of 0.1 mL/min with sterile water by a low-compliance perfusion system. The pressure channels were attached to external pressure transducers (Baxter,

Deerfield, IL, United States). The signals were amplified, analogue-to-digital converted and stored on a computer for later analysis (Openlab, Ditens A/S, Aalborg, Denmark).

Study protocol

The patients were fasting for 12 h prior to the experiment due to the well known delayed gastric emptying in this patient group (Table 1). During the fasting period the blood glucose concentrations were monitored every hour and adjusted to approximate the normal range (below 6 mmol/L) using intravenous glucose infusions and subcutaneous injection of fast-acting insulin (Actrapid, Novo, Bagsværd, Denmark). The healthy controls were fasting for six hours (Figure 1, bottom).

The probe was swallowed and the subject was positioned supine with the upper part of the body 30 degrees tilted. The lower esophageal sphincter was identified guided by the pressure recordings and the ultrasound image. The bag was positioned 10 cm above the lower esophageal sphincter. Distension was done by inflating the bag at a rate of 15 mL/min. Several preconditioning distensions to the pain threshold were done prior to experimental distensions to the perception of moderate pain^[34,35]. The preconditioning distensions were repeated until the obtained data were reproducible. The bag was deflated between distensions at a rate of 15 mL/min. Then 20 mg of butylscopolamine (Buscopan, Boehringer, Ingelheim, Germany) was administrated intravenously to abolish esophageal contractility followed

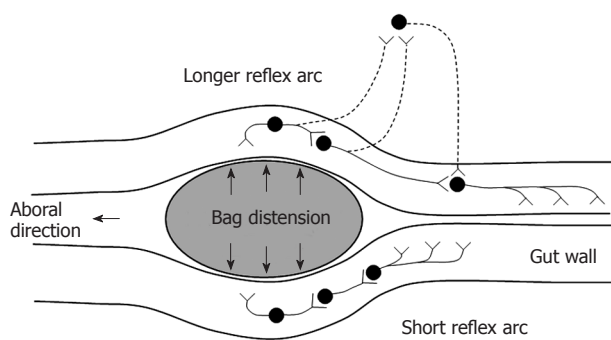


Figure 2 Schematic illustration of the neural pathways involved in distension-induced contraction. The primary afferent neurons, the excitatory interneurons and efferent neurons are shown. The dotted neurons illustrate extrinsic pathways. The induction of contraction on the bag depends on the local reflex arc (bottom). The measurement 6 cm proximal to the bag depends on a longer reflex arc including extrinsic pathways (top).

by one distension to the perception of moderate pain.

The probe was then advanced into the horizontal part of the duodenum guided by transabdominal ultrasound (SonoSite 180, SonoSite Inc, Bothell, WA, USA), endoscopic ultrasound and by the motility pattern observed. Approximately 30 min after the esophageal testing, and when duodenal phase II activity was observed, duodenal distensions were performed before and after administration of butylscopolamine as described above but at an inflation rate of 25 mL/min.

The total examination time was approximately two hours and the blood glucose concentrations in all patients and controls were measured before, after one hour, and at the end of the study. The blood glucose level was adjusted to approximate the normal range if it deviated during the study.

Analysis of the distension-induced contractile activity

Contractions were defined as having amplitude ≥ 10 cm H₂O and duration ≥ 3 s. Contractile activity at the distension site and proximal to it were analyzed for the filling phase of all distensions. The time from start of the distension to induction of the first contraction during the distension procedure was noted. The time to induce the first contraction at the distension site is generally thought to be dependent on a local short enteric reflex arc. The time to induce the first contraction 6 cm proximal to the bag depends on a longer reflex arc that is more likely affected by extrinsic pathways (Figure 2). Hence, the ratio between these contractile responses serves as a proxy of the function of the neural pathway between the bag and proximal pressure measurement site. The number of contractions during the first minute and the frequency of all contractions were also noted. Furthermore, the pressure amplitude and duration of the strongest contraction were calculated. A proxy of the contractile work was computed by multiplying the amplitude and the duration of the strongest contraction. In the duodenum the frequency of contractions at the proximal pressure measurement site was also calculated before and after the distension procedure.

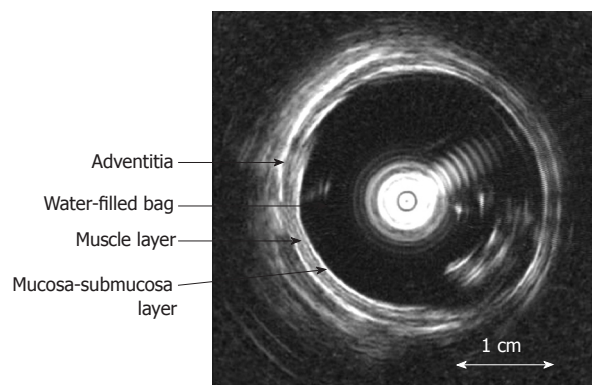


Figure 3 The cross-sectional ultrasound image of the distended distal esophagus allows identification of the esophageal layers, i.e. mucosa-submucosa, muscle and adventitia layers. The white round shadow in the centre is caused by the intraluminal ultrasound probe.

Biomechanical analysis of esophageal and duodenal distensions

The biomechanical properties including stress-strain and tension-strain relations, esophageal wall thickness and multidirectional deformation were measured and calculated from the intraluminal ultrasound images (Figure 3) and pressure recordings (see Appendix).

Statistical analysis

Data are given as mean \pm SE unless otherwise stated. The contractile activity of the patients and controls was analyzed using one-way analysis of variance (ANOVA). The contraction frequencies in the duodenum in relation to the distension and the duodenal wall stiffness were analyzed using two-way ANOVA, which was also used for the esophageal stress, strain, stress-strain ratio, multidirectional deformation and wall thickness, and duodenal tension, strain and tension-strain ratio at maximum perception. The esophageal wall stiffness was analyzed using three-way ANOVA with the factors: (1) controls *vs* patients, (2) before *vs* after butylscopolamine, and (3) mucosal surface *vs* submucosa-muscle interface *vs* outer surface. The association between the clinical data and the contractile activity and biomechanical properties was analyzed using Spearman correlation test. *P*-values less than 0.05 were considered significant. If the data were not normal-distributed they were logarithmically transformed before the parametric tests were performed. SPSS version 11.0 was used for the statistical analysis.

RESULTS

The study was completed in 12 (10 males, 2 females, mean age 40 years, range 25-62 years) of the 15 diabetic patients. Three patients interrupted the study in its initial phase due to severe throat irritation, nausea and vomiting. All healthy controls completed the study.

Blood glucose levels

At the beginning of the 12 h fasting period the diabetic patients had a mean blood glucose level of 10.6 (range

Table 2 The contractile activity on the bag and at the proximal pressure recording site are given for both the esophagus and duodenum (mean \pm SEM)

	Esophagus				Duodenum			
	Bag		Proximal		Bag		Proximal	
	Controls	Diabetes	Controls	Diabetes	Controls	Diabetes	Controls	Diabetes
Time to first contraction (s)	17.5 \pm 6.0 <i>F</i> = 3.8	9.9 \pm 2.7 <i>P</i> = 0.07	35 \pm 9.0 <i>F</i> = 16	6.6 \pm 2.3 <i>P</i> < 0.001	16.5 \pm 5.9 <i>F</i> = 0.4	15.9 \pm 5.2 <i>P</i> = 0.6	28 \pm 8 <i>F</i> = 2.2	6.5 \pm 2.5 <i>P</i> = 0.16
Ratio bag/proximal, time to first contraction	0.5 \pm 0.1 <i>F</i> = 5.8	1.5 \pm 0.6 <i>P</i> = 0.03			0.6 \pm 0.2 <i>F</i> = 3.6	1.2 \pm 0.2 <i>P</i> = 0.08		
Number of contractions in first minute	2.6 \pm 0.5 <i>F</i> = 14	5.9 \pm 0.7 <i>P</i> = 0.001	2.2 \pm 0.6 <i>F</i> = 5.8	4.5 \pm 0.8 <i>P</i> = 0.03	1.6 \pm 0.4 <i>F</i> = 0.2	1.9 \pm 0.5 <i>P</i> = 0.7	1.2 \pm 0.2 <i>F</i> = 2.9	2.1 \pm 0.7 <i>P</i> = 0.09
Frequency (min ⁻¹)	3.3 \pm 0.5 <i>F</i> = 13	6.0 \pm 0.6 <i>P</i> = 0.001	3.0 \pm 0.7 <i>F</i> = 2.6	4.6 \pm 0.8 <i>P</i> = 0.11	1.7 \pm 0.4 <i>F</i> = 0.03	1.7 \pm 0.5 <i>P</i> = 0.9	1.2 \pm 0.3 <i>F</i> = 0.5	1.7 \pm 0.4 <i>P</i> = 0.5
Maximum amplitude (mm H ₂ O)	95 \pm 11 <i>F</i> = 4.9	63 \pm 10 <i>P</i> = 0.04	37 \pm 7 <i>F</i> = 0.09	34 \pm 5 <i>P</i> = 0.8	19.1 \pm 4.8 <i>F</i> = 0.01	19.5 \pm 5.1 <i>P</i> = 0.9	15.9 \pm 2.6 <i>F</i> = 0.08	14.6 \pm 3.8 <i>P</i> = 0.8
Contractile work (mm H ₂ O x s)	1470 \pm 257 <i>F</i> = 7.8	592 \pm 165 <i>P</i> = 0.01	386 \pm 57 <i>F</i> = 7.5	181 \pm 43 <i>P</i> = 0.01	106 \pm 50 <i>F</i> = 0.9	88 \pm 43 <i>P</i> = 0.8	60 \pm 15 <i>F</i> = 0.6	42 \pm 16 <i>P</i> = 0.4

P- and *F*-values are given, and bold *P*-values indicate significant difference between the diabetes patients and healthy controls.

7.0-16.5) mmol/L with fluctuations during the first hours. The glucose level was adjusted and stabilized to a mean level of 8.1 (range 5.2-10.5) mmol/L during the study period. The controls had a mean blood glucose level (after 6 h fasting) of 4.6 (range 3.5-5.2) mmol/L during the study period.

Distension-induced contractile activity

Data on the contractile activity induced by the bag distensions are provided in Table 2.

Esophagus: The diabetic patients had shorter time to the first contraction on the bag (borderline), increased number of bag contractions during the first minute, increased frequency of contractions, reduced pressure amplitudes and reduced contractile work of the bag contractions. Similar findings were found 6 cm proximal to the bag where the diabetic patients had shorter time to the first contraction, increased number of contractions during the first minute, and reduced contractile work of the contractions. The ratio between the time until the first contraction on the bag and the time until the first contraction 6 cm proximal to the bag was increased in the diabetic patients. This likely reflects dysfunction of local intestinal neural pathways.

Duodenum: The contractile activity between the diabetic patients and controls did not differ in the duodenum. The ratio between the time until the first contraction on the bag and the time until the first proximal contraction tended to increase in the diabetic patients. Furthermore, the frequency of contractions in the diabetic duodenum was 2.3 ± 1.0 min⁻¹ before, 2.1 ± 0.8 min⁻¹ during and 2.8 ± 0.6 min⁻¹ after the distension and in the controls the numbers were 1.5 ± 0.4 min⁻¹, 1.6 ± 0.6 min⁻¹ and 1.9 ± 0.8 min⁻¹, respectively. Hence, no difference between the patients and controls (*F* = 0.8, *P* = 0.4) and in relation to the distension (*F* = 0.5, *P* = 0.6) were found.

Esophageal wall thickness

The thickness of the entire esophageal wall, the muscle

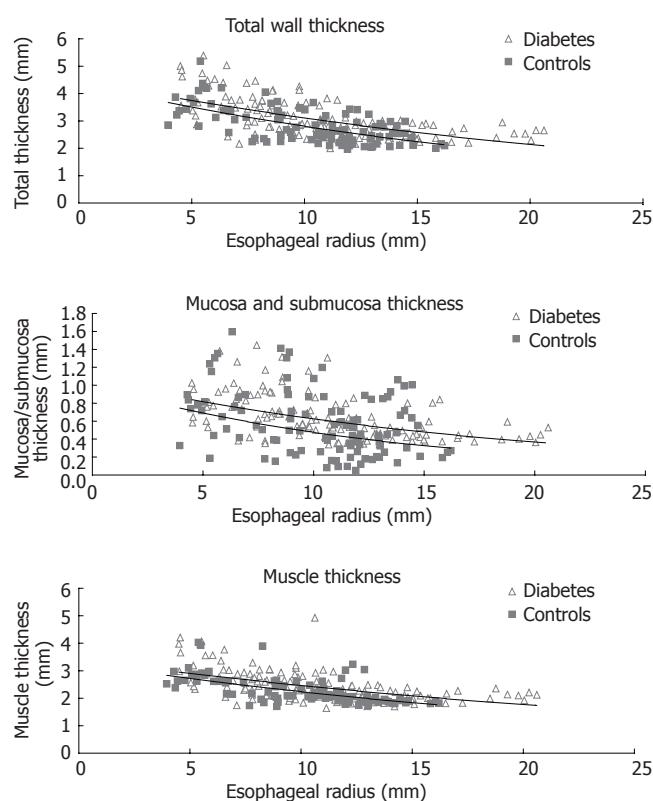


Figure 4 The distension-induced change in thicknesses of the total esophageal wall structure, the muscle layer, and mucosa-submucosa layer during smooth muscle relaxation with butylscopolamine are illustrated as function of the esophageal radius. The data points represent the multiple measuring points during each distension of the patients and controls. Exponential trend lines (solid lines) of the patients and controls are shown. The total wall thickness and the mucosa-submucosa layer were increased in the diabetic patients.

layer and mucosa-submucosa layer obtained during the distensions (after administration of butylscopolamine) are illustrated in Figure 4. The wall and the mucosa-submucosa layer were thicker (0.2-0.3 mm) in the diabetic patients compared to the control subjects (*F* = 13, *P* < 0.001, and *F* = 13, *P* < 0.001). The muscle layer thickness showed a borderline increase (*F* = 3.7, *P* = 0.055).

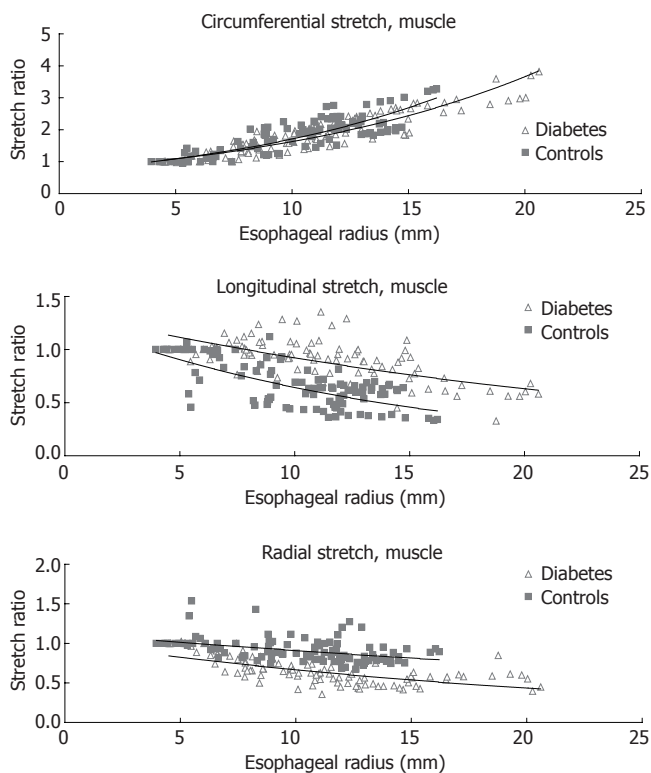


Figure 5 The distension-induced change in circumferential, longitudinal and radial stretch ratios are illustrated as function of the esophageal radius. The curves were obtained during smooth muscle relaxation with butylscopolamine. The data points represent the multiple measuring points during each distension of the patients and controls. Exponential trend lines (solid lines) of the patients and controls are shown. The shortening during distension was clearly reduced in the diabetic patients while the radial stretch was decreased.

Multidirectional deformation of the esophagus

The circumferential, longitudinal and radial stretch ratios during distension in both the diabetic patients and control subjects are shown in Figure 5. The curves were obtained from the muscle layer and after administration of butylscopolamine. The same pattern was seen in all sub-layers both before and after administration of butylscopolamine. During distensions the patients tended to stretch less in the circumferential direction ($F = 0.007$, $P = 0.1$). The compressive deformation (shortening) in the longitudinal and radial directions during distension was clearly reduced in the diabetic patients ($F = 150$, $P < 0.001$ for longitudinal direction and $F = 180$, $P < 0.001$ for the radial direction). Thus, the esophageal wall appeared to be deformed less in all normal directions in diabetics compared to healthy volunteers.

Stress-strain and tension-strain relations

The tissue stiffness (approximated by the mechanical alpha-constant, see Appendix) in circumferential direction of the different esophageal layers and the duodenum are provided in Figure 6.

Esophagus: The circumferential stiffness increased throughout the esophageal wall ($F = 14$, $P < 0.001$). The difference in tissue stiffness between the patients and the control subjects was non-significant ($F = 0.3$, $P = 0.6$) and unaffected by butylscopolamine ($F = 2.2$, $P = 0.15$).

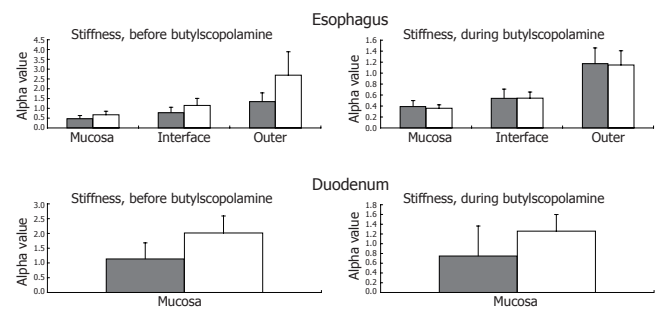


Figure 6 The computed esophageal stiffness (circumferential [alpha] constant) of the mucosa, submucosa-muscle interface and outer surface are illustrated. The solid grey bars represent the controls, while the grey-white bars represent the diabetic patients. The stiffness tends to be higher in the diabetic patients and is normalized during smooth muscle relaxation with butylscopolamine. This indicates an increased esophageal resting tone in diabetes. The computed duodenal stiffness is also illustrated. Mean and SEM values are shown.

At moderate induced pain, the circumferential stress, the circumferential strain and the ratio between them (which also indicates the wall stiffness) obtained in the patients were not significantly different from those obtained in the control subjects ($F = 0.7$, $P = 0.4$; and $F = 1.3$, $P = 0.3$; and $F = 0.7$, $P = 0.4$), and unaffected by butylscopolamine (all $P > 0.4$).

Duodenum: The difference in tissue stiffness (Figure 6) between the patients and the controls was non-significant ($F = 2.2$, $P = 0.14$). The stiffness was not changed by butylscopolamine ($F = 1.1$, $P = 0.3$). At moderate pain the circumferential tension, the circumferential strain and the ratio between them (indicating the wall stiffness) did not differ between patients and control subjects ($F = 1.0$, $P = 0.3$; and $F = 0.02$, $P = 0.9$; and $F = 2.3$, $P = 0.14$), and was unaffected by butylscopolamine (all $P > 0.5$).

Correlation to clinical data

The distension induced contractile activity in the diabetic patients was not affected by the mean glucose level during the study ($P > 0.2$ for all comparisons). The disease duration was clearly associated with the distension-induced contractile activity. Thus, the disease duration correlated with increased frequency of the contractions and with the number of contractions during the first minute proximal to the esophageal bag (correlation coefficient $r_s = 0.71$, $P = 0.009$ and $r_s = 0.69$, $P = 0.01$), and to increased duration of the strongest contraction on the esophageal bag ($r_s = 0.71$, $P = 0.009$). In the duodenum the disease duration correlated with increased frequency (bag: $r_s = 0.58$, $P = 0.04$; proximal: $r_s = 0.68$, $P = 0.01$), increased number of contractions during the first minute (bag: $r_s = 0.57$, $P = 0.04$; proximal: $r_s = 0.68$, $P = 0.04$), increased pressure amplitude (bag: $r_s = 0.59$, $P = 0.04$; proximal: $r_s = 0.70$, $P = 0.01$), duration (bag: $r_s = 0.62$, $P = 0.03$; proximal: $r_s = 0.69$, $P = 0.01$) and contractile work (bag: $r_s = 0.63$, $P = 0.03$; proximal: $r_s = 0.72$, $P = 0.01$) of the strongest contraction both at and proximal to the bag.

DISCUSSION

An ultrasound based testing approach was applied to

assess the contractile activity, geometry and biomechanical properties of the esophagus and duodenum in patients affected by gastrointestinal symptoms and diabetic autonomic neuropathy. Overall, this study illustrates that the patients had increased reactivity to standardized esophageal distension including dyscoordination and reduced contractile work of the contractions. The disease duration was associated with increased contractile reactivity. Finally, the esophageal wall thickness and the pattern of deformation in longitudinal and radial directions were affected in diabetic patients indicating remodeling.

Both acute and chronic hyperglycemia is known to impair the GI motor responses to stimulation^[12,36] and to reduce the perceived sensation^[37]. This study was designed to minimize the influence of the blood glucose level on the contractile activity. However, the patients in the present study typically suffered from severe fluctuating glucose levels and fluctuations in GI symptoms with intermittent nausea and vomiting which makes glycemic control very challenging. Even though the mean blood glucose level during the study was higher in the diabetic patients, the glucose level itself did not seem to affect the contractile activity. Hence, the differences found in this study are more likely due to neuromuscular changes. However, comparison of data obtained in different studies of diabetic patients must be done with caution.

The present study utilizes a *cross-sectional imaging technique*. Conventional methods based on *pressure-volume measurements* obtained during bag distension do not directly measure variables used in analysis of tissue deformation and stiffness^[38]. Since the esophagus is thick-walled, only cross-sectional ultrasound imaging with good temporal and spatial resolution provides data for computation of the circumferential stress, wall thickness and multi-directional deformation^[30].

All patients included in the study had, as per inclusion criteria, severe and long-lasting upper GI symptoms (nausea, vomiting, bloating and pain). The observed esophageal contractile hyperreactivity may be explained by both primary neuropathic changes of the gut nerves and secondary changes due to reflex mechanisms caused by increased sensitivity to the distensions (central sensitization)^[39]. However, esophageal motor abnormalities in terms of decreased contraction amplitude, decreased number of esophageal peristaltic contractions^[13,23,24,40] and impaired coordination^[41,42] have also been recorded in diabetic patients without GI symptoms. Disordered esophageal motility and acid reflux may be related to diabetic neuropathy^[43]. Such changes correspond to the changes observed during distension in the present study. The observed reduction in time until the first contraction on the bag in the diabetic patients suggests that a local neuronal dysfunction is responsible for the hyperreactivity. This is consistent with the finding that the hyperreactivity is associated with the disease duration. A neuronal dysfunction can theoretically be addressed to the mechanoreceptor, afferent fibers, interneurons, or efferent fibers located in the gut wall. Impaired balance of the inhibitory and excitatory pathways from the central nervous system can potentially also affect the motor response. The time until the first contraction 6 cm proximal to the bag was also reduced in the diabetic

patients, which may indicate hyperactivity and dysfunction of local intestinal neural pathways (a long reflex arc which may also include extra-intestinal pathways, Figure 2). Since this long reflex arc is more affected than the short arc (evidenced in Table 2 and by the computed ratio), the neuronal pathways rather than the mechanoreceptors seem to be affected. Even though neuropathic damage to the nerve fibers is expected, central (and peripheral) neuronal hyperexcitability in response to the distensions may counteract the response. Such hyperreactivity and hyperexcitability have for example been shown in patients with non-cardiac chest pain^[44]. In contrast, in structural GI disorders such as systemic sclerosis, the local mechanoreceptors in the gut wall seem to be affected (resetting) more than the neuronal pathways^[45]. However, the present study cannot definitively distinguish between neuronal changes restricted to the enteric nervous system and the effect of changes in the central inhibitory and excitatory neuronal pathways. Advanced methods using evoked brain potentials and functional magnetic resonance imaging may shed more light on this important aspect. Also one should take into consideration that the neuronal changes observed could be due to both diabetes-induced autonomic neuropathy and changes in the afferent visceral nervous system (sensitization and hypersensitivity) evoked by the long-standing GI symptoms^[39]. Finally, the vagal innervation of different organs and central *vs* peripheral levels can easily be affected to different degrees^[46]. As an indicator of dysfunction of the esophageal neuromuscular apparatus, the diabetic patients also seem to have an increased resting tone of the muscle component in the esophagus. This is indicated by the mechanical constants in Figure 6 where smooth muscle relaxation is shown to abolish/normalize the increased stiffness observed in the diabetic patients.

Studies of the small intestinal contractile activity in diabetes have revealed a wide spectrum of motor patterns ranging from normal to grossly abnormal^[5]. The distension-induced duodenal contractile activity recorded in this study was not clearly affected in diabetes compared to the control subjects. However, the duodenal reactivity increased with the disease duration.

The question is if the disordered contractile activities observed in these studies are only due to the neuronal changes and dysfunction (diabetes-induced autonomic neuropathy) or if primary diabetes-induced remodeling in the GI tract may also play a role. Animal studies have shown that diabetes may induce crypt hyperplasia, change in the villous microvasculature and increase in the mucosal and muscle mass^[14,17,19,47]. A histopathological study of the human stomach in diabetic patients with severe gastroparesis showed prominent collagenization and smooth muscle atrophy of the muscle layer^[48]. Studies on diabetes and aging show that advanced glycation end-products are causing cross linking of collagen molecules responsible for basement membrane thickening and loss of matrix elasticity^[15,16,49]. Animal studies support the presence of structural and biomechanical changes with increased stiffness, weight per unit length and wall thickness (i.e. increased stiffness and thickness of the GI wall)^[19-22]. Even though the wall stiffness and circumferential deformation induced by the circumferential distensions

in the present study were not significantly affected in diabetes, the esophageal deformations in longitudinal and radial directions and the wall thickness were abnormal with reduced deformation. The fact that the deformation is reduced but the stiffness appears unchanged can be attributed to the stress-dependent growth law for soft tissues stating that tissue remodeling is determined by the stress and remodels towards a tissue-specific stress level^[35]. The esophageal wall and the mucosa-submucosa layer were thickened in diabetes, which indicates growth processes of the intestinal wall. The observed increase in esophageal wall thickness can theoretically be caused by increased muscle tone, even though an attempt to abolish smooth muscle contractions was done and repeated if contractions were still present. Butylscopolamine diminishes cholinergic mediated tone and from the tracings it appeared that relaxation was obtained. Also the decreased ability of esophageal shortening during distension and the change in radial deformation supports that structural changes occur in diabetic esophagus. The contractile work of the esophageal contractions was decreased in spite of increased neuronal activity, increased muscle thickness and the increased esophageal resting tone. This indicates that the ability of the muscle to contract could be restricted by structural remodeling (accumulation of connective tissue). The fact that the esophageal wall deformed less in diabetic patients supports this hypothesis. Alternatively, myopathic abnormalities with diabetes-induced reduced action of the muscle fibers may also be important^[48].

The present study shows that both the neuronal function of the contractile system and structural apparatus of the GI tract are affected in patients with long-standing diabetes and autonomic neuropathy. This may contribute to the understanding of the pathophysiology of diabetic gut dysfunction and may have impact on future management of these patients.

ACKNOWLEDGMENTS

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APPENDIX

Analysis of esophageal distensions

Ultrasound images were captured corresponding to the start of the distension (the zero-pressure state) and between contractions during the distensions (tonic state) by a video converter (jpg format, 787 × 576 pixels, A4 Video Converter v. 2.3, www.a4video.com)^[35]. The mucosal surface (bag-mucosa interface), the submucosa-muscle interface, and the outer surface (muscle-adventitia interface) contours of the esophageal wall were identified by visual inspection by an experienced radiologist using image measurement software (SigmaScan Pro v. 5.0.0, SPSS Inc., Chicago, IL, USA) (Figure 3). This allowed computation of the cross-sections encircled by the mucosal surface (CSA_{muc}), submucosa-muscle interface (CSA_{in}) and outer surface (CSA_{out}), and the corresponding

circumferences (C_{muc}, C_{in} and C_{out}).

For each of the three sur- and interfaces the *circumferential stretch ratio* (a deformation measure) was computed as the relative elongation of each circumference: $\lambda_\theta = c/c_{\text{zero-pressure}}$, where $c_{\text{zero-pressure}}$ denotes the circumference of the unloaded segment. The $c_{\text{zero-pressure}}$ was measured directly from ultrasound images at the start of the butylscopolamine distension. The *circumferential strain* at each circumference (mucosa, submucosa-muscle and outer, respectively) was represented by the Green strain:

$$\varepsilon_\theta = \frac{c^2 - c_{\text{zero-pressure}}^2}{2c_{\text{zero-pressure}}^2} \quad (1)$$

The radial deformation of the mucosal and muscle layers were computed as *radial stretch ratio*: $\lambda_r = h/h_{\text{zero-pressure}}$, where h and $h_{\text{zero-pressure}}$ represents the thickness of the distended and butylscopolamine relaxed layers.

Assuming a circular shape, the wall thicknesses were calculated as:

$$h_{\text{total}} = r_{\text{out}} - r_{\text{muc}} = \sqrt{\text{CSA}_{\text{out}}/\pi} - \sqrt{\text{CSA}_{\text{muc}}/\pi}, \quad (2a)$$

$$h_{\text{muc}} = r_{\text{in}} - r_{\text{muc}} = \sqrt{\text{CSA}_{\text{in}}/\pi} - \sqrt{\text{CSA}_{\text{muc}}/\pi}, \text{ and} \quad (2b)$$

$$h_{\text{muscle}} = r_{\text{out}} - r_{\text{in}} = \sqrt{\text{CSA}_{\text{out}}/\pi} - \sqrt{\text{CSA}_{\text{in}}/\pi}. \quad (2c)$$

The *longitudinal stretch ratio* of each layer which indicate the degree of longitudinal deformation was calculated as $\lambda_z = \frac{1}{\lambda_\theta \lambda_r}$ assuming incompressibility ($\lambda_\theta \lambda_r \lambda_z = 1$) of the tissue^[35].

Since the esophagus is thick-walled the pressure and stress decay through the wall is expected to be non-linear^[50]. Hence, the distribution of the circumferential stress through the wall was computed as:

$$\tau_{\theta,r} = \frac{r_{\text{out}}^2 r_{\text{muc}}^2 \Delta p}{r^2 (r_{\text{out}}^2 - r_{\text{muc}}^2)} + \frac{\Delta p r_{\text{muc}}^2}{r_{\text{out}}^2 - r_{\text{muc}}^2} \quad (3)$$

where r denotes the radial location inside the wall^[30,50]. The radii r_{out} and r_{muc} was calculated as: $r_{\text{out}} = \sqrt{\text{CSA}_{\text{out}}/\pi}$ and $r_{\text{muc}} = \sqrt{\text{CSA}_{\text{muc}}/\pi}$. Δp represents the bag pressure corrected for the baseline pressure (representing the mediastinal resting pressure) before the butylscopolamine distensions.

To obtain mechanical constants of each layer the circumferential stress-strain relationship of the mucosal surface, the interface between submucosa and muscle layer, and the outer surface layers for each subject were plotted. α and β constants were obtained using non-linear curve fitting (Microcal Origin 6.0, Microcal Software Inc., Northampton, MA, USA) approximating the equation^[51]:

$$\tau_\theta = \beta(e^{\alpha \varepsilon_\theta} - 1) \quad (4)$$

The computed stiffness parameter is dependent on passive stretch and the contribution from active muscle contraction or tone. Administration of butylscopolamine abolishes smooth muscle contractions whereby the passive properties can be assessed.

Analysis of duodenal distensions

The duodenal ultrasound images were captured as

described at the start of each distension and between the distension-induced contractions (see above). This allowed computation of the luminal duodenal *CSA* and mucosal circumference *c*. The *circumferential stretch ratio* was computed as the relative elongation of the mucosal surface (circumference *c*) during distension:

$$\lambda_\theta = c / c_{\text{zero-pressure}} \quad (5)$$

Since the ultrasound imaging not always allowed measurements at the start of the distension (artifacts due to air), $c_{\text{zero-pressure}}$ was in some cases approximated by a double logarithmic fitting of the circumference-pressure data. The validity of the fitting procedure was verified from the experiments with good imaging quality. A good agreement between fitted and measured values was found^[51]. The *circumferential strain* was computed as the Green strain, see Eq. 1.

As an approximation the wall thickness was not taken into account^[35] and consequently the *circumferential tension* was computed using Laplace's law:

$$T_\theta = \Delta p r \quad (7)$$

where *r* denotes the inner duodenal radius assuming circular shape: $r = \sqrt{CSA/\pi}$. The wall tension is the integration of the stresses through the wall (the stress moment).

The circumferential tension-strain relationships for each subject were plotted to obtain the curve fitting constants. The α and β constants were obtained using non-linear curve fitting (Microcal Origin 6.0, Microcal Software Inc., Northampton, MA, USA) using a modification of Fung's approach^[51]:

$$T_\theta = \beta(e^{\alpha\lambda_\theta} - 1) \quad (8)$$

Limitation of the ultrasound technique

The ultrasound technique suffers from limitations too. At low degrees of distension convolutions of the plastic bag resulted in artifacts (air and plastic folds) and in many cases only the bag-mucosa, submucosa-muscle and muscle-adventitia interfaces could be clearly identified. At low degrees of distension it was difficult to clearly identify the inner circumference of the duodenum. This was due to small amounts of air outside the bag and folds in the bag resulting in artifacts and the fact that the bag might not always be in contact with the wall in the entire circumference at low degrees of distension. To compensate for this problem, the mucosal circumference at low degrees of distension was approximated using double-logarithmic curve fitting. Other limitations were that the ultrasound system used in this study did not provide sufficient image quality for accurate measurement of the wall thickness in the entire duodenal circumference, especially at high degrees of distension.

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Ferucarbotran *versus* Gd-DTPA-enhanced MR imaging in the detection of focal hepatic lesions

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Abstract

AIM: To evaluate the efficacy of ferucarbotran-enhanced MR imaging in the detection of focal hepatic lesions compared to plain and Gd-DTPA-enhanced MR imaging.

METHODS: Fifty-nine patients with suspected focal hepatic lesions were admitted to the study. Plain MR imaging (FSE T₂WI with fat suppression and GRE T₁WI sequences) and Gd-DTPA dynamic enhanced MR of the liver were initially performed followed by ferucarbotran-enhanced MR imaging 48 h later (including GRE T₁WI, FSE T₂WI with fat suppression, and GRE T₂*WI sequences). Images were reviewed independently by three observers. Results were correlated with surgery and pathologic examination or reference examination, and sensitivity was statistically calculated for the different MR imaging sequences.

RESULTS: Among all confirmed lesions ($n = 133$), ferucarbotran-enhanced MR imaging revealed 130 lesions on FSE T₂WI with fat suppression, 115 lesions on dynamic T₁WI GRE, and 127 lesions on GRE T₂*WI. Pre-contrast MR imaging revealed only 84 lesions on GRE T₁WI and 106 lesions on FSE T₂WI with fat suppression, while Gd-DTPA dynamic enhanced GRE T₁WI revealed 123 lesions. For 44 micro-lesions (< 1.0 cm) in all patients the detection rates were as follows: ferucarbotran-enhanced FSE T₂WI with fat suppression, 93.2% (41/44); ferucarbotran-enhanced GRE T₂*WI, 88.6% (39/44); Gd-DTPA dynamic-enhanced GRE T₁WI, 79.5% (35/44); pre-contrast FSE T₂WI with fat suppression, 54.5% (24/44); and pre-contrast GRE T₁WI, 34.1% (15/44). In detecting micro-lesions, statistically significant difference was found for Ferucarbotran-enhanced FSE T₂WI with fat suppression and GRE T₂*WI sequences compared to the other sequences ($P < 0.05$).

CONCLUSION: Ferucarbotran-enhanced FSE T₂WI with fat suppression and GRE T₂*WI sequences are superior in detecting micro-lesions (< 1 cm) in comparison with plain and Gd-DTPA dynamic-enhanced MR imaging.

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Key words: Liver disease; Contrast media; Superparamagnetic iron oxide; Magnetic resonance imaging

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INTRODUCTION

The detection of focal liver lesions in patients with liver cancer is a very important challenge because failure to detect cancerous lesions can have major clinical consequences. High accuracy in liver cancer detection can improve the efficacy of treatment, including partial hepatectomy, liver transplantation, radiofrequency ablation, percutaneous ethanol injection, transarterial chemoembolization, or (commonly) a combination of these methods^[1-5]. Currently, magnetic resonance (MR) is increasingly used in the detection of hepatic lesions^[6]. With the use of conventional extracellular contrast agents such as gadolinium chelates, analysis of enhancement patterns on T₁-weighted dynamic imaging during the different vascular phases is an important tool in the detection and characterization of focal hepatic lesions. Post-contrast imaging has been shown to be superior to conventional plain MR imaging in detecting hepatic lesions^[7]. It is known, however, that 40%-60% of cancer nodules, especially those in cirrhotic liver that are smaller than 10 mm, are missed at ultrasonography (US), computed tomography (CT)^[8], and MR^[9]. Given this background, a possible solution to this problem is the use of liver-specific MR contrast materials: that is, agents that are targeted to either the hepatocytes or the Kupffer cells. Ferucarbotran is available as a new superparamagnetic iron oxide (SPIO) agent for liver imaging in most European countries and some Asian countries^[10,11]. The present study was designed

as an open-label, within-patient comparison of the diagnostic performance of non-enhanced, gadolinium-enhanced, and ferucarbotran-enhanced MR imaging, in terms of lesion detection and characterization according to the Phase III clinical trial of ferucarbotran in China. This article is mainly concerned with evaluating efficacy in the detection of hepatic lesions.

MATERIALS AND METHODS

Patients

The study protocol was approved by the Zhongshan Hospital Ethics Committee. A total of 59 patients (40 men, 19 women; mean age, 48.9 year; age range, 26-68 year) with at least one suspected focal hepatic lesion were enrolled in the study and received test contrast material between December 2003 and July 2004. Each patient gave written or witnessed informed consent and was evaluated for eligibility. Each patient underwent a reference-standard examination. The best method to confirm imaging findings—that is, pathologic confirmation—was unavailable for every patient in this study.

Contrast material

Ferucarbotran (Resovist, Schering, Berlin, Germany) was preloaded into a 2.25 mL connecting intravenous tube (Connection Tubing; Clinico, Bad Hersfeld, Germany) and manually injected as a bolus through a filter with 5- μ m pore size; the connecting catheter was flushed with 10 mL of saline solution within 3 s of injection (injection rate, approximately 2-3 mL/s). Patients with a body weight of 60 kg or more received 1.4 mL of ferucarbotran, while those with a body weight of less than 60 kg received 0.9 mL (range, 7.0-12.9 μ mol iron/kg); 1 mL ferucarbotran contains 28 mg of iron. Gd-DTPA (Magnevist, Schering, Erlangen, Germany) was manually administered as an intravenous bolus injection at a dose of 0.2 mL/kg (corresponding to 0.1 mmol/kg) with a flow rate of 2-3 mL/s.

MR imaging protocol

MR imaging was performed with a superconducting magnet operating at 1.5T (Signa, GE Medical Systems, Milwaukee, USA). The imaging protocol consisted of pre- and post-contrast imaging at 25, 60, 180, and 480 s after the administration of each contrast material, with an interval of 48 h between the injection of each. Every examination consisted of T2-weighted fast spin-echo (FSE) with fat suppression, T1-weighted fast multiplanar spoiled gradient-recalled (SPGR), and T2* GRE sequences (Table 1). The imaging factors were the same for all patients, and all sequences were performed before and after Gd-DTPA contrast agent and ferucarbotran administration.

Imaging assessment

According to the surgical and pathologic confirmation or reference examination, all images from the MR imaging sequences were interpreted independently by three radiologists; the number of lesions was then calculated, excluding cysts. Consensus reading was necessary to ensure

that both observers assessed the same lesion. Lesions were divided into three groups according to lesion size (< 1 cm, 1-3 cm, > 3 cm).

Statistical analysis

The detection rates for the different sequences (FSE T2WI with fat suppression, GRE T1WI, and GRE T2*WI) were compared using Pearson's chi-square test and the Fisher exact probability test. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

According to pathologic confirmation and the reference examinations, a total of 133 lesions were present among the subjects (85 lesions were confirmed by pathology; 75 hepatocellular carcinoma lesions, 35 metastatic lesions, 7 dysplastic nodules, 6 hemangiomas, 5 cases of focal nodular hyperplasia, 2 tuberculomas, 1 inflammatory pseudotumor, 2 angioleiomyolipomas). Twenty-one lesions were larger than 3.0 cm, 68 were 1-3 cm, and 44 were smaller than 1 cm. On ferucarbotran-enhanced MR imaging, FSE T2WI with fat suppression revealed 130 lesions, dynamic T1WI GRE revealed 115 lesions, and GRE T2*WI revealed 127 lesions (Figure 1 A-H). On pre-contrast MR imaging, GRE T1WI revealed only 84 lesions and FSE T2WI with fat suppression revealed 106 lesions. Gd-DTPA dynamic-enhanced GRE T1WI revealed 123 lesions (Table 2). Three lesions were not found by any observer on any sequence: All were smaller than 0.8 mm in diameter and were metastases from mammary adenocarcinoma and colorectal cancer. In another patient, a 0.3 mm-diameter subcapsular metastasis was detected only by ferucarbotran-enhanced FSE (Figure 2A-F).

Table 2 shows the detectability among 44 micro-lesions (< 1.0 cm) for all patients as follows: ferucarbotran-enhanced FSE T2WI with fat suppression, 93.2% (41/44); ferucarbotran-enhanced GRE T2*WI, 88.6% (39/44); Gd-DTPA dynamic-enhanced GRE T1WI, 79.5% (35/44); pre-contrast FSE T2WI with fat suppression, 54.5% (24/44); and pre-contrast GRE T1WI, 34.1% (15/44). The detectability of ferucarbotran-enhanced FSE T2WI with fat suppression and ferucarbotran-enhanced GRE T2*WI sequences was significantly greater than that of the other sequences ($P < 0.01$).

DISCUSSION

Ferucarbotran contains superparamagnetic iron oxide nanoparticles (maghemite [γ -Fe₂O₃] and magnetite [γ -Fe₃O₄]) coated with a carboxydextran shell, and was developed as a new intravenous liver-specific contrast agent. Its T1 relaxivity is 25.4 mmol/L per second, with T2 relaxivity of 151.0 mmol/L. second. The blood half-life is similar to that of ferumoxides (another SPIO agent), but the mean particle size is smaller (60 nm)^[10,11]. Ferucarbotran is taken up by reticuloendothelial system (RES) cells in the liver, spleen, bone marrow, and lymph nodes. Hydrolytic enzymes degrade intracellular SPIO particles causing a loss of R2 relaxivity as the iron loses its

Table 1 MR sequences and parameters

Sequence	TR	TE	NEX	Matrix	FOV (cm)	Thickness	Intersection gap (mm)	Flip angle (mm)
T2-FSE/TSE with fat suppression	3333-4000	98	2	256 × 160	36 × 36	8	2	-
GRE T1WI	150	2	1	256 × 128	36 × 36	8	2	90°
GRE T2*WI	150	11.5	1	256 × 160	36 × 36	8	2	10°

Table 2 Comparative analysis of lesion detection for 133 lesions in 59 patients using pre-contrast, ferucarbotran-enhanced, and Gd-DTPA-enhanced sequences

Sequence	n (%)		
	< 1 cm (n = 44)	1-3 cm (n = 68)	> 3 cm (n = 21)
1 Pre-contrast T1WI GRE	15 (34.1) ^b	48 (70.6) ^b	21 (100.0)
2 Pre-contrast FSE T2WI with fat suppression	24 (54.5) ^b	61 (89.7)	21 (100.0)
3 Dynamic Gd-DTPA-enhanced T1WI GRE	35 (79.5) ^a	67 (98.5)	21 (100.0)
4 Dynamic ferucarbotran-enhanced T1WI GRE	32 (72.7)	62 (91.2)	21 (100.0)
5 Delayed ferucarbotran-enhanced FSE T2WI with fat suppression	41 (93.2)	68 (100)	21 (100.0)
6 Delayed ferucarbotran-enhanced GRE T2*WI	39 (88.6)	67 (98.5)	21 (100.0)

^a $P < 0.05$, ^b $P < 0.01$, *vs* (5), (6).

crystalline structure^[12]. Within minutes of administration, 80% of the injected dose of SPIO agent efficiently accumulates in the liver, while approximately 5%-10% of the injected dose accumulates in the spleen^[13-15]. On T2-weighted sequences there is a marked decrease in the signal intensity of normal liver and spleen; because malignant tumor tissue typically lacks a substantial number of Kupffer cells or has a lower activity of phagocytic cells, it appears as hyperintense/bright lesions that are contrasted against the hypointense/black liver^[11,16].

In the detection of micro-lesions (< 1.0 cm) in the present study, delayed T2-weighted ferucarbotran-enhanced FSE with fat suppression and T2*-weighted ferucarbotran-enhanced GRE provided higher sensitivity in MR imaging than pre-contrast (T1-weighted GRE) images (93.2% and 88.6%, respectively, *vs* 34.1%). More lesions were detected on T2-weighted ferucarbotran-enhanced FSE than on dynamic Gd-DTPA-enhanced images in 16%-20% of our patients. It had statistically significant difference in detecting micro-lesions ($P = 0.021$), which is in agreement with previous studies^[11,16-18]. In the present study, even a 0.3 mm-diameter metastasis was detected by T2-weighted ferucarbotran-enhanced FSE with fat suppression. The loss of liver parenchyma signal intensity after ferucarbotran administration improves lesion-to-liver contrast, which in turn improves detection (especially in micro-lesions), visualization, delineation, and overall diagnostic confidence^[19] (Figure 3A-D). Although diagnostic confidence is a subjective parameter, it is relevant in the clinical situation because a radiologist must determine the confidence with which findings observed on images are correct. Evaluation of diagnostic certainty was also recommended by Thornbury in his six-tier model

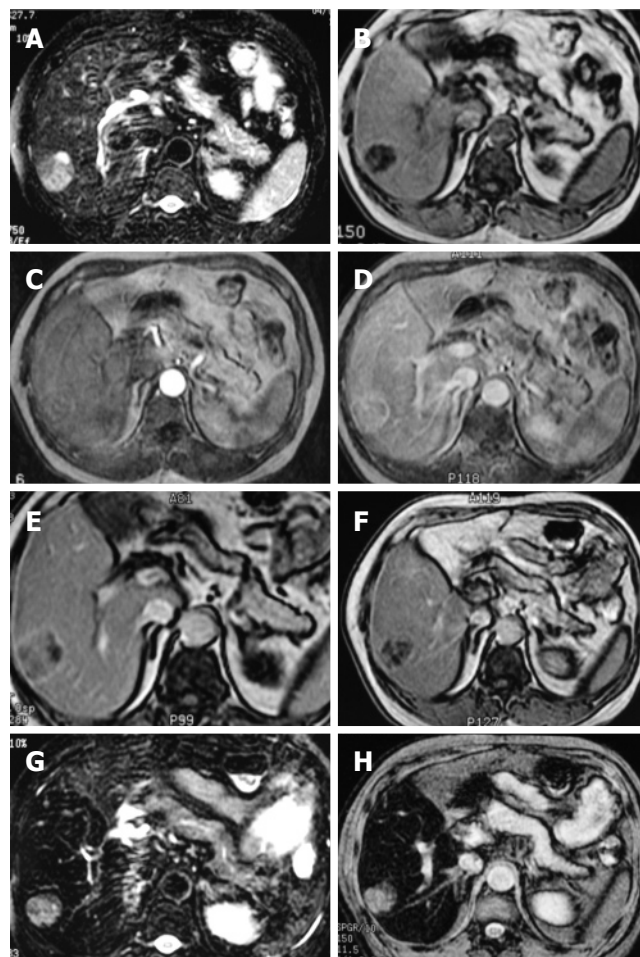


Figure 1 Images of HCC. **A:** The lesion appears hyperintense on the pre-contrast T2WI FSE image; **B:** The lesion appears hypointense on the pre-contrast T1WI GRE image; **C:** The lesion enhances inhomogeneously during the hepatic arterial phase; **D:** Ring-enhancement is observed during the portal venous phase; **E, F:** Dynamic T1WI GRE images obtained after administration of ferucarbotran show ring-enhancement of an asymmetric moderately hyperintense lesion; **G, H:** A markedly hyperintense lesion with surrounding hypointense liver parenchyma is shown during the accumulation phase on ferucarbotran-enhanced T2WI and T2*WI images.

of efficacy^[20]. Diagnostic confidence conceptualizes the diagnostic thinking efficacy (one tier of the Thornbury model) and links the technical and diagnostic efficacy of a contrast agent to the therapy of the patient. Higher accuracy in liver cancer detection potentially changes the clinical treatment of patients with liver cancer. The report of Ros *et al* states that the proportionate change in clinical treatment was as high as 59% after examination using SPIO-enhanced MR^[21]. For detection of lesions larger than 1 cm, there was no significant difference among the findings of the different sequences (excluding pre-

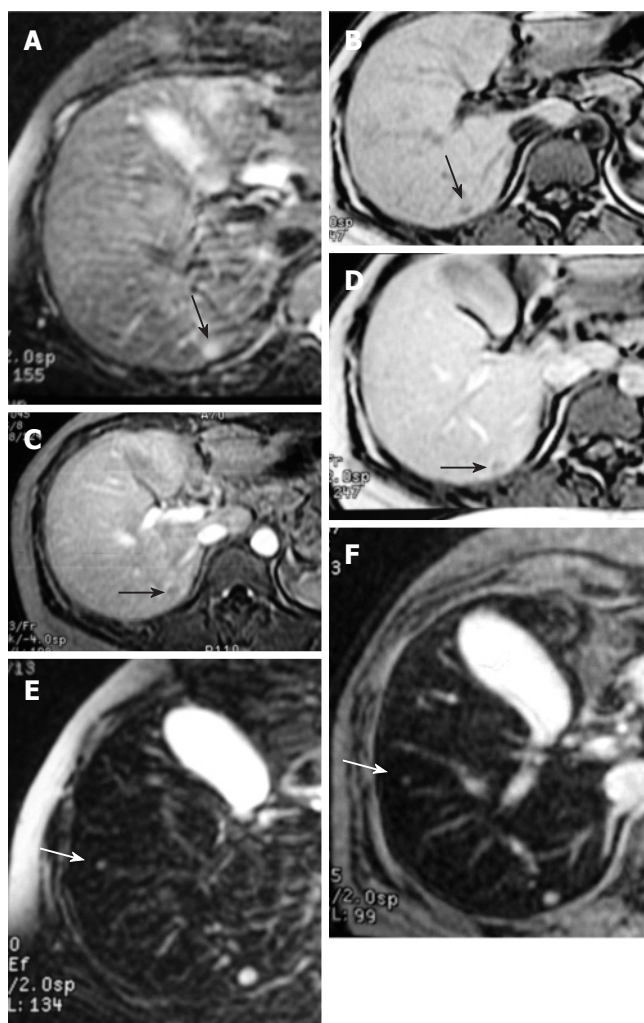


Figure 2 Liver metastasis from mammary adenocarcinoma. **A, B:** Only one lesion (black arrow) was found on the pre-contrast T2WI and T1WI GRE images; **C, D:** Only one lesion (black arrow) was found on dynamic T1WI GRE images obtained after administration of Gd-DTPA; **E, F:** An additional small metastasis (0.3 mm, white arrow) was detected on T2WI and T2*WI images during the accumulation phase.

contrast sequences); however, diagnostic confidence was determined for the same reason as that described above.

Ferucarbotran is an SPIO agent that can be injected as a bolus^[10]. It contains particles with smaller mean hydrodynamic diameter than that of the stock solution, which has a stronger T1-effect and longer blood half-life because of slower uptake into the RES. Previous studies using animals found that iron particles begin attaching to the Kupffer cells 3 min after administration^[22]. The R1 relaxivity of ferucarbotran varies considerably within the range of diagnostically applied proton Larmor frequency (i.e., field strength); however, R1 relaxivity at 40 MHz with 12.3 mmol/L per second is still four times higher than the R1 relaxivity of low molecular gadolinium chelates. The very high R2/R1 ratio is characteristic of superparamagnetic colloids of the SPIO type. This ratio varies from 6 to 15 as the Larmor frequency increases from 10 to 40 MHz, a fact which might be in favor of the use of lower imaging fields to better utilize the T1 effect of these materials^[12]. In fact, this subfraction contributes

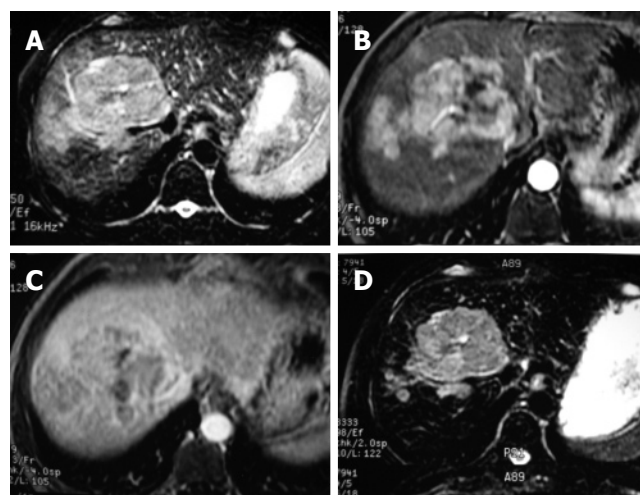


Figure 3 HCC image. **A:** The lesion appears hyperintense on the pre-contrast T2WI FSE image, but is not clearly delineated; **B:** The lesion is markedly enhanced during the hepatic arterial phase; **C:** The lesion appears hypointense during the portal venous phase, but is not clearly delineated; **D:** The main lesion and satellite nodules are clearly delineated from the surrounding hypointense liver parenchyma on the ferucarbotran-enhanced T2WI image during the accumulation phase.

to the signal change on T1-weighted images and is the reason why the signal characteristics are comparable to those of angiographic agents with a blood-pool effect. This subfraction also explains the biexponential blood half-life of ferucarbotran, which has also been tested for its capability in contrast-enhanced MRA^[23]. Enhancement increased with the degree of T1-weighting and shifted towards higher concentrations with shorter echo times. The T1 effect of ferucarbotran is a function of dose and concentration in plasma, and can be monitored during a time window in which the plasma concentration stays below a certain level depending on pulse sequence parameters. Nevertheless, in the present study, dynamic ferucarbotran-enhanced T1-weighted GRE/FLASH showed no superiority over dynamic Gd-DTPA-enhanced T1-weighted GRE/FLASH in the detection of lesions. The benefit of dynamic T1-weighted MR imaging techniques is in the characterization of lesions; however, in many respects dynamic imaging with ferucarbotran is different from that with Gd-DTPA. In dynamic T1-weighted images obtained with ferucarbotran, the signal increase is observed to be lower but longer lasting, as described by Amelie *et al*^[24].

Ferucarbotran has an advantage over ferumoxides (Feridex, Advanced Magnetix; Guerbet), which is the only marketed SPIO in most countries. Although it is well known that ferumoxides have relatively high R1 values (up to four or five times higher than those of gadolinium-based contrast agents)^[10,25,26], these T1 effects cannot be readily exploited for dynamic T1-weighted MR imaging. Bolus administration of ferumoxides has not been recommended because of the possible side effects (lumbar pain and cardiovascular problems, including dose-dependent hypotensive reactions) that positively correlate to the rate of injection; therefore, this compound must be administered as a slow drip infusion^[13]. Ferucarbotran used in this study has few side effects^[10,11] and can be given as a

rapid bolus injection. No adverse reactions were observed in any of the patients in the present study; however, as for any SPIO contrast agent, the efficacy of ferucarbotran depends on the number of Kupffer cells or their activity. For example, some well-differentiated hepatocellular carcinoma lesions still contain a certain amount of Kupffer cells and which could confuse us to make the correct diagnosis^[27,28].

In conclusion, ferucarbotran is a safe contrast material for MR imaging of local liver lesions. The use of ferucarbotran-enhanced images improves diagnostic confidence and lesion detection, especially in the detection of micro lesions (< 1 cm). Ferucarbotran can be rapidly injected without clinically important adverse events, enabling dynamic MR imaging to be performed. As a dual-function and complementary MR imaging agent, ferucarbotran-enhanced images in combination with dynamic Gd-DTPA-enhanced images are helpful in improving the accuracy of differential diagnoses for focal hepatic lesions.

COMMENTS

Background

In patients with liver cancer, the detection of focal liver lesions is one of the most important challenges. High accuracy in liver cancer detection can improve the efficacy of treatment. It is known, however, that 40%-60% of cancer nodules, especially those that are smaller than 10 mm in cirrhotic liver, are missed on ultrasonography (US), computed tomography (CT) and magnetic resonance (MR).

Research frontiers

Ferucarbotran, a liver-specific MR contrast material targeted to the Kupffer cells, is available as a new superparamagnetic iron oxide (SPIO) agent for liver imaging. A current research hotspot involves the evaluation of two contrast media for their efficacy in detecting hepatic lesions.

Innovations and breakthroughs

Ferucarbotran is a safe contrast material for MR imaging of focal liver lesions. The use of ferucarbotran-enhanced images improves diagnostic confidence and lesion detection, especially in the detection of micro lesions.

Applications

Ferucarbotran-enhanced images combined with dynamic Gd-DTPA-enhanced images aid in improving the accuracy of differential diagnosis for focal hepatic lesions.

Terminology

SPIO agent is a liver-specific contrast agent that contains superparamagnetic iron oxide nanoparticles coated with a carboxydextran shell. Ferucarbotran is taken up by reticuloendothelial system (RES) cells in the liver, spleen, bone marrow, and lymph nodes.

Peer review

The effectiveness of ferucarbotran-enhanced MR imaging in the detection of hepatic lesions is known. The present study compares the diagnostic efficiency of three MR imaging sequences in patients with focal hepatic lesions. The scientific value of this manuscript is demonstrated.

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Endoscopic management of gastrointestinal smooth muscle tumor

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2 cm. The "digging" biopsy technique would be a good option for histologic diagnosis of SMTs.

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Key words: Gastrointestinal; Smooth muscle tumor; Endoscopy; Endoscopic ultrasonography; Management

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Abstract

AIM: To systematically evaluate the efficacy and safety of endoscopic resection of gastrointestinal smooth muscle tumors (SMTs, including leiomyoma and leiomyosarcoma) and to review our preliminary experiences on endoscopic diagnosis of gastrointestinal SMTs.

METHODS: A total of 69 patients with gastrointestinal SMT underwent routine endoscopy in our department. Endoscopic ultrasonography (EUS) was also performed in 9 cases of gastrointestinal SMT. The sessile submucosal gastrointestinal SMTs with the base smaller than 2 cm in diameter were resected by "pushing" technique or "grasping and pushing" technique while the pedunculated SMTs were resected by polypectomy. For those SMTs originating from muscularis propria or with the base size ≥ 2 cm, ordinary biopsy technique was performed in tumors with ulcers while the "Digging" technique was performed in those without ulcers.

RESULTS: 54 cases of leiomyoma and 15 cases of leiomyosarcoma were identified. In them, 19 cases of submucosal leiomyoma were resected by "pushing" technique and 10 cases were removed by "grasping and pushing" technique. Three cases pedunculated submucosal leiomyoma were resected by polypectomy. No severe complications developed during or after the procedure. No recurrence was observed. The diagnostic accuracy of ordinary and the "Digging" biopsy technique was 90.0% and 94.1%, respectively.

CONCLUSION: Endoscopic resection is a safe and effective treatment for leiomyomas with the base size \leq

INTRODUCTION

Gastrointestinal smooth muscle tumors (SMTs, including leiomyoma and leiomyosarcoma) represent relatively common lesions that are thought to originate from a muscular layer of gastrointestinal tract. They can be found in the esophagus, stomach, small intestine, and colon^[1-3]. The most common symptoms of gastrointestinal SMTs are gastrointestinal bleeding, as a result of overlaying mucosa ulceration, and pain. Other symptoms may include anorexia, dysphagia, obstruction, perforation, or fever^[4-6].

Gastrointestinal SMTs are difficult-to-cure gastrointestinal tumors when compared with polyps and the complete surgical resection is still considered to be the most definitive therapy for gastrointestinal SMTs. In recent years, several reports^[7-10] suggest that endoscopic treatment of GI submucosal leiomyoma is a valid alternative to invasion surgery. However, these reports cannot provide enough convincing evidence for the efficiency and safety of the treatment they used because lack of enough cases (majority of these reports include only one single case). Meanwhile, the endoscopic resection is inappropriate for leiomyosarcoma and those leiomyomas with either the base ≥ 2 cm in diameter or originating from muscularis propria because of the risk of hemorrhage and perforation^[7-10]. Therefore, a safe and efficient therapeutic strategy for endoscopic resection of leiomyoma is worth being explored.

From 1986-2006, more than 100 cases of gastrointestinal submucosal tumors (including leiomyoma) were found and successfully resected under endoscopy in our unit. Enlightened by these cases, we prospectively explored the feasibility, efficacy, and safety for endoscopic removal of

leiomyoma. During the last fifteen years, 69 cases of SMTs have undergone the endoscopic examinations and finally proven pathologically at our hospital. Within these, 32 cases of submucosal leiomyoma were successfully removed under endoscopy. The present study evaluated the efficacy and safety of our technique for endoscopic resection of submucosal leiomyoma. Meanwhile, our preliminary experience on diagnosis of SMT based on endoscopy and endoscopic ultrasonography (EUS) was also reviewed in this study.

MATERIALS AND METHODS

Patients

From January 1992 to January 2006, 69 cases of SMT were found under endoscopy and identified by further pathological examination at First Affiliated Hospital of Nanchang University (Nanchang, China). Of these, 39 were male and 30 were female. The age range was 15-74 (average 45.6) years. All the patients complained of at least one of the GI symptoms such as gastrointestinal bleeding, abdominal pain, anorexia, and dysphagia, which could be attributed to the SMT. Written informed consent was obtained from every patient. The locations and types of these SMTs are presented in Table 1. Under immunohistochemical staining, all these SMTs were positive for smooth muscle actin (SMA) but negative for CD117 (C-kit).

All the patients underwent routine gastrointestinal endoscopy (Olympus GF/CF 230 or 240I; PENTAX-2901) to assess the location, appearance, extent, and overlaying mucosa integrity of the SMTs. After February 2005, endoscopic ultrasonography (EUS, Olympus GF-UM20) was utilized to detect the size and shape, echodensity, and the originating layer of tumor in the wall structure. Interpretation was based on the five-layer structure of the wall. For those SMTs with overlaying mucosa ulcerations, the biopsy specimens were obtained from the bottom of the ulcer. The "digging" biopsy technique was employed for those protrusive lesions without overlaying mucosa ulceration but with the base ≥ 2 cm in diameter or originating from muscularis propria.

The criterion for choice of therapy was: (1) the pedunculated submucosal SMTs with the base smaller than 2 cm in diameter were resected by polypectomy; (2) the sessile submucosal SMTs with the base smaller than 2 cm in diameter were removed using a "pushing" technique or "grasping and pushing" technique; (3) those SMTs pathologically identified malignant, originated from muscularis propria, or with the base size ≥ 2 cm were surgically resected. Histopathologic features of both endoscopically and surgically removed SMTs were reviewed by two experienced histopathologists. In addition, all specimens underwent immunostaining of SMA and CD117 (C-kit). Histological examination was also used to determine whether the tumor was removed completely.

After endoscopic removal of SMT, patients were required to remain in the hospital for at least 2 d. Bed rest was necessary for the patients with colonic SMT. Patients with upper gastrointestinal SMTs fasted for 2 d. Endoscopy was performed one week after resection to

Table 1 The sites of the GI SMT identified by endoscopy and histological examination

	Location	Leiomyoma	Leiomyosarcoma
Esophageal	Upper	4	0
	Middle	11	0
	Lower	13	0
Stomach	Cadiac	4	0
	Fornix	4	6
	Corpus	5	4
	Antrum	6	2
Duodenum	Bulb	2	1
	Descending part	1	2
Colon	Ascending colon	2	0
	Transverse colon	2	0
Total		54	15

assess healing and examine hemorrhagic signs such as exposed vessels. Follow-up endoscopic examination was performed every six months for the first year and annually thereafter. Each case was followed up by endoscopic examination for 1-2 years.

Polypectomy

The technique for the resection of pedunculated SMTs was the same as initiated for epithelial polyps. In brief, the snare was placed around the stalk of the SMT, tightened and lifted toward the cavity of the GI tract. The snare was tightened gradually and the SMT was resected by coagulation current.

"Pushing" technique

For a sessile SMT, the snare was placed around the lesion (Figure 1). The head (gastroscopy) or anal (colonoscopy) side of the lesion was pushed by the insulated cannula of snare to form a semipedunculation. The snare was tightened gradually at the top of the semipedunculation and total SMT was captured and then resected completely by a high-frequency electrosurgical current.

"Grasping and pushing" technique

This technique was performed with a double channel endoscopy. In brief, a polypectomy snare inserted through the accessory channel was first placed around the submucosal tumor. The body of the tumor was lifted by a grasping forceps inserted from the other channel to form a semipedunculation. The submucosal tumor was then captured by tightening the snare gradually at the top of the semipedunculation. Finally, the tumor was resected by a high-frequency electrosurgical current.

"Digging" biopsy technique

Initially, a biopsy forceps was used to open a hole in the overlaying mucosa leaving the SMT exposed. At least 4 biopsy specimens were then obtained from the exposed SMT.

RESULTS

During the last 15 years, 54 cases of leiomyoma were

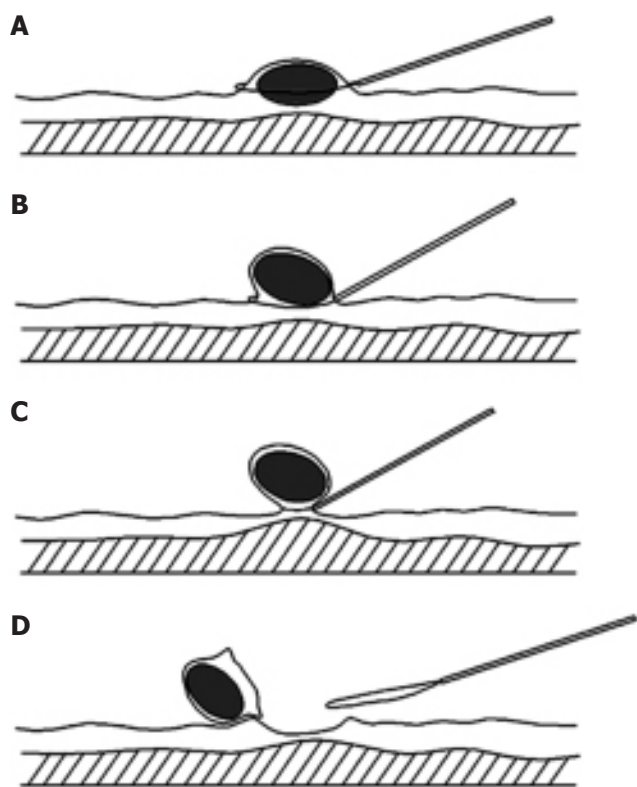


Figure 1 Schematic diagram for “pushing” technique. **A:** The snare was placed around the leiomyoma; **B:** The head (gastroscope) or anal (colonoscopy) side of leiomyoma was pushed by the insulated cannula of snare to form a semipedunculation; **C:** The snare was tightened gradually and total leiomyoma was captured; **D:** The leiomyoma was resected completely.

identified at our hospital. Under endoscopy, most leiomyomas appeared as a red and smooth sessile protrusions with normal overlying mucosa. Only 3 cases of leiomyoma were shown as pedunculated lesions. The esophagus was the most frequent site (51.8%) for leiomyoma (Table 1), followed by stomach (35.2%), colon (7.4%), and duodena (5.6%). No correlation was found between the occurrence and either age or gender in this study. The average base size of leiomyomas measured under endoscopy was 1.2 ± 0.2 cm (standard deviation, SD). The smallest one was 0.8 cm in diameter while the largest was 3.4 cm in diameter. From February 2005 to January 2006, EUS was performed in 6 cases of leiomyoma. All these leiomyoma were shown as an echolucent mass with sharp margin and originating from muscularis mucosa (3 cases) or muscularis propria (3 cases) of gastrointestinal tract. Average size of these 6 cases of leiomyomas measured under EUS was 1.1 ± 0.3 cm (SD).

Among the above-mentioned 54 cases of leiomyoma, 19 cases of submucosal leiomyoma were resected by “pushing” technique (Figure 2) while 10 cases were removed by “grasping and pushing” technique (Table 2). Only 3 cases pedunculated submucosal leiomyoma were resected by polypectomy (Table 2). All these resected leiomyomas were confirmed by the following histopathologic examination. Immediate endoscopic observation after all these resections showed a 1.2–1.5 cm cauterization burn without other abnormalities. No complications such as perforation and hemorrhage

Table 2 The amount and location of the GI leiomyomas resected by “pushing” technique, “grasping and pushing” technique, and polypectomy, respectively

	Location	Pushing	Pushing and grasping	Polypectomy
Esophageal	Upper	2	0	0
	Middle	5	2	0
	Lower	3	3	1
Stomach	Cadiac	1	1	0
	Fornix	1	1	0
	Corpus	1	0	1
	Antrum	3	0	1
Duodenum	Bulb	0	2	0
	Descending part	0	1	0
Colon	Ascending colon	1	0	0
	Transverse colon	2	0	0
Total		19	10	3

developed during or after the procedure in most of these patients. Oozy bleeding occurred in 4 patients and easily controlled after epinephrine or thrombin spraying. After a follow-up period of one to two years with repeated endoscopy, no recurrence was found.

Among the remaining 22 cases of leiomyoma with the base size ≥ 2 cm or originating in muscularis propria, 9 cases were observed with occurrence of overlying mucosa ulceration. Of these, 8 cases (88.9%) were confirmed pathologically by obtaining biopsy specimens from the bottom of the ulcer while one case failed to report by this method. For those leiomyomas without ulcer, 12 cases (92.3%) were confirmed by “digging” biopsy while only one case (7.7%) failed to report by this method. All 22 cases of leiomyoma were successfully removed by surgery.

In this study, 15 cases of leiomyosarcoma were surgically resected and confirmed by following histopathologic examination. No correlation was found between the occurrence and either age or gender in this study. Of these, endoscopy revealed the lesion as an intraluminal protuberant tumour with ulcer (Figure 3A) in 7 cases and without ulcer in 4 cases. Another 4 cases appeared as an ulcer alone. The occurrence frequency of ulcer in leiomyosarcoma is 73.3% (11/15), which is obviously higher than that in leiomyoma (16.7%, 9/54). Leiomyosarcoma were observed in stomach (80.0%) and duodena (20.0%). The average base size of leiomyosarcoma measured under endoscopy was 6.8 ± 2.3 cm (SD). A significant difference was found between the base size of leiomyoma and leiomyosarcoma ($P < 0.001$, non-paired *t* test). From February 2005 to January 2006, three cases of leiomyosarcoma underwent EUS examination. All three tumours were found to arise from the fourth echo poor layer (muscularis propria); EUS showed that one gastric tumour disrupted all the wall layers. The tumour echostructure and margins were inhomogeneous and irregular in all three cases (Figure 3B).

In those 11 cases of leiomyosarcoma with occurrence of ulcer, 10 cases (90.9%) were confirmed pathologically by obtained biopsy at the bottom of the ulcer. One case failed to report by this technique and finally confirmed

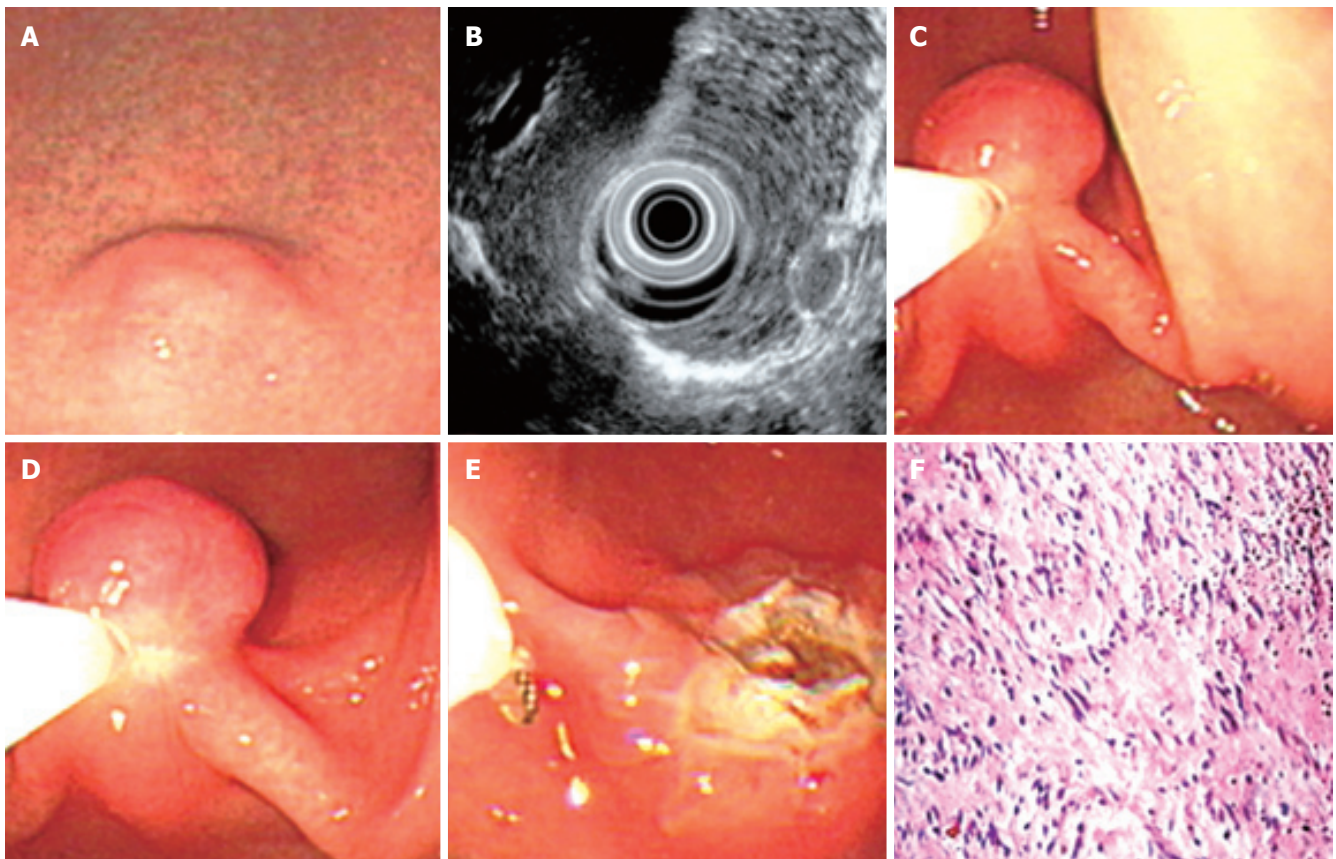


Figure 2 Endoscopic views for "pushing" resection of a leiomyoma. **A:** A sessile leiomyoma at antrum of stomach; **B:** EUS revealed that the mass originated from muscularis mucosa; **C:** The leiomyoma was pushed by cannula to form a semipedunculation and then captured by snare; **D:** The captured leiomyoma was resected by high-frequency electrosurgical current; **E:** The endoscopic view for the cauterization burn of leiomyoma after resection; **F:** The histologic view of leiomyoma after resection (HE, x 200).

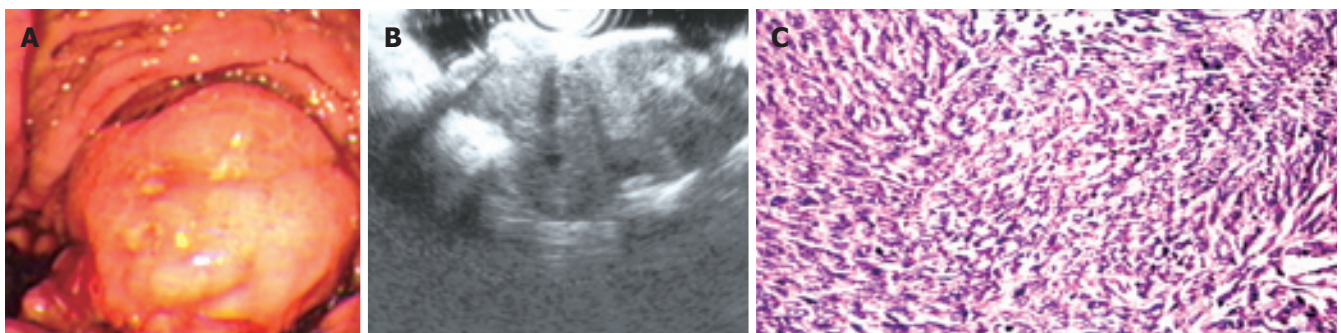


Figure 3 **A:** Endoscopic view of a leiomyosarcoma at corpus of stomach; **B:** The view of EUS; **C:** The histologic examination after "digging" technique (HE, x 200).

after surgical resection. The remaining 4 cases of leiomyosarcoma without ulcer were confirmed by "digging" biopsy.

DISCUSSION

To resect submucosal SMT by endoscopy, it is crucial to determine the originating layer of the lesion. If the tumor arises from muscularis propria, complete resection should be avoided because of the risk of perforation^[7-12]. Recently, EUS was considered to be very helpful in determining the size, consistency, extension of submucosal tumors, and the layer from which tumors originate^[12-15].

Therefore, the assistance of EUS greatly increases the safety of endoscopic resection of submucosal SMTs^[9,12,13]. In our unit, EUS were employed in evaluating 6 cases of leiomyoma. Of these, three cases were found originating from muscularis mucosa, and then were resected completely under endoscopy. Another 3 cases, originating from muscularis propria, were successfully performed via surgical resection. However, the EUS were equipped in our department only after January 2005. Before 2005, we determined the location of submucosal tumor through detecting the mobility of tumor by closed biopsy forceps. In brief, if the forceps pushed the tumor to slide under mucosa, it suggested that the tumor originated superficially

in muscularis mucosa and was resectable by means of endoscopy. Whereas, an immobile tumor revealed that the tumor had its roots in muscularis propria and could not be removed by endoscopic resection. Although we admit that this criterion is somewhat imprecise, all submucosal leiomyomas (26 cases) determined by this method were successfully performed endoscopic resections, no perforations or severe bleeding occurred after resection. In addition, in those 6 cases examined by EUS, the conclusions drawn by this method fit well with those by EUS. All these were enough to prove the reliability of our method.

The “Pushing” technique has been employed by our group for resection for various gastrointestinal submucosal tumors including leiomyoma, fibroma, lipoma, carcinoid. All these submucosal tumors were successfully resected, with the exception of one case of fibroma, which underwent severe bleeding after resection. In the present study, 19 cases of leiomyoma were successfully and safely removed by the “pushing” technique. No recurrence was observed after 1-2 years follow-up. The crucial step for this technique is the movement of pushing. Because of the pushing by insulated cannula of snare, a semipedunculation forms and then the whole body of the tumor is easily captured by pre-placed snare. This ensures that the tumor can be resected completely. Meanwhile, because the tumor body is lifted toward cavity before cauterization and the line of resection is at the bottom of the tumor body and the top of the semipedunculation, the muscularis propria has already separated from the place of cauterization and then will not be injured by the high-frequency electrosurgical current. Additionally, to minimize potential severe hemorrhage and perforation, we avoid undergoing endoscopic resection of those lesions with the base ≥ 2 cm. All these fully demonstrate the efficiency and safety of the “pushing” technique utilized. When compared with other techniques such as En Bloc^[9,15-17], the pushing technique is much easier to be operated and takes less time.

The En bloc technique has been used by several groups for endoscopic resection of submucosal SMTs^[9,15-17]. By our understanding, there are at least two advantages for this technique. First, this technique contains an important step-injection of the saline solution into the submucosa, which is to separate the line of resection from muscularis propria and then prevent the injury of muscle layer. This step greatly increases the safety of the operation. Second, the removed tumors can be easily captured for further histological examination. However, we consider that this technique also has the shortcomings of complicated operation and time-consuming. In fact, it is a tough job for an endoscopist to inject the solution exactly into the base of the leiomyoma without injuring the wall of the gastrointestinal tract. Furthermore, injection of saline may make the margin of the lesion unclear. To overcome these problems, we tried to explore the possibility of deleting the procedure of saline injection. Normally, 19.4 mm grasping forceps can easily grasp the body of tumor and lift it up towards the cavity of the gastrointestinal tract to form a pseudo-pedunculation. In fact, this step has already separated the tumor from muscularis propria and then is

enough to prevent the injury of muscle layer when the captured tumor is removed by pre-placed snare. In some cases, the surface of the tumor is too slippery and difficult to grasp. To solve this problem, we first grasped the overlaying mucosa, and then pushed the tumor by insulated cannula of snare to form a semipedunculation, which also prevented the injury of muscularis propria when captured tumors were removed by high-frequency electrosurgical current. In the last 15 years, 10 cases of leiomyoma have been safely removed in our department by using this “grasping and pushing” technique. No recurrence was observed after 1-2 years follow-up. All these fully support the efficiency and safety of the grasping and pushing technique we utilized.

For those SMTs with the base size ≥ 2 cm and/or originating in muscularis propria, to differentiate malignant from benign is crucial for further treatment. Histological diagnosis is necessary not only to ascertain whether a lesion is benign or malignant (usually larger lesions with irregular borders, inhomogeneous areas, or eroded surfaces), but also to detect smaller lesions without malignant morphologic features. In recent years, several methods have been developed for this histological diagnosis. Matsui *et al.*^[18] have described a biopsy technique-endoscopic ultrasonography-guided fine needle aspiration biopsy (EUS-FNAB). In this technique, the biopsy materials are obtained from a needle, which is inserted into the lesions guided by EUS. Open biopsy, developed by Kojima *et al.*^[9], is another effective biopsy technique for gastrointestinal submucosal lesions. In this technique, the covering mucosa is resected to expose the tumor and then several tissues are obtained by ordinary forceps at the bottom of the artificial ulcer. The techniques we used in this study were the “digging” and ordinary biopsy techniques. In order to evaluate the diagnostic accuracy of these two techniques, we selected those cases which were finally removed by surgery. In our series, the diagnostic accuracy of “digging” biopsy is 94.1% for those SMTs without ulcer (leiomyoma: 92.3%; leiomyosarcoma: 100%). This result is very close to the above-mentioned two techniques, but the “digging” biopsy is much easier and cheaper than EUS-FNAB and open biopsy. In this series, no severe hemorrhage developed after the “digging” biopsy. In addition, the diagnostic accuracy of ordinary biopsy in the SMTs with ulcer is 90.0%, similar to that of “digging” biopsy.

Although EUS is very helpful in deciding the technique for endoscopic resection of submucosal SMTs, it is difficult to differentiate the malignant from benign SMTs by means of EUS unless there is local extension or metastasis, because no significant difference has been found between malignant and benign lesions with regard to homogeneity of internal echo pattern or marginal echo pattern^[3,19,20]. However, EUS is considered to be reliable in predicting the potential malignancy of SCTs^[3]. The three most predictive EUS features described by Palazzo *et al.*^[3] are irregular margins, cystic spaces, and lymph nodes with a malignant patterns. Palazzo *et al.*^[3] concluded that (1) the presence of at least one of these criteria had a sensitivity of 91%, a specificity of 88%, a positive predictive value of 83%, and a negative predictive value of 94% for

potential malignancy; (2) a combination of two of these three criteria had a positive predictive value and specificity of 100%; (3) tumors of 30 mm or less, with regular extraluminal margins and a homogeneous pattern, are likely to be benign. Our series also support predictive EUS features although only 6 cases of leiomyoma and 3 cases of leiomyosarcoma were investigated.

In conclusion, endoscopic resection is a safe and effective therapy for submucosal leiomyoma with the base size ≤ 2 cm. The guidance of EUS greatly increases the safety of endoscopic resection of submucosal leiomyoma. The “digging” biopsy technique would be a good option for the histologic diagnosis of SMT.

COMMENTS

Background

Gastrointestinal smooth muscle tumors (SMTs, including leiomyoma and leiomyosarcoma) represent relatively common lesions. The complete surgical resection was considered to be the most definitive therapy for SMTs in the past. In recent years, some researchers reported that endoscopic treatment of GI submucosal leiomyoma is a valid alternative to invasion surgery. However, they failed to provide enough convincing evidences for the efficiency and safety of the treatment they used because lack of enough cases.

Research frontiers

In the last few decades, gastrointestinal endoscopy has been widely used in the treatment of gastrointestinal diseases, it is very important to elucidate the efficiency and safety of endoscopic treatment of SMTs.

Innovations and breakthroughs

The authors revealed that endoscopic treatment of SMTs is efficient and safe through a prospective research with many cases. Meanwhile, the “Pushing” technique and “Grasping and pushing” technique were put forward and analyzed.

Applications

The current study will guide the clinical application of endoscopic treatment of SMTs.

Peer review

This paper may show us endoscopic management of smooth muscle tumor in a single hospital for more than 10 years. However, this study is only a descriptive study of the experienced cases. The authors should consider again the novel findings obtained from the experienced cases.

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Does protracted antiviral therapy impact on HCV-related liver cirrhosis progression?

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Abstract

AIM: To study the outcomes of patients with compensated hepatitis C virus-related cirrhosis.

METHODS: Twenty-four grade A5 and 11 grade A6 of Child-Pugh classification cirrhotic patients with active virus replication, treated for a mean period of 31.3 ± 5.1 mo with moderate doses of interferon-alpha and ribavirin, were compared to a cohort of 36 patients with similar characteristics, without antiviral treatment. Salivary caffeine concentration, a liver test of microsomal function, was determined at the starting and thrice in course of therapy after a mean period of 11 ± 1.6 mo, meanwhile the resistive index of splenic artery at ultra sound Doppler, an indirect index of portal hypertension, was only measured at the beginning and the end of study.

RESULTS: Eight out of the 24 A5- (33.3%) and 5 out of the 11 A6- (45.45%) treated-cirrhotic patients showed a significant improvement in the total overnight salivary caffeine assessment. A reduction up to 20% of the resistive index of splenic artery was obtained in 3 out of the 8 A5- (37.5%) and in 2 out of the 5 A6- (40%) cirrhotic patients with an improved liver function, which showed a clear tendency to decrease at the end of therapy. The hepatitis C virus clearance was achieved in 3 out of the 24 (12.5%) A5- and 1 out of the 11 (0.091%) A6-patients after a median period of 8.5 mo combined therapy. In the cohort of non-treated cirrhotic patients, not only the considered parameters remained unchanged, but 3 patients (8.3%) had a worsening of

the Child-Pugh score ($P = 0.001$).

CONCLUSION: A prolonged antiviral therapy with moderate dosages of interferon-alpha and ribavirin shows a trend to stable liver function or to ameliorate the residual liver function, the entity of portal hypertension and the compensation status at acceptable costs.

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Key words: Liver cirrhosis; Hepatitis C virus infection; Antiviral therapy

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INTRODUCTION

Hepatitis C virus (HCV) infection is a leading cause of liver disease worldwide. Infected patients usually develop chronic hepatitis, which may progress producing diffuse disorganization of normal hepatic structure, to liver cirrhosis over a long period. Approximately 10%-20% of patients with chronic HCV infection have cirrhosis at the first clinical presentation, and near 20%-30% of those who do not present liver cirrhosis will eventually develop this illness and its complications^[1-3].

To date, the most effective treatment to prevent disease progression and eventually cure chronic hepatitis C (CHC) virus infection is the combined therapy of pegylated (PEG) interferon (IFN) alpha and ribavirin (RBV). The rate of sustained viral response (SVR) in immunocompetent patients undergoing this antiviral regimen ranges from 42% to approximately 80%, depending on the HCV genotype, with an acceptable safety profile^[6,7]. Thus, PEG-IFN alpha has substituted standard IFN alpha in the regimens used to treat CHC. However, the advantage of such an approach, with the additional cost it entails, still remains to be demonstrated^[8]. In patients with compensated HCV-related cirrhosis, standard IFN alpha or combination therapy offers an interesting SVR^[9]. Neither substantial difference has been reported between PEG-IFN alpha and standard IFN alpha in combination with

RBV, nor PEG-IFN has a higher risk of complications^[10]. Indeed, also in the decompensated form of liver cirrhosis, favourable results have been achieved after a 24 wk-course of combined therapy^[11]. Since liver cirrhosis progression is related to other factors beyond the viral infection, the therapeutic efficacy should be evaluated under a wider perspective. Actually, data on the liver residual function and hepatic hemodynamics assessment, following the route of compensated liver cirrhosis after antiviral therapy, are limited, having prevalently studied the survival rate and hepato-carcinoma appearance.

Expecting a modification of the liver parenchymal structure and vascular bed (as partial gain following the reduced cellular damage, fibrosis and nodular regeneration) after a long-term antiviral treatment at a moderate-dosage, our principal aim was to investigate the impact of this schedule on eventually modifying the progression of liver cirrhosis. Then, we started weighing three characteristic prognostic aspects of the illness, i.e., the residual liver function (index of intact cellular mass) by a quantitative liver function test (QLFT), the presence of liver decompensation by regular assessment of Child-Pugh (C-P) severity class, and the altered hemodynamics (consequence of a cyto-architectural alteration) by a non-invasive parameter reflecting the portal hypertension entity.

MATERIALS AND METHODS

Population

Among the 121 patients (regularly followed up at our out-patient clinic) suffering from HCV-related liver cirrhosis, with HCV load > 800 000 IU/mL and genotype 1, 71 were enrolled in the study, well matched for sex, age and severity of the disease. These 71 patients were divided into treated group (35) and non-treated group (36) (Table 1). Of the patients (including 13 females) in the first group, 24 (mean age 58.9 ± 5.7 years) belonged to C-P grade A5 cirrhotics and 11 (mean age 61 ± 5.0 years, 7F) to C-P grade A6 cirrhotics. A further characteristic was that 18 underwent previous antiviral therapy during the past years (14 non-responders and 4 relapsers). These patients were treated for a mean period of 31.3 ± 5.1 mo with moderate doses of IFN alpha (8 with 1.5 MU daily, 13 with 3 MU on alternate days and 14 with 1 mcg/kg body weight of Peg-IFN alpha 2b weekly) in combination with oral RBV (400 or 600 mg per day for body weight < or > 75 kg). One was excluded from the study because he spontaneously ended the therapy and two had a suspension period of more than six weeks. Collaterally, a cohort (without antiviral treatment) of 36 initially compensated cirrhotics (25, A5 C-P patients including 12 females, and 11 including 6 females, A6 C-P patients) was studied for the same period (9 patients were suffering from various grades of mood disorders, 4 were affected by microcythemia, 6 presented with thyroid laboratory abnormalities, 17 refused the treatment by choice. Of these patients, 15 were non-responders and 2 relapsers to previous antiviral therapy).

No patient in the two groups received mono- or combined treatment in the last two years.

The diagnosis of liver cirrhosis was made by histology

(25 patients), laboratory data (34 patients), and combined clinical and laboratory parameters plus ultra sound (US) findings (12 patients).

Main outcome measures

As QLFT, we chose to probe the microsomal activity of enzymes, e.g., cytochrome P450 1A2, with the total overnight salivary caffeine assessment (TOSCA), comparable to the well-known caffeine clearance according to a recent study^[12] and with the normal values set at ≤ 1 mcg/mL. Briefly, patients were allowed to drink coffee in late afternoon and on the day after overnight intake-washout, a sample of saliva, was collected in the morning, roughly centrifuged, frozen and stored until analysis. TOSCA was performed at first and then thrice during the course of therapy, at a mean interval period of 11 ± 1.6 mo. Caffeine was determined by an enzyme multiplied immunoassay technique (Dade Behring Liederbach, Germany) at the end of the follow-up period. Clinical assessment combined with other laboratory parameters, i.e., serum albumin, prothrombin time and bilirubin levels, was performed between periods of TOSCA evaluation. Occult blood in stools was detected to exclude gastrointestinal haemorrhage due to portal hypertension, as a possible cause of decompensation.

The resistive index of the splenic artery (SARI) at US Doppler was used to indirectly ascertain the entity of portal hypertension as previously described^[13]. SARI was taken at the beginning and the end of the observation period using a 3.5 convex probe of an ESAOTE (Genoa, Italy), by two operators who were blind to each other and the laboratory results.

A slightly modified C-P classification was adopted to define the severity of liver cirrhosis (Table 2). A worsening total score was set at three points when patients belonged to the A5 class and two points in case of the A6 class.

Exclusion criteria

The exclusion criteria included: (a) alcohol abuse screening according to recent recommendation^[8], (b) metabolic syndrome following the adult treatment panel III classification^[9], (c) detection of iron overload, (d) serum positivity for HBsAg, (e) previous chronic use of potentially toxic drugs as amiodarone, methotrexate and high doses of vitamin A.

Statistical analysis

Chi square test was used to study the frequencies. Differences between means were studied by unpaired T test or Mann-Whitney test. Wilcoxon signed ranks test was carried out for paired observations. ANCOVA (repeated measure analysis of variance corrected for the basal values) was carried out to analyze the differences in TOSCA data at various time intervals. Association was evaluated by applying Pearson correlation. Logistic regression was used to verify if the IFN formulation and the schedule of antiviral therapy affected the severity of liver cirrhosis, based on C-P classification. The inter-observer agreement at US was appraised by the Cohen's Kappa. Kaplan-Meier (K-M) survival curves were used for comparing

Table 1 Laboratory and sonographic parameters of the population at entry

	Treated									
	Not (36 patients)					Yes (35 patients)				
	Mean	SD	Median	Minimum	Maximum	Mean	SD	Median	Minimum	Maximum
ALT ¹	61.25	9.7				61.3	13.6			
Chol ²			133.5	59	190			132	89	200
Plts ³			141	67	191			134	90	193
SLD at US ⁴			134	122	151			133	120	146

ALT: alanine aminotransferase; Chol: total cholesterol; Plts: platelets; SLD: splenic longitudinal diameter; US: ultra sound; SD: standard deviation. ¹*P* = 0.97 at unpaired T test; ²*P* = 0.72 at Mann-Whitney (M-W) test; ³*P* = 0.15, at M-W test; ⁴*P* = 0.65 at M-W test.

Table 2 Child-Pugh classification (slightly modified)

Clinical and laboratory data	Points for increasing abnormality ¹		
	1	2	3
HE (grade ²)	None	(SHE)0-1-2	3-4
Ascites	None at US	Mild or controlled by diuretics	Present despite diuretics
PT (% of activity) ³	> 70	70-40	< 40
Albumin (g/dL)	> 3.5	2.8-3.5	< 2.8
Bilirubin (mg/dL)	< 2	2-3	> 3

¹Scoring system: 5-6 points, grade A; 7-9 points, grade B; 10-15 points, grade C. ²HE: hepatic encephalopathy; Grade 0: sub-clinical hepatic encephalopathy, SHE, unravelled by retain tests A or B; Grade 1: anxiety, irritability, depression, impaired concentration, sleep disturbances; Grade 2: disorientation, poor short-term memory, disinhibited behaviour, drowsiness; Grade 3: somnolence, bizarre behaviour, confusion, amnesia, paranoia; Grade 4: Coma. ³PT, prothrombin time, also expressed in seconds prolonged < 4; 4-6; > 6, or as INR < 1.7; 1.7-2.3; > 2.3. US: ultra sound.

C-P classification worsening probability curves in the two groups of patients. Cox proportional-hazards regression was employed to analyze the effect of non treatment (risk factor) on the C-P score aggravation. MedCalc version 9.0.2.1. and SYSTAT version 11.00.01 were used.

The study was conducted following the International Conference on Harmonization for Good Clinical Practice, in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki).

RESULTS

Whenever neutrophil count was < 750 cells/ μ L or haemoglobin < 10 g/dL, granulocyte colony-stimulating factor (filgrastim, Neupogen, Amgen Inc., California, 300 μ g to eight patients, weekly) or erythropoietin analog (Eprex, Janssen-Cilag, 10 000 units to seven patients, twice/thrice weekly) was administered (after the blood count increased, the growth factor administration was prolonged for at least two weeks). In the treated group, six out of the 24 A5 C-P (25%) and four out of the 11 A6 C-P (36.4%) cirrhotic patients showed an improvement (> 20% in respect to the base-line determination) in TOSCA (Figure 1). A clear association was found between the post-treatment TOSCA values and the prothrombin time ($r = -0.76$, *P* = 0.0001). No patient showed a worsening liver function. In the comparative cohort of initially

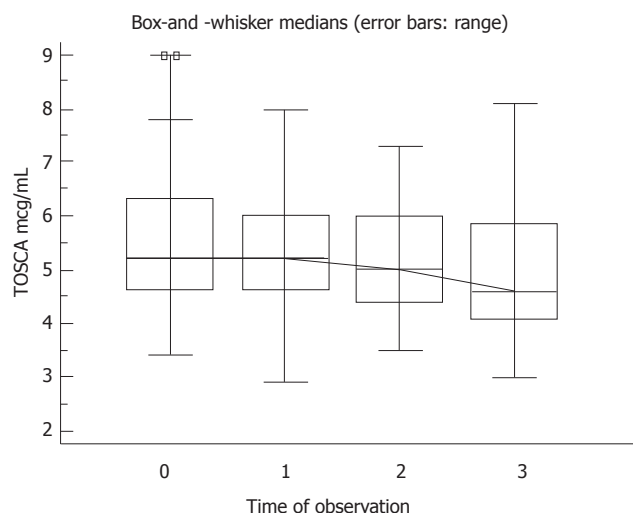


Figure 1 Behaviour of the applied quantitative liver function test in 35 patients treated with combined antiviral therapy. TOSCA: total overnight salivary caffeine assessment. The knurled line shows which groups are significantly different in the values of TOSCA, in respect to the basal ones; ANCOVA (repeated measure) test, *P* = 0.0001.

compensated cirrhotic patients without antiviral treatment, five out of the 36 patients (13.9%) had an exacerbation in respect to previous TOSCA values. A higher C-P score was found in non-treated than with treated patients (8/36 versus 1/35, *P* = 0.04).

Taking the US operator-dependent variability into consideration (although our inter-observer agreement was high, i.e., 89%), an appreciable reduction (> 20% compared with the pre-treatment measurements) in the SARI was obtained in 3 of 6 A5 C-P and 2 of 4 A6 C-P cirrhotic patients, showing an improvement in liver function. Furthermore, a significant decrease in the median SARI was present in the whole population compared to the pre- and post-treatment values.

HCV viral clearance was achieved in 3 out of the 24 (12.5%) A5 C-P and 1 out of the 11 (.91%) A6 C-P patients after a median (range) period of 8.5 (8-10) mo combined therapy.

No independent risk factor for C-P worsening was highlighted among the type of IFN, doses and period of treatment.

In our series during the entire period of study, no liver failure, hepato-cellular carcinoma and non-Hodgkin's lymphoma were discovered. There was a significant difference in the K-M curves of liver cirrhosis during

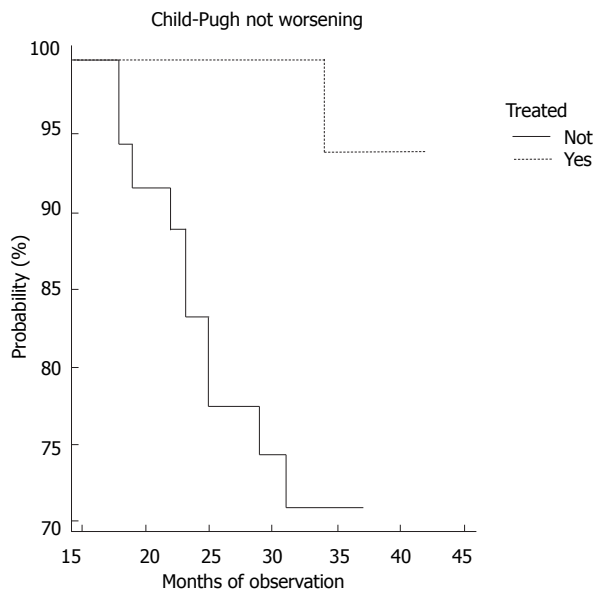


Figure 2 Behaviour of Child-Pugh classification in patients on (n 35) or not on (n 36) combined antiviral therapy. Comparison of the probability curves between treated and non-treated patients was made (Log-rank test). Chi-square test = 8.3, $P = 0.004$; Hazard ratio = 10.9; 95% CI = 1.75-18.9.

the four year-observation period between the two groups. About 30% of patients in the non-treated group experienced a worsening C-P score than the opposite cohort (Figure 2).

A small percentage of side-effects was registered in the treated group, e.g., two patients showed onset or enlargement of psoriatic plaques, three asthenia, one repeated low urinary tract infection, six anorexia, two low-grade fever, seven local erythema, one mild alopecia.

The cost of combined therapy plus growth factors per patient was estimated to be approximately 6000 US\$ for standard IFN and 15 000 US\$ for Peg-IFN, respectively.

The final values of TOSCA in samples were assessed for each group of patients ($\alpha = 0.05$, $\beta = 0.01$).

DISCUSSION

The main outcomes of antiviral therapy for chronic hepatitis C detected are the viral clearance and its lasting time. Actually, IFN therapy seems also be able to determine a decrease in the rate of functional disease progression in patients with the same disease, even in non-responders and relapsers^[14].

On the other hand, previous data have emphasised the scarce response to IFN therapy when dealing with advanced chronic liver diseases. This finding has not been further supported by a recent study^[11]. However, an uncertainty still exists, because it is not clear if a reduction in viral load or its clearance is of some benefit for any of the main processes, i.e., fibrosis and nodular regeneration, the extent of which determines the severity of liver cirrhosis. Variation exists among individuals regarding the rate and time of the progression of fibrosis to cirrhosis. There is evidence that the Ito cells are activated in response to HCV-induced hepatocellular

damage initiating fibrosis^[15]. These cells proliferate and become myofibroblasts, enhancing degradation of the normal matrix and producing excess abnormal matrix. Reactive oxygen species (ROS)^[16] and inflammatory cytokines (mainly transforming growth factor and platelet-derived growth factor) speed up fibrosis. These cells play a key role in the alteration of metalloproteinase enzymes that regulate matrix collagen metabolism^[17]. Fibrous tracts join branches of afferent portal veins and efferent hepatic veins, bypassing the hepatocytes and limiting their blood supply. Since the same myofibroblasts stimulated by endothelin-1, contribute to the increased portal vein resistance, fibrosis leads to "hepatocyte ischemia" with successive hepatocellular dysfunction and portal hypertension. Cirrhosis resulting from HCV infection has a slowly progressing course. Then, using the classic ones as therapeutic end-points^[18], could result in the short-medium ones. Furthermore, an eventual change in some prognostic aspects of liver cirrhosis has not been reported, especially if long-term, moderate-dosage schedules were used.

Having this in mind, we tracked the progression of liver cirrhosis, using three combined parameters, all indices of the severity of the illness.

We found an improvement in residual liver function as assessed by TOSCA, a lack of C-P classification variation and a concomitant constancy of the indirect assessment of portal hypertension grade. All of them showed a stable severity of the illness in treated patients. Antiviral therapy could affect the ongoing liver cirrhosis by influencing the inflammation- or apoptosis-mediated fibrosis process, working on HCV viral replication, lessening ROS formation. The last mechanism is of paramount importance because it alters reparative processes. Interestingly, a reduced cellular damage could also justify the improvement in QLFT following therapy. Obviously, SARI is a surrogate marker, reflecting the hyper-dynamic, high-flow circulatory state as a consequence of splanchnic vasodilatation caused by portal hypertension. Actually, the only supra-hepatic vein catheterization could ultimately give valid information on hypertensive status. In any case, this non-invasive tool offers its best resources for serial measurements of hypertension and it has not yet been criticized as other Doppler US parameters^[19]. In our study, a stable sonographic parameter or a slightly-reduced sonographic parameter which non invasively weighs the portal hypertension, was evidenced. Furthermore, the present study has other limitations in its methodology. The first is that it was not a randomized study, but a pilot study. In fact, only patients who accepted the treatment or had no contra-indications were enrolled. The second is that a four-year-interval was not representative of the natural history of this chronic disease, although it could foresee a certain trend. The third is that even if we tried to exclude the presence of co-factors, we were not sure that occult causes such as low intake of alcohol or episodic drug toxicity could have influenced the progression of liver cirrhosis in the non-treated patients. Moreover, we could have measured a serum fibrosis marker, a procollagen-III peptide, even if a consolidated study has failed to assess its utility^[20]. We stress that the follow-up and work-up

intervals were similar in the two groups. The fourth is that the quality of life was not opportunely evaluated, which is very important for the patient, although the non-standard dosage plays a key role in reducing side-effects. One positive aspect of this approach could be the health-care-cost in light of a likely procrastination of the need for hospitalization and liver transplantation.

In conclusion, our results favour the hypothesis that a stability (lack of fatal progression) or a trend to improve the severity of liver cirrhosis based on the clinical status and laboratory-instrumental profile is a reliable end-point after antiviral therapy. A prolonged combined antiviral course at reduced doses may slow down the progression of liver disease to cirrhosis. Further well-designed studies are needed to corroborate the present observations.

COMMENTS

Background

Hepatitis C virus (HCV) infection is a major health care problem. Recently combined antiviral therapy has been ascertained to improve the non-progressive form of chronic liver disease, eliminating the viral load and reducing fibrosis. However, whether HCV-related-liver cirrhosis characterized by a reduced hepatocellular mass and an increased pressure of portal vein can be cured still remains a challenge.

Research frontiers

The hot spots discussed in the article are the main outcomes of this controlled study, i.e., the assessment of real liver function with a simple and repeatable test (TOSCA) that probes the microsomal enzymatic activity and the evaluation of an Ultrasonographic index of portal hypertension.

Innovations and breakthroughs

The differences from the other related or similar studies relay on the particular schedule of combined antiviral therapy for the treatment of HCV-related liver cirrhosis (low long-protracted doses).

Applications

The perspectives of future application of this research could be expanded by larger and double-blinded studies to validate the good cost/benefit ratio.

Terminology

Total overnight salivary caffeine assessment (TOSCA) is comparable to the well-known caffeine clearance. Briefly, patients were allowed to drink coffee up to late afternoon and after overnight intake-washout, a sample of saliva, was collected in the morning of the next day, roughly centrifuged, frozen and stored until analysis.

Peer review

The manuscript describes that antiviral therapy in patients with hepatitis C-related cirrhosis shows a trend to stabilize or even to ameliorate residual liver function. Although the number of patients was rather small, the study provides evidence for how to treat these patients.

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Secondary pancreatic involvement by a diffuse large B-cell lymphoma presenting as acute pancreatitis

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Abstract

Diffuse large B-cell lymphoma is the most common type of non-Hodgkin's lymphoma. More than 50% of patients have some site of extra-nodal involvement at diagnosis, including the gastrointestinal tract and bone marrow. However, a diffuse large B-cell lymphoma presenting as acute pancreatitis is rare. A 57-year-old female presented with abdominal pain and matted lymph nodes in her axilla. She was admitted with a diagnosis of acute pancreatitis. Abdominal computed tomography (CT) scan showed diffusely enlarged pancreas due to infiltrative neoplasm and peripancreatic lymphadenopathy. Biopsy of the axillary mass revealed a large B-cell lymphoma. The patient was classified as stage IV, based on the Ann Arbor Classification, and as having a high-risk lymphoma, based on the International Prognostic Index. She was started on chemotherapy with CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone). Within a week after chemotherapy, the patient's abdominal pain resolved. Follow-up CT scan of the abdomen revealed a marked decrease in the size of the pancreas and peripancreatic lymphadenopathy. A literature search revealed only seven cases of primary involvement of the pancreas in B-cell lymphoma presenting as acute pancreatitis. However, only one case of secondary pancreatic involvement by B-cell lymphoma presenting as acute pancreatitis has been published. Our case appears to be the second report of such a manifestation. Both cases responded well to chemotherapy.

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Key words: Non-Hodgkin's lymphoma; Acute pancreatitis; Pancreatic lymphoma

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INTRODUCTION

Non Hodgkin's lymphoma (NHL) frequently arises in extra-nodal sites, with about 50% of patients having extra-nodal involvement^[1]. The gastrointestinal tract is the most frequent site of involvement, with the stomach and intestines being involved in most cases^[2]. Involvement of the pancreas by NHL has been infrequently reported^[3,4]. Only about 0.2%-2% of patients with NHL have pancreatic involvement at the time of presentation^[1,4,5]. The present report describes a unique case of NHL with secondary pancreatic involvement presenting as acute pancreatitis. A literature search has revealed seven cases of primary involvement of the pancreas by B-cell lymphoma presenting as acute pancreatitis^[6,7]. However, only one case of secondary pancreatic involvement by B-cell lymphoma presenting as acute pancreatitis has been reported^[8].

CASE REPORT

A 57-year-old Caucasian female presented to her primary medical doctor with complaints of abdominal pain. Pain was located in the epigastrium, was dull in character, constant, and grade 3-4/10 in severity. On examination, the patient was also found to have a painful right axillary mass, which appeared to be matted lymph nodes. A biopsy of the matted lymph nodes was undertaken.

However, 4 d later, the patient presented to our emergency department because of worsening abdominal pain. Pain had increased to a severity of 9-10/10 and was associated with nausea and bilious vomiting. On physical examination, she was found to have extreme epigastric tenderness. Laboratory investigations showed an elevated serum amylase level of 623 U/L (normal range 30-110 U/L) and a serum lipase level of 4963 U/L (normal range 23-300 U/L). The patient was initially diagnosed with acute pancreatitis and admitted to the hospital. Past medical history was negative for alcohol consumption or medication use. Abdominal ultrasound done at the time of admission did not reveal any gallstones. Electrolytes and lipid panel were completely normal.

A subsequent abdominal computed tomography (CT) scan showed a diffusely enlarged pancreas due to an infiltrative neoplasm, along with bulky retroperitoneal lymphadenopathy, bulky peripancreatic, mesenteric adenopathy, ascites and peritoneal carcinomatosis

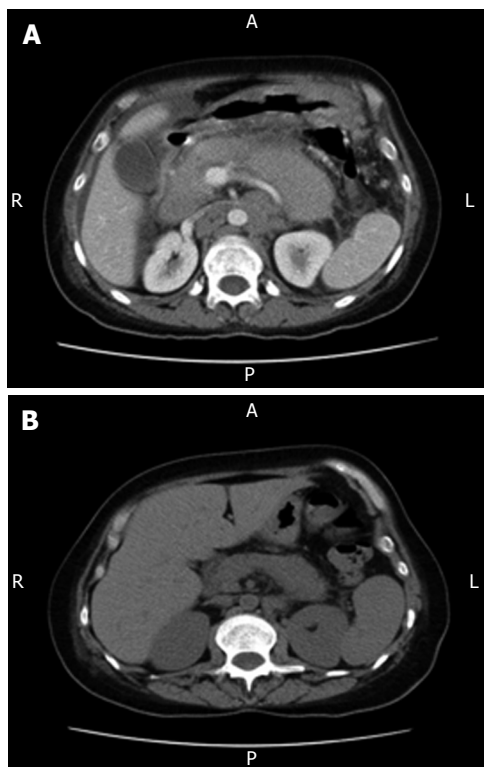


Figure 1 A: Baseline CT scan (pre-therapy). Abdominal CT scan shows diffusely enlarged pancreas due to infiltrative neoplasm, along with bulky retroperitoneal lymphadenopathy, bulky peripancreatic, mesenteric adenopathy, ascites and peritoneal carcinomatosis; B: Post-therapy CT Scan (non-enhanced). A follow-up CT scan of the abdomen reveals a marked decrease in size of the pancreas and retroperitoneal lymph nodes.

(Figure 1A). A report of the biopsy of the axillary mass was available the day after admission, and showed fragments of mononuclear cells with features consistent with lymphocytes, predominantly large in size, with frequent mitotic figures and focal necrosis (Figure 2). Immunohistochemical analysis showed features consistent with a large B-cell lymphoma. Eleven immunohistochemical stains were performed, including CD20, which was positive. Serum lactic dehydrogenase level was elevated to 1227 IU/L (normal range 300-600 U/L) at the time of admission.

Bone marrow biopsy showed evidence of bone marrow involvement by the diffuse large B-cell lymphoma. The patient was classified as stage IV A, based on the Ann Arbor classification designed for Hodgkin's and non-Hodgkin's lymphoma. She was classified as high risk on the International Prognostic Index for diffuse large B-cell lymphoma.

The patient received one cycle of chemotherapy with cyclophosphamide (750 mg/m² Intravenous), doxorubicin (50 mg/m² intravenous), vincristine (1.4 mg/m² intravenous) and prednisone (100 mg/m² orally daily for 5 consecutive days) (CHOP regimen). Within a week after chemotherapy, the patient's abdominal pain had resolved and nausea and vomiting had significantly decreased. Her serum amylase and lipase levels returned to normal (Table 1). A follow-up CT scan of the abdomen revealed a marked decrease in the size of the pancreas and retroperitoneal lymph nodes (Figure 1B).

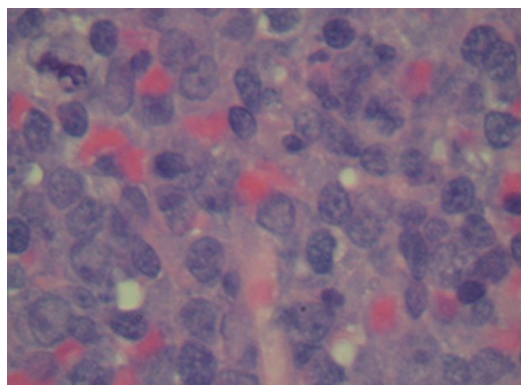


Figure 2 Biopsy of the axillary mass reveals a diffuse large B-cell lymphoma.

Table 1 Differences in serum amylase and lipase levels before and after treatment

	Serum amylase level (U/L)	Serum lipase (U/L)
Before chemotherapy	623	4963
Two days after chemotherapy	72	122
Four days after chemotherapy	45	97

DISCUSSION

Malignant lymphoma infrequently involves the pancreas. The estimated frequency of primary NHL of the pancreas is about 2.2%^[4]. Secondary involvement of the pancreas by NHL is rare. Approximately 0.2%-2% of patients with NHL have pancreatic involvement at presentation^[1,4,5]. An autopsy series of 1269 cases of NHL found pancreatic involvement in 28.9% of the cases^[8]. A literature search of the PubMed database has revealed seven cases of primary involvement of the pancreas by B-cell lymphoma, presenting as acute pancreatitis^[6]. However, only one case of secondary involvement of the pancreas by B-cell lymphoma presenting as acute pancreatitis has been described^[6]. As in our case of secondary pancreatic involvement, that previous patient also presented with acute abdominal pain radiating to the back. He was found to have elevated serum amylase and serum lipase. A CT scan showed diffuse swelling of the pancreas with two masses, one in the corpus and one in the tail. There was also lymphadenopathy near the pancreas. CT of the thorax showed a large lymph node in the mid mediastinum. This lymph node was biopsied and revealed a large B-cell lymphoma. This case of secondary pancreatic involvement also responded well to chemotherapy. A standard regimen of chemotherapy led to normalization of serum lipase and amylase levels, as well as a decrease in the size of the pancreas and the peripancreatic and retroperitoneal lymph nodes.

Diffuse large B-cell lymphoma is the most common NHL, and makes up 30% of newly diagnosed cases. The lymphoma can present with enlarged lymph nodes at either the primary or extra-nodal sites. More than 50% of patients have some extra-nodal involvement at diagnosis^[1]. The most common sites are the gastrointestinal tract and

bone marrow, each being involved in 15%-20% of cases.

The presenting symptoms of pancreatic lymphoma are usually non-specific and include abdominal pain (83%), abdominal mass (58%), weight loss (50%), jaundice (37%), acute pancreatitis (12%), small bowel obstruction (12%) and diarrhea (12%). These symptoms help distinguish pancreatic lymphoma from pancreatic carcinoma^[2]. Imaging plays an important role in the diagnosis and staging of pancreatic masses^[5,9,12]. This is particularly true for pancreatic lymphoma, as treatment and prognosis are significantly different from those for pancreatic adenocarcinoma^[9]. A CT scan is the modality commonly used for the detection of pancreatic lymphoma. Two types of morphological presentation have been reported on CT; one is a tumor-like, localized, well-circumscribed mass presenting as a hypoechogenic mass, and the second is a diffuse enlargement infiltrating the pancreas^[9,10]. The well-circumscribed tumoral form is distinguished from pancreatic adenocarcinoma by the absence of pancreatic duct involvement and the presence of surrounding lymphadenopathy^[9,10]. The imaging findings in the second type are similar to those in acute pancreatitis. Ultrasound- or CT-guided fine needle biopsy of the pancreatic mass can also help distinguish pancreatic lymphoma from pancreatic adenocarcinoma^[4]. In the present case, pancreas biopsy was not necessary to diagnose the lymphoma because sufficient tissue was obtained from the axillary mass.

Anthracycline-based chemotherapy is the standard treatment for NHL, and includes six to eight cycles of R-CHOP for patients of all ages^[11]. High-grade pancreatic lymphoma generally responds well to standard chemotherapy^[11].

Our patient presented with acute abdominal pain and was in the high-risk category of the International Prognostic Index, and was therefore treated as an emergency with high-dose inductive chemotherapy. She responded very well to the chemotherapy and following the first cycle, there was a decrease in the size of the pancreas, as well as the peripancreatic and retroperitoneal lymph nodes. The patient's abdominal pain resolved and her serum amylase and lipase levels normalized.

Secondary involvement of the pancreas by B-cell lymphoma is a rare occurrence. It is important to include such secondary involvement in the differential diagnosis of patients that present with acute pancreatitis. Pathologic

diagnosis is important in distinguishing pancreatic lymphoma from pancreatic carcinoma. Most cases of pancreatic lymphoma respond very well to chemotherapy.

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Meetings

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Meeting Canadian Digestive Diseases Week (CDDW)
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Meeting Falk Symposium 159: IBD 2007 - Achievements in Research and Clinical Practice
4-5 May 2007
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Meeting European Society for Paediatric Gastroenterology, Hepatology and Nutrition Congress 2007
9-12 May 2007
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espghan2007@colloquium.fr

Digestive Disease Week
19-24 May 2007
Washington Convention Center, Washington DC

Meeting Gastrointestinal Endoscopy Best Practices: Today and Tomorrow, ASGE Annual Postgraduate Course at DDW
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Meeting ESGAR 2007 18th Annual Meeting and Postgraduate Course
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From bed to bench: Which attitude towards the laboratory liver tests should health care practitioners strike?

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Abstract

There is a general consensus in re-interpreting the so-called liver function tests in the light of novel discoveries. At the same time, recent evidence favours the use of different laboratory data to assess liver damage, fibrosis or regenerative process, but this point is not always shared. Actually, balancing the need for diagnosis, prognostic evaluation and therapy response of liver disease with a good cost/benefit ratio is very difficult. New tests are probably not needed but the aim should be for better utilization of existing tests to contain the increasing cost of health care.

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SCENARIO

The liver being a multifunctional organ (specialized in detoxification, metabolism and defence), the reliability of a single laboratory test is relatively limited (obviously, chemistry is the first approach followed by imaging and histology, not necessarily in this order). So far, the hepatic diagnostic evaluation is based on several contextual or in sequence analyses. Different algorithms have been proposed to improve the detection, the differentiation and the severity of liver diseases. Actually,

the favourable relationship between hepatologists and the clinical chemistry experts is jeopardized by an overuse of laboratory services. This attitude does not reduce mortality, does not shorten hospital stays, nor contribute to the quality of medical care. Actually, balancing the need for diagnosis, prognostic evaluation and therapy response of liver disease with a good cost/benefit ratio is quite difficult. But, is it true that those never ending reports that advice the practitioners about the clinical utility of various diagnostic tools greatly broaden the health care costs?

Until now, physicians evaluate the type and the entity of hepato-cellular damage. Generally, it is considered to appear as inflammation and colliquative necrosis. In this direction the laboratory liver tests (LLTs) moved since the discovery of the transaminases usefulness in diagnosing acute hepatitis, anciently known as “icterus catharralis”, in the mid 1950s, and published some years later^[1].

These tests are erroneously named as liver function tests, and alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are considered the most important. They are present in high concentration in hepatocytes, where they catalyse the transfer of amino groups from alanine and aspartate to the α -keto group of ketoglutaric acid in order to produce pyruvic acid and oxaloacetic acid, respectively. These enzymes leak into the blood when the hepatocytes cell membranes are damaged.

IS THE ROLE OF APOPTOSIS DEFINITELY UNDERESTIMATED IN EVALUATING THE ENTITY OF LIVER INJURY?

As previously stated, the hepatic damage evaluation is abundantly unbalanced toward the inflammation/necrosis process. Apoptosis may occur in the absence of significant transaminases increase as happens in hepatocellular necrosis. Apoptosis describes the process by which damaged or senescent cells are eliminated from the organism. The expression “the falling of old leaves from trees in the autumn” clearly explains the apoptosis meaning. Apoptosis occurs by two mechanisms: death receptor or extrinsic mechanism and mitochondrial or intrinsic mechanism. Hepatocytes express different death receptors, i.e., Fas, tumor necrosis factor-receptor 1 (TNF-R1), TNF-related apoptosis-inducing ligand receptor 1 (TRAIL-R1), and receptor 2 (TRAIL-R2); stellate cells express Fas and TRAIL when are activated into myofibroblast-like phenotype and undergo apoptosis during resolution of liver injury *in vivo*. Cholangiocytes seem to be a type of

cells in which the mitochondrial mechanism to apoptotic is essential. Apoptosis has a major role in hepatic injury and subsequent progression to fibrosis as it has been well established in different hepatic diseases. In fact, apoptosis is one of the most important mechanisms leading to hepatocyte elimination in non-alcoholic fatty liver disease (NAFLD) and contribute to intensify inflammation in the same disease inducing proapoptotic protein p53 with the inhibition of antiapoptotic Bcl-2^[2].

Apoptosis results in the extracellular release of a limited number of proteins including: cytochrome c, some caspases and cleaved cytokeratin-18 and possibly a few other proteins. Mitochondrial cytochrome c release is the key event, which is critical for the initiation of the formation of the apoptosome complex that is essential for the activation of caspase-9 and initiation of the mitochondria-dependent apoptotic cascade^[3,4]. Following this pattern of speculation, a new (not completely!) marker in detecting Non-Alcoholic Steato Hepatitis (NASH)^[5], has been studied.

MORE SUBTLE TESTS OF LIVER CELL DAMAGE WILL PROBABLY BECOME AVAILABLE IN THE FUTURE, BUT IN THE MEANTIME, DOES MEASUREMENT OF SERUM AMINOTRANSFERASE CONCENTRATIONS FULFILL THE PRECISION PURPOSE?

ALT is the more specific marker of hepatocellular injury because it occurs exclusively in the liver, whereas AST occurs to some extent also in heart, skeletal muscle, kidney and pancreas. AST is prevalently found in mitochondria (near 80%) and to a lesser extend in cytoplasm (20%). Meanwhile, ALT is bound to cytoplasm. It is necessary to stress that, although transaminases are sensitive indicators of hepatocyte damage, they could not be considered reliable markers. The likelihood that abnormal aminotransferase levels reflect liver disease is exponentially increased by the presence superior to six months; the association with signs or symptoms of liver disease; the high enzyme levels; the plurality of abnormal liver enzymes.

Serum ALT/AST concentrations are raised in almost all forms of liver disease, but to different degrees. Very high concentrations ($> 10 \times$ Upper limit of normality, ULN) are typical of acute viral, drug-induced hepatitis, ischemic injury to the liver. ALT/AST levels may be unexpectedly high in the early stage of biliary obstruction and, rarely, in flares of autoimmune chronic hepatitis. Those levels do not necessarily mirror the severity of hepatocyte necrosis or the patients' prognosis. This point is evident in massive hepatic necrosis, when a decreasing serum concentration signifies not recovery, but a fewness of hepatocytes from which the enzymes can leak. In the patient in state of shock, serum ALT and AST concentrations typically rise abruptly and return to normal within days after haemodynamic stability is restored. Return to previous

normal values may be sufficiently rapid in drug-induced hepatitis. These laboratory abnormalities patterns contrast with those in acute viral hepatitis, in which the decrease is far more gradual. Serum ALT and AST concentrations are moderately raised ($2-10 \times$ ULN) in chronic and asymptomatic cases of acute viral or drug-induced hepatitis, autoimmune hepatitis, and alcoholic liver disease. In these disorders the enzyme concentrations generally correlate with the activity of the disease. Post-viral cirrhosis, the whole spectrum of NAFLD, cholestatic liver disease, and hepato-cellular carcinoma are characterised by slightly raised serum aminotransferase levels ($< 2-3 \times$ ULN). The serum AST/ALT ratio may help diagnose some liver diseases. In most patients with acute liver injury the ratio is 1 or less, whereas in alcoholic hepatitis it is generally about 2. Deficiency of pyridoxal-5'-phosphate, a necessary coenzyme for both aminotransferases, is common in alcoholic liver disease. This deficiency decreases hepatic ALT to a greater extent than AST, ending up in changes in serum concentrations. Although diagnostic laboratories scarcely offer measurement of AST isoenzymes, a high ratio in the serum of mitochondrial to total AST indicates mitochondrial damage and provides further evidence of alcoholic liver disease. Paradoxically, in patients suffering from liver cirrhosis of any etiology without ongoing liver injury the values may be normal.

COULD THE "MEASUREMENT LOCATION" AFFECT THE ALT AND AST ELEVATION DEGREE?

This point is clearly supported by some evidence. The causes of very high serum aminotransferase concentrations in a particular centre are influenced by its location, as noted in Swansea, Wales, where viral hepatitis accounts for only 3.5% of AST concentrations of over 400 U/L. Ischaemic/hypoxic liver injury accounts for a half, extrahepatic biliary obstruction for a quarter, and drug toxicity for 8.5% of the cases. This pattern reflects a highly developed community. In developing countries viral hepatitis is still prevalent, and toxic hepatitis is more likely and biliary obstruction from gallstones less likely to be encountered than they are in Swansea^[6].

ARE CLUES IN THE HISTORY AND CLINICAL EXAMINATION NECESSARY TO ASSESS ALT/AST LEVELS?

A recent study sought for identifying any benefit of routine LLTs, i.e., AST, ALT, total bilirubin, or alkaline phosphatase, in 268 consecutive, chronically ill, geriatric patients presenting for acute care from a long-term care facility. All were without jaundice, right upper quadrant pain, pruritus, bruising, or evident signs of chronic liver disease. The degree of LLTs abnormality during admission was compared to the clinical diagnosis at the time of discharge. The levels normalized within two days in 26 of these patients, 25 of whom had a history of vascular disease (96%). All but one of the 268 patients were

discharged without further evaluation. Over one year of follow-up, no patient returned for a liver-related problem. Based on these findings, only those patients with LLTs levels that are twice normal and which do not normalize within two days warrant further evaluation. Authors concluded that transient LLTs abnormalities may be due to decreased liver perfusion^[7].

CARDIOVASCULAR DISEASE EVALUATED BY A VINTAGE TEST

ALT is a marker of NAFLD presence (be aware of silent NASH) and predicts incident type 2 diabetes mellitus. Lately, ALT was shown to be also associated with endothelial dysfunction and carotid atherosclerosis^[8].

SHOULD NEW PERSPECTIVES BE APPLIED TO THE PROGNOSIS EVALUATION OF LIVER DISEASES?

In westernized countries metabolic, alcoholic and chronic viral disease represent the major part of hepatic illnesses. The search for markers of alcohol abuse has been dynamic for the past years. Several laboratory abnormalities based on liver enzymes, metabolic substances, hormone levels and haematological characteristics have been observed to be associated with the problem of drinking.

Due to its short half life, breath, urine or blood ethanol analyses provide scarce information about the entity of alcohol drinking, being an increased tolerance possibly identified. Blood or breath alcohol levels > 1.5% without great evidence of intoxication or > 3% at any time have been reported to be the first-level criterion of alcoholism.

An elevated serum level of membrane-bound enzyme, gamma-glutamyl transpeptidase (GGT) has been widely used as a marker of alcohol abuse. The half life of elevated GGT is between two and three weeks. Unfortunately, the sensitivity of GGT in detecting alcohol abuse has been reported to greatly vary (34%-85%).

GGT does not increase after acute alcohol intake, but needs continuous alcohol consumption of 80-200 g/d for several weeks. In addition to alcohol abuse, increased GGT is frequently found in NAFLD, heart failure and in subjects using antiepileptics, anticoagulants or barbiturates.

Mean corpuscular volume (MCV) is an index of red blood cell size. Increased MCV values have been observed in 34%-89% of alcohol abusers. Increased MCV values are also found in cases of vitamin B₁₂ and folic acid deficiency, chronic liver diseases, hypothyroidism, haematological disorders as well as among smokers. MCV responds slowly to abstinence and up to 40% may have an elevated MCV value even after three months of abstinence.

Other generally used markers are serum amino-transferase. These enzymes are more indicative of liver damage than of alcohol abuse. The overall sensitivity of AST has been estimated to be 35% as a marker of alcohol abuse^[9]. The sensitivity for ALT is even lower. One of the modern laboratory tests for alcohol abuse is serum carbohydrate deficient transferrin (CDT). The marker consists of the asialo, mono-sialo and di-sialo isoforms of

transferrin that are deficient in their terminal trisaccharides. False-positives have been reported in patients with severe liver diseases (primary biliary cirrhosis, chronic hepatitis C, hepato-cellular carcinoma) and in patients with genetic D-variant of transferrin. During abstinence, the CDT values normalize with a mean half-life of 15 d. CDT values seem to increase after one week of drinking at a level of at least 80 g ethanol per day. Obviously, the diagnosis of alcoholic liver disease (ALD) is based on alcohol consumption, physical signs and symptoms, and laboratory tests. In a recent study, the sensitivity and specificity of CDT for ALD was 93.4% and 71.9%, respectively^[10].

Alcoholism plays a key role in worsening any other liver disease! An up-to-date research using population-based mortality data to investigate whether heavy drinking affected the age of death among individuals with HCV was analyzed a total of 7263163 death records in the United States between 2000 and 2002. This study provided evidence to establish heavy alcohol consumption as one of the key risk factors contributing to premature deaths from HCV in the United States. Still, it pointed out that alcohol consumption affects men and women differently in HCV mortality^[11].

From a similar point-of-view, laboratory tests reflecting the "metabolic co-factor" linked to HCV-related chronic hepatitis should not be neglected.

WHICH LIVER DISEASE IS UNRAVELLED BY CHRONIC ABNORMAL ALT LEVELS?

Near 10% of the patients with chronically abnormal ALT levels show no evident relationship with any illness. The prognosis of this finding, the link with liver fibrosis and the need for a liver biopsy are largely unknown. The aim of a recent retrospective study in 67 patients with accidentally detected chronically elevated ALT levels, who had a liver biopsy, was to evaluate the prevalence of NASH, and the biological factors associated with this entity. Fibrosis scores were: F0, 37.3%; F1, 32.8%; F2, 26.9%; F3, 1.5%; and F4, 1.5%. NASH was absent in 59.7% and present in 40.3%. Significant differences were observed between F < 2 and F ≥ 2 fibrosis patients for AST and ALT and between patients with NASH or without for body mass index. The prevalence of F ≥ 2 fibrosis and NASH in patients with unexplained chronic abnormal ALT were 30% and 40%, respectively. Since the risk of F ≥ 2 fibrosis was significantly increased in patients with AST > N and/or ALT > 2N, liver biopsy, concluded the Authors, should be performed only in patients with AST > N or ALT > 2N^[12].

WHICH IS THE NORMAL RANGE OF ALANINE-AMINOTRANSFERASE?

The ULN of serum ALT was in these years challenged, because patients diagnosed with liver diseases may have 'normal' or near-'normal' ALT levels, and because possible modulators are often ignored in determining normal range. Some researchers reviewed medical records of subjects aged 15-90, who underwent standard panels of laboratory tests, including serum ALT, over 6 mo at a

central laboratory. Three groups were defined: Group 1, comprised total study population ($n = 272$). Group 2 ($n = 87$) comprised total study population, excluding those receiving potentially hepatotoxic drugs, or diagnosed with liver disease, or had any abnormal laboratory test results other than for triglycerides, cholesterol, glucose, or HbA1c. Group 3 ($n = 17$) the 'healthy' population, from whose ALT values we established the new ULN, comprised Group 2 subjects with normal triglycerides, cholesterol, glucose, and HbA1c levels. The results were intriguing. The 95th percentile ALT values, corresponding to the ULN, in groups 1, 2, and 3 were 50.1, 40, and 37.5 U/L, respectively. 6.2% of subjects whose ALT was below ULN listed by the test manufacturer (52 U/L), had ALT level above in their new ULN. Linear and logistic-regression analyses showed that ALT levels were significantly modified by gender, age, glucose, cholesterol, triglycerides, and overweight/obesity diagnosis^[13].

Elevated liver enzymes are infrequent in patients with Hepatitis C virus (HCV) infection undergoing chronic hemodialysis (HD), suggesting that ALT is a poor predictor of hepatocellular damage in this population. The objective of this research was to establish a more appropriate cut-off value of ALT to identify biochemical activity due to HCV infection in HD patients. A total of 217 patients, with an average age of 51.2 years, were evaluated in a single year; 130 were males (60%). Serum ALT was measured by a kinetic method in five consecutive monthly blood samples, from which an average was obtained and divided by ULN. The cut-off value of ALT was obtained from a ROC curve. Within the 217 patients, 18 (8.3%) were anti-HCV-positive, 17 (7.8%) of whom were also HCV-RNA-positive. Genotype distribution was: 1a = 47%; 1b = 18%; 3a = 35%. Mean ALT/ULN (0.77 ± 0.57) of the 18 anti-HCV-positive cases was higher than the negative group (0.38 ± 0.23). The mean ALT/ULN (0.81 ± 0.57) of the 17 HCV-RNA-positive cases was also higher than the negative cases (0.37 ± 0.23). The cut-off value of ALT to distinguish the anti-HCV-positive from negative patients was 0.50% or 50% of the ULN (sensitivity = 67%; specificity = 83%). According to the HCV-RNA, the cut-off value of ALT was 0.45% or 45% of the ULN (sensitivity = 71%; specificity = 80%). The Authors concluded that reducing the cut-off of ALT by half, enabled a better identification of biochemical activity in patients with HCV infection on chronic HD^[14].

ALT activity is the most widely used laboratory test for the recognition of liver disease. As aforementioned, normality limits for values of serum ALT activity have been questioned lately. One reason for this recent uncertainty may be an unrecognized decline in aminotransferase levels in the aging population. To verify such hypothesis a cross-sectional evaluation of the association between age and ALT activity was performed. Laboratory data of residents in single home for the aged and of adult subjects in three general practice clinics in Israel were reviewed, excluding subjects with known liver disease. One hundred and twenty-eight individuals from the home for the aged and 207 individuals from three family practices were included. The study ended up in finding out that ALT activity regressed with age creating

an inverted U curve with a peak at 40-55 years, (polynomial regression). According to age groups, serum ALT level was 19 ± 13 U/L in those under 40 year, 25 ± 19 U/L in 40-55 year olds, 22 ± 10 U/L in 56-72 year olds, 17 ± 9 U/L in 73-83 year olds, and 13 ± 5 U/L in 83-100 year olds. Gender also showed different levels for ALT, i.e., 22 ± 15 U/L in men, and 17 ± 11 U/L in women. Multiple regression analysis including age, gender revealed that age and gender retained association with ALT activity but not interestingly BMI. No such associations were noted for AST activity. These data suggest a significant inverted-U-like association between age and serum ALT activity. Thus, when interpreting the laboratory results of a subject suspected of liver disease, age should probably be taken into account^[15].

Despite the association between elevated ALT levels and liver diseases, the entity of the ALT elevation does not always correlate with the extent of liver cell damage. Accordingly, the ALT levels are of scarce prognostic value. It is noteworthy to stress that, currently, measurement of serum ALT levels is the most frequently used test to identify patients with liver diseases.

Other reports have questioned whether established values to define normal ALT/AST range are accurate and have suggested that the upper limit of normal should be revised. A study by Prati^[16], screening 6835 first-time blood donor candidates, demonstrates that ALT activity was independently related to body mass index (BMI) and to laboratory indicators of abnormal lipid or carbohydrate metabolism, as well as to sex. Still, the authors calculated "healthy" ranges for serum ALT level in 3927 donors who had a normal BMI and normal levels of serum glucose, cholesterol, triglyceride, and who were not taking medications. The upper limit of normal for ALT level decreased from 40 U/L to 30 U/L in men and from 30 U/L to 19 U/L in women. The new normal ranges increased the sensitivity for detection of patients with liver injury from 55% to 76% but decreased the specificity from 97% to 88%. But, should these more realistic values for normal ALT levels be widely adopted? Probably yes, because this would sensibly increase the number of asymptomatic patients with abnormal ALT values and would identify far more patients with NAFLD and clinically mild or occult HCV infection^[17].

The normal range for ALT/AST levels was set a half century ago and has never changed since then!

PROFILING THE OLD CUMBERSOME TESTS ON A NEW LIGHT: A RIGHT CHOICE?

An occurrence could be by the following study implanted to investigate if and to what extent antiviral therapy influenced a broad panel of quantitative testing of liver function (QTLF). Fifty patients with chronic hepatitis C were either treated with interferon, interferon/ribavirin or peg-interferon/ribavirin. QTLF, including aminopyrine breath test (ABT), galactose elimination capacity (GEC), sorbitol clearance (SCI) and indocyanine green clearance

(ICG) was performed before and 3 mo after initiation of antiviral therapy. After three months of antiviral treatment, 36 patients showed normal transaminases and were negative for HCV-RNA, 14 patients did not respond to therapy. ABT and GEC as parameters of microsomal and cytosolic liver function were reduced in all patients before therapy initiation and returned to normal values in the 36 therapy responders. Parameters of liver perfusion (SCI and ICG) were not affected by antiviral therapy. In the 14 non-responders, no changes in QTLF values were observed during the treatment period. Early determination of ABT and GEC may differentiate responders from non-responders to antiviral treatment in hepatitis C^[18].

An effort to simplify the “real” liver function tests, favouring their application on a large scale, at least in advanced forms, was present-day made by evaluating the TOSCA^[19].

SHOULD LABORATORY LIVER TESTS BE PERFORMED AFTER THE IMAGING RESULTS?

Some Authors speculated about the association between the severity of liver steatosis and MS in apparently healthy Korean adults. They examined 1022 men and women, aged 30-79 years, who participated in a health screening test. A standard interview, anthropometrics, biochemical studies, and abdominal UltraSonography (US) were conducted for each participant. Metabolic syndrome (MS) was defined according to the National Cholesterol Education Program Adult Treatment Panel III, lightly modified. The severity of liver steatosis was evaluated using liver US, and AST, ALT, and GGT levels were determined. The results clearly evidenced that the MS showed a stronger association with the severity of US steatosis than with the serum liver enzyme levels. The researchers advised that the degree of fatty infiltration detected on US could be used as an indicator of liver dysfunction attributable to metabolic abnormalities^[20].

IS GAMMA-GLUTAMYL TRANSPEPTIDASE A SERUM MARKER OF CHOLESTASIS/ALCOHOL-INDUCED DAMAGE/ ENZYMATIC INDUCTION ONLY?

Oxidative stress plays a crucial role in a variety of clinical settings including atherogenesis, and mediates many pathways linked to inflammation. GGT, an enzyme responsible for the extracellular catabolism of antioxidant glutathione, may directly take part in atherogenesis and evolve as a potential biochemical risk indicator of cardiovascular morbidity and mortality. Classically, GGT has been thought of as a diagnostic tool for hepatobiliary disorders and alcohol abuse. More recently, growing body of data points out that serum GGT levels can aid detection of individuals at high risk for subsequent cardiovascular events, and thus have an application in primary and secondary prevention of cardiovascular disease. Although

several investigations have shown that some drugs are effective in decreasing both serum lipids and GGT, and concomitantly the incidence of subsequent cardiovascular disease (CVD). Based on current experimental and epidemiological studies, Turgut postulates that GGT present in the serum, even within its laboratory reference intervals regarded as physiologically normal, is a promising biomarker for cardiovascular risk^[21].

An increase in serum GGT predicts onset of MS, incident CVD, and death suggesting that GGT is a marker of metabolic and cardiovascular risk^[22]. An unchanged or increased GGT level over time, even when GGT is in the normal range, is correlated with increasing insulin resistance and is associated with a risk of incident type 2 diabetes in both sexes, independently of baseline GGT, which is itself a diabetes risk factor^[23]. Also for other Authors, the role of GGT is different. In fact, it has been proposed that elevated serum GGT is an independent marker of the activation of systemic inflammation and increased oxidative stress, independent of their relationship to MS, and that the presence of MS and elevations of this liver enzyme may additively worsen the atherogenic state^[24].

ARE THERE ANY INTERESTING MARKERS ON THE EDGE?

A recent study, zeroing in on novel parameters, assessed the role of transforming growth factor-beta1 (TGF-beta1, as anti-apoptotic marker) and vascular endothelial growth factor (VEGF, as angiogenetic factor) in the pathogenesis of fibrosis associated with HCV-related chronic hepatitis and evaluated the influence of the antiviral therapy on above parameter levels depending on the treatment results^[25]. This research showed that a complete response in these patients is associated with significant changes in TGF-beta1 and VEGF.

FIBROSIS MARKERS: ARE THEY REALLY USEFUL?

Liver fibrosis is the excessive deposition and redistribution of extracellular matrix (ECM) in this organ induced by chronic injury. It leads to ultimately cirrhosis, characterized by progressive liver insufficiency/portal hypertension, and hepato-cellular carcinoma. There is a certain need for reliable, non-invasive, repeatable markers of fibrosis and fibrogenesis to substitute the invasive method (liver biopsy, which is burdened by a high degree of sampling error). A systematic literature search, using relevant papers, was timely performed to weigh the usefulness of non-invasive biomarkers of liver fibrosis^[26].

Serum biomarkers are differentiated in those reflecting ECM turnover (fibrogenesis and fibrolysis) and/or fibrogenic cell changes, mainly of Ito cells. They are procollagen peptides, hyaluronan, and laminin. The remainders are based on algorithmic evaluation of commonly observed liver alterations that do not necessarily reflect ECM metabolism or fibrogenic cell changes. Several numerical scores or indices are reported for parameters,

which are mostly routine laboratory tests and frequently multiparametric, e.g., panels. In good substance, although several serum markers and panels offer the opportunity to noninvasively assess the extent of liver fibrosis and spare some patients the risks associated with percutaneous liver biopsy, only a few of them allow the determination of different stages of fibrosis on a continuum similar to that achieved with liver biopsy^[27].

Indeed, hyaluronic acid is considered an accurate variable for the severity of hepatic inflammation and fibrosis^[28].

Conclusively, the impact of both classes of biomarkers for diagnosis and monitoring of fibrosis, fibrogenesis, and fibrolysis is limited. They cannot replace needle biopsy even though some of them, generally expensive, might be complementary in follow-up studies. Transient elastography could be a valid alternative choice.

FINAL CONSIDERATION

Unfortunately, it is true that new reports that advice the practitioners about the clinical utility of various diagnostic tools, when embraced, make the health care costs increase without expanding the diagnostic-prognostic horizon, with very few exceptions.

To make the hepatologists' life a little more complicated, some computations have been advanced. For example, the model of end-stage liver disease (MELD) score has gained acceptance over the Child-Pugh (C-P) score in predicting survival in patients with decompensated cirrhosis, although it is more sophisticated. A suggestive research compared the predictive values of MELD, C-P and creatinine-modified C-P scores in decompensated cirrhosis. The areas under the receiver operating characteristic curves did not differ significantly among the four scores. In Cox regression analysis, all four scores were significantly associated with survival, while MELD and creatinine-modified C-P scores had better predictive values than C-P score. Adjustment for GGT (*de novo*) levels increased the predictive values of all systems. Thus, MELD compared to the C-P classification does not appear to offer a clear advantage in predicting survival in patients with decompensated cirrhosis in daily clinical practice^[29].

Still, the MELD model is only slightly superior to the Child-Pugh classification for the prediction of long-term survival in TIPS patients^[30].

The "ancient" serum total bilirubin, present in MELD as well as in C-P scores, offers the best single prognostic factor in evaluating liver cirrhosis progression, except for patients suffering from Gilbert syndrome.

Prothrombin activity (expressed in time or percentage or as INR) should be regarded as a clue of disseminated intravascular coagulation.

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S- Editor Liu Y L- Editor Li M E- Editor Yin DH



TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, Series Editor

Alcoholic liver injury: Pathological features and models

From the editor

In this 9-topic review series, we will provide an insight into the pathogenesis of alcohol-mediated liver damage. The aspects addressed in the reviews cover the most important pathological features induced by ethanol/or ethanol metabolism: dysregulation of cell cycle progression, immune response, impaired protein hydrolysis by proteasome and steatosis and apoptosis induction. The mechanisms of ethanol-elicited liver injury are related to compromised transmethylation, involvement of ethanol in iron metabolism and emerging roles of mitochondria and free radicals in establishment and progression of liver damage. These events are observed not only in hepatocytes, but also in some other liver cells, including the sinusoidal endothelial cells and immune cells. At the molecular level, these pathological symptoms are progressing due to the altered signal transduction processes, which further impair cell functions. To study the alcohol-induced cell damage, certain adequate cell models, including polarized hepatic cells, are developed. The following reviews written by well-established scientists with a long-term expertise in the field of alcohol-induced liver injury.

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Natalia A Osna, MD, PhD, Series Editor

Role of alcohol in the regulation of iron metabolism

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Abstract

Patients with alcoholic liver disease frequently exhibit increased body iron stores, as reflected by elevated serum iron indices (transferrin saturation, ferritin) and hepatic iron concentration. Even mild to moderate alcohol consumption has been shown to increase the prevalence of iron overload. Moreover, increased hepatic iron content is associated with greater mortality from alcoholic cirrhosis, suggesting a pathogenic role for iron in alcoholic liver disease. Alcohol increases the severity of disease in patients with genetic hemochromatosis, an iron overload disorder common in the Caucasian population. Both iron and alcohol individually cause oxidative stress and lipid peroxidation, which culminates in liver injury. Despite these observations, the underlying mechanisms of iron accumulation and the source of the excess iron observed in alcoholic liver disease remain unclear. Over the last decade, several novel iron-regulatory proteins have been identified and these have greatly enhanced our understanding of iron metabolism. For example, hepcidin, a circulatory antimicrobial peptide synthesized by the hepatocytes of the liver is now known to play a central role in the regulation of iron homeostasis. This review attempts to describe the interaction of alcohol and iron-regulatory molecules. Understanding these molecular mechanisms is of considerable clinical importance because both alcoholic liver disease and genetic hemochromatosis are common diseases, in which alcohol and iron appear to act synergistically to cause liver injury.

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Key words: Alcoholic liver disease; C/EBP alpha; Divalent metal transporter 1; Ferroportin; Hepcidin

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INTRODUCTION

Iron is essential for an array of key biological processes including erythrocyte production, DNA synthesis and cellular respiration^[1-3]. The normal iron content of the body in an adult male is 35 to 45 mg of iron per kilogram of body weight^[2]. The majority of the iron is bound to hemoglobin in erythrocytes. The rest is incorporated into myoglobin in the muscle, the tissue enzymes and plasma transferrin^[2]. Parenchymal cells of the liver and reticuloendothelial macrophages serve as depots for excess iron storage^[2,4]. However, due to its potential to take part in the Fenton reaction as a transition metal, iron can also be toxic to the cell^[5,6]. Hepatic iron overload is common in many liver diseases, where iron is a risk factor in disease progression^[7-13].

IRON AND ALCOHOL

Alcoholic liver disease (ALD) patients frequently display evidence of iron overload^[14-18]. Recently, even mild to moderate alcohol consumption has been shown to elevate the indices of iron stores^[19]. Suzuki *et al*^[20] have demonstrated elevated expression of transferrin receptor-1 in ALD patients by immunohistochemical analysis of liver biopsy samples. Moreover, Kupffer cells isolated from experimental animal models of ALD also display increased iron content^[21,22]. It is a well established fact, that both iron and alcohol individually cause oxidative stress and lipid peroxidation^[6,23-26]. Hence, alcohol induced iron overload enhances the production of free radicals and proinflammatory cytokines, thereby leading to liver injury^[11,12,27]. Elegant studies with experimental animal models of ALD have demonstrated, that increased iron content in Kupffer cells leads to the activation of the transcription factor, nuclear factor-kappa (NF- κ B), and increased expression of the proinflammatory cytokine, tumor necrosis factor-alpha (TNF- α)^[21,22,28]. These effects were abolished by iron chelation^[29], thereby indicating a role for iron-mediated cell signaling in the pathogenesis of experimental alcoholic liver disease.

The iron overload disorder, genetic hemochromatosis (GH), is one of the most prevalent genetic diseases in

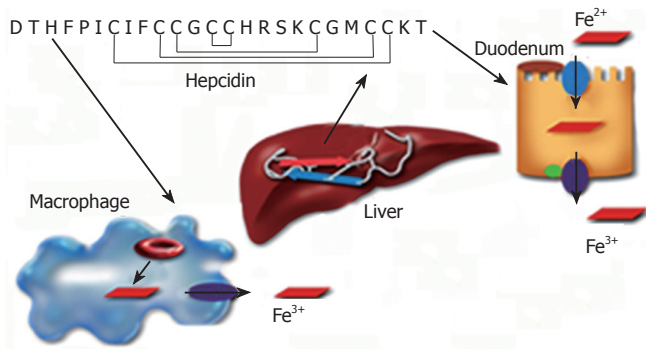


Figure 1 Regulation of iron metabolism by hepcidin. Hepcidin peptide, which is synthesized by the liver and released into the circulation, regulates iron homeostasis by inhibiting duodenal iron transport and the release of iron from macrophages.

individuals of Caucasian origin^[30-32]. The majority of GH patients are homozygous for a C282Y mutation in their Hfe gene^[33,34]. Excessive alcohol consumption has been reported to exacerbate liver injury in GH patients homozygous for the C282Y mutation of Hfe gene^[35]. However, despite all these findings, the underlying mechanisms of iron accumulation observed in alcoholic liver disease, and the source of the excess iron remain elusive. *In vivo* whole-body retention studies have demonstrated a two-fold increase in intestinal iron absorption in chronic alcoholics^[36]. Changes in intestinal permeability are thought to be the underlying mechanism of enhanced intestinal iron absorption^[36]. Of note, hepatocytes are the primary site of iron storage in the liver, and iron may also leak out of injured hepatocytes. However, it is also feasible that the iron stores in alcoholic patients are increased through recognized regulatory mechanism(s). Significant advances have been made with the discovery of novel iron-regulatory molecules in recent years, which have improved our understanding of iron metabolism. Studying the regulation of these molecules by alcohol is important for understanding the underlying mechanisms of iron overload in alcoholic liver disease.

IRON-REGULATORY MOLECULES

Since there is no physiological pathway of excretion for excess iron in the body, the uptake, transport and storage of iron must be tightly regulated^[37-41]. A series of novel iron-regulatory molecules including iron transporters and soluble mediators have recently been identified. Divalent metal transporter 1 (DMT1, also known as Nramp2) is a multi-transmembrane protein^[42], responsible for importing dietary non-heme iron through the apical site of absorptive enterocytes in the duodenum^[42]. Studies employing mice with the targeted deletion of DMT1 in the duodenum have confirmed the role of DMT1 in intestinal iron absorption^[43]. Conversely, ferroportin (also known as MTP1, Ireg1) exports iron into the bloodstream^[44-46]. As a transition metal, iron undergoes reduction and oxidation reactions during these cellular uptake and export processes. Understanding the mechanisms involved in these reactions and identifying the candidate enzymes will require further investigation^[47-49]. Iron circulates in the plasma by binding

to the glycoprotein, transferrin (Tf). Iron-laden Tf is taken up into the cell by forming complexes with transferrin receptor-1, TrfR1^[2,50]. Recently, another homologous receptor, TrfR2 has been identified^[51]. Unlike TrfR1, which is ubiquitously expressed, TrfR2 is mainly expressed in the liver^[51].

The regulation of iron metabolism involves multiple organs including the duodenum, liver and bone marrow. Hence, the presence of soluble mediators has long been suspected. The discovery of hepcidin peptide has not only confirmed these notions but also highlighted the importance of the liver in the regulation of iron homeostasis. Hepcidin is an antimicrobial peptide, which was first isolated from human urine and blood^[52,53]. It is synthesized in the hepatocytes of the liver as an 84 amino acid precursor protein, which is subsequently cleaved into the 25 amino-acid disulphide-bridged active peptide form^[54,55]. Mice express two copies of the hepcidin gene, Hpc1 and Hpc2, resulting from a tandem duplication of the hepcidin gene^[54,56,57]. Transgenic mice studies have confirmed a role for hepcidin in the regulation of iron metabolism. Hepcidin knockout mice develop iron overload in the liver, pancreas and heart^[58], whereas mice overexpressing hepcidin display severe iron deficiency and anemia^[59]. Hepcidin synthesis in the liver is sensitive to body iron levels; increasing with iron overload and decreasing in the case of iron deficiency^[54,60,61]. Interestingly, hepcidin is also regulated by inflammatory signals, and the inflammatory cytokines, IL-1 and IL-6^[62-65]. However, the role of Kupffer cells in the regulation of hepcidin expression by inflammation is controversial^[66,67].

Hepcidin plays a central role in the regulation of iron metabolism by inhibiting intestinal iron transport and the release of iron from macrophages (Figure 1)^[60,61]. Hepcidin achieves this by binding to the iron exporter ferroportin and inducing its internalization and degradation^[68,69]. Studies with both hemochromatosis patients and transgenic mouse models have identified candidate proteins, which modulate hepcidin synthesis in the liver (Figure 2A)^[70-74]. Hemochromatosis patients, homozygous for the expression of TrfR2 mutations display increased transferrin saturation but reduced urinary hepcidin levels^[70]. Despite the iron overload phenotype, expression of hepcidin in the livers of TrfR2 knockout mice is similar to that of control littermates^[72,75]. Similarly, both genetic hemochromatosis patients with Hfe mutations and Hfe transgenic mice display significantly reduced hepcidin expression in the liver^[73,74]. Taken together, these studies demonstrate a role for both TrfR2 and Hfe in the regulation of hepcidin expression in the liver. However, the underlying mechanisms are unknown. Recently, the juvenile hemochromatosis gene hemojuvelin, has been shown to regulate hepcidin expression via the bone morphogenetic protein (BMP) signaling pathway in the liver (Figure 2A)^[76,77].

The promoter of the hepcidin gene harbors consensus binding sites for several transcription factors including C/EBP- α , HNF4- α , Stat3 and Smad4^[78-81]. CCAAT/enhancer-binding protein alpha (C/EBP- α) plays a role in the iron-mediated regulation of hepcidin gene transcription^[78].

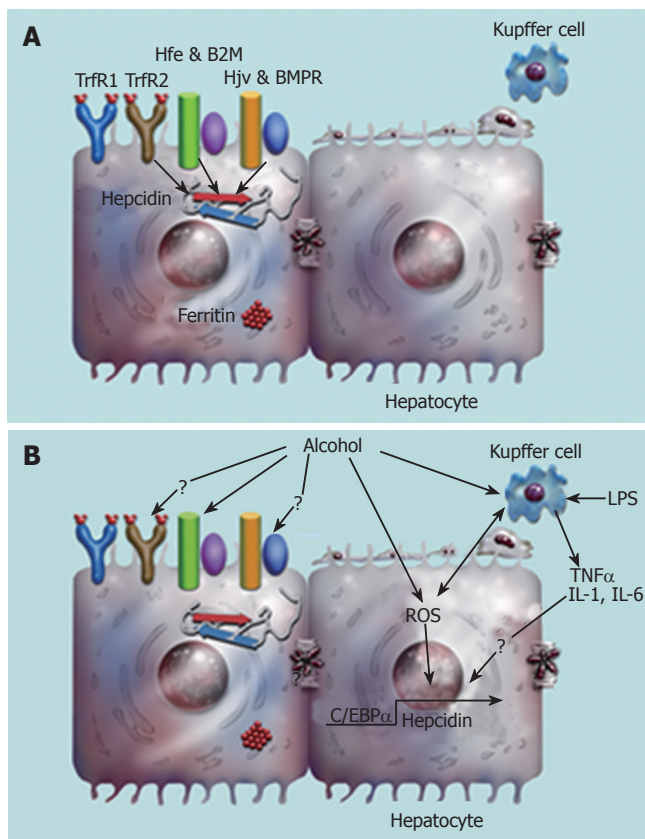


Figure 2 A: Modulators of hepcidin synthesis in the liver. Transferrin receptor 2 (TrfR2), Hfe and beta-2 microglobulin (B2M) complex; hemojuvelin (HJV) and bone morphogenetic protein receptor (BMPR) complex. B: Alcohol-mediated regulation of hepcidin transcription in the liver may involve both parenchymal and non-parenchymal cells of the liver.

ALCOHOL AND HEPCIDIN

Alcohol consumption increases the transfer of both iron and endotoxin from the intestine into the circulation^[36,82]. Hepcidin synthesis in the liver is regulated by iron and inflammation, with hepcidin playing a central role in iron homeostasis^[54,58,59,62,63,69,83]. Hence, a role for alcohol is implicated in the regulation of hepcidin expression in the liver. Indeed, alcohol was found to down-regulate hepcidin expression both *in vitro* with alcohol metabolizing hepatoma cells, and *in vivo* with mice subjected to short-term alcohol exposure^[84]. The effect of alcohol on hepcidin expression in hepatoma cells was abolished by 4-methylpyrazole, a specific inhibitor of the alcohol metabolizing enzymes^[84]. Furthermore, alcohol did not alter the expression of transferrin receptor-1 and the iron storage protein, ferritin, or the activation of iron regulatory RNA-binding proteins, IRP1 and IRP2^[84]. These findings demonstrate that alcohol does not regulate hepcidin expression by altering the iron status of the cell but rather acts on hepcidin directly. Short-term alcohol exposure down-regulated hepcidin 1, but not hepcidin 2 mRNA expression in mice^[84]. Similarly, iron has also been shown to up-regulate hepcidin 1 gene expression in mice^[57]. Furthermore, rats with chronic alcohol exposure also display reduced hepcidin expression^[85,86]. Collectively, these studies demonstrate a role for alcohol in the regulation of hepcidin expression in the liver.

Alcohol-mediated down-regulation of hepcidin expression in the liver leads to elevated expression of the iron transporter proteins, DMT1 and ferroportin in the duodenum^[84]. This effect is abolished by injecting mice with the hepcidin peptide confirming a role for hepcidin in the alcohol-mediated increase in duodenal iron transporter protein expression^[84]. The increase in intestinal iron transporter expression leads to increased intestinal iron absorption, and hence to increased body iron indices. A recent study has also reported increased serum iron in mice exposed to alcohol^[87]. We have observed an increased expression of the iron storage protein, ferritin in the livers of rats with chronic alcohol exposure^[86]. Taken together, these findings suggest that iron overload observed in patients with alcoholic liver disease is mediated by regulatory mechanisms, and that the alcohol-mediated down-regulation of hepcidin synthesis in the liver may be one of the underlying mechanisms of iron overload.

Hepcidin synthesis in the liver responds to body iron levels, and is up-regulated by iron overload *in vivo*^[54,61,83]. This raises the question of whether the alcohol-induced decrease in liver hepcidin expression would be sustained while the body iron levels progressively increase through continued alcohol consumption. It is feasible that the decrease in liver hepcidin expression may only be an initial response to alcohol, which may eventually be negated by elevated iron levels. On the other hand, it is also possible, that hepcidin expression in the liver is continuously suppressed by alcohol despite the iron overload state, which will eventually lead to liver injury. Thus, we investigated the combined effect of iron and alcohol in the regulation of hepcidin expression. Despite iron overload, alcohol down-regulated the expression of hepcidin in the liver^[86] demonstrating that alcohol renders liver hepcidin synthesis insensitive to body iron levels. A further decrease in hepcidin expression was also observed in Hfe knockout mice exposed to alcohol^[86]. It is worth noting that hepcidin protects the body from iron overload by inhibiting duodenal iron uptake and iron release from macrophages (Figure 1)^[60,61,69]. These findings suggest that the mechanisms which protect the body from the harmful effects of iron overload (e.g. increased hepcidin expression and decreased iron uptake and storage) are compromised by alcohol^[86].

Both iron and alcohol induce oxidative stress and oxidative stress plays an important role in alcoholic liver disease^[23-25,88-90]. Treatment with antioxidants abolished the effect of alcohol on hepcidin expression in the liver and on duodenal iron transporter expression in the duodenum^[84]. These findings strongly suggest a role for acute alcohol-induced oxidative stress in the regulation of hepcidin expression. Alcohol down-regulated both hepcidin promoter activity and the DNA-binding activity of the transcription factor, C/EBP alpha. This effect was abolished by treating mice with antioxidants^[84]. Furthermore, alcohol also inhibited the iron-mediated up-regulation of C/EBP activity in the liver^[86]. These findings demonstrate that redox changes and oxidative stress associated with alcohol metabolism regulate hepcidin gene transcription by altering C/EBP alpha activity. Oxidative stress is therefore one of the mechanisms by which

moderate alcohol consumption regulates liver hepcidin expression and hence iron homeostasis, without causing steatosis or apparent liver injury (Figure 2B)^[84].

Hepcidin is expressed in hepatocytes of the liver^[54]. However, we observed a more prominent down-regulation of hepcidin expression *in vivo* compared to *in vitro* in hepatoma cells suggesting a role for non-parenchymal cells of the liver in the regulation of hepcidin expression^[84]. Kupffer cells play an important role in the progression of alcoholic liver disease^[13,91]. Since alcohol down-regulates, and inflammation up-regulates hepcidin expression^[62,63,84,85], this raises the question of whether Kupffer cells and inflammation play a role in alcohol-mediated regulation of hepcidin expression. Recently, we have shown a role for oxidative stress in the regulation of hepcidin transcription by alcohol^[84]. Alcohol metabolism induces oxidative stress in the hepatocytes and Kupffer cells of the liver^[13,90,91]. Oxidative stress leads to the release of the pro-inflammatory cytokine, TNF- α from activated Kupffer cells^[12,13,92,93]. TNF- α has been reported to down-regulate hepcidin expression *in vitro*^[62]. Thus, alcohol-mediated regulation of hepcidin expression may involve both the hepatocytes and Kupffer cells of the liver (Figure 2B). The role of parenchymal and non-parenchymal cells of the liver, and the proinflammatory cytokines in the regulation of hepcidin expression by alcohol requires further investigation.

CONCLUSION

A role has been established for alcohol in the regulation of hepcidin expression in the liver. Alcohol-mediated oxidative stress inhibits C/EBP- α DNA-binding activity and down-regulates hepcidin transcription in the liver. Increased down-regulation of hepcidin expression by alcohol may play a role in the disease severity of genetic hemochromatosis patients in combination with alcohol intake. Down-regulation of hepcidin expression in the liver leads to increased intestinal iron transporter expression. Moreover, alcohol also abrogates the protective effect of hepcidin in iron overload by rendering the synthesis of hepcidin in the liver insensitive to body iron levels. Deregulation of hepcidin synthesis in the liver may be one of the underlying mechanisms by which alcohol consumption leads to iron overload. Ultimately, iron acts as a secondary risk factor in alcoholic liver disease. A better understanding of the molecular mechanisms underlying the regulation of iron homeostasis by alcohol may help us to develop therapeutic strategies or diagnostic tools to detect alcohol-induced liver injury at earlier stages before it develops into a chronic disease with irreversible liver damage.

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Implication of altered proteasome function in alcoholic liver injury

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Abstract

The proteasome is a major protein-degrading enzyme, which catalyzes degradation of oxidized and aged proteins, signal transduction factors and cleaves peptides for antigen presentation. Proteasome exists in the equilibrium of 26S and 20S particles. Proteasome function is altered by ethanol metabolism, depending on oxidative stress levels: low oxidative stress induces proteasome activity, while high oxidative stress reduces it. The proposed mechanisms for modulation of proteasome activity are related to oxidative modification of proteasomal proteins with primary and secondary products derived from ethanol oxidation. Decreased proteolysis by the proteasome results in the accumulation of insoluble protein aggregates, which cannot be degraded by proteasome and which further inhibit proteasome function. Mallory bodies, a common signature of alcoholic liver diseases, are formed by liver cells, when proteasome is unable to remove cytokeratins. Proteasome inhibition by ethanol also promotes the accumulation of pro-apoptotic factors in mitochondria of ethanol-metabolizing liver cells that are normally degraded by proteasome. In addition, decreased proteasome function also induces accumulation of the negative regulators of cytokine signaling (I- κ B and SOCS), thereby blocking cytokine signal transduction. Finally, ethanol-elicited blockade of interferon type 1 and 2 signaling and decreased proteasome function impairs generation of peptides for MHC class I-restricted antigen presentation.

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Key words: 20S proteasome; 26S proteasome; PA28;

CYP2E1; Apoptosis; Liver

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INTRODUCTION

The steady state level of proteins depends on their rates of synthesis and degradation. The proteasome is a major protein-degrading enzyme, which has a predominant role in intracellular protein catabolism. This enzyme influences various cell functions, as the enzyme not only hydrolyzes obsolete and oxidized proteins, thereby preventing cellular toxicity, but also regulates signaling events by destroying short-lived signal transduction factors and by controlling apoptotic cell death. Another important function of the proteasome is the generation of peptides for MHC class I-restricted antigen presentation. Recently, the proteasome has been shown as a potent regulator of the circadian cycle^[1] by its role in degradation of Cryptochrome and Period proteins. Besides proteolysis, the proteasome has non-proteolytic functions, regulating transcription, DNA repair and chromatin remodeling^[2].

PROTEASOME STRUCTURE AND FUNCTIONS

In mammalian cells, proteasome is localized mainly in the cytosol and the nucleus^[3]. It plays an important role in proteolytic centers within the cells, such as the centrosome, a perinuclear structure. Targeted proteins are transported to the centrosome for degradation, which is carried out by two proteasome structures, the 26S and 20S proteasomes. The 26S proteasome consists of core proteasome (20S proteasome) and two 19S (PA700) regulatory particles^[4]. The 20S proteasome is a large complex, which has four stacks containing 14 different gene products and is organized as outer alpha (α) subunits that "shape" 20S proteasome and beta (β) subunits that constitute the 20S catalytic core subunits (β 1, β 2 and β 5, or Y, Z and X). β subunits have various activities, based on the ability to cleave either hydrophobic bonds (chymotrypsin-like

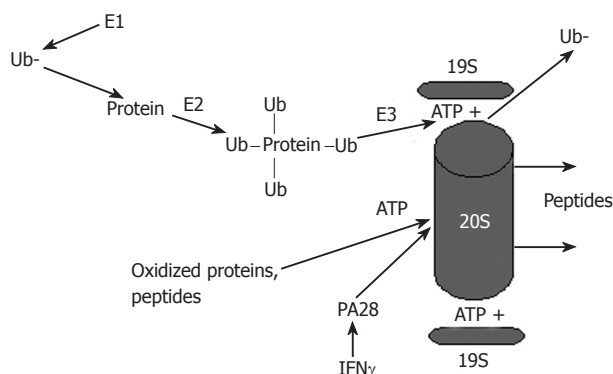


Figure 1 Proteasome-dependent degradation of proteins by the 26S and the 20S proteasome. Proteins undergo ubiquitylation and are degraded in an ATP-dependent manner by the 26S proteasome, which activity is regulated by the 19S particle. Alternatively, oxidized proteins can be degraded by the 20S proteasome. This degradation is ATP-independent and is regulated by the 20S proteasome activator, PA28.

activity), basic bonds (trypsin-like activity), or acidic bonds (caspase-like activity). The 20S proteasome exists in two forms: constitutive proteasome and immunoproteasome. The immunoproteasome subunits, LMP2, LMP7 and MECL-1, replace the constitutive subunits, usually under the influence of IFN γ ^[2]. The immunoproteasome is involved in generation of MHC class I-restricted peptides for antigen presentation. The efficiency of peptide cleavage by the immunoproteasome is higher than by the constitutive proteasome^[5]. Mice lacking the LMP7 subunit have reduced antigen presentation^[6]. Recently, interferon type 1 (IFN α) has been reported as an effective inducer of immunoproteasome in liver cells^[7]. Unpublished results from our laboratory confirmed this finding in hepatoma cell lines stimulated with IFN α .

ATP-DEPENDENT AND INDEPENDENT HYDROLYSIS OF PROTEINS BY PROTEASOME

Degradation of proteins by proteasome may or may not require ATP. The 26S proteasome catalyses protein degradation in an ATP-dependent manner, while the 20S proteasome does it ATP-independently. The most important question is how the proteasome “senses” the proteins, which are to be degraded. 26S proteasome usually recognizes proteins, which are marked with ubiquitin, a small 8.5-kDa protein that is covalently attached to protein substrate. To be attached, ubiquitin is activated by E1, the ubiquitin-activating enzyme (Figure 1). Activated ubiquitin is transferred *via* a high-energy thiol ester intermediate, E2-ubiquitin-conjugating enzyme, to the protein substrate destined for degradation. Protein covalently attaches multiple ubiquitin molecules that generate polyubiquitin chain binding to internal lysine (lysine 48) residue. If the substrate protein binds to monoubiquitin (i.e. one ubiquitin molecule per residue) instead of polyubiquitin (K48), it is targeted for degradation by the lysosome. Linkage through Lys 48, is associated with the ubiquitin-proteasome pathway, while Lys 63 linkage has a role in the

inflammatory response, endocytic pathway, and ribosomal protein synthesis^[8]. The ubiquitin-conjugated protein is subjected for degradation by the 26S proteasome with a help of E3 ligase. To be ubiquitylated and recognized by proteasome, the substrates usually undergo post-translational modification, such as phosphorylation and oxidation. For recognition by E3, in certain cases proteins may require association with molecular chaperones^[9].

The important functions of the 19S particle in 26S proteasome are to recognize ubiquitylated proteins and to open the aperture of the 20S proteasome α -rings to allow substrate entry into the catalytic core. The 19S particle unfolds ubiquitylated substrates, dissociates from ubiquitin with the help of de-ubiquitylating enzymes for further ubiquitin recycling and inserts the proteins into the 20S proteasome for degradation. This insertion needs ATP hydrolysis and is controlled by ATPases, which forms 19S sub-complexes known as the base and the lid.

Alternatively, degradation of non-ubiquitylated proteasome substrates can be carried out in an ATP- and ubiquitin-independent manner by the 20S proteasome, which requires the gate opening by the activators, PA28 α , β and γ (Figure 1). PA28 α and β are predominantly located in the cytosol, whereas PA28 γ has a nuclear localization. PA28 enhances hydrolysis of short peptides. In mice, disruption of PA28 α and β genes leads to defective generation of MHC class-restricted peptides^[10], while mice with disrupted PA28 γ gene have slower cell proliferation and enhanced susceptibility to apoptosis. Functions of the PA28 are antagonized by proline-rich proteins, PI31 and Pr39, which, *in vitro*, block proteasome activity^[11,12].

20S proteasome can be simultaneously activated by both 19S and PA28 *via* the two outer rings. This structure is called a hybrid proteasome, which displays the catalytic properties of both the 26S and 20S forms of the proteasome. These hybrid proteasomes generate a unique spectrum of antigenic peptides for presentation in an ubiquitin-dependent as well as ubiquitin-independent ways^[13].

ETHANOL METABOLISM AND LIVER PROTEASOME FUNCTION

The liver is the main site of ethanol metabolism. Hepatocytes express the major ethanol metabolizing enzymes, alcohol dehydrogenase (ADH) and cytochrome P450E1 (CYP2E1), which catalyze the generation of acetaldehyde and reactive oxygen/nitrogen species when liver cells are exposed to ethanol. The level of intracellular oxidative stress depends on the balance between oxidative and protective (antioxidant systems) factors. The level of oxidative stress differentially regulates proteasome function (i.e. low oxidative stress enhances proteasome activity, while high oxidative stress suppresses it).

There is a reciprocal relationship between CYP2E1 and the proteasome. Studies on HepG2 cells that overexpress CYP2E1 showed that ethanol and other CYP2E1 ligands increase the content and activity of CYP2E1 without affecting CYP2E1 mRNA^[14,15]. Pulse-chase experiments demonstrated that ethanol and other ligands stabilize the

enzyme against proteolysis. The proteasome is responsible for CYP2E1 degradation since treatment with the proteasome inhibitor, PSI, which blocks its chymotrypsin-like activity, caused a dose-dependent increase in the CYP2E1 apoprotein. Heat shock proteins 70 and 90 provide the communication between CYP2E1 and the proteasome^[16,17].

Stabilization of CYP2E1 by ethanol, in turn, results in increased production of ethanol metabolites, which block proteasome function. *In vitro* treatment of VL-17A cells (ADH/CYP2E1-positive HepG2 cells) with 100 mmol/L ethanol significantly reduces proteasome activity^[18,19] as well as the ability of crude proteasome preparations (cytosols) from VL-17A cells to hydrolyze HBV peptide cleavage^[20]. The effects of ethanol exposure are blocked by treatment with inhibitors of ethanol metabolism and CYP2E1 activity. *In vivo*, proteasome inhibition is common in rodents intragastrically fed with ethanol^[21,22]. The proteasomal chymotrypsin-like activity reduction in intragastrically fed rats was blunted in animals treated with a CYP2E1 inhibitor, chlormethazole^[23]. Furthermore, suppression of proteasome activity by ethanol was established in wild-type mice, but not in ethanol-fed CYP2E1 knockout mice^[24]. In orally fed mice, the effect on the proteasome depends on blood ethanol levels achieved after chronic ethanol feeding: usually when blood ethanol exceeds 40 mmol/L, there is a suppression of proteasome function. However, lower blood ethanol levels sometimes result in proteasome activation^[21].

The proposed mechanisms for modulation of proteasome activity are related to oxidative modification of proteasomal proteins with primary and secondary products derived from ethanol oxidation. Thus, CYP2E1 activation, reactive oxygen species formation and subsequent adduction of proteasome with protein carbonyls in E47 cells (CYP2E1⁺ HepG2 cells) caused a decline in the trypsin-like, proteasome activity^[25]. Furthermore, *in vivo* ethanol studies on intragastrically fed rats demonstrated significant induction of CYP2E1 and an increase in 4-hydroxynonenal (4-NHE) concentrations after one month of ethanol feeding, which resulted in decreased proteasome activities^[26]. While a 3.5-fold CYP2E1 induction occurred after 15 d of feeding, there was no loss in proteasome activity, indicating that modulation of proteasome activity is a delayed response to CYP2E1-induced proteasome oxidation. Proteasome suppression was attributed to 4-HNE adducts of the 19S (PA700) proteasome regulator specifically an ATPase Rpt4 subunit. It was postulated that this adduction caused disrupted association between the 19S and 20S proteasome particles. In addition, our laboratory discovered the regulating effects of nitration on proteasome function. Nitration is caused by peroxynitrite, a reaction product of superoxide (generated by CYP2E1) and nitric oxide (generated by nitric oxide synthase), which is released as a result of ethanol metabolism and is involved in alcohol-induced liver injury^[27]. Massive nitration of 20S proteasome with subsequent 3-NT adduct formation caused a reduction of chymotrypsin-like proteasome activity, primarily due to reduced interaction between

PA28 and the 20S proteasome. Interestingly, low levels of nitration activated proteasome function and enhanced proteasome activation by PA28^[28]. Similar effects of the peroxynitrite donor, SIN-1, were observed using highly purified proteasome, cultured hepatoma cells and rodent livers. Immunoproteasome has been shown to be more sensitive to the effects of peroxynitrite than the constitutive form of the enzyme^[29]. Studies on the 26S proteasome (4-HNE adducts) and on the 20S proteasome (3-NT adducts) revealed that in oxidatively modified proteasome, the ability of 20S proteasome to respond to activating stimuli of its regulators, 19S proteasome and PA28 is limited, which impedes proteasome function. The 26S proteasome is more susceptible to oxidative stress than 20S proteasome^[30].

Another potent oxidant generated by ethanol metabolism, acetaldehyde, which is produced by ADH and CYP2E1 has also been shown to affect proteasome function. *In vitro* adduction of acetaldehyde to cytosolic proteins inhibits proteasome function in a dose-dependent manner^[31]. We also observed suppression of proteasome activity in hepatoma cells treated with 400 mmol/L acetaldehyde (Osna, unpublished observation). Thus, not only CYP2E1, but also ADH by generating ethanol metabolites can contribute to proteasome inhibition.

OXIDATIVE MODIFICATION OF PROTEINS: AGGREGATION AND PROTEASOME FUNCTION

The inability for ubiquitylated proteins to be degraded by proteasome is based not only on impaired proteasome activity but also on the relative susceptibility of substrates to degradation. Proteasomal substrates (proteins) can be modified by oxidation of amino acid residues by intracellular oxidants, such as H₂O₂ or by covalent binding to reactive species, such as peroxynitrite. Depending on their degree of modification, these substrates become either more or less susceptible to proteasome-catalyzed hydrolysis. Thus, when aconitase was treated with peroxynitrite and hydrogen peroxide, low concentrations of oxidants (up to 2 mmol/L for hydrogen peroxide and up to 1 mmol/L for peroxynitrite) enhanced susceptibility of aconitase to degradation, while higher concentrations of oxidants decreased proteolysis^[32].

Ubiquitin is essential for ATP-dependent protein degradation by the proteasome. Under certain pathological conditions or due to genetic predisposition, defective ubiquitin + 1 (Ub + 1) is generated by a dinucleotide deletion in Ub mRNA. Proteins ubiquitylated with Ub + 1 are the substrates for proteasomal degradation, but at high concentrations of Ub + 1, the degradation is inhibited^[33]. Ub + 1 forms tight noncovalent interactions with target proteins and cannot be removed from the substrate (Luders *et al.*^[34], 2003). The accumulation of Ub + 1-labeled undegraded proteins is observed in neurological disorders (e.g. Huntington's Disease, Alzheimer's Disease and Down's Syndrome).

Decreased proteolysis by the proteasome also results in the accumulation of insoluble protein aggregates, which

cannot be degraded by proteasome and which further inhibit proteasome function. In patients with alcoholic cirrhosis, the aggregates are known as Mallory bodies or so-called alcoholic hyaline^[35,36] and are a common signature of alcoholic liver diseases. Mallory bodies are formed by liver cells when proteasomes are unable to remove cytokeratins, mainly cytokeratins 7 and 19^[37]. Experimental studies revealed that the blockade of proteasome with specific inhibitors or by ethanol metabolism induces overexpression of heat shock proteins and Mallory body formation, which was observed both *in vivo* and *in vitro*^[38,39]. Ub + 1 has also been detected in Mallory bodies, which is a common histological signature of alcoholic liver disease. *In vitro* delivery of Ub + 1 to mouse hepatocytes caused formation of aggresomes and Mallory body formation^[38].

THE UBIQUITIN-PROTEASOME SYSTEM AND ETHANOL-INDUCED CELL DEATH

Proteasome function controls cell death and survival under the normal and pathological conditions. Thus, suppression of proteasome function with specific proteasome inhibitors results in increased cell death. The link between the proteasome function and cell survival is supported by the fact of degradation of certain cell cycle controlling and pro- or anti-apoptotic factors by the 26S proteasome. Because apoptotic events are downstream from proteasome function and ethanol suppresses proteasome activity, it may be possible that proteasome inhibition would promote the accumulation of pro-apoptotic factors in mitochondria of ethanol-metabolizing liver cells. Ultimately, it might stimulate liver cell apoptosis.

Inhibition of the 26S proteasome causes hepatotoxicity *in vitro* and *in vivo*. In Hep G2 cells transfected with CYP2E1, the amount of cell death by apoptotic mechanism depended on actual CYP2E1 activity^[40]. We also observed elevation of caspase 3 activity in VL-17A cells exposed to 100 mmol/L ethanol^[19]. In addition, a proteasome inhibitor, MG132, injected to mice 4 h prior to TNF α injection, sensitized liver cells to TNF α -induced apoptosis^[41]. In ethanol non-metabolizing cells, there is a negative feedback between chemotrypsin-like activity of proteasome and caspase-3 activation, when cells were treated with staurosporin. Furthermore, thymocytes, which normally are rich with proteasome, have declined proteasome activity when they underwent apoptosis after dexamethasone treatment^[42].

Ethanol promotes cell death by inducing both apoptotic and necrotic events. Mitochondria, the primary site of energy (ATP) generation, are critical for induction of cell death by ethanol. Ethanol metabolism causes mitochondrial dysfunction, including impairment of fatty acid β -oxidation, inhibition of mitochondrial respiration and damage of mitochondrial DNA. The opening of mitochondrial pores (mitochondria permeability transition, MPT), normally blocked by anti-apoptotic factor, Bcl-2, results in release of cytochrome C that initiates the further death events. The scenario of the death events depends on ATP levels. In the case of ATP depletion, cells die *via* necrosis. However, if ATP levels are not changed,

cytochrome C release causes activation of the mediators of apoptosis, caspases, and subsequent DNA fragmentation. Certain pro-apoptotic factors (Bid, Bax) closely associated with the mitochondrial membrane are degraded by the proteasome^[43]. Thus, ethanol plays a dual role in regulating cell death: on one hand, it has a tendency to deplete ATP and facilitates necrosis; on the other hand, due to the proteasome suppression, it promotes the accumulation of pro-apoptotic factors in mitochondria of ethanol metabolizing liver cells and initiates apoptosis.

UBIQUITIN-PROTEASOME INVOLVEMENT IN ALCOHOL-INDUCED TISSUE INFLAMMATION

The outcome of acute inflammation depends on the balance of pro- and anti-inflammatory events initiated by mediators of inflammation, which can be “turned on or off” *via* certain signal transduction pathways. To be effective and non-toxic, the signals must be short enough to allow switching to the next stage of the process (such as the transition from inflammation to regeneration). These changes in gene activation repertoire occur with the help of transduction factors, which, after their job is done, are ubiquitinated and degraded by the proteasome. Alcohol consumption seems to interfere with the transduction of pro-inflammatory signals.

Many important pro-inflammatory and proliferative signals are regulated *via* the NF- κ B pathway. Activation of this pathway occurs by cytokines (such as tumor necrosis factor α , TNF α), phosphorylation of cytoplasmic inhibitory protein I κ B and subsequent translocation of NF- κ B to nucleus to initiate gene transcription. I κ B can be “marked” by ubiquitin upon phosphorylation and then is degraded by proteasome; alternatively, non-phosphorylated I κ B is modified by small ubiquitin-like modifier (SUMO) and is not targeted for degradation^[44]. In ethanol non-metabolizing cells, ethanol enhances NF- κ B activation; however, in ethanol metabolizing cells, I κ B dissociation is blocked and NF- κ B activation does not happen. Acetaldehyde, a product of ethanol metabolism, is responsible for I κ B stabilization and non-degradation by the proteasome^[45]. Acetaldehyde combines with the product of lipid peroxidation, malondialdehyde, and form the adducts with numerous proteins (malondialdehyde-acetaldehyde adducts, MAA). The MAA adducts reduce the susceptibility of lysozyme to proteasome degradation. MAA-modified proteins can serve as the targets of autoimmune reactions, enhancing liver injury^[46].

In liver, ethanol treatment modulates activity of various signal transduction pathways and induces certain downstream events. Thus, feeding of animals for 16 wk with ethanol diet causes up-regulation of chemokines and neutrophil infiltration in the liver. It also induces elevation of IL-8 levels, which results in suppression of the 26S proteasome, due to activated JNK/AP1 pathways^[41].

The Jak-STAT pathway, which plays a critical role in inflammation, proliferation, repair, and antiviral defense and usually supports proteasome activation, is also

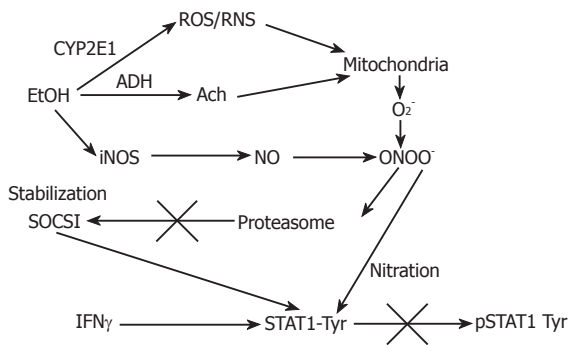


Figure 2 The proposed effects of EtOH metabolism on IFN γ -induced STAT1 phosphorylation in VL-17A cells. EtOH is metabolized by ADH and CYP2E1 to Ach and ROS/RNS and increase a formation of PN (peroxynitrite, ONOO \cdot), a reaction product of O $_2^{\cdot-}$ and NO, which at high concentrations blocks proteasome function. This causes stabilization of SOCS1, a negative regulator of Jak-STAT1 signaling as well as prevents STAT1 phosphorylation on tyrosine residues.

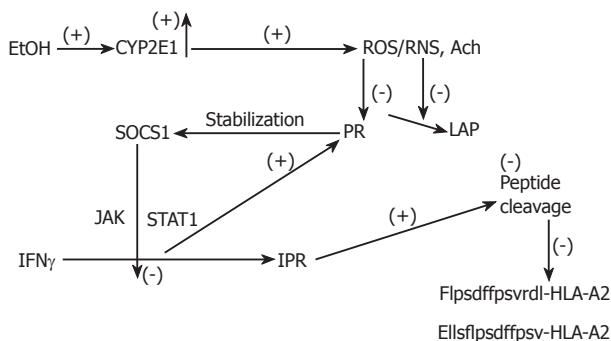


Figure 3 The proposed mechanism of regulation of peptide cleavage by ethanol metabolism. Ethanol (EtOH) treatment induces CYP2E1 activity, which catalyzes production of oxidants. These products block proteasome (Pr) and leucine aminopeptidase (LAP) activities, stabilizing SOCS1 and preventing IFN γ -dependent activation of PA28 and immunoproteasome (I Pr). Finally, generation of C- and N-extended peptides for MHC class I-restricted antigen presentation is blocked.

regulated by ethanol metabolism. Thus, acute ethanol exposure inhibited STAT3 activation by leptin in human Huh7 hepatoma cells^[47]. Inhibition of MAP kinase, protein kinase A and protein kinase C partially enhance IFN γ -induced STAT1 phosphorylation reduced by ethanol^[48]. We also reported inhibition of STAT1 phosphorylation in VL-17A hepatoma cells^[49], which was attributed to ethanol-mediated induction of SOCS1, due to the limited degradation of SOCS1 by suppressed proteasome (Figure 2). Peroxynitrite formation with subsequent nitration of tyrosine residues seems to play a critical role in this process. Similarly, inhibition of proteasome has also been shown to inhibit IL-6 signaling *via* stabilization of SOCS3^[50], which is supposed to be proteasome dependent as well.

Except liver tissue, some other types of tissues (brain, heart) induce the inflammation in response to ethanol and its metabolism. In these tissues, ethanol metabolism occurs predominantly *via* CYP2E1 and catalase, a peroxisomal enzyme, which catalyze ethanol oxidation by using H $_2$ O $_2$ as a hydrogen acceptor. Hepatocytes transfected with catalase, are also protected from lipid peroxidation after glutathione

depletion^[51]. In all ethanol-affected tissues, ethanol can also undergo non-oxidative metabolism and form fatty acid ethyl esters, which also induce mitochondrial dysfunction, inhibit protein synthesis and toxically affect cultured cells^[52].

ETHANOL-ELICITED IMPAIRMENT OF PROTEASOME FUNCTION AND MHC CLASS I -RESTRICTED ANTIGEN PRESENTATION

Processing of peptides for MHC class I-restricted antigen presentation is necessary for elimination of liver cells that express viral and other non-self proteins. At the most downstream level, these non-self proteins are cleaved by the proteasome in the cytoplasm^[10,53]. Further N-terminal cleavage is carried by aminopeptidases^[54]. These cleavages are regulated by IFN γ *via* the Jak-STAT1 pathway. Generated 8-10 amino acid peptides are presented on the cell surface, for recognition by cytotoxic T-lymphocytes (CTLs) and elimination. Escaping surveillance of infected cells by CTLs ultimately causes chronic persistence of viral infection in the liver, as is often observed in case of viral hepatitis B and C. Recently, we have shown that ethanol treatment of VL-17A cells suppresses proteasome and leucine aminopeptidase functions, which results in a partial blockade of HBV peptide hydrolysis and suppression of IFN γ -induced expression of PA28 and immunoproteasome^[20] (Figure 3). Ethanol-elicited impairment in IFN γ signaling is partially responsible for the latter event. This situation might be observed in chronically HBV-infected alcohol consumers, who are unable to clear viral infection in the liver.

CONCLUSION

Ethanol exposure creates oxidative stress in liver cells and that bi-phasically affects proteasome activity: low oxidative stress up-regulates proteasome function, while high oxidative stress suppresses it. Both acetaldehyde and CYP2E1-induced ROS and RNS contribute to this inhibition. Suppression of proteasome function results in enhanced cell death, mainly due to increased apoptosis. It also induces inflammation, by modulating various signal transduction pathways. Low proteasome activity interferes with efficient MHC class I-restricted presentation of antigenic peptides, which lowers antiviral defense.

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TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, Series Editor

Immunological response in alcoholic liver disease

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Abstract

The development of alcoholic liver disease (ALD) can be attributed to many factors that cause damage to the liver and alter its functions. Data collected over the last 30 years strongly suggests that an immune component may be involved in the onset of this disease. This is best evidenced by the detection of circulating autoantibodies, infiltration of immune cells in the liver, and the detection of hepatic aldehyde modified proteins in patients with ALD. Experimentally, there are numerous immune responses that occur when proteins are modified with the metabolites of ethanol. These products are formed in response to the high oxidative state of the liver during ethanol metabolism, causing the release of many inflammatory processes and potential of necrosis or apoptosis of liver cells. Should cellular proteins become modified with these reactive alcohol metabolites and be recognized by the immune system, then immune responses may be initiated. Therefore, it was the purpose of this article to shed some insight into how the immune system is involved in the development and/or progression of ALD.

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Key words: Alcoholic liver disease; Liver endothelial cells; Aldehyde adducts; Oxidative stress; Immune system; Cytokines; Metabolism

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INTRODUCTION

It has become commonly accepted that immune mechanisms are partially responsible for the onset and/or progression of alcoholic liver disease (ALD). This is demonstrated by alcohol abuse increasing the immune deficiency in numerous diseases including: pneumonia, tuberculosis, human immunodeficiency virus (HIV), hepatitis C virus (HCV), hepatitis B virus (HBV), and many other less common infections^[1]. This observation is often overlooked because of the many other complications of alcohol abuse such as: malnutrition, ingestion of high saturated fats, vitamin deficiency, drug abuse, and smoking^[2]. The immune system has been suggested as playing a role in ALD because of the many clinical manifestations that have been observed and are thought to contribute to the damage of liver tissue^[3,4]. However, the combination of immune function, metabolism, genetics, nutrition, and environmental factors most likely together play a role in the development of ALD. This review will begin to digest some of the potential hypothesis as to how the immune system, in combination with other factors contribute to the onset or progression of alcoholic liver disease.

LIVER TOXICITY DUE TO ETHANOL AND ITS METABOLITES

General byproducts

After ethanol is ingested, it is absorbed in the gut and transported to the liver by the bloodstream where it is metabolized by either the alcohol dehydrogenase or cytochrome-P450 pathways. The first and most important pathway is alcohol dehydrogenase, which is isolated in the mitochondria and oxidizes ethanol to acetaldehyde. Acetaldehyde is further oxidized to acetate by acetaldehyde dehydrogenase. These reactions help form NADH and NAD, which alter the redox state of the cell, causing harmful effects on lipid and carbohydrate metabolism when the normal ratio is altered^[5]. Elevated levels of NADH inhibits fatty acid metabolism leading to the possible formation of acute fatty liver^[6] or they may play a role in the formation of scar tissue in the liver (i.e. fibrosis)^[7]. In the second method of ethanol metabolism, CYP2E1 (an isoenzyme of the cytochrome-P450 system) is involved. However, it is very limited at low doses, but can be induced by continuous exposure to higher levels of ethanol when the need arises^[8]. As a general rule,

alcoholics tend to have higher levels of this enzyme than non alcoholics^[9].

Studies have found that CYP2E1 can metabolize certain fatty acids to exacerbate the CYP2E1 pathway causing a build-up of fatty infiltrate into the liver^[10]. During CYP2E1 breakdown of alcohol, there is a release of highly reactive oxygen species (ROS). These ROS molecules damage the liver by altering the degradation of fat molecules, resulting in oxidative stress and free radical build up^[11]. Therefore, both ethanol metabolism pathways contribute to the build-up of fats and oxidative stress in the liver, providing a source for highly reactive molecules capable of modifying or altering proteins in the liver.

Metabolite adducts

In the livers of chronic alcoholics, a number of metabolites are produced that could potentially become reactive to form stable protein adducts. Previous findings have demonstrated that acetaldehyde (AA) protein adducts form during the metabolism of ethanol to acetaldehyde^[12-15]. These acetaldehyde adducts are immunogenic and have been found in the serum of both alcohol fed rats^[16] and alcoholic patients^[17]. Researchers have found that these AA-adducts interact with specific amino acids, particularly lysine, during their formation^[18,19], as lysine residues on proteins tend to be highly reactive when protonated, and the level of modification depends on the concentration of acetaldehyde.

One AA-adduct that was investigated for a number of years is the N-ethyl lysine adduct that can be formed when AA is incubated under reducing conditions. However, the development of monoclonal and affinity purified polyclonal antibodies to this adduct have shown that it is not present in the tissue of rats chronically consuming ethanol or human alcoholics. This now makes sense, since the levels of reduction necessary to form this product do not occur in the liver during alcohol metabolism (which is an oxidative mechanism)^[20,21].

As mentioned above, the metabolism of ethanol occurs through two pathways, both of which are oxidative and induce oxidative stress. Lipid peroxidation metabolizes fats to form malondialdehyde (MDA) and 4-hydroxy-2-nonenal (HNE), which can react with proteins to generate still other adducts^[19,22]. Both of these adducts have been suggested to be detected in patients with alcoholic liver disease, with their concentrations possibly correlating with the severity of the disease^[23,24].

The increased concentration of acetaldehyde levels and the oxidation of the fats in the liver due to chronic ethanol intake, increase the oxidative state of the liver, providing a key component in the formation of another adduct that "molds" these two observations. Recent studies have shown that both malondialdehyde (from lipid peroxidation) and acetaldehyde (from ethanol metabolism) react with lysines on proteins in a synergistic manner to form hybrid adducts different from either of these two aldehydes alone^[25]. This adduct has been called Malondialdehyde-Acetaldehyde adduct (MAA) and has been shown to form two different components on the lysine residue. The first is a relatively stable compound formed on lysine with two molecules of MDA and 1 molecule of AA. This 2:1 adduct

is extremely stable and fluorescent due to its characteristic ring structure. The second compound is formed on lysine with one molecule of MDA and one molecule of AA. This 1:1 adduct is very unstable and most likely serves at the precursor to the 2:1 adduct^[26]. The MAA-adduct has been found in both rats^[27] and human subjects^[28] chronically consuming alcohol and in human studies, it has been shown that increased levels of adducts in the serum correlated with severity of liver disease^[28].

Immune response to MAA-adducts

The MAA-adduct has been the focus of numerous studies to determine its role in the onset of alcoholic liver disease. One thing that is clear is that these MAA-protein adducts, when injected into animals (in the absence of adjuvant), produce strong antibodies to the adduct, carrier protein, and the protein carrier conjugate^[21]. Studies performed in our laboratory have demonstrated that MAA-adducts initiate the immune system to respond. These responses are achieved by binding to scavenger receptors, up-regulating adhesion molecules, inducing pro-inflammatory cytokines, producing antibody and T-cell responses, and increasing the pro-fibrotic response.

Clearance of MAA-adducts

Recent evidence has shown that MAA-adducts bind to cells expressing scavenger receptors on their surface^[29,30]. Chronic ethanol feeding studies with rats has shown that ethanol impairs the receptor(s) on liver endothelial cells impairing their ability to clear acetaldehyde and MAA adducts^[31-33]. The inability to clear these molecules out of the circulation leaves them available to other cells of the immune system. These immune cells bind modified adducts and induce inflammatory responses.

MAA-adducts upregulate adhesion molecules

In alcoholic liver disease, there is an increase in the infiltration of immune cells, which accounts for the necrosis of hepatocytes^[34]. The recruitment of these cells occurs by the increase in adhesion molecule expression on liver sinusoidal endothelial cell (SECs) surface^[35]. This action occurs when expression of selectins on SECs mediate loose attachment of leukocytes to the vessel walls. Chemoattractants cause the activation of integrins on leukocytes and these integrins bind to intercellular adhesion molecules (ICAMs) on the surfaces wall of the SECs. The leukocytes then respond to the amount of chemoattractant and diapedese through the SECs cell junction^[36]. It has been shown that metabolites of alcohol can increase the expression of these adhesion molecules on the surface of SECs^[37-41].

The ability of these adducts to increase chemoattractants (TNF-Alpha, MCP-1, and MIP-2) and adhesion molecules (ICAM, p-selectin, and L-selectin) provide evidence for the recruitment of immune cells into the liver. Damage to the liver results from the movement of these cells into the liver parenchyma subsequently, causing an increase in cell death.

MAA-adducts induce pro-inflammatory cytokines

Aldehyde adducts have also been shown to induce the release of pro-inflammatory cytokines and chemokines in

kupffer, endothelial, and stellate cells^[42-45]. These adducts most likely bind to scavenger receptors located on these cells and signal the release of cytokines. Since these adducts are not cleared efficiently, they are able to be re-circulated and induce pro-inflammatory responses. Also, T-cells and antibody can be elicited to exacerbate this response.

It has been determined that levels of the cytokines TNF-alpha, IL-1 and IL-6 are elevated in alcohol liver disease patients^[46-48]. One possible mechanism for the increased levels of TNF-alpha is that increased gut permeability due the ethanol (leaky gut), causes the translocation of lipopolysaccharide (LPS) from the lumen into the blood stream^[49]. LPS generated from bacterial cell walls has been shown to stimulate macrophages to release TNF-alpha and other cytokines^[50-53]. Data generated from our laboratory has shown that LPS (in extremely small amounts) in conjunction with MAA-modified proteins actually increases the release of TNF-alpha, MCP-1, and MIP-1 by rat liver kupffer and endothelial cells^[42]. One could postulate that both the metabolites of ethanol (adduct formation on proteins) and the increased levels of LPS in the blood stream (gut derived) provide the necessary components for endothelial and kupffer cells to begin releasing pro-inflammatory cytokines. Stellate cells have also been shown to increase the release of these pro-inflammatory cytokines in response to aldehyde adducts^[45,54,55].

Increase in antibody and T-cell responses to MAA-adducts

Immune responses to MAA-adducts have been shown to be induced in the absence of adjuvants^[19,21]. This response may represent an important mechanism by which T and B-cells respond to soluble proteins. It has been shown that with as little as 25 micrograms of MAA-modified protein, a tremendous antibody response is demonstrated; both to the MAA-adduct and the protein carrier conjugate^[21].

Involvements of T-cells in these responses are shown by the strong expansion of T-cells to the MAA-adduct in *in vitro* studies^[56]. These responses were determined to be to the adduct and protein carrier conjugate. It was also determined that scavenger receptors were involved in the uptake of the MAA-adduct and presentation to T-cells^[30]. Recent studies in our laboratory have shown that MAA-modified self proteins can become immunogenic, potentially modifying liver proteins, and increasing the risk of specific organ damage.

Pro-fibrotic response to MAA-adducts

Hepatic fibrosis is the start of the wound-healing process resulting from the injury to the liver caused by years of alcohol consumption. This process can be reversed should ethanol consumption be eliminated. However, if ethanol consumption continues, fibrosis will occur, followed by scarring and finally the development of cirrhosis^[57,58]. Fibrosis is the build up of excessive depositions of extracellular matrix (ECM) proteins, which consists of collagen and fibronectin^[59]. These ECM proteins may cause the characteristic scar tissue formed in the liver after an injury has occurred. Byproducts of ethanol

metabolism have been shown to increase the release of these products. Hepatic stellate cells up-regulate collagen genes in response to stimulation with acetaldehyde^[60,61]. Also of interest is the finding that SECs secrete the isoform (EIII A) of fibronectin in response to MAA-adducts, which are capable of causing stellate cells to release collagen^[62]. Activation of the stellate cells is the key component in the fibrogenic process, providing the main production of fibrillar collagen. The build up of modified protein adducts causes disability of effective clearance, thereby increasing the activation of SECs and stellate cells, resulting in a fibrogenic response. The only way to intervene in the continual response from fibrosis to cirrhosis is to remove the ethanol from the system. Some anti-fibrotic drugs have been experimentally tested, yet there are still problems concerning delivery and concentration^[63-65].

IMMUNE FUNCTION AND ALCOHOL

Innate immune response

Ethanol affects many functions of the innate immune system. The cell types involved in this early response include: macrophage, neutrophils, and natural killer cells. One of the most active cells, the macrophage, is designed to respond to bacterial cell wall antigens by releasing cytokines, and engulfing foreign agents. In alcoholic liver disease, kupffer cells in the liver are activated by lipopolysaccharide (LPS) caused from a breakdown in the intestinal wall permeability. This phenomenon has been called "leaky gut" and occurs when alcohol increases gut permeability, causing bacteria from the intestinal tract to escape into the blood stream^[66,67]. When LPS is present, it activates kupffer cells to release TNF-alpha and superoxides that result in an inflammatory response. Recent studies in our laboratory have shown that adducts and very small amounts of LPS can stimulate kupffer cells and SECs to release these pro-inflammatory cytokines^[42]. Once these cytokines are initiated, inflammation and necrosis occurs to hepatocytes and other cell types of the liver.

Neutrophils are the cell type that is predominantly recruited to the liver in response to the increase in cytokine concentrations. When these cells infiltrate into the site of infection, they phagocytose antigens and release proteolytic enzymes capable of destroying cell walls. They also release more chemoattractants to expedite the inflammatory process^[68]. In alcoholic liver disease, these cells play a role in the propagation of the disease by infiltrating and cleaning up dead or dying cells. In fact, it has been shown that IL-8, a known neutrophil chemoattractant, is elevated in patients with alcoholic liver damage^[69,70]. These cell types can be seen microscopically infiltrating the liver in patients with alcoholic hepatitis^[1].

Adaptive immune system

The adaptive immune system consists of cells types designed to induce memory to specific antigens. It is characterized by the presentation of antigen to T-cells and clonal expansion of these cells to increase specificity to proteins, further increasing disposal of foreign agents.

More importantly, there is a tolerance to self antigens, protecting the organism from self elimination^[71]. When a breakdown in the adaptive immune response occurs, autoimmune diseases are sometimes initiated.

T-cells and NK T-cells have been implicated in the development of alcoholic liver disease by the increased numbers found in human livers following ethanol injury^[72]. Studies have demonstrated that co-culturing lymphocytes (from alcoholic cirrhotic patients) with ethanol increased the expression of adhesion molecules and TNF-alpha as compared to control patients^[38]. In this same study, the control patient lymphocytes when cultured with ethanol had a suppressive effect on the release of these molecules. The T-cells in the liver help drive the inflammatory process by releasing more cytokines including: TNF-alpha and INF-gamma. Another study implicating lymphocytes in ALD was done by transferring T cells from ethanol-fed rats to control recipient rats. In this experiment, damage to the naive livers was shown using reactive T-cells from alcohol-fed animals^[73]. Cytotoxic T cells can be generated in response to acetaldehyde-modified spleen cells^[74], giving aldehyde-modified proteins a role in the activation of these cells. Additionally, we have shown that aldehyde-modified proteins at high levels (non-physiological) can cause cell death and apoptosis to these cells^[75]. Therefore, the build up of these adducts in the liver could potentially increase the level of cell death/apoptosis, increase cytokine production in all immune cell types, and cause increased inflammation in the liver leading to cirrhosis.

Alcoholic liver disease has often been associated with circulating antibodies and lymphocytes specific to hepatic antigens^[76,77]. Circulating antibodies specific to acetaldehyde adducts and hydroxyethyl-free radicals have also been found to correlate with ALD^[78,79]. Malondialdehyde-acetaldehyde (MAA-adducts) have been found to be significantly increased in patients with ALD, which correlated with the severity of liver damage^[28]. Work done in animals has shown that MAA-adducted proteins are immunogenic without the use of adjuvants^[21]. Antibodies directed against these proteins were specific for both the MAA-adduct and the carrier protein. When hen egg lysozyme (HEL) was used as the carrier antigen, an antibody response to the carrier protein and not the adduct was observed^[56]. This provides a possible mechanism of autoantibody production wherein metabolites of ethanol modify hepatic self antigens and induce an autoimmune response against the liver.

In order to induce an autoimmune response, the right set of circumstance must be present to override the fail-safe mechanism(s) put in place by immune cells of the organism. In the adaptive immune response, this means that tolerance must be broken. How is tolerance broken in alcoholic liver disease? When metabolites of alcohol are present in the liver (as described above), the immune response is tremendously increased in the liver. Toxicity due to the chemical breakdown of these metabolites and the inflammation of immune cells increase the number of hepatocyte damage. Spilling of cellular material into the liver is cleaned up by macrophage and neutrophils. Self proteins from hepatocytes could become modified with acetaldehyde, malondialdehyde, or both. These self

proteins could bind to and be taken up by macrophage, endothelial cells, or dendritic cells and presented to T-cells. If this occurs, reactive cytotoxic T-cells or the production of antibody could aid in damaging the liver. Experimental evidence to support this hypothesis has been demonstrated in animal models^[80,81].

Previous studies with maleylated mouse serum albumin (MSA) have shown the breaking of tolerance by inducing an antibody response to the carrier protein (MSA)^[82]. These proteins were found to bind to scavenger receptors on the surface of macrophage and presented to T-cells from the spleen. In fact, many different aldehyde-modified proteins have been shown to bind to scavenger receptors^[29,83-85]. Studies performed in our laboratory have demonstrated similar findings when MSA was modified with acetaldehyde and malondialdehyde^[21]. These MAA-modified self proteins appear to bind scavenger receptors, are processed and presented in the cell, and increase the proliferative response in T-cells^[30]. Further proof for these observations is the increase in the co-stimulatory molecules B7-1 and B7-2 by splenocytes stimulated with MAA-modified proteins^[86]. These co-stimulatory molecules are the key component in autoimmune regulation and the breaking of tolerance^[87]. When the T-cell binds an antigen presenting cell, the T-cell receptor binds to the matching peptide presented in the groove of the MHC molecule. If co-stimulatory molecules are ligated, then the cell is signaled to respond and the T-cells begin to proliferate. Specificity is to the peptide in the groove, and if this is a self protein, the immune system sees these proteins (liver specific) as a foreign invader and eliminates the threat. This threat is eliminated with cytotoxic T-cells destroying the cell with their powerful enzymes^[71]. If these proteins happen to be presented to multiple B-cells and crosslinking of receptors occurs, antibody production specific for the self-protein is initiated with the help of T-helper cells. Antibodies would then bind to the self proteins and be taken up by Fc receptors located on NK cells and attacking the liver cells^[71]. See Figure 1 for a description of this hypothesis.

INTERACTION OF DIET, ETHANOL, AND IMMUNE FUNCTION

Diet and ethanol intake

Alcoholics have always been considered to be malnourished due to the exchange of food calories for alcohol, leaving a reduction in valuable nutrients. One explanation for this is that increased alcohol intake interferes with absorption of these nutrients from the intestine, making them unavailable to the body^[8]. Thus, a decrease in body fat is the case for a number of individual alcoholics. However, metabolic syndrome in individuals has become a risk factor with the increase in high fat diets^[88]. Metabolic syndrome is a chronic inflammatory condition that promotes insulin resistance and fat accumulation in the liver^[89]. This disease, known as fatty liver, is quickly becoming an epidemic in our society as a result of increased dietary fat intake^[90]. Taken into account the chronic inflammatory condition in alcoholic liver disease alone, one could assume that the two

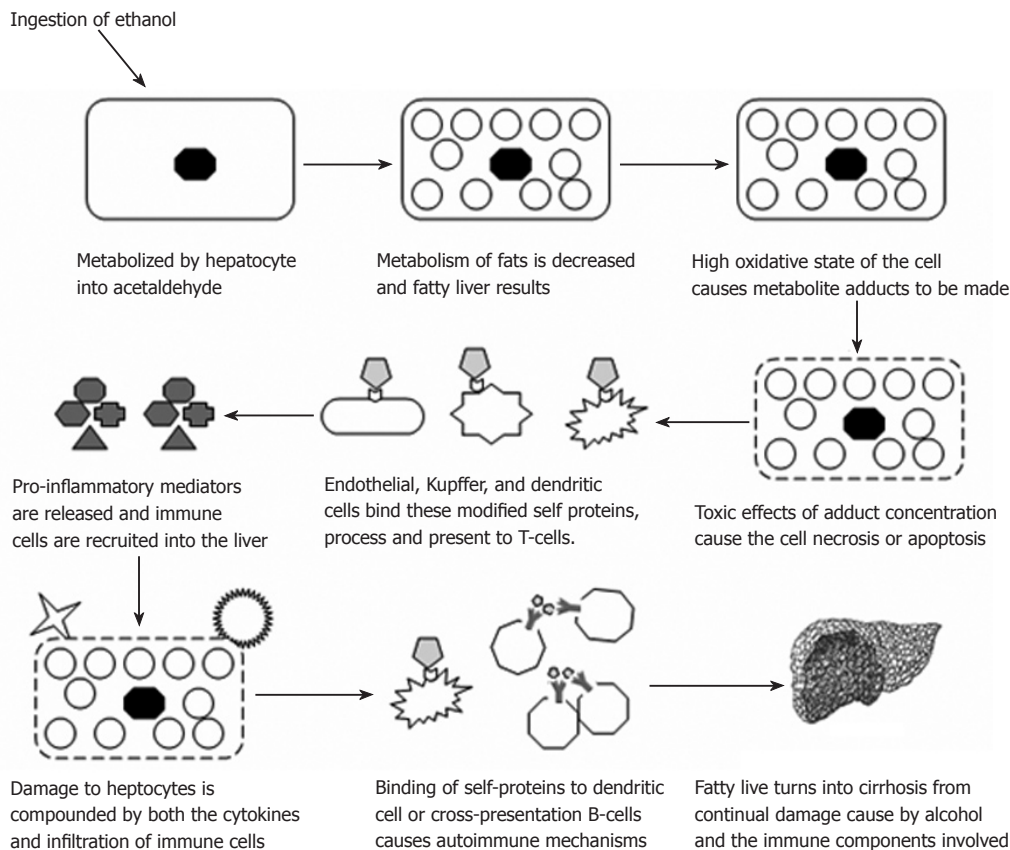


Figure 1 The proposed involvement of immune mechanisms in the development and/or progression of alcoholic liver disease. A schematic depicting the involvement of the immune system in the progress of fatty liver to end stage cirrhosis.

diseases together would greatly increase the severity of the disease. To support this claim experimentally, investigators have shown in a rat model that incorporates an ethanol diet with polyunsaturated fats, there is an increase in the severity of liver damage^[91].

As mentioned above, alcohol metabolism increases the accumulation of fats in the liver. This is caused by suppression in lipid oxidation as a result of the increased production of acetate produced from the metabolism of ethanol^[92]. Fatty liver can also occur when levels of NADH are increased. This enzyme is increased when alcohol is broken down by alcohol dehydrogenase and accounts for a number of metabolic conditions including: hyperlipemia, hypoglycemia, hyperlactacidemia, hyperuricemia, and gout attacks^[8]. In looking at the increased inflammatory state and oxidative stress of the liver under these conditions, one could speculate that lipoproteins and other proteins are modified by the metabolites of ethanol.

Lipoproteins are oxidized under certain conditions. These lipoproteins are under investigation for contributing to coronary heart disease^[93]. However, there is some indication that these modified lipoproteins may play a role in damage to the liver. They have been shown to bind to scavenger receptors on stellate cells and stimulate extracellular matrix proteins^[94]. Aldehyde-modified proteins are similar in nature to these modified LDL proteins, in that they bind to some of the same receptors^[95]. Interestingly, in other studies, MAA-adducted proteins were found in atherosclerotic aortas, implicating a similar connection between MAA-modified proteins and oxidized LDL^[96]. These data indicate that modified proteins may not be limited to the liver and may reach the blood stream

and travel throughout the body. Since these modified proteins bind to scavenger receptors on endothelial cells, it seems likely that excess presence of these adducts could be harmful.

Malondialdehyde, one of the breakdown products of lipid metabolism, has been implicated in autoimmune disease^[97]. Anti-MDA adducts have been found in patients with systemic lupus erythematosus (SLE) and correlated with antibody markers specific for this disease. MDA-adducts have also been found to correlate with atherosclerosis and many other diseases by their ability to form lipofuscin^[98,99]. MDA-adducts in our laboratory has been found in atherosclerotic plaques using immunohistochemical techniques^[96].

These adducts formed from the metabolism of fats or alcohol and have been shown experimentally in human subjects to have many harmful effects. The fact that they can be toxic to cells at high levels increases the cell death or apoptotic numbers in the organ^[75,100,101]. This increases the amount of pro-inflammatory cytokines in the organ and calls immune cells in to clean up the debris. If aldehyde adducts bind to the cellular debris, and this material is picked up by an antigen-presenting cell, the risk for an increased immune response is prevalent. If these molecules happen to be presented to an activated dendritic cell, the immune response is generated that is specific for the self protein.

Taken altogether, the observation that many individuals are overweight or already have fatty liver without the consumption of alcohol^[102], there is an increased risk of liver damage when alcohol is consumed in large quantities. Metabolism of fats and ethanol together constitute a fine balance between clearance and build

up of reactive metabolites. Acetaldehyde from alcohol and malondialdehyde from lipids increase in number until the product is eliminated from the body. If and when individuals continue to consume either ethanol, fats, or both in large quantities, they run a risk of adduct formation. As discussed above, adduct formation can lead to a number of diseases including: autoimmunity, atherosclerosis, alcoholic liver disease, non-alcoholic liver disease, and many others.

Management or treatment options

In looking at treatment options for alcohol abuse and obesity, the fastest way to resolve these diseases is to eliminate the source. Alcoholic steatosis can be reversed if withdrawal from ethanol is accomplished. In fact, abstinence from ethanol can improve cirrhosis and/or fibrosis within only several weeks if eliminated from the diet^[103]. As for obesity, any loss in weight has large implications in improving disease state of any kind. However, individuals will continue to consume fats and ethanol so treatment using other methods could help in the quest for improved disease state.

Nutrition of patients with alcoholic liver disease has been a key component, as many of these individuals are malnourished. This puts pressure on the immune system in its ability to clear infections and aid in liver regeneration^[103]. Increasing proteins, vitamins, essential amino acids, and decreasing saturated fat intake can aid in the recovery of many liver disease patients^[91,104]. However, diet change is hard for individuals so administration of certain vitamins has been somewhat successful. These include injections of vitamin B1, supplements of vitamins B2 and B6, and increasing folic acid levels^[8].

When nutritional changes are not working for these patients, various compounds have had some success. These include: steroids, anti-TNF, S-adenosylmethionine, betaine, antibiotics, and possibly statins. Corticosteroids have been able to reduce the inflammatory response in numerous diseases. In alcoholic liver disease, steroids have been shown to reduce cytokine production, suppress acetaldehyde adducts, and inhibit the production of collagen^[105]. Studies have demonstrated that TNF-alpha levels are greatly increased in the liver following chronic alcohol consumption^[106]. This cytokine is toxic to cells and is the major source of inflammation in the liver. Treating with anti-TNF antibodies immobilizes the effects of TNF-alpha on hepatocytes and other cells in the liver^[107].

Treatment with anti-oxidants has been somewhat successful in the quest for alcoholic liver disease therapy. S-adenosylmethionine (SAM) and betaine have been shown to restore glutathione levels, which increase the ability of the liver to metabolize fats^[108-110]. SAM and betaine work by correcting alterations in methionine metabolic pathways, restoring methylation reactions in the oxidative liver. Use of antibiotics for treatment of liver cirrhosis in individuals with bacterial infections is also beneficial^[111,112]. Bacterial infections in these patients are due in part to the increase in gut permeability, where by alcohol cause the release of normal flora into the blood stream. Lipopolysaccharide from the cell walls of these bacteria increase levels of

cytokines greatly enhancing toxicity to cells in the liver^[48]. By decreasing the level of bacteria in the system, activation of the immune system by these cytokines can be reduced.

Statins are commonly used to lower harmful cholesterol in the blood stream^[113]. These drugs are mainly used to prevent atherosclerosis and heart disease. However, recent evidence for preventing other diseases has become available. For example, statins have been postulated to have an anti-inflammatory and immunomodulatory role in protecting the body against endotoxin from bacteria^[114]. Evidence has also shown that these drugs may have implications in the treatment or prevention of metabolic syndrome^[115]. The cholesterol lowering and anti-inflammatory properties of these drugs might be helpful in the future to lower the fatty liver associated with alcoholic liver disease. Studies will need to be performed to determine the efficacy of statins.

There is some suggestion that ethanol in moderation might have some beneficial effects to certain individuals. Decreased risk of coronary heart disease has been associated with individuals who drink as little as one to six alcoholic beverages per week^[116]. Experts think that small doses of alcohol may increase the level of good cholesterol (HDL) in the blood stream regardless of the beverage consumed^[117]. However, the overall benefit of alcohol does not outweigh the risk of the toxic effects of this drug. Like all things in life, moderation is the key to living a healthy lifestyle.

CONCLUSION

While there are a number of contributing factors resulting in the development and/or progression of alcoholic liver disease, the immune system appears to be a major player. Immune function in alcoholics has shown circulating autoantibodies, hypergammaglobulinemia^[3,118], antibodies to metabolite adducts^[24,28,79], and the infiltration of immune cells into the liver^[34,37,72]. One other factor to support the immune system is seen in alcoholic patients who have received a liver transplant. If these individuals start consuming ethanol, hepatic fibrosis or cirrhosis occurs much more quickly than the years it took to develop prior to transplant^[119]. This quick memory response to the liver suggests that the adaptive immune system is involved.

Other factors that contribute to the development of ALD include: metabolism, toxicity, genetics, and nutritional factors. The metabolism of excess ethanol causes many harmful effects that could contribute to the formation of metabolite adducts. If these adducts form while cells are dying from the toxic effects of ethanol, self-proteins could become modified, providing the necessary mechanism for autoimmune disease. If you figure in the susceptibility of certain individuals and their genetic make up, the risk for liver disease becomes higher. Also factored into to this is the observation that many alcoholics are malnourished, consuming many of their calories from alcohol^[8]. It has been shown that the immune system works better when a healthy balanced diet is followed^[120]. One other common issue is the consumption of high levels of fatty foods in the diets of most individuals. Alcohol metabolism already

contributes to the build-up of fats in the liver (fatty liver); compound that with the addition of more fats in the diet and the potential exists for a double-edged sword of damage and oxidative stress.

The fact remains that alcohol consumption has many harmful effects on the body. However, getting people to stop drinking completely is an unrealistic expectation. The need for understanding the mechanisms of alcoholic liver disease remains necessary for determining better therapy and intervention strategies.

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Role of transmethylation reactions in alcoholic liver disease

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Abstract

Alcoholic liver disease is a major health care problem worldwide. Findings from many laboratories, including ours, have demonstrated that ethanol feeding impairs several of the many steps involved in methionine metabolism. Ethanol consumption predominantly results in a decrease in the hepatocyte level of S-adenosylmethionine and the increases in two toxic metabolites, homocysteine and S-adenosylhomocysteine. These changes, in turn, result in serious functional consequences which include decreases in essential methylation reactions *via* inhibition of various methyltransferases. Of particular interest to our laboratory is the inhibition of three important enzymes, phosphatidylethanolamine methyltransferase, isoprenylcysteine carboxyl methyltransferase and protein L-isoaspartate methyltransferase. Decreased activity of these enzymes results in increased fat deposition, increased apoptosis and increased accumulation of damaged proteins—all of which are hallmark features of alcoholic liver injury. Of all the therapeutic modalities available, betaine has been shown to be the safest, least expensive and most effective in attenuating ethanol-induced liver injury. Betaine, by virtue of aiding in the remethylation of homocysteine, removes both toxic metabolites (homocysteine and S-adenosylhomocysteine), restores S-adenosylmethionine level, and reverses steatosis, apoptosis and damaged proteins accumulation. In conclusion, betaine appears to be a promising therapeutic agent in relieving the methylation and other defects associated with alcoholic abuse.

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Key words: Transmethylation; S-adenosylhomocysteine; Alcohol; Betaine; Liver; Steatosis; Apoptosis;

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INTRODUCTION

Biological transmethylation reactions utilize methyl groups derived from dietary methyl donors and from cofactors carrying 1-carbon units. A pathway that is central to many of these reactions is the metabolic cycling of methionine (Figure 1).

The significant role of methionine metabolism in liver is underscored by the fact that nearly half of the daily intake of methionine in humans and rats is metabolized there *via* methionine adenosyltransferase (MAT)-catalyzed reaction to generate S-adenosylmethionine (SAM), the principal methylating agent in the body. Under normal conditions, most of the 6-8 g SAM generated per day serves as a methyl donor for many transmethylation reactions. The normal estimated rate of these reactions, corrected to 70 kg body weight, is 16.7-23.4 mmol/d in young normal adults and 15.5-21.7 mmol/d in elderly subjects^[1]. At least 50 of such SAM-dependent methylation reactions have been identified in mammals^[2]. These reactions catalyzed by diverse methyltransferases result in the production of methylated biomolecules, such as phospholipids, proteins, nucleic acids and small molecules that have vital biological roles in biosynthesis, regulation, repair and detoxification. The other product is S-adenosylhomocysteine (SAH), which is hydrolyzed to adenosine and homocysteine by S-adenosylhomocysteine hydrolase (SAHH). This series of reactions is referred to as transmethylation. The methionine cycle is completed when homocysteine is remethylated back to methionine. The biochemical step between homocysteine and methionine occurs through two reactions, both of which are equally important in converting homocysteine to methionine in the liver^[3]. In the first, a methyl group is transferred from betaine (which in turn, is derived from the oxidation of choline) to homocysteine to form methionine and dimethylglycine (DMG) *via* betaine-homocysteine methyltransferase (BHMT). The second reaction utilizes folate and through the action of methionine synthetase (MS), a methyl group is transferred from 5-methyltetrahydrofolate (MTHF) to vitamin B₁₂ to form

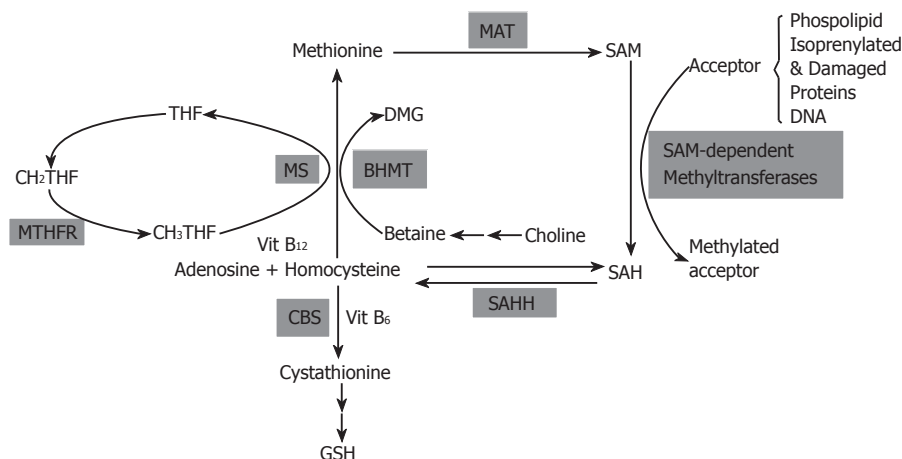


Figure 1 Methionine metabolic pathway in the liver highlighting the importance of the methyltransferase-catalyzed reactions maintained by the cycling of the main participants, methionine, S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH) and homocysteine. The hepatocellular ratio of SAM to SAH is critical in controlling many transmethylation reactions.

methylcobalamine. The methylcobalamine in turn transfers the methyl group to homocysteine to produce methionine. The remethylation of homocysteine by the folate pathway is ubiquitously distributed but the BHMT-mediated pathway is tissue-specific existing primarily in the liver and kidney^[14]. Another enzyme that links the one-carbon pool and methionine metabolic pathway is 5, 10 methylene tetrahydrofolate reductase (MTHFR) that is involved in the production of MTHF. Folate also functions to transfer one carbon units for the synthesis of thymidylate, a key step in DNA synthesis.

Homocysteine can also be irreversibly catabolized through the transsulfuration pathway by the action of cystathionine β -synthase (CBS). The transsulfuration pathway superimposes on the methionine pathway and is important for the synthesis of cysteine and glutathione (GSH) as well as the removal of homocysteine. The transsulfuration occurs in a restricted number of mammalian tissues—liver, kidney, pancreas and the intestine, because the rest of the organs lack expression of one or more enzymes of this pathway.

Effects of alcohol on liver methionine metabolism

Many laboratories have shown that alcohol consumption induces alterations in methionine metabolic pathways. A major defect elicited by ethanol consumption appears to be inhibition of liver MS activity, which results in impaired remethylation of homocysteine to form methionine^[5-8]. Concomitantly, to compensate for the decreased MS activity, the activity of BHMT, an alternate enzyme for homocysteine remethylation, is induced after ethanol ingestion. A major outcome of this increase is a marked lowering in liver betaine levels in an effort to maintain SAM levels for transmethylation reactions^[9]. However, SAM levels diminish upon prolonged ethanol exposure^[10]. This does not appear to be due to a loss in liver-specific MAT level or activity since no decrease was observed despite 9 wk of intragastric feeding with ethanol and high fat^[11]. Similar results were also reported for micropigs fed chronic ethanol for up to a year^[7]. However, subsequent studies done by the same group have revealed decreased liver SAM levels in micropigs fed ethanol for 14 wk^[12]. Data from human studies also demonstrates subnormal

MAT function in liver biopsies from alcoholic patients^[13,14].

Our laboratory and others have further reported that despite the compensatory increase in BHMT, alcohol-induced impairment of liver MS activity results in an enhanced generation of the potentially toxic agent homocysteine^[12,15,16]. This in turn results in an increased intracellular level of SAH^[7,8,12,17,18] as the equilibrium of the SAH hydrolase reaction energetically favors SAH generation over SAH hydrolysis. We further reported that the intracellular ratio of SAM:SAH levels was significantly lower (2.5) in freshly isolated hepatocytes and livers of ethanol-fed rats as compared to the ratio of 5 observed in hepatocytes and livers of control-fed rats^[17, 19]. Although many laboratories have shown comparable ratio values and decreases with ethanol as we report^[20,21], studies with mice and micropigs reveal a similar trend of ratio decrease but lower absolute ratio values^[22,23].

It is very crucial to maintain SAM:SAH ratios because SAH is a competitive inhibitor for many SAM-dependent methyltransferases. The K_i value for SAH is in the submicromolar to low micromolar range and is often less than the K_m value for SAM for many of the methyltransferases^[24]. Therefore, any significant decrease in the ratio negatively affects many transmethylation reactions.

Betaine maintains normal SAM:SAH ratios after ethanol consumption

Ours and other laboratories have also shown that betaine (a naturally occurring tertiary amine, trimethylglycine) can enhance the remethylation of homocysteine *via* BHMT-catalyzed reaction^[15,16] to alleviate ethanol-induced changes in intracellular SAH levels^[17] as well as cause elevations in SAM levels^[19,25]. Similar increases in hepatic SAM levels have also been reported in mice fed intragastrically an ethanol diet supplemented with betaine^[26]. Betaine, by modulating SAM and SAH levels, results in normalization of the alcohol-induced alterations in the hepatocellular SAM:SAH ratios^[17,19].

We have further observed that while ethanol treatment resulted in a 2-fold elevation of hepatic BHMT activity, this increase was further elevated in the livers of rats fed ethanol diet supplemented with 1% betaine. This increase in activity correlates with the enzyme level of BHMT

quantified by western blot analysis using an antibody to rat BHMT as well as with quantitative RT-PCR studies^[19]. These results imply that the inclusion of betaine in the diet promotes the alternate remethylation pathway to ultimately maintain hepatocellular SAM:SAH ratios by not only providing the substrate, betaine, for the BHMT-catalyzed reactions, but also further inducing BHMT at the gene and protein level. Further studies indicate an interplay between ethanol and not only the diet composition, but also its presentation^[27].

Effects of ethanol and betaine on transmethylation reactions

Researchers over the years have attempted to determine which transmethylation reactions are affected by acute or chronic ethanol consumption as well as to understand how these altered reactions in turn, contribute to alcohol-induced liver injury. Further attempts have been made to determine the therapeutic effects of some participants of the methionine cycle, such as betaine and SAM, in minimizing the adverse effects.

Our laboratories and others have shown that there are many SAM-dependent methylation reactions that are adversely affected by ethanol consumption and normalized by co-administration by betaine. Of particular interest to us is three such reactions that clearly demonstrates a close relationship between the activities of methyltransferases with hallmark features of alcoholic liver injury. These three liver methyltransferases are: (1) Phosphatidylethanolamine methyltransferase (PEMT), the methyltransferase involved in the generation of phosphatidylcholine (PC); (2) isoprenylcysteine carboxyl methyltransferase (ICMT), the methyltransferase that carboxyl methylates isoprenylated proteins; (3) Protein L-isoaspartate methyltransferase (PIMT), the methyltransferase that catalyzes the repair of isoaspartyl sites of spontaneously damaged proteins.

Association between PEMT activity and steatosis

PEMT catalyzes three successive methyl group transfers from SAM to phosphatidylethanolamine (PE) to generate PC. This pathway has been reported to be responsible for about 50% of total SAM utilized^[28]. Although PC can also be synthesized by the Kennedy Pathway in the liver^[29], it has recently been shown that the PC derived *via* the PEMT pathway is preferentially used in the assembly of very low density lipoproteins (VLDL) and is necessary for its normal secretion^[30]. As the liver exports triglycerides and cholesterol only as constituents of VLDL particles, any impairment in the processes of either the synthesis or export of VLDL particles could lead to fat accumulation within the hepatocyte. Indeed, experimentally inhibiting PC synthesis *via* the PE methylation pathway or targeted PEMT gene disruption has been shown to impair the incorporation of triglycerides into VLDL and reduce its secretion by hepatocytes^[30,31]. Conversely, stable expression of PEMT has been shown to enhance this secretion^[31]. The ultimate substantiation of the critical role of methylation reactions catalyzed by PEMT in the prevention of fat accumulation in hepatocytes is provided

by the observations that homozygous PEMT knockout mice spontaneously develop hepatic steatosis despite adequate choline in the diet^[32].

PEMT and alcoholic steatosis

While earlier studies have implicated decreased hepatic VLDL secretion to play a role in the development of alcoholic steatosis^[33], the association of PEMT activity with alcoholic liver injury was suggested by reports that identified decreased PEMT activities in livers of cirrhotics and a modulation of PEMT activities by altered SAM:SAH ratios^[14,34,35]. However, the relationship between PEMT activity, SAM:SAH ratios and alcoholic steatosis became apparent after our observations that the SAM:SAH ratios in livers and hepatocytes of ethanol-fed rats is much lower than the ratios in livers and isolated hepatocytes of control diet fed rats^[17,19]. Our further understanding of the relationship between ratios, PEMT activities and steatosis became evident from experiments conducted in our laboratory using liver microsomal fractions, isolated hepatocytes and in an *in vivo* rat model of ethanol abuse.

Using microsomal fractions isolated from liver of Purina-fed rats, PEMT activities measured in the presence of varying amounts of SAH (such that the ratio of SAM:SAH in the incubation mixture varied to mimic ratios seen in hepatocytes from ethanol, control or *in vitro* betaine-supplemented conditions) revealed increasing inhibitions of PEMT activity with decreases in the SAM:SAH ratio. A 30-40% inhibition of PEMT activity was observed when the SAM:SAH ratio was 2.5 (which corresponds to the intracellular ratio observed in isolated hepatocytes from ethanol-fed rats) as compared to the activity observed at the ratio of 5.0 (the ratio observed in the controls). A ratio as seen in the betaine-supplemented hepatocytes essentially resulted in restored enzyme activity^[17]. Identical experiments were also performed on fractions prepared of hepatocytes obtained from both control and ethanol-fed rats. While similar activities were noticed in isolated microsomal fractions obtained from both cell types under basal conditions^[36], the PEMT activities were also equally impaired by the decreasing SAM:SAH ratios. These results implied: (1) that the PEMT enzyme level appears to be unaffected by alcohol consumption, but it is the alcohol-induced decrease in the hepatocellular SAM to SAH content ratio that causes impaired PEMT activity, and (2) betaine, by virtue of its ability to increase the ratio, would restore (increase) the activity of PEMT.

To examine the physiological relevance of the direct inhibition of PEMT activity in isolated microsomal fractions by increasing SAH amounts, the influence of ethanol on PC generation *via* the PEMT pathway was also analyzed. It was observed that ethanol feeding resulted in decreased PC production by a PEMT catalyzed reaction. Betaine either as *in vitro* supplementation to isolated hepatocytes obtained from ethanol-fed rats or fed to rats on ethanol diet corrected the ethanol-induced decrease in SAM:SAH ratios^[19] and by normalizing the PC production *via* the PEMT-catalyzed reaction, significantly reduced fatty infiltration associated with ethanol consumption^[19,36]. It has

also been shown that the PC to PE ratio is a key regulator of cell membrane integrity and plays a role in progression of steatosis to steatohepatitis^[37].

ICMT-catalyzed transmethylation reactions and apoptosis

The substrates of ICMT are proteins that terminate with the CAAX motif (where C = cysteine, A = aliphatic amino acid, and X = undefined). Such proteins first undergo prenylation of the cysteine residues, followed by proteolytic cleavage of the AAX sequence. ICMT subsequently catalyzes the reversible carboxyl methylation of the newly exposed isoprenylcysteine using SAM as the methyl donor. This post-translational modification is required for the normal activity of proteins such as GTPases that play a crucial role in the signaling of many complex biological processes, including the prevention of apoptosis^[38,39].

The functional importance of normal ICMT in preventing apoptosis is demonstrated in many studies. Apoptosis in cultured cells can be induced by inhibiting the carboxyl methylation of endogenous physiological substrates of ICMT^[40-42] or by elevated intracellular SAH levels^[43,44]. That SAH-induced apoptosis could be prevented by over-expression of ICMT emphasizes an involvement of impaired ICMT in the apoptotic cascade^[41]. The physiological importance of ICMT is further reiterated by studies on homozygous ICMT knockout mice. These mice present with developmental defects of the liver that are so severe that virtually all of the knockout embryos die by mid-gestation^[45], possibly due to liver agenesis/apoptosis^[46]. Subsequent studies revealed that ICMT-catalyzed carboxyl methylation of its substrates, Ras and Rho GTPases, are very critical for prevention of caspase activation and programmed cell death^[41].

ICMT, alcoholic apoptosis and betaine

It has been demonstrated that ethanol administration is linked to increased hepatocyte apoptosis in both clinical and experimental alcohol-induced liver injury in a variety of species of animals including rats, mice and minipigs^[7,16,47-52]. Compelling evidence from our laboratory implicates altered hepatocellular SAM:SAH ratios and subsequent decreased ICMT activity to be responsible for alcoholic apoptosis. The first supporting data for this conjecture is that betaine supplementation (which normalizes homocysteine and hepatocellular SAM:SAH ratios) is effective in preventing hepatocyte apoptosis^[53]. While Ji *et al*^[16,26], using the mouse model of intragastric ethanol feeding, have also shown the protective effect of betaine on alcoholic apoptosis, their focus has been in pursuing a link of homocysteine-induced ER stress to apoptosis. But further studies in our laboratories using biochemical approaches have confirmed that indeed it is the increased intracellular SAH levels (that were achieved by using adenosine or tubercidin to specifically increase SAH levels without a change in homocysteine levels) that cause increases in apoptosis of hepatocytes. Further, these induced increases in apoptosis and increased SAH levels could both be significantly attenuated (40%-50%) by simultaneous betaine exposure^[53]. Verification that

the protective effect of betaine was primarily *via* the BHMT-catalyzed methyl group transfer, and not due to osmoregulation^[54], was illustrated by using DMG^[53], a product feedback inhibitor of BHMT^[55].

We have also shown increasing inhibitions of ICMT activity with decreases in the SAM:SAH ratio similar to our observations with PEMT. Almost 30% less ICMT activity was observed when the SAM:SAH ratio was 2.5 (intracellular ratio in hepatocytes from ethanol-fed rats) as compared to the activity observed at the ratio of 5.0 (the ratio observed in the controls or betaine-fed ethanol rats). We have also shown that hepatocellular ICMT enzyme levels are not affected by ethanol consumption (Kharbanda and Tuma, unpublished observations). There is evidence that the ICMT activity may be enhanced in non-parenchymal hepatic cells after ethanol consumption^[56,57], which suggests that the alterations in methylation reactions may be limited to alcohol-metabolizing cells.

Final support for a role of normal ICMT in preventing apoptosis has been derived from experiments using inhibitors of ICMT such as cysmethynil^[58], N-acetyl-L-farnesyl cysteine (AFC) and N-acetyl-geranylgeranyl cysteine (AGGC). Data shows that exposure to cysmethynil, AFC or AGGC induce hepatocytes to undergo apoptosis (Kharbanda and Tuma, unpublished observations). These observations clearly implicate a role of normal carboxyl methylation of endogenous ICMT substrate(s) in hepatocytes to play crucial roles in the prevention of apoptosis as has been shown for other cell types^[40-42]. Efforts are in progress to determine the downstream effector(s) of ICMT-catalyzed transmethylation reactions associated with inhibition of apoptosis of hepatocytes.

There are also reports that caspase-8 gene expression is sensitive to intracellular methylation status^[59]. Alcohol-induced increased SAH levels could directly increase caspase-8 expression/activity to result in enhanced apoptosis^[18].

PIMT and damaged proteins

Proteins undergo spontaneous thermodynamically driven changes over time that can result in decreased functionality. A common form of such damage is the spontaneous and non-enzymatic modification of aspartate and asparagine (which are among the most unstable residues in proteins and peptides) to form atypical isoaspartyl residues. The enzyme PIMT catalyzes the transfer of the methyl group of SAM to these isoaspartyl sites, allowing reisoimerization and restoration of the original alpha peptide linkage after repeated rounds of methylation^[60]. This physiological repair reaction helps prevent the accumulation of the protein molecules containing these abnormal aspartyl residues. In mammals, PIMT is expressed in all tissues with the highest level in the brain and blood tissues^[61]. Homozygous PIMT knockout mice accumulate damaged proteins in their tissues and organs, including the liver^[61].

Isoaspartate residues have been identified in a variety of cellular structural and functional proteins such as tubulin, calmodulin, histone 2B and collagen type-I. Evidence has been accumulating that the modified

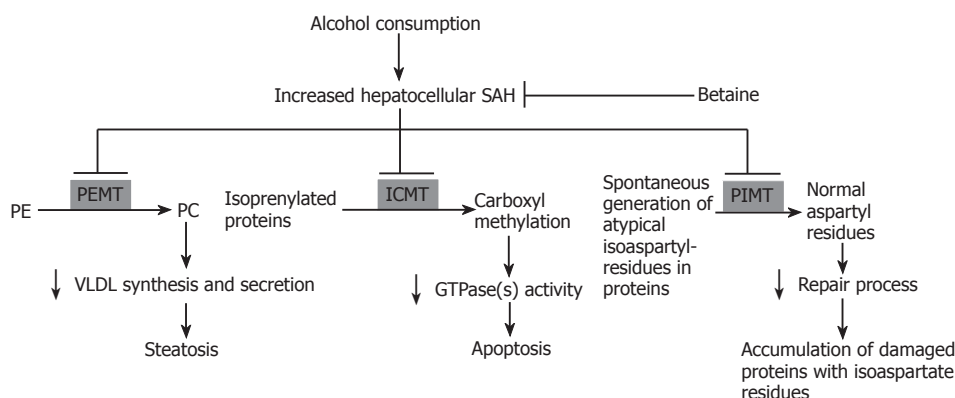


Figure 2 Schematic of the effects of ethanol and betaine on transmethylation reactions. Overall, it had been determined the deleterious effects of ethanol on impaired transmethylation reactions can be prevented by betaine supplementation.

proteins have impaired biological activity that can be substantially restored *via* methylation by PIMT^[62]. The physiological relevance of this repair by PIMT has also been demonstrated *in vivo* by studies using PIMT-deficient mice. These animals show aberrant vesicular transport which is caused by microtubule disorganization resulting from increased amounts of isoaspartyl residues in β -tubulin, a major substrate of PIMT and a component of microtubules^[63].

Environmental factors such as heat shock, photochemical stress and oxidative stress have been reported to result in an enhanced accumulation of damaged proteins with isoaspartate residues^[64,65].

Alcohol, PIMT, accumulation of damaged proteins and betaine

Our recent data shows that in addition to the above mentioned factors, chronic alcohol ingestion results in the accumulation of isoaspartyl-bearing damaged proteins in rat liver homogenates, particularly in the cytosol and microsomal fractions. This accumulation correlates to the lower SAM:SAH ratios and consequent impaired PIMT activity in livers of these rats as no change in the PIMT enzyme level by alcohol was observed. Furthermore, rats fed the betaine-supplemented ethanol diet showed similar isoaspartyl residue quantities per unit protein as controls. This restoration of the protein repair process was related to the effect of betaine to normalize the SAM:SAH ratios, which in turn, normalizes the PIMT-catalyzed protein repair methylation reaction^[66].

We also determined the effect of varying SAM:SAH ratios on the activity of purified PIMT. We observed 30% less PIMT activity at a ratio of 2.5 (which corresponds to the ratio in livers of ethanol-fed rats) as compared to a ratio of 5 as seen in livers of control or betaine-supplemented fed ethanol rats^[66]. These results were similar to those obtained with PEMT and ICMT activity measurements.

We are currently focusing on identifying some of the unique cellular structural and functional proteins susceptible to isoaspartyl damage following ethanol consumption by a proteomic approach and in understanding their role in altering the hepatocellular dynamics that could contribute to the progression of alcoholic liver injury.

Effects on other methyltransferases

DNA Methyltransferases and Alcohol: Modulation of

gene expression by alterations in DNA methylation can result in epigenetic changes and functional consequences. Current investigations are focused in understanding the molecular steps involved in the effects of ethanol on epigenetic modifications in relation to pathological changes in the liver. The inhibition of SAM-dependent DNA methyltransferase activities by ethanol-induced decreased SAM:SAH ratio could be responsible for the impaired DNA methylation reported in rats fed intragastrically with ethanol and a high fat diet^[11]. The reduced hepatic DNA methylation patterns by ethanol consumption has been reported to be associated with increased expression of c-myc and increased genome-wide DNA strand break accumulation^[11]. Other studies have shown no effects of alcohol consumption on O⁶-methylguanine DNA methyltransferase levels in the liver of cirrhotic^[67], however its activity may be affected by altered SAM:SAH ratios. More information can be obtained from an excellent editorial of the epigenetic effects of ethanol on liver injury that was recently published^[68].

Glycine-N-Methyltransferase and Alcohol: This is an abundant cytosolic SAM-dependent methyltransferase involved in the synthesis of sarcosine. But GNMT, unlike PEMT, ICMT, PEMT and the DNA methyltransferases, is less sensitive to inhibition by SAH^[24]. However, it is allosterically inhibited by MTHF^[69] and plays a role in regulating SAM:SAH ratios^[70]. Ethanol has been shown to decrease GNMT transcript levels, however an increase in its activity was observed after alcohol consumption, possibly related to decreased MTHF levels^[71].

CONCLUSION

Alcohol-induced elevation of intracellular SAH levels can impair the methylation reactions catalyzed by three enzymes, PIMT, PEMT and ICMT, which consequently contribute to the accumulation of damaged proteins with isoaspartyl residues and the induction of hepatic steatosis and apoptosis, respectively. We further provide data to support that these pathologies can be significantly attenuated by betaine administration. We believe that this protection is *via* remethylation of homocysteine that leads to normalized intracellular SAH levels and maintenance of normal methylation reactions of these three and other methyltransferases. A schematic of this working scheme is

depicted as Figure 2. Overall, we have identified that the increased hepatocellular SAH levels generated by chronic ethanol exposure can negatively affect the activities of the three methyltransferases, PEMT, ICMT and PIMT. These methylation defects, in turn, cause decreased synthesis and secretion of VLDL, impaired activation of GTPases and diminished protein repair process on molecules such as tubulin. These deficiencies ultimately contribute to the induction of steatosis, apoptosis and the accumulation of damaged proteins which could exaggerate hepatocellular injury by disrupting normal cellular function including trafficking events. Betaine administration can prevent the increase in SAH and thereby prevent these ethanol-induced pathologies.

In addition to its effects in ameliorating alcoholic liver injury, betaine has also been shown to prevent carbon tetrachloride-induced liver injury^[72] as well as to considerably decrease indices of steatosis in nonalcoholic steatohepatitis (NASH) patients^[73-75]. These properties along with the low cost and easy use and availability of betaine underscore the advantages of choosing this metabolite for the treatment of liver injury.

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Effects of ethanol on hepatic cellular replication and cell cycle progression

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Abstract

Ethanol is a hepatotoxin. It appears that the liver is the target of ethanol induced toxicity primarily because it is the major site of ethanol metabolism. Metabolism of ethanol results in a number of biochemical changes that are thought to mediate the toxicity associated with ethanol abuse. These include the production of acetaldehyde and reactive oxygen species, as well as an accumulation of nicotinamide adenine dinucleotide (NADH). These biochemical changes are associated with the accumulation of fat and mitochondrial dysfunction in the liver. If these changes are severe enough they can themselves cause hepatotoxicity, or they can sensitize the liver to more severe damage by other hepatotoxins. Whether liver damage is the result of ethanol metabolism or some other hepatotoxin, recovery of the liver from damage requires replacement of cells that have been destroyed. It is now apparent that ethanol metabolism not only causes hepatotoxicity but also impairs the replication of normal hepatocytes. This impairment has been shown to occur at both the G1/S, and the G2/M transitions of the cell cycle. These impairments may be the result of activation of the checkpoint kinases, which can mediate cell cycle arrest at both of these transitions. Conversely, because ethanol metabolism results in a number of biochemical changes, there may be a number of mechanisms by which ethanol metabolism impairs cellular replication. It is the goal of this article to review the mechanisms by which ethanol metabolism mediates impairment of hepatic replication.

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Key words: Cyclin-dependent kinases; Cell cycle; Liver regeneration; Ethanol metabolism; Acetaldehyde;

INTRODUCTION

The association between alcohol consumption and liver disease has been recognized for centuries. It is now well established that ethanol is a hepatotoxin. It is thought that the liver is a target of ethanol-induced toxicity because the hepatocytes of the liver are the primary ethanol metabolizing cells in the human body. The metabolism of ethanol results in many biochemical changes that have been proposed to contribute to liver disease. Among these changes are the generation of the reactive intermediate acetaldehyde, increased generation of reactive oxygen species, anoxia, changes in mitochondrial functions and bioenergetics, as well as an increase in the redox-state of hepatocytes.

There are two major ethanol-metabolizing pathways in hepatocytes; the first is mediated by the cytosolic enzyme alcohol dehydrogenase. This enzyme is responsible for the vast majority of the ethanol metabolism by hepatocytes^[1]. The second major pathway is mediated by the membrane bound enzyme cytochrome P450 2E1. The activity of this enzyme has been shown to be induced by ethanol and therefore, it has a greater contribution to the metabolism of ethanol in cases of chronic ethanol abuse^[2]. Even though there is a tremendous amount of evidence indicating that ethanol metabolism is required for many ethanol-induced dysfunctions, the mechanism(s) by which ethanol metabolism causes hepatic damage are not entirely clear. Although the mechanisms of ethanol-induced liver damage are equivocal, it is obvious that recovery from ethanol-induced liver injury is dependent on the ability of cells to replicate, replacing those damaged or killed as a consequence of ethanol metabolism.

Cellular replication is a process in which a tightly orchestrated series of sequential events must occur for successful completion. This series of events is collectively referred to as the cell cycle. The ultimate goals of the cell cycle are accurate replication of DNA and cell division.

The cell cycle can be broadly divided into five stages or phases. G0 is a stage in which cells are metabolically active but are not actively involved in the replication process (hepatocytes are normally in this stage). G1 is the initial stage of the replicative cycle, this is the stage in which enzymes and substances required for DNA synthesis are produced. S is the stage where DNA synthesis occurs. G2 is the stage in which the enzymes and substances required for mitosis are synthesized or activated. M phase is the stage in which cells divide and cellular replication is completed.

Tissue repair is a balance between damage and repair. Tissue damage occurs when injury dominates the ability of the tissue to replace cells lost to injury. In the case of ethanol-induced liver damage, it appears that ethanol metabolism is not only responsible for tissue damage but also impairs the ability of hepatocytes to respond normally and replace dead or damaged cells. Whether cellular injury to the liver has been caused by the effects of ethanol metabolism or by some other means, recovery is dependent, at least in part, on the capacity of the liver to replace cells lost to toxic insult. It is the focus of this article to review the evidence and potential mechanisms by which ethanol metabolism impairs the replication of hepatic cells and initiates or enhances alcoholic liver disease.

G1 phase inhibition

The liver has a tremendous capacity to replace cells that are lost or damaged by cytotoxic injury. In fact, if part of the liver is surgically removed, the remaining portion of the liver will increase in size to replace the mass of the original organ. This surgical procedure, performed with experimental animals has been termed partial hepatectomy and has become a standard model to investigate hepatic regeneration. Following partial hepatectomy, a synchronized wave of cellular replication occurs; thus, this model of liver damage is extremely amenable to the study of individual aspects of the cell cycle.

Much of what we have learned about the effects of ethanol on the cell cycle and hepatic replication has been derived through the use of the partial hepatectomy model of liver injury. In early studies using this model of liver injury, Wands *et al*^[3] found that the timing of ethanol administration after partial hepatectomy was important in determining the effects of ethanol on hepatic regeneration.^[12] If the animals were administered a single dose of ethanol 4 h before, or up to 8 h after partial hepatectomy, no effect of ethanol was detected on DNA synthesis 24 h after the surgical procedure. Conversely, if ethanol was administered 12-16 h after partial hepatectomy, DNA synthesis at 24-h post surgery was significantly impaired. If the animals received multiple doses of ethanol 0-8 h after partial hepatectomy, the effects of ethanol were altered and more severe inhibition of DNA synthesis was observed. These results led the authors to suggest that the inhibitory effects of ethanol on liver regeneration were cell cycle dependent; hepatocytes appeared to be most sensitive to the effects of ethanol early in G1, prior to the initiation of DNA synthesis. Once DNA synthesis was initiated, hepatocytes appeared to be relatively resistant to the effects of ethanol^[3]. It has also been shown that chronically feeding

ethanol to experimental animals impairs the regenerative capacity of the liver^[3,4]. This impairment was characterized by a delay in the peak, as well as a reduction in the magnitude of DNA synthesis, after partial hepatectomy^[4].

Although these initial *in vivo* investigations established the fact that ethanol affected the regenerative capacity of the liver, they did not address the possible mechanisms involved in this impairment. Higgins and Borenfreund, using the rat liver tumor cell line 32 II A6/2d, showed that addition of pharmacological concentrations of ethanol (10-100 mmol/L) for up to three days resulted in a concentration-dependent reduction in the final population density of the culture^[5]. Additionally, these authors demonstrated that substitution of acetaldehyde, the reactive byproduct of ethanol metabolism, for ethanol in the growth medium reproduced the ethanol-mediated impairments. This indicated that ethanol metabolism was responsible for the observed impairments and that acetaldehyde had a role in this dysfunction. To further implicate the role of ethanol metabolism, addition of the alcohol dehydrogenase inhibitor, pyrazole, ameliorated the ethanol-mediated impairments^[5]. Extending these findings, Higgins and Borenfreund analyzed 32 II A6/2d cells exposed to ethanol by flow cytometry and found an increase in the percentage of cells in the G1 phase of the cell cycle. The authors concluded that the inhibition of cellular proliferation associated with acute ethanol exposure in these cells was a result of this accumulation of cells in the G1 phase of the cell cycle^[6].

In the normal liver, the vast majority of hepatocytes are arrested in the G0 phase of the cell cycle. Hepatocytes require stimulation of hormones and growth factors to traverse G1 and begin cellular replication^[7]. Using isolated rat hepatocytes, Carter *et al*^[8] demonstrated that inclusion of ethanol to the growth medium dampened the proliferative response induced by hormonal and growth factor stimulation. These results indicated that the signals required to induce completion of G1 are also affected by ethanol.

Ethanol metabolism results in the production of reactive oxygen species; accumulation of reactive oxygen species results in oxidative stress^[9,10]. After partial hepatectomy there is an increase in the expression of TNF α and a number of mitogens^[7]. Interestingly, TNF α (which is necessary for induction of hepatocyte DNA synthesis) and mitogens both cause transient increases of H₂O₂ (a species of reactive oxygen). Transient increases in reactive oxygen species induce the expression of three important mediators of hepatocyte replication, the mitogen activated protein kinase (MAPK), extracellular regulated kinases (ERK) 1 and 2, and cyclin D1. Prolonged exposure to reactive oxygen species not only induces ERK 1 and 2, but also induces the cyclin dependent kinase inhibitor p21, and p38 MAPK, an inhibitor of cyclin D1. The combined effects of reduced cyclin D1 and expression of p21 efficiently inhibits the proliferation of cells^[11].

Investigating the effect of chronic ethanol administration on liver regeneration after partial hepatectomy, Koteish *et al*^[12] demonstrated that p38 MAPK and p21 were significantly increased in the livers of animals chronically fed ethanol, compared to controls after partial hepatectomy. These increases were accompanied by decreased expression

of cyclin D1, increased expression and activity of the cyclin dependent kinase inhibitor p27, and an increase in the activity of the transcriptional activator signal transducer and activator of transcription-3 (STAT-3). STAT-3 can be involved in cell cycle progression or growth arrest^[13] and is capable of inducing the expression of the cyclin-dependent kinase inhibitors p21 and p27. Normally, cyclin D1 binds to and sequesters p21, effectively nullifying its inhibitory affects. When cyclin D1 is deficient, as it is after partial hepatectomy in ethanol-fed animals, the activity of p21 as a cyclin-dependent kinase inhibitor is favored and cells can be arrested at the G1/S transition. Because ethanol metabolism produces reactive oxygen species in hepatocytes, the authors suggested that the increased activity of STAT-3, induction of p21, and cell cycle arrest at the G1 stage of the cell cycle may be a form of cytoprotection, protecting the cells from the acute increases in reactive oxygen species resulting from partial hepatectomy or other oxidative injury^[12].

Fat accumulation and impaired hepatic replication

The first pathologic change that occurs to the liver as a result of ethanol consumption is the appearance of fat in the liver, a condition known as steatosis. The accumulation of fat in the liver was once thought to be benign, but is now recognized as a factor that can be involved in more serious liver injury^[14]. It has been proposed that ethanol sensitizes the liver to a “second hit”, and that this “second hit” then induces the damage associated with alcoholic liver disease^[15]. In the model proposed above, fat accumulation in the liver, as a result of ethanol consumption, sensitizes the liver to additional oxidative injury.

To determine if the accumulation of fat in the liver was sufficient to sensitize the liver to oxidative damage, Torbenson *et al*^[16] investigated the effects of fatty liver on liver regeneration after partial hepatectomy. Mice with mutations in their leptin genes (leptin deficient ob/ob mice) spontaneously develop fatty livers. Using ob/ob mice, Torbenson *et al*^[16] found that liver regeneration was delayed in the ob/ob mice compared with lean littermates. The authors also found that the accumulation of the activated, phosphorylated form of STAT-3 (pSTAT-3) was increased in the livers of ob/ob mice compared with lean littermates. Additionally, an inverse correlation between the nuclear location of pSTAT-3 and DNA synthesis was observed, as well as decreased percentages of nuclei labeled with the DNA analog 5-bromo deoxyuridine (BrdU), increased expression of the cyclin-dependent kinase inhibitors p21 and p27, and expression of cyclin D1^[16]. These authors also suggested that this cell cycle arrest could act as a cytoprotective mechanism to protect hepatocytes from reactive oxygen species.

These results were very reminiscent of those observed in ethanol-fed animals, and leads one to speculate whether the induction of fatty liver by ethanol consumption is a causative agent of cell cycle impairment and aberrant cellular replication.

Mitochondrial dysfunction

Mitochondrial dysfunction has also been implicated as a mechanism by which ethanol metabolism mediates

hepatocyte injury. The mitochondria are the sites where oxidative phosphorylation occurs and ATP is produced. The process of oxidative phosphorylation inherently generates the reactive oxygen species, superoxide anion, and therefore, is a major source for the production of reactive oxygen species. Interestingly, mitochondrial dysfunction has also been shown to have a role in impaired hepatic regeneration.

Mice deficient in the inner mitochondrial anion carrier uncoupling protein-2 (UCP2), a negative regulator of superoxide production^[17], show impairment in their hepatic regenerative response after partial hepatectomy^[18]. The characteristics associated with this impairment in liver regeneration are reminiscent of those observed in ethanol-fed and leptin deficient ob/ob animals, and include elevated levels of reactive oxygen species, as well as increased and sustained expression of p21 and p38 MAPK.

UCP2 is induced by fatty acids and its expression is upregulated in the livers of ethanol-fed and ob/ob animals^[19,20]. Although one would expect that increased UCP2 would inhibit reactive oxygen-mediated impairment of liver regeneration, this does not appear to be the case^[18]. It has been suggested that the excess fuel supply present in the fatty liver promotes increased production of superoxide to levels beyond the capacity of the increased UCP2. This results in a net increase in reactive oxygen production that leads to the reactive oxygen species-mediated activation of signals, cell cycle inhibition, and impaired hepatic regeneration^[18].

G2/M transition

Using cells based on the well-differentiated hepatoblastoma cell line, Hep G2, that were genetically engineered to express alcohol dehydrogenase, our group investigated the effects of ethanol on cellular replication. Initially, it was shown that culturing these cells, termed VA-13 cells, in the presence of 25 mmol/L ethanol resulted in a decrease in the accumulation of the number of cells in the culture, and that this effect could be ameliorated by inhibiting alcohol dehydrogenase activity^[21]. Additionally, it was shown that inclusion of cyanamide, an inhibitor of aldehyde dehydrogenase, accentuated this impairment and that culturing the VA-13 cells in the presence of isopropanol, had no effect on cell accumulation. Alcohol dehydrogenase-mediated metabolism of isopropanol results in the production of acetone, not acetaldehyde. These results led to the proposal that acetaldehyde was responsible for the impairment in cell accumulation^[21].

Further investigation of the mechanisms of this impairment revealed an increase in the percentage of cells at the G2/M transition of the cell cycle in cultures of VA-13 cells maintained in ethanol^[22]. The activity of the cyclin-dependent kinase, CDK1, is required for mitosis to occur^[23]. Catalytic activity of CDK1 is negatively regulated by phosphorylation at threonine 14 (Thr 14) and tyrosine 15 (Tyr 15) and positively regulated by its interaction with cyclin B1. It was shown that the inactive phosphorylated form of CDK1 was increased by ethanol metabolism. Additionally, it was shown that there was no impairment in the ability of CDK1 to associate with cyclin B1. These findings indicated

that the G2/M cell cycle arrest was, at least in part, the result of an increase in the phosphorylated form of CDK1 and decreased activity of this cyclin-dependent kinase, the activity of which is required for mitosis^[22].

Recently we have begun studies investigating the mechanisms by which ethanol metabolism results in increased phosphorylation of CDK1, and have found that ethanol metabolism activated the checkpoint kinases Chk1 and Chk2. A number of stages or transitions at which the cell cycle is delayed or arrested have been described and have been termed checkpoints^[24]. These checkpoints are highly conserved evolutionarily. It is generally thought that the checkpoints have been conserved because of the importance of repairing damaged or inaccurately replicated DNA, thereby ensuring genomic integrity.

Cell cycle checkpoints result from the activation of signal transduction pathways that are mediated by ataxia telangiectasia mutated (ATM) and ATM-and Rad3-related (ATR), two closely related, highly conserved kinases with catalytic domains related to the phosphatidylinositol 3-kinase^[25]. ATM and ATR are thought to share the responsibility as the apical protein kinases of all cell cycle checkpoints with the possible exception of the mitotic spindle checkpoint^[25]. A variety of stimuli can initiate activation of the ATM/ATR regulated signal transduction pathways these include: DNA damage, delayed or incomplete DNA replication, viral infection, or oxidative stress^[25,26]. In cells of human origin, ATM and ATR primarily mediate their actions through two downstream effector kinases Chk1 and Chk2^[27]. One of the cell cycle transitions that can be regulated by this signal transduction cascade is the transition from the G2 to the M phase of the cell cycle. Although Chk1 and Chk2 are structurally unrelated serine/threonine protein kinases that are activated through phosphorylation by either ATM or ATR^[28], functionally they exhibit significant overlap with respect to substrate specificity, which appears in some circumstances to function redundantly.

Chk1 and Chk2 react with a variety of substrates involved in the G2/M cell cycle arrest. Among the more important are the phosphatase Cdc25c, and the tumor suppressor p53^[29-31]. Cdc25c is a phosphatase that removes the inhibitory phosphates at Tyr 15 and Thr 14 of CDK1^[32]. Both Chk1 and Chk2 phosphorylate Cdc25c at Ser 216. This phosphorylation creates a 14-3-3 protein-binding site; binding of 14-3-3 to Cdc25c results in Cdc25c being sequestered in the cytoplasm, inhibiting its transport to the nucleus where it is required to dephosphorylate CDK1^[32]. Using the VA-13 cells we have found that ethanol metabolism results in activation of both Chk1 and Chk2 and an increase in the phosphorylation of Cdc25c at serine 216. This may inhibit the dephosphorylation of CDK1, and contribute to the cell cycle arrest at the G2/M transition.

The tumor suppressor p53 is a transcriptional activator and therefore, binds specific DNA sites and modulates the transcriptional activity of target genes. The activity of p53 is primarily regulated by post-translational modification^[33]. Chk1 and Chk2, as well as ATM and ATR, phosphorylate p53. Phosphorylation and acetylation of p53 enhances p53 activity by increasing its stability, making it a less

desirable substrate for Mdm2-mediated degradation, and increasing its affinity to its target sites^[34-36]. Therefore, p53 mediates its actions by modulating the expression of its downstream targets. Among the cell cycle transcriptional targets that are up-regulated by p53 are p21 and 14-3-3^[37]. Additionally, expression of cyclin B1, the cyclin required for the G2/M activity of CDK1, is repressed^[38]. We have shown that the phosphorylation of p53 is increased in VA-13 cells exposed to ethanol, and that there is an increase in the expression of the cyclin dependent-kinase inhibitor p21 indicating that p53 may also be involved in the ethanol metabolism-mediated G2/M cell cycle arrest. Thus, multiple p53-dependent and-independent pathways regulate the G2/M transition.

Cell cycle arrest and liver disease

There is little doubt that ethanol impairs the replication of hepatocytes by a number of mechanisms, and that this impairment may have detrimental consequences not only in terms of inhibiting cellular replication, but also perhaps by aberrant cellular replication. The replacement of damaged or injured hepatocytes lost from the liver parenchyma normally occurs by the replication of mature hepatocytes^[39]. The normal replication of mature hepatocytes can be inhibited by cell cycle delay or arrest^[11,12,40,41]. Under conditions where normal mature hepatocyte replication is inhibited, the replication of the bipotential progenitor cells known as oval cells is increased to compensate for the inhibition of normal hepatocyte replication^[40,42,43]. Because oval cells are bipotential and can differentiate into either hepatocytes or bile ductular epithelium, it has been proposed that impairment of the primary pathway of hepatocyte replacement not only leads to regeneration of hepatocytes but also to increased proliferation of ductals^[44]. Importantly, it has been shown that there is a very strong correlation between the increased proliferation of bile ducts and liver fibrosis in patients suffering from alcoholic liver disease^[42,45]. This has led to the suggestion that impairment of mature hepatocyte replication and increased replication of oval cells contributes to portal fibrogenesis^[44].

CONCLUSION

Alcoholic live disease is a complex multifactorial disease. It appears that the biochemical changes that occur in the liver as a result of ethanol are primarily responsible for the ethanol-induced toxicity to this organ. The liver has a tremendous capacity to replace cells lost to toxic insult. It is clear that the biochemical changes that occur as a result of ethanol metabolism can affect the replication of hepatic cells and potentially the recovery of the liver. The impairment in cellular replication may initiate or potentate liver damage by impairing the regeneration of cells lost to ethanol metabolism-mediated toxic injury or induction of aberrant replicative pathways. Further investigation of these dysfunctions is required to fully delineate the role of impaired cellular replication in alcoholic liver disease.

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TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, Series Editor

Effect of ethanol on pro-apoptotic mechanisms in polarized hepatic cells

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Abstract

Chronic ethanol consumption is associated with serious and potentially fatal alcohol-related liver injuries such as hepatomegaly, alcoholic hepatitis and cirrhosis. Moreover, it has been documented that the clinical progression of alcohol-induced liver damage may be associated with an increase in hepatocellular death that involves apoptotic mechanisms. Although much information has been learned about the clinical manifestations associated with alcohol-related diseases, the search continues for a better understanding of the molecular and/or cellular mechanisms by which ethanol exerts its deleterious effects such as the induction of pro-apoptotic mechanisms and related cell damaging events. As part of the effort to enhance our understanding of those particular cellular pathways and mechanisms associated with ethanol toxicity, researchers over the years have utilized a variety of model systems. Recently, work has come forth demonstrating the utility of a hybrid cell line (WIF-B) as a cell culture model system for the study of alcohol-associated alterations in hepatocellular mechanisms. Success with such emerging model systems could aid in the development of potential therapeutic treatments for the prevention of alcohol-induced apoptotic cell death that may ultimately serve as a significant target in delaying the onset and/or progression of clinical symptoms of alcohol-mediated liver disease. This review article summarizes the current understanding of ethanol-mediated modifications in cell survival and thus the promotion of pro-apoptotic events with emphasis on analyses made in various experimental model systems, particularly the more recently characterized WIF-B cell system.

INTRODUCTION

It has been well established that chronic ethanol consumption can lead to a variety of pathological consequences (ethanol-related liver injuries such as hepatomegaly, fatty liver, alcoholic hepatitis and cirrhosis)^[1,2], yet the mechanism(s) by which ethanol causes hepatotoxicity requires further clarification. From several years of investigation, certain factors have been identified as having a role in ethanol-induced toxicity, such as changes in redox status (NAD⁺/NADH ratio), the accumulation of acetaldehyde (a product of ethanol oxidation), depletion of antioxidants such as glutathione, and the generation of reactive oxygen species (ROS)^[3-8]. As a consequence, it has been demonstrated that amongst the various deleterious effects that can result from ethanol administration, it has been shown that alcohol-induced diseases are accompanied by morphological liver changes that include the increased production of apoptotic cells^[9-11]. More specifically, it has been demonstrated that ethanol administration is linked to hepatocyte apoptosis^[12-14] and that the number of apoptotic cells detected in the liver correlates with the development of ethanol-induced pathological liver injury^[15].

Apoptosis is a regulated mode of cell death, which is characterized by specific biochemical and morphological changes in the cell. Morphologically, during induction of apoptosis, the affected cells shrink, lose cytoskeletal contacts and undergo chromatin condensation. During the terminal phase of the apoptotic process, the nucleus collapses followed by fragmentation of the entire cell into apoptotic bodies which are recognized and eliminated through phagocytosis by neighboring cells^[16-18]. The search for the biochemical and signal transduction pathways involved in producing the characteristic apoptotic morphological cellular changes has been underway for several years. The current dogma is that apoptosis is

thought to occur by two main pathways involving either an extrinsic route (which utilizes death receptors), and/or an intrinsic pathway that involves mitochondrial intracellular stress signals^[19]. Once triggered, the apoptotic machinery is set into motion through the activation of a family of intracellular cysteine proteases known as caspases, which amplify appropriate signals by acting as initiators as well as executioners in the death program. For instance, there are downstream executioner enzymes (caspases 3, 6, and 7) that are activated by upstream initiator caspases (caspases 8, 2, 9, and 10) that are in turn regulated by specific protein-protein interactions. As an example, caspases may be activated in an extrinsic manner by membrane signaling events (death domain transmembrane receptors) and/or by intracellular intrinsic changes resulting in the release of specific proteins from the mitochondria. Intertwined in these pathways are the proteins that control the intrinsic or extrinsic routes of apoptosis that often belong to families that have specific domains that mediate their action. Some of the regulatory proteins that have been identified include those of the Bcl-2 family, which possess anti-apoptotic as well as pro-apoptotic activity *via* the *Bcl-2* and *Bax* genes respectively.

Normally, in a healthy individual, the highly regulated apoptotic system is counterbalanced by cytoprotective signals that maintain tissue homeostasis. However, when an organism is subjected to repeated or exacerbated insults of a pathological stimulus such as alcohol, pro-apoptotic death factors can be inappropriately expressed shifting the balance towards enhancement of the apoptotic machinery and subsequent deleterious effects. Hence, it has become clinically relevant that a better understanding of the mechanisms and factors involved in apoptosis be ascertained which has lead to an emergence of studies concerning the role of apoptosis in the initiation and progression of alcohol-induced liver injury.

ETHANOL-MEDIATED PATHOLOGICAL FACTORS AND APOPTOSIS

Over the past decade, significant progress has been made concerning the identification of contributing factors that are involved in the initiation and progression of apoptosis in both clinical and experimental alcoholic liver disease states. In general, it has been shown that alcohol-mediated apoptosis is a multi-factorial process that could involve: (1) oxidative stress mechanisms^[20]; (2) the effects of various cytokines, particularly TNF- α and TGF- β ^[21,22]; (3) the involvement of death receptor pathways (TNF-receptor 1 and Fas/CD95)^[7,23]. How and when these factors elicit hepatotoxic effects is not completely understood, however it has been noted that a direct correlation exists between the ethanol concentration and time of exposure in the ability of these factors to promote apoptotic and/or necrotic cell death. For instance, it has been shown that higher amounts of alcohol resulted in a decline in apoptosis with an increase in promotion of necrotic cell death, presumably from the induction of microsomal cytochrome components, specifically cytochrome P450 2E1 (CYP2E1). Conversely, at lower concentrations of

ethanol, apoptosis is the preferential mode of injury as death was found to be triggered by the Fas receptor system^[24]. Thus, ethanol administration is related to cellular injury mechanisms, yet a complete understanding of the presumably intertwined factors that can be involved in hepatotoxicity is still sought. The following is a brief review of current knowledge concerning the factors identified in alcohol-mediated apoptotic liver damage.

Oxidative stress and alcohol-induced liver apoptosis

Following the recognition that ethanol and its metabolites induce the formation of reactive oxygen species (ROS) in liver cells; studies were able to link the ethanol-mediated induction of oxidative stress to the observed enhancement of apoptosis. Specifically, it has been demonstrated that one of the mechanisms responsible for ethanol-induced hepatotoxicity appears to involve the induction of intracellular enzymes, such as alcohol dehydrogenase (ADH) and the induction of CYP2E1, that oxidize ethanol to reactive metabolites, producing reactive oxygen species and lipid peroxides^[20]. In linking the ethanol-induced oxidative stress with liver cell apoptosis, it was determined that ROS cause damage to the mitochondria by altering mitochondrial membrane potential and/or membrane permeability. This in turn can initiate the release of pro-apoptotic factors, such as cytochrome c, thus activating the caspase cascade^[25-27]. Identified targets in this process include mitochondrial DNA and specific proteins that are promoters of apoptosis (Bax, Bad, and Bid), which are related to the Bcl-2 oncogene family^[7].

Ethanol treatment was also shown to impair antioxidant levels in hepatocytes, resulting in ROS generation and increased oxidative stress. For example, the balance of glutathione is impaired by ethanol treatment and was found to be involved in the regulation of mitochondrial function in alcohol-associated apoptosis^[28]. In particular, it was determined that the mitochondrial glutathione (mGSH) pool itself is depleted after ethanol administration, leading to the induction of the apoptotic cell death program^[29-31].

Overall, in reviewing the data, it is clear that ethanol administration results in enhanced oxidative stress; that mitochondria are intimately involved in the survival of the cell as both a producer and target of ROS; and that apoptotic death can be the consequential end of these changes.

The role of cytokines in ethanol-induced apoptosis

Cytokines are produced by multiple cell types in the liver and are integral in cellular signaling processes. Normally, liver cells use well-developed defense mechanisms as protection against cytokine-mediated damage. However, when toxins such as ethanol stress the cells, they lose their survival protection and become susceptible to the effects of the various cytokines^[21].

This scenario has been shown as the ethanol-related release of cytokines [e.g. tumor necrosis factor- α (TNF- α), interleukin-1 beta (IL-1 β), IL-6, IL-8, IL-10, and transforming growth factor-beta 1, TGF- β 1] is associated with the promotion of pro-apoptotic mechanisms^[22,32]. Furthermore, TNF- α and TGF- β in particular have

been strongly implicated in ethanol-induced apoptosis. Specifically, it has been well documented that the chronic exposure of ethanol can result in an elevation of hepatic TNF- α , which in turn has been related to the activation of caspases and the apoptotic program^[8,33,34]. One pathway in which TNF- α -associated apoptosis occurs is through the interaction with the death receptor family, Fas ligand/Fas, and TNF/TNF receptor (TNFR) systems. Within this family are three characterized receptors (TNFR1, TNFR2, and the Fas antigen, which are structurally related with shared homology for a death domain sequence, a C-terminal intracellular domain that is involved in apoptosis pathways. In another mechanism of TNF- α -induced cell death, the role of TNF- α and mitochondrial GSH depletion in ethanol-related apoptosis was further defined. As mentioned above, ethanol impairs the transport of cellular glutathione (particularly mGSH) resulting in its depletion and the subsequent sensitization of the cell to undergo an apoptotic death. This ethanol-related depletion of mGSH was also found to be associated with TNF-mediated cell death in hepatocytes, as the cells became apoptotic as a result of TNF-related events (such as the generation of hydrogen peroxide). Validation of this association came through studies demonstrating that TNF- α -induced oxidative stress and subsequent apoptosis could be prevented by incubating hepatocytes with antioxidants as well as S-adenosyl-L-methionine, a substance that increases mGSH levels^[35]. Overall, the data obtained from numerous studies has demonstrated that ethanol exacerbates TNF-related hepatotoxicity, in part, through the induction and perpetuation of apoptotic cell death pathways.

Participation of TGF- β 1 in ethanol-induced apoptosis has also been widely studied. TGF- β 1 is the prototype member of the TGF- β family that is involved in a diverse array of biological activities including development, differentiation, tissue remodeling, and apoptosis^[36,37]. Several liver cells (including hepatocytes and hepatic stellate cells) can produce TGF- β 1 in response to alcohol toxicity, and this production is considered to have a significant impact in the initiation and progression of alcoholic liver disease^[38,39]. Researchers have identified some of the pro-apoptotic pathways that have been induced by the up-regulation of TGF- β 1 in response to ethanol treatment. It has been demonstrated utilizing a fetal hepatocyte model that TGF- β 1 induces apoptosis by producing oxidative stress in the cells by increasing ROS production and decreasing the level of a natural antioxidant, glutathione^[40,41]. In addition, the increase in TGF- β 1-mediated signaling, resulting in caspase activation, can be associated with the enhanced cleavage of the caspase substrate PARP [poly (ADP)-ribose polymerase]^[42]. It has also been identified that TGF- β 1 can activate the caspase cascade by either of the two primary pro-apoptotic mechanisms (death receptor-mediated pathway or intracellular stress-signaled pathway involving mitochondrial changes and the release of cytochrome c), and either pathway can involve the formation of an apoptosome complex that activates the proteolysis of the cell^[43].

Involvement of the Fas/Fas ligand system in ethanol-induced liver cell apoptosis

Of the death receptor-mediated pathways, the involvement of the Fas/Fas ligand system has proven to play a significant role in alcohol-related apoptosis in the liver. The pro-apoptotic signaling of Fas (a glycosylated cell-surface protein) is similar to other death receptors in that a specific interaction of the oligomerized receptor with an associated ligand (i.e. Fas ligand) or antibody stimulates the recruitment of the cytoplasmic adapter protein (Fas associated death domain, FADD) that mediates caspase activation and the signaling of the apoptotic death program^[44]. In the liver, increases in the expression of membrane-bound Fas as well as the levels of soluble Fas and Fas ligand have been associated with pathological conditions associated with alcoholic hepatitis^[13,45]. Also, the up-regulation of Fas ligand in hepatocytes following ethanol treatment is thought to induce apoptotic death of neighboring cells by interacting with the Fas receptor on the surface of those cells^[45]. Overall, it is thought that the activation of Fas results in the promotion of the apoptotic program that can include the induction of the caspase cascade and permeabilization of the mitochondrial membrane^[46,11]. Moreover, the importance of the Fas/Fas ligand system in caspase 3 activation and apoptosis in the liver following ethanol treatment was substantiated as the administration of a caspase 3 inhibitor was shown to block ethanol-induced caspase 3 activity along with apoptosis^[47]. Thus, there is a growing amount of evidence that the Fas/Fas ligand system is a critical element in the activation of the caspase cascade and the subsequent demise of liver cells following chronic ethanol abuse.

MECHANISMS OF ALCOHOL-INDUCED APOPTOSIS

The enhancement of our understanding of the mechanisms involved in the apoptotic cascade is becoming exceedingly important and relevant to disease states such as ALD. However, studies concerning the effect of ethanol on cellular processes such as apoptosis are often limited due to the applicability of the model system. As an example, the dose required for ethanol-induced hepatotoxicity *in vitro* has been found to vary between cell strains presumably due to the differences in the metabolism of ethanol by ADH and CYP2E1^[25]. Also, the use of freshly isolated hepatocyte models were found to produce confounding results when searching for mechanistic parameters as the cells dedifferentiate (i.e. lose cell polarity and many liver-specific characteristics such as the ability to metabolize ethanol) within a few hours after isolation, making the cells useful for short-term culture conditions only. Despite these limitations, many studies have been performed yielding useful information concerning pro-apoptotic mechanisms that are induced following ethanol administration.

Around fifteen years ago, interest began to develop concerning the relevance of apoptotic cell death in liver pathology with particular interest in hepatocellular injury associated with ethanol toxicity^[48]. Since the

acceptance that apoptosis may play a significant role in the development of liver injury, studies have searched for the potential mechanisms and pathways that are involved. In animal models, it was demonstrated that ethanol feeding resulted in an increase in apoptotic cells in the liver^[11,49] that was subsequently shown to be related to the induction of reactive oxygen species (ROS) as a result of ethanol metabolism and the generation of acetaldehyde^[50]. Furthermore, it was concluded that the ethanol-induced ROS production was driven by redox changes that ultimately lead to mitochondrial dysfunction and caspase activation. These intrinsic events were not, however, the only mechanism by which ethanol was found to induce apoptosis. Specifically, it was determined that ethanol can also induce apoptosis through the involvement of the extrinsic death receptor pathway via the Fas/CD95 receptor system^[50].

In other works, acute studies involving ethanol treatment to cells isolated from animals also demonstrated that oxidative stress was involved in mitochondrial membrane changes that were found to result in cytochrome c release and caspase activation^[51,52]. Additionally, studies using developed recombinant cell lines demonstrated the role ethanol-inducible CYP2E1 has in alcohol-mediated apoptosis. Particularly, it was shown that ethanol-induced apoptosis was related to oxidative stress and subsequent lipid peroxidation in CYP2E1 over expressing cells^[53], confirming the central role ROS and CYP2E1 have in ethanol-mediated cell death as hypothesized in previous works^[25,54-56]. Additional evaluations demonstrated that such activation of intrinsic apoptotic pathways could be related to alcohol-mediated glutathione depletion which enhances the sensitivity of the cell to succumb to death, especially when faced with additional insults (e.g. increases in TGF- β expression or the presence of Hepatitis virus)^[52,57-59]. Furthermore, continued searches for pro-apoptotic mechanisms involved in ethanol-mediated cell injury have provided additional information regarding the potential contribution regulatory mechanisms have in hepatocellular apoptosis, such as the role of NF- κ B and ethanol-mediated alterations in proteasome function^[60,61], whether the processes are p53 dependent^[62,63], and what role pro-apoptotic Bcl-2 family proteins have in mediating mitochondrial permeability and apoptosis during alcohol cytotoxicity^[59].

Overall, substantial information has been gained linking oxidative stress from alcohol metabolism *via* ADH and CYP2E1 to the induction of several pro-apoptotic mechanisms involving both intrinsic and extrinsic apoptosis pathways. However, the development and use of additional model systems, such as a recently described polarized hepatic cell line, may significantly contribute to our understanding of pro-apoptotic mechanisms induced as a consequence of ethanol administration.

ETHANOL-INDUCED APOPTOSIS IN A POLARIZED LIVER CELL MODEL

Many of the studies performed previously concerning the effect of ethanol on cellular processes have used alcohol-

fed animal models as sources of isolated hepatocytes in order to provide more compatible models to human pathology than most cell culture systems provide. However, limitations were often observed in the model systems used. For example, the study of protein trafficking events in cultured systems has been hampered by the lack of a well-polarized cell that adequately mimics the complexity of *in vivo* hepatocyte protein delivery systems (i.e. the indirect transport of apical membrane proteins). Recently, our laboratory has demonstrated that the use of the WIF-B hepatoma hybrid cell line (in which polarity is a stable and dominant trait)^[64,65] is an ideal *in vitro* model for studying the effects of ethanol on cellular processes^[66]. Specifically, the WIF-B cells were found to exhibit alcohol dehydrogenase (ADH) activity allowing for the efficient metabolism of ethanol. Also, increases were observed in cellular triglyceride levels in the WIF-B cells following ethanol treatment, similar to the reported fat accumulation that is observed in human alcoholic liver injury. In addition, treatment of the WIF-B cells with alcohol resulted in morphological changes in the cells as demonstrated by decreases in bile canaliculi formation and cell-cell contacts^[66]. Thus, the use of the WIF-B cell line to study ethanol-induced cellular mechanisms is quite appropriate since the cells present a unique culture system that has the ability to metabolize ethanol and is a system that more closely resembles human morphology than other known cell lines.

The WIF-B hybrid cell is a cross between a human fibroblast (WI 38) and a Fao rat hepatoma cell line^[64,67]. This clone represents a polarized and differentiated cell of hepatic origin that exhibits long-term viability in culture, develops a hepatocellular-polarized phenotype and expresses human genes coding for liver-specific proteins (albumin, fibrinogen, and ADH)^[68]. One of the unique characteristics of the WIF-B clone is that the cells grow in monolayers and acquire a polarized phenotype as the cells form bile canaliculi-like spaces (BC) that have concentrated apical membrane proteins. Therefore, these cells are useful for the functional studies of hepatocyte-specific properties such as the transport of membrane proteins. Indeed, several studies have utilized the advantages of the maintained polarized cell to study intercellular communication, bile acid transport systems, and membrane trafficking pathways^[69-71].

In recent studies using this hepatic model, it was demonstrated that ethanol treatment induces apoptosis via signals emanating from the pro-apoptotic death receptor systems (i.e. Fas/CD95) as well as from intrinsic signals that resulted in mitochondrial changes^[72]. Specifically, it was demonstrated that ethanol treatment of WIF-B cells resulted in a significant increase in apoptotic-related morphological changes (cells that contained condensed chromatin) that were also associated with pro-apoptotic biochemical features (indicated by the induction of caspase-3 activity). Also, it was shown that caspase-3 activation in ethanol-treated WIF-B cells was related to mitochondrial permeability transition (MPT) as ethanol treatment resulted in cytochrome c release that was found to be sensitive to cyclosporine A (an inhibitor of MPT). Additionally, it was determined that in the ethanol-induced

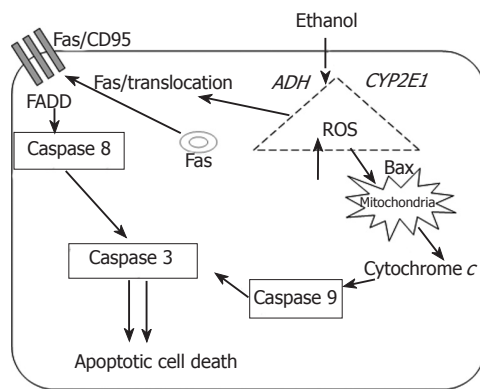


Figure 1 Schematic representation of the potential pathways involved in the initiation and propagation of ethanol-induced apoptosis. Ethanol metabolism and the consequential generation of reactive oxygen species are implicated in the activation of pro-apoptotic mechanisms via death receptor (i.e. Fas/CD95) as well as as intrinsic apoptotic pathways.

apoptosis in WIF-B cells, the activities of upstream initiator caspases (caspase-2 and caspase-8), that are directly related to membrane signaling events of death receptors such as Fas, were increased. Concurrently, it was determined that Fas protein levels in the membrane fraction of the cell were found to be enhanced without a corresponding change in mRNA levels.

Overall, it was shown that ethanol treated WIF-B cells were induced to undergo apoptosis via protease activation and this was found to be related to death receptor signaling as well as mitochondrial stress events (Figure 1). These results are consistent with those reported in other model systems, especially the work by Minana *et al*^[60]. In those studies, the investigators demonstrated that ROS (produced as a consequence of ethanol metabolism) induced apoptosis by two different mechanisms that involved MPT and the Fas receptor system. However, characterization of CYP2E1-mediated events in ethanol-mediated apoptosis in WIF-B cells has not been completed. In preliminary work, it has become evident that the WIF-B model system may be quite useful as CYP2E1 activity and protein levels can be reproducibly evaluated in these cells by ethanol as well as pyrazole treatment (unpublished data). Therefore, the utilization of WIF-B cells, a model which allows manipulation of the ethanol metabolizing systems, may significantly increase the potential of acquiring novel and clinically relevant information. Thus, further studies using WIF-B cells aim to enhance our understanding of crosstalk mechanisms that may exist between the major apoptotic-inducing pathways (extrinsic and intrinsic). Also, a more complete understanding of how apoptotic signals are favored and triggered over survival or compensatory signals is sought. Additionally, information as to how necrotic cell death mechanisms overcome apoptotic signals as the duration and amount of ethanol consumption is increased could potentially be determined using the WIF-B model system.

CONCLUSION

Apoptotic hepatotoxicity has been implicated in the pathogenesis of several liver diseases including that

involving ethanol abuse. Insights into the cellular mechanisms involved in the initiation and propagation of apoptosis will significantly impact our understanding of alcohol-induced liver disease and may lead to the potential development of therapeutic interventions. The use of an emerging model system, polarized hepatic WIF-B cells, could significantly impact the study of alcohol-related hepatocellular injury, especially concerning the delineation of mechanisms involved in ethanol-induced cell death. In addition, polarized hepatic cell cultures may aid in the acquisition of translational information as the WIF-B cells offer a more compatible model system that better correlates to human pathology for analysis of potential therapeutic targets that could modulate apoptotic mechanisms induced by ethanol.

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Novel interactions of mitochondria and reactive oxygen/nitrogen species in alcohol mediated liver disease

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Abstract

Mitochondrial dysfunction is known to be a contributing factor to a number of diseases including chronic alcohol induced liver injury. While there is a detailed understanding of the metabolic pathways and proteins of the liver mitochondrion, little is known regarding how changes in the mitochondrial proteome may contribute to the development of hepatic pathologies. Emerging evidence indicates that reactive oxygen and nitrogen species disrupt mitochondrial function through post-translational modifications to the mitochondrial proteome. Indeed, various new affinity labeling reagents are available to test the hypothesis that post-translational modification of proteins by reactive species contributes to mitochondrial dysfunction and alcoholic fatty liver disease. Specialized proteomic techniques are also now available, which allow for identification of defects in the assembly of multi-protein complexes in mitochondria and the resolution of the highly hydrophobic proteins of the inner membrane. In this review knowledge gained from the study of changes to the mitochondrial proteome in alcoholic hepatotoxicity will be described and placed into a mechanistic framework to increase understanding of the role of mitochondrial dysfunction in liver disease.

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Key words: Mitochondria; Alcohol; Liver; Oxidative stress; Nitric oxide; Proteomics; Post-translational modifications

INTRODUCTION

Long term heavy alcohol consumption is the most prevalent cause of liver-related morbidity and mortality in the United States. Excessive alcohol consumption is estimated to be the third leading cause of preventable death in the United States with up to 12 000 deaths each year attributed to alcoholic liver disease^[1]. While it has long been held that the severity of alcoholic liver disease is dependent on the dose and duration of alcohol consumption, it has become increasingly clear that other factors may play a significant role in the development of liver disease^[2]. Studies show that only 25% of heavy drinkers will develop alcoholic steatohepatitis and even less than 10% will progress to cirrhosis^[3]. These observations have led to the general hypothesis that environmental, genetic, metabolic and/or viral factors, also referred to as "hits", may influence the progression from simple fatty liver (steatosis) to more serious liver diseases^[2].

Evidence supports a "multi-hit" hypothesis in the pathophysiologic sequelae of alcoholic liver disease wherein the "first-hit" involves lipid accumulation in hepatocytes (steatosis), followed by "second-hits" that lead to more serious conditions such as alcoholic steatohepatitis, fibrosis, cirrhosis, and cancer. Examples of "second-hits" include metabolic stressors such as obesity, hypercholesterolemia, and hyperglycemia, which are components of the cardiometabolic syndrome, and environmental stressors, which may include dietary factors and pollutants such as tobacco smoke. Recent epidemiologic and clinical studies indicate that environmental tobacco smoke, a widespread toxicant, may accelerate fibrogenesis and increase the severity of a number of chronic liver diseases including hepatocellular carcinoma, hepatitis C-mediated liver injury, and primary biliary cirrhosis^[4-8]. Moreover, alcohol, tobacco smoke, and obesity have been shown to be synergistic risk factors for hepatocellular carcinoma in patients^[9]. Taken together,

these studies clearly highlight the need to identify the molecular targets and mechanisms through which these potential stressors or "hits" interact to accelerate and worsen alcoholic liver disease.

Studies suggest a potential mechanistic link among chronic alcohol consumption and many of the proposed secondary stressors, which include increased oxidative damage, hypoxic stress, and disrupted redox cellular signaling. Interestingly, the mitochondrion is a primary target for many of these metabolic derangements, which can also differentially exacerbate hepatic pro-inflammatory responses. Furthermore, the mitochondrial proteome is exquisitely sensitive to modifications by reactive oxygen and nitrogen species (ROS/RNS) and thus offers a unique opportunity to investigate the molecular mechanisms underlying hepatic pathobiology from chronic alcohol consumption. Accordingly, this review article will present an overview of the emerging new roles of mitochondria and reactive species in the development of alcoholic liver disease.

MITOCHONDRIAL DYSFUNCTION IN ALCOHOLIC LIVER DISEASE: BIOENERGETIC DEFECTS AND OXIDANT PRODUCTION

The effects of chronic alcohol consumption on liver have been extensively studied and have advanced our understanding of the molecular mechanisms responsible for alcohol hepatotoxicity. In general, chronic consumption of alcohol causes liver disease *via* oxidative stress, hypoxia, upregulation of pro-inflammatory cytokines, and bioenergetic defects involving the interactions of multiple liver cell types. Early in the disease process gut-derived endotoxin^[10] activates Kupffer cells, which release a variety of potentially toxic substances including cytokines and ROS/RNS that negatively affect hepatic stellate cells and hepatocyte functions^[11]. Consequently, a number of responses occur in hepatocytes including increased ROS/RNS production, mitochondrial damage, and altered nitric oxide (NO)-dependent control of respiration^[12-14]. This profound disruption in mitochondrial metabolism contributes, in part, to liver disease by placing hepatocytes under bioenergetic stress. This is important because the inability to maintain hepatic ATP levels will predispose the liver to permanent damage due to a depression in the ATP requiring anabolic pathways responsible for replacing damaged and/or lost cellular macromolecules.

One critical change to hepatic mitochondria after chronic consumption of alcohol is a decrease in the rate of ATP synthesis. Conclusive evidence shows that chronic alcohol exposure depresses the activities of all the oxidative phosphorylation complexes by approximately 30%-50%, except Complex II^[15,16]. Inhibition of mitochondrial protein synthesis due to alcohol-mediated damage to the mitochondrial DNA^[13,17] and ribosomes^[18,19] is proposed to contribute to decreased functioning of the oxidative phosphorylation system and depressed rates of ATP synthesis^[20,21]. Recently, studies by Cunningham and colleagues have shown that both hepatic energy charge and

NADH-linked respiration are decreased in mitochondria isolated from liver of non-human primates allowed to consume alcohol for 18 mo^[22]; thus recapitulating the findings observed in rodent studies. This alcohol-dependent loss of mitochondrial function is also predicted to cause further increases in ROS/RNS production and oxidative damage to the organelle and liver.

During electron transport in the respiratory chain, electrons can "leak" from the respiratory complexes and be passed one at a time to molecular oxygen (O_2) to form low amounts of superoxide anion ($O_2^{\bullet-}$), which is proposed to be increased in hepatocytes due to alcohol-dependent alterations in mitochondria. Mitochondria contribute to the production of ROS in hepatocytes when alcohol is consumed through the metabolism of alcohol *via* distinct oxidative mechanisms and chronic alcohol-related alterations to the oxidative phosphorylation system^[23,24]. First, ethanol metabolism increases the availability of reducing equivalents (i.e. NADH) to the mitochondrion, which results in the redox active semiquinone intermediates within Complexes I and III to be in a more "reduced" state, thereby facilitating the reduction of O_2 to $O_2^{\bullet-}$ ^[25,26] (Figure 1). Moreover, in the chronic alcohol consumer, it is postulated that mitochondrial production of ROS is elevated not only due to increased NADH delivery to the respiratory chain, but also as a consequence of molecular defects to the respiratory complexes caused by chronic alcohol consumption^[13,27-29].

The mechanism of $O_2^{\bullet-}$ production by Complex III (ubiquinone cytochrome *c* reductase) is well understood, as it is linked to the reactions of the ubiquinone or "Q"-cycle^[30]. As the levels of cytochrome *b* are decreased by chronic alcohol consumption^[27,29] this defect is postulated to decrease the rate of re-oxidation of the ubisemiquinone anion ($Q^{\bullet-}$). This resulting increase in the steady-state levels of $Q^{\bullet-}$ would subsequently lead to increased $O_2^{\bullet-}$ in mitochondria from alcohol exposed subjects (Figure 1A). In contrast, the mechanism responsible for increased $O_2^{\bullet-}$ production by Complex I (NADH dehydrogenase) in response to chronic alcohol consumption remains undefined because the sites responsible for generating $O_2^{\bullet-}$ are not known. For example, the semiquinone in the flavin mononucleotide and the various iron-sulfur centers of Complex I have all been implicated as potential sites of $O_2^{\bullet-}$ production (Figure 1B). Recent studies have demonstrated that the majority of $O_2^{\bullet-}$ generated in mitochondria respiring on the Complex II-linked substrate succinate occurs *via* reverse electron transport into Complex I^[31-33]. This mechanism involves reverse electron flow from succinate to NAD^+ providing reducing equivalents to the redox carriers of Complex I, which then function as sites of $O_2^{\bullet-}$ production (Figure 1C). The contribution of reverse electron flow through Complex I may be enhanced as a consequence of alcohol-dependent defects to the complex^[27,28]. Similarly, mitochondrial levels of ROS may be increased by chronic alcohol consumption as a consequence of increased mitochondrial CYP450 2E1 levels^[34] and as a by-product from matrix enzymes such as α -ketoglutarate dehydrogenase^[35,36]. These higher rates of ROS production in the alcohol-damaged mitochondria

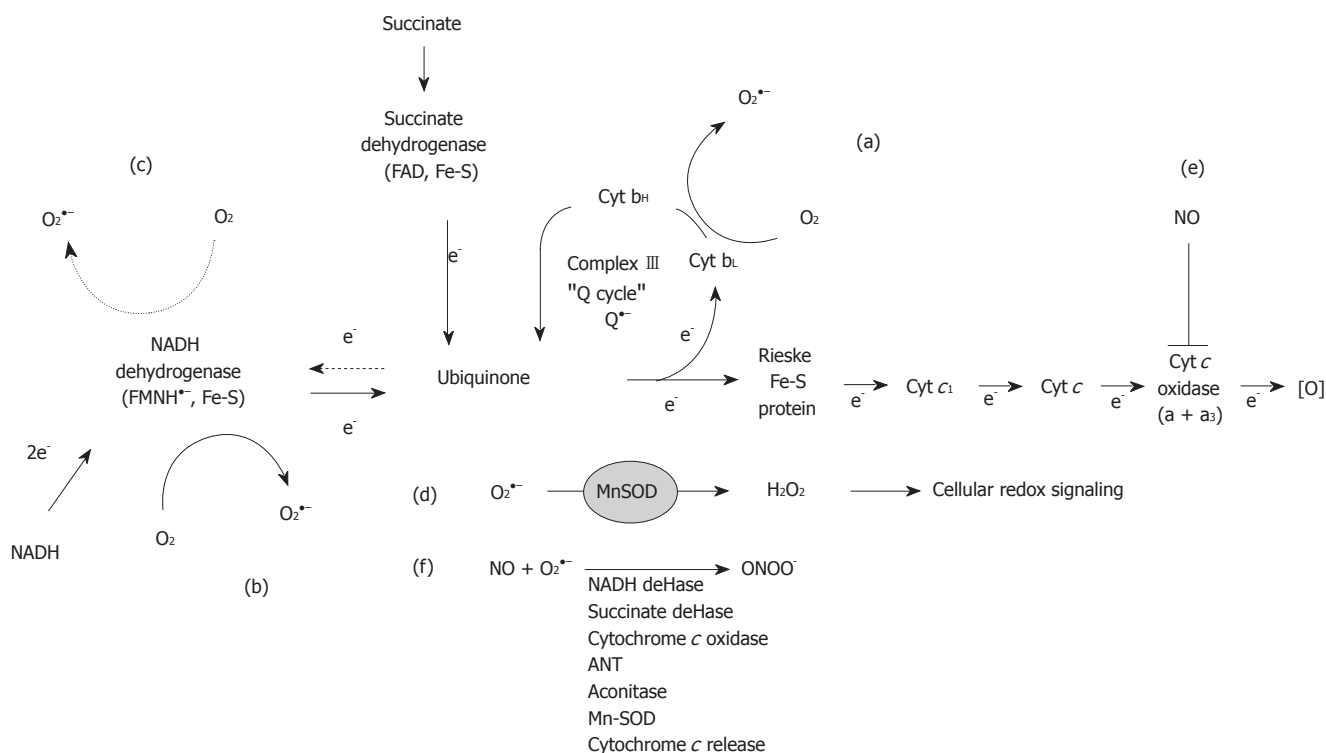


Figure 1 Superoxide production and reactive nitrogen species reactions in mitochondria. During electron transfer superoxide ($O_2^{\bullet-}$) is generated within Complexes I (b, c) and III (a) due to the presence of stable semiquinone anion species. Solid arrows illustrate forward electron flow, whereas dashed arrows indicate reverse electron flow through Complex II to increase $O_2^{\bullet-}$ production in Complex I (c). $O_2^{\bullet-}$ dismutation to hydrogen peroxide (H_2O_2) by manganese superoxide dismutase (MnSOD) affects cellular redox signaling pathways (d). Increased iNOS expression leads to diffusion of nitric oxide (NO) into mitochondria where it inhibits cytochrome c oxidase (e), which increases $O_2^{\bullet-}$ generation.

are predicted to negatively affect mitochondrial function through oxidative damage to mitochondrial macromolecules and cellular function through modulation of cellular redox signaling pathways (Figure 1D).

NITRIC OXIDE, REACTIVE NITROGEN SPECIES, AND MITOCHONDRIA PHYSIOLOGY: IMPACT ON ALCOHOL HEPATOTOXICITY

In this section some of the key characteristics by which nitric oxide (NO) and RNS affect mitochondrial function in the context of alcoholic liver injury will be presented. As discussed above, several studies have demonstrated that chronic alcohol consumption increases hepatocyte ROS production presumably from the mitochondrial respiratory chain^[12,25,37]. As these increased levels of ROS exceed those required for signal transduction and detoxification *via* antioxidant systems, mitochondrial DNA, protein, and lipid damage is predicted to be enhanced as a consequence of chronic alcohol exposure. In addition, NO production is increased in response to chronic alcohol *via* induction of inducible nitric oxide synthase (iNOS)^[12,38]. This has important ramifications for toxicity because NO and its metabolite peroxynitrite (ONOO⁻) have been implicated as key mediators of mitochondrial dysfunction^[39,40]. Indeed, the detrimental effects of NO largely stem from excess NO diffusing into the mitochondrion and reacting

with $O_2^{\bullet-}$ to produce ONOO⁻, a reactive metabolite that can directly or indirectly participate in reactions leading to inactivation of mitochondrial proteins *via* post-translational modifications^[41]. Moreover, it is known that NO can regulate mitochondrial function through reversible binding at the heme site in cytochrome c oxidase, which inhibits oxygen consumption^[42,43] and through cross-talk mechanisms with soluble guanylate cyclase^[44]. Potential impacts of NO and ONOO⁻ on mitochondria are illustrated in Figure 1E and F.

Exposure of mitochondria to low concentrations of NO results in the reversible inhibition of cytochrome c oxidase activity due to the competition of NO with O_2 at the binuclear center of the enzyme^[45-48]. Thus, NO has the effect to inhibit mitochondrial respiration. Studies from our laboratories have shown a novel role of NO in the pathogenesis of alcohol hepatotoxicity. We have demonstrated that the response of respiration to NO is altered by chronic alcohol consumption such that mitochondria from alcohol-fed animals are more sensitive to NO-dependent inhibition of respiration^[14,38]. It is proposed that this loss of control of NO signaling per se, results in excessive inhibition of the respiratory chain leading to bioenergetic dysfunction (i.e. decreased ATP synthesis) and increased ROS production^[49], which contribute to the development of alcoholic liver injury. The critical role of NO in alcohol hepatotoxicity is further supported because the increased sensitivity of mitochondrial respiration to inhibition by NO is absent in iNOS knockout mice fed alcohol chronically^[38]. Therefore,

this finding supports the hypothesis that the early induction of iNOS modifies the control of NO-dependent respiration, which contributes to the development of alcohol-dependent steatosis and inflammation^[38,50].

It has been shown that NO-mediated inhibition of respiration alters activation of hypoxia responsive targets in cells such that increased NO interferes with the upregulation of molecules required by cells to adapt to hypoxic stress^[51,52]. This may also have the effect of changing oxygen gradients in tissues. Indeed, this is precisely the series of events that we propose occurs with NO in alcohol hepatotoxicity^[49], which has been shown to be associated with tissue hypoxia^[53,54]. Several studies have clearly demonstrated that acute alcohol exposure "steepens" the hepatic oxygen gradient as a consequence of ethanol oxidation^[55,56], thus rendering the pericentral regions of the liver lobule hypoxic^[53]. Thus, the effect of acute hypoxia in an individual actively drinking is proposed to be exaggerated in the chronic alcohol consumer as a consequence of the alcohol dependent increase in inhibition of mitochondrial respiration by NO^[14]. In fact, studies by Arteel and colleagues demonstrate that chronic alcohol consumption increases liver hypoxia^[54]. This concept is important to consider because it suggests that the interaction of hypoxia and disrupted NO signaling in mitochondria may accelerate the progression from steatosis to more severe liver pathologies. Moreover, it is these conditions that will lead to the irreversible post-translational modification and inactivation of mitochondrial proteins, which are proposed to contribute to alcohol-mediated mitochondrial dysfunction and hepatic pathobiology.

MODIFICATION TO THE LIVER MITOCHONDRIAL PROTEOME IN RESPONSE TO CHRONIC ALCOHOL CONSUMPTION

From the discussion above it is clear that increased NO and altered control of respiration by NO can lead to excessive ROS/RNS formation and the post-translational modification of mitochondrial proteins. Specifically, it has been shown that ROS, RNS, and reactive lipid species can modify critical amino acid residues thereby disrupting the catalytic function of proteins. As stated above, these toxic effects on proteins largely stem from the diffusion of NO into mitochondria and its reaction with $O_2^{\bullet-}$ to generate more reactive species, such as ONOO⁻, other secondary RNS, and reactive lipid products. Because alterations in the redox state of protein thiols are important in regulating mitochondrial function such as respiration and oxidant production^[57,58], the identification of proteins with oxidized and/or modified thiol groups is critical for elucidating the mitochondrial defects that contribute to alcoholic liver disease.

A number of reversible and irreversible modifications to cysteine residues are known to occur upon interaction of free sulfhydryl groups (-SH) with ROS, RNS, and reactive lipids. Reversible modifications to thiols include the formation of nitrosothiols (P-SNO), sulfenic acids (P-SOH) and mixed disulfides (P-SSG). Cysteine residues can also be irreversibly oxidized to higher oxidation states such as sulfinic (P-SO₂H) and sulfonic (P-SO₃H)

acids by ROS and RNS. Each of these modifications has the potential to elicit a unique biological response that may disrupt normal mitochondrial functions. Studies have linked the oxidation and modification of protein thiols with induction of the mitochondrial permeability transition, alterations in energy metabolism, and oxidant production^[59]. Our laboratories have shown an alcohol-dependent loss of function of the mitochondrial low K_m aldehyde dehydrogenase (ALDH) as a consequence of oxidative modification of thiols^[60]. This is significant because inactivation of this important detoxification enzyme could potentially lead to increased levels of acetaldehyde and other reactive aldehyde species, which themselves have been shown to inactivate proteins through cysteine modifications^[61,62]. Recently, Song and colleagues also demonstrated alcohol-dependent inactivation of ALDH and several β -oxidation enzymes *via* oxidation and nitrosation of thiols^[63]. Taken together, these findings are consistent with the concept that modification of protein thiols may contribute to alcoholic steatosis and mitochondrial dysfunction through inactivation of proteins critical to the energy conservation pathways in liver.

While changes in the redox status of cysteine residues are known to affect mitochondrial activities, other types of post-translational modification should be considered as well. Tyrosine nitration (P-NO₂) is generally thought to induce permanent loss of protein function^[64] however recent studies suggest that tyrosine nitration may be oxygen-regulated, target-selective, and reversible such that it may function as a "nitrative signaling" process controlling mitochondrial energy metabolism^[65,66]. Studies have also established that 4-hydroxynonenal (4-HNE), a reactive lipid, may play an important role in alcohol hepatotoxicity *via* modification of key signaling proteins^[67,68] and inhibition of cytochrome *c* oxidase activity^[69,70]. 4-HNE has also been shown to inhibit several of the matrix dehydrogenase enzymes^[71,72] and proteins of the β -oxidation system^[73]. Moreover, electrophilic lipids like 4-HNE and the cyclopentenone 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂ have been shown to co-localize to mitochondria and induce mitochondrial ROS production^[74]. These results provide strong evidence suggesting that reactive lipids may disrupt mitochondrial function through selective targeting of mitochondrial enzymes.

In addition to post-translational modifications, it is also proposed that changes in the levels (i.e. abundances) of proteins that comprise the mitochondrial proteome may also negatively affect mitochondrial bioenergetics leading to liver injury. Early studies by Coleman and Cunningham established a key link between the chronic alcohol-related defects in Complexes I, III, IV, and V and losses in the 13 mitochondrial encoded polypeptides and redox centers that comprise the oxidative phosphorylation system complexes^[27,75,76]. Similarly, using a variety of proteomics approaches our laboratories have extended these findings to demonstrate a coordinated decrease in both mitochondrial and nuclear encoded subunits of the respiratory complexes, particularly those that comprise cytochrome *c* oxidase^[12,13]. Moreover, proteomic analyses revealed that 40 additional mitochondrial proteins had altered levels in response

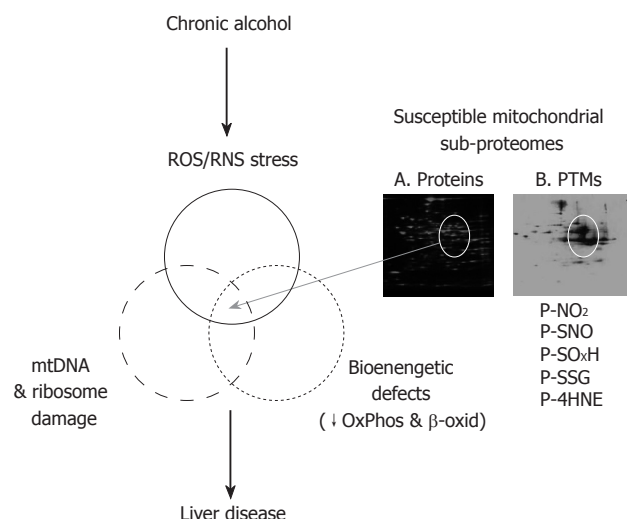


Figure 2 Overlapping alterations to the liver mitochondrial proteome contribute to the development of alcohol-induced liver disease. This figure illustrates the concept that there is a unique mitochondrial sub-proteome that when altered by chronic alcohol mediated mtDNA/ribosome damage, increased ROS/RNS production, and energy deficits contribute to the development of alcohol hepatotoxicity. These alterations involve changes in both protein levels (panel A) and post-translational modifications (PTMs, panel B) to this susceptible population of mitochondrial proteins.

to chronic alcohol consumption^[13]. These studies are interesting in that, previously unidentified alterations in several key energy metabolism enzymes of β -oxidation, the TCA cycle, and amino acid metabolism, as well as several mitochondrial chaperones were found to be altered by chronic alcohol exposure. Importantly, these changes can also be linked to pathways with a clear impact on one of the primary pathologies of alcoholic liver disease, i.e. steatosis. These findings highlight the power of proteomics to detect previously unidentified alterations to "vulnerable" mitochondrial sub-proteomes following chronic alcohol consumption that contribute, in part, to the development of alcoholic liver disease (Figure 2). These alterations may include changes in protein levels as well as post-translational modifications to this susceptible sub-set of mitochondrial proteins.

In summary, mitochondria play a variety of roles in a number of essential cellular functions including energy production and homeostasis, redox cell signaling, and apoptosis. Disruption of mitochondrial function, manifested by the inability to maintain cellular ATP levels is critical to the development of chronic alcohol-induced liver injury. Moreover, as a primary source for the formation of ROS and RNS, the mitochondrion is recognized as a critical component involved in cellular stress responses. While the sequence of events leading to alcohol induced mitochondrial dysfunction and liver injury remains undefined, emerging evidence suggests that: (1) interaction of NO with the respiratory chain may predispose hepatocytes to hypoxic injury and (2) post-translational modifications of critical residues within mitochondrial proteins by reactive species may alter cellular functions including energy metabolism and redox signaling. Similarly, an alcohol-mediated increase in ROS/RNS can damage mitochondrial DNA and ribosomes

resulting in decreased mitochondrial protein synthesis, which ultimately translates into a severe mitochondrial dysfunction. Investigations have provided novel mechanistic information regarding the impact of chronic alcohol consumption on the mitochondrial proteome; providing unique insight into the molecular mechanisms responsible for disease. This knowledge will clearly advance the design and testing of novel mitochondria-specific therapeutics in the treatment of alcoholic liver disease.

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TOPIC HIGHLIGHT

Natalia A Osna, MD, PhD, Series Editor

Alcohol-induced steatosis in liver cells

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Abstract

Alcohol-induced fatty liver (steatosis) was believed to result from excessive generation of reducing equivalents from ethanol metabolism, thereby enhancing fat accumulation. Recent findings have revealed a more complex picture in which ethanol oxidation is still required, but specific transcription as well as humoral factors also have important roles. Transcription factors involved include the sterol regulatory element binding protein 1 (SREBP-1) which is activated to induce genes that regulate lipid biosynthesis. Conversely, ethanol consumption causes a general down-regulation of lipid (fatty acid) oxidation, a reflection of inactivation of the peroxisome proliferator-activated receptor- α (PPAR- α) that regulates genes involved in fatty acid oxidation. A third transcription factor is the early growth response-1 (Egr-1), which is strongly induced prior to the onset of steatosis. The activities of all these factors are governed by that of the principal regulatory enzyme, AMP kinase. Important humoral factors, including adiponectin, and tumor necrosis factor- α (TNF- α), also regulate alcohol-induced steatosis. Their levels are affected by alcohol consumption and by each other. This review will summarize the actions of these proteins in ethanol-elicited fatty liver. Because steatosis is now regarded as a significant risk factor for advanced liver pathology, an understanding of the molecular mechanisms in its etiology is essential for development of effective therapies.

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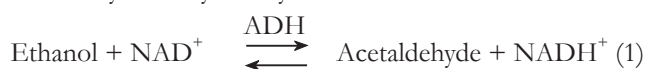
Key words: Ethanol metabolism; Fatty liver; Sterol regulatory element binding protein; Peroxisome proliferator activated receptor; Early growth response-1;

INTRODUCTION

Fatty liver (steatosis) is the earliest, most common response of the liver to moderate or large doses (i.e. binge drinking) of alcohol as well as to chronic ethanol consumption^[1]. Steatosis was previously considered a relatively harmless side effect of heavy alcohol use. However, recent evidence now indicates that alcohol-induced fatty liver enhances susceptibility of the liver to more advanced pathology if drinking continues. Individuals with alcohol-induced steatosis are vulnerable to developing alcoholic steatohepatitis (ASH), hepatic fibrosis, cirrhosis and even hepatocellular carcinoma. Obesity, which may potentiate alcoholic fatty liver, is also considered an additional risk factor for alcoholic cirrhosis^[2] and for advanced liver disease in obese nondrinkers^[3]. The latter is most often presented clinically as nonalcoholic steatohepatitis (NASH). The reason(s) why some individuals progress from fatty liver to the more advanced stages of liver disease is not clear. The current concept adopted by many investigators is the two hit hypothesis in which the first hit is steatosis. This is followed by a second "hit" in the form of cytokine (e.g. TNF- α) production, mitochondrial dysfunction and/or oxidative stress. All three of these "hits" are widely believed to be major contributors to alcohol-induced liver injury and may compound the initial steatosis. An evolving concept that is gaining acceptance is that certain accumulated fatty acids are toxic to the liver, but that their toxicity is blunted by esterification with glycerol to form triacylglycerols (triglycerides). Thus ethanol-elicited hepatic lipid accumulation as well as that caused by dietary sources, has prompted renewed interest as a cornerstone of liver toxicity, as it may not only initiate but enhance the progression of alcoholic liver disease. This review will discuss both the metabolic and the molecular factors that contribute to ethanol induced steatosis. It will also consider the possible mechanism(s) by which liver injury is initiated to produce advanced liver pathology.

ETHANOL METABOLISM AND FATTY LIVER

The liver is the primary site of ethanol metabolism, as hepatocytes express the major ethanol metabolizing enzymes, alcohol dehydrogenase (ADH), and cytochrome P450 2E1 (CYP2E1) at higher levels than any other tissue^[4-6]. ADH, with a Michaelis constant of 1 to 2 mmol/L, catalyzes ethanol oxidation, using NAD as a coenzyme in a reaction that forms acetaldehyde and reduced NAD (i.e. NADH⁺) in reaction (1) below. This and the subsequent oxidation of acetaldehyde to acetate with the formation of another reducing equivalent of NADH⁺ in a reaction catalyzed by aldehyde dehydrogenase (ALDH) in reaction (2) below were shown to cause significant changes in cellular redox potential and provide more substrate availability for fatty acid synthesis.



Enhanced generation of reduced NAD also disrupts mitochondrial β -oxidation of fatty acids thereby diminishing the rate of lipid oxidation. This metabolic explanation for fatty liver was the prevailing concept for a number of years, but it was not sufficient to explain the rapid formation of fatty liver after acute ethanol administration. In addition, the degree of change in liver redox potential that occurs *in vivo* after chronic ethanol administration to rodents was significant but rather modest. Thus, the metabolic explanation for alcohol-induced fatty liver became insufficient to account for all changes that occurred in hepatic lipids after ethanol consumption. Later discoveries in cell signaling and characterization of specific transcription factors prompted investigations of specific factors that are operative in the pathophysiology of ethanol-induced steatosis.

UPDATED MECHANISMS OF ETHANOL-INDUCED FATTY LIVER

Enhancement of lipid synthesis by ethanol consumption

Hepatic fatty acid triglyceride and phospholipid synthesis are accelerated after acute and chronic ethanol consumption^[7]. Enhanced lipogenesis is a reflection of higher expression of lipogenic enzymes including fatty acid synthase, acyl CoA carboxylase (ACC), ATP citrate lyase (ACL), stearoyl CoA desaturase and malic enzyme.^[8,9] These enzymes are encoded by genes regulated by the transcription factor, sterol regulatory element binding protein-1 or SREBP-1.

Activation of SREBP-1 and enhanced expression of lipogenic enzymes

By activating SREBP, a member of a family of transcription factors that target genes encoding enzymes involved in cholesterol metabolism, ethanol consumption can short circuit normal hepatic lipid metabolism,

converting the liver from a lipid burning to a lipid storing organ. There are three isoforms of SREBP: SREBP-1a, 1c and 2. SREBP 1a and 1c are alternative forms of the protein encoded by the same exon. Forms 1c and 2 are principally involved in regulating fatty acid and cholesterol synthesis, respectively. All SREBP proteins reside in the endoplasmic reticulum. To function as a transcription factor, the amino terminal domain of SREBP is proteolytically cleaved from the carboxy terminal domain in the Golgi apparatus and the fragment translocates to the cell nucleus. This is accomplished by proteolytic cleavage through the action of two proteases; site-1 protease (S1P) and site-2 protease (S2P), both located in the Golgi apparatus. SREBP is initially translocated from its site in the ER to the Golgi by SREBP activating protein (SCAP). SCAP activity is sensitive to intracellular cholesterol levels and possesses a cholesterol sensing domain in its primary structure. When cholesterol levels are low, SCAP translocates SREBP from the ER to the Golgi where S1P cleaves the precursor form of SREBP, producing a membrane-bound intermediate. The intermediate is then cleaved by S2P to release the active transcription factor domain from the membrane, enabling it to bind to specific promoter elements on the DNA. Under normal circumstances, when intracellular cholesterol levels rise, SCAP activity is suppressed, thereby preventing transport and activation of SREBP by S1P and S2P. Ethanol metabolism, on the other hand, disturbs the regulatory loop of SREBP activation and causes increased cleavage of SREBP-1c, even when intracellular levels of cholesterol and/or fatty acids are high. Active SREBP-1c is detected experimentally in nuclear fractions as a -65 kDa protein, derived originally from a -125 kDa precursor. Thus, SREBP-1c has an essential function in the development of ethanol-elicited fatty liver. Nuclear fractions from livers of ethanol-fed rodents or from cultured cells that are capable of ethanol metabolism have elevated levels of the active form of SREBP-1c. A requirement for ethanol metabolism in SREBP induction has been reported using cultured cells and there is considerable evidence that the activating factor is acetaldehyde^[10]. The mechanism of ethanol-elicited SREBP-1 activation is also partly due to inhibition of the AMP-activated protein kinase (AMPK), described in greater detail below. AMPK catalyzes the phosphorylation of target enzymes, some of which are important rate-limiting enzymes in hepatic lipid metabolism. By inhibiting the activity of AMPK, ethanol relieves suppression of SREBP-1, causing its activation and increasing its levels in the nucleus.

Ethanol-elicited decline in fatty acid oxidation: Regulation of the peroxisome proliferator activated receptor alpha

Ethanol-elicited decline in fatty acid oxidation: Regulation of the peroxisome proliferator activated receptor alpha is a nuclear receptor that regulates expression of genes that possess peroxisome proliferator response elements in their promoter regions. Several such genes are those that encode fatty acid transporters, including carnitine palmitoyl transferase I, proteins involved in export,

such as apolipoprotein B, the microsomal triglyceride transfer protein, fatty acid binding protein, and acyl CoA dehydrogenase. PPAR- α interacts with the retinoid X receptor (RXR). The protein complex is activated by binding to fatty acids, thereby enhancing expression of proteins involved in the fatty acid transport and oxidation.

Ethanol administration to rodents or ethanol exposure to ethanol-metabolizing cultured cells causes a general down-regulation of PPAR- α , as reflected by reduced binding by the RXR-PPAR- α complex to a PPAR- α -specific promoter sequence^[11]. This effect of ethanol is enhanced by exposure of ethanol-treated hepatoma cells to the aldehyde dehydrogenase inhibitor, cyanamide and is abolished by the alcohol dehydrogenase inhibitor, 4-methylpyrazole. Further, RXR-PPAR- α extracted from hepatoma cells previously exposed to ethanol or acetaldehyde binds poorly to an oligonucleotide containing peroxisome proliferator response elements, indicating that acetaldehyde is responsible for the action of ethanol. It is possible that acetaldehyde, because of its ability to covalently bind proteins^[12] can form adducts with the PPAR- α transcription complex, thereby preventing its ability to bind the promoter element(s). It is noteworthy; however, that the effect at the level of transcription does not universally down-regulate all PPAR- α -regulated enzymes. *In vivo* studies have shown that the steady state level of mRNA encoding medium chain acyl CoA dehydrogenase is decreased by ethanol, but mRNAs encoding other PPAR- α -sensitive enzymes, including those mentioned above are minimally affected, unaffected, and in the case of fatty acid binding protein, actually induced by ethanol. An explanation for this, as described by You and Crabb^[13] is that the regulation of fatty acid oxidation is not solely controlled by the activities of the fatty acid oxidative enzymes. There is additional allosteric regulation of fatty acid transport into the mitochondrion by the intramitochondrial levels of malonyl CoA, which inhibits the carnitine palmitoyltransferase I (CPT I), thereby preventing transport of fatty acyl CoA derivatives into this organelle. With the concomitant up-regulation of fatty acid biosynthesis by ethanol, this is an important step in the down-regulation of fatty acid oxidation. An additional natural regulatory factor is the adipocyte hormone adiponectin. Chronic ethanol administration causes a significant reduction in circulating adiponectin, which is associated with the ethanol-elicited reduction in fatty acid oxidation^[14]. Restoration of adiponectin to ethanol-fed animals by treatment with the recombinant form of the hormone restores fatty acid oxidation to normal. Moreover, such treatment decreases the levels of TNF- α in these animals, as the hormone and the cytokine are known to regulate each other's production^[15].

It is also noteworthy that ethanol-elicited inhibition of fatty acid oxidation can be reversed, as demonstrated by experiments in which ethanol-fed animals were co-treated with the PPAR- α agonist, WY14,643. Fatty liver was blocked in these animals due to induction of PPAR- α , which, in turn, accelerated fatty acid oxidation thereby preventing ethanol-elicited fatty liver^[15]. These findings underscore the metabolic importance of fatty acid oxidation in the elimination of steatosis and emphasize

the therapeutic potential of this agent and other PPAR- α agonists as adjuncts to the natural hormone, adiponectin.

Adenosine monophosphate-activated protein kinase and alcohol-induced fatty liver

Hepatic lipid metabolism has been shown to be tightly regulated by adenosine monophosphate-activated protein kinase (AMPK). This enzyme, a heterotrimeric protein, is, itself, activated by AMP as well as by phosphorylation by another kinase, LKB-1. Elevated AMP levels are indicators of low intracellular energy charge. Therefore, when AMP activates the AMPK it down-regulates energy requiring pathways, presumably as a means of energy (ATP) conservation. Such ATP-utilizing pathways are generally biosynthetic and include lipid, RNA and protein synthesis. Conversely, AMPK activates ATP-generating catabolic pathways, such as fatty acid oxidation, the TCA cycle and glycolysis. The rate-limiting enzyme in lipid biosynthesis is the acetyl CoA carboxylase (ACC), the activity of which is down-regulated *via* phosphorylation by AMPK^[16]. ACC catalyzes the carboxylation of acetyl CoA to malonyl CoA. The latter is a potent inhibitor of lipid oxidation and its declining levels potentiate mitochondrial transport and fatty acid oxidation.

When ethanol is added to cultured hepatoma cells that oxidize ethanol, it induces transcription of an SREBP-regulated promoter and increases the levels of the mature form of SREBP-1. Both these effects are blocked by the inclusion in the culture medium of 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) or metformin, both AMPK activators^[17]. Furthermore, overexpression of AMPK in these cells blocks the ethanol-induced increase in promoter activity and SREBP protein. Exposure of cells to ethanol reduces the activity of AMPK. *In vivo* studies confirm these findings. Feeding mice an ethanol diet significantly reduces hepatic AMPK activity, while increasing the activity of the rate-limiting enzyme, ACC^[17]. At the same time, fatty acid oxidation is reduced. These findings demonstrate that AMPK regulates the ethanol-elicited SREBP-1 activation and the development of steatosis. They also demonstrate the therapeutic value of AICAR and of metformin in preventing and/or reversing fatty liver caused not only by ethanol but also by dietary induction. The latter has been demonstrated in metformin-treated leptin-deficient experimental animals^[18]. Thus the central role of AMPK in regulating these activities is well illustrated by ethanol's effect in the genesis of fatty liver.

Role of Egr-1 in ethanol-induced steatosis

Another transcription factor, early growth response-1 (Egr-1) also appears to have a role in ethanol-elicited steatosis. Egr-1, also known as nerve growth factor 1-A (NGF1-A), is an immediate early gene and transcription factor that regulates genes involved in response to cellular stress. Early work demonstrated that Egr-1 is induced in response to growth factors^[19,20]. Several genes relevant to alcohol-induced liver injury have promoter regions that bind Egr-1. Among these genes are those that encode platelet-derived growth factor (PDGF), transforming growth factor beta (TGF- β), and intercellular adhesion molecule-1 (ICAM-1)^[21]. Specifically, with regard to ethanol-induced fatty liver, Egr-1

also binds the gene encoding tumor necrosis factor alpha (TNF- α)^[22]. This latter finding is noteworthy as this cytokine is considered lipogenic, causing the activation of SREBP-1, thereby enhancing lipid biosynthesis^[23]. Perhaps the most compelling evidence for the essentiality of Egr-1 in ethanol-elicited steatosis is that reported by McMullen *et al*^[24], who showed that, in contrast to wild type mice, which develop fatty liver in response to chronic ethanol administration, that Egr-1 null mice exhibit neither hepatic steatosis nor elevated TNF- α , after four weeks of chronic ethanol feeding. These workers also reported enhanced production of Egr-1 in ethanol-fed mice treated with lipopolysaccharide (LPS), compared with similarly-treated pair-fed control mice. The data suggest that the absence of Egr-1 prevents ethanol-elicited fatty liver and reduces sensitivity to LPS. Studies by Zhou *et al*^[1] demonstrated that 90 min after an acute dose of ethanol (6 g/kg body wt), hepatic TNF- α levels rise three-fold, followed by elevated levels of hepatic triglycerides. While these investigators did not measure Egr-1, they showed a close association between elevated levels of plasma endotoxin, oxidative stress, and TNF- α in ethanol-fed mice, suggesting that endotoxin, *via* oxidative stress, stimulates the production of TNF- α . This latter may result from enhanced transcription by Egr-1.

While Egr-1 is rapidly synthesized, its levels are also regulated through degradation by the proteasome^[25]. It has been postulated that inhibition of proteasome activity by ethanol administration has a distinct role in the increase in SREBP-1 protein, which is also a proteasome substrate^[17]. Similarly, PPAR- α and RXR levels are controlled by the ubiquitin-proteasome pathway^[26,27]. So, too proteasome inhibition may also contribute to the ethanol-induced rise in Egr-1. Recent experiments in our laboratory have demonstrated that acute ethanol administration to wild type mice causes enhancement of both Egr-1 mRNA and protein levels in livers of these animals one hr after ethanol administration. These increases precede the ethanol-elicited rise in triglycerides, detected after 3 h. In cultured hepatoma cells that metabolize ethanol, overexpression of Egr-1 following transfection with an adenoviral Egr-1 expression vector, enhances triglyceride accumulation in these cells over those of control cells. Furthermore, exposure of transfected cells to ethanol causes a further rise in the level of Egr-1 with a concomitant rise in cellular triglycerides (Donohue, unpublished). These findings provide further evidence for an essential role of Egr-1 in ethanol-induced fatty liver and the possibility that it may initiate downstream events. They also suggest that in cultured cells, there may be post-translational regulation of Egr-1 *via* ethanol-elicited suppression of proteasome activity. While Egr-1 regulates the expression of TNF- α , it also binds to promoter regions of other genes, including that encoding transforming growth factor- β (TGF- β), which has a major role in hepatic fibrosis^[28]. Thus, Egr-1 may not only be a major factor in steatosis, but also in the more advanced stages of alcohol-induced liver injury, as suggested by Pritchard and Nagy^[20].

Alcohol-elicited steatosis: What is the actual toxin?

The preceding sections have described the molecular

mechanisms by which ethanol consumption disrupts hepatic lipid metabolism, resulting in fatty liver. As stated earlier, an evolving concept that is gaining acceptance is that certain accumulated fatty acids are toxic to the liver, but that their toxicity is blunted by esterification with glycerol to form triacylglycerols (triglycerides). Ironically, triglyceride accumulation is the principal means by which fatty liver is quantified. Thus the question of whether there is a generalized toxicity of accumulated fatty acids or that specific fatty acids are hepatotoxic is important in determining whether alcoholics are at risk for more advanced disease. Recent work by Yamaguchi *et al*^[30,31] used antisense oligonucleotide (ASO) to block the synthesis of diacylglycerol acyltransferase (DAG), thereby inhibiting triglyceride synthesis. Blockade of DAG, which catalyzes the final step in triglyceride synthesis, reduced hepatic steatosis in these animals when they were fed a methionine-choline-deficient diet to induce NASH. However, such treatment with the ASO exacerbated liver injury to cause fibrosis, despite the fact that TNF- α levels were decreased, adiponectin levels were increased, and insulin sensitivity was improved. The explanation for these findings is that the prevention of triglyceride synthesis enhanced the intracellular levels of free fatty acids in the liver. While the specific fatty acids were not identified, the fact that enhanced lipid peroxidation occurred in liver-injured animals suggests that accumulation of unsaturated fatty acids would most likely be incriminated in causing further liver damage. With regard to ethanol-elicited steatosis, there is a substantial body of evidence that unsaturated fatty acids from corn oil or fish oil cause greater liver damage than saturated fatty acids when either of these are fed in combination with alcohol.

CONCLUSION

The foregoing review has summarized both the formerly and currently accepted models of alcohol-induced fatty liver. In summary, ethanol consumption causes activation of SREBP-1, which induces genes involved in lipid biosynthesis. Conversely, ethanol consumption down-regulates the PPAR- α transcription factor, which regulates enzymes involved in fatty acid oxidation, and simultaneously prevents the import of fatty acids into the mitochondrion for oxidation. The regulation of Egr-1 by ethanol is a less well characterized but an important area of investigation, as its rapid induction may well be upstream of the latter two factors or it may exert coordinate regulation along with SREBP-1 and PPAR- α . Finally, it should be emphasized that in sections of this review, comparisons of ASH were made to other fatty liver diseases, including NASH. The latter was used simply as an analogous disorder, but there was no intent to imply that the two (ASH and NASH) are the same. Clearly, further investigations are warranted into the pathophysiology of ethanol-induced steatosis, but recent investigations have demonstrated considerable progress toward therapeutic measures. These latter discoveries will most certainly prompt further ones in preventing or abating liver damage caused by alcohol abuse.

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Signaling mechanisms in alcoholic liver injury: Role of transcription factors, kinases and heat shock proteins

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Abstract

Alcoholic liver injury comprises of interactions of various intracellular signaling events in the liver. Innate immune responses in the resident Kupffer cells of the liver, oxidative stress-induced activation of hepatocytes, fibrotic events in liver stellate cells and activation of liver sinusoidal endothelial cells all contribute to alcoholic liver injury. The signaling mechanisms associated with alcoholic liver injury vary based on the cell type involved and the extent of alcohol consumption. In this review we will elucidate the oxidative stress and signaling pathways affected by alcohol in hepatocytes and Kupffer cells in the liver by alcohol. The toll-like receptors and their down-stream signaling events that play an important role in alcohol-induced inflammation will be discussed. Alcohol-induced alterations of various intracellular transcription factors such as NF κ B, PPARs and AP-1, as well as MAPK kinases in hepatocytes and macrophages leading to induction of target genes that contribute to liver injury will be reviewed. Finally, we will discuss the significance of heat shock proteins as chaperones and their functional regulation in the liver that could provide new mechanistic insights into the contributions of stress-induced signaling mechanisms in alcoholic liver injury.

Key words: TNF α ; Toll-like receptors; NF κ B; Heat shock proteins; Mitogen-activated protein kinases

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INTRODUCTION

Alcohol induced liver injury is marked by pathological changes in the liver ranging from steatosis, steatohepatitis to cirrhosis and sometimes hepatocellular carcinoma. The complex pathogenesis of acute and chronic alcohol consumption is multifactorial with diverse consequences in different tissues and cell types. Alcohol consumption leads to elevated endotoxin in the blood and liver leading to activation of immune cells that produce inflammatory mediators (i.e. cytokines). Abnormal cytokine production is a major feature of alcoholic liver disease. Elevated serum concentrations of TNF α , IL-6 and IL-8 have been reported in alcoholic patients and correlated with liver injury and clinical outcome^[1]. Among inflammatory cytokines, TNF α is a critical factor in alcoholic liver injury, a hypothesis that has been confirmed in animal models and human studies^[2,3]. Resident macrophages/Kupffer cells in the liver increase their production of cytokines in patients with alcoholic liver disease. Cultured monocyte/macrophages from alcoholic hepatitis patients produce TNF α spontaneously that is enhanced further in response to lipopolysaccharide (LPS)^[1]. In alcoholic liver injury, studies have shown that it is TNF α that is responsible for hepatocyte killing resulting from increased sensitivity of otherwise resistant cells to TNF-induced killing/apoptosis^[4]. It appears that early alcoholic liver injury involves interactions of cytokine over-production due to induction of the "hyper-inflammatory" state in monocytes/macrophages and sensitization of hepatocytes to cell death. The intracellular molecular mechanisms in response to alcohol exposure leading to inflammatory gene expression in the innate immune cell compartment and its down-stream effect on parenchymal cells of the liver are of considerable current interest.

In this article we will review the key components involved in alcohol-induced sensitization to TLR-signaling pathways and the pivotal role of transcription factors and mitogen-activated protein kinases (MAPK) contributing to alcohol-induced inflammation and hepatocyte injury. Furthermore, we will highlight the possible role of stress-induced heat shock proteins as chaperones in alcoholic liver injury.

CELL TYPES INVOLVED IN ALCOHOLIC LIVER INJURY

The currently accepted model of alcoholic liver injury

is characterized by increased gut permeability due to prolonged alcohol consumption resulting in increased endotoxin levels in portal circulation^[5]. Endotoxin is recognized by the resident macrophages/Kupffer cells in the liver via the toll-like receptor-4 (TLR4) leading to activation of intracellular signaling pathways in the macrophages and production of pro-inflammatory cytokines (TNF α , IL-1), chemokines (IL-8, MCP-1) and TGF β ^[6,7]. Kupffer cell-derived mediators then activate the other cell types in the liver resulting in damage. Chemokines recruit polymorphonuclear neutrophils and other inflammatory cells such as macrophages and T cells which contribute to amplification of the inflammatory response. Hepatocytes undergoing oxidative stress due to ROS generation and CYP2E1 induction are sensitized to TNF α induced apoptosis and necrosis^[3,8]. Furthermore, mediators such as TGF β and LPS activate stellate cells to proliferate and produce collagen leading to fibrosis and progression of liver injury^[9]. Alcohol-related injury of liver sinusoidal endothelial cells and their role in progression of disease has not been well studied. Some studies suggest that liver sinusoidal endothelial cells can be activated to produce cytokines and chemokines by malondialdehyde-acetaldehyde adduct proteins, generated by alcohol metabolism and thus could further contribute to amplification of alcohol-induced inflammation^[10]. Collectively, alcoholic liver injury involves various liver cell types during progression of disease.

ALCOHOL, OXIDATIVE STRESS AND INFLAMMATION

Chronic alcohol induced inflammatory responses in the liver are thought to be central to alcoholic liver injury. Excessive generation of reactive oxygen species (ROS) called free radicals plays an important role in alcohol-induced cellular damage^[8]. A number of studies have shown that alcohol increases generation of ROS *in vitro*^[11-13]. However, to determine the *in vivo* generation of ROS by alcohol has been rather challenging. Nevertheless, ROS-induced cellular responses are critical in alcohol-induced inflammation as well as TNF-induced hepatocyte killing^[2,13]. The ROS-related intracellular mechanisms leading to the sensitization of cellular injury by alcohol are underway in various laboratories. In the liver, Kupffer cells produce ROS in response to chronic alcohol exposure as well as endotoxin^[13]. Recent evidence shows that direct interaction of NADPH oxidase isozyme 4 with TLR4 is involved in LPS-mediated ROS generation and NF κ B activation^[14]. In alcohol fed rats, pretreatment with diphenyliodonium (DPI), which inhibits NADPH oxidase and normalizes ROS production, decreased LPS-induced ERK1/2 phosphorylation and inhibited increased TNF α production in Kupffer cells^[13]. Thurman and his group have shown that p47 phox-/- mice are resistant to alcohol-induced liver injury, indicating an important role for NADPH oxidase in not only inflammatory responses but also liver injury^[15]. Furthermore, dilinoleoylphosphatidylcholine (DPC) also prevented LPS induced NF κ B and ERK1/2 activation and TNF α production

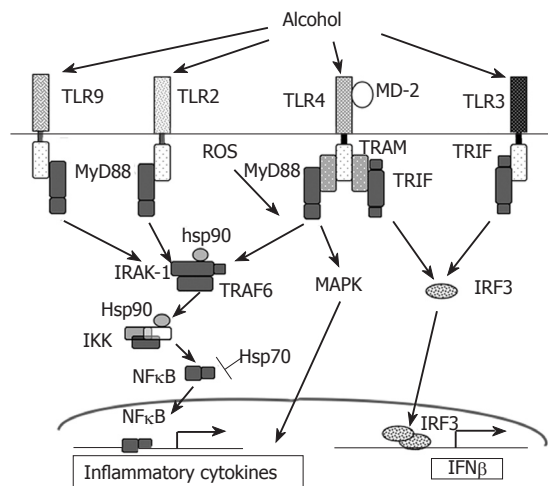


Figure 1 TLR signaling pathways affected by alcohol.

in Kupffer cells of chronic alcohol fed rats^[16]. It is now widely accepted that ROS not only plays a critical role in direct hepatocyte injury but also contributes to increased inflammatory responses further enhancing liver injury. Hence, the mechanisms affecting interaction of ROS and inflammatory responses as well as alcohol-induced sensitization mechanisms leading to hepatocyte death by alcohol need further elucidation.

ALTERATION OF TLR INDUCED SIGNALING PATHWAYS BY ALCOHOL

A major role for TLR mediated signaling, via endotoxin, in alcoholic liver disease (ALD) (Figure 1) was established by studies of Thurman and colleagues^[7,17]. Innate immune responses activated *via* the Kupffer cells, the primary effector cell in the liver, play a key role in the early pathogenesis of alcohol-induced liver injury^[18]. Increased levels of circulating lipopolysaccharide (LPS) in alcoholic patients have been shown^[19]. The currently accepted model of alcoholic liver injury elucidates that LPS promotes hepatic injury *via* induction of Kupffer cell activation resulting in production of TNF α and other inflammatory mediators. The Kupffer cells respond to stimulation by gut-derived endotoxins and apoptotic dead cells in the tissue resulting in increased inflammatory responses. Studies in knock out mouse models have shown that chronic alcohol feeding in CD14, TLR4 and LPS-binding protein (LBP) deficient mice results in alleviation of alcohol-induced liver injury indicating an important role for the TLR4 pathway^[7,20]. Furthermore, LPS recognition by TLR4 expressed on hepatic stellate cells and sinusoidal epithelial cells may also contribute to the progression of ALD^[21,22].

Circulating TNF α is increased in chronic alcoholics as well as in mouse chronic alcohol feeding models^[1,23]. Alcohol sensitizes Kupffer cells and monocytes/macrophages to produce increased TNF α in response to endotoxin^[24]. Although studies on effects of alcohol on membrane proximal events using mutant and knock out mice have shown an important role for CD14^[20]

and TLR4^[7], recent studies show hepatic expression of TLR2 or TLR4 mRNA was not changed by chronic alcohol feeding or by acute alcohol administration^[25]. Upon activation of TLR4, IRAK is recruited to the TLR4 complex *via* interaction with MyD88 (Figure 1). Acute and chronic alcohol exposure affects activation and recruitment of the IRAK-1 and IKK kinase activation^[28] Mandrekar *et al*^[26-28] unpublished. In contrast to chronic alcohol consumption, acute alcohol exposure inhibits TLR4 signaling in monocytes and macrophages after *in vitro* as well as *in vivo* alcohol treatment in mice leading to decreased LPS-induced TNF α production. Acute alcohol administration also suppressed TLR3 downstream signaling^[29]. *In vitro* acute alcohol exposure of human monocytes or macrophages suppresses LPS-induced IRAK-1 phosphorylation^[28] and inhibits poly I:C induced IRAK-1 degradation^[29] (Figure 1). Furthermore, acute alcohol exposure of murine macrophages inhibits TLR2, TLR4 and TLR9 ligand-induced IL-6 and TNF α production^[30]. Thus, it is evident that TLR-associated molecules such as CD14, TLR4 and LPS-binding protein (LBP) as well as their intra-cytoplasmic mediators TLR receptor associated kinases (IRAKs), I κ B kinase (IKK) and NF κ B are altered by alcohol and contribute to alcoholic liver injury.

TRANSCRIPTION FACTORS INDUCED BY ALCOHOL

In response to alcohol exposure, multiple signaling transduction pathways are activated in the liver. Receptors such as TLRs, TNF α , *etc.* in various liver cell types culminating in nuclear events involving binding of transcription factors to the promoter elements of target genes. The progression of alcoholic liver disease is characterized by initial appearance of fatty liver and inflammation, necrosis and apoptosis followed by fibrosis. It is generally accepted that the molecular mechanisms regulating the different stages of alcoholic liver disease from fatty liver and inflammation to fibrosis and cirrhosis are diverse. Since early alcoholic liver injury, a reversible condition, which comprises of development of fatty liver and inflammation, research on intracellular mechanisms has been focused primarily on these early stages. Chronic alcohol exposure increases expression of genes regulating fatty acid synthesis and suppresses genes involved in fatty acid oxidation resulting in increased fatty acid accumulation or steatosis. Transcription factors regulating fatty acid metabolism including sterol regulatory element binding protein (SREBP) and peroxisomal proliferating factor α (PPAR α) that is involved in fatty acid oxidation play a pivotal role in early alcoholic liver injury. Liver specific overexpression of SREBP1 α or SREBP1c results in fatty liver with significantly increased hepatic triglyceride content^[31]. In hepatoma cultures and ethanol-consuming mice, SREBP mRNA and active SREBP1 protein levels were significantly increased^[32,33] and accompanied by hepatic triglyceride accumulation. PPAR α , an essential regulatory factor up-regulating fatty acid oxidation also plays an important role in alcoholic fatty liver induction.

French and colleagues reported that chronic alcohol feeding decreased PPAR α mRNA^[34]. Further, exposure of primary hepatocytes and hepatoma cells to chronic alcohol resulted in impaired PPAR α DNA binding activity that was prevented by 4-methylpyrazole, an inhibitor of alcohol dehydrogenase^[35]. Although chronic alcohol decreased PPAR α activity, treatment of mice with WY14643, a PPAR α agonist restored DNA binding activity without affecting quantities of PPAR α , indicating additional mechanisms affected by alcohol to regulate fatty acid oxidation.

Similar to the effects of chronic alcohol on transcription factors involved in lipid homeostasis, alcohol-induced inflammatory mediators that play an important role in disease progression, are induced by key transcription factors. The most well studied example is the activation of NF κ B in monocytes and macrophages controlling pro-inflammatory cytokine induction. Studies have shown increased LPS-induced NF κ B DNA binding activity in monocytes of patients with alcoholic hepatitis compared to controls^[36]. While chronic alcohol exposure increases LPS-induced NF κ B binding in monocytes and macrophages, acute alcohol exposure decreases LPS-induced NF κ B binding resulting in different regulation of pro-inflammatory cytokine genes based on duration of alcohol exposure. While several animal models have shown increased hepatic LPS-induced NF κ B DNA binding activity, some studies have failed to observe any effect of chronic alcohol feeding on NF κ B activity^[37-39]. Many studies have described a positive effect of ROS in the regulation of NF κ B activation^[38,40,41]. Recent studies have shown that alcohol-induced TNF α production due to activation of the NADPH oxidase system and interaction with adapters of the TLR4 signaling pathway influence NF κ B DNA binding and promote pro-inflammatory cytokine production^[14]. It is likely that chronic alcohol exposure may activate NF κ B *via* ROS-dependent mechanisms since treatment of liver macrophages with dilinoleoylphosphatidylcholine (DPC) protects liver injury and prevents NF κ B activation^[16].

Activator protein-1 (AP-1) another transcription factor is also regulated by acute and chronic alcohol exposure in monocyte/macrophages and hepatocytes. Acute and chronic alcohol exposure increases AP-1 DNA binding activity^[28,42,43] in monocytes/macrophages whereas isolated Kupffer cells do not show any effect on AP-1 activity after chronic ethanol feeding^[44]. Furthermore, chronic alcohol increases AP-1 expression and induces activation in livers of chronic alcohol fed mice and in isolated primary hepatocytes^[42,45]. Increased activation of AP-1 could influence pro-inflammatory and anti-inflammatory cytokine gene induction and hence could contribute to the amplification of the inflammatory response after chronic alcohol exposure. Since AP-1 regulates collagen synthesis, increased AP-1 activation could also be implicated in alcohol-induced fibrotic changes in the liver^[46].

PPAR γ , another transcription factor known to inhibit inflammatory responses is also regulated by chronic alcohol exposure in macrophages. PPAR γ expression was increased in Kupffer cells and hepatocytes during chronic alcohol exposure^[47]. Treatment with PPAR γ agonists prevented development of chronic alcohol induced

steatosis and inflammation^[48]. The exact mechanism by which PPAR γ exerts its effect to resolve alcohol-induced liver injury remains to be studied.

Early growth response factor-1 (Egr-1), a zinc finger transcription factor induced in response to environmental stress and shown to regulate cellular growth and proliferation is up-regulated during chronic alcohol exposure in Kupffer cells^[44,49]. Increased LPS-stimulated Egr-1 expression is dependent on ERK1/2 activation in Kupffer cells of chronic alcohol fed mice compared to pair-fed controls^[44]. Furthermore, recent data show that chronic alcohol feeding induced liver injury is blocked in Egr-1 knock out mice, indicating a role for the ERK1/2-Egr-1 pathway in the pathogenesis of alcoholic liver injury^[50]. These studies illustrate that based on the cell type involved and duration of alcohol exposure, regulation of transcription factors is highly complex and requires further evaluation. Future investigations on regulation of transcription factors during the different stages of alcoholic liver injury will aid in designing effective therapeutic strategies.

ALCOHOL AND MAP KINASES

LPS recognition also activates MAPK family members including extracellular receptor activated kinases 1/2 (ERK1/2), p38 and c-jun-N-terminal kinase (JNK) resulting in TNF α production^[51]. Chronic alcohol increases LPS-induced ERK1/2 activation which contributes to TNF α expression in macrophages^[44]. Similarly, LPS stimulation of Kupffer cells exposed to chronic alcohol showed increased p38 activity whereas decreased JNK activity was observed in livers after chronic alcohol feeding^[39]. Activation of p38 MAPK by LPS has been shown to contribute to TNF α mRNA stability via interaction with tristetraprolin (TTP)^[52]. Inhibition of p38 activation completely abrogated alcohol-mediated stabilization of TNF α mRNA^[39]. On the other hand, ERK1/2 inhibition did not affect TNF α mRNA stability but affected its transcription^[44]. LPS stimulation of JNK leads to phosphorylation of c-jun and subsequent binding of c-jun to the CRE/AP-1 site in the TNF α promoter^[51]. Although chronic alcohol feeding decreased JNK activity without any effect on TNF α mRNA, acute alcohol exposure increased JNK phosphorylation as well as AP-1 binding in the presence of combined TLR4 plus TLR2 stimulation^[28] in human monocytes. Furthermore, LPS-induced ERK1/2 phosphorylation was decreased in acute alcohol exposed monocytes^[28], whereas p38 MAPK activity was increased contributing to anti-inflammatory mediators such as IL-10 after acute alcohol exposure in monocytes^[43]. Increased oxidative stress in chronic alcohol exposed rats promotes hepatocyte apoptosis and necrosis and is implicated in the alcohol-induced sensitization to the pro-apoptotic action of TNF α ^[2]. Besides modulation of MAPK activity in macrophages, potentiation of alcohol induced hepatocyte death has been attributed to increased mitochondrial permeability transition and caspase-3 activation in hepatocytes and depends on p38 MAPK activation but is independent of caspase-8^[4,53].

ALCOHOL AND CHAPERONES: ROLE OF HSP70 AND HSP90

Mammalian heat shock proteins (hsps) induced in response to cellular oxidative stress serves as chaperones in refolding, disaggregation and degradation of damaged polypeptides^[54,55]. Amongst the family of heat shock proteins, Hsp70, Hsp60, Hsp90 and Hsp32 (also termed HO-1) have been implicated in protective mechanisms against increased oxidative stress in liver injuries. Upregulation of hsps in liver cells in culture has been shown to diminish the toxicity of a number of hepatotoxicants. Immunohistochemical detection revealed elevated Hsp70 in livers of alcoholic patients^[56]. Male Wistar rats fed with acute as well as chronic ethanol for 12 wk showed induction of Hsp70 in various regions of the brain and to a small extent in the liver^[57,58]. However, the intensity of induction of Hsp70 in the liver, the principal organ of ethanol oxidation was much less pronounced than the hippocampus or striatal areas of the brain^[57,58]. Recent studies also reveal that acutely and chronically ethanol-treated primary astrocyte cultures showed increased Hsp70 expression at 50 mmol/L, but not at 200 mmol/L ethanol concentration suggesting that severe toxicity of a 200 mmol/L ethanol concentration seems to exceed the power of inducible protective mechanisms elicited by heat shock proteins in astrocytes^[59]. The mechanism by which Hsp70 exerts its protective role is not clear. Oxidative stress due to depletion of glutathione (GSH) and induction of Hsp70 has been closely linked^[60]. Antisense-Hsp70 experiments in rat astrocyte cultures resulted in moderate oxidative damage in control astrocytes and a consequent drastic decrease in the viability of ethanol-treated cells, with mitochondrial functionality being affected^[59]. Thus, heat shock proteins confer a survival advantage to the cells preventing oxidative damage. Hsp70 induction using geranylgeranylacetone showed subsequent inhibition of alcohol-induced apoptosis of hepatocytes^[61]. Induction of hsp72 by hyperthermia pre-conditioning increased hsp70 and reduced TNF α responses in CCl₄-induced cirrhotic rats^[62]. Use of drugs that elevate intracellular hsp70 may serve to exert a cytoprotective effect in alcoholic liver disease. In addition to its role in cytoprotection, hsp70 also inhibits inflammatory responses in immune cells^[63]. Hsp70 interacts with various components of the NF κ B signaling pathway (Figure 1). Overexpression of Hsp70 leads to repression of NF κ B mediated gene expression^[63]. Further, nuclear translocation of NF κ B is also affected in cells transfected with the Hsp70 gene^[64]. Hsp70-induced NF κ B inhibition is attributed to both increased I κ B α expression and attenuated I κ B α degradation^[65]. In addition, Hsp70 can directly interact with NF κ B p65 and NF κ B p50 to influence NF κ B mediated responses^[66].

Hsp90 also plays an important role in regulating the TLR signaling pathway and can thus influence inflammatory responses (Figure 1) by chaperoning key signaling molecules. Inhibition of Hsp90 impairs function of its client signaling proteins and thus alters cell function^[67]. Treatment of cells with inhibitors of Hsp90 such as the

benzoquinone ansamycin, geldanamycin activates a heat shock response without the stress^[68]. Geldanamycin also inhibits LPS-induced NF κ B activity and TNF α production in macrophages^[69,70]. Hsp90 is crucial for biogenesis and activity of IKK α and IKK β , kinases responsible in I κ B α phosphorylation and activation of NF κ B, and inhibition of Hsp90 results in IKK α and IKK β depletion^[71]. Thus Hsp90 can play a crucial role in maintaining the activity of various components of the NF κ B signaling pathway. In alcoholic liver disease, ethanol induced oxidative stress using the intragastric feeding model induces thiol modification of Hsp90 in the liver^[72]. Whether the thiol modification of Hsp90 contributes to progression of alcoholic liver disease needs further evaluation. Studies have shown that chronic alcohol exposure modulates endothelial cell function by increasing NO production *via* PI3 K-dependent up-regulation of eNOS and its interaction with Hsp90^[73]. Furthermore, Hsp90 and Hsp70 have been shown to form insoluble aggregates with cytokeratins to form Mallory bodies^[74] in alcoholic liver disease. Selective enhancement of cytochrome P450 activity in rat hepatocytes can be achieved by heat shock treatment^[75]. Alcohol inducible cytochrome P450 2E1 (CYP2E1) is also shown to be a Hsp90 “client” protein and regulation of Hsp90 can profoundly affect enzyme turnover^[76]. Thus, taken together, it is tempting to speculate that chaperoning activity of Hsp90 and Hsp70 could be modulated using pharmacological inhibitors to alleviate alcohol-induced oxidative stress and inflammation and reversal of liver injury.

CONCLUSION

In conclusion, the biological effects of acute and chronic alcohol exposure and the net result on intracellular signaling pathways in liver cell types are complex. It is evident that the innate immune response plays a significant role in the development of alcoholic liver injury. A wealth of information is available on the contribution of pro-inflammatory responses in alcohol-induced liver damage. Studies on interactions of various signaling mechanisms in the different cell types of the liver and their outcomes in the liver microenvironment will provide a better understanding of the pathogenesis of alcoholic liver disease. Future studies performed to delineate novel molecular pathways are necessary and will provide a better understanding of liver injury induced by chronic alcohol consumption.

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LIVER CANCER

Nerve growth factor involvement in liver cirrhosis and hepatocellular carcinoma

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Abstract

AIM: To define NGF (nerve growth factor) and its high-affinity receptor $trkA^{NGF}$ presence and distribution in fibrotic liver and in HCC, and to verify if NGF might have a role in fibrosis and HCC.

METHODS: Intracellular distribution of NGF and $trkA^{NGF}$ were assessed by immunohistochemistry and immunoelectron microscopy in liver specimens from HCC, cirrhosis or both. ELISA was used to measure circulating NGF levels.

RESULTS: NGF and $trkA^{NGF}$ were highly expressed in HCC tissue, mainly localized in hepatocytes, endothelial and some Kupffer cells. In the cirrhotic part of the liver they were also markedly expressed in bile ducts epithelial and spindle-shaped cells. Surprisingly, in cirrhotic tissue from patients without HCC, both NGF and $trkA^{NGF}$ were negative. NGF serum levels in cirrhotic and/or HCC patient were up to 25-fold higher than in controls.

CONCLUSION: NGF was only detected in liver tissue with HCC present. Intracellular distribution suggests paracrine and autocrine mechanisms of action. Better definition of mechanisms may allow for therapeutic and diagnostic/prognostic use of NGF.

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Key words: Nerve growth factor; Hepatocellular carcinoma; Liver cirrhosis; Confocal microscopy; Transmission electron microscopy

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INTRODUCTION

NGF (nerve growth factor) is a prototypical member of the neurotrophin family essential for survival, differentiation, and maintenance of neuronal cells in the central and peripheral nervous system^[1]. In recent years, many findings have indicated that NGF could also have a role outside the central and peripheral nervous system. In particular, it may be involved in lung and skin tissue repair^[2] as well as in allergic inflammation and fibrosis^[3]. Increased levels of circulating NGF were observed in several autoimmune, chronic inflammatory and fibrotic disorders^[4,5]. Numerous data also indicate that NGF is involved in tumour growth, invasion and metastasis^[6-14].

Two types of receptors mediate NGF effects: $trkA^{NGF}$, the high-affinity receptor with protein kinase activity, specific for NGF, and $p75^{NTR}$, the low-affinity glycoprotein receptor, also binding other neurotrophins^[15,16]. Most of the biological activities elicited by NGF are mediated by binding to $trkA^{NGF}$ receptor^[17,18]. $trkA^{NGF}$, $p75^{NTR}$ receptors or both, together with NGF, are expressed in various cancers, including lung^[7], breast^[12] and prostate cancer^[10,11,13], suggesting that the NGF autocrine or paracrine pathway may have a role in tumorigenesis. NGF is also shown to be over expressed in lung and skin fibrotic process^[2] as well as in liver during experimental fibrotic injury^[19]. However, the role of NGF in tissue remodelling, fibrotic process and cancer progression is still controversial.

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world, showing a rapid progressive clinical course, poor response to pharmacological

treatment and a severe prognosis^[20,21]. A major risk factor for HCC is hepatitis C virus (HCV)-related cirrhosis^[22-25]; many chronically infected patients remain asymptomatic for a long period, with liver cirrhosis developing after approximately 30 years^[26,27]. The lack of predictive markers that can detect the beginning of liver cirrhosis in chronic HCV patients may also contribute to the late diagnosis of HCC, its progression and the poor prognosis.

The expression of NGF and its receptors in liver tissue during fibrotic injury and HCC has been previously investigated mostly in animal models with contradictory results. Some authors demonstrated that hepatocytes expressed NGF but not trkA^{NGF} during profibrotic liver injury, or in early preneoplastic lesions and HCCs. TrkA^{NGF} was only expressed in the walls of arteries associated with tumors^[28,29]. These data suggest that in hepatocytes NGF may not be an autocrine factor, but may rather act in a paracrine fashion. On the contrary, other authors^[19] reported the presence of trkA^{NGF} mRNA, but not of NGF mRNA in hepatocytes of fibrotic rat liver. It is not clear from the literature which cell types (apart the hepatic stellate cells) constituting the whole liver tissue are actually involved in the cross-talk mediated by NGF during liver tissue remodelling processes and HCC progression.

In this study we investigated the expression of NGF and its high-affinity receptor trkA^{NGF} in patients suffering from HCC, cirrhosis or both. This was performed to verify if the expression of this neurotrophin may be correlated with tissue remodelling processes and HCC progression. We also studied the expression of NGF and trkA^{NGF} in the different cell types inside the fibrotic and HCC tissues, analyzing the intracellular distribution in each cell type at an ultrastructural level, attempting to clarify their involvement in the hepatic damage. Circulating NGF levels were also measured.

MATERIALS AND METHODS

Human liver specimens

20 human liver specimens, taken for diagnostic purposes or resected before transplantation, were used in this study: 5 near normal liver biopsy specimens, 4 with cirrhosis without HCC from transplanted patients (Child-Pugh A), 11 with cirrhosis and HCC post-hepatitis C [Child-Pugh A ($n = 4$); Child-Pugh C ($n = 7$)]. All the patients had voluntarily signed a written consent to participate the study. The diagnosis was based on histopathologic examination of routinely processed tissue together with clinical and laboratory data. Each specimen was received fresh, and fixed in part in 10% buffered formalin solution for immunohistochemistry, and the other part in 1% or 2.5% glutaraldehyde for immunogold labelling or morphological analysis by transmission electron microscopy observation, as described below.

Criteria used for identification of immuno-positive cell types in liver tissues

Cell types in liver tissues from cirrhotic and HCC patients have been identified on the basis of microscopic and ultramicroscopic morphological feature. In particular:

(1) Endothelial cells: elongated narrow cells composing the wall of sinusoids; (2) Kupffer cells: macrophage cells, exhibiting considerable phagocytic ability, bulging into sinusoidal lumen; (3) Biliary epithelial cells: tightly arranged cuboid or columnar cells, constituting the wall of bile ducts, lying on a basement membrane and bearing microvilli on the luminal surface; (4) Spindle-shaped cells: elongated fibroblast-like cells embedded in fibrous tissue, possessing characteristics of myofibroblast, as shown by positive staining for smooth muscle actin (SMA).

Immunohistochemistry and Confocal Laser Scanning Microscopy (CLSM)

Expression of NGF and trkA^{NGF} in normal liver, cirrhotic and HCC tissues, obtained as described above, was investigated by Confocal Laser Scanning Microscopy (CLSM) using an indirect immunofluorescence technique. Immuno labelling was performed on sections obtained from paraffin embedded tissues using rabbit polyclonal antibodies against NGF (Chemicon Int., Temecula, CA, USA) or trkA^{NGF} (Santa Cruz Biothech., Santa Cruz, USA). Primary antibodies detection was obtained by a reaction with TRITC-conjugated goat anti-rabbit IgG. To test the specificity of the immunoreaction, for each liver specimen examined and on a section close to that used for NGF or trkA^{NGF} staining, negative controls were performed by substitution of primary antisera with non-immune rabbit serum. Fluorescently labelled samples were imaged by the confocal microscope LEICA TCS 4D (Leica, Heidelberg, Germany) supplemented with an Argon/Krypton laser and equipped with 40×1.00 -0.5 and 100×1.3 -0.6 oil immersion lenses. The excitation/emission wavelengths employed were 488 nm/510 nm, for green auto-fluorescence (used to visualize liver tissue morphology), and 568 nm/590 nm for TRITC-labelling. During the observation, both fluorescent signals were obtained simultaneously. To better visualize the intracellular distribution of NGF or trkA^{NGF} along the thickness of tissue sections, confocal sections were taken at intervals of 0.5 μm , a 3D reconstruction image for each fluorescent signal was recorded, and merged images of the two signals were obtained using the confocal microscope software.

To determine the nature of spindle-shaped cells, double staining using rabbit polyclonal antibodies against NGF (Chemicon Int.) and mouse monoclonal antibody against smooth muscle actin (SMA, Sigma-Aldrich Co., St. Louis, Mo, USA) was performed. Anti-SMA detection was obtained by a reaction with Alexa Fluor 488-conjugated rabbit anti-mouse IgG (Molecular Probes Inc., Eugene, OR).

For the assessment of staining intensity, a minimum of three section for each sample were examined and the different level of positive immunoreaction was evaluated on the basis of number of positive cells and fluorescence intensity. The following criteria were used for evaluation of staining: (-), same as the background obtained in control performed by substitution of specific anti-NGF or trkA^{NGF} antibodies with non-immune rabbit serum; (\pm), moderately higher than the background; (+), higher than the background; (++), much higher than the background.

Table 1 Expression of NGF and trkA^{NGF} HCC and cirrhotic tissues and in normal liver

N°11 Patients			Cirrhotic liver		HCC	
ID#	Etiology	Child-Pugh	NGF	trkA^{NGF}	NGF	trkA^{NGF}
2	HCV	A	++	++	+	+
4	HCV	A	+	+	±	±
5	HCV	A	+	+	+	±
10	HBV	A	±	+	±	±
3	HCV	C	++	++	+	+
11	HCV	C	±	+	+	+
14	HCV/HBV/HDV	C	+	++	±	+
16	HCV	C	+	±	+	+
15	HCV	C	na	na	+	++
17	HCV	C	na	na	±	++
18	HCV	C	na	na	±	+
N° 4 Patients			Cirrhotic liver			
ID#	Etiology	Child-Pugh	NGF	trkA^{NGF}		
20	HCV	C (transplantation)	-	-		
21	HCV	C (transplantation)	-	-		
22	HCV	C (transplantation)	-	-		
23	HCV	C (transplantation)	±	-		
N° 5 patients			Normal liver			
ID#			NGF	trkA^{NGF}		
1			-	-		
7			-	-		
8			-	-		
9			-	-		
13			-	-		

na: not available (HCC tissue without cirrhosis).

For histological observation, sections obtained from paraffin embedded tissues and close to that used for immunohistochemistry were stained with haematoxylin-eosin (HE) and observed by an optical microscope.

Transmission Electron Microscopy (TEM)

For ultrastructural observation, samples of normal liver, cirrhotic and HCC tissues were fixed with 2.5% glutaraldehyde in 0.1 mol/L Millonig's phosphate buffer (MPB), and then post-fixed with 1% OsO_4 in the same buffer. Samples were dehydrated in increasing concentrations of ethanol and embedded in Spurr epoxy resin (Agar Scientific LTD, Stansted, Essex, UK). For immuno-electron microscopy, tissue samples were fixed with 1% glutaraldehyde in 0.1 mol/L MPB, dehydrated in increasing ethanol concentrations and embedded in LR White acrylic resin (Agar Scientific LTD). Immunogold labelling was performed on ultrathin sections using the same rabbit polyclonal antibodies against NGF or trkA^{NGF} used for immunohistochemistry. Negative controls were performed by substitution of primary antisera with non-immune rabbit serum. Detection of primary antibodies was obtained by the reaction with 10 nm gold particles-conjugated goat anti-rabbit IgG. For morphological and immunogold labelling observation, ultrathin sections were stained with uranyl acetate and lead citrate and observed under a Philips CM12 transmission electron microscope operating at 80 kV.

NGF determination

Circulating NGF levels were measured in sera from 27 patients with a documented cirrhosis, HCC or both, by

a modified highly sensitive two-site immunoenzymatic assay (ELISA), using anti-NGF antibodies (clone 27/21, Chemicon Int.)^[30,31]. This assay specifically identifies human NGF but not brain-derived neurotrophic factor, with a detection limit of 0.5 pg/mL.

RESULTS

Expression of NGF in liver biopsies

The results of NGF and trkA^{NGF} immunostaining in all the human liver specimens examined are summarized in Table 1.

Analysis of NGF distribution by immunohistochemistry and CLSM observation indicated that all 11 patients with cirrhosis and HCC were positive, while the 5 normal liver biopsy specimens as well as the 4 samples from transplanted patients with cirrhosis without HCC, were negative. In HCC tissues NGF was detectable in a high number of cells at different levels of intensities, depending on the patient (Table 1, Figure 2) but never in normal liver tissue (Table 1, Figure 1). Interestingly, in liver from patients with cirrhosis (Child-Pugh C, undergoing transplantation) but without HCC, NGF and trkA^{NGF} were negative (Table 1 and Figure 3A₂ and 3A₃). Conversely NGF and trkA^{NGF} were markedly positive in patients with cirrhosis that had evolved into HCC, already at early staging (Child-Pugh A; Table 1 and Figure 3B₂ and 3B₃).

Furthermore, both the number of positive cells and the intensity of immunoreaction were significantly elevated in cirrhotic tissues with respect to the HCC tissues obtained from the same liver (Table 1 and Figure 4). Moreover in HCC tissues, NGF was mainly localized in the cytoplasm and on the nuclei of hepatocytes, and

to a lesser extent on endothelial and Kupffer cells and some lymphocytes (Figure 2). However, in cirrhotic tissue, it was also markedly expressed on biliary epithelial cells constituting the wall of bile ducts, and on spindle-shaped cells embedded in fibrous tissue (Figure 4). Double staining using anti-NGF and antibody against SMA (Figure 4J and K) indicated that these spindle-shaped cells presumably correspond to the hepatic stellate cells described by other authors^[19].

Ultrastructural observation by transmission electron microscopy after immunogold labelling showed that in hepatocytes of HCC tissue (Figure 5), positive reaction for NGF mainly localized on cytoplasmic vesicles (Figure 5B and C) and on endoplasmic reticulum (Figure 5E), suggesting that synthesis and accumulation of this molecule may occur. In some cases, immunogold labelling was also present on nuclei, beneath the nuclear membrane (Figure 5D). Furthermore, in cirrhotic tissue (Figure 6) a more intense immunoreaction, with respect to HCC tissue, was observed on cytoplasmic vesicles (Figure 6B), free in the cytoplasm (Figure 6B) and along endoplasmic reticulum (Figure 6B) of hepatocytes, indicating that in cirrhotic tissue NGF may be actively produced by hepatocytes. Positive immunogold reaction was also observed on cytoplasmic vesicles of endothelial cells (Figure 6C) and of spindle-shaped cells (Figure 6D). In biliary epithelial cells NGF localized in large cytoplasmic vacuoles that were present in the portion of cells near the ductal lumen (Figure 6E).

Expression of TrkA^{NGF} in liver biopsies

Immunohistochemical staining and CLSM observation showed that in HCC tissue trkA^{NGF} localized on the cell membrane of few hepatocytes (Figure 7A₂), while in cirrhotic tissue an intense immunoreactivity was also observed on biliary epithelial cells (Figure 7B₃ and 7B₆). This was particularly evident beneath the cell membrane near the ductal lumen (Figure 7B₃), and on spindle-shaped cells scattered throughout fibrotic tissue surrounding reactive bile ducts (Figure 7B₆). Positive immunoreaction was also observed on endothelial cells constituting the wall of blood vessels (Figure 7B₆).

Furthermore, in cirrhotic tissue, immunogold labelling showed that in biliary epithelial cells (Figure 8A) trkA^{NGF} localized on cell membrane and on cytoplasmic vesicles beneath it (Figure 8A₁), suggesting a possible receptor recycling after receptor-ligand reaction. Immunoreactivity was also observed in the cytoplasm of some endothelial cells (Figure 8B₁) and on cell membrane of some lymphocytes (Figure 8B₂).

NGF detection in sera

NGF levels were measured in sera obtained from 27 patients with documented cirrhosis, HCC, or both by immunoenzymatic assay. All patients disclosed with circulating NGF levels elevated 25-fold over the normal with (range 73-520 pg/mL, compared to a mean of 20 pg/mL in healthy donors, Figure 9).

DISCUSSION

The expression of NGF and its receptors in human and

rodent liver cells has been investigated for some time in previous studies with contradictory results. Oakley *et al.*^[29] demonstrated for example, that hepatocytes expressed NGF, but not trkA^{NGF} during profibrotic liver injury, in early preneoplastic lesions and HCCs, suggesting that in the hepatocytes NGF may not be an autocrine factor but may rather act in a paracrine manner. Other studies^[28] reported that in HCC tissue from male B6C3F1 mice, trkA^{NGF} is expressed exclusively in the walls of arteries associated with tumors, presumably in smooth muscle cells, whereas it is negative in other cell types including tumor cells. The same authors also demonstrated that NGF was expressed not only in HCCs but also in early preneoplastic lesions, possibly representing a very early change during mouse hepatic carcinogenesis. On the contrary, Cassiman *et al.*^[19] provided evidence that in human and rat cirrhotic tissues, NGF and other neurotrophins, as well as their receptors, were expressed not only in hepatic stellate cells, but also in regenerating bile ducts and in some hepatocytes with a cytoplasmic or nuclear staining patterns. Recently it has been reported that NGF is over expressed in approximately 60% of human HCC tissues compared to the surrounding liver tissue with cirrhosis and chronic hepatitis, suggesting a role for NGF in the progression of HCC^[32]. Also, until now, to our knowledge, it has not yet been clarified what role NGF plays and which cell types in the liver tissue are actually involved in the NGF mediated cross-talk during liver fibrosis and HCC progression. In addition, even if increased levels of circulating NGF have been reported in several autoimmune, chronic inflammatory and fibrotic disorders^[4,5], meanwhile data concerning circulating NGF levels in HCC or cirrhotic patients are not known.

In this study we provide immunohistochemical evidence that NGF and its high-affinity receptor trkA^{NGF} are over expressed in patients suffering from HCC and to a greater extent from HCC with cirrhosis. Surprisingly, NGF and trkA^{NGF} were negative in liver specimens from patients with cirrhosis undergoing transplantation but without HCC, supporting the hypothesis of an active role for NGF in HCC insurgence and progression. The elevated circulating NGF levels recorded in sera from patients with documented cirrhosis, HCC or both, as well as the tissue distribution of NGF and its receptor further support the correlation between NGF activity and the progression of liver fibrosis towards HCC. We also have been able to identify the NGF and trkA^{NGF} immuno positive cell types in liver tissues from cirrhotic and HCC patients, comparing the results obtained by immunohistochemistry and immuno-electron microscopy, as summarized in Table 2.

The evidence that hepatocytes in HCC and cirrhotic tissues from the same liver produce NGF (as suggested by the localization on cytoplasmic vesicles and on endoplasmic reticulum) and express its receptor strongly support the hypothesis that NGF may act by both autocrine and paracrine mechanisms, rather than a prevalent paracrine one, as previously suggested^[29].

NGF and trkA^{NGF} have been reported over expressed *in vivo* in proliferating cholangiocytes after bile duct ligation. NGF also stimulates rat cholangiocyte proliferation *in vitro*^[33]. We observed that in cirrhotic tissue both NGF and its receptor are over expressed on proliferating biliary

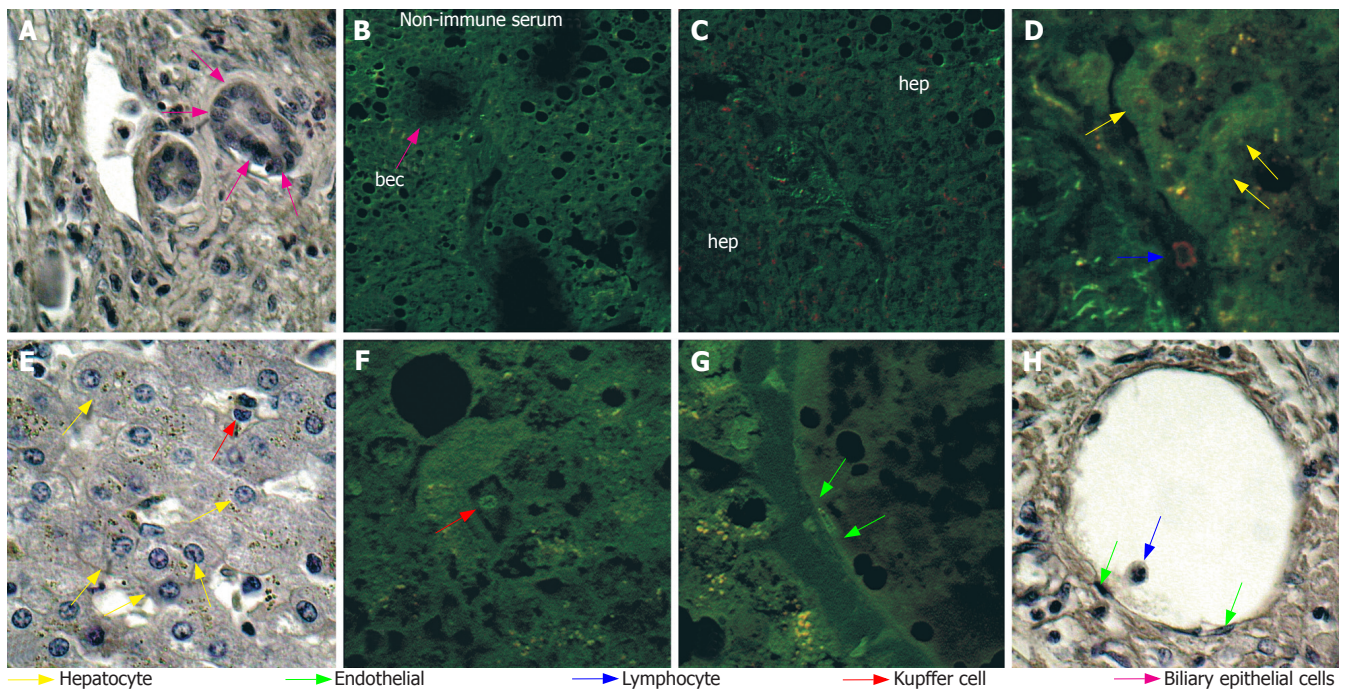


Figure 1 NGF distribution in tissues from healthy donors. **B, C, D, F, G:** Confocal microscopy images of immuno-stained sections; in negative hepatocytes, endothelial cells, biliary epithelial cells and Kupffer cells the immunoreaction is the same as the background obtained in control performed by substitution of primary antisera with non-immune rabbit serum; positive immunoreaction (red hue) is only observed on lymphocytes in blood vessels. Green hue represents the auto-fluorescence used to visualize liver tissue morphology; **A, E, H:** Images of H&E stained sections close to that used for immunohistochemistry. Differently coloured arrows indicate the different cell types (see legend). bec: biliary epithelial cells; hep: hepatocytes.

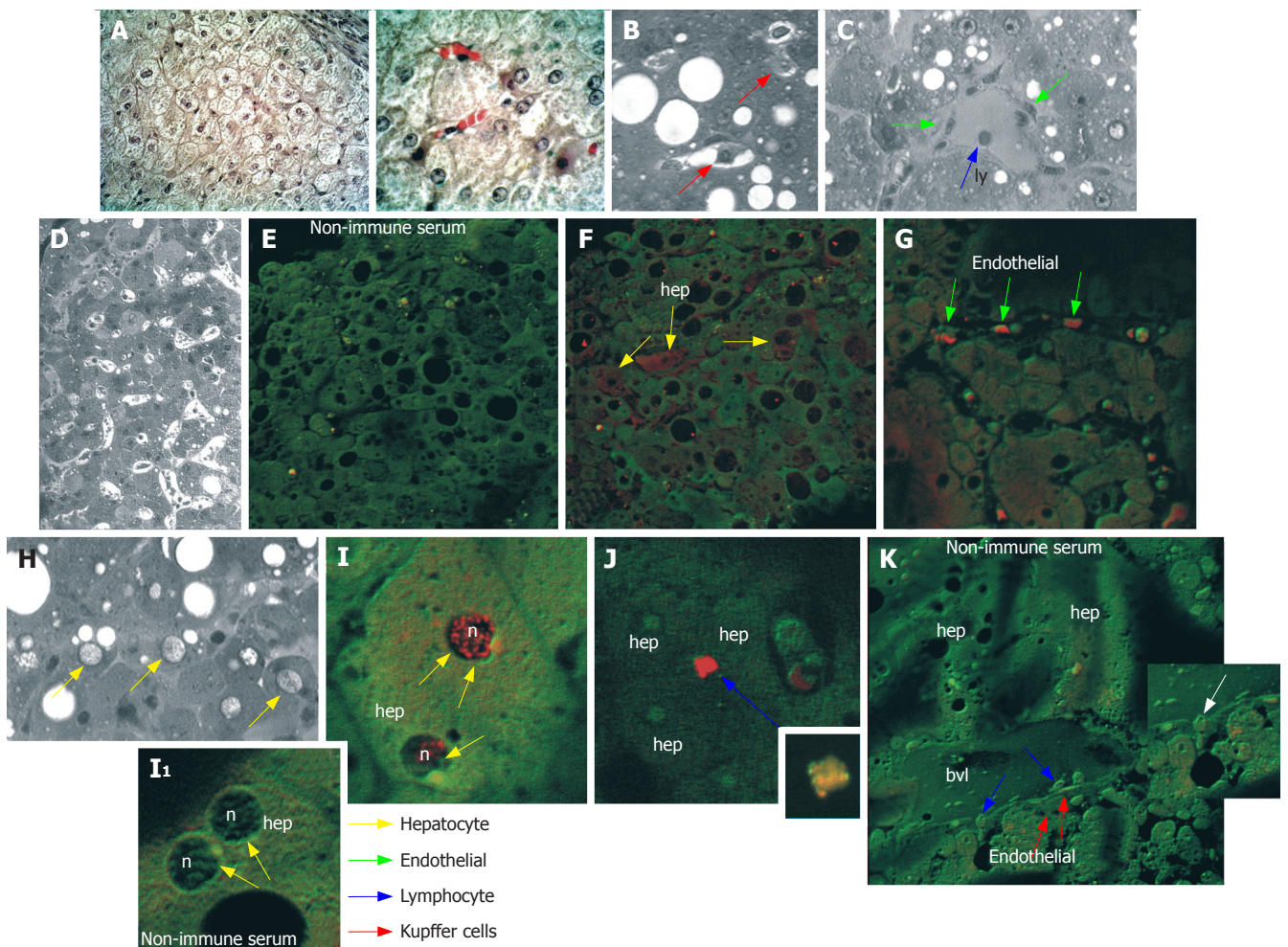


Figure 2 NGF distribution in HCC tissue. **E, F, G, I, J, K:** Confocal microscopy images of immuno-stained sections showing NGF immunoreaction (red hue) in the cytoplasm (**F**) and on nuclei (**I**) of some hepatocytes, on endothelial cells, on lymphocytes and on Kupffer cells; no immunoreaction was observed in controls performed by substitution of primary antisera with non-immune rabbit serum. **A:** Images, at two different magnification, of H&E stained sections; **B, C, D, H:** Images of semithin sections from samples embedded in Spurr epoxy resin. Differently coloured arrows indicate the different cell types (see legend). n: nucleus; hep: hepatocyte; bvl: blood vessel lumen.

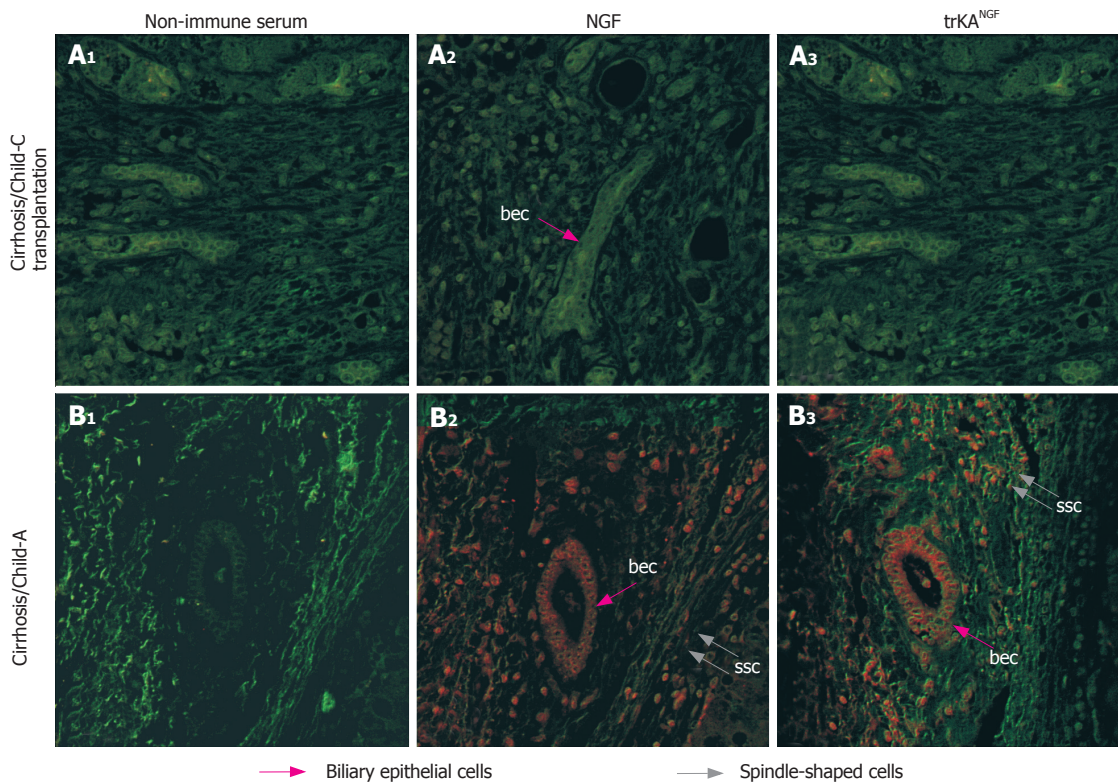


Figure 3 NGF and trkA^{NGF} distribution in cirrhotic tissues by confocal microscopy. **A₁**: Liver specimens obtained before transplantation from patients with cirrhosis but without HCC (Child-Pugh C); **A₂**: NGF distribution; **A₃**: trkA^{NGF} distribution; **B₁**: Specimens from patients with cirrhosis, also suffering from HCC, at early staging (Child-Pugh A); **B₂**: NGF distribution; **B₃**: trkA^{NGF} distribution. NGF or trkA^{NGF} immunoreaction (red hue) is particularly evident on biliary epithelial cells and on spindle-shaped cells, in cirrhotic tissue from patient at early staging (Child-Pugh A), but in presence of HCC, while no immunoreaction is observed in liver specimens resected before transplantation (Child-Pugh C) from patients without HCC. No immunoreaction is observed in controls performed by substitution of primary antisera with non-immune rabbit serum. bec: biliary epithelial cells; ssc: spindle-shaped cells.

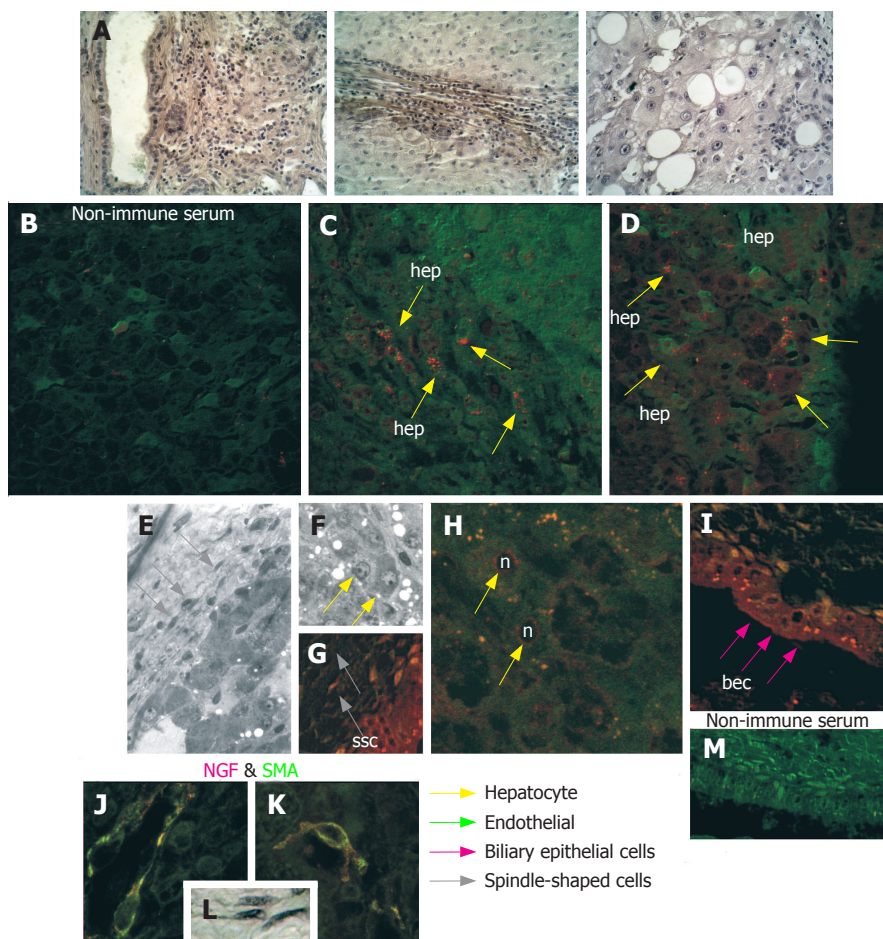


Figure 4 NGF distribution in cirrhotic tissue from patient also suffering from HCC. **B, C, D, G, H, I, M, J, K**: Confocal microscopy images of immuno-stained sections showing NGF immunoreaction (red hue) in the cytoplasm of some hepatocytes, on biliary epithelial cells and on spindle-shaped cells; no immunoreaction is observed in controls performed by substitution of primary antisera with non-immune rabbit serum. **J, K**: Double staining on spindle-shaped cells using anti-NGF and antibody against the myofibroblast marker smooth muscle actin (SMA); **A, L**: Images of H&E staining; **E, F**: Images of semithin sections from samples embedded in Spurr epoxy resin. Differently coloured arrows indicate the different cell types (see legend). bec: biliary epithelial cells; n: nucleus; hep: hepatocyte; ssc: spindle-shaped cells.

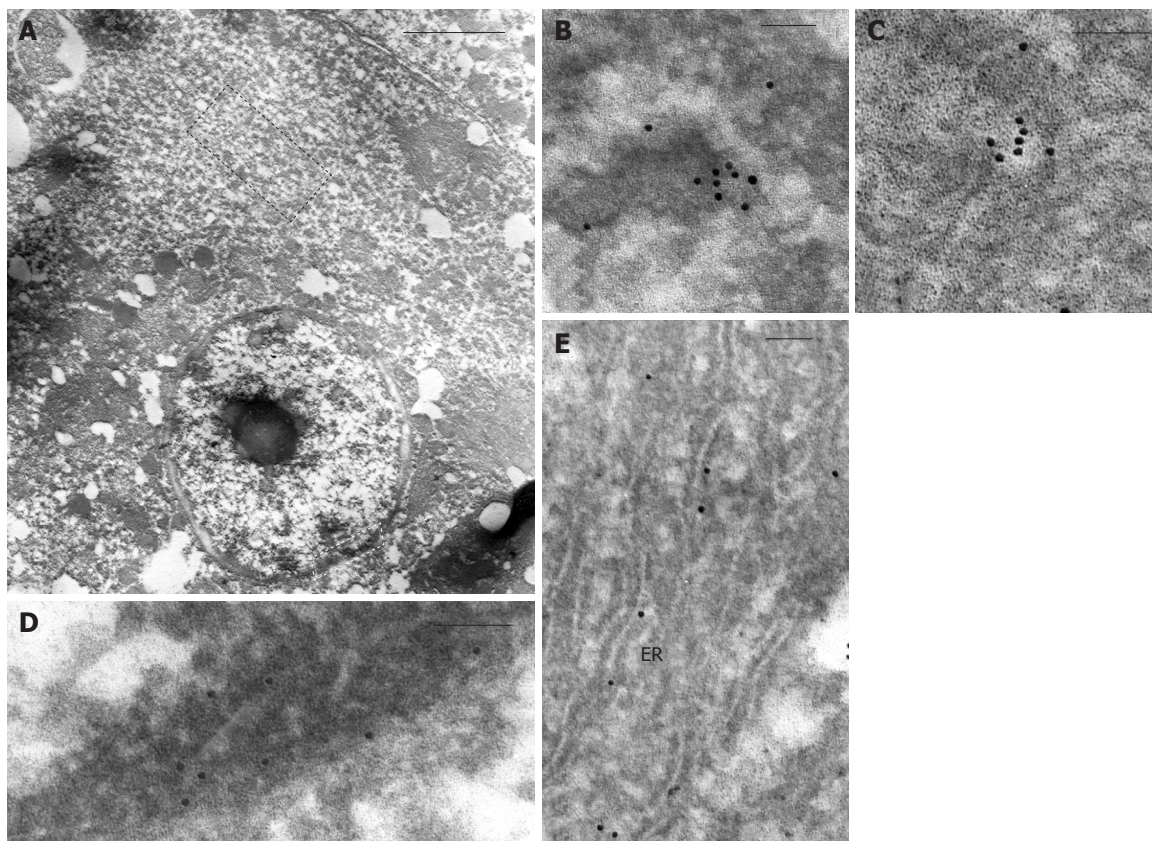


Figure 5 NGF distribution in HCC tissue by immunogold labelling. **A**: Transmission electron micrographs of hepatocyte; **B, C**: Positive immunogold reaction on cytoplasmic vesicles; **D**: Positive immunogold reaction on nuclei; **E**: Positive immunogold reaction on endoplasmic reticulum. ER: endoplasmic reticulum. Scale bars = **A**: 2 μ m; **B-E**: 100 nm.

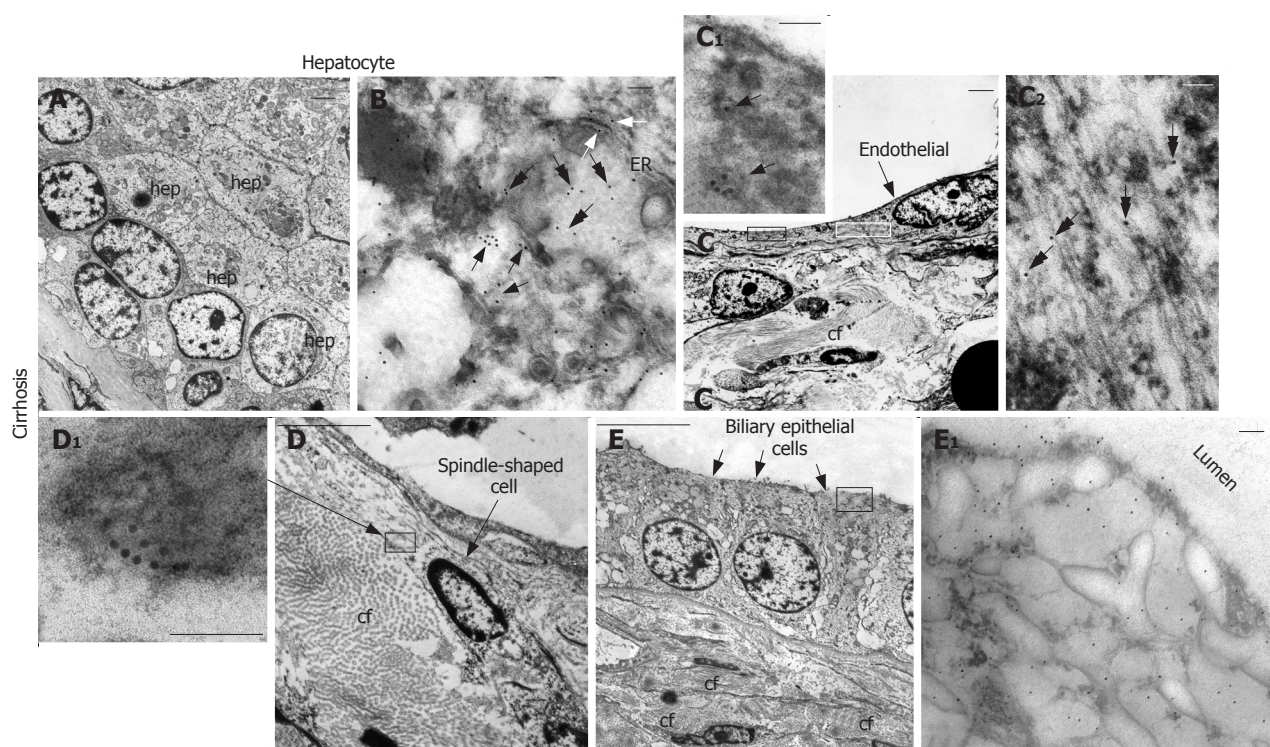


Figure 6 NGF distribution in cirrhotic tissue from patient with HCC by immunogold labelling. **A, B**: Transmission electron micrographs of hepatocytes showing positive immunogold reaction on cytoplasmic vesicles (black arrows), free in the cytoplasm (double pointed arrows) and along endoplasmic reticulum (white arrows); **C, C1, C2**: Endothelial cells; positive immunogold reaction on cytoplasmic vesicles (black arrows) and free in the cytoplasm (double pointed arrows); **D, D1**: Spindle-shaped cells; **E, E1**: Biliary epithelial cells. In **C1, C2, D1** and **E1** details at higher magnification are shown. hep: hepatocytes; cf: collagen fibers; ER: endoplasmic reticulum. Scale bars = **A, C, D, E**: 2 μ m; **B, C1, C2, D1, E1**: 100 nm.

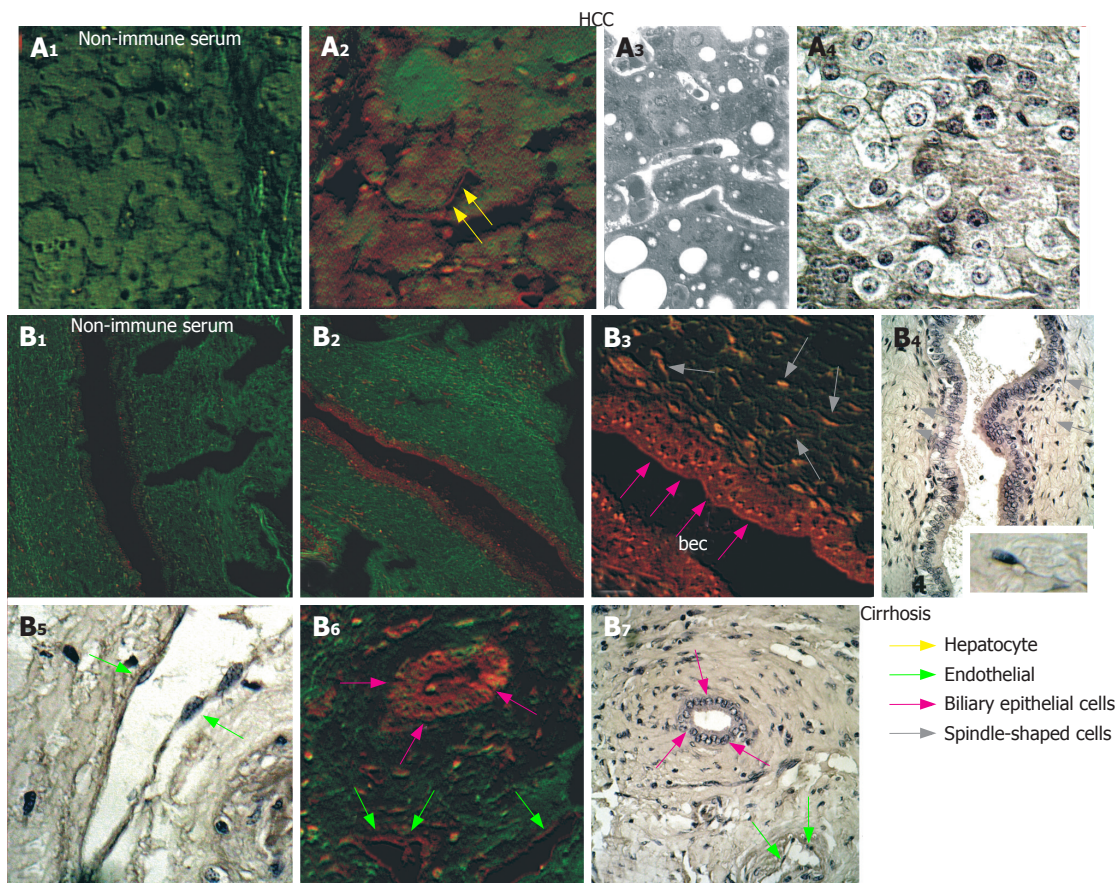


Figure 7 TrkA^{NGF} distribution in HCC and cirrhotic tissues. **A:** HCC tissue; **B:** Cirrhotic tissue. **A₁, A₂, B₁, B₂, B₃, B₆:** Confocal microscopy images of immuno-stained sections. No immunoreaction was observed in controls performed by substitution of primary antisera with non-immune rabbit serum; **A₄, B₄, B₅, B₇:** Images of HE stained sections; **A₃:** Images of semithin section from samples embedded in Spurr epoxy resin. Differently coloured arrows indicate the different cell types (see legend). bec: biliary epithelial cells.

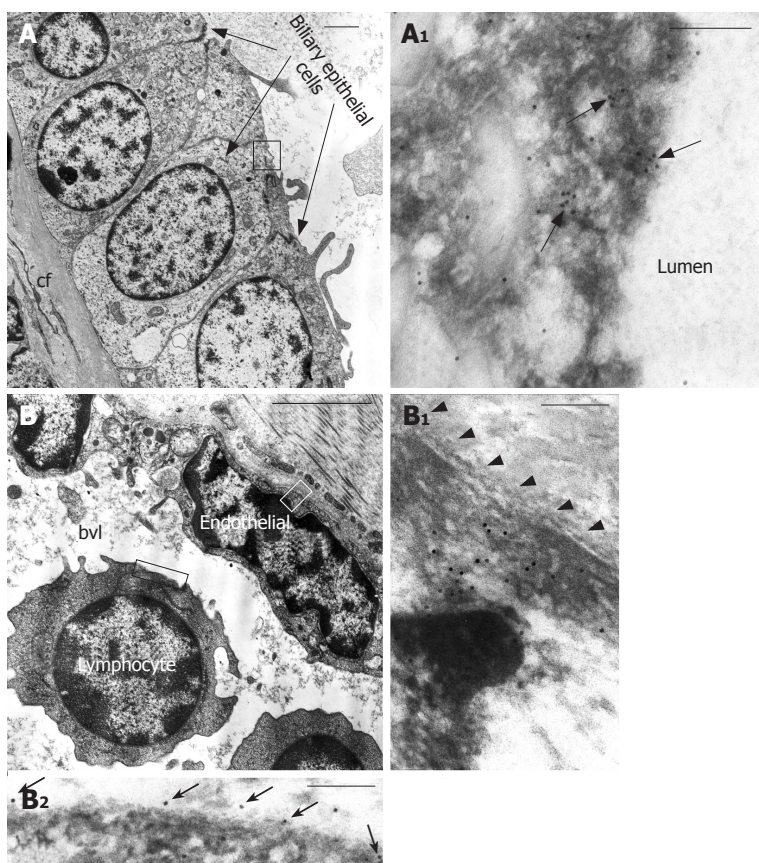


Figure 8 TrkA^{NGF} distribution in cirrhotic tissue from patient with HCC by immunogold labeling. **A, A₁:** Transmission electron micrographs of biliary epithelial cells showing positive immunogold reaction on cell membrane and cytoplasmic vesicles (black arrows) near the ductal lumen; **B:** Transmission electron micrographs of endothelial cells and lymphocytes; **B₁:** Immunoreactivity free in the cytoplasm of endothelial cells beneath the cell membrane (arrowheads); **B₂:** Gold particles on cell membrane of a lymphocyte. In **A₁, B₁** and **B₂** details at higher magnification are shown. bvl: blood vessel lumen; cf: collagen fibers. Scale bars = **A, B:** 2 μm; **A₁, B₁, B₂:** 200 nm.

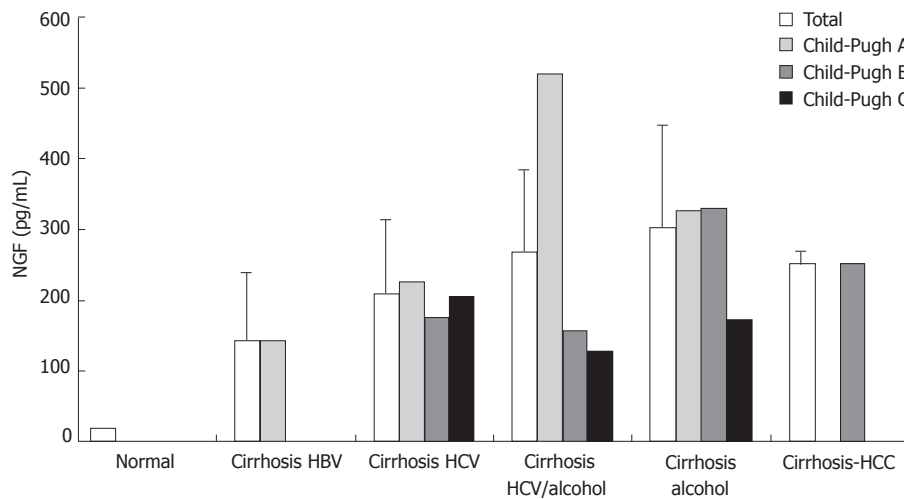


Figure 9 Bar diagram illustrating the circulating NGF levels, determined by test ELISA, in patients with documented cirrhosis/HCC. NGF amounts, reported with regard to the etiology, is calculated either as mean \pm SD (all patients examined) or as mean values for Child-Pugh class A (score = 5-6), for Child-Pugh class B (score = 7-9) and for Child-Pugh class C (score = 10-15). As a control, mean \pm SD of circulating NGF levels from some healthy individuals is also reported.

Table 2 Expression of NGF and trkA^{NGF} in liver cell types from healthy donors and from patients with cirrhosis and HCC

	Hepatocytes		Biliary epithelial cells		Endothelial cells		Spindle-shaped cells		Lymphocytes		Kupffer cells		Determination methods
Health	NGF	-	NGF	-	NGF	-	NGF	nd	NGF	+	NGF	-	CLSM (IF)
	trkA^{NGF}	-	trkA^{NGF}	-	trkA^{NGF}	-	trkA^{NGF}	nd	trkA^{NGF}	nd	trkA^{NGF}	-	
HCC	NGF	$^{+1}$	NGF	\pm	NGF	$^{+}$	NGF	nd	NGF	$^{+}$	NGF	$^{+}$	CLSM (IF)
	trkA^{NGF}	\pm	trkA^{NGF}	\pm	trkA^{NGF}	\pm	trkA^{NGF}	nd	trkA^{NGF}	nd	trkA^{NGF}	\pm	TEM (IG)
CIRR	NGF	$^{++1}$	NGF	$^{++2}$	NGF	$^{++}$	NGF	$^{+}$	NGF	$^{+}$	NGF	\pm	CLSM (IF)
	trkA^{NGF}	$^{++}$	trkA^{NGF}	$^{++2}$	trkA^{NGF}	$^{+}$	trkA^{NGF}	$^{+}$	trkA^{NGF}	$^{+}$	trkA^{NGF}	$^{+}$	TEM (IG)

IF: immunofluorescence labelling; IG: immunogold labelling; nd: not determined. ¹Immunoreaction mainly localized on cytoplasmic vesicles and endoplasmic reticulum. ²Immunoreaction mainly localized in the portion of cells near the ductal lumen.

epithelial cells, mainly localized on large cytoplasmic vacuoles in the ductal lumen portion of cells suggesting an intraductal lumen secretion during the progression from cirrhosis to HCC. Other studies^[34,35] have shown that binding of NGF to its cognate receptor trkA^{NGF} induces formation of signalling endosomes containing both the NGF and activated trkA^{NGF} receptor, with the latter exhibiting very high dynamic trafficking between the cell surface and internal cell compartments. Therefore, the predominant staining on cytoplasmic vacuoles observed for trkA^{NGF} on biliary epithelial cells in cirrhotic tissue may represent the receptor endocellular trafficking. Taken together, these findings indicate that NGF and its related receptor play an important role also in modulating the physiopathology of the intrahepatic biliary epithelium in the course of liver tissue remodelling processes and HCC progression.

The mechanism of NGF involvement in liver tissue remodelling processes and HCC progression remain unclear. However, our observation, defining NGF distribution both inside the liver and in the intracellular compartments, indicate that it may function, in a paracrine and an autocrine manner, as a messenger molecule in the cross-talk between different cell types. This, in addition to the reported lack of NGF expression in cirrhotic tissue without concomitant HCC even if present at high level in serum, strongly support the need to further clarify the mechanism and to define the role of this neurotrophin.

This opens up an interesting perspective for the possible use of NGF, not only as a marker of progression

and transformation, but also as an attractive target for a new therapeutic approach.

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COLORECTAL CANCER

Correlation of IL-8 with induction, progression and metastatic potential of colorectal cancer

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association between IL-8 expression, induction and progression of colorectal carcinoma and the development of colorectal liver metastases.

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Key words: Interleukin-8; Gene expression profiling; RNA and protein expression; Colorectal cancer; Colorectal liver metastases

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Abstract

AIM: To investigate the expression profile of IL-8 in inflammatory and malignant colorectal diseases to evaluate its potential role in the regulation of colorectal cancer (CRC) and the development of colorectal liver metastases (CRLM).

METHODS: IL-8 expression was assessed by quantitative real-time PCR (Q-RT-PCR) and the enzyme-linked immunosorbent assay (ELISA) in resected specimens from patients with ulcerative colitis (UC, $n = 6$) colorectal adenomas (CRA, $n = 8$), different stages of colorectal cancer ($n = 48$) as well as synchronous and metachronous CRLM along with their corresponding primary colorectal tumors ($n = 16$).

RESULTS: IL-8 mRNA and protein expression was significantly up-regulated in all pathological colorectal entities investigated compared with the corresponding neighboring tissues. However, in the CRC specimens IL-8 revealed a significantly more pronounced overexpression in relation to the CRA and UC tissues with an average 30-fold IL-8 protein up-regulation in the CRC specimens in comparison to the CRA tissues. Moreover, IL-8 expression revealed a close correlation with tumor grading. Most interestingly, IL-8 up-regulation was most enhanced in synchronous and metachronous CRLM, if compared with the corresponding primary CRC tissues. Herein, an up to 80-fold IL-8 overexpression in individual metachronous metastases compared to normal tumor neighbor tissues was found.

CONCLUSION: Our results strongly suggest an

INTRODUCTION

Colorectal cancer (CRC) is one of the most common cancers, with nearly one million estimated new patients per year world wide in 2006. Despite continuous improvements in diagnosis and therapy, approximately 60% of colon cancer patients develop metastases. While there is a good survival rate for patients diagnosed with localized disease, the CRC prognosis worsens dramatically with advancing stages and distant metastases and only 5% of patients diagnosed with distant metastases survive 5 years^[1,2]. Colorectal specimens and cell lines derived from them have been shown to express a variety of chemokines, including the multifunctional cytokine Interleukin (IL)-8, a member of the CXC chemokine superfamily of structurally and functionally related inflammatory cytokines that stimulate the migration of distinct subsets of cells^[3,4]. IL-8 and other anti-inflammatory cytokines have been shown to act as potent chemoattractants for leucocytes, such as neutrophils and natural killer cells, thus contributing to healing processes but also stimulating inflammatory diseases such as rheumatoid arthritis, sepsis and inflammatory bowel disease^[5-7]. Subsequent studies revealed that IL-8 also promotes the movement of cells of different lineages, such as fibroblasts and keratinocytes^[8,9]. In recent years, IL-8 was reported to induce the migration of tumor cells and its expression was correlated with tumor growth, angiogenesis and metastatic potential in various human carcinomas and animal models^[10-16].

Yet, up to now, the pathophysiological role of IL-8 in

tumor development and metastasis is not fully understood and still a matter of debate. For example, various recent studies point out that IL-8 expression correlates with disease progression in human melanoma^[17-19]. On the other hand, IL-8 expression in melanoma is only associated with early malignancy, whereas metastatic melanoma as well as dysplastic naevi specimens showed little or no IL-8 up-regulation as shown by immunohistochemical staining^[20]. Although constitutive IL-8 expression was determined in melanoma cells from surgical specimens, no correlation was found between the degree of cytokine expression and the clinical tumor stage^[21]. Accordingly, there is no distinct relationship between IL-8 expression and metastatic potential in various cell lines representing different clinical stages^[22,23] and some studies have even reported on antitumoral IL-8 effects^[24].

However, increasing data suggest that constitutive expression of IL-8 in colon carcinoma cell lines is associated with the metastatic potential and that IL-8 might act as an autocrine/paracrine growth factor in colon cancer progression and metastasis^[25,26]. Still it remains unclear, whether IL-8 expression is related to cancer progression and the metastatic potential in colorectal carcinoma tissues. Therefore, the purpose of this study was to examine the expression profile of IL-8 in inflammatory as well as non-malignant and malignant colorectal diseases and colorectal liver metastases (CRLM) to evaluate its role in the regulation of colorectal cancer (CRC) and the development of CRLM.

MATERIALS AND METHODS

Patients

Surgical specimens and corresponding normal tissue from the same samples were collected from patients with ulcerative colitis (UC, $n = 6$), colorectal adenomas (CRA, $n = 8$), colorectal carcinomas of different cancer stages ($n = 48$) and synchronous or metachronous CRLM ($n = 16$) who underwent surgical resection at our department between 2003 and 2006. Informed consent for tissue procurement was obtained from all patients. The study was approved by the ethics commission of the Ärztekammer of the Saarland. The clinical variables were obtained prospectively from the clinical and pathological records and are in accordance with the UICC/TNM classification^[27] (Tables 1 and 2).

Tissue preparation

Tissue samples were collected immediately after resection, snap frozen in liquid nitrogen and then stored at -80°C until they were processed under nucleic acid sterile conditions for RNA and protein extraction. Tumor samples were taken from vital areas of histopathologically confirmed (M.W.) adenocarcinomas and liver metastases, respectively. As corresponding normal tissue we used adjacent unaffected mucosa, 2-3 cm distal to the resection margin from the same resected adenocarcinoma or liver specimen, respectively. All tissues obtained were reviewed by a minimum of two experienced pathologists (M.W. *et al*) and examined for the presence of tumor cells. As

minimum criteria for usefulness for our studies we only chose tumor tissues in which tumor cells occupied a major component ($> 80\%$) of the tumor sample.

Single-strand cDNA synthesis

Total RNA was isolated using RNeasy columns from Qiagen (Hilden, Germany) according to the manufacturer's instructions. RNA integrity was confirmed spectrophotometrically and by electrophoresis on 1 g/L agarose gels. For cDNA synthesis 5 μg of each patient total RNA sample were reverse-transcribed in a final reaction volume of 50 μL containing 1 \times TaqMan RT buffer, 2.5 $\mu\text{mol/L}$ random hexamers, 500 $\mu\text{mol/L}$ each dNTP, 5.5 mmol/L MgCl_2 , 0.4 nakt/L RNase inhibitor, and 1.25 nakt/L Multiscribe RT. All RT-PCR reagents were purchased from Applied Biosystems (Foster City, CA). The reaction conditions were 10 min at 25°C , 30 min at 48°C , and 5 min at 95°C .

Real-time PCR

All Q-RT PCR assays containing the primer and probe mix were purchased from Applied Biosystems, (Applied Biosystems, Foster City, CA) and utilized according to the manufacturer's instructions. PCR reactions were carried out using 10 μL 2 \times Taqman PCR Universal Master Mix No AmpErase[®] UNG and 1 μL gene assay (Applied Biosystems, Foster City, CA), 8 μL Rnase-free water and 1 μL cDNA template (50 mg/L). The theoretical basis of the qRT assays is described in detail elsewhere^[28]. All reactions were run in duplicates along with no template controls and an additional reaction in which reverse transcriptase was omitted to assure absence of genomic DNA contamination in each RNA sample. For the signal detection, ABI Prism 7900 sequence detector was programmed to an initial step of 10 min at 95°C , followed by 40 thermal cycles of 15 s at 95°C and 10 min at 60°C and the log-linear phase of amplification was monitored to obtain C_T values for each RNA sample.

Gene expression of IL-8 was analyzed in relation to the levels of the slope matched housekeeping gene Cyclophilin C (CycC)^[29]. Since reporting of data obtained from raw C_T values falsely represent the variations, we converted the individual C_T values to the linear form as follows:

$$\text{Fold difference} = 2^{-(\text{mean } C_{T, \text{pathological tissue}} - \text{mean } C_{T, \text{calibrator}})} = 2^{-\Delta C_T}$$

Hence, the normal tissue became the 1 \times sample, and all other quantities were expressed as an n-fold difference relative to this tissue.

Isolation of total protein

Protein lysates from frozen tissues were extracted with the radioimmunoprecipitation (RIPA) cell lysis and extraction buffer from Pierce (Pierce, Rockford, USA). Total protein content was assessed by using the Pierce BCA protein assay reagent kit (Pierce, Rockford, USA).

Enzyme-linked immunosorbant assay

IL-8 tissue concentrations in the lysates were determined by sandwich-type Enzyme-linked immunosorbant assay (ELISA) according to the manufacturer's protocol: R&D systems (R&D Systems Inc. Minneapolis, Minnesota, USA)

Table 1 Clinical characteristics of patients with colorectal carcinomas and colorectal liver metastases

Factor	CRC (n = 48)	CRLM (n = 16 ¹)
Localization of primary tumor		
Colon	22	6
Rectum	26	8
Gender		
Male	29	7
Female	19	7
Age, yr ²	63.7 (47-78)	60.3 (41-76)
Hepatitis (A, B or C)		
Positive	6	2
Negative	42	12
Liver cirrhosis		
Positive	1	0
Negative	47	14
Adipositas		
Positive	12	4
Negative	36	10
Largest tumor diameter (cm) ²	4.6 (1.2-9.1)	4.2 (1.5-5.5)
TNM stage of primary tumor		
I	8	1
II	15	2
III	15	10
IV	10	1
Grading		
I	0	0
II	21	4
III	27	10
Lymphatic permeation		
Positive	27	10
Negative	21	4
Vascular invasion		
Positive	6	5
Negative	42	9
Chemotherapy before operation	0	2
Radiotherapy before operation	0	2

TNM: Tumor-node-metastasis; CRC: Colorectal carcinoma; CRLM: Colorectal liver metastases. ¹16 CRLM originating from 14 CC patients; ²Median with range in parentheses.

for the quantification of IL-8. The absorbance was read at 450 nm.

Laser Capture Microdissection

Laser microbeam microdissection (LMM) was employed for obtaining pure tumor cell and pure normal cell samples for subsequent genetic analysis. LMM was performed on three samples for each tissue type for IL-8. Histochemical staining was used on cryo sections before microdissection. Specimen preparation, microdissection and catapulting were performed following a laser pressure catapulting protocol according to the manufacturer's instructions (P.A.L.M. Microlaser Technologies, Bernried, Germany). RNA was extracted using the P.A.L.M. RNA extraction kit and for reverse transcription the invitrogen reverse transcription kit (Invitrogen Life Technologies, Karlsruhe, Germany) was applied. Subsequently quantitative PCR analysis was performed.

Statistical analysis

IL-8 expression profiles in the different groups are presented as mean and standard error of the mean (SEM). Statistical calculations were done with the MedCalc

Table 2 Clinical characteristics of patients with ulcerative colitis and colorectal adenomas

Factor	UC (n = 6)	CRA (n = 8)
Localization of biopsy		
Colon	6	6
Rectum	0	2
Gender		
Male	4	5
Female	2	3
Age (yr) ¹	51.7 (23-78)	65.3 (41-75)
Adipositas		
Positive	0	5
Negative	6	3

UC: Ulcerative colitis; CRA: Colorectal adenoma. ¹Median with range in parentheses.

software package (MedCalc software, Mariakerke, Belgium)^[30]. Where appropriate, either the parametric *t*-tests or the Wilcoxon's rank sum test were applied to test for group differences of continuous variables. $P \leq 0.05$ was deemed significant.

RESULTS

IL-8 expression in colorectal carcinomas related to tumor stage

Recently, we demonstrated the involvement of the murine macrophage inflammatory protein (MIP)-2, a functional murine analogue of the human IL8, in angiogenesis and tumor growth^[16]. Here, we analysed IL-8 expression in human tissue specimens of various colorectal diseases and cancer stages using Q-RT-PCR and ELISA. Our tissue specimens descended from 6 patients with UC, 8 patients with CRA and 48 patients with CRC of various cancer stages with different metastatic potentials (Tables 1 and 2). Adjacent, disease and tumor free tissue samples of the UC, CRA and CRC patients served as control groups, respectively.

Q-RT-PCR analysis revealed significant up-regulation of IL-8 mRNA expression in all pathological tissue specimens analysed, if compared with the unaffected corresponding UC-, CRA- and CRC neighbor tissues, as presented in Figure 1A. In UC- and CRA tissue specimens IL-8 mRNA expression was on average 11- and 10-fold up-regulated ($P < 0.05$, respectively). Yet, in the different T- stages of the CRC specimens IL-8 expression was more than 25-fold up-regulated ($P < 0.05$). The results shown in Figure 1A represent the pooled mean expression levels and the SEMs from 6 UC-, 8 CRA-, 8 T1-, 15 T2-, 15 T3- and 10 T4 stage CRC patients, respectively. Since averaging out the C_t values or ratios could mask significant differences between individual paired samples, we also analysed the differences between gene expression from matched normal/cancer samples. The results obtained from this level of analysis corresponded widely with the results presented in Figure 1A thus confirming the correlation between the degree of IL-8 expression and advanced tumor stage. Sections of tumor and normal cells have been microdissected in three specimens of each tissue type, followed by subsequent RT-PCR gene expression analysis

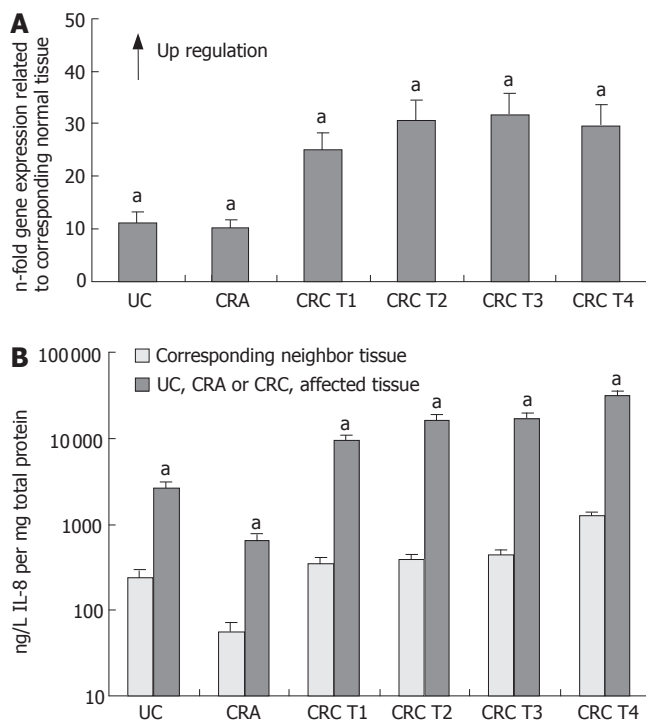


Figure 1 IL-8 expression in ulcerative colitis (UC), colorectal adenoma (CRA) and colorectal carcinomas (CRC). **A:** Q-RT-PCR; **B:** ELISA (mean \pm SE, $^aP < 0.05$, $n = 6, 8, 8, 15, 15$ and 10 , respectively).

to ensure that the expression data corresponded well with the results presented in Figure 1A.

In consistence with our results obtained at the RNA level, IL-8 protein expression, as assessed by ELISA, revealed also significant up-regulation of IL-8 protein expression in all pathological tissue specimens in comparison to the unaffected corresponding UC-, CRA- and CRC mucosa tissues, respectively (Figure 1B). Analysis of IL-8 tissue protein concentrations showed a similar profile with an average 12- and 11-fold overexpression in the UC- and CRA tissue specimens in relation to normal mucosa tissues ($P < 0.05$), and on average 30-fold higher IL-8 expression in the different T- stages of the CRC specimens, if compared with the respective tumor neighboring mucosa tissues ($P < 0.05$). Moreover, absolute expression on the protein level revealed a distinct increase in IL-8 expression levels in tumor- and tumor-free neighbor tissues of CRC patients with T-stage 4 in comparison to CRC patients with T-stages 1-3 ($P < 0.05$). However, most notably, we observed that IL-8 protein concentrations were significantly higher expressed in all CRC specimens compared to non-malignant and inflammatory tissue samples ($P < 0.05$, Figure 1B), showing on average 30-fold IL-8 protein up-regulation in the CRC specimens in comparison to the CRA tissues. For example, comparative analysis of the absolute protein levels between the CRC specimens and the CRA tissues revealed a significantly higher IL-8 expression of almost 30000 ng/L IL-8 in the CRC tissues of tumor- stage 4 compared to approximately 600 ng/L IL-8 in the CRA tissues ($P < 0.05$, Figure 1B).

IL-8 expression in colorectal liver metastases

In the next set of experiments, we analysed IL-8 mRNA

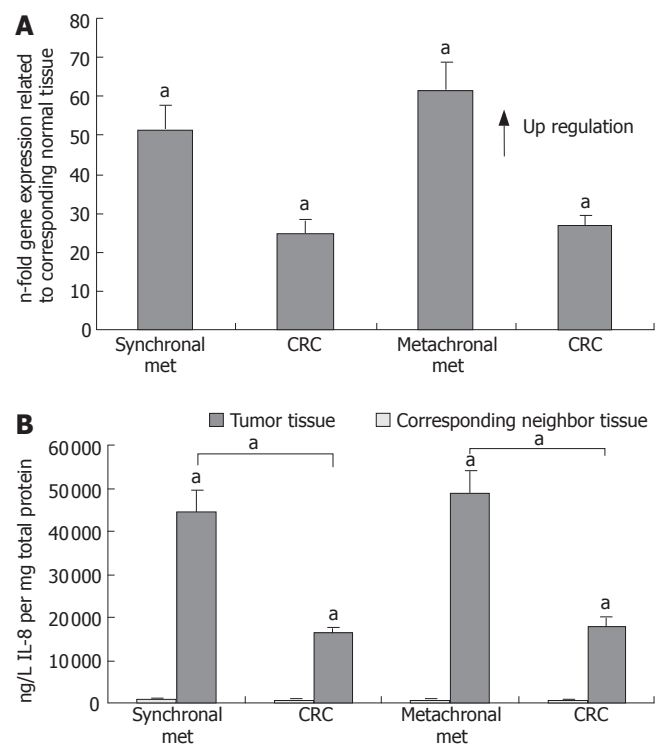


Figure 2 IL-8 expression in colorectal liver metastases of synchronous (Synchronous Met) and metachronal (Metachronal Met) origin and corresponding colorectal carcinomas (CRC). **A:** Q-RT-PCR; **B:** ELISA (mean \pm SE, $^aP < 0.05$, $n = 9$ and 7 , respectively).

and protein expression in 16 CRLM of synchronous and metachronous origin and the corresponding colorectal cancer tissues where the metastases originated from (Table 1). Healthy tumor neighbor tissues of the CRLM and corresponding CRC tissues served again as control groups. On the RNA level we found on average a 52-fold increase in IL-8 expression in the synchronous metastases compared with the corresponding tumor neighbor tissues and a 62-fold increase in IL-8 expression in the metachronous metastases ($P < 0.05$, respectively, Figure 2A). These findings were well in line with the results obtained on the protein level, which showed a 47-fold increase in IL-8 expression in synchronous metastases and a 60-fold increase in metachronous metastases ($P < 0.05$, respectively) with up to 80-fold IL-8 overexpression in individual metastases, when compared to the corresponding tumor-free hepatic neighbor tissues, respectively (Figure 2B). In our survey, neither gender nor the specific tumor localization (colon versus rectum) influenced IL-8 expression profiles in our CRC patient series. However, CRC patients with synchronous or metachronous metastases beyond the age of 60 expressed significantly more IL-8 than CRC patients younger than 60 years ($P < 0.05$). Most interestingly, the highest IL-8 overexpression was detected in synchronous and metachronous colorectal metastases, if compared to their corresponding primary CRC tissues ($P < 0.05$, respectively Figure 2B). For example, comparative analysis of the absolute protein quantities between the CRLM specimens and their corresponding CRC tissues revealed an IL-8 expression of almost 50000 ng/L in the CRLM tissues of metachronous metastases compared to approximately

18000 ng/L in the corresponding CRC primary tumor tissues ($P < 0.05$, Figure 2B).

DISCUSSION

In this study, we present an unprecedented complete and comparative analysis of the IL-8 RNA and protein expression profile of various inflammatory, non-malignant and malignant histopathological colorectal entities comprising ulcerative colitis and colorectal adenomas, both representing known premalignant conditions often preceding the development of colorectal malignancies. We further included colorectal carcinomas of different tumor stages as well as synchronous and metachronous colorectal liver metastases along with their related primary colorectal tumors.

The major findings of this study demonstrate that the magnitude of IL-8 expression in surgical CRC tissue specimens correlates with colorectal cancer progression and the development of synchronous and metachronous liver metastases. Moreover, to our knowledge we could demonstrate for the first time that irrespective of the tumor stage IL-8 is significantly higher expressed in CRC tissues compared to inflammatory colorectal conditions and adenomas of the colon/rectum. Since such conditions often constitute prevalent pre-existing disease states in the etiopathogenesis of colorectal cancer, our results strongly suggest a close association of IL-8 up-regulation and the development of CRC. In line with this conclusion is the concept that inflammation is a critical component of tumor progression. Chronic inflammation can activate cell actions that increase the induction of irreversible DNA damage and the risk of cancer and it is now becoming clear that tumor cells have co-opted some of the signalling molecules of the innate immune system, such as chemokines and their receptors for invasion, migration and metastasis^[31]. In addition, we demonstrated significant IL-8 overexpression in CRLM in comparison to the related primary colorectal tumors, indicating a correlation between IL-8 expression and the malignant status of colorectal cancer cells. Thus, our results not only suggest an association between IL-8 expression and the induction and progression of colorectal carcinoma, but also clearly point to a correlation between IL-8 expression and the development of CRLM. As our results are based on expression data, they will need further evaluation. Therefore, the next step in our investigations will comprise the functional analysis in cell culture assays. However, even at this point, the clinical significance of our findings has meaningful consequences. Examining the IL-8 expression profile in CRC patients may potentially help to assess more precisely the course of the cancerous condition. In this respect it is conceivable to monitor the IL-8 expression level in CRC patients that show no diagnosable symptoms of CRLM at the time of presentation, but may still carry a high risk for developing such metastases. A significantly up-regulated level of IL-8 might thus be a useful tool to evaluate the prognosis of patients with CRC with an important impact on future treatment strategies. Thus, patients with a higher risk of developing CRLM may receive a different treatment from patients with a lower

risk of developing CRLM.

Our findings are supported by previous studies^[32], exploiting the potential clinical significance of serum IL-8 measurements in untreated CRC patients in comparison to healthy volunteers. Circulating IL-8 serum concentrations revealed a significant correlation with the clinical stage of CRC, bowel wall invasion and liver metastasis^[32,33]. Our results are also well in line with the outcome of recent surveys which compared the expression of a number of different chemokines and growth factors in a panel of arbitrarily chosen CRC specimens with normal mucosa by use of ELISA. The authors also found significant IL-8 over-expression in the malignant tissues that correlated with tumor size, depth of infiltration, Dukes stage and shorter overall survival times^[34,35]. Accordingly, Li and colleagues^[25] studied IL-8 expression in vitro in human colon carcinoma cells with different metastatic potentials and found an association between constitutive IL-8 expression and aggressiveness of human colon carcinoma cells, which was assigned to autocrine growth and angiogenic properties of IL-8 in the regulation of multiple biological activities in endothelial cells^[36,37]. In animal studies inhibition of IL-8 reduced tumorigenesis of human non-small cell lung cancer in SCID mice^[38], and our group^[16,39] reported that MIP-2 dose-dependently promotes angiogenesis as well as hepatic engraftment and tumor growth of colon cancer cells. In other tumor types a direct role of IL-8 was demonstrated, e.g. by enhancing proliferation and invasive potential of metastatic melanoma cells^[19]. Although several authors investigated possible pathways^[40,41], the precise molecular mechanisms underlying the IL-8 mediated regulation of tumor cells still remain obscure. Recently, IL-8 was reported to promote cell proliferation and migration through metalloproteinase-cleavage in human colon carcinoma cells^[42], and other studies indicated that IL-8 mediated tumor progression may be regulated by tumor-associated stress factors^[43].

In spite of the increasing number of studies that outlined an important role and potential mechanisms of IL-8 contributing to cancer progression in various cancer types through its regulative, autocrine and paracrine functions, its interference with respect to the development of malignancies, metastasis and angiogenesis still remains controversial. For example, Kassim and colleagues demonstrated an association between IL-8 expression and a dismal prognosis in epithelial ovarian cancer patients^[44], while other studies demonstrated that IL-8 reduced tumorigenicity of human ovarian cancer *in vivo* due to neutrophil infiltration^[45]. Accordingly, in a model of colon cancer in rats, IL-8 was found to have a highly reproducible antitumoral effect that was associated with a systemic activation of T lymphocytes^[24,46]. Likewise, various metastatic tumor cell lines were shown to produce IL-8 constitutively^[19,25], while other cell lines like some metastatic prostate tumor cell lines do not express IL-8 at detectable levels^[22]. Finally, in some studies no correlation between IL-8 secretion and tumor growth kinetics or metastatic potential was found at all^[47].

Despite still ongoing controversies regarding the biological relevance of IL-8 in cancer biology, our clinical and experimental findings strongly support

a multifunctional role for IL-8 in tumor induction, progression and metastasis. Taken together, our data outline a significant correlation between IL-8 expression and the induction and progression of CRC as well as the development of CRLM. The predominance of high IL-8 expression levels in surgical colorectal carcinoma specimens might thus be a useful indicator of poor prognosis. In addition to its prognostic significance we therefore propose IL-8 as a putative target for the development of novel treatment concepts in the therapy of CRC. Herein, neutralization of IL-8 or inhibition of its production and activity might be a future basic treatment strategy in the management of CRC.

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COMMENTS

Background

In recent years, an increasing number of studies suggested an involvement of IL-8 in the migration of tumor cells and correlated its expression with tumor growth, angiogenesis and metastatic potential in various human carcinomas and animal models. Yet, up to now, the pathophysiological role of IL-8 in tumor development and metastasis is not fully understood and discussed controversially. Moreover, it remains unclear, whether IL-8 expression is related to cancer progression and the metastatic potential in colorectal carcinoma tissues.

Research frontiers

The research hotspot is to elucidate a potential pathophysiological role of IL-8 in CRC development and metastatic spread and to clarify the precise molecular mechanisms underlying the IL-8 mediated regulation of colorectal carcinoma (CRC) cells.

Innovations and breakthroughs

In recent years, IL-8 expression was reported to be associated with colon cancer cell proliferation and the metastatic potential in colon carcinoma cell lines. Moreover, IL-8 was suggested to act as an autocrine/paracrine growth factor in colon cancer progression. In order to further evaluate the role of IL-8 in the regulation of CRC and the development of colorectal liver metastases (CRLM), we examined the expression profile of IL-8 in ulcerative colitis (UC) and colorectal adenoma (CRA) as conditions often preceding the development of colorectal malignancies, and moreover, in CRC tissues of different tumor stages as well as in synchronous and metachronous CRLM along with their related primary colorectal tumors. We demonstrated a significant correlation between IL-8 expression and the induction and progression of CRC as well as the development of CRLM.

Applications

Our findings suggest that monitoring the IL-8 expression level in CRC patients may potentially help to assess the course of the cancerous condition and the prognosis of patients with respect to the development of CRLM. Thus, we propose IL-8 as a useful indicator of poor prognosis and a putative target for the development of drugs in the therapy of CRC.

Terminology

UC is a disease that causes ulcers in the lining of the rectum and colon. It is one of a group of diseases called inflammatory bowel disease. CRA means a benign epithelial neoplasm of the colon or rectum, which extends from the ileocecal junction to the sigmoid colon. CRC develops as a locally invasive cancer in the colon and rectum often evolving from polyps with a very high incidence of metastatic spread.

Peer review

This is very nice work looking at IL-8 and the correlation with induction, progression and metastatic potential colorectal cancer. The scientific and innovative contents

as well as readability can reflect the advanced levels of the clinical and basic researches in gastroenterology.

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Protective effects of ursodeoxycholic acid on chenodeoxycholic acid-induced liver injury in hamsters

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Abstract

AIM: To investigate the effects of ursodeoxycholic acid (UDCA) on chenodeoxycholic acid (CDCA)-induced liver injury in hamsters, and to elucidate a correlation between liver injury and bile acid profiles in the liver.

METHODS: Liver injury was induced in hamsters by administration of 0.5% (w/w) CDCA in their feed for 7 d. UDCA (50 mg/kg and 150 mg/kg) was administered for the last 3 d of the experiment.

RESULTS: At the end of the experiment, serum alanine aminotransferase (ALT) increased more than 10 times and the presence of liver injury was confirmed histologically. Marked increase in bile acids was observed in the liver. The amount of total bile acids increased approximately three-fold and was accompanied by the increase in hydrophobic bile acids, CDCA and lithocholic acid (LCA). UDCA (50 mg/kg and 150 mg/kg) improved liver histology, with a significant decrease (679.3 ± 77.5 U/L vs 333.6 ± 50.4 U/L and 254.3 ± 35.5 U/L, respectively, $P < 0.01$) in serum ALT level. UDCA decreased the concentrations of the hydrophobic bile acids, and as a result, a decrease in the total bile acid level in the liver was achieved.

CONCLUSION: The results show that UDCA improves oral CDCA-induced liver damage in hamsters. The protective effects of UDCA appear to result from a decrease in the concentration of hydrophobic bile acids, CDCA and LCA, which accumulate and show the cytotoxicity in the liver.

INTRODUCTION

It is generally accepted that ursodeoxycholic acid (UDCA) can improve values for clinical and biochemical indices in patients with cholestatic liver disease, such as primary biliary cirrhosis (PBC)^[1-4], primary sclerosing cholangitis (PSC)^[5,6] and viral hepatitis^[7,8]. UDCA exerts its choleretic action on the cholestatic liver and consequently decreases the values for serum indices of hepatotoxicity, such as alanine aminotransferase (ALT).

Cholestasis, defined as impairment of bile flow in hepatobiliary circulation, is accompanied by the retention of bile acids in the liver^[9]. As mentioned above, cholestasis is observed widely not only in cholestatic liver diseases such as PBC and PSC, but also in viral hepatitis such as hepatitis C^[10]. It is necessary to determine the changes in the bile acid amounts in the liver in liver diseases and how UDCA affects the bile acid pool changes. Such studies are limited clinically and interpretation of the data from experimental animals is required. In studies using mice and rats, however, care should be taken in interpreting the data because bile acids have different metabolic profiles^[11,12]. For example, β -muricholic acid is one of the major bile acids, which is not produced in humans. On the other hand, hamsters have the advantage that their profile of the bile acid metabolism resembles that in humans^[13,14].

In this study, we fed CDCA, a hydrophobic bile acid, to hamsters to induce liver injury, and to investigate the efficacy of UDCA administration using the model for the first time. We also evaluated the correlation between bile acid concentration in the liver and the protective effects of UDCA in the liver.

MATERIALS AND METHODS

Materials

Ursodeoxycholic acid (UDCA) was synthesized at

Mitsubishi Pharma Corporation (Osaka, Japan), and its purity was confirmed to be higher than 99%. Chenodeoxycholic acid was purchased from CALBIOCHEM (San Diego, California).

Animals

Six-week-old Syrian golden hamsters (weighing 89-117 g) were purchased from Japan SLC Inc. (Hamamatsu, Japan). The animals were maintained in a 12-h day/night cycle, with standard powder chow (MF, Oriental Yeast Co., Ltd. Tokyo, Japan) and tap water given *ad libitum*. All animal experiments were approved by the Animal Ethical Committee of Mitsubishi Pharma Corporation and performed in accordance with guidelines of the Japanese Pharmacological Society.

Experimental protocol

After acclimation for 5 d, the hamsters were randomly allocated to four groups, 10 animals each. For 7 d, the animals in each group were fed a standard powder chow (MF) with or without 0.5% (w/w) CDCA as follows: chow without 0.5% (w/w) CDCA (normal group); chow with 0.5% (w/w) CDCA (control group and UDCA-treated groups). UDCA was orally administered (50 or 150 mg/kg) to the CDCA-fed hamsters at a volume of 5 mL/kg between 3 pm and 5 pm, once a day for the last 3 d of the experiment. Purified water was administered to hamsters in the normal group and control groups.

All animals were fasted for 18 h before sacrifice. The animals were weighed, and then anesthetized with diethyl ether. Blood was collected by cardiac puncture for the determination of serum ALT which was analyzed with an autoanalyzer (FUJI DRI-CHEM 7000, FUJIFILM, Tokyo). Then the liver was rapidly removed, weighed and rinsed with saline. The liver was processed for histopathological examination and for bile acid analysis (other than the UDCA 50 mg/kg group).

Histopathological examination

Part of the rinsed liver tissue was fixed in 100 mL/L formalin and embedded in a paraffin block. The paraffin section was stained with hematoxylin-eosin (HE) and examined microscopically. Histological findings were assessed blindly and independently by two pathologists and graded from (-) to (+++) for the severity of vacuolation, cell infiltration and focal necrosis as follows: -: no change, ±: slight change, +: mild change, ++: marked change, and +++: severe change.

Bile acid analysis

Bile acids in the liver were analyzed according to a method described elsewhere^[15]. Briefly, 100 µL each of methanol and 23-nordeoxycholic acid (the internal standard), and 1 mL of 0.2 mol/L NaOH (maintained at 80°C) were added to about 50 mg of liver tissue homogenate. The mixture was immediately heated at 80°C for 20 min to dissolve the liver tissue, then mixed with 3 mL of water, and left to cool to room temperature. A 3-mL n-hexane extraction was performed three times to remove neutral lipids. Bile acids were then extracted from the remaining aqueous phase with a BondElut C18 cartridge. The methanol

Table 1 Body weight changes and food intake during the experiment (mean ± SE)

Group	Body weight (g)		Weight gain (g)	Food intake (g/d)
	Before the experiment	End of the experiment		
Normal	104.8 ± 2.5	110.6 ± 3.3	5.8 ± 1.8	7.1 ± 0.2
Control	103.7 ± 2.5	106.9 ± 1.9	3.2 ± 1.2	6.5 ± 0.3
UDCA 50 mg/kg	103.1 ± 2.9	104.7 ± 3.1	1.6 ± 1.3	6.2 ± 0.4
UDCA 150 mg/kg	104.7 ± 2.1	107.2 ± 1.9	2.5 ± 1.6	6.2 ± 0.2

UDCA: Ursodeoxycholic acid. There was no significant difference between normal and control groups (Student's *t*-test). There was no significant difference between the control group and UDCA groups (Dunnett's multiple comparison test).

eluate was evaporated and the bile acids were analyzed by HPLC (Inertsil ODS-2 column). Examined bile acids were as follows: cholic acid (CA), CDCA, deoxycholic acid (DCA), LCA and UDCA. Concentrations of these 5 bile acids were calculated with summations of the 3 different types of conjugates (i.e., unconjugated, glycine and taurine conjugated bile acid) and expressed per gram of tissue weight. They were also expressed as composition percentages of total bile acids detected.

Statistical analysis

During the experiment, data on one out of every ten animals of each group were removed due to experimental treatment failures. Data on 9 animals of each group were processed as results in this study. Results were expressed as mean ± SE except the case for the bile acid analyses, which were expressed as mean ± SD. Differences among the group means were tested using Student's *t*-test or Dunnett's multiple comparison test. *P* < 0.05 was considered statistically significant.

RESULTS

Body weight and food intake changes during the experiment

The results of body weight and food intake are shown in Table 1. No significant differences were found in those parameters among the hamsters in the four groups during the experiment.

Effect of UDCA on serum ALT in CDCA-induced liver injury in hamsters

As shown in Figure 1, feeding of CDCA for 7 d induced liver injury, and the serum ALT level (679.3 ± 77.5 U/L) in the CDCA-fed group (control group) was significantly (*P* < 0.01) elevated as compared with that of the normal group (61.4 ± 8.7 U/L). UDCA administration for the last 3 d of the experiment significantly (*P* < 0.01) reduced the serum ALT values (50 mg/kg: 333.6 ± 50.4 U/L, 150 mg/kg: 254.3 ± 35.5 U/L) as compared to the control group (679.3 ± 77.5 U/L).

Effect of UDCA on hepatic pathology

Induction of hepatic injury was confirmed histopathologically. Slight to mild vacuolation in a broad area was

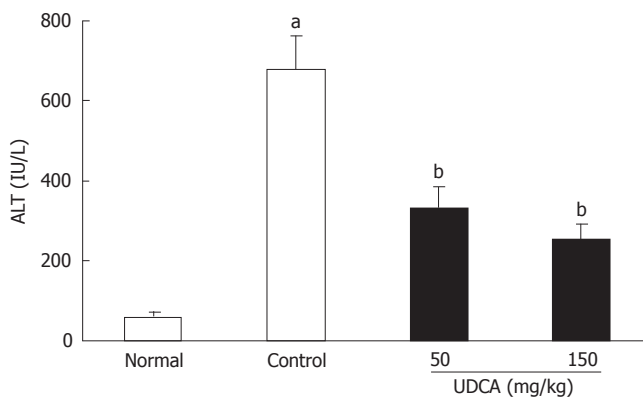


Figure 1 Effect of UDCA on serum ALT in hamsters fed CDCA. Data are expressed as mean \pm SE ($n = 9$). ^a $P < 0.01$ vs normal group (Student's *t*-test); ^b $P < 0.01$ vs control group (Dunnett's multiple comparison test).

Table 2 Effect of UDCA on histological extent of CDCA-induced liver injury in hamsters

	Findings	Grade		
		-	\pm	+
Normal		9	0	0
Control	Vacuolation	0	2	7
UDCA 150 mg/kg	(Hydropic swelling)	9	0	0
Normal		8	1	0
Control	Cell infiltration	8	1	0
UDCA 150 mg/kg		7	2	0
Normal		8	1	0
Control	Focal necrosis	8	1	0
UDCA 150 mg/kg		7	2	0

UDCA: Ursodeoxycholic acid; CDCA: Chenodeoxycholic acid. Grades are as follows: -: no change, \pm : slight change, +: mild change.

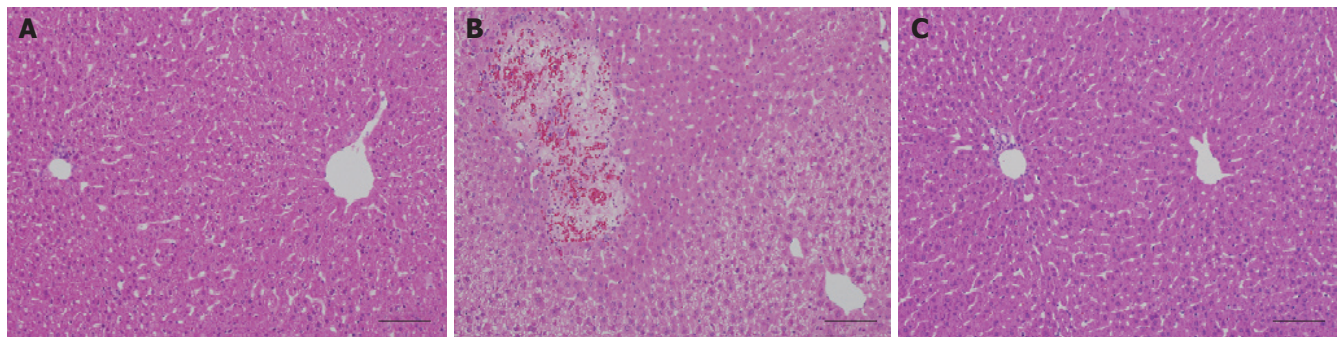


Figure 2 Effect of UDCA on light micrographic changes of the liver in hamsters fed CDCA. A: Normal; B: Control; C: 150 mg/kg UDCA (Hematoxylin-eosin staining). Bars indicate 100 μ m.

observed in the livers in the CDCA-fed group (control group), which was not observed in the normal group (Table 2, Figure 2A and B). UDCA treatment (150 mg/kg) led to a significant reduction in the grades of vacuolation observed in the control group (Table 2, Figure 2C). No changes were observed in cell infiltration and focal necrosis by CDCA feeding.

Bile acid concentration and composition in the liver

Concentrations and compositions of 5 major bile acids in the liver in each group are shown in Table 3. Livers from the normal group contained CA 102.0 ± 41.7 nmol/g ($68.2\% \pm 3.2\%$ of total bile acids), CDCA 36.0 ± 17.1 nmol/g ($23.8\% \pm 3.6\%$), DCA 10.3 ± 4.4 nmol/g ($6.8\% \pm 1.3\%$), and UDCA 1.9 ± 2.2 nmol/g ($1.2\% \pm 1.3\%$). The concentration of LCA was less than 1.0 nmol/g. In CDCA-fed hamsters, the total bile acids level was increased more than three-fold (484.7 ± 189.7 nmol/g). Analysis of each bile acid concentration revealed that CDCA concentration increased ten-fold (364.9 ± 141.5 nmol/g), and LCA concentration also markedly increased (89.4 ± 32.4 nmol/g). Compositions of the two hydrophobic bile acids were increased to $75.3\% \pm 2.7\%$ and $18.7\% \pm 2.0\%$, respectively. The concentration of UDCA was increased to 4.0 ± 0.9 nmol/g, whereas its composition did not change ($0.9\% \pm 0.3\%$) by CDCA feeding. On the other hand, concentrations and compositions of CA and DCA were

decreased significantly (CA: 20.9 ± 13.8 nmol/g and $4.0\% \pm 1.1\%$; DCA: 5.6 ± 3.2 nmol/g and $1.1\% \pm 0.3\%$).

When 150 mg/kg of UDCA was administered, the total bile acids level was significantly decreased ($P < 0.01$) to 296.0 ± 88.0 nmol/g, which suggests the improvement of cholestasis. Increase of UDCA (27.4 ± 12.8 nmol/g) level was observed accompanied by decrease of CDCA (196.4 ± 65.3 nmol/g) and LCA (56.0 ± 15.7 nmol/g) level. Concentrations of CA (12.8 ± 3.8 nmol/g) and DCA (3.3 ± 2.2 nmol/g) did not change statistically from those of the control group by the administration of UDCA.

DISCUSSION

The liver injury model was produced by a 7-d diet of 0.5% (w/w) CDCA administered to hamsters, which have a similar metabolic profile of bile acids as in humans. An increase in serum ALT was observed and the appearance of liver injury was confirmed histopathologically, which was assessed (Figures 1 and 2, Table 2). These phenomena were in accordance with the results of previous studies^[16,17] which showed the toxicity of CDCA in hamsters. First of all, we determined the change in concentrations of major bile acids in liver of the hamsters to clarify the correlation of those bile acids and the liver injuries. We also administered UDCA in the hamsters to examine the improvement of liver damage caused by CDCA feeding,

Table 3 Effects of UDCA on liver bile acids concentration and composition in hamsters fed CDCA ($n = 9$, mean \pm SD)

	Normal		Control		UDCA 150 mg/kg	
	nmol/g liver	%	nmol/g liver	%	nmol/g liver	%
UDCA	1.9 \pm 2.2	1.2 \pm 1.3	4.0 \pm 0.9 ^a	0.9 \pm 0.3	27.4 \pm 12.8 ^d	9.5 \pm 4.9 ^d
CA	102.0 \pm 41.7	68.2 \pm 3.2	20.9 \pm 13.8 ^b	4.0 \pm 1.1 ^b	12.8 \pm 3.8	4.4 \pm 1.2
CDCA	36.0 \pm 17.1	23.8 \pm 3.6	364.9 \pm 141.5 ^b	75.3 \pm 2.7 ^b	196.4 \pm 65.3 ^d	66.0 \pm 4.7 ^d
DCA	10.3 \pm 4.4	6.8 \pm 1.3	5.6 \pm 3.2 ^a	1.1 \pm 0.3 ^b	3.3 \pm 2.2	1.1 \pm 0.7
LCA	< 1	0.0	89.4 \pm 32.4 ^b	18.7 \pm 2.0 ^b	56.0 \pm 15.7 ^c	19.1 \pm 1.9
Total	150.2 \pm 62.9		484.7 \pm 189.7 ^b		296.0 \pm 88.0 ^c	

UDCA: Ursodeoxycholic acid; CA: Cholic acid; CDCA: Chenodeoxycholic acid; DCA: Deoxycholic acid; LCA: Lithocholic acid. ^a $P < 0.05$, ^b $P < 0.01$ vs normal group; ^c $P < 0.05$, ^d $P < 0.01$ vs control group (Student's *t*-test).

using the changes in the concentrations of bile acids.

In terms of bile acid concentration, CDCA feeding increased the amounts of CDCA and LCA in the hamster liver; on the other hand, it decreased the amounts of CA and DCA (Table 3). This might be explained as follows: At first, CDCA feeding itself increases CDCA in the liver. The increased CDCA is transported from the liver to the digestive tract through the bile duct. CDCA is metabolized to LCA by the enterobacteria there. LCA, which is a potent hydrophobic bile acid^[18], migrates to the liver by enterohepatic circulation. In contrast, a decrease in CA and DCA would be explained as follows: Oda *et al*^[16] showed that CDCA was a potent inhibitor of cholesterol-7 α -hydroxylase. Based on this inhibition of the enzyme activity, feeding of CDCA inhibited biosynthesis of CA which is synthesized from cholesterol, and DCA which is formed by the bacterial 7 α -hydroxylation of CA in the intestine and migrates to the liver by enterohepatic circulation, resulting in decreased proportions of these bile acids in the liver.

It is reported that cholestasis is believed to be an impairment of bile transport or the molecular mechanism from the liver to intestine, consequently leading to intrahepatic accumulation of hydrophobic bile acids, such as CDCA, DCA and LCA. Increase in the hydrophobic bile acids is reported to induce cytotoxicity in hepatocytes^[16,19]. The mechanism of hepatic damage by these bile acids has not been clarified completely; however, several possibilities have been presented. It has been reported that some bile acids induce mitochondrial perturbation^[20,21]. More recently hydrophobic bile acids have been shown to regulate the expression of several genes by acting as ligands of some nuclear receptors in the liver. Through these mechanisms, they affect bile salt synthesis^[22,23], detoxification^[24,25], and transporting molecules^[26,27].

UDCA is widely used for the treatment of liver dysfunction in patients with primary biliary cirrhosis and acute and chronic intrahepatic cholestatic disorders^[1-8]. The powerful choleric effect of the drug was also confirmed in this hamster model. Improvement of liver histology associated with a significant decrease in serum ALT level was observed. The 150 mg/kg UDCA-administered group showed a significant decrease in the concentrations of total bile acids in the liver as comparison with the control group. The concentration of UDCA in the liver became 27.4 \pm 12.8 nmol/g liver by the administration of 150 mg/kg UDCA. Setchell *et al*^[28] have shown that concentration of UDCA became 40.1 \pm 9.0 nmol/g liver when clinical

dosage of the drug (600 mg/d) was administered for 4 d in patients suffering chronic hepatitis C. These findings suggested that liver UDCA concentration after UDCA administration in hamster was not extremely different from that in human.

In the liver of this group, the concentration of UDCA was raised, whereas that of the hydrophobic bile acids, CDCA and LCA was significantly decreased. UDCA is more hydrophilic compared to CDCA and LCA^[12]. It was confirmed that the reduction in the concentration of hydrophobic bile acids and the replacement with hydrophilic bile acids in liver might be the mechanism of UDCA.

In recent years, several studies have been conducted to determine the mechanisms of action for UDCA at the molecular level. Schuetz *et al*^[29] showed that UDCA activated the pregnane X receptor (PXR), and the reversal of cholestasis in humans by UDCA might include PXR-mediated activation of CYP3A4 and perhaps drug transporter targets that lead to enhanced metabolism and efflux of hepatotoxic bile acids. Rost *et al*^[30] showed that UDCA might prevent impairment of hepatic function by restoring the expression of the hepatic transporter. Our next study will be the simultaneous investigation of the transporter expression and the bile acid concentration in this hamster model.

In conclusion, this study showed that the liver injury model was successfully produced by a 7-d diet of 0.5% (w/w) CDCA administered to hamsters. In this model, liver accumulations of two hydrophobic bile acids, CDCA and LCA, were observed. UDCA improved liver damages, which was confirmed with the decrease in serum ALT, and improvement of the liver histology. The protective effects of UDCA seem to result from a decrease in the concentration of the hydrophobic bile acids which accumulate in the liver.

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COMMENTS

Background

Ursodeoxycholic acid (UDCA) is widely used for the therapy of liver dysfunction in many types of liver disease. The mechanisms of this drug, however, have not

been fully clarified yet. Abnormal retention of bile acids in the liver is a common finding when hepatic function is impaired. In this study we investigated the hepatoprotective effects of UDCA, using chenodeoxycholic acid (CDCA)-induced liver injury in hamsters to elucidate a correlation between liver injury and bile acid profiles in the liver.

Research frontiers

It has been reported that the profile of bile acid metabolism in hamsters resembles that in humans. Administration of CDCA is known to cause liver injury in hamsters. Thus, we considered that it was possible to evaluate the relationship between hepatoprotective action of UDCA and liver bile acid profiles in hamsters.

Innovations and breakthroughs

There are some reports that the rate of UDCA increased and those of CDCA and DCA decreased in serum and bile by UDCA treatment. One of the mechanisms of action of UDCA for improvement of liver dysfunction is replacement hydrophobic bile acids [CDCA, deoxycholic acid (DCA) and lithocholic acid (LCA)] with hydrophilic bile acids including UDCA. Because the liver is thought to be the major target organ of UDCA, changes of bile acid concentrations in the liver must be very important. There have been, however, few reports to study the correlation between liver bile acids concentrations and the hepatoprotective effects of UDCA.

Applications

UDCA is widely used for the therapy of liver dysfunction in many types of liver disease, especially those with the accumulation of bile acids. Cholestasis is one of the typical diseases accompanied by the phenomenon. The results obtained in this study have shown that decrease of hydrophobic bile acids in the liver are correlate with the hepatoprotective action of UDCA and it may be one of the mechanisms of the drug.

Terminology

Individual bile acids differ in hydrophobicity and hepatotoxicity. CDCA, DCA and LCA are more hydrophobic and hepatotoxic than UDCA.

Peer review

The manuscript written by Iwaki *et al* describes the protective effect of UDCA on liver injury induced by CDCA. Although UDCA is clinically used for many liver diseases, the mechanisms for actions have not been fully understood. Therefore, their study is important, and the results are interesting.

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Liver angiogenesis as a risk factor for hepatocellular carcinoma development in hepatitis C virus cirrhotic patients

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0.001950; $P = 0.6692$). MVD only was able to predict the occurrence of HCC in these patients. Among other known risk factors for HCC, only male sex was statistically associated with an increased risk.

CONCLUSION: Liver angiogenesis has a role for in HCV-related liver carcinogenesis and for defining patients at higher risk.

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Key words: Liver cancer; Hepatitis C virus; Angiogenesis; Proliferating cell nuclear antigen

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Abstract

AIM: To evaluate the predictive value of hepatocyte proliferation and hepatic angiogenesis for the occurrence of Hepatocellular carcinoma (HCC) in hepatitis C virus (HCV) cirrhotic patients.

METHODS: One hundred-five patients (69 males, 36 females; age range, 51-90 year; median 66 year) with biopsy proven HCV cirrhosis were prospectively monitored for HCC occurrence for a median time of 64 mo. Angiogenesis was assessed by using microvessel density (MVD), hepatocyte turnover by MIB1 and PCNA indexes at inclusion in liver biopsies.

RESULTS: Forty six patients (43.8%) developed HCC after a median time of 55 (6-120) mo while 59 (56.2%) did not. Patients were divided into two groups according to the median value of each index. The difference between patients with low (median MVD = 3; range 0-20) and high (median MVD = 7; range 1-24) MVD was statistically significant ($\chi^2 = 22.06$; $P < 0.0001$) which was not the case for MIB1 or PCNA (MIB-1: $\chi^2 = 1.41$; $P = 0.2351$; PCNA: $\chi^2 = 1.27$; $P = 0.2589$). The median MVD was higher in patients who developed HCC than in those who did not. HCC-free interval was significantly longer in patients with the MVD ≤ 4 ($P = 0.0006$). No relationship was found between MIB1 or PCNA and MVD (MIB-1 $r^2 = 0.00007116$, $P = 0.9281$; PCNA: $r^2 =$

INTRODUCTION

Hepatocellular carcinoma (HCC) is nowadays the leading cause of death in patients with cirrhosis and its incidence is continuously increasing in the west^[1]. Although cirrhosis is present in about 80%-90% of cases in western countries, other risk factors have been well defined such as age, sex, platelet count, alpha-fetoprotein (AFP) level and the cause of the underlying liver disease^[2]. Cirrhosis is considered to cause HCC independently of etiology and HCC from different individuals may have different phenotypes^[3]. This could be due to individual host response to etiologic agent, but there are features that unify HCC occurring in a background of a specific etiologic agent^[3]. HCC is the result of a multi step process that proceeds from the initiation of one or more hepatocytes to a diffuse disease^[4]. HCC due to chronic viral infection may be an indirect result of hepatocyte proliferation that occurs during chronic hepatitis in an effort to replace infected cells that have been immunologically attacked^[5]. Changes due to viral factors may also play a more direct role in liver oncogenesis^[6,7]. Most cases of HCC in the West occur in patients with cirrhosis mainly due to hepatitis C virus (HCV) infection which is the second cause of HCC in the world^[8]. HCV-infected patients have a high risk for developing HCC but mechanisms leading to the increased

risk for liver cancer are still matter of debate.

Chronic HCV infection may lead to a high hepatocyte turnover, resulting in replicative senescence of liver cells, accumulation of DNA alterations^[9,10] and cancer. This phenomenon appears to be hardly specific to HCV infection^[3].

Angiogenesis is known to play a pivotal role in almost every kind of malignancy favoring growth and metastasis of several types of cancer including HCC^[11]. Many angiogenesis-related factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived endothelial cell growth factor (PD-ECGF), thrombospondin (TSP), angiogenin, pleiotrophin and endostatin levels have been evaluated and shown to be of prognostic significance in various types of cancer. Quantification of microvessel density (MVD) inside the tumour has been widely demonstrated to be a reliable tool of evaluating angiogenesis^[12]. The exact role of these factors is still unclear although the crucial importance of VEGF, cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) overexpression are widely recognized^[13].

The prognostic significance of MVD independently of other pathologic predictors was demonstrated in several malignancies, i.e. breast, gastric, colorectal, pancreatic carcinoma, testicular germ tumour, malignant melanoma and even hematological malignancies^[14-17].

HCC is a highly vascular tumour characterized by a propensity for vascular invasion. Angiogenesis increases in the early phases of HCC development^[18]. MVD is reported to be correlated to the size of the tumour and may predict early recurrence after resection^[19,20]. Therefore, it is conceivable that the high density of new vessels observed in the liver of patients with chronic HCV infection may contribute to the early development of cancer cells and may explain the high incidence of HCC in these patients^[18].

In a previous study, we demonstrated that liver angiogenesis as measured by MVD was significantly more common and intense in patients with chronic HCV infection in comparison to matched patients with chronic HBV infection or controls^[21]. Experiments *in vitro* using endothelial cells exposed to liver homogenates and sera of patients with different hepatic diseases confirmed that HCV positive biologic tissues stimulated in a greater extent migration and proliferation of endothelial cells^[21]. Based on these results, liver angiogenesis due to chronic HCV infection was argued to be independent of inflammation. These observations indicated that liver angiogenesis is most intense in HCV infected patients, suggesting a possible mechanism linking HCV infection to the increased risk of developing HCC.

The present study aimed at investigating whether increased liver angiogenesis as estimated by the MVD in liver biopsies could be predictive of the future occurrence of HCC in patients with HCV related cirrhosis. Furthermore, since PCNA staining, a marker of hepatocyte proliferation, was previously reported to be predictive of HCC, in this study also two proliferative indexes, PCNA and MIB₁ and their correlation to MVD were studied together other known risk factors for HCC.

MATERIALS AND METHODS

Patients

One hundred and five cirrhotic patients (69 males and 36 females; age range, 51-90 year; mean 62.5 ± 8.3 year) were included and monitored (120 mo) from January 1993 to January 2003. All enrolled patients met the following criteria: (1) chronic HCV infection documented by the presence of anti-HCV antibodies and HCV RNA in the serum; (2) histologically proven HCV related cirrhosis; (3) absence of other causes of viral or non-viral liver disease including alcohol abuse or any kind of genetic disease; (4) absence of contemporary or previous antiviral treatment. All patients were investigated at the beginning of the study by routine liver function test assessment, liver ultrasound scan, and determination of serological markers of viral infections and autoimmune diseases. All patients had a homogenous liver at ultrasound (US) examination and serum alfafetoprotein (AFP) level under 20 µg/L. All patients were affected by Child-Pugh A well compensated liver cirrhosis according to international criteria^[22,23]. They were thereafter included in a screening program for HCC consisting in periodic US examination and determination of serum AFP levels at least every 6 mo.

This study was approved by the local Ethical Committees of both Azienda Ospedaliero-Universitaria Careggi, Firenze, Italy and Hopital Jean Verdier, Assistance Publique-Hopitaux de Paris et UFR SMBH-Universite Paris XIII, Bondy, France. All enrolled subjects gave their informed consent to undergo liver biopsy for diagnostic purposes and to participate in the study.

Virological assessment

All patients were tested for anti-HCV antibodies by a second generation enzyme-linked immunosorbant assay (Ortho Diagnostic Systems, Inc., Raritan, NJ) and radio immunoblotting assay (Chiron Corporation, Emeryville, CA). Confirmation of the presence of serum viral HCV-RNA in anti-HCV positive patients was performed by a standard polymerase chain reaction technique. Serum hepatitis B markers and HBV DNA (Abbott Laboratories, North Chicago, IL) were performed in addition.

Immunohistochemistry

Liver biopsies were coded, formaldehyde-fixed, paraffin-embedded, cut into 4 µm thick sections, and stained with standard techniques. Immunohistochemical stainings were performed on 4 µm thick formaldehyde-fixed paraffin embedded sections, using the streptavidin-biotin immunoperoxidase technique. Antigen retrieval with microwave treatment with citrate buffer, pH 6.0, for 10 min was used. After blocking endogenous peroxidase the primary antibodies were applied.

Monoclonal antibody anti-CD34 (Immunotech, Mar-siglia, Fr) was used at a 1:100 dilution at 4°C for 12 h. Sections were incubated for 12 h at room temperature with MIB-1 monoclonal antibody (Immunotech, Marseille, Fr) at 1:50 dilution or were incubated for 2 h at room temperature with PCNA monoclonal antibody (Dako, Carpinteria CA) at 1:200 dilution. For negative controls, sections were treated the same way except they were incubated with Tris-

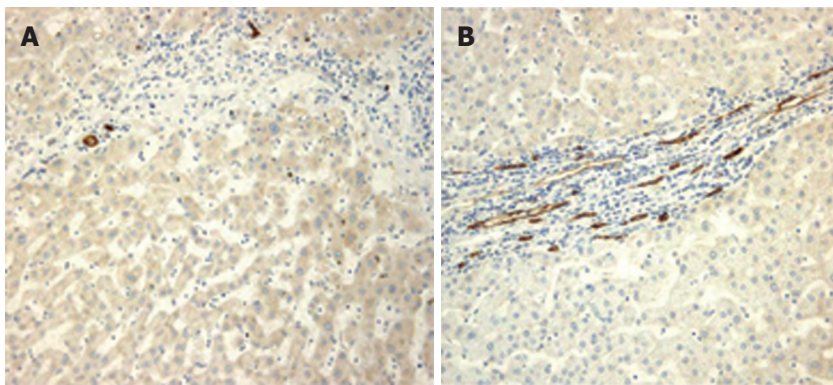


Figure 1 Immunohistochemical staining for CD 34 expression, assessed by QB-END/10 monoclonal antibody. A: Low panel (MVD ≤ 4); B: High panel (MVD > 4).

Table 1 The 105 patients divided in two groups according to the MIB-1 or PCNA score

	HCC incidence during follow-up	No HCC during follow-up	Total
MIB-1 > 1	40	46	86
MIB-1 ≤ 1	6	13	19
PCNA > 4	23	23	46
PCNA ≤ 4	23	36	59
Total	46	59	105

buffered saline instead of the primary antibody.

The percentage of tumor cells positive for MIB-1 as well as PCNA was determined semiquantitatively by assessing the whole tumor section, and each sample was scored by 0.1 points for every 1% of positive cells.

MVD evaluation

MVD was assessed by light microscopy using the counting method introduced by Weidner *et al.*^[24], CD34 was used to identify newly formed microvessels. As explained elsewhere^[21] liver tissue sections were scanned at low magnification ($\times 40$ and $\times 100$) to find the areas that showed the most intense vascularization (hot spots). Individual microvessels were counted in three fields at $200\times$ magnification ($20\times$ objective lens and $10\times$ ocular lens; 0.7386 mm^2 per field). The final MVD was the mean value obtained from counts of the 3 fields. MVD was expressed as mean \pm standard deviation (vessels per mm^2). Any immunostained endothelial cell or endothelial cell cluster that was clearly separated from hepatocytes and other connective tissue elements was considered a single and countable microvessel. Vessel lumens were not necessary for a structure to be defined as a microvessel, and red cells were not used to define a vessel lumen. Evaluation of MVD was performed without knowledge of any clinical and pathological data.

Statistical analysis

Statistical analysis was performed using Yates' corrected analysis to compare proportions. Correlation between continuous variables was performed with linear regression analysis. Disease-free survival rates were computed by the Kaplan-Meier method and were compared by the log-rank test. Student's *t*-test, one-way ANOVA, χ^2 analysis were also used where indicated. $P \leq 0.05$ was considered

significant. Cox regression method was used to analyze the effect of single risk factors on HCC occurrence risk. SPSS 10.0 software (SPSS, USA) was used to analyze the data.

RESULTS

Serum AFP levels of patients were $7.55 \pm 4.87\text{ }\mu\text{g/L}$, and their platelets levels of patients were $113 \pm 25 \times 10^8/\text{L}$ (mean \pm SD). The median time of follow-up in the whole group of patients was 64 months. Forty-six out of the 105 (43.8%) patients developed HCC during the follow-up. The median time to HCC occurrence was 55 (range 6-120) mo. Fifty-nine patients (56.2%) did not develop HCC the observation time. Year incidence of HCC was 4.4 %. CD 34 staining as a marker of newly formed microvessels was observed within hepatic tissue along vascular channels, in groups of cells that apparently did not border vascular lumina and in groups of cells located in portal tracts and septa. Figure 1 shows examples of low (MVD ≤ 4 , Panel A) and high (MVD > 4 , Panel B) CD 34 expression, assessed by QB-END/10 monoclonal antibody. The highest number of newly formed microvessels observed in one single microscopic field ($250\times$) was 24. A microvessel density score less than or equal to 4 was considered to be low angiogenesis expression while one greater than 4 as high. The cut-off score of 4 was chosen as it was the median of the MVD scores in our study group.

When considering the whole group of 105 patients, 32 out of the 46 patients who developed HCC showed a high (> 4) MVD score while only 14 patients of the 59 patients who did not develop HCC showed a high MVD score ($\chi^2 = 22.26$; $P < 0.0001$).

The median MVD score of those patients developing HCC was 7 (range 1-24) while the median score of those who remained HCC-free was 3 (range 0-20). Proliferating cells were identified by immunohistochemistry for monoclonal antibodies, MIB-1 and PCNA. No difference was observed when comparing immunostaining for Ki-67 (MIB-1 high score: > 1 and low score: ≤ 1 and PCNA: high score: > 4 and low score: ≤ 4 as score 1 and 4 were the medians of the MIB-1 and PCNA scores in our study group, respectively) in the group of patients which developed HCC during the study as compared to other patients who did not develop HCC (MIB-1: $\chi^2 = 1.24$; $P = 0.2351$; PCNA: $\chi^2 = 1.27$; $P = 0.2589$) (Table 1).

No correlation was found between the MVD score and MIB-1 (Panel A, MIB-1: $r^2 = 0.00007116$; $P = 0.9281$) or

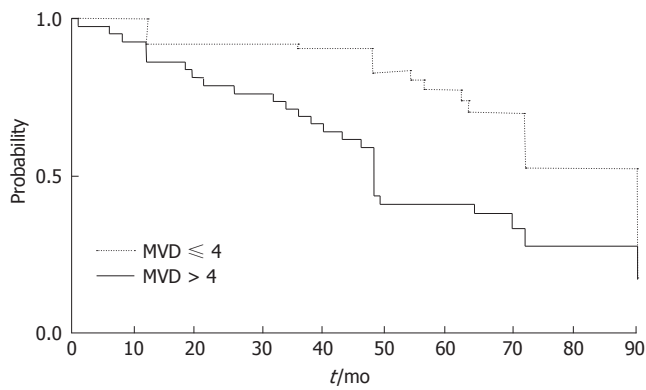


Figure 2 HCC-free time during follow-up of patients.

PCNA score (Panel B; PCNA: $r^2 = 0.001950$; $P = 0.6692$). As previously shown, the presence of cirrhosis had no significant effect on immunostaining results for either QB-END/10 or MIB-1 or PCNA antibodies (data not shown)^[21].

The HCC-free interval during follow-up turned out to be significantly longer in the group of patients having an MVD score < 4 ($P = 0.0006$) (Figure 2). On the contrary, no difference was shown by chi-squared analysis of the 105 patients who were divided in two groups according to the PCNA score ($\chi^2 = 1.27$; $P = 0.2589$). Among known risk factors for HCC such as sex, age, platelets and AFP only male sex was a risk factor for HCC development ($\chi^2 = 3.91$; $P = 0.048$).

DISCUSSION

HCC is a lethal malignancy and its incidence ranks fifth but fourth in terms of mortality^[2,4]. It is well known that the growth of tumours and metastatization depend on adequate angiogenesis. HCC is a hyper-vascular tumour and neo-vascularization is a hallmark of HCC. At least two studies demonstrated that angiogenesis, as assessed by counting of microvessels, should be considered as an important prognostic factor in HCC surgical therapy (resection) outcome^[25,26]. Intense angiogenesis expressed as high microvessel count is associated with neoplastic disease spread and poor survival. The present study is the first one that analyzes the predictive value of MVD for occurrence of HCC in patients with HCV cirrhosis. Results shown here suggest that HCV positive cirrhotic patients with more intense hepatic angiogenesis are at higher risk for HCC occurrence. This data contribute to a more accurate definition of risk factors in HCV positive cirrhotic patients together with other simple epidemiological parameters such as sex and age. According to this findings, hepatic MVD could be added to other of histologic parameters such as large cell dysplasia that could help to a more accurate definition of high risk groups of patients for HCC. Furthermore, it gives a contribute to our understanding of liver cancerogenesis in HCV positive patients.

Several studies considered angiogenesis in HCC patients, even in early stages, but none provided evidence of a relationship between angiogenesis and the probability

of HCC developing during chronic HCV infection. Although a relationship between MVD and HCC free survival time and prognosis in patient surgically treated for HCC was already proven, this is the first study that analyze the prognostic value of MVD in patients with chronic HCV infection and liver cirrhosis. Many angiogenesis-related immunohistochemical markers, such as VEGF, b-FGF, PD-ECGF, TSP, pleiotrophin and endostatin levels as well as the identification of angiogenesis measured as MVD have been found to be of prognostic significance in cancer disease. Particularly, MVD has been widely demonstrated to be a suitable assay to evaluate cancer related angiogenesis^[12,15].

A prognostic value of MVD independently from conventional pathologic predictors was demonstrated in many types of cancer. It was shown that angiogenesis as measured by MVD correlates significantly with tumour size in patients undergoing HCC resection and may predict early recurrence after liver resection. Increased angiogenesis assessed by MVD can be considered as an hallmark of HCV chronic hepatitis as we emphasized years ago^[21]. In that study, it was shown that MVD was 14 fold increased in patients with chronic HCV hepatitis compared to controls and 5 fold compared to patients with matched chronic HBV hepatitis. Patients with non viral liver diseases such as primary biliary cirrhosis and inflammatory pseudo tumour were considered as controls and had a mild and non significant increase in MVD. Studies performed in vitro confirmed those findings showing enhanced HUVEC migration and proliferation when endothelial cells were exposed to sera or liver homogenates from HCV infected patients.

In the present study we used the MVD count to quantify angiogenesis in the liver of 105 patients with HCV related cirrhosis. Forty-six of them have developed HCC after a median time of 55 mo whilst 59 patients did not develop HCC. The whole group of patients was divided in two groups according to the MVD count. Patients with a MVD count higher than 4 were exposed to a higher risk of developing HCC during follow-up. A high MVD score in fact, was associated with a significantly higher probability to develop HCC during follow-up. It is noteworthy that the median time of follow-up of those who remained free from HCC was significantly longer than that of the group of patients that would have developed HCC during follow-up. The mean annual incidence of HCC occurrence was 4.4% corresponding to what is usually observed in this type of patients.

Among other known risk factors for HCC, only male sex was associated with a higher risk to develop HCC. The difference between male and female in the occurrence of HCC was statistically significant (69 males *vs* 36 females; $P = 0.048$). However, it must be said that to establish risk factors for multifactorial diseases as is cancer are usually needed a much larger number of subjects included in a survey study than what included in the present study. In addition, in the present study only patients with histology proven HCV related liver cirrhosis with a mean age older than what is commonly reported in the literature as mean age of HCC occurrence were considered. We think that these facts played a big role in explaining why

some of other known risk factors for HCC did not appear statistically related to liver cancer in the present study. Interestingly, different geographic areas did not play any role in affecting HCC risk in the present work, in fact French and Italian patients who developed HCC did not show a significant difference in mean age (68.5 years *vs* 65.3 years, French *vs* Italian, respectively) at the moment of diagnosis. In addition, dividing French from Italian patients the MVD score remained predictive for HCC development ($\chi^2 = 13.18$; $P < 0.0001$; and 5.60; $P < 0.0179$ French and Italians, respectively).

In this study, besides the MVD counts, we considered the predictive value of 2 proliferative indexes, PCNA and MIB-1, that have been proposed as risk factors for the occurrence of HCC^[12]. In our hands, PCNA and MIB-1 staining were not predictive of the occurrence of HCC.

No one of patients included in the present study had had treatment with antiviral drugs before entering the study neither had during it. After that it was shown^[27,28] that antiviral treatment resulted in a reduction of HCC risk the study could not go on for ethical reasons. These findings, although have brought to the end our study, on the same time, they indirectly confirmed the validity of the observation we did in the present study. In fact, lymphoblastoid IFN alpha has antiangiogenic activity in addition to its antiviral activity and therefore our findings support the role of IFN alpha treatment in patients with HCV positive liver cirrhosis.

Recent epidemiological studies have showed that the annual incidence of HCC is greater in HCV related cirrhosis than in other types of liver diseases due to non viral agents but also HBV liver disease at least in Western countries and in Japan^[29]. In geographic areas such as Africa and South-East Asia the occurrence of HCC is greatly influenced by aflatoxin exposure and p53 mutations in addition to HBV and HCV infection. In Western countries and in Japan where exposure to aflatoxin is negligible the incidence of HCC in patients with HBV cirrhosis has always been found lower than in patients with HCV cirrhosis leading to endless speculations about the oncogenic role of a RNA virus unable to integrate in the hepatocyte genome^[29]. We think that intense liver angiogenesis might provide a plausible explanation of this phenomenon and explains why patients with mixed causes of chronic liver diseases such as alcoholic or HBV liver disease are at very high risk of HCC when super-HCV infection occurs. The mechanism by which increased angiogenesis in HCV patients is unknown but COX-2 expression in hepatocytes might play a role as it is correlated to the degree of angiogenesis in liver tumours and is up-regulated by viral core and NS5A protein in patients with chronic hepatitis^[30]. This hypothesis is consistent with data where cancer related angiogenesis was evaluated. In particular, it was demonstrated the association between HCC vascularity and COX-2 and iNOS expression^[31]. The Authors concluded that higher COX-2 and iNOS expression in HCC was correlated to angiogenesis and to a worst prognosis of patients^[31].

Novel therapies have been proposed for HCC^[32-34]. Based on *in vitro* data, chemoprevention of HCC by COX-2 inhibitors has been recently proposed although further studies should be carried out.

In conclusion, the increased hepatic angiogenesis assessed by the MVD counting is a hallmark of HCV liver disease, it can play a role in liver carcinogenesis and, most importantly, can be considered as a risk factor for HCC occurrence in patients with HCV cirrhosis. If confirmed, this observation could help to make a better definition of high risk patients, giving a clue to understand the mechanism leading to high incidence of cancer in these patients and a potential therapeutic target in the future.

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COMMENTS

Background

Chronic HCV infection is one of the most important causes for HCC, however hepatic oncogenic mechanisms related to HCV infection are still not fully elucidated. Since it was shown that HCV is angiogenic, we hypothesised that HCV related angiogenesis is a risk factor for HCC development.

Research frontiers

Years ago we showed that patients with chronic HCV hepatitis had more common and intense liver angiogenesis as compared to matched patients with chronic HBV hepatitis and patients such as those with PBC or pseudo-inflammatory tumor who were used as controls for inflammation (1). Angiogenesis is a well recognized risk factor for cancer, at least for its growth and diffusion (2). Patients with chronic HCV infection show expression of COX-2 an inducible enzyme that is angiogenic and anti apoptotic. Thus, the goal of our manuscript was to show that in cirrhotic patients, with chronic HCV infection, those with the most intense hepatic angiogenesis were at greater risk for HCC development.

Innovations and breakthroughs

The manuscript improves our understanding on how chronic HCV infection may be responsible for the higher incidence of HCC as compared to other patients with chronic liver diseases. In fact, the demonstration that active liver angiogenesis increases the risk for HCC not only can explain why interferon treatment reduce the risk of HCC acting directly on angiogenesis (interferons are compounds with substantial anti angiogenic activity), but it also opens new perspectives for designing new therapeutic strategies to prevent HCC in cirrhotic HCV patients. With regards this point, for instance, the recent demonstration that sorafenib (ASCO 2007, abstract book), a new anti tyrosine kinase agent whose main action is antiangiogenic, can be very active and helpful in the treatment of advanced HCC, support data of the present manuscript.

Terminology

Angiogenesis is the phenomenon that occurs in several physiological and pathological conditions in the body and it is characterized by the production of new vessels starting from pre-existing endothelial cells. The micro vessel density counting (MVD) is a quite diffuse manner to measure angiogenic activity and it correlates quite well with bad prognosis of cancer patients. PCNA and MIB-1 are two indexes used to measure proliferative activity in a tissue.

Peer review

The manuscript deals with an important issue in clinical oncology and gastroenterology: To improve our understanding on how chronic HCV infection increases the risk of HCC in addition to the fact to cause chronic inflammation and liver cirrhosis.

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Coinfection of hepatitis B and hepatitis C virus in HIV-infected patients in south India

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hepatotropic viruses, as a consequence, infections with HBV and HCV are expected in HIV infected patients. Therefore, it would be advisable to screen for these viruses in all the HIV infected individuals and their sexual partners at the earliest.

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Key words: Hepatitis B virus; Hepatitis C virus; Human immunodeficiency virus; Co-infection; Hepatotropic viruses; HBV and HCV India; HBV and HCV and HIV

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Abstract

AIM: To screen for the co-infection of hepatitis B (HBV) and hepatitis C virus (HCV) in human immunodeficiency virus (HIV) infected patients in southern India.

METHODS: Five hundred consecutive HIV infected patients were screened for Hepatitis B Virus (HBsAg and HBV-DNA) and Hepatitis C virus (anti-HCV and HCV-RNA) using commercially available ELISA kits; HBsAg, HBeAg/anti-HBe (Biorad laboratories, USA) and anti-HCV (Murex Diagnostics, UK). The HBV-DNA PCR was performed to detect the surface antigen region (pre S-S). HCV-RNA was detected by RT-PCR for the detection of the constant 5' putative non-coding region of HCV.

RESULTS: HBV co-infection was detected in 45/500 (9%) patients and HCV co-infection in 11/500 (2.2%) subjects. Among the 45 co-infected patients only 40 patients could be studied, where the detection rates of HBe was 55% (22/40), antiHBe was 45% (18/40) and HBV-DNA was 56% (23/40). Among 11 HCV co-infected subjects, 6 (54.5%) were anti-HCV and HCV RNA positive, while 3 (27.2%) were positive for anti-HCV alone and 2 (18%) were positive for HCV RNA alone.

CONCLUSION: Since the principal routes for HIV transmission are similar to that followed by the

INTRODUCTION

Human immunodeficiency virus (HIV) and Hepatitis B and C viruses (HBV and HCV) are the three most common chronic viral infections documented world-wide^[1,2]. These viruses have similar routes of transmission, namely through blood and blood products, sharing of needles to inject drugs and sexual activity, enabling co-infection with these viruses a common event^[3-5]. HBV and HCV co-infections in HIV positive individuals is of utmost importance due to the underlying consequences such as the hepatological problems associated with these viruses, which have been shown to decrease the life expectancy in the HIV-infected patients^[5].

Given the epidemiological similarities of HBV and HIV infections, it is not surprising that markers of past HBV infections namely hepatitis B surface antibody (anti-HBs) or hepatitis B surface antigen (HBsAg) positivity, as the reported evidence of past HBV infection among people living with AIDS is about 10%^[6,7]. HIV accounts for 38.6 million infections world-wide at the end of 2005^[8] while HBV and HCV account for 400 million and 170 million chronic infections respectively^[9,10]. Moreover, among the HIV-infected patients, 2-4 million are estimated to have chronic HBV co-infection while 4-5 million are co-infect-

ed with HCV^[9]. Hepatitis B is a significant public health hazard in the subcontinent, where the average carrier rates in the general population is estimated to be 4%^[11,12]. Further, reports on the prevalence of HCV infection in the Indian subcontinent are scarce. A community-based Indian study indicated a seroprevalence of 0.87%^[13] and that the rate reportedly increased from 0.31% for children < 10 years to 1.85% among subjects > 60 years of age^[13]. In addition, the rate of HBV and/or HCV co-infection in HIV patients have been variably reported depending on the geographic regions, risk groups and the type of exposure involved^[14-20]. The literature regarding the prevalence of HIV co-infection with HBV and/or HCV in India is sparse. Hence, we investigated the co-infection pattern of HBV and HCV among HIV infected south Indian subjects with various risk factors and analyzed the association of viral markers of HBV and HCV infection among subjects with various stages of HIV disease.

MATERIALS AND METHODS

Study population

Patients attending clinics at YRG Centre for AIDS Research and Education (YRG CARE) were screened for HIV based on suspicion on clinical grounds after performing pre-test counseling and informed consent. Only the confirmed HIV positive serum samples (as per World Health Organization testing strategies) were included in this study and were anonymously tested for hepatitis B and C virus markers.

Virological assays

The patients were screened for HBV and HCV using ELISA kits; Hepatitis B surface antigen (HBsAg), Hepatitis B envelop antigen (HBeAg), antibody to envelop antigen (anti-HBe) using Biorad laboratories, USA and antibody to hepatitis C virus (anti-HCV) using Murex Diagnostics, UK. The HBV-DNA PCR was performed as per the methods of Shih *et al*^[21]. Primers specific for the surface antigen region (pre S-S) were used for the molecular diagnosis of HBV infection. For the diagnosis of HCV RNA, extracted RNA was subjected to reverse transcriptase-nested polymerase chain reaction assays (RT-PCR) for the constant HCV 5' untranslated region (5'UTR)^[22].

Statistical analysis

Descriptive statistics are presented with mean \pm standard deviation (SD) or proportions, for continuous or categorical variables, respectively. The Mann-Whitney test was used to compare continuous variables between groups. Statistical package for social sciences (SPSS, Version 13.0) software was used for analyzing the data. A *P* value < 0.05 was considered statistically significant.

RESULTS

Epidemiological characteristics

Of the 500 HIV infected participants investigated, 56 (11.1%) were co-infected with hepatitis viruses [45 (9%) HBV and 11 (2.2%) HCV positive]. Male gender predominance was observed (86%), (48 males and 8

females) and the median age was 37 years (95% CI \pm 3.6) (range from 20-55 years). The main clinical, virological and epidemiological characteristics are presented in Table 1. Data on the risk factors for HIV seroconversion were available for all patients; 359 (72%) were heterosexual, 38 (8%) were intra venous drug users (IVDs), 46 (9%) were blood transfusion recipients and 57 (11%) were unnoticed. Among the co-infected patients the predominant risk factor observed was heterosexual (70%) rather than parental risk (14%) as shown in Table 1.

Majority of the HIV-infected patients were from the 31-40 years age group (39 %) followed by the 41-50 years age group (27%). Mean age of the HIV positive patients was 35 years (95% CI \pm 6.8 years), 37 years (95% CI \pm 3.6 years) among the co-infected patients. HBV-HIV co-infection was high in the 31-40 years age group (58%) while HCV-HIV co-infection was predominant among subjects with > 51 years of age (55%) (Figure 1).

Immunological characteristics

The stage of HIV infection according to the revised 1993 Centers for Disease Control and Prevention (CDC) classification was determined in all patients. Among 45 HIV/HBV co-infected patients only 40 patients were classified according to HIV disease groups namely Group A 4 (10%), Group-B 19 (48%) and Group-C 17 (42%) whereas all the 11 HIV/HCV co-infected subjects could be classified as Group-A, 1 (9%), Group-B, 3 (27%) and Group-C, 7 (64%). Nonetheless, the CD4 lymphocyte profile between HIV and hepatotropic virus co-infected patients was not significant (Table 1, Table 2, Table 3).

HBV and HIV co-infection

Of the 45 HIV/HBV co-infected patients, only 40 could be classified under the various HIV disease groups. The HBeAg positivity was seen in 25%, 47% and 71% whereas antiHBe positivity was seen in 75%, 53% and 29% in the HIV disease groups A, B, and C respectively (Table 2). The HBV DNA positivity was seen in 25%, 47% and 76% of the patients in groups A, B and C respectively (Table 2). The HBe/antiHBe was inversely proportional to each other and significantly associated with the stage of HIV disease progression (*P* < 0.01). Out of 23 total HBV-DNA positive cases, significantly higher level of HBV-DNA positivity (87%) was observed in HBe positive cases compared to HBe seroconverted patients (13%). In the overall HBV-DNA positivity among HIV/HBV co-infected patients, the stage of HIV disease progression was significantly associated the positivity pattern of HBV DNA (*P* < 0.01). Randomly selected 250 HBsAg seronegative cases were also tested for qualitative HBV-DNA by PCR and none of the patients revealed "occult" HBV infection.

HCV and HIV co-infection

Of the 11 HIV/HCV coinfecting patients (i.e. either positive for anti-HCV or HCV RNA or both) (Table 1), 6 were anti-HCV and HCV RNA positive, 3 were anti-HCV alone positive and 2 were HCV RNA alone positive. The HCV-RNA positivity was 100%, 66%, and 71% in group-A, group-B, and group-C respectively (Table 3).

Table 1 Baseline characteristics of HIV and hepatitis coinfected patients

Groups	Total	Age	Sex		Probable route of transmission				Mean CD4 counts (cells/mm ³)	CDC status		
			Male	Female	Sexual	IVDs	Blood	Not known		A	B	C
HIV alone	444	36 ± 9	298	146	320	35	41	48	334 ± 199 ¹ 294 ± 173 ²	26 ¹	17 ¹	20 ¹
HIV + HBV	45	32 ± 6	39	6	35	-	2	8		4 ²	19 ²	17 ²
HIV + HCV	11	38 ± 8	9	2	4	3	3	1	231 ± 104	1	3	7

CDC: Centre for Disease Control; IVD: Intra-venous drug users. ¹Based on the available clinical data, only 63 out of the total 444 HIV alone infected patients could be classified and ²only 40 out of the total 45 HIV/HBV coinfected could be classified.

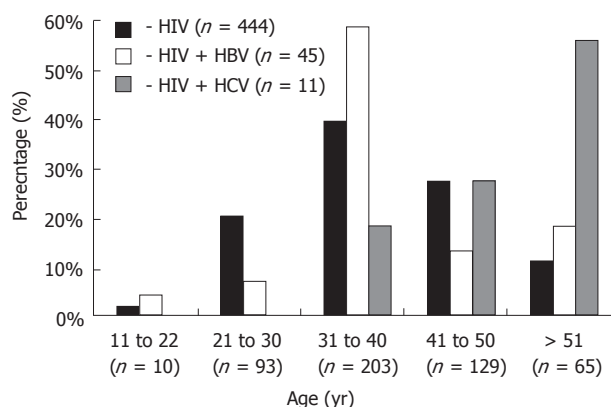


Figure 1 Age-related distribution of HBV and HCV co-infection in HIV infected patients.

From the remaining 489 anti-HCV seronegative cases, 300 were randomly selected for qualitative HCV-RNA testing by PCR, in which only 2 cases (0.6%) were positive for HCV-RNA, the CD4 counts were 58 and 205 cells per mm³ respectively. The RNA positivity in anti-HCV positive cases was highly significant (73% *vs* 0.6%) than the anti-HCV seronegative cases ($P < 0.001$).

DISCUSSION

India has the second highest number of people living with HIV^[23]. Moreover, among the HIV infected patients, 2-4 million are estimated to have chronic HBV co-infection while 4-5 million are co-infected with HCV^[9]. Co-infection of HBV and/or HCV with HIV complicates the clinical course, management and may also adversely affect therapy for HIV infection. The reported co-infection rates of HBV and HCV in HIV patients have been variable worldwide depending on the geographic regions, risk groups and the type of exposure involved^[24-26]. Within India HBV and HCV co-infection among HIV infected patients have been reported infrequently from region to region^[15-20]. However, our study indicated that HIV-infected patients are at a high-risk of viral co-infections, as evident from the high prevalence of HBV (9%) and HCV (2.2%), which is fairly higher than the HBV and HCV prevalence reported in the Indian general population^[11,12].

Our findings showed that study group predominantly comprised of heterosexually acquired HIV infections than other mode of transmission and the male gender were

Table 2 HBV marker profile of HBV/HIV co-infected patients (n = 40)

HIV/HBV coinfection group (CDC 1993 revised) (n = 40) ¹	HBV marker			
	HBsAg	HBeAg	Anti-HBe	HBV DNA
Group A	4 (10%)	1 (25%)	3 (75%)	1 (25%)
Group B	19 (48%)	9 (47%)	10 (53%)	9 (47%)
Group C	17 (42%)	12 (71%)	5 (29%)	13 (76%)

¹Based on the available clinical data, only 40 out of the total 45 HIV/HBV co-infected could be classified according to HIV disease group as per CDC-1993 revised classification.

significantly (86% *vs* 14%) higher than female ($P < 0.01$). This concurs previous report that male subjects were significantly at a higher risk to develop HBV co-infection^[14,18], justified by the age group against the pattern of co-infection analyzed in the present investigation. This data shows that the maximum levels (58%) of co-infection for HBV/HIV occurred in the 31-40 age-group, which is the normal age group where the HIV positivity is reportedly higher as per Indian literatures^[15-20]. This also suggests that sexual route could also be the common mode of transmission for both HBV and HIV. Further, the chronic HCV co-infection rate in our study is in line with Padmapriyadarsini *et al.*, from South India^[18]. In contrast to the HIV/HBV co-infection observed among the sexually active age group viz. (31-40 years), the HIV/HCV co-infection was higher (55%) among the > 50 years age group, which speculates that HCV transmission could have been non-sexual and/or parenteral.

The frequency of anti-HCV among our HIV subjects (2.2%) is much lower than that reported previously amongst HIV/HCV co-infected Indian subjects^[15-20] and much higher than from the general Indian community^[12]. The low frequency of HCV could be due to the low incidence of IVD use and infrequent transfusion in our study groups, which are relatively different from that reported from other parts of India where IVDs and transfusion history were the main risk factors identified for HCV infection among HIV patients^[15-20]. In our cohort, two individuals co-infected with HIV/HCV neither had transfusion history, IVDs use, tattooing, piercing nor sexual promiscuity with IVDs users; nevertheless presented details of high risk sexual behaviors and prior STI history, which suggests that sexual intercourse could have been the route of infection. In addition, both subjects had active HCV infection (positive HCV-RNA) but failed to

Table 3 HCV marker profile of HCV/HIV co-infected patients ($n = 11$)

HIV Disease Group of HIV/HCV coinfectd cases (CDC 1993 revised)	HCV coinfection ($n = 11$)	Anti- HCV and HCV RNA positive ($n = 6$)	Anti HCV alone positive ($n = 3$)	HCV RNA alone positive ($n = 2$)
Group A	1 (9%)	1	0	0
Group B	3 (27%)	1	1	1
Group C	7 (64%)	4	2	1

seroconvert to anti-HCV. The CD4 T-cell counts (58 and 205 cells per mm^3) are suggestive of HCV detection by ELISA among patients with very low CD4 counts may not be useful for screening. In agreement with earlier reports our study propose that prior HIV infection facilitates HCV transmission much easier, though this mode is not widely documented through sexual contact, which however needs more studies with more number of cohorts under the various risk groups and matched controls.

Co-infection of hepatotropic viruses in HIV disease reportedly leads to massive impairment of cell mediated responses and enhances the kinetics of hepatotropic viral replication^[27-30]. Furthermore, HBV co-infection in HIV disease considerably complicates its diagnosis and management. Patients with AIDS apparently are less likely to clear HBV infection after exposure or more likely to reactivate latent HBV infection or both^[30,31]. Our study showed that co-infection with HBV or HCV is frequent in HIV disease, as evident from higher HBV-DNA and HCV-RNA positivity rates. The effects of HIV on the course of chronic HBV and HCV infection have largely been assessed^[1-5]. Our investigations are also suggestive of a higher degree of immunodeficiency, (CDC 1993 revised classification of HIV disease group (group A, B and C) concurrently with a higher rate of HBV and/or HCV replication and HIV disease progression. HBsAg-negative “occult HBV” was not seen in our HIV population. Further, the HBeAg and anti-HBe positivity pattern of our study cohort clearly shows that, both the HBe and anti-HBe status were inversely proportional to HIV disease progression among the HIV/HBV co-infected cases. Statistically there is a significant trend between HBsAg positivity and HBV DNA positivity between the three CDC defined HIV disease groups ($P < 0.01$).

We observed that the incidence of HBV co-infection rises with disease progression. Significant difference of co-infection existed between the symptomatic and asymptomatic groups of HIV infected patients ($P < 0.01$). The co-infection seems to have pronounced effect on the natural history of these infections. Although the effect of HBV infection on HIV is uncertain, HIV appears to have marked influence on the natural history of HBV infection. The increased viral replication of HBV in AIDS patients indicates that HIV significantly affects the HBV life cycle and the host ability to clear HBV infection. If this holds true, more HBV infection and more chronic carriers would be expected as the AIDS epidemic expands in this part of the country. Such a profile would have worrisome public health implications since more chronic liver diseases, including HCC, would be expected as the mortality rate associated with HIV is reduced^[32]. Chronic

HBV infection can be associated with severe liver damage in HIV positive drug abusers and homosexuals. HIV infection does not seem to attenuate and may even worsen HBV associated chronic liver damage^[30,33]. The long-term effect of immunodeficiency on the out come of hepatitis B infection remains to be evaluated. The HIV-RNA viral load difference was however not significant in our study groups. In regard to CD4 and CD8 counts, the HIV/HCV co-infected patients revealed a comparatively lower levels in the above parameters with the HIV and HIV/HBV co-infected patients, albeit statistically insignificant (data not shown). This could probably be due to small number of cases, wide range of CD4, CD8 counts and the HIV cases being in different stages of HIV disease. In the HIV/HCV co-infected groups, the HCV-RNA positivity was found to be higher in Group-C (71%) than group B (66%) but only one patient in group A showed HCV-RNA positivity. These observations are also concordant to previous reports of increased hepatotropic viral replication in immunocompromised subjects^[34,35]. However, this needs to be confirmed with adequate number of patients with different stages of HIV disease.

The present study has certain limitations. Firstly, this is a cross-sectional study unable to adequately establish a casual relationship between the time of exposure and subsequent infection. Secondly, the study was conducted with patients limited to a tertiary HIV referral hospital setting and not of a community setting. However the results can be implied to approximate and prepare for clinical care of our HIV-infected patients. Moreover, at the time of this study, HIV negative group was not available. In addition, the HCV-RNA testing used to screen HCV infection in terms of cost was quite prohibitive in our resources-limited setting. Immunosuppression from HIV infection may impair antibody formation, and false-negative HCV antibody tests have been reported in individuals co-infected with HIV^[36,37]. It is unlikely that the low seroprevalence of HIV/HCV co-infection was due to selection bias because subjects in the present investigation were selected from one tertiary referral hospital that covers a wide range of socio economic strata.

Uncertainties remain regarding the real effect of co-infection with HIV and HBV and HCV on the progression and outcomes of theses viral infections. The situation is largely due to difficulties in performing accurate natural history studies, particularly in the constantly developing field of HIV medicine. However, an increased infectivity for chronic HBV infections in HIV positive persons regardless of their clinical state or laboratory evidence of immune suppression that may have implications for hepatitis policies, and epidemiologic studies should be

considered to monitor a possible increase in the spread of HBV among population at risk for HIV and HBV. Prolonged survival of HIV infected patients co-infected with HBV or HCV may become an important clinical problem. Our findings strengthen the evidence for the significance of HIV infection on the natural history of chronic HBV infection, which by prolonging the period of infectivity could have influenced the epidemiology of HBV infection in India^[30,31]. It is thus far clear that apart from other infections, HIV infected individuals have a high probability of getting co-infected with HBV and/or HCV. HIV disease progression and enhanced immunosuppression has a direct bearing on the natural history and pathogenesis of these infections. Sexual transmission of both HBV and HCV also appears to be significant and is of epidemiological importance in the light of high heterosexual transmission of HIV in India. Monitoring of HIV infected patients for concurrent infection with HBV and HCV is therefore necessary.

The implication of HBV and/or HCV co-infection in HIV patients is of serious concern to the growing Indian economy as there is an overt increase in the trend of number of patients diagnosed with HIV disease in recent years. The knowledge of co-infection in a HIV positive patient is vital since these patients, as they live longer on antiretroviral treatment will also need to be managed for their co-infection with HBV and/or HCV. Hence, there is an urgent need to conduct, detailed studies on the interplay of HIV and hepatotropic viruses in the Indian community with a plethora of multifaceted approaches to investigate the real crisis of HIV/hepatotropic viral infection pattern at the earliest to efficiently control and manage the situation.

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COMMENTS

Background

The influence of HBV or HCV co-infections among subjects with HIV disease is of serious concern to the development of the Indian economy as an increase in the overall trend in the number of patients diagnosed with HIV disease is raising in the recent years. Therefore, scientific knowledge of hepatotropic co-infections in the HIV/AIDS community is important, as they live longer after prompt antiretroviral treatment, in addition to concurrent effective management of HBV or HCV co-infections. Hence, there arises an urgent need to carry out elaborate studies on the interplay of HIV and hepatotropic viruses in the community with a multifaceted approach to promptly explore the scientific facts behind the real crisis to efficiently control and manage the circumstances.

Research frontiers

Uncertainties remain regarding the real effect of co-infection with HIV and HBV or HCV on the progression and outcome of these infections due to the foreseeable difficulties in performing accurate natural history studies, particularly in the persistently developing field of HIV medicine. Although the effect of HBV infection on HIV is indecisive, HIV appears to have a marked role on the natural history of HBV infection in concordant to earlier reports. Therefore, we propose that HIV

influences the natural history of HBV and/or HCV replication, which may have implications for development of hepatitis policy guidelines and epidemiologic studies need to be aimed at strengthening approaches to monitor the possible increase in the spread of HBV and HCV among population at risk for HIV.

Innovations and breakthroughs

The low frequency of HCV in our cohort could be attributed to the low incidence of intravenous drug usage and the infrequent incidence of transfusion, which are substantially different to other reports from parts of India, where IDU and prior transfusion history are reportedly the main risk factors identified among HCV/HIV co-infection. On the contrary, two individuals co-infected with HIV/HCV never had the aforementioned predominant risks; nevertheless presented details of high risk sexual behaviors and prior STI history, which suggestive of the fact that sexual intercourse could also have been the route infection. In addition, both subjects had active HCV infection (evident from positive HCV-RNA) but failed to seroconvert to anti-HCV. The CD4 T-cell counts (58 and 205 cells per mm³) were suggestive of anti-HCV detection with very low CD4 counts and may not be useful for screening. In line with earlier reports, we propose that prior HIV infection could facilitate HCV transmission much easier, though this has not been widely described, and needs more prospective investigations with more number of cohorts with matched controls in the context of HIV/AIDS.

Applications

Though there are few available data on the prevalence of these infections in the Indian general population, information in the HIV positive community is inadequate. In view of the major mode of HIV spread in India being heterosexual, and parenteral route accounting for < 5% of the infections, screening the high-risk population for these infections would aid early detection of co-infections. Hence, initiation of prompt diagnosis and treatment would help decrease the further spread of these chronic viral infections.

Terminology

Hepatitis 'e' antigen (HBeAg) is a peptide and normally detectable in the bloodstream when the hepatitis B virus is actively reproducing, this in turn leads to the person being much more infectious and at a greater risk of progression to liver disease; however, some variants (precore mutant) of the hepatitis B virus do not produce the 'e' antigen at all, so this rule does not always hold true; Anti-HBe is an antibody produced in response to the Hepatitis B e antigen. In those who have recovered from acute hepatitis B infection, anti-HBe will be present along with anti-HBc and anti-HBs. In those with chronic hepatitis B, usually anti-HBe becomes positive when the virus goes into hiding or is eliminated from the body and this phase is generally taken to be a good sign and indicates a favourable prognosis; A positive (or reactive) HBV-DNA or HCV-RNA by PCR (qualitative) indicates the presence of virus that can be passed to others, whereas the negative result (non-reactive) usually means the virus cannot be spread to others; "Occult hepatitis B virus" (HBV) infection is generally defined as the detection of HBV-DNA in the serum or liver tissue of patients who test negative for hepatitis B surface antigen.

Peer review

The authors reported the prevalence rates of HBV or HCV in HIV-positive patients in south Indian area and suggested the higher replication status of HBV or HCV as the disease status of HIV infection progressed. The paper is well written.

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Three novel missense germline mutations in different exons of *MSH6* gene in Chinese hereditary non-polyposis colorectal cancer families

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families, may play an important role in the development of HNPCC.

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Key words: Hereditary non-polyposis colorectal cancer; *MSH6*; Missense mutation; Colorectal cancer

Yan SY, Zhou XY, Du X, Zhang TM, Lu YM, Cai SJ, Xu XL, Yu BH, Zhou HH, Shi DR. Three novel missense germline mutations in different exons of *MSH6* gene in Chinese hereditary non-polyposis colorectal cancer families. *World J Gastroenterol* 2007; 13(37): 5021-5024

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Abstract

AIM: To investigate the germline mutations of *MSH6* gene in probands of Chinese hereditary non-polyposis colorectal cancer (HNPCC) families fulfilling different clinical criteria.

METHODS: Germline mutations of *MSH6* gene were detected by PCR-based DNA sequencing in 39 unrelated HNPCC probands fulfilling different clinical criteria in which *MSH2* and *MLH1* mutations were excluded. To further investigate the pathological effects of detected missense mutations, we analyzed the above related *MSH6* exons using PCR-based sequencing in 137 healthy persons with no family history. The clinicopathological features were collected from the Archive Library of Cancer Hospital, Fudan University and analyzed.

RESULTS: Four germline missense mutations distributed in the 4th, 6th and 9th exons were observed. Of them, three were not found in international HNPCC databases and did not occur in 137 healthy controls, indicating that they were novel missense mutations. The remaining mutation which is consistent with the case H14 at c.3488A>T of exon 6 of *MSH6* gene was also found in the controls, the rate was approximately 3.65% (5/137) and the type of mutation was not found in the international HNPCC mutational and SNP databases, suggesting that this missense mutation was a new SNP unreported up to date.

CONCLUSION: Three novel missense mutations and a new SNP observed in the probands of Chinese HNPCC

INTRODUCTION

Hereditary non-polyposis colorectal cancer (HNPCC), also known as Lynch syndrome, is an autosomal dominant inherited disease characterized by susceptibility to a wide spectrum of cancers, including cancer of the colon, rectum, endometrium, small bowel, and urinary tract^[1]. HNPCC syndrome accounts for 5%-10% of all colon-rectum cancer cases^[2]. Germline mutations in the *bMLH1* (MIM#120436; GDB: 249617) and *bMSH2* (MIM#120435; GDB: 203983) genes are detected in 30%-70% of HNPCC families^[3-5]. Recently, mutations in another MMR gene (*MSH6*), accounting for 10% of HNPCC kindreds, have also been shown to result in HNPCC^[6]. The number of mutations in the *MSH6* gene keeps increasing^[7] (<http://www.nfdht.nl>). Germline mutations of *MSH6* gene are mainly associated with patients with no *MSH2* and *MLH1* mutations. This study was to investigate the *MSH6* gene germline mutations by DNA sequencing in 39 Chinese HNPCC kindreds fulfilling the criteria in which *MSH2* and *MLH1* mutations are excluded, in order to identify HNPCC families and provide experimental information for HNPCC database.

MATERIALS AND METHODS

Materials

From January 1998 to October 2005, 39 Chinese HNPCC families fulfilling the HNPCC clinical criteria were registered at the Department of Abdominal Surgery

in Shanghai Cancer Hospital/Institute. Eleven families fulfilled Amsterdam criteria (AC I) and II (AC II)^[8,9], 11 additional families Japanese criteria^[10] and the remaining 17 kindreds Bethesda guidelines (BG)^[11]. Germline mutations of *MSH2* and *MLH1* were excluded by based-PCR sequencing in the probands of all the Chinese HNPCC families. Each proband was asked to give 10 mL peripheral blood samples and consent for access to archival tumor tissue. A total of 137 control blood samples were taken from healthy persons after obtaining informed consent, none of the individuals in the control group had a family history suggesting HNPCC or development of colon cancer in earlier age. This study was proved by the Medical Ethical Committee of Cancer Hospital, Fudan University, and the procedures of the study were in accordance with the international rules and regulations.

DNA extraction

Genomic DNA was extracted with the QIAGEN (Hilden, Germany) DNA extraction kit following the manufacturer's instructions. Concentrations of the genomic DNA were determined by an ultraviolet spectrophotometer (Beckman, DU640 type).

PCR amplification and DNA sequencing

According to the exon/intron boundary sequences of *MSH6* (GenBank accession number: NM_000179.1), 18 sets of primers were designed to amplify the entire coding region, including 10 exons and each splicing site of *MSH6* (Table 1). The primer pairs used to amplify the 18 fragments of *MSH6* have either M13 forward 5' primers or M13 reverse 3' primers to facilitate sequencing after amplification. PCR was carried out with Taq DNA polymerase (Promega, USA) as described elsewhere^[12]. The PCR conditions were as follows: preheating at 94°C for 7 min, followed by 30-38 cycles of denaturation at 94°C for 45 s, annealing at 56°C-68°C for 45 s and extension at 72°C for 45 s, and a final elongation at 72°C for 10 min (Table 1). PCR products were subjected to 2% agarose gel electrophoresis. After observation of clear and expected size bands, the products were purified and used as a template for sequencing reactions with BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). The sequencing primers were M13F or M13R. Automated fluorescence analysis was performed on a 3700 DNA sequence system (ABI, USA).

Bioinformatic analysis

Each result of sequencing was analyzed by DNASTar5.08 bioanalysis software. The type of mutations and potential significance were determined by comparing the corresponding amino acids and proteins in the following databases (<http://www.ncbi.nlm.nih.gov/>, <http://www.ensembl.org/homo-sapiens> and <http://www.insight-group.org>).

RESULTS

Four missense mutations were found in 39 probands of different Chinese HNPCC families, at codons 468 (CGT>CAT, Arg>His), 1163 (GAA>GTA, Glu>Val) (Figure 1), 666 (TCT>CCT, Ser>Pro) (Figure 2) and 1284

Table 1 Primer sequences and PCR condition of different exons of *MSH6* gene

EN	Primer sequence (5'-3')	Size (bp)	AT (°C)	CN (C)
Exon1	M13F-AGCTCCGTCCGACAGAAC M13R-CTGTGCGAGCCTCCCT	381	68	38
Exon2	M13F-TGCCAGAAGACTTGGAAATTG M13R-CAAACACACACATGGCAG	325	63	32
Exon3	M13F-GATGGGGTTTGCTATGTTGC M13R-TACACCTCCCCCTTTCTTC	341	67	36
Exon4.1	M13F-GGCTGCACGGGTACCATAT M13R-CATTCTCTTCCGCTTTCGAG	390	60	34
Exon4.2	M13F-GCCAGACACTAAGGAGGAAGG M13R-TAGATGCATCAAAATCGGGG	386	59	35
Exon4.3	M13F-TGGCTTAAGGAGGAAAAGAGA M13R-TCTACATCGTGCCTCCATCA	378	60	34
Exon4.4	M13F-TTCTGGCTTTCCTGAAATTG M13R-TAAATCTCGAACAATGGCGA	374	60	34
Exon4.5	M13F-TCTGGCCATACTCGTGCATA M13R-AGCACCTGGGTAACATCAC	329	60	34
Exon4.6	M13F-TCAGGAAGGTCTGATACCCG M13R-GCACCATTGCTTGATAGGCT	353	62	33
Exon4.7	M13F-AAGTGAATTGGCCCTCTCTG M13R-TGGTTCTGACTCTTCAGGGG	483	62	30
Exon4.8	M13F-TTTTGGTAAGCGGCTCCTAA M13R-TTTCGAGCCTTTTCATGGTC	465	62	35
Exon4.9	M13F-TTCTGCTCTGGAAGGATTC M13R-TCGTTTACAGCCCTTCTTGG	440	62	30
Exon4.10	M13F-TGAACAGAGCCTCCTGGAAT M13R-CAGCTGGCAAACAGCACTAC	390	62	32
Exon5	M13F-CTGATAAAACCCCAAAACGA M13R-CTGTGTTTGGAAAATGATCACC	403	62	30
Exon6	M13F-CCAGTCATAAAAGACCTTTCC M13R-GACTGAATGAGAACTTAAGTGGG	192	56	34
Exon7	M13F-AAGGTGAAAGTACATT M13R-TTCAAATGAGAAGTTAATG	119	61	33
Exon8/9	M13F-CCTTTGAGTTACTTCTCTT M13R-TCATAGTGCATCATCCCTCC	573	61	32
Exon10	M13F-GGAAGGGATGATGCACTATG M13R-AAGAAAATGGAATAATGGTCA	254	61	35

Abbreviation: EN, Exon name. AT, Anneal temperature. CN, Cycle number. The sequence of M13F was 5'-GTAAACGACGCGCCAGT-3'. The sequence of M13R was 5'-AACAGCTATGACCATG-3'.

(ACG>ATG, Thr>Met), respectively (Table 2). To further investigate the pathological effects of these four missense mutations, we analyzed the four related *MSH6* exons using PCR-based sequencing in 137 healthy persons with no family history, showing that the mutation of codon 1163 which is consistent with the case H14 at c.3488A>T of exon 6 of *MSH6* gene was also found in the control persons, the rate was approximately 3.65% (5/137). The remaining three missense mutations did not occur in the 137 healthy controls. None of these four mutations was found in the *MSH6*-SNP database (www.ensembl.org/homo-sapiens). Thus, except that the mutation at c.3488A>T of *MSH6* gene in the H14 HNPCC case was an unreported new single nucleotide polymorphism (SNP), the remaining ones were novel missense mutations.

DISCUSSION

Hereditary non-polyposis colorectal cancer is an autosomal dominant inherited syndrome^[13]. Although its clinical diagnostic criteria were established in 1990, known as

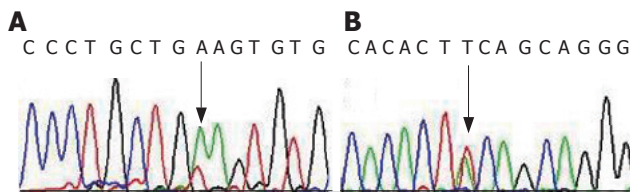


Figure 1 Missense germline mutation of exon 6 of *MSH6* gene in the proband of H14 HNPCC kindreds. Arrow indicates the mutation site. The single basyl substitution was transversed from A to T (A>T) at the codon 1163, the codon from GAA to GTA, causing the amiod acid changes from glutamine to valine, the change was identified as a new SNP. **A** and **B** represent the forward sequence and reverse, respectively.

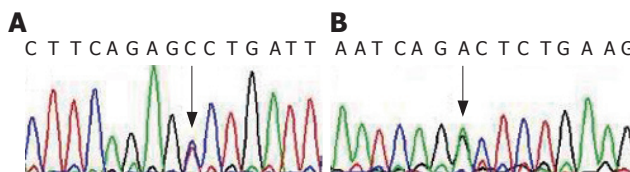


Figure 2 Missense germline mutation of exon 4.6 of *MSH6* gene in the proband of H40 HNPCC kindreds. Arrow indicates the mutation site. The single basyl substitution was transversed from T to C (T>C) at the codon 666, the codon from TCT to CCT, causing the amiod acid changes from serine to proline. **A** and **B** represent the forward and reverse sequence, respectively.

Amsterdam criteria, Japanese Criteria and Bethesda guidelines, a certain number of HNPCC families may be neglected for lacking characteristic clinical manifestations and family history. Molecular genetic screening is been regarded as a standard method for its diagnosis. Mutations of MMR genes are considered golden criteria for molecular diagnosis and monitoring family members. These mutations can be used to select persons who would benefit from genetic counseling and clinical surveillance programs for their relatives to reduce morbidity and mortality due to HNPCC-related tumors^[14]. Germline mutations in the coding regions of *MSH2* and *MLH1* are known to be responsible for up to 45%-64% of all HNPCC families^[15]. We have previously detected germline mutations of the entire coding regions of *MSH2* and *MLH1* genes in 24 AC probands, 15 JC probands and 19 BG patients using PCR-gene-sequencing with 17 germline mutations detected including two mutations occurred in a same patient^[16]. Three new mutations have been found by mRNA-based PCR sequencing^[17]. The remaining 39 probands without *MSH2* and *MLH1* might be associated with the other abnormal MMR genes such as *MSH6*.

MSH6 mutations are involved in the development of colorectal cancer^[18]. Germline mutations of *MSH6* have been reported in two atypical HNPCC Japanese families lacking mutations in *MSH2* and *MLH1*^[19,20]. Some researchers believe that *MSH6* gene might be the first candidate gene for detecting germline mutations in HNPCC families in which *MSH2* and *MLH1* mutations are excluded^[21]. It was reported that *MSH6* mutations account for 10% of kindreds in which *MSH2* and *MLH1* mutations are excluded^[6]. Our study has demonstrated four missense mutations of *MSH6* gene in the probands

Table 2 Germline mutations of *MSH6* gene in 4 probands of HNPCC families

Family No	Exon	Position of mutation	Base change	Result	Mutation type
H3	4.3	Codon 468	c.1403G>A	Arg>His	Missense mutation
H14	6	Codon 1163	c.3488A>T	Glu>Val	New SNPs
H40	4.6	Codon 666	c.1996T>C	Ser>Pro	Missense mutation
H61	9	Codon 1284	c.3851C>T	Thr>Met	Missense mutation

Table 3 Clinical characteristics of 4 mutational probands of HNPCC families

No	Sex	Age (yr)	Criteria	Site of cancer	Age at diagnosis of first CRC (yr)	Metachronous tumor
H3	M	50	AC	Sigmoid	46	VA
H14	F	26	BG	Rectum	26	NMT
H40	F	39	BG	Rectum	39	NMT
H61	F	51	BG	Descending	33	EC

Abbreviation: AC, Amsterdam criteria. JC, Japanese criteria. BG, Bethesda guidelines. CRC, Colorectal cancer. VA, Villiform adenoma. NMT: No Metachronous tumor. EC, Endometrial cancer.

of 39 Chinese HNPCC families, which have not been reported (<http://www.nfdht.nl>). However, missense mutations in MMR genes are common and often pose a formidable problem of interpretation, because these changes do not necessarily affect the function of the protein. Further functional studies are required in order to determine whether the missense mutations are neutral polymorphisms or clinically relevant mutations. We detected the exons of these four missense mutations by direct sequencing of the *MSH6* gene using genomic DNA from blood samples of 137 healthy persons, the mutational rate was approximately 3.65% (5/137). Since “polymorphism” is a term that is usually used for genetic variants with a minor allele frequency $\geq 1\%$ in a given population^[22], single basyl transversion at c.3488A>T of exon 6 might represent a new SNP, although the changes are not found in SNP (<http://www.ensembl.org>). The remaining three missense mutations were not detected in genomic DNA from 137 healthy persons, suggesting that they are three novel missense mutations of *MSH6* gene in Chinese HNPCC families. The clinical features of the probands of these three novel missense mutations and one new SNP are shown in Table 3. In brief, the above mutation carriers occur more frequently in the left colon than in *MLH1* or *MSH2* mutation carriers. However, the relationship between the above typical features and germline mutations of *MSH6* gene in the probands of Chinese HNPCC families still remains unclear. To date, the International Collaborative Group on HNPCC (ICG-HNPCC) has found over 30 potentially pathogenic *MSH6* mutations. A significant proportion (35%) of them results in a single amino acid substitution, which is difficult to interpret. Since the pathogenicity of HNPCC mutations is linked to malfunction of MMR, whether the above three novel missense mutations are involved in human MMR needs to be further investigated.

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COMMENTS

Background

Germline mutations in mismatched repair genes, such as *MLH1*, *MSH2* and *MSH6*, lead to hereditary nonpolyposis colorectal cancer (HNPCC) syndrome. Germline mutations of *MLH1* and *MSH2* gene have been reported in Chinese HNPCC families. However, the germline mutation of *MSH6* has not yet been reported.

Research frontiers

Now many researchers are engaged in studies of HNPCC, especially in germline mutations of MMR genes such as *MSH2*, *MLH1* and *MSH6*. These studies can contribute to the early diagnosis of HNPCC and screening of HNPCC families. Few studies on germline mutations of *MSH6* gene are available.

Innovations and breakthroughs

Three novel germline mutations of *MSH6* gene have been found in 39 probands of Chinese HNPCC families by PCR-based sequencing, and a new SNP has been found by screening the missense mutations of genomic DNA in 137 healthy persons.

Applications

Germline mutations in genes can be used to diagnose early HNPCC and enrich international HNPCC mutation and SNP databases.

Terminology

HNPCC is an abbreviation of hereditary nonpolyposis colorectal cancer. Germline mutations are the mutations in genomic DNA.

Peer review

This paper is an interesting manuscript, the authors detected new HNPCC-related mutations and discovered three novel mutations and an additional SNP in 39 unrelated HNPCC probands. Data on patient characteristics, such as gender and age at diagnosis of colorectal cancer in the patient (or in the family) should be provided in detail.

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¹⁸F-DG PET/CT in detection of recurrence and metastasis of colorectal cancer

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Abstract

AIM: To evaluate the value of ¹⁸F-DG PET/CT in detecting recurrence and/or metastasis of colorectal cancer (CRC).

METHODS: Combined visual analysis with semiquantitative analysis, the ¹⁸F-DG PET/CT whole-body imaging results and the corresponding clinical data of 68 postoperative CRC patients including 48 male and 20 female with average age of 58.1 were analyzed retrospectively.

RESULTS: Recurrence and/or metastasis were confirmed in 56 patients in the clinical follow-up after the PET/CT imaging. The sensitivity of PET/CT diagnosis of CRC recurrence and/or metastasis was 94.6%, and the specificity was 83.3%. The positive predictive value (PPV) was 96.4% and the negative predictive value (NPV) was 76.9%. PET/CT imaging detected one or more occult malignant lesions in 8 cases where abdominal/pelvic CT and/or ultrasonography showed negative findings, and also detected more lesions than CT or ultrasonography did in 30.4% (17/56) cases. Recurrence and/or metastasis was detected in 91.7% (22/24) cases with elevated serum CEA levels by ¹⁸F-DG PET/CT imaging.

CONCLUSION: ¹⁸F-DG PET/CT could detect the recurrence and/or metastasis of CRC with high sensitivity and specificity.

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Key words: Colorectal cancer; Recurrence; Metastasis; Positron emission tomography; Computed tomography

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<http://www.wjgnet.com/1007-9327/13/5025.asp>

INTRODUCTION

Colorectal cancer (CRC) is the most common gastrointestinal malignancy, and its incidence and mortality is still rising in China^[1,2]. Radical resection and postoperative chemotherapy remains to be the major management of CRC, but recurrence and/or metastasis occurs in 30%-50% of the patients after surgery^[3-6]. An early diagnosis and accurate staging of postoperative metastasis is crucial for prescribing an optimal individualized chemotherapy regimen and thus elevating the survival rate. Combined positron emission tomography and computed tomography (PET/CT) is currently widely used in clinical diagnosis of cancer, showing great superiority in tumor staging^[7-9]. In the present research, we investigated the diagnostic significance of fluorine 18 fluorodeoxyglucose (¹⁸F-DG) PET/CT in postoperative recurrence and/or metastasis of CRC by conducting a retrospective study of 68 patients.

MATERIALS AND METHODS

Subjects

A total of 68 postoperative CRC patients, 48 male and 20 female, examined in the PET/CT center of Jinling Hospital between August 2004 and August 2006 were included in the study cohort. The age of the patients ranged from 27 to 77 years, and the average was 58.1. A whole-body ¹⁸F-DG PET/CT was conducted in each case. The interval time between the operation and the examination ranged from 4 mo to 8 years, and the average was 2.5 years. The diagnosis of recurrence and/or metastasis was based on pathological evidence, colonoscopy findings, medical imaging results, and clinical follow-up outcome. The follow-up time ranged from 5 to 28 mo.

PET/CT imaging

Biography sensation 16 PET/CT (Siemens, Germany) was used. ¹⁸F-DG was produced by TR19 cyclotron (EBCO, Canada) and automatic chemical synthesizer (Beijing Patborn Biotech, China). The synthesis efficiency was 701%, and the radiochemical purity was higher than

95%. Prior to the FDG injection, blood samples were drawn from all patients to assure fasting glucose levels to be less than 6.60 mmol/L. Afterwards, 296-444 MBq of FDG was administered intravenously, and patients waited 50-60 min before undergoing PET and CT scanning. PET emission images were obtained with a weight-based protocol for 5-7 bed positions, with 3 min of acquisition time per bed position. A delayed imaging of the region of interest (ROI) or suspicious lesion region was conducted 3 h later. High resolution CT (HRCT) was conducted if any lesion was detected in the lung. Traverse, sagittal and coronary sections of CT, PET and combined PET/CT were obtained, with a section thickness of 5 mm, by reconstruction using an iterative algorithm, with CT-based attenuation correction applied.

Image interpretation had been individually performed by more than 2 experienced board-certified nuclear medicine physicians and radiologists. Visual assessment and semi-quantitative analysis were adopted in the image interpretation. ROI was determined at the section most clearly showing the hyper-intensive radioactivity, and the maximum standardized uptake value (SUV_{max}) was accessed. The diagnosis of a positive lesion was based on a SUV_{max} higher than 2.5 in the early imaging, while a typical positive lesion on a SUV_{max} higher than 2.5 and also ΔSUV_{max} ($\Delta SUV_{max} = SUV_{max, delayed} - SUV_{max, early}$) higher than 20% in the delayed imaging. The diagnosis of a malignancy lesion should be simultaneously based on the CT findings including the shape, size, intensity and distribution of the lesion, which could differentiate the physiological uptake.

RESULTS

Diagnosis of recurrence and/or metastasis of CRC with ^{18}F -DG PET/CT

Among the 68 examinees, recurrence and/or metastasis were later diagnosed in 56 patients, including 8 recurrences, 46 metastases, and 2 recurrence and metastases. The sensitivity of PET/CT diagnosis of CRC recurrence and/or metastasis was 94.6%, and the specificity was 83.3%. The positive predictive value (PPV) was 96.4% and the negative predictive value (NPV) was 76.9%. In 8 cases which abdominal/pelvic CT and/or ultrasonography showed negative findings, PET/CT imaging detected one or more occult malignant lesions, resulting in altered clinical staging. In 30.4 % (17/56) cases, PET/CT imaging detected more lesions than CT and ultrasonography did, showing disseminated metastases. PET/CT imaging displayed a superior sensitivity in detecting stump recurrence, local and distant metastasis compared with non-contrast CT scanning. Metastases were commonly detected in liver (18/56, 32.1%), lung (15/56, 26.8%), abdominal cavity and mesentery (14/56, 25%), retroperitoneum and psoas major (11/56, 19.6%). Others included bone, mediastinum lymph node, peritoneum, adrenal gland, ovary and brain. Liver and lung were the preferred metastasis foci, and blood metastasis was the major route of metastasis, followed by lymph node metastasis.

Influence of the ^{18}F -DG PET/CT diagnosis on clinical treatment

The treatment plans of 11 cases (11/68, 16.2%) were

altered based on the ^{18}F -DG PET/CT findings. Local metastasis focus was detected in the liver or lung in 3 cases, and surgical resections were conducted instead of intravenous chemotherapy and biotherapy. Conversely, disseminated metastases were detected in 6 cases, thus intravenous or oral chemotherapy was prescribed instead of surgery. Elevated serum carcinoembryonic antigen (CEA) level was detected in 2 cases while no specific lesion was found, as a result, intravenous chemotherapy was about to be prescribed. With the ^{18}F -DG PET/CT imaging however, the treatment plan was subsequently changed. In one case, PET/CT detected a local recurrence, so a reoperation was preformed in time. In the other case, metastases were detected in both the lung and bone, hence intravenous chemotherapy and local radiotherapy were performed. Second primary cancer was found in another 2 postoperative CRC cases. In one case of colon cancer, ^{18}F -DG PET/CT detected a rectal cancer as the second primary cancer. In the other case, rectal cancer was the first primary cancer while hyper-intensive ileocecal radioactivity was detected in the ^{18}F -DG PET/CT imaging, and colon cancer was confirmed after the reoperation.

^{18}F -DG PET/CT in postoperative CRC patients with elevated serum CEA levels

Elevated serum CEA level (363.7-863.2 μ g/L, average 185.9 μ g/L) was found in 24 cases, and recurrence and/or metastasis was detected in 22 of them by ^{18}F -DG PET/CT imaging with specific lesion observed. Diagnoses of recurrence and/or metastasis were confirmed in all of the 22 cases during the clinical follow-up. However, the negative findings of PET/CT in the remained 2 cases were concluded to be false negative in clinical follow-up. Multiple nodi and small metastasis focus were detected in the mesentery and vagina respectively, both with the diameter around 1 cm, and postoperative pathological evidence confirmed the diagnosis of metastasis. As a result, the positive rate of recurrence and/or metastasis detection with PET/CT in postoperative CRC patients with elevated serum CEA levels was 91.7%.

DISCUSSION

The prognosis of CRC is closely related to the histological type, intestinal wall invasion, malignant lymph node, operation manner and postoperative recurrence and/or metastasis. An emphasis of the postoperative follow-up is the local recurrence and distant metastasis, which could not be detected by traditional imaging techniques such as CR, magnetic resonance imaging (MRI) and ultrasonography until the lesion reaches a considerable size. Monitoring by tumor markers such as CEA and CA19-9 was not always accurate, and could not provide an orientation indication of the possible lesion either. PET/CT imaging provides whole-body overview at one examination, and could detect abnormal glucose metabolism before the morphological change of a lesion could be identified. Consequently, it has become an efficient and accurate noninvasive examination technique in the postoperative follow-up of CRC^[10]. In the present study, PET/CT imaging showed high sensitivity (94.6%), specificity (83.3%) and PPV (96.4%) in the

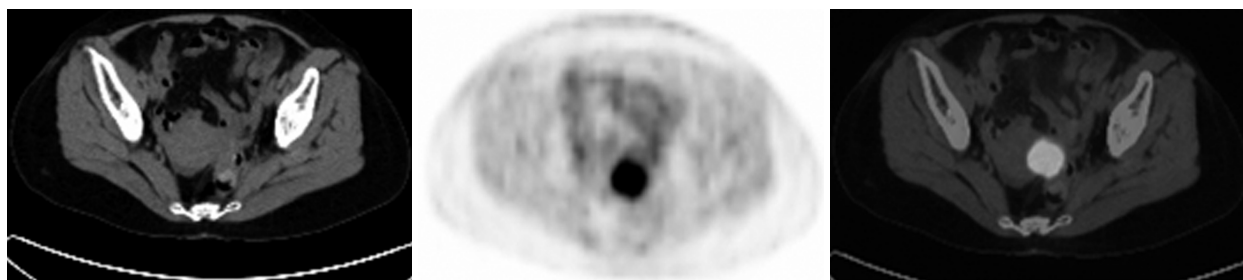


Figure 1 PET/CT imaging showing local recurrent rectal carcinoma in a 49-year-old female postoperative rectal cancer patient. CT scan did not find definite recurrence signs. PET imaging detected hyper-intensive radioactivity by the left side of uterus. The SUV_{max} was 7.5 and the T/N was 9.4. Combined PET/CT imaging indicated elevated ^{18}F -DG uptake of the anastomosis. The reoperation confirmed a recurrent tubular adenocarcinoma of the rectum.

diagnosis of the recurrence and/or metastasis of CRC. These findings are consistent with the Meta-analysis results of Huebner *et al.*^[10]. It is suggested that application of PET/CT imaging to detect recurrence and/or metastasis of CRC is appropriate. It is commonly recognized that PET has become an effective technique in detecting postoperative recurrence and/or metastasis of CRC. The sensitivity and specificity reported were over 90% and 70% respectively, both higher than that of traditional imaging techniques.

^{18}F -DG PET/CT imaging is more accurate than traditional imaging in detecting hepatic metastases. Kinkel *et al.*^[11] performed a meta-analysis to compare current noninvasive imaging methods in the detection of hepatic metastases from colorectal, gastric, and esophageal cancers. In studies with a specificity higher than 85%, the mean weighted sensitivity was 55% (95% CI: 41, 68) for ultrasonography, 72% (95% CI: 63, 80) for CT, 76% (95% CI: 57, 91) for MRI, and 90% (95% CI: 80, 97) for FDG PET. Thus they demonstrated a greater sensitivity of FDG PET than ultrasonography ($P = 0.001$), CT ($P = 0.017$), and also MRI ($P = 0.055$). Delbeke *et al.*^[12] demonstrated in their study on the effect of PET on management that PET helped clarify the final diagnosis misinterpreted on CT and/or CT portography in 28% of the patients ($n = 17$), leading to a change in surgical management. PET helped to plan surgery in 6 patients by identifying a resectable site of recurrence and helped to avoid unnecessary surgery in 11 patients by identifying unresectable disease.

In our study, PET/CT imaging detected one or more occult malignant lesions in 8 cases where abdominal/pelvic CT and/or ultrasonography showed negative findings, resulting in altered clinical staging. PET/CT imaging also detected more lesions than CT and ultrasonography did in 30.4% (17/56) cases, showing disseminated metastases and hence resulting in altered clinical treatment plans. PET/CT imaging increases the accuracy and certainty of locating lesions in colorectal cancer. The fused imaging of PET and CT is especially important for identifying lesions in abdominal and pelvic cavity. PET itself is insufficient to identify anatomic structures clearly and also incapable to interpret the nonspecific uptake of the gastrointestinal and muscles and the excretion of the urinary system. CT provides an attenuation correction, and its morphologic imaging is helpful to differentiate among physiological uptake, anastomosis inflammation, and the cancer recurrence. It also helps to detect tumors which showed

a negative uptake of ^{18}F -DG in PET imaging. Cohade *et al.*^[13] compared the diagnostic roles of ^{18}F -FDG PET and PET/CT in a population of patients with colorectal cancer. The frequency of equivocal and probable lesion characterization was reduced by 50% (50 to 25) with PET/CT, in comparison with PET. The frequency of definite lesion characterization was increased by 30% (84 to 109) with PET/CT. The number of definite locations was increased by 25% (92 to 115) with PET/CT. Overall correct staging increased from 78% to 89% with PET/CT on a patient-by-patient analysis. Our study also shows the superiority of combined PET and CT imaging in detecting recurrent and isolated hepatic metastasis of CRC (Figures 1 and 2).

When local recurrence and/or distant metastasis are diagnosed and the reoperation is planned, PET/CT imaging is greatly helpful in preoperative staging and evaluating the feasibility of the operation. It could detect the unresectable and additional occult lesions, and thus avoid unnecessary surgical resections and also economic expenditures. It could also provide further information regarding the surgical resection details based on the imaging findings in those cases which reoperation is needed. A better decision of clinical management based on preoperative PET/CT imaging results in better clinical outcome. Zubeldia *et al.*^[14] concluded that integration of FDG-PET into the presurgical evaluation of patients with hepatic metastases could substantially reduce overall costs and patients' morbidity. This substantial net saving, 5269 dollars averagely, results from the unique ability of FDG-PET in excluding patients with extrahepatic disease, and avoiding unnecessary surgical expenses. In our study, unnecessary surgeries were avoided in 7 cases where multiple distant metastases were found in PET/CT imaging, accounting for 70% (7/10) of the cases where reoperation was planned before PET/CT imaging. These imaging findings directly resulted in decreased medical expenses. In those cases with elevated serum CEA levels, PET/CT is helpful in detecting recurrence and/or metastasis based on both functional and morphological imaging. In the study of Shen *et al.*^[15], whole-body FDG-PET was performed in 50 patients suspected of having recurrent colorectal cancer and asymptotically increased serum level of CEA ($> 5 \mu\text{g/L}$), and the final diagnosis of recurrent colorectal cancer was established in 64 lesions of 45 patients. The diagnostic sensitivity and positive predictive value of FDG-PET was 95.3% on a patient-

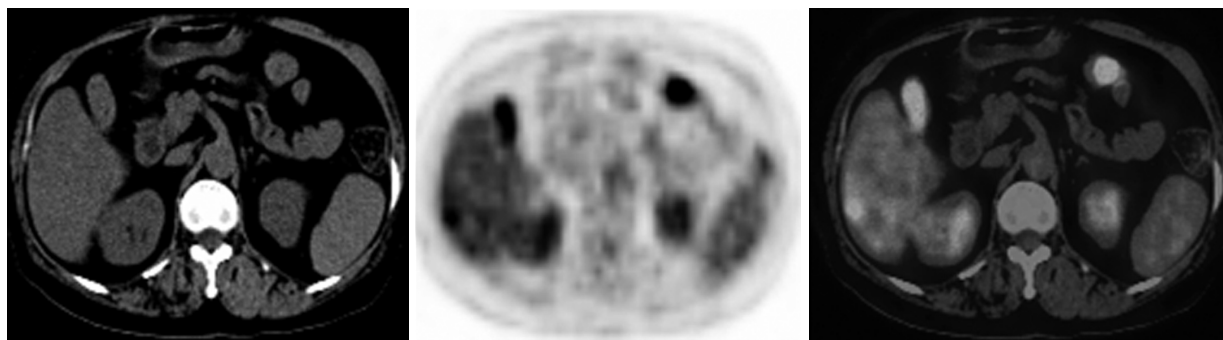


Figure 2 PET/CT imaging showing isolated hepatic metastasis in a 69-year-old female postoperative sigmoid carcinoma patient. CT scan did not find definite recurrence signs. PET imaging detected localized hepatic subcapsular hyper-intensive radioactivity. Local resection of liver lobe was performed in the reoperation, and metastatic adenocarcinoma was confirmed.

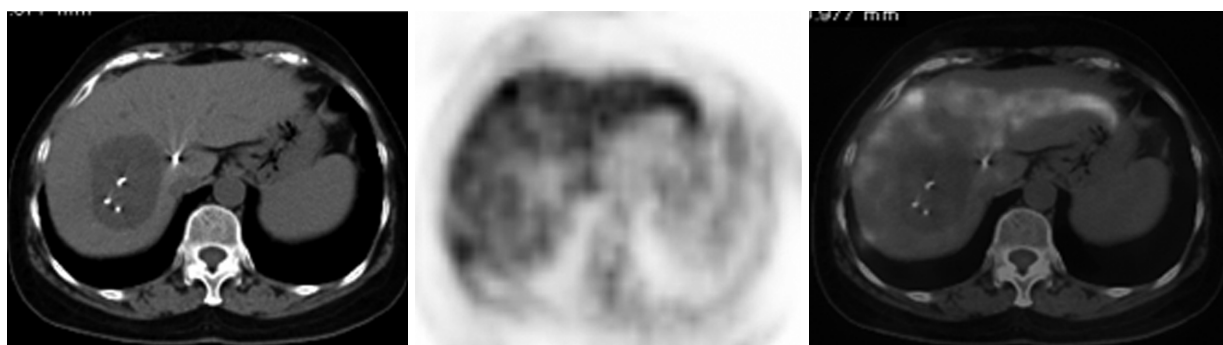


Figure 3 PET/CT imaging showing hepatic metastasis after local therapy in a 60-year-old male postoperative colon cancer patient. ^{125}I seeds brachytherapy was performed in the metastatic lesion of the liver, causing necrosis and ^{18}F -DG uptake decrease of the lesion. However, additional localized hepatic subcapsular hyper-intensive radioactivity was detected, indicating that new liver metastasis had occurred.

based analysis, and 96.9% on a lesion-based analysis. In our study, PET/CT detected one or more possibly malignant lesions in 91.7% (22/24) postoperative CRC patients with increased serum level of CEA. It was obvious that PET/CT is clinically significant in the detection of postoperative malignant lesions in CRC patients.

PET/CT could also be used in monitoring the treatment effect of local therapy of isolated liver and lung metastatic lesions. Local therapy approaches of hepatic metastasis include selective transcatheter arterial chemoembolization (TACE), radio frequency ablation (RFA), Argon-Helium cryoablation, gamma knife radiosurgery (GKS), ^{125}I seeds brachytherapy, and so on. Traditional imaging techniques could not discriminate between tissue edema and tumor remnant, and could not exclude the interference of iodinated oil retention either. By contrast, PET provides a functional imaging of the metabolism levels within the suspected foci and also the whole-body, so it substantially helps the clinicians in recognizing tumor remnants and evaluating whole-body status, which are crucial in making treatment decisions. In our study, 7 patients underwent local therapy of isolated liver or lung metastasis. Figure 3 shows the typical PET/CT imaging of one case underwent ^{125}I seeds brachytherapy of the localized hepatic metastasis.

In conclusion, our study demonstrated the superiority of ^{18}F -DG PET/CT in the imaging diagnosis of postoperative recurrence and/or metastasis of CRC.

It could detect not only recurrent tumor and/or occult metastasis in early stage, but also any other lesions showing abnormal metabolism level within the whole-body of postoperative CRC patients. The PPV was 96.4% and the negative NPV was 76.9%. The major causes of false positive were granulomatous inflammation at the anastomosis and inflammatory lymph node, while those of false negative were small lesion sizes (usually less than 1 cm in diameter). As a result, interpretation of ^{18}F -DG PET/CT imaging should also be based on clinical findings in order to minimize the chance of misdiagnose.

COMMENTS

Background

Combined positron emission tomography and computed tomography (PET/CT) is currently widely used in clinical diagnosis of cancer, however, the value of PET/CT in detection of recurrence and/or metastasis of colorectal cancer (CRC) remains to be confirmed furtherly.

Research frontiers

In the area of application of PET/CT in CRC, the research hotspot is to evaluate the value of this technique in pre-surgery staging, predicting therapy effects and detecting postoperative recurrence and/or metastasis of the disease.

Innovations and breakthroughs

In the present study, recurrence and/or metastasis were confirmed in 56 patients in the clinical follow-up after the PET/CT imaging. The sensitivity of PET/CT diagnosis of CRC recurrence and/or metastasis was 94.6%, and the specificity was 83.3%. The positive predictive value (PPV) was 96.4% and the negative predictive

value (NPV) was 76.9%. PET/CT imaging detected one or more occult malignant lesions in 8 cases where abdominal/pelvic CT and/or ultrasonography showed negative findings, and also detected more lesions than CT or ultrasonography did in 30.4% (17/56) cases. Recurrence and/or metastasis was detected in 91.7% (22/24) cases with elevated serum CEA levels by ¹⁸F-DG PET/CT imaging.

Applications

¹⁸F-DG PET/CT imaging is a useful technique for detecting recurrence and/or metastasis of CRC with high sensitivity and specificity. It could detect not only recurrent tumor and/or occult metastasis in early stage, but also any other lesions showing abnormal metabolism level within the whole-body of postoperative CRC patients.

Terminology

PET/CT: Combined the technologies of positron emission tomography and spiral computed tomography into a highly sensitive and quantitative molecular imaging modality. The "fused" image of PET and CT scan can provide complete information on the location, nature and extent of lesions.

Peer review

This is a good article in which authors demonstrated the superiority of ¹⁸F-DG PET/CT in the imaging diagnosis of postoperative recurrence and/or metastasis of CRC by conducting a retrospective study of 68 patients. The result is valuable for choice of imaging techniques for detecting recurrence and/or metastasis of CRC.

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RAPID COMMUNICATION

Thymidylate synthase and thymidine phosphorylase gene expression as predictive parameters for the efficacy of 5-fluorouraci-based adjuvant chemotherapy for gastric cancer

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Abstract

AIM: To investigate the prognostic role of thymidylate synthase (TS) and thymidine phosphorylase (TP) mRNA levels in T3 or T4 gastric cancer treated with 5-fluorouraci-based adjuvant chemotherapy.

METHODS: Fifty-one patients with T3 or T4 gastric cancer received systemic 5-fluorouraci-based adjuvant chemotherapy, and intratumoral expression of TS and TP in 51 gastric cancer tissue samples was tested by real-time quantitative PCR.

RESULTS: The median disease-free survival (DFS) time was 10.2 mo in the patients. There were no significant differences in DFS between the groups with high and low levels of TP. However, the group with low level of TS had a longer DFS (14.4 mo vs 8.3 mo, $P = 0.017$). The median overall survival (OS) time was 18.5 mo, and there were significant differences in OS between the groups with high and low levels of TS or TP (for TS, 17.0 mo vs 21.3 mo, $P = 0.010$; for TP, 16.6 mo vs 22.5 mo, $P = 0.009$). Moreover, the coupled low expression of these two genes was strongly associated with a longer survival time of patients as compared with that of a single gene.

CONCLUSION: Expression of TS and TP mRNA is a useful predictive parameter for the survival of postoperative gastric cancer patients after 5-fluorouracil-based adjuvant chemotherapy.

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Key words: 5-fluorouracil; Gastric cancer; Thymidine phosphorylase; Thymidylate synthase

Hua D, Huang ZH, Mao Y, Deng JZ. Thymidylate synthase and thymidine phosphorylase gene expression as predictive

INTRODUCTION

Since originally synthesized in 1957, 5-fluorouracil (5-FU) has been widely applied in clinical practice. Although 5-FU is considered one of the most effective agents against gastrointestinal carcinoma, its efficacy rate is only 10%-15% for gastric and colon cancer, and differs greatly among patients^[1,2]. As a pyrimidine analog, 5-FU exerts its antitumor effects through anabolism, which is determined by the rate of catabolism. Thus, the expression level of genes coding for key enzymes in metabolism may play a pivotal role in the sensitivity and efficacy of 5-FU^[3].

Thymidylate synthase (TS) is the target enzyme for 5-FU and catalyzes methylation of fluorodeoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), which is an important process of DNA biosynthesis^[4,5]. Several reports indicate that TS expression in clinical tumor samples is significantly related to the response to 5-FU in gastric cancer patients^[6]. Recent studies demonstrate that TS mRNA level may be an important independent prognosticator of survival in patients with gastric cancer^[7].

Thymidine phosphorylase (TP) is an enzyme involved in pyrimidine nucleoside metabolism. TP is expressed in a wide variety of solid tumors (carcinomas of the breast, stomach, colon, pancreas and lung), and its content is higher in tumor tissues than in adjacent normal tissues^[8]. TP levels might affect the sensitivity of 5-FU, and transfection of TP cDNA into cancer cells increases their sensitivity to 5-FU^[9]. It was also reported that high expression of TP in tumors is indicative of a poor prognosis^[10].

In this study, we examined the mRNA level of TS/TP in a panel of 51 patients with T3 or T4 gastric adenocarcinoma to probe its predictive value for the efficacy of 5-FU-based adjuvant chemotherapy. In addition, we tested if combination of expression levels of the analyzed genes has an increased effect on the prediction of patient survival.

MATERIALS AND METHODS

Patients and tissue samples

A total of 51 patients with T3 or T4 gastric adenocarcinoma who underwent gastrectomy at the 4th Affiliated Hospital of Suzhou University from August 2003 to April 2005 were enrolled in this study. Tumor tissue samples were obtained during surgery and stored in liquid nitrogen until preparation of RNA extracts. Inclusion criteria for patients included: (1) patients with histologically proved gastric cancer, (2) patients without early recurrence or non-curable resection, (3) patients receiving no other adjuvant treatments, such as radiotherapy or immunotherapy, (4) patients with their performance status score of 0-1 and a life expectancy over 6 mo. All patients gave their informed consent, and the study was approved by the Institutional Ethics Review Board.

Chemotherapy schedule

After surgery, all patients underwent 6 courses of 5-FU-based adjuvant chemotherapy. The chemotherapy protocol included intravenous infusion of 5-FU (375-500 mg/m² per day, d 1-5) and leucovorin (LV) (100-200 mg/m² per day, d 1-5) at three-week interval between two courses. The therapy was terminated or shifted to second-line chemotherapy when disease progression was observed or unacceptable toxicity appeared. All patients were treated in our hospital from 2003 to 2005.

Total RNA isolation and cDNA synthesis

RNA was extracted by using Trizol (Invitrogen Corporation, CA) according to the manufacturer's instructions. The amount of total RNA was estimated by ultraviolet absorbance at 260 nm, and the quality was determined by agarose gel electrophoresis in the presence of formaldehyde. cDNA strand synthesis was performed using a reverse transcription system (Promega Corporation, US).

Real-time quantitative PCR assay for TS and TP

Quantitative PCR was performed with the LightCycler TS/TP mRNA quantification kit^{PLUS} (Roche Diagnostics, Germany) as follows: denaturation of cDNA at 95°C for 5 min; 40 cycles of amplification of cDNA at 95°C for 10 s, at 62°C for 10 s, at 72°C for 10 s; cooling the rotor and thermal cycler at 40°C for 30 s. LightCycler TS/TP calibrator RNA was also used to compensate for the constant differences between detection of the target (TS/TP) and reference gene (glucose- 6-phosphate dehydrogenase, G6PDH) and to provide a constant calibration point between PCR runs. The amount of mRNA encoding for TS/TP was calculated using the LightCycler relative quantification software (Roche). All gene expression analyses were performed in a blinded fashion by the laboratory investigators.

Statistical analysis

To evaluate the association of TS and TP with disease-free survival (DFS) and overall survival (OS), TS and TP expression levels were categorized into a low and high value, using the median concentration as a cut point. The maximal χ^2 method was used to determine which expression value best segregated patients into poor and

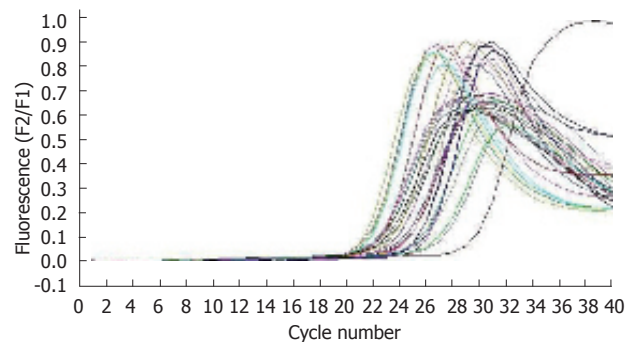


Figure 1 Amplification curve of RT-PCR on the target (TS/TP) and reference gene (G6PDH). TS: thymidylate synthase; TP: thymidine phosphorylase; G6PDH: glucose- 6-phosphate dehydrogenase.

good prognosis subgroups. The survival rate was calculated by the Kaplan-Meier method and statistical analysis was performed using the log-rank test. Cox's proportional hazard model was used to assess the prognostic importance of gene expressions adjusted for the following clinicopathologic features: age, gender, histological type, lymphatic metastasis. $P < 0.05$ was considered statistically significant. All analyses were performed using SPSS software (version 10.0.5; SPSS Inc. Chicago, IL).

RESULTS

Expression of 5-fluorouracil-related genes TS and TP in gastric tumor

A total of 51 eligible patients were enrolled in the study between August 2003 and April 2005. The quantitation of TS and TP mRNA levels in gastric cancer tissues was successfully performed in all specimens (Figure 1). Intratumoral expression of TS and TP genes ranged from 0.09 to 4.60 (median: 0.94) and 3.99 to 196.28 (median: 21.20), respectively.

No significant differences were observed between the mRNA levels of TS/TP and clinicopathological features such as gender, age and stage. The relationship between TS and TP expression levels and clinicopathologic factors are summarized in Table 1.

DFS in relation to expression of TS and TP

The median DFS of the 51 eligible patients was 10.2 mo. When the median value (21.20) of TP was assigned as the cut-off value, there was no significant difference in DFS between the high and low level groups (9.5 mo *vs* 14.0 mo, $P = 0.068$). Patients with a low expression level of TS showed a trend to correlate with prolonged DFS (14.4 mo *vs* 8.3 mo, $P = 0.017$).

OS in relation to expression of TS and TP

The median DFS was 16.2 mo in patients with low TS and TP expression, but only 8.30 mo in patients with high TS and TP expression. The median OS was 18.5 mo. When the median value of 5-fluorouracil-related TS and TP genes was designated as a cut-off value, there were significant differences in OS between the groups with high TS (TP) expression and low TS (TP) expression (17.0 mo *vs* 21.3 mo,

Table 1 Relationship between TS/TP mRNA levels and clinicopathologic factors

Factor	n	TS		P value	TP		P value
		High value	Low value		High value	Low value	
Age (yr)							
≥ 60	22	9	13	0.313	12	10	0.492
< 60	29	16	13		13	16	
Gender							
Male	39	19	20	0.938	18	21	0.460
Female	12	6	6		7	5	
Histological Type							
II	25	15	10	0.124	11	14	0.482
III	26	10	16		14	12	
Lymphatic metastasis							
Positive	41	22	19	0.323	21	20	0.777
Negative	10	3	7		4	6	

TS: thymidylate synthase; TP: thymidine phosphorylase.

Table 2 Coupled expression of TS + TP and patients survival

	TS + TP		P-value
	High value	Low value	
n	16	17	
DFS	8.3 (3.7-23.7)	16.2 (5.8-30.7)	0.013
OS	16.2 (5.3-29.7)	25.0 (9.2-32.1)	0.003

DFS: disease-free survival; OS: overall survival; TS: thymidylate synthase; TP: thymidine phosphorylase.

$P = 0.010$; 16.6 mo *vs* 22.5 mo, $P = 0.009$), (Figure 2A-B). Tumors tended to be associated with a low TS/TP mRNA expression level and a prolonged patient survival.

Coupled expression of TS and TP and patients survival

The relationship between the expression levels of TS and TP was determined for the survival of patients (Table 2). As Figure 2C shows, the survival of 16 patients with high expression of TS and TP was significantly shorter than that of 17 patients with low expression of TS and TP (for OS, 16.2 mo *vs* 25.0 mo, $P = 0.003$; for DFS, 8.3 mo *vs* 16.2 mo, $P = 0.013$). Thus, the coupled low expression level of the two genes resulted in longer OS as well as DFS in gastric adenocarcinoma patients in this study.

Analysis of clinicopathological features and prognosis

No significant differences were observed in OS and clinicopathological features. However, there existed a significant negative correlation between OS and TS or TP expression. Histological type, lymphatic metastasis and TS gene expression were analyzed by multivariate regression analysis, showing that TS expression level could be identified as a potential predictive marker for OS with a 2.52 hazard ratio for high TS expression (95% confidence interval = 1.30-4.862, $P = 0.006$).

DISCUSSION

Since the response rate of gastrointestinal carcinoma

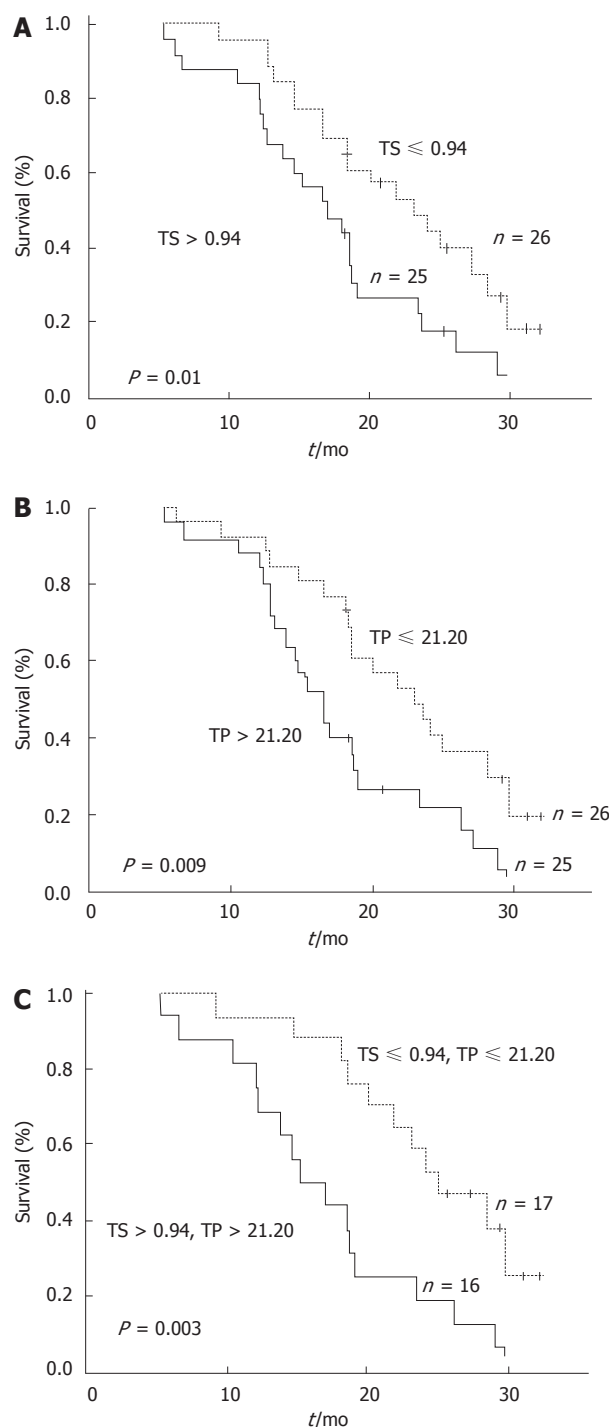


Figure 2 Kaplan-Meier survival curve for patients with intratumoral TS (A), TP (B), TS + TP mRNA levels above and below the median level of TS/TP (C). TS: thymidylate synthase; TP: thymidine phosphorylase.

to 5-FU as a single agent, is less than 30% and differs greatly among patients^[3], it is imperative to identify some indexes, which can be used to predict the efficacy of 5-FU in clinical settings. In the present study, we analyzed the mRNA levels of two genes in 51 patients with T3 or T4 gastric adenocarcinoma involved in 5-FU metabolism before 5-FU-based adjuvant chemotherapy to see if it is associated with survival. Our results showed that there was a significant correlation between TS/TP expression levels and survival of gastric cancer patients receiving postoperative FU-based adjuvant chemotherapy. The

identification of predicative markers for chemosensitivity by pharmacogenomics means could clarify which subset of patients might gain benefit, and enable clinicians to design individualized chemotherapy regimens^[11,12].

The primary biochemical mechanism responsible for cytotoxicity of 5-FU is the formation of 5-fluorouridine monophosphate (FdUMP), which can bind tightly to and inhibit thymidylate synthase in the presence of 5, 10-methylene tetrahydrofolate. TS catalyzes the reductive methylation of deoxyuridine-5'-monophosphate (dUMP) to deoxythymidine-5'-monophosphate (dTMP), which is the only pathway for de novo synthesis of dTMP, so inhibition of TS by FdUMP disrupts intracellular nucleotide pools necessary for DNA synthesis^[13,14]. As the main target of fluoropyrimidines, the expression level of TS is assumed to influence the efficacy of chemotherapy, although the amount of TS is not unanimously recognized as a determinant factor for 5-FU sensitivity^[7,15-18]. For example, Lenz *et al*^[7] showed that TS mRNA level influences response to 5-FU-based chemotherapy and survival of patients with primary gastric cancer. However, in two other studies^[16,17], TS mRNA levels did not reach statistically significant association with survival. In this work, patients with a lower TS expression level showed a trend to correlate with a longer DFS ($P = 0.017$) and a longer OS ($P = 0.010$) than those with a high TS expression.

TP is known to be higher in tumor tissue than in surrounding normal tissue^[19]. When 5-FU is administered, it is anabolized to FdUMP by TP in tumors^[20,21]. It was reported that the expression of TP can predict the efficacy of fluoropyrimidine chemotherapy and survival of patients^[21,22]. In the present study, although there was no significant difference in DFS in relation to TP expression, there existed significant differences in OS between the patients with high and low TP expression. Additionally, there would have an increased effect on prediction of survival in gastric cancer patients when TS and TP expressions were analyzed. These results indicate that measurement of TS and TP expression can identify a better survival prognosis of patients.

In summary, mRNA expression in TS and TP is a potential indicator in predicting the survival of patients with T3 or T4 gastric adenocarcinoma treated with 5-FU-based adjuvant chemotherapy. Further study is required to confirm our results and to establish the advantages of pre-treatment tumor biopsy for TS/TP screening, which permits a more rational decision on whether to precede a FU-based therapy.

COMMENTS

Background

5-fluorouracil (5-FU) is one of the most effective agents against gastrointestinal cancer. Its efficacy differs greatly among patients. As a pyrimidine analog, 5-FU exerts its antitumor effects through anabolism, which is determined by the rate of catabolism. Thus, the expression level of genes coding for key enzymes (such as TS and TP) in metabolism may play a pivotal role in the sensitivity and efficacy of 5-FU.

Research frontiers

As the key enzymes of fluoropyrimidines, the expression levels of TS and TP are assumed to influence the efficacy of chemotherapy, although TS or TP is not unanimously recognized as a determinant factor for 5-FU sensitivity. Some

studies showed that TS or TP mRNA level may serve as an important independent prognostic factor of survival in patients with solid tumor, including gastric cancer. However, there are no related reports about the relationship between TS/TP mRNA expressions and the clinical outcome of gastric cancer patients treated with 5-FU-based adjuvant chemotherapy.

Innovations and breakthroughs

The mRNA expression in TS and TP is a potential indicator in predicting the survival of patients with gastric adenocarcinoma. To our knowledge, it is the first report on the effect of TS/TP mRNA expression on the prognosis of Chinese patients with gastric cancer treated with 5-FU-based adjuvant chemotherapy.

Applications

Prospective controlled clinical trials are required to confirm our results and to establish the advantages of pre-treatment tumor biopsy for TS/TP screening, which permits a more rational decision on whether to precede a fluoropyrimidine-based therapy.

Terminology

5-fluorouracil (5-FU) is a widely used medicine in treatment of solid tumor. Thymidylate synthase (TS) is the target enzyme of 5-FU and catalyzes methylation of fluorodeoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), which is an important process of DNA biosynthesis. Thymidine phosphorylase (TP) is an enzyme involved in pyrimidine nucleoside metabolism.

Peer review

The authors investigated the level of thymidylate synthase (TS) and thymidine phosphorylase (TP) mRNA in T3 or T4 gastric cancer patients treated with 5-fluorouracil-based adjuvant chemotherapy, and found that they were associated with overall survival and disease-free survival of the patients. They concluded that mRNA level of TS and TP may be a useful predictive parameter for the survival of postoperative gastric cancer patients after 5-fluorouracil-based adjuvant chemotherapy.

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Laparoscopic hemicolectomy in a patient with situs inversus totalis

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Abstract

As among persons with normal anatomy, occasional patients with situs inversus develop malignant tumors. Recently, several laparoscopic operations have been reported in patients with situs inversus. We describe laparoscopic hemicolectomy with radical lymphadenectomy in such a patient. Careful consideration of the mirror-image anatomy permitted safe operation using techniques not otherwise differing from those in ordinary cases. Thus, curative laparoscopic surgery for colon cancer in the presence of situs inversus is feasible and safe.

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Key words: Situs inversus; Colon cancer; Laparoscopic surgery; Hemicolectomy; Radical lymphadenectomy

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INTRODUCTION

Situs inversus (SI) is an uncommon anatomic anomaly. Situs inversus totalis denotes complete right-left inversion of thoracic and abdominal viscera^[1-7]. Accordingly, surgical procedures are considered more difficult in

patients with SI in other patients because of different anatomic positions of organs, especially in laparoscopic surgery. While laparoscopic colorectal surgery recently has become a standard procedure, only two previous reports of laparoscopic surgery for colonic disease in patients with SI are available^[3,8]. Here, we display some figures of laparoscopic hemicolectomy with radical lymphadenectomy in a patient with SI, highlighting differences from the ordinary situation.

CASE REPORT

A 53-year-old woman known since early childhood to have situs inversus totalis was referred to her personal physician upon detection of anemia. She underwent ovariectomy for ovarian cancer at the age of 48 years. With a diagnosis of ascending colon cancer according to barium enema radiograph and colonoscopy, the patient was admitted to our hospital for further evaluation and surgical treatment. Laboratory examination confirmed anemia (red blood cell count, $377 \times 10^4/\text{mm}^3$; hemoglobin, 9.3 g/dL; hematocrit, 29.9%). The serum concentration of carcinoembryonic antigen was elevated (89.8 ng/mL; reference range, 0 to 4.9 ng/mL). A chest radiograph showed dextrocardia and a right subphrenic gastric bubble (Figure 1). Abdominal ultrasonography and computed tomography (Figure 2) showed complete transposition of abdominal viscera, confirming situs inversus totalis. Furthermore, the superior mesenteric vein was located to the left of the superior mesenteric artery. Barium enema and colonoscopy showed an ulcerated lesion in the ascending colon. Histologic examination of a specimen from colonoscopic biopsy indicated adenocarcinoma. According to the findings above, laparoscopic hemicolectomy with radical lymphadenectomy was performed. With the patient in a lithotomy position, the operator was situated between the legs, the first assistant on the left, and the endoscopist on the right with a reversal of assistants' location for orthotopic patients. Trocars also were placed in a mirror-image manner, including a 12-mm umbilical trocar for the camera, a 12-mm trocar in the right iliac fossa and a 5-mm trocar in the left iliac fossa as working ports for the operator, and a 5-mm trocar in the left flank and a 12-mm trocar in the epigastrium for traction. The liver was located on the left and the spleen on the right. The mesentery was incised just caudal to the ileocolic vessels, and the fusion fascia was mobilized searching the anterior

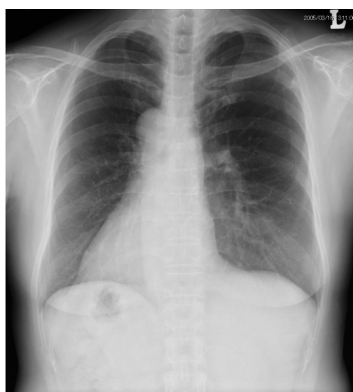


Figure 1 A chest radiograph showing dextrocardia and a right subphrenic gastric bubble.

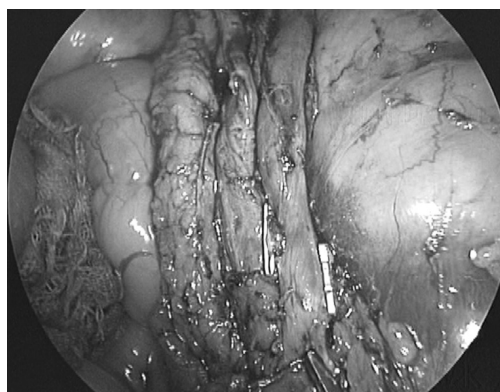


Figure 4 Radical lymphadenectomy along the superior mesenteric artery.



Figure 2 Computed tomography disclosing complete transposition of abdominal viscera.

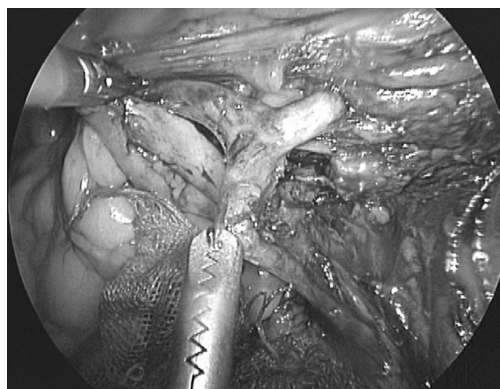


Figure 5 Division of the left branch of the middle colic artery.

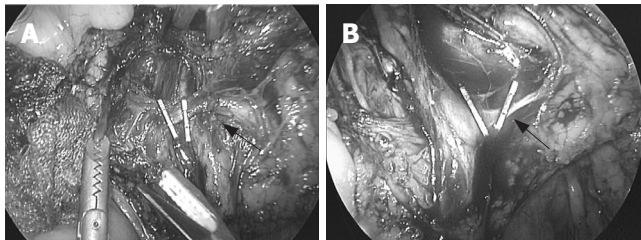


Figure 3 Initial laparoscopic vascular procedures identifying the ileocolic artery and dividing it at its root (arrow) (A) and ileocolic vein after exposure of the superior mesenteric vein (arrow) (B).

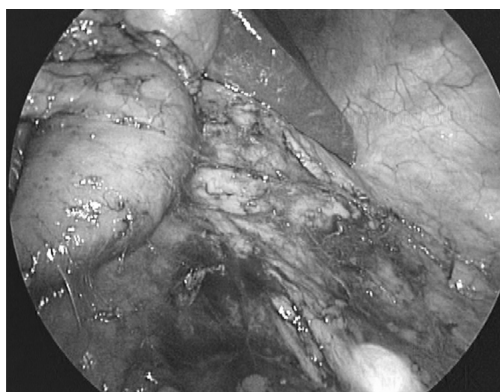


Figure 6 Full mobilization of the ascending colon including the tumor.

surface of the transverse portion of the duodenum. The ileocolic vessels were identified and divided after the superior mesenteric vein was exposed (Figure 3). Radical lymphadenectomy was continued up to the root of the middle colic artery (Figure 4), the left branch of the artery was divided (Figure 5). Mobilization of the ascending colon including the tumor (Figure 6) was followed by reconstruction using an end-to-end triangulating stapling method^[9] carried out extracorporeally through a 5-cm skin incision continued to the umbilical port. No additional ports were needed. Operation time was 191 min and blood loss was 60 mL, similar to typical findings in orthotopic patients. The procedure was not complicated apart from a need to appreciate mirror-image aspects of anatomy and surgery, including some awkwardness in handling surgical or endoscopic instruments in the presence of right-left inversion. Macroscopically, the tumor was a 45 mm × 25 mm ulcerated lesion in the ascending colon. Histologic

examination of the resected specimen disclosed a well-differentiated adenocarcinoma with serosal, lymphatic, and venous invasion, as well as lymph node metastasis. After an uneventful postoperative course, the patient was discharged on the 10th postoperative day.

DISCUSSION

Predisposition to situs inversus (SI), a rare congenital condition present in 1 of 5000 to 10 000 live births, is inherited in a simple autosomal recessive manner. SI may involve transposition of thoracic or abdominal organs,

or both. Situs inversus totalis denotes complete inversion of thoracic and abdominal viscera^[1-7]. Apart from genetic predisposition, no etiologies have been established, and SI itself has no pathophysiologic significance^[1,5]. However, surgical techniques need to allow for spatial implications of this condition. Cardiac and intestinal malformations as well as other visceral and vascular anomalies that can be associated with SI follow variable patterns^[1]. Careful preoperative anatomic assessment is vitally important in the presence of SI^[1,5]. Careful preoperative planning of laparoscopic procedures (positions of operator, assistants, and trocar sites as well as instrumentation) is needed, as mentioned in several previous reports of laparoscopic surgery in patients with SI^[2-4,6-8]. No specific intraoperative complications have been described in the reports above. Likewise, no unanticipated problems were encountered in our case. Patients with SI who develop malignant tumors in lung, liver, stomach, or colon have been encountered^[1,5]. In the present case, the patient was operated on by a surgeon with experience in over 400 laparoscopic colorectal operations. The position of the operator did not differ from that in orthotopic patients, but positions of assistants and trocars were reversed from the usual locations. Endoscopic instruments were used as they were ordinarily used, although mechanical awkwardness was noted in handling surgical or endoscopic instruments in the presence of reversed spatial relationship. Careful recognition of the mirror-image anatomy permitted safe radical lymphadenectomy and mobilization of the colon, while the surgical technique itself did not differ from the usual situation. Operative time and blood loss were comparable to those in orthotopic patients. Laparoscopic surgery for colon cancer in SI thus should be considered a

feasible, safe and curative procedure that surgeons should not hesitate to perform.

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Severe acute pancreatitis: Clinical course and management

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Abstract

Severe acute pancreatitis (SAP) develops in about 25% of patients with acute pancreatitis (AP). Severity of AP is linked to the presence of systemic organ dysfunctions and/or necrotizing pancreatitis pathomorphologically. Risk factors determining independently the outcome of SAP are early multi-organ failure, infection of necrosis and extended necrosis (> 50%). Up to one third of patients with necrotizing pancreatitis develop in the late course infection of necroses. Morbidity of SAP is biphasic, in the first week strongly related to early and persistence of organ or multi-organ dysfunction. Clinical sepsis caused by infected necrosis leading to multi-organ failure syndrome (MOFS) occurs in the later course after the first week. To predict sepsis, MOFS or deaths in the first 48-72 h, the highest predictive accuracy has been objectified for procalcitonin and IL-8; the Sepsis-Related Organ Failure Assessment (SOFA)-score predicts the outcome in the first 48 h, and provides a daily assessment of treatment response with a high positive predictive value. Contrast-enhanced CT provides the highest diagnostic accuracy for necrotizing pancreatitis when performed after the first week of disease. Patients who suffer early organ dysfunctions or at risk of developing a severe disease require early intensive care treatment. Early vigorous intravenous fluid replacement is of foremost importance. The goal is to decrease the hematocrit or restore normal cardiocirculatory functions. Antibiotic prophylaxis has not been shown as an effective preventive treatment. Early enteral feeding is based on a high level of evidence, resulting in a reduction of local and systemic infection. Patients suffering infected necrosis causing clinical sepsis, pancreatic abscess or surgical acute abdomen are candidates for early intervention. Hospital mortality of SAP after interventional or surgical debridement has decreased in high volume centers to below 20%.

INTRODUCTION

Dealing with the clinical course of acute pancreatitis (AP) and the management of severe acute pancreatitis (SAP) are complicated by limited understanding of pathogenesis and multi-causality of the disease, uncertainties to predict outcome and a few effective treatment modalities. AP comprises clinically a mild oedematous-interstitial inflammation, which is a self-limiting disease, and a severe type of AP with a local necrotizing inflammation and systemic complications. Despite the importance of recognizing severe disease early in the course, many patients initially identified as having mild disease, progress to severe pancreatitis over the initial period of disease. Clinical studies and experimental new data have led to considerable progress in understanding the pathophysiological events of the early period of human AP, but the underlying processes leading to acinus cell necroses and the propagation of the necrotizing inflammation by impaired microcirculation of pancreatic tissue compartments in the initial 48-72 h, are still unknown to a large extent. Hence, management of human AP has been empiric and conflicting opinions are still present regarding medical and surgical management concepts.

PATTERN OF INFLAMMATION

The tissue response of the pancreas to an injury like acinus cell necrosis leads to production and liberation of proinflammatory cytokines, chemokines and other biological active compounds^[1-4]. Clinical and experimental studies have verified activation of local macrophages and attraction of activated polymorphonuclear cells (PMNs) as first-line players in the defense and limitation of pancreatic tissue injury^[5-7]. Inflammatory mediators are primarily released from the splanchnic area and gain access to the systemic compartment mainly by lymphatic, portal vein and suprahepatic circulation^[1,8]. The lungs are the first

Table 1 Severe acute pancreatitis: Gut barrier dysfunction causes local changes and systemic complications

Local	Systemic consequences
Mucosal ischemia ^[10,15,16]	Priming of neutrophils ^[20-22]
Disruption of mucosal epithelial integrity ^[17]	Endotoxemia ^[14,23,24]
Reperfusion injury of mucosal epithelia ^[18]	Bacterial translocation ^[25-27]
Increase of intestinal permeability ^[19]	Cytokine overproduction ^[1,2,28]
Gram-negative intestinal bacterial overgrowth ^[11]	Impaired systemic immunity ^[29,30]
Impaired mucosal immunity ^[11]	

pass taker of the porto-hepatic blood and lymph of the splanchnic compartments enriched of activated PMNs, cytokines and other biological active compounds. Gut barrier failure, with the ensuing translocation of bacteria and endotoxin, has been proposed as a major contributor to the development of local infection and multi-organ failure in SAP^[9]. Evidence of the association between gut injury and the subsequent development of infected necroses and distant organ failure continues to increase. Intestinal permeability disturbances have been found in humans with SAP 72 h after onset, correlating strongly with clinical outcome^[10]: the increase of permeability was significantly higher in patients who developed multi-organ failure and/or died compared to patients suffering from mild attacks^[11]. Intestinal permeability increases gradually during the course of SAP reaching a maximum at the end of the first week^[12]. In a recent prospective study of patients with AP, endotoxemia as a consequence of increase of gut permeability was found on the day of admission to the hospital significantly more common and of greater magnitude in severe attacks than in those with mild attacks, in non-survivors than in survivors, and in patients who developed multi-organ failure than in those who did not^[13,14] (Table 1).

The peritoneal compartment is the site of pro-inflammatory reaction to pancreatic necrosis, whereas an anti-inflammatory response dominates in the lymph collected from the thoracic duct, as well as in the systemic circulation during the first week after onset of systemic complications^[1]. The cytokine levels in the blood and lymph are closely associated with the severity of illness on admission, the magnitude of multi-organ failure syndrome (MOFS) and the outcome as well^[1,2]. Correlated with local and systemic complications, a compartmentalization of the inflammatory responses has been objectified. Local inflammatory cytokines at high concentrations are found in the portal and splanchnic circulation at the same time when in the systemic blood compartment anti-inflammatory compounds prevail over inflammatory cytokines. In SAP, a compensatory anti-inflammatory response affects the immunocompetence^[30,31]. Patients with SAP show impaired immune response in regard to reduced HLA-DR expression of monocytes and macrophages^[29,32], reduced numbers of CD4- and CD8-positive T-cells^[33,34], an impairment of mononuclear phagocytic capacity^[35] and an increase of the anti-inflammatory cytokine IL-10 and IL-1 receptor antagonist^[36]. Reduced immune competence,

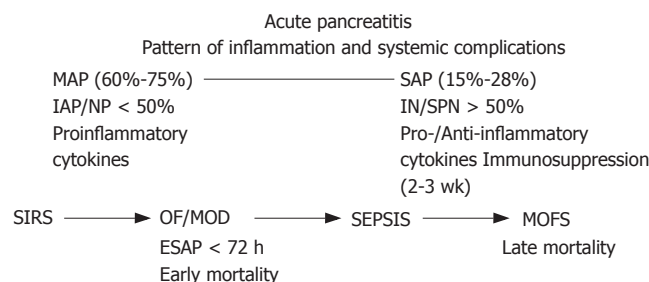


Figure 1 Pattern of inflammation and systemic complications in acute pancreatitis. MAP: Mild acute pancreatitis; SAP: Severe acute pancreatitis; IAP: Intestinal oedematous pancreatitis; NP: Necrotizing pancreatitis; IN: Infected necrosis; SPN: sterile pancreatic necrosis; SIRS: severe inflammatory response syndrome; OF: Organ failure; MOD: Multiorgan failure; SEPSIS: Leukocytes > 10 000/mm³ + fever > 38.5 rectal/> 48 h + metabolic acidosis base excess > -4 mmol/L; MOFS: Multiorgan failure syndrome; ESAP: Early severe acute pancreatitis.

as objectified by reduced expression of HLA-DR of monocytes and macrophages, predicts the development of organ failure and is associated with secondary infection^[29] (Figure 1).

CLASSIFICATION OF ACUTE PANCREATITIS

At the beginning of the 1980s, the morphologic feature of SAP was established by the definition of infected and sterile necrosis, pancreatic abscess and postacute pseudocysts as the principal morphologic and bacteriologic criteria for clinical severity and considered to be determinants of the clinical course^[37]. Macroscopically, necrotizing pancreatitis is characterized by focal or diffuse areas of devitalized parenchyma frequently associated with peripancreatic fatty tissue necroses extending sometimes to retroperitoneal spaces up to the pelvis. Intrapaneatic hemorrhage is variably present and may lead to an acute abdominal compartment syndrome. Infection of necrosis occurs in up to 30% of all patients with necrotizing pancreatitis^[27,38]. In the clinical setting of AP, post-acute pseudocysts and pancreatic abscess are late consequences of the disease^[39]. In both subgroups of AP, an inflammatory wall is developed which separates the inflammatory processes from the surrounding tissues. Both features have differences in clinical symptomatology and associated morbidity. Peripancreatic fluid collection that arises early during the course of AP is frequently a sign of severity. However, in most instances peripancreatic fluid collection disappears without any treatment^[40].

The Atlanta Classification is accepted worldwide as the first clinical reliable classification system of AP. But the accumulation of clinical data forces a revision of the Atlanta criteria of severity. Organ failure has been recognized as a more important determinant of survival than the extent of pancreatic necroses. Particularly early multi-organ failure at admission or in the first days predicts strongly the clinical course and the outcome. Severity of organ failure using multi-step criteria as introduced for septic patients by the SOFA-score is considered clinically relevant and increasingly applied for severity scoring and predicting outcome^[41]. The SOFA criteria for systemic

Table 2 Clinical course of AP/SAP

	Clinical	Pathophysiologic process
Early: d 1-10 after HA	Hypovolemia Abdominal pain	Fluid sequestration Liberation of pro- and anti-inflammatory cytokines
ESAP in about 20% of SAP	Dysfunction Pulmonary Renal Cardiocirculatory	Endotoxemia Liberation of vasoactive substances
	Liver Intestine	Disturbance of blood coagulation Translocation of endotoxin and bacteria
Late > 2 wk after HA	Local and systemic septic complications IN, SPN	Bacterial translocation CARS Anti-inflammatory reaction Immunosuppression

AP: Acute pancreatitis; SAP: Severe acute pancreatitis; ESAP: Early severe acute pancreatitis; HA: Hospital admission; IN: Infected necrosis; SPN: Sterile pancreatic necrosis; CARS: Compensatory antiinflammatory syndrome.

organ dysfunctions are clinically more reliable for decision making than the Atlanta criteria.

CLINICAL COURSE OF SAP

Acute pancreatitis is not a stable disease. Increasing amounts of intrapancreatic and retroperitoneal necroses are closely related to the frequency and severity of local and systemic complications^[27]. About 70%-80% of AP takes a mild course and is associated only with minimal organ dysfunctions. First clinical signs are abdominal pain located in the epigastrium, frequently radiating into the midback (Table 2). Clinical improvement can easily be achieved by fluid replacement, a pain treatment and re-institution of regular food intake. The initial 2-4 d after onset of symptoms are most important, when about 15%-25% of patients with AP take the course of a severe disease. Based on clinical and experimental data, this period is characterized by an initial hypovolaemic state^[42-44]. In SAP, hypotension or even shock occurs as a consequence of sequestration of protein-rich fluids into the pancreas, the retroperitoneal spaces and the abdominal cavity. The initial systemic inflammatory response syndrome causes a hyperinflammatory reaction exerting systemic organ dysfunctions of the lungs, kidneys, cardiocirculatory system and splanchnic intestinal compartments^[45,46]. Acute fluid collections arise early in the course of severe acute pancreatitis, lack a well-defined wall and are usually peripancreatic in location, and usually resolve without sequelae but may evolve into pancreatic pseudocysts or abscesses. Acute fluid collections rarely require drainage. About 60%-70% of fluid collection resolves spontaneously and has no connection with the pancreatic duct system^[47].

EARLY SEVERE ACUTE PANCREATITIS

About 20% of patients with SAP develop in the 72 h after onset of the disease organ failure or even have organ- or multi-organ failure at admission to hospitals^[48]. Despite application of maximum intensive care treatment,

Table 3 Severe acute pancreatitis-early organ failure

Admission	Dynamic of organ failure	Hospital mortality
ESAP (n = 47)	SOF 25 (53%) MOF 22 (47%)	Reversible 9 develop MOF 14 (30%) Reversible 1 progress to MOFS 21 (95%)
SAP (n = 111)	OF (-) 30 (27%) SOF 26 (23%) MOF 55 (50%)	14%

ESAP: Early severe acute pancreatitis; SOF: Single organ failure; MOF: Multiorgan failure; SAP: Severe acute pancreatitis; OF: Organ failure. Reversibility or assistance in spite of maximum intensive care treatment of early organ failure or early organ failure syndrome (n = 158)^[48].

30%-50% of the patients with early severe acute pancreatitis do not promptly respond to ICU treatment and take a complicated course with persistence of multi-system organ dysfunctions. Patients suffering from early and persistent multi-organ insufficiency syndrome have a high risk of mortality^[49]. Recently it has been shown that severe organ failure within the first week after onset of AP before any kind of intervention is closely linked to clinically relevant pancreatic infection which occurs two weeks later^[50]. Early multi-organ dysfunction syndrome (MODS) obviously triggers additional mechanisms that render bacterial translocation into clinically manifested sepsis. Early onset MODS > 2 organs has proved to be the predominant risk factor for death. Early mortality in the first 6-10 d of SAP is caused by severe inflammatory response syndrome (SIRS) associated with early multi-organ insufficiency syndrome (Table 3). Early mortality was reported between 42% and 60%^[51-53].

Presence of necrosis and infection

Gross destruction of the pancreatic gland by tissue necroses is observed in about 20% of patients with AP and takes place in the first week after onset^[54]. Experimental and clinical observations reveal that development of pancreatic necrosis is accompanied by an increase of local and systemic organ complications, increasing the risk of morbidity and mortality compared to patients with interstitial-oedematous pancreatitis^[55,56] (Figure 2). Most patients who develop early or late organ failure suffer from necrotizing pancreatitis. Autopsy data and surgical results have verified that more than 80% of deaths are correlated with the presence of necroses. The highest risk for local and systemic complications is seen in patients who show extended necrosis of more than $\geq 50\%$ of the pancreas by magnetic resonance tomography (MRT) or contrast-enhanced computer tomography (CECT)^[57,58]. Patients with sterile extended pancreatic necroses (> 50%) display clinically signs of sepsis including organ dysfunctions, septic fever, leucocytosis, hyperdynamic cardiocirculatory state and intestinal motility disorder. To discriminate clinical infection from sterile necrosis, the use of sepsis criteria is not reliable in patients with extended sterile necrosis.

In addition to the presence of pancreatic parenchymal necrosis, the occurrence and extent of the necrotizing process into extrapancreatic retroperitoneal fatty tissue

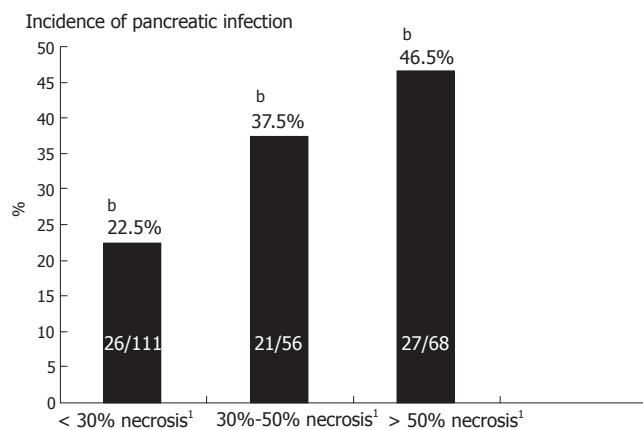


Figure 2 Relation between bacterial infection and extent of necrosis in 225 patients with severe acute pancreatitis. ¹On the basis of contrast-enhanced CT. ² $P = 0.008$ between the groups, Cochran-Armitage trend test.

spaces including tissue compartments of the mesentery of the small and large bowel, the peri-renal fat and the para- and retrocolic compartments, are important factors influencing the course of the disease and strongly affect the clinical severity^[59]. The overall infection rate of pancreatic tissue in necrotizing pancreatitis is up to 30% and may increase to 70% in the 3rd wk^[27] (Table 4).

The setting of pancreatic infection includes infected necrosis, pancreatic abscess and infected pancreatic pseudocysts. The bacteriological analysis of puncture aspirates or of intraoperative smears reveals predominantly gram-negative microbes deriving from the intestine. *Escherichia coli* was the most frequent pathogen followed by *Enterococcus* and *Klebsiella*^[27]. However, in recent years a shift of the bacterial pattern has been observed towards more gram-positive bacteria like *Staph aureus* and *Enterobacteriaceae*^[60,61]. The presence of candida species in infected necroses has been observed in 5%-15%^[62]. Candida patients have a higher mortality and experience more systemic complications than patients without candida infections of necroses. Recent data about the routine use of prophylactic antibiotics provided evidence that application of antibiotics contributes to the development of candida infection and to changes in bacterial spectrum of infected necroses with an increased incidence of gram-positive infections^[62,63].

CT-guided fine needle aspiration (FNA) of the necrotic area is a safe procedure to diagnose infection, identify bacteria and institute appropriate therapy. To distinguish pancreatic inflammation from secondary infection, gram staining and culture must be performed after guided aspiration^[64]. The knowledge of the bacteria and candida species and the pattern of chemo-resistance may lead to a rational antibiotic treatment.

MANAGEMENT OF SAP: PREDICTION OF SEVERITY AND OUTCOME

The management of patients with AP is challenging due to late hospitalization after onset of the acute attack and difficulty in distinguishing mild from severe disease in the first 48-72 h. Identification of risk factors for the

Table 4 Frequency of pancreatic infection in 427 patients¹ with necrotizing pancreatitis²

		NP (%)	AP (%)
Infected necrosis	99	23.2	6.9
Pancreatic abscess	40	9.4	2.8
Infected pseudocyst after AP	7	1.6	0.5
Total	146	34.2	10.1

¹Pancreatic necrosis/extrapancreatic fatty tissue necrosis, pancreatic abscess, postacute pseudocyst. ²5/1982-12/1996 Department of General Surgery, University of Ulm.

development of necrotizing pancreatitis within the initial 24 h of hospitalization is of potential clinical importance. Patients who display at admission organ dysfunctions or an Apache II score ≥ 8 ^[65] or C-reactive protein (CRP) > 120 mg/dL^[66] or procalcitonin > 1.8 ng/mL^[67] or a hematocrit > 44 ^[44] should have early intensive care for optimal surveillance and ICU treatment. The use of early CECT or MRI for determination of severity is limited by several factors: (a) only a quarter of patients with acute pancreatitis develop necroses; (b) pancreatic necroses may not develop in the first 48 h; and (c) the presence of pancreatic necroses and the amount of necroses does not strongly correlate with the development of organ failure (Figure 3)^[68]. The CECT based Balthazar classification shows the highest diagnostic and predictive accuracy when performed after the first week of disease. The APACHE II scoring and the sequential Sepsis-Related Organ Failure Assessment (SOFA) have a highly reliable sensitivity and specificity and positive predictive value for the degree of severity of SAP. APACHE II-, Marshall- and SOFA score can objectify the responses of the patients to intensive care measures (Table 5) on a daily basis. The biochemical parameters, CRP, procalcitonin and IL-8 have a high predictive accuracy for the degree of severity of necrotising pancreatitis in the first days. Procalcitonin > 1.4 ng/mL has a diagnostic accuracy of 70% for infection of necrosis; and procalcitonin of > 3.8 ng/mL predicts MODS with a diagnostic accuracy of 92%^[71] (Table 6).

First line treatment of SAP

Admission hematocrit of > 47 and a failure of admission hematocrit to decrease at 24 h has been identified as reliable criteria of hemoconcentration of SAP in the very early period of the disease^[42-44]. Vigorous intravenous fluid resuscitation is required to overcome systemic hypovolemia caused by intravascular fluid loss^[72,73]. Intravenous fluid substitution for patients with predicted SAP should be established with 250-350 mL/h for the first 48 h^[42]. Restoration of normal cardiocirculatory functions objectified by heart-rate, systolic or mean arterial blood pressure, an oxygen saturation of venous blood of $> 95\%$, absence of a base deficit > 5 μ mol/L and urine flow of ≥ 50 mL/h are decisive criteria of treatment response.

In mild biliary acute pancreatitis, endoscopic retrograde cholangiopancreatography (ERCP) and removal of common bile duct stones do not change the natural course

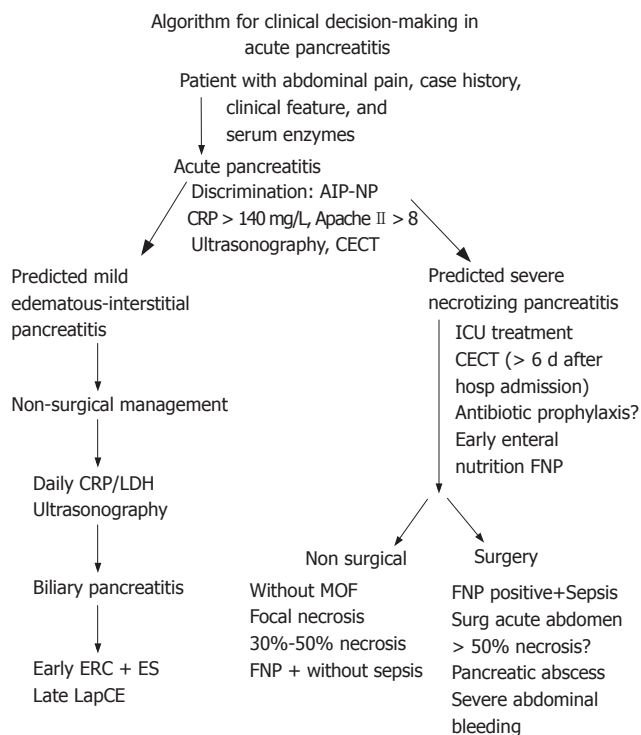


Figure 3 Algorithm for clinical decision making in acute pancreatitis. AIP: Acute interstitial pancreatitis; NP: Necrotizing pancreatitis; CRP: C reactive protein; CECT: Contrast-enhanced computer tomography; LDH: Lactate dehydrogenase; ERC: Endoscopic retrograde cholangiography; ES: Endoscopic sphincterotomy; CE: Laparoscopic cholecystectomy; FNP: Fine needle procedure; MOF: Multiorgan failure syndrome.

Table 5 Severe acute pancreatitis: Clinical systems to predict prognosis

	Cut-off	Time	Reference
Ranson	> 2 points	> 48 h	SGO 1974 ^[71]
Apache II	> 9 points	daily	Br J Surg 1990 ^[65]
Balthazar	C, D, E	> first week	Radiology 1990 ^[69]
Marshall score	> 3 points	72 h	Crit Car Med 1995 ^[45]
MOF/Goris	> 1 point	48 h	Arch Surg 1985 ^[46]
SOFA	> 4 points	48 h/d	Crit Car Med 1996 ^[41]

MOF: Multiorgan failure; SOFA: Sepsis-related organ failure assessment.

of pancreatitis. ERCP, endoscopic sphincterotomy and stone removal are applied after subsidence of clinical signs of AP. In severe biliary pancreatitis, **early sphincterotomy** and stone extraction are **beneficial when common bile duct stone has been diagnosed to be associated with SAP**. Early endoscopic extraction of common bile duct stones brings about disappearance of cholestasis and decompression of the pancreatic main duct. A significant reduction of biliary and systemic morbidity has been objectified by two **randomized controlled trials (RCTs)**^[74-76]. However, ERCP and sphincterotomy in SAP increases the risk of an additional pancreatic trauma in up to 10% of patients and may increase the risk of additional cholangitis episodes during the course of SAP.

Antibiotic prophylaxis turned out to be not very effective in regard to avoidance or reduction of infection of necrosis and associated systemic complications^[77]. Two randomized

Table 6 Early prediction of infected necrosis, infected necrosis + MODS and death using biochemical parameters

	Cut-off	Sensitivity (%)	Specificity (%)	Accuracy (%)
Prediction of infected necrosis				
PCT	≥ 1.4 ng/mL	75	68	69 ^a
CRP	≥ 400 mg/L	29	92	76
Prediction of infected necrosis and MODS				
PCT	≥ 3.8 ng/mL	80	93	92 ^a
CRP	≥ 410 mg/L	35	93	87
Prediction of death				
PCT	≥ 3.8 ng/mL	82	88	88 ^a
Prediction of IN and MODS or death				
PCT	≥ 3.8 ng/mL	76	94	92 ^a
CRP	≥ 400 mg/L	35	92	84

PCT: Randomized controlled trial; CRP: C reactive protein; MODS: Multiorgan dysfunction syndrome; IN: Infected necrosis. Receiver operating curve-analysis based on d 3 and 4 onset of symptoms, ^a*P* < 0.05-0.004, Rau, Annals of Surgery 2007^[72].

Table 7 Severe acute pancreatitis-antibiotic prophylaxis is inefficient in severe acute pancreatitis; results of two randomized controlled double-blind multicentric trials

	Isenmann ^[79]		Dellinger ^[80]	
	2004	<i>P</i> value	2005	<i>P</i> value
Patients (<i>n</i>)	114		100	
Treatment (<i>n</i>) ¹	48		40	
Placebo (<i>n</i>)	41		40	
Infection of necrosis				
Treatment	12% ¹	NS	23% ²	NS
Placebo	14% ¹		15% ²	
Need for surgery				
Treatment	17% ¹	NS	23% ²	NS
Placebo	11% ¹		24% ²	
Hospital mortality				
Treatment	12% ¹	NS	20% ²	NS
Placebo	9% ¹		18% ²	

¹Statistical comparison of treatment and placebo group data 2004; ²Data 2005.

double blinded prospective controlled multi-center trials proved antibiotic prophylaxis ineffective in regard to reduction of infection of necrosis and hospital mortality^[78,79] (Table 7). But patients with **pulmonary infection** and who show a positive blood culture associated with signs of sepsis should be **treated with antibiotics**. **Enteral feeding (EN)** in SAP reduces significantly the infection rate of necrosis and lowers the need for surgical interventions^[80-84]. However, hospital mortality and non-infectious complications are not altered by enteral feeding compared to parenteral nutrition (Table 8). The beneficial effect of EN may be more pronounced if it is **instituted early**^[85].

Non-surgical ICU-management is successful in most patients with AP who have **sterile pancreatic necroses** and who do **not develop organ failure** (Table 9). Patients having pancreatic necroses and who are fine needle procedure (FNP)-positive but do **not show clinical signs of sepsis**, do not need surgical intervention^[86-88].

Interventional treatment of infected necrosis

Surgical debridement has been documented to be

Table 8 Severe acute pancreatitis-enteral feeding reduces infection in the need for surgical intervention

Benefits of enteral nutrition	Lower infections ($P = 0.004$) Reduced surgical interventions ($P = 0.05$) Reduced LHS-2.9 d ($P < 0.001$)
Differences	Hospital mortality ($P = 0.3$) Non-infectious complications ($P = 0.16$)

LHS: Length of hospital stay. Enteral feeding *vs* parenteral nutrition, results of 6 RCTs, meta-analysis of 263 patients^[81-86].

Table 9 Severe acute pancreatitis-surgical and non-surgical treatment: Ulm Experience: 1568 patients¹ n (%)

	Patients	Conservative	Surgery/Intervention
Interstitial-oedematous	1071 (68.3)	1056 (98.6)	15 (1.4) ²
Necrotizing pancreatitis	359 (22.9)	95 (26.5)	264 (73.5)
Sterile necrosis	227	85 (37.5)	142 (62.5)
Infected necrosis	132	10 (7.6)	122 (92.4)
Pancreatic abscess	42 (2.7)	3 (7.1)	39 (92.9)
Postacute pseudocyst	96 (6.1)	22 (22.9)	74 (77.1)

¹5/1982-12/1999 Department of General Surgery, University of Ulm, Germany. ²Biliary tract surgery not included.

effective for patients with proven infected necrosis and progressive clinical sepsis. Patients with SAP who develop a surgical acute abdomen during the course of ICU treatment need emergency surgery to avoid development of abdominal compartment syndrome^[57] or consequences of intestinal perforations. Patients with extended sterile necrosis (> 50% of the pancreas) are at high risk for infected necrosis with the consequence of progressive MODS. These patients are candidates for surgical and interventional measures after their clinical signs show non-response to maximum intensive care treatment. Patients with infected necrosis are managed by surgical and interventional treatment modalities (Table 10).

A variety of surgical treatment modalities are currently in use. The advantages of minimally invasive interventional debridement, whether performed by laparoscopic techniques or by a retroperitoneal approach, are up to now not based on results of controlled clinical trials. By use of minimal invasive techniques for infected necrosis in the late course of disease, the morbidity remains high for several days. Two to 7 reoperations with lavage are necessary to interrupt systemic complications of the local inflammatory process^[95-102] (Table 11). An open surgical debridement combined with continuous short-term lavage of the lesser sack interrupts clinical sepsis in patients suffering from extended necrosis with infection accompanied by multi-system organ failure. The early treatment related morbidity is much lower in patients treated with open surgery than after first pass of minimal invasive debridement. The frequency of reoperation is between 25% and 40% and up to 100% in patients after minimal access intervention. Hospital mortality in high volume centers is below 20% after open necrosectomy plus bursa lavage and after minimal invasive surgical approach as well.

Table 10 Results of open surgical debridement of necrotizing pancreatitis using surgical debridement and local bursa lavage

	Complication			Hospital mortality
	n	Postop, n (%)	Reop, n (%)	n (%)
Pederzoli 1990 ^[90]	191	55 (29)	34 (18)	40 (21)
Beger 1999 ^[92]	221	122 (55)	93 (42)	46 (21)
Mai 2000 ^[61]	27	10 (37)	6 (22)	5 (18)
Hungness 2002 ^[93]	26		4 (15.4)	6 (23)
Farkas 2006 ^[94]	220	43%	48 (22)	17 (7.7)
Howard 2007 ^[95]	102	83 (81)	69 (68)	12 (11.8)
1990-2007	787	43%	29.60%	14.70%

Table 11 Results of minimal invasive interventional treatment of necrotizing pancreatitis: Minimal invasive debridement + local lavage

	n	Infect. necrosis (%)	Apache II	Time O-S	Early morbidity (%)	OP/ pts	Hospital mortality (%)
Freeny 1998 ^[96]	34	100	-				20
Goizu 1999 ^[97]	32	81	26				15
Carter 2000 ^[98]	10	90		24 d	10	3	20
Horvath 2001 ^[99]	6	100			33		0
Castellanos 2002 ^[100]	15	100			40		27
Connor 2003 ^[101]	24	58	8		88	4	25
Zhou 2003 ^[102]	12	58		72/102			0
Connor 2005 ^[103]	47	81	9	28 d	92	3	19
1998-2006	156 pts	83			70.6		18.3

OP: Operation per patient; O: Onset of disease; S: Surgery.

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REVIEW

Surgical management of polycystic liver disease

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Abstract

Adult polycystic liver disease (PCLD) is an autosomal dominant condition commonly associated with autosomal dominant polycystic kidney disease (ADPKD). However in the last decade, it has been recognized that there is a distinct form of autosomal dominant PCLD that arises without concomitant ADPKD. Early knowledge of the pathogenesis was gained from the study of hepatic cysts in patients with ADPKD. Bile duct overgrowth after embryogenesis results in cystic hepatic dilatations that are known as biliary microhamartomas or von Meyenburg complexes. Further dilatation arises from cellular proliferation and fluid secretion into these cysts. There is a variable, broad spectrum of manifestations of PCLD. Although PCLD is most often asymptomatic, massive hepatomegaly can lead to disabling symptoms of abdominal pain, early satiety, persistent nausea, dyspnea, ascites, biliary obstruction, and lower body edema. Complications of PCLD include cyst rupture and cyst infection. Also, there are associated medical problems, especially intracranial aneurysms and valvular heart disease, which clinicians need to be aware of and evaluate in patients with PCLD. In asymptomatic patients, no treatment is indicated for PCLD. In the symptomatic patient, surgical therapy is the mainstay of treatment tailored to the extent of disease for each patient. Management options include cyst aspiration and sclerosis, open or laparoscopic fenestration, liver resection with fenestration, and liver transplantation. The surgical literature discussing treatment of PCLD, including techniques, outcomes, and complication rates, are summarized in this review.

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Key words: Polycystic liver disease; Fenestration; Laparoscopy; Liver resection; Liver transplantation

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INTRODUCTION

Adult polycystic liver disease (PCLD) was first described in 1856 by Bristowe in association with autosomal dominant polycystic kidney disease (ADPKD)^[1]. PCLD is a rare (incidence < 0.01%) dominantly inherited disorder characterized by multiple diffuse cystic lesions of the liver parenchyma. An asymptomatic enlarged liver is usually the hallmark of the disease. However with more effective treatment of renal disease, increasing numbers of patients are living long enough to experience symptoms from their associated polycystic liver disease. Significant symptoms or complications from liver involvement can occur in up to 20 percent of cases^[2,3]. In symptomatic PCLD patients, surgical therapy is the mainstay of therapy including laparoscopic or open fenestration with or without hepatic resection and orthotopic liver transplantation. The surgical therapy should be tailored to the extent of disease in each patient. In this review, we will summarize the literature addressing the clinical presentation, associated medical problems, and appropriate surgical management of patients with adult polycystic liver disease.

PATHOGENESIS AND GENETIC BASIS OF POLYCYSTIC LIVER DISEASE

Although there is an isolated form of polycystic liver disease, knowledge concerning the pathogenesis of hepatic cysts was gained from the study of hepatic cysts in ADPKD. These lesions have been attributed to bile duct overgrowth after the arrest of embryogenesis and failure of the intralobar bile ducts to involute. This involutional failure results in cystic dilations that are known as biliary microhamartomas or von Meyenburg complexes (VMC)^[4]. Further study of these VMC confirmed that they maintain communication with the biliary tree^[5,6]. The growth of cysts in the liver is thought to arise from cell proliferation, solute and fluid secretion into the cysts, and expansion of abnormal cell matrices. Perrone and colleagues demonstrated, via culture derived epithelial cell lines, that these cysts are of biliary origin^[7]. Morphological studies demonstrate that the peripheral cysts arise from biliary microhamartomas, but the centrally located cysts arise from dilatation of the peribiliary glands in the liver^[8].

ADPKD is one of the most commonly inherited diseases with an incidence of 1 in 400 to 1 in 1000. It is



Figure 1 A: Type I PCLD; B: Type II PCLD; C: Type III PCLD.

a cause of 8%-10% of all chronic end-stage renal failure requiring dialysis^[9]. The number of ADPKD patients with hepatic involvement appears to be rising, likely due to increased life expectancy from improved renal replacement therapy and renal transplantation. Early literature suggested that 40%-50% of patients with ADPKD had polycystic liver disease^[10,11], but in more recent literature, this figure has increased to 75%-90%^[12]. Independent risk factors for hepatic involvement in the ADPKD include advancing patient age, female gender, and severity of renal disease. The increased prevalence in females may be due to stimulatory effects of estrogen. The reported prevalence of hepatic cysts in female patients with ADPKD ranges from 58% to 75% while the prevalence in male patients ranged from 42% to 62%^[13]. Further support for the stimulatory effect of estrogen comes from studies showing an increase in liver cyst volume in pregnant women and women receiving postmenopausal estrogen therapy^[14]. Finally, a correlation has been established between an increasing burden of hepatic cysts in patients as the severity of renal cystic disease increases^[13].

The first suggestions of an isolated form of polycystic liver disease were made in the mid-1980's^[15,16] and confirmation that there was a distinct autosomal dominant polycystic liver disease (ADPLD) occurred in the late 1990's^[17,18]. ADPLD, much rarer than its PKD counterpart, has a reported incidence of less than 0.01%. ADPLD is linked to a mutation on chromosome 19 that leads to a mutated protein hepatocystin which may play a role in abnormal biliary cell proliferation and differentiation^[19,20].

CLINICAL PRESENTATION

In 80% of patients, PCLD is asymptomatic^[2,3] and often diagnosed incidentally during work-up of other clinical problems. In patients with long-standing disease, the most frequent symptoms are abdominal pain, early satiety, nausea and vomiting, supine shortness of breath, lower body edema, biliary obstruction, and ascites. These symptoms are usually indicative of significant hepatic enlargement or compression of adjacent structures, but rarely can be from infection or malignant transformation^[21,22]. Laboratory studies including bilirubin, hepatic transaminases (AST and ALT), alkaline phosphatase, and gamma glutamyl

transferase (GGT) are usually normal in asymptomatic patients. In symptomatic patients undergoing evaluation, alkaline phosphatase and GGT may be elevated in up to 47% and 70% of patients respectively^[21,23-25]. AST may be elevated in up to 27% of patients and bilirubin levels may be elevated in up to 15%^[21,23].

CLASSIFICATION OF APLD

Gigot and coauthors have described a detailed classification scheme for patients with polycystic liver disease based on pre-operative computed tomography (CT). This description is based on the number and size of cysts as well as the amount of residual normal liver parenchyma between the cysts^[25]. Type I patients have a limited number (< 10) of large cysts with large areas of non-cystic parenchyma (Figure 1A). Patients with Type II PCLD have diffuse involvement of liver parenchyma by medium sized cysts with remaining large areas of non-cystic parenchyma (Figure 1B). Finally, Type III patients are characterized by massive, diffuse involvement of liver parenchyma by small and medium sized liver cysts and only a few areas of normal liver parenchyma between cysts (Figure 1C). This classification system offers a good platform for comparison of morphological disease between patients and their classification can aid us in formulating appropriate plans for therapy.

HEPATIC COMPLICATIONS OF POLYCYSTIC LIVER DISEASE

Hepatic complications from polycystic liver disease (PCLD) typically occur only in the setting of significant hepatomegaly. These cases usually present with a palpable abdominal mass, significant abdominal pain, early satiety, or dyspnea. Occasionally, severe abdominal pain will result from rupture of a hepatic cyst, hemorrhage into a cyst or if a cyst becomes infected^[2,26]. Despite longstanding polycystic liver involvement, only rarely does this entity lead to hepatic insufficiency or failure. The displaced hepatic parenchyma still functions quite well.

An infected hepatic cyst is a serious, but rare, complication. These patients will usually present with fever, leukocytosis, and right upper quadrant pain. This

acute presentation should be suspected in this population and treated early to prevent progression to bacteremia, sepsis, and death. The morbidity and mortality rates of a cyst infection have been reported at 3% and 2% respectively^[27]. Thus there should be early institution of antibiotics and a drainage procedure. Telenti and coauthors reviewed outcomes of patient's with infected hepatic cysts and strongly supported the use of antibiotics and a drainage procedure^[28]. In their review of 14 patients with infectious complications involving hepatic cysts, seven of the fourteen patients received antibiotics alone. Of these patients, two died without having a drainage procedure, four patients underwent drainage after their symptoms persisted through antibiotic therapy, and one patient was not offered drainage due to the difficult location of the infected cyst. In the seven patients treated with combined drainage and antibiotics, one patient died from post-operative sepsis and one died from unrelated causes, while five patients had no reported morbidity from their procedures. While this literature favors antibiotic therapy along with drainage, it is often difficult to determine which cyst(s) is/are actually infected. Imaging studies can often direct us toward an area of suspicion. On CT scanning, the finding of fluid-fluid levels within cysts, cystic wall thickening, or intracystic bubbles have been associated with infection of a hepatic cyst^[28,29]. Ultimately drainage of the suspected cyst provides the definitive answer whether this is from the open approach or percutaneously. In those cases where the cyst is too small or cannot be accurately identified on preoperative imaging, broad spectrum antibiotics should be instituted with narrowing of the spectrum if blood cultures implicate a specific organism^[28].

Rarely, jaundice occurs as a result of cystic compression of the biliary system. This manifests as a result of the normal progression of cyst growth and compression of the common hepatic duct or common bile duct^[30]. Relief of this obstruction is accomplished by surgical decompression or resection of the offending cysts.

ASSOCIATED MEDICAL CONDITIONS

Intracranial aneurysms and their association with ADPKD have been described^[9,31]. There also may be increased risk for intracranial aneurysm, rupture, or dissection with ADPLD. Geevargheese and colleagues estimated the prevalence of intracranial aneurysms within this population to be 10%. Because cerebral aneurysms can be a source of morbidity and mortality in these patients, they recommended screening by magnetic resonance angiography (MRA) or computed tomographic angiography (CTA) in all patients with PCLD^[32]. Also, Qian and coauthors reported an association between ADPLD and increased risk for intracranial aneurysms. In their cohort, six percent of patients with genetically confirmed ADPLD were found to have either intracranial aneurysm or dissection^[33]. Although there are no definitive recommendations, these reports should encourage screening radiography and treatment prior to any surgical intervention for their PCLD.

Other associated medical conditions with ADPKD and ADPLD that clinicians should be aware of are valvular

heart disease and pancreatic cysts. Classically, the valvular disease most frequently described has been mitral valve prolapse and mitral valve incompetence. In their clinical profile of ADPLD patients, Qian *et al* estimated that mitral valve prolapse occurred in up to 26% of ADPLD patients and mitral valve incompetence in up to 31% of this population^[33]. This indicates cardiac evaluation in patients with ADPLD. Finally, patients with ADPKD may have asymptomatic cysts within multiple organs, including the pancreas, spleen, ovaries, and lungs. Pancreatic cysts are the most common of the extrarenal cysts with a reported incidence of 9% among ADPKD patients over 30 years^[34-36].

THERAPEUTIC OPTIONS FOR APLD

The primary aims of surgical therapy for polycystic liver disease should be to significantly reduce the size of the polycystic liver without compromising liver function, and to provide long-term relief of symptoms. There is no clear consensus regarding the optimum timing of intervention and the surgical approach is based in part on the number, size, and location of the cysts. All patients should be carefully evaluated for significant symptoms and degree of disability, as well as the degree of hepatic and renal dysfunction that could affect morbidity and mortality. In addition, patients should be made aware of the risk and limitations of the surgery prior to proceeding with any surgical management.

In high risk patients and those with a large dominant cyst, percutaneous aspiration and sclerosis of cysts has been proposed as a feasible option but is associated with higher recurrence rates^[37-39]. Surgical options include: laparoscopic fenestration and/or resection, open fenestration and/or resection, and liver transplantation.

FENESTRATION

Prior to the advent of laparoscopic techniques, open fenestration was the standard therapy for patients with symptomatic PCLD. The technique of fenestration was first described by Lin and coauthors in 1968^[40]. This technique involves de-roofing and performing the widest possible excision of the cyst wall back to the interface of the liver parenchyma. This approach allows visualization, fenestration, and drainage of superficial and deeply seated cysts within the hepatic parenchyma and internal drainage within the peritoneal cavity. The site of fenestration must be carefully selected to avoid any bleeding or leakage of bile. Destruction of the fluid producing epithelial cyst lining, with cautery or Argon beam coagulation, may be helpful to reduce continual fluid loss from the fenestrated cysts. Patients, having type I PCLD, with superficial and large cysts of limited number are the best candidates for this procedure.

With the introduction of laparoscopy, there are increasing numbers of reports of laparoscopic fenestration of patients with PCLD^[24,25,41-46]. It can be performed with similar morbidity and mortality as the open fenestration, but this approach must be utilized in the appropriate population. Patients with majority of their cysts in segments VI, VII, and often VIII (when there is marked

Table 1 Open and Laparoscopic fenestration for polycystic liver disease

Reference	No. of patients	Technique (n)	Mortality (%)	Morbidity (%)	Mean follow-up (mo)	Rate of symptom recurrence (%)	Re-operation (%)
Lin ^[40]	3	Open	0	0	32	0	0
Van Erpecum ^[21]	9	Open	1 (11)	0	48	0	0
Turnage ^[47]	5	Open	1 (20)	1 (20)	10	40	0
Sanchez ^[67]	7	Open	0	NR	18	57	0
Farges ^[68]	13	Open	0	9 (69)	84	23	0
Gigot ^[25]	10	Open (9)	0	6 (60)	73	11	11
		Lap (1)	0	0		0	0
Koperna ^[41]	39	Open (34)	0	NR	75	21	21
		Lap (5)	0	NR		0	0
Morino ^[42]	9	Lap (9)	0	4 (44)	NR	40	NR
		Conv. (2)					
Kabbej ^[24]	13	Lap (13)	0	7 (54)	26	72	23
Martin ^[43]	13	Open (6)	0	2 (33)	96	20	20
		Lap (7)	0	2 (29)	37	71	71
Katkhoua ^[44]	9	Lap (9)	0	3 (33)	30	11	11
		Conv. (1)					
Fiamingo ^[45]	6	Lap	0	3 (50)	1-67 ¹	25	0
Marks ^[46]	6	Lap	0	4 (67)	2-72 ¹	14	0

Lap: Laparoscopic; Conv: Converted from Laparoscopic to Open; NR: Not reported. ¹Follow-up range in months.

hepatomegaly) and patients with deeply seated cysts that are difficult to visualize and fenestrate with laparoscopy may be better candidates for open fenestration. From the published series, these patients have a higher recurrence rate after laparoscopic fenestration due to the inability to adequately fenestrate all of their cysts^[42,43,46]. The 13 published series describing open and/or laparoscopic fenestration are summarized in Table 1.

Koperna and colleagues reported the largest series of patients who underwent open or laparoscopic fenestration for PCLD. In their series, thirty-nine out of forty-four patients underwent a fenestration (34 open and 5 laparoscopic) for their symptomatic polycystic liver disease, while the other four underwent hepatic resection. In their experience, those patients with multiple cysts of 5 cm or greater had a higher likelihood of recurrence as compared with patients having fewer and smaller cysts (27% *vs* 13%). They performed both techniques of fenestration with no mortalities and commented that the most common morbidity was post-operative ascites. They had a mean follow-up of seventy-five months with similar overall recurrence rates between the open and laparoscopic groups (13% *vs* 11%). They concluded that in the appropriate PCLD patients, laparoscopic fenestration should replace open fenestration because it has similar rates of success along with similar morbidity and mortality rates^[41]. Hepatic resection should be reserved for those patients with massive enlargement of the liver that would not benefit from simple fenestration. Combining all patients undergoing open or laparoscopic fenestration (Table 1), there was an overall high morbidity and low mortality rate of 30% and 1%, respectively. Despite an adequate fenestration, there is a moderate recurrence of symptoms and rate of re-operation (Table 1). There must be a careful evaluation of the extent of each patient's disease to determine whether fenestration alone or resection with fenestration should be recommended.

HEPATIC RESECTION WITH FENESTRATION

The combination of hepatic resection with fenestration appears to be a valuable option for those patients with symptomatic PCLD and more severe parenchymal involvement. Most of these patients are classified as Type II or III PCLD, based on Gigot's classification^[25]. Fenestration alone in this group is rarely successful because the liver parenchyma is more rigid due to the fibrosis around the cysts and the cysts do not collapse as expected after fenestration. However, combined fenestration and resection allows for the removal of multiple segments that are grossly affected and allows for reduction in liver mass. Likewise, the large superficial and deep-seated cysts within remnant segments with more normal parenchyma can also undergo fenestration. The 10 published series are reviewed in Table 2.

The largest experience is reported by Que *et al* in a long-term follow up of 31 patients. The majority of the patients in this group had more severe parenchymal involvement (type II and III PCLD) necessitating resection combined with fenestration. The extent and type of liver resection depended on severity of disease with 13 patients undergoing lobectomies, 2 undergoing extended liver resections, and 16 non-anatomic liver resections. An average of 4 liver segments were resected per patient with an average weight of the resected tissue being 3.9 kg (8.6 lbs)^[23]. Their mortality rate was 3% which is consistent with the other larger reported series which range from 3%-10% (Table 2). Despite a low mortality rate, the morbidity rates associated with this procedure are high and must be considered. This series reported a morbidity rate of 58%, while in other series the morbidity rates range from 20% to 100%. The most commonly reported morbidities are ascites, pleural effusions, transient biliary leaks, bleeding, and wound

Table 2 Hepatic resection with and without fenestration for polycystic liver disease

Author	No. of patients	Technique (n)	Mortality (%)	Morbidity (%)	Mean follow-up (mo)	Rate of symptom recurrence (%)	Re-operation (%)
Turnage ^[47]	3	Fen & Res	2 (67)	2 (67)	9.6	33	0
Vauthey ^[2]	5	Fen & Res	0	5 (100)	14	0	0
Henne-Bruns ^[48]	8	Fen & Res	0	3 (38)	15	50	0
Que ^[23]	31	Fen & Res	1 (3)	18 (58)	28	3	0
Soravia ^[49]	10	Fen & Res	1 (10)	2 (20)	68	33	11
Koperna ^[41]	5	Fen & Res	0	NR	NR	0	0
Martin ^[43]	9	Res	0	6 (67)	9	33	0
Vons ^[50]	12	Res	1 (8)	10 (83)	34	17	0
Hansman ^[51]	2	Res	0	0	NR	100	0
Yang ^[52]	7	Fen & Res	0	7 (100)	20	100	0

Fen: Fenestration; Res: Resection; NR: Not reported.

infection^[2,43,47-52]. Que and coauthors had excellent results with an extremely low recurrence rate with 30 out of 31 patients remaining asymptomatic at a median follow-up of 28 mo. Importantly, they felt the extent of resection and fenestration was important for good long term outcomes. Overall, most of these patients had an improvement in their quality of life and functional status without deterioration in their hepatic or renal function^[23]. Although there are high morbidity rates, resection and fenestration provides patients' with severe parenchymal involvement an opportunity for symptomatic and clinical improvement with an acceptable recurrence rate.

LIVER TRANSPLANTATION

Liver transplantation as treatment for advanced PCLD, while more accepted in recent literature, still has a limited role in management of these patients. Although a majority of PCLD patients have normal liver function, orthotopic and living donor liver transplantation have been successfully utilized in the treatment of symptomatic PCLD^[53-64]. Aspiration, fenestration, or surgical resection can provide adequate palliation to those patients with large single cysts or dominant disease in one lobe, but the treatment of small, truly diffuse, cystic type PCLD may well require transplantation. Total hepatectomy and liver transplantation offers the chance of definitive treatment for this disease, but may be considered drastic, considering the absence of liver failure, the potential morbidity and mortality, and the organ shortage. In their early report of transplantation for PCLD, Starzl and colleagues described a "syndrome of lethal exhaustion" as the major indication to offer transplantation to these patients^[53]. These patients often reach the end of their functional lives, have intractable pain, and have a severely diminished quality of life. Indications for transplantation include cachexia, weight loss, recurrent cyst infections, portal hypertension, and ascites. Early reports have proposed these patients not wait until end-stage complications of their PCLD become manifest before offering the option of transplantation^[56,58,59]. Transplantation in those with end-stage PCLD, exhibited by severe disability, weakness, and malnutrition, has been shown to have higher infection-related mortality in early liver transplant series^[56,59]. Performing earlier

transplantation in appropriate candidates would seem to offer a greater chance of improved outcomes, meaningful recovery, and return to their prior functional status and quality of life. Furthermore, patients who have undergone prior more conservative therapies (aspiration, sclerosis, fenestration, or resection) may have post-surgical changes that make transplantation much more difficult^[53,58,59].

The option of transplantation should be balanced against the risks of surgery, long-term immunosuppression, and the need for concurrent or subsequent renal transplantation in those with ADPKD. Thus, transplantation should be limited to those patients with Type II/III PCLD with diffuse, small cystic disease that would not benefit from previously described therapies. Although the first reports of transplantation for PCLD by Kwok *et al*^[65] and Starzl *et al*^[53] occurred in the early 1990's, eleven to reflect studies in Table 3 describing transplantation for PCLD (Table 3). Two of the largest series reported by Lange *et al* and Pirenne *et al* report the outcomes of transplantation for PCLD in 17 and 16 patients, respectively^[56,59]. Lang and coauthors reported symptomatic relief in all patients following transplantation; however they did have 5 mortalities (29%) in their series. All five of these patients had severe anorexia, physical exhaustion, and evidence of malnutrition from end-stage PCLD prior to transplant and had postoperative infectious complications leading to their mortality. These deaths occurred at a mean of 41 d^[56]. Pirenne and colleagues reviewed their experience of 16 patients undergoing liver transplantation for severe PCLD. They reported two mortalities (12.5%): one intra-operative death from bleeding and air emboli in a patient who had undergone previous resection, and a second late death from post-transplant lung cancer. Patient and graft survival rates were 87.5% with follow-up from 3 mo to 10 years^[59]. In summary, liver transplantation offers the chance of immediate, complete, and definitive treatment in those patients with massive hepatomegaly secondary to diffuse PCLD. In these patients, fenestration and resection only offers temporary palliation, puts them at risk for potential morbidity and mortality, and jeopardizes the chances of further definitive treatment by transplantation. Several other series and their results are

Table 3 Liver Transplantation for polycystic liver disease

Author	No. of patients	Previous surgical procedure	Combined liver/Kidney Tx	Mortality	Mean follow-up (mo)
Kwok ^[65]	1	1	1	1	-
Starzl ^[53]	4	0	1	2	26
Uddin ^[54]	3	0	0	0	NR
Washburn ^[55]	5	4	1	1	38
Lang ^[56]	17	6	8	5	12
Swenson ^[58]	9	4	3	1	26
Pirenne ^[59]	16	4	1	2	18-120 ²
Takegoshi ^[60]	1 ¹	0	0	0	8
Koyama ^[61]	1 ¹	0	0	0	18
Gustafsson ^[63]	7	4	3	0	4
Becker ^[62]	17	NR	17	3	49
Ueda ^[64]	3 ¹	NR	0	0	32
Kirchner ^[66]	36	NR	15	5	62

NR: Not reported. ¹Indicates living-donor transplantation, ²Range of follow-up in months.

reviewed in Table 3.

As more patients undergo transplantation for PCLD, it is important to assess their long term outcomes, especially quality of life. Kirchner and colleagues reviewed the quality of life, *via* the SF-36 and a self-designed questionnaire, in 23 of 36 patients who underwent liver or combined liver-kidney transplantation for PCLD. Of the respondents, 91% of patients felt “much better” or “better”, while only 9% felt “worse” than before. Fatigue, physical fitness, anorexia, vomiting, physical attractiveness, and interest in sex improved significantly after transplantation. Overall, patients with advanced PCLD have an improved quality of life after liver or combined liver-kidney transplantation^[66].

CONCLUSION

The management of patients with PCLD continues to be challenging. In the past several decades, there have been great advances in the knowledge of the pathogenesis, genetics, and effective treatment for PCLD. Understanding this disease, potential complications, associated medical conditions, and successful treatment strategies is essential for gastroenterologists and hepatobiliary surgeons. The ability to risk-stratify these patients by severity of disease can lead to earlier interventions and attempts at prevention of massive hepatomegaly that can be so debilitating. In patients with symptomatic PCLD, invasive management strategies should be based on the degree of symptoms, the severity of associated medical conditions, and the extent of their disease. Those symptomatic patients with large cysts or limited hepatic involvement would likely benefit from laparoscopic fenestration. Adequate hepatic resection with fenestration should be favored in patients with diffuse involvement of certain areas of hepatic parenchyma with remaining large areas of non-cystic parenchyma. Finally in the patient with diffuse, small cysts, transplantation is a valid option and should be pursued as primary therapy prior to development of debilitating disease that can increase complication rates.

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GASTRIC CANCER

Rationales for expression and altered expression of apoptotic protease activating factor-1 gene in gastric cancer

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Abstract

AIM: To elucidate the relationship between apoptotic protease activating factor-1 (Apaf-1) gene and gastric cancer.

METHODS: Thirty-five postoperative cancer and adjacent normal tissue samples were collected in the present study. Expression of the Apaf-1 gene in these samples was analyzed by semi-quantitative RT-PCR. Loss of heterozygosity (LOH) was used to determine whether there was loss of Apaf-1 gene in domain of 12q22-23 in the samples. Promoter methylation of Apaf-1 gene in the samples was analyzed by methylation specific (MSP) PCR.

RESULTS: The expression of Apaf-1 mRNA in gastric cancer tissue samples was 51%. The LOH frequency of D12S346, D12S1706, D12S327, D12S1657 and D12S393 was 33%, 8%, 58%, 12% and 42%, respectively. Fifty percent LOH was found at two sites and 17% LOH at three sites. Apaf-1 mRNA expression decreased significantly in 13 cases ($r_s = 0.487$, $P = 0.003$). The rate of Apaf-1 promoter methylation was 49% in gastric cancer tissue samples and 23% in para-cancerous tissue samples. Promoter methylation occurred significantly in 16 of 18 gastric cancer tissue samples with decreased expression of Apaf-1 mRNA ($r_s = 0.886$, $P = 10^{-6}$).

CONCLUSION: The expression of Apaf-1 gene is low in gastric cancer tissues. Methylation of Apaf-1 gene promoter and LOH in domain of 12q22-23 are the main reasons for the expression and altered expression of Apaf-1 gene.

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Key words: Gastric cancer; Apaf-1 gene; Loss of

heterozygosity; Methylation

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INTRODUCTION

Formation and progression of gastric cancer are a continuous multiple-step process. Cell apoptosis is one of the main mechanisms for tumor genesis^[1-3], and Apaf-1 protein is an important apoptosis factor which is expressed abnormally in a series of tumor studies^[4-7]. It was recently reported that Apaf-1 gene is closely related to several cancer-inducing genes and tumor suppressor genes, such as p53 and Bcl2^[8-10]. However, the expression condition of Apaf-1 gene in gastric cancer and its correlation with the genesis of gastric cancer remain unclear. This study was to study the relation between Apaf-1 gene expression and gastric cancer genesis in promoter domain.

MATERIALS AND METHODS

Materials

Thirty-five postoperative cancer tissue and adjacent normal tissue samples (> 5 cm away from the center of cancer) were collected from gastric cancer patients from January 2005 to September 2005 at No.1 and No.2 Affiliated Hospitals of China Medical University. All these patients were pathologically diagnosed according to the new TNM classification criteria of Union Internationale Contre le Cancer^[11]. Of the 35 patients with progressive gastric cancer (25 males and 10 females with a mean age of 58.51 ± 12.24 years), 10 had moderately differentiated gastric cancer and 25 had poorly-differentiated gastric cancer, while 25 had lymphatic metastasis and 10 had no lymphatic metastasis. Tumor and adjacent normal tissues were isolated and maintained at -70°C . Total RNA extracting reagent Trizol (Invitrogen Co.), RT-PCR kit (TaKaRa Co.), methylating reagent (Sigma Co.) and Wizard DNA clean-up (Promega Co.) were used in the study.

Semi-quantitative RT-PCR analysis

Total RNA was extracted with Trizol reagent and cDNA

Table 1 Polymorphic sites, primers and amplification conditions (12q22-23)

Site	Repeat	Heterozygosity	Amplification length (kb)	Primer sequence	Reaction conditions
D12S327	Dinucleotide	0.8501	0.1820-0.2010	Aaa gtt tct gga tgg taa tat cg Aga gca aga cct tgt ctg aa	54°C 40 s 72°C 45 s
D12S1657	Dinucleotide	0.6439	0.1500-0.1600	Tcc taa aga tgg tgt gca t Aag ttc caa tgt tag tga acc	58°C 40 s 72°C 45 s
D12S393	Tetranucleotide	0.6410	0.2490	Att att gcc agg aca tta aac g Cct cac aca atg ttg taa ggg	58°C 30 s 72°C 40 s
D12S1706	Dinucleotide	0.8916	0.1190-0.1390	Cct atg att tcc cat caa gtt t Att att agg aga gcc ctg gg	57°C 40 s 72°C 40 s
D12S346	Dinucleotide	0.8400	0.1660	Tgc cc acct gcc tgt aac Aat gga ggg taa atg ccc g	58°C 30 s 72°C 40 s

was synthesized with reverse transcriptase and Oligo (dT)₂₀ primer. Apaf-1 primer sequence was designed with primer 3, including functional caspase recruitment domain (CARD), upstream primer (5'-TTGCTGCCCTTCTCC ATG AT-3'), downstream primer (5'-TCCCAACTGAAA CCCAATGC-3') and amplification length (334 bp). The internal reference primer was β -actin, including upstream primer (5'-GTGGGGCGCCCCAGGCACCA-3'), downstream primer (5'-CTCCTTAATGTCACGCAC GATTTC-3') and amplification length (498 bp). Both pairs of primers were added into 25 μ L reaction system to react under the proper PCR reaction conditions (denatured at 94°C for 45 s, renatured at 58°C for 45 s and elongated at 72°C for 60 s, for 35 cycles). RT-PCR products were isolated with 2% agarose gel, imaged through Alpha Image 2000 automatic imager and analyzed using Fluorchem V 2.0 Stand Alone software.

LOH analysis

Down-regulation of Apaf-1 gene expression was correlated with allele loss at its 5 sites (D12S327, D12S1657, D12S393, D12S1706 and D12S346). These 5 polymorphic sites were selected for LOH analysis. DNA was extracted from tumor and normal tissues using phenol/chlorine method. Primer sequences derived from genome database (GDB) and amplification conditions are listed in Table 1. Two percent agarose gel electrophoresis revealed target genes but no hybrid band in each specimen. PCR products were added into the denaturing buffer (98% formamide, 10 mmol/L EDTA, 0.025% bromophenol blue and 0.025% xylene cyanol) at a ratio of 1:1, mixed uniformly, denatured at 97°C for 10 min and immediately placed into ice. Fifteen μ L of specimens was loaded in each hole, separated through 9% non-denaturing PAGE (in a 100 V low-temperature water bath for 2-3 h) and the electrophoresis results were analyzed through silver staining.

Methylation analysis at Apaf-1 promoter domain

DNA extracted from gastric cancer and adjacent normal tissues was treated with Na₂SO₃, purified and recovered with Wizard DNA clean-up (Promega Co.) for MSP analysis. A 49-378 base zone rich in CpG at Apaf-1 cDNA promoter domain was selected to design MSP primers, including upstream primer (5'-GAGGTGTCGTAG CCGTATTTC-3'), downstream primer (5'-CGAAAATTA

ACGAAATAAACGTC-3'), PCR reaction conditions (denatured at 94°C for 45 s, renatured at 58°C for 45 s and elongated at 72°C for 60 s, for 38 cycles) and amplification length (221 bp). Methylating primers included upstream primer (5'-ATTTGAGGT GTGTAGTGGTATTT G-3'), downstream primer (5'-ACCTCCAAAAATTAACAA AATAAACAT-3'), PCR reaction conditions (denatured at 94°C for 45 s, renatured at 56°C for 45 s, elongated at 72°C for 60 s, for 38 cycles) and amplification length (221 bp).

Statistical analysis

Statistical analyses were performed by the SPSS13.0 software package (SPSS, Inc, Chicago, IL). Continuous Data were expressed as mean \pm SD. Continuous variables were compared by *t*-test when appropriate, whereas categorical variables were compared by χ^2 test or Fisher's exact test when appropriate. Spearman rank correlation was analyzed. *P* < 0.05 was considered statistically significant.

RESULTS

Gene expression

The gene expression was increased 30%-70% in cancer tissue samples with an increased difference between Apaf-1/ β -actin cDNA optical density ratios in gastric cancer and adjacent normal tissue samples. In the present study, Apaf-1 expression decreased in 48.57% (17/35) of gastric cancer tissue samples (including expression loss in 2 cases), paired *t* test with SPSS13.0 statistical software showed that the relative Apaf-1 content was 0.96 ± 0.40 in adjacent normal tissue samples and 0.69 ± 0.36 in cancer tissue samples, respectively (*t* = 3.518, *P* < 0.01), and Apaf-1 expression was significantly higher in adjacent normal tissue samples than in cancer tissue samples. Apaf-1 mRNA expression did not increase in tumor tissue samples (Figures 1 and 2).

LOH analysis

The data from 2 fragments (i.e. genome DNA was a certain marked heterozygote in normal tissue or adjacent normal tissue samples) were sufficient for LOH analysis, but the data from one fragment were not sufficient for LOH analysis. LOH meant that there was 1 band with less genome DNA PCR amplification product or 1

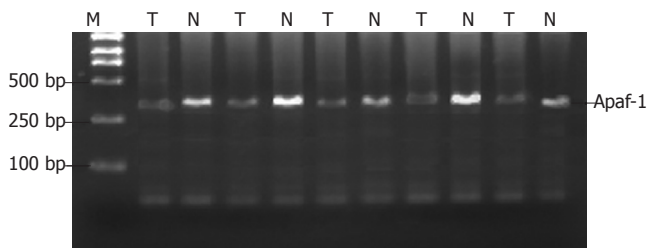


Figure 1 Expression of Apaf-1 mRNA. M: Marker, T: Tumor, N: Adjacent normal tissue, Apaf-1 mRNA: 334 bp, marker: DL2000.

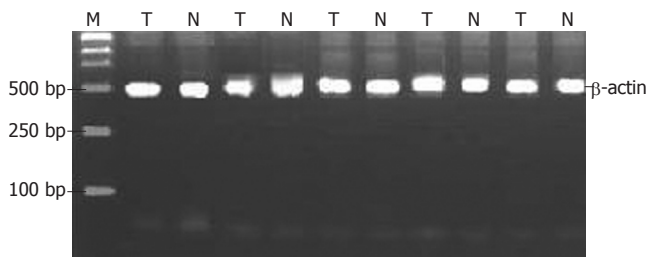


Figure 2 Expression of β -actin mRNA. M: Marker; T: Tumor; N: Normal adjacent tissue; β -actin mRNA: 498 bp; marker: DL2000.

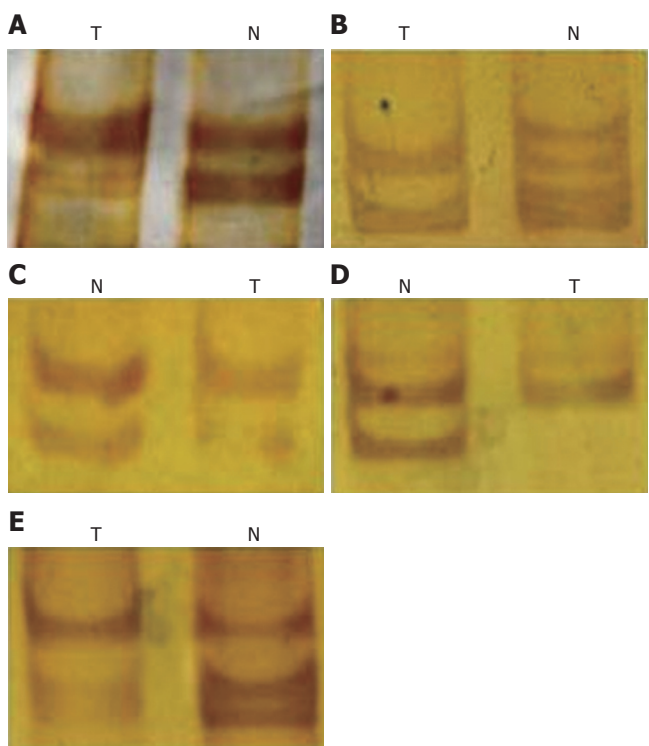


Figure 3 LOH analysis of polymorphic sites of D12S327 (A), D12S1657 (B), D12S393 (C), D12S1706 (D), and D12S346 (E). T: Tumor tissue; N: Adjacent normal tissue. Silver staining diagram showing Apaf-1 loss and LOH analysis showing allele loss at 5 polymorphic sites of Apaf-1 gene in gastric cancer tissue.

band with significantly weaker signal of up to 50% in cancer tissue samples than that in adjacent normal tissue samples. As shown by LOH analysis of 5 polymorphic sites for Apaf-1 gene in the present study, the LOH detection rate was 34.29% (12/35), 8.57% (3/35), 57.14% (20/35), 11.43% (4/35) and 42.86% (15/35) at single D12S346, D12S1706, D12S327, D12S1657 and

Table 2 Correlation of Apaf-1, LOH and Apaf-1 mRNA expression in gastric cancer tissue samples

		mRNA expression of Apaf-1 gene (n)		<i>r_s</i>	<i>P</i>
		Down-regulation	Up-regulation		
More than 2 sites (n)	+	13	5	0.487	0.003
	-	4	13		

Table 3 Correlation of Apaf-1 methylation and Apaf-1 mRNA expression in gastric cancer tissue samples

		mRNA expression of Apaf-1 (n)		<i>r_s</i>	<i>P</i>
		Down-regulation	Up-regulation		
Apaf-1 gene methylation (n)	+	16	1	0.886	10 ⁻⁶
	-	1	17		

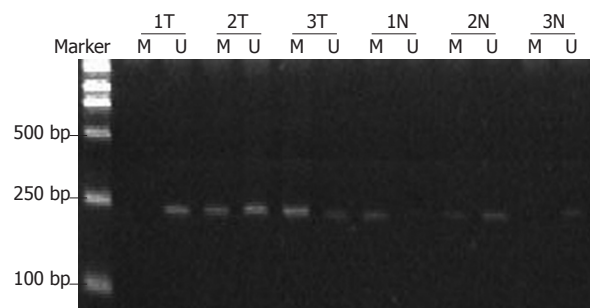


Figure 4 Methylation of Apaf-1 promoter. M: 212 bp methylation band; U: 221 bp non-methylation band; T: Gastric cancer tissue.

D12S393, respectively. However, the LOH detection rate was 51.43% (18/35) at more than 2 sites (16.67% at 3 sites) (Figure 3), suggesting that the LOH of Apaf-1 gene was correlated with the genesis of gastric cancer. Apaf-1 mRNA expression decreased significantly in 13 cases ($r_s = 0.487$, $P = 0.003$), indicating that the LOH of Apaf-1 gene was correlated with its decreased expression in gastric cancer (Table 2 and Figure 3).

Promoter methylation

MSP analysis of methylation conditions for Apaf-1 gene promoter showed that the methylation rate of Apaf-1 gene was 48.57% (17/35) in gastric cancer tissue samples and 17.14% (6/35) in adjacent normal tissue samples, respectively ($P < 0.05$), demonstrating that Apaf-1 methylation was correlated with the genesis of gastric cancer. Significant promoter methylation occurred in 16 of 18 gastric cancer tissue samples while the expression of Apaf-1 mRNA was decreased ($r_s = 0.886$, $P = 10^{-6}$), displaying that promoter methylation was correlated with decreased expression of Apaf-1 gene (Table 3 and Figure 4). Promoter methylation and LOH of Apaf-1 gene occurred at polymorphic sites in 17 of 18 gastric cancer tissue samples and the expression of Apaf-1 mRNA was decreased.

DISCUSSION

Human Apaf-1 gene is located at chromosome 12q23 to

encode cytoplasm protein (130 KD). As an important apoptosis factor, Apaf-1 gene participates in the pathway of mitochondria-mediated apoptosis^[12]. However, the condition of Apaf-1 gene expression in gastric cancer and the correlation between expression of Apaf-1 gene and genesis of gastric cancer remain unclear. Although Apaf-1 is an important apoptosis factor and a tumor suppressor gene^[12-16], few studies on the relationship between Apaf-1 gene and gastric cancer are available.

In the present study, Apaf-1 gene expression in gastric cancer tissue samples was significantly lower than that in adjacent normal tissue samples, suggesting that Apaf-1 gene plays an important role in genesis and progression of gastric cancer.

According to the epigenetics theory on gene regulation, methylation of gene fragments (especially promoter domain) inhibits gene transcription, and acetylation of gene-related histone up-regulates gene expression. One of the carcinogenic mechanisms is gene silencing caused by hyper-methylation of CpG islet at tumor suppressor gene promoter domain and deacetylation of histone^[17-23]. It was reported that Apaf-1 gene is a tumor suppressor gene and seldom mutates, but has functional loss due to LOH and promoter methylation^[24]. Apaf-1 gene has different effects and expression patterns in cancer tissue of different sources. The lower (or inactive) expression of Apaf-1 gene is related to methylation silencing in acute leukemia and laryngeal squamous carcinoma^[24,25], and LOH in colon cancer, ovarian cancer and malignant melanocarcinoma^[26-28].

In the present study, Apaf-1 gene promoter was methylated in 14 gastric cancer tissue samples with a decreased expression of Apaf-1 as detected by the MSP technique. Apaf-1 expression decreased in 13 gastric cancer tissue samples with LOH at more than 2 sites (including promoter methylation in 6 cases) as demonstrated by 9% non-denaturing PAGE. These findings show that decreased expression of Apaf-1 gene in gastric cancer tissue is due to some complex reasons, among which, however, promoter methylation and LOH at polymorphic sites play a major role.

COMMENTS

Background

The expression condition of Apaf-1 gene in gastric cancer and its correlation with the genesis of gastric cancer remain unclear. The purpose of this study was to make it clear.

Research frontiers

The epigenetics theory on gene regulation is the highlight in recent tumor studies. This article describes the role of Apaf-1 gene eigenetics in gastric cancer.

Innovations and breakthroughs

This article focuses on the Apaf-1 expression condition in gastric cancer, and its relation with loss of heterozygosity and methylation in promoter domain.

Applications

Apaf-1 gene is an important apoptosis gene. Its status of methylation and LOH may contribute to the study on the etiology of gastric cancer.

Terminology

Methylation is a term used in chemical sciences to denote the attachment or substitution of a methyl group on various substrates. DNA methylation profiling

is gaining momentum as an epigenetic approach to understanding the effects of aberrant methylation (either hyper- or hypomethylation) both in basic research and in clinical applications.

Peer review

Low expression of Apaf-1 gene in gastric cancer tissue was shown in this study, indicating that methylation of Apaf-1 gene promoter and LOH in domain of 12q22-23 are the main reasons for expression and altered expression of Apaf-1 gene in gastric cancer

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Modifications produced by selective inhibitors of cyclooxygenase and ultra low dose aspirin on platelet activity in portal hypertension

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Abstract

AIM: To study the mechanism involved in the potentially beneficial effect of ultra low dose aspirin (ULDA) in prehepatic portal hypertension, rats were pretreated with selective COX 1 or 2 inhibitors (SC-560 or NS-398 respectively), and subsequently injected with ULDA or placebo.

METHODS: Portal hypertension was induced by portal vein ligation. Platelet activity was investigated with an *in-vivo* model of laser induced thrombus production in mesenteric circulation and induced hemorrhagic time (IHT). Platelet aggregation induced by ADP and dosing of prostanoid products 6-keto-PGF_{1α}, TXB₂, PGE₂ and LTb₄ were also performed.

RESULTS: The portal hypertensive group receiving a placebo showed a decreased *in vivo* platelet activity with prolonged IHT, an effect that was normalized by ULDA. SC-560 induced a mild antithrombotic effect in the normal rats, and an unmodified effect of ULDA. NS-398 had a mild prothrombotic action in portal hypertensive rats, similar to ULDA, but inhibited a further effect when ULDA was added. An increased 6-keto-PGF_{1α} was observed in portal hypertensive group that was normalised after ULDA administration. TXA₂ level after ULDA, remained unchanged.

CONCLUSION: These results suggest that the effect of ULDA on platelet activity in portal hypertensive rats, could act through a COX 2 pathway more than the COX 1, predominant for aspirin at higher doses.

INTRODUCTION

Portal hypertension is a major complication of chronic liver disease. In its pathophysiology, increased hepatic resistance is followed by a hyperdynamic circulatory state^[1]. This hyperdynamic state, in which nitric oxide (NO) and prostacyclin (PGI₂) are important vasoactive substances, induces a decreased platelet activity, even in the absence of hepatic damage. Although NO plays an important role in modifying platelet adhesion in portal hypertension^[2], PGI₂, a powerful vasodilator prostanoid with antithrombotic properties, was also found increased in mesenteric vascular bed^[3].

Ultra low dose aspirin (ULDA) has shown prothrombotic activity when analysed in humans and in the interface platelet-endothelial cell^[4,5]. Previous papers have shown the potentially beneficial effect of ULDA in portal hypertensive animals normalizing altered platelet activity and induced hemorrhagic time^[6]. Further experiments were performed to clarify if this effect was mediated mostly by a cyclooxygenase (COX) pathway or by modifying NO synthesis, the two aspirin mechanisms of action^[7], although a previous study with a different model suggested that ULDA could decrease PGI₂ synthesis^[8]. The same *in vivo* Laser induced thrombus formation, was used in portal hypertensive rats to investigate the effects of Indomethacin, a non selective COX inhibitor, and L-Nitro Arginin Methyl Ester (NAME), a non selective inhibitor of NO production. The results suggested that the effects of ULDA were more influenced by COX pathway than by NO synthesis inhibition^[9]. Addition of ULDA in portal hypertensive rats, when previously inhibiting COX with Indomethacin, increased number of emboli and duration

of embolization but blunted the normalization of induced hemorrhagic time, suggesting probably a more selective action on COX 1 or COX 2 pathway. TXA₂, the main product of arachidonic acid *via* the activity of COX 1 in platelets, increases platelet aggregation and its synthesis is inhibited by Aspirin. PGE₂ and PGI₂ are produced by COX 1 and 2 mainly in endothelial cells. PGE₂ has no probable role in portal hemodynamic changes observed in portal hypertensive rats^[10], but depending on its concentration, it can modulate platelet aggregation by regulating intracellular levels of cAMP^[11].

The present investigation was designed to clarify the mechanism of the effects of ULDA in portal hypertension, by using previous selective inhibition of cyclooxygenase COX 1 or COX 2 (with SC-560 and NS-398 respectively). Models of Laser induced Thrombosis and IHT were used and plasmatic levels of 6-keto-PGF_{1α} (the stable metabolite of PGI₂), PGE₂, TXB₂ (the stable metabolite of TXA₂) and LTB₄ were determined.

MATERIALS AND METHODS

Animals

Male Wistar rats (200-250g) purchased from Delpre Breeding Center (St. Doulchard, France) were housed separately and acclimatized before use under conditions of controlled temperature (25 ± 2°C) and illumination (12 h light/dark cycle). They were fed with standard rat chow and water *ad libitum*. Animals received care in compliance with the European Convention of Animal Care.

Surgical procedures

After one week of acclimatization, rats were randomized and separated into two groups: one consisted of sham-operated (Sh) rats and the other formed by portal hypertensive (PH) rats. Portal hypertension was induced by a calibrated portal vein stenosis, according to the procedure described by Vorobioff *et al*^[1].

Rats were anesthetized with Ketamine (Panpharma, Fougères, France) 90 mg/kg body weight, i.m. and then a midline abdominal incision was made. The portal vein was located and isolated from the surrounding tissues. A ligature of 3-0 silk was placed around the vein and snugly tied to a 20 gauge blunt end needle placed along side the portal vein. The needle was subsequently removed to yield a calibrated stenosis of the portal vein. Sham-operated rats underwent an identical procedure except that portal vein was isolated but not stenosed.

Animals were housed during fourteen days after the operation to develop portal hypertension in the corresponding group.

Thrombus induction

Animals were anaesthetized with 200 mg/kg of thiopental sodium (Pentothal[®], Laboratories Abbott, Rungis, France) a median laparotomy was performed. The intestinal loop was placed on the microscope table and vascular lesions were induced by Argon laser (Stabilite 2016, Spectra Physics, France). The wavelength used was 514 nm and the energy was adjusted to 120 mW. The laser beam was

applied during 1/15 s. The dynamic-course of thrombus formation was continuously monitored and recorded by placing the laser beam coaxially into the inverted light beam path of the microscope (Axiovert, Zeiss, France). Microscopic images were recorded through a digital camera (DX L107, color camera CCD, Basler, Vision Technologies) to visualize and digitalize data coupled to a Dell monitor. A schematic view of the apparatus used has been previously described^[12]. Arterioles between 15 and 25 μm diameter were used. The parameters assessed were the number of emboli removed by blood flow and the duration of embolisation (time between the first and the last emboli occurring during a 10 min observation period).

Induced hemorrhagic time

An experimental model of induced hemorrhagic time (IHT) was performed 10 min before thrombosis induction by laser. The tail of the rat was immersed in water for 5 min at 37°C and sectioned 6 mm from the extremity. IHT measured, corresponded to the time between the tail section and the end of bleeding, expressed in seconds.

Biological analysis

Platelet aggregation study: Platelet aggregation was made according to the method of Cardinal and Flower on a Chrono Log 500 *V/S* aggregometer (Coultronics, Margency, France) on the whole blood obtained from the rat after laser experimentation. Platelet aggregation was induced by ADP final concentration 5 μmol/L (Laboratoire Diagnostica, Stago, France). Two parameters were determined: Impedance, representing the maximum amplitude of aggregation expressed in Ohms. Velocity of aggregation expressed in ohms/min.

Prostanoids determinations: At the end of each experiment, blood was collected by cardiac puncture and centrifuged for 20 min at 4000 r/min to obtain Platelet Poor Plasma (PPP). Concentrations of 6-keto-PGF_{1α}, TXB₂, PGE₂ and LTB₄ were determined in plasma samples using competitive binding Elisa tests (R&D Systems Europe, Abingdon, UK) according to the manufacturer's instructions.

Drugs tested

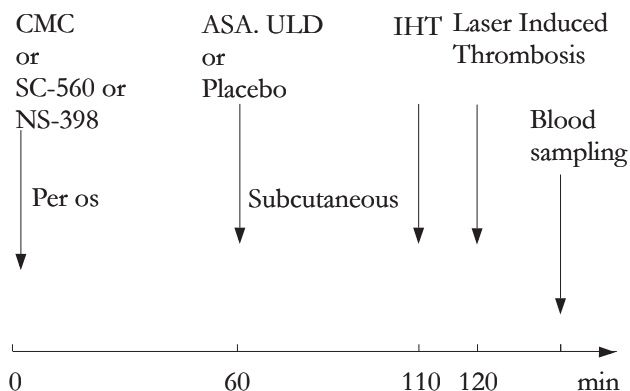
The Aspirin solution was purchased from Boiron Laboratories (Sainte-Foy-Les-Lyon, France). ULDA was prepared as follows: 1 g of pure, finely powdered aspirin was suspended in 99 mL of alcohol (70°). After being vigorously shaken, 1 mL of this dilution was then mixed with 99 mL of distilled water and vigorously shaken. The last process was repeated 13 more times^[11]. Alcohol and sterilized water following the above cited procedures without adding the Aspirin was used as control. ULDA or placebo were subcutaneously administered at a final volume of 1 mL/kg rat weight.

Selective inhibitors of COX 1, SC-560 and of COX 2, NS-398 were purchased from Cayman Chemical, (Ann Arbor Michigan, USA). They were administered per os at a dose of 10 mg/kg rat weight, suspended in Carboxymethylcellulose (CMC) 5 g/L at a final volume

of 1 mL/kg rat weight. The CMC solution was used as placebo.

Protocol

Fourteen days after the corresponding operation, 216 rats were randomly assigned in 12 groups and treated as follows:



Groups

Groups, procedures and treatments are detailed in Table 1.

Statistical analysis

Data are expressed as mean \pm SEM and compared using one way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test. A value of $P < 0.05$ was considered significant. Statistical calculations were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com).

RESULTS

Thrombus induction and IHT

Groups with placebo (CMC): Portal hypertension decreased the number of emboli ($P < 0.05$) and the duration of embolisation ($P < 0.05$) and prolonged the Induced Hemorrhagic Time ($P < 0.01$). ULDA induced significant modifications to normalize these values ($P < 0.01$, $P < 0.001$ and $P < 0.001$ for Number of Emboli, Duration of Embolisation and IHT respectively). An increased number of shots needed to start the embolisation was observed in the portal hypertensive group, and this effect also normalised after ULDA ($P < 0.05$).

Effects of SC-560, selective inhibitor of COX 1: After the inhibition of COX 1, a non significant antithrombotic effect was observed in the sham operated group, and no further change in cirrhotic group. After ULDA, the prothrombotic effect remained active for Number of Emboli ($P < 0.05$) and IHT ($P < 0.01$) despite the opposite effect of pre-treatment with SC-560. No changes were observed in number of shots.

Effects of NS-398, selective inhibitor of COX 2: The pre-treatment with NS-398 induced a decreased induced hemorrhagic time in portal hypertensive rats, similar to the effect of ULDA without COX inhibitors. No effect of ULDA was seen after COX 2 inhibition (Figure 1A-D).

Table 1 Experimental groups

Group	Procedure and treatments		
	Phase 1: 14 d before treatments	Phase 2: 120 min before experiment	Phase 3: 60 min before experiment
ShP	Sham operated	Placebo (CMC)	Placebo (H ₂ O)
PHP	Portal hypertension	Placebo (CMC)	Placebo (H ₂ O)
PHPAs	Portal hypertension	Placebo (CMC)	ULDA
ShP	Sham operated	SC-560	Placebo (H ₂ O)
PHP	Portal hypertension	SC-560	Placebo (H ₂ O)
PHPAs	Portal hypertension	SC-560	ULDA
ShP	Sham operated	NS-398	Placebo (H ₂ O)
PHP	Portal hypertension	NS-398	Placebo (H ₂ O)
PHAs	Portal hypertension	NS-398	ULDA

ULDA: Ultra low dose aspirin; CMC: Carboxymethylcellulose; ShP: Sham-placebo; PHP: Portal hypertension-placebo; PHPAs: Portal hypertension-ULDA.

Biological analysis

Platelet aggregation induced by ADP (Figure 2A and B): Platelet aggregation has shown a decreased velocity in portal hypertensive animals and in sham operated animals pretreated with SC-560. An increased velocity was observed in portal hypertensive animals pretreated with SC-560 or NS-398 and with ULDA. None of the observed alterations were found in Amplitude and in Velocity.

Variations in metabolites of arachidonic acid (Figure 3A-D): 6 keto PGF_{1α} (Figure 3A): This stable metabolite of PGI₂ was found increased in portal hypertensive rats ($P < 0.01$). This increase was normalised by ULDA ($P < 0.05$) or SC-560 ($P < 0.05$).

TXB₂ (Figure 3B): Inhibition of COX 2 with NS-398 produced an increased level of TXB₂ in portal hypertensive animals (XI group), that was not modified by ULDA ($P < 0.05$ and 0.001 respectively).

PGE₂ (Figure 3C): An increase in PGE₂ was observed in portal hypertensive (PHP) group, treated with SC-560 ($P < 0.05$).

LTB₄ (Figure 3D): Dosage of LTB₄, a lipoxygenase (LOX) metabolite of arachidonic acid, was performed as control of experiment. No variations of production were found when comparing the different groups.

DISCUSSION

ULDA has shown prothrombotic effects with an *in vivo* model, testing the interaction between platelet and endothelial cells^[5,12]. Exploration of this effect in portal hypertensive rats revealed a decreased number of emboli as well as duration of embolisation and a prolonged IHT that were normalised by ULDA administration. In the search of an explanation for the mechanism involving this effect, previous publications of L-NAME (an inhibitor of Nitric Oxide synthesis) and Indomethacin (a nonselective COX inhibitor) effects on portal hypertensive rats showed that the effect of ULDA was more affected by Indomethacin than by NAME⁹. Moreover, Indomethacin seemed to

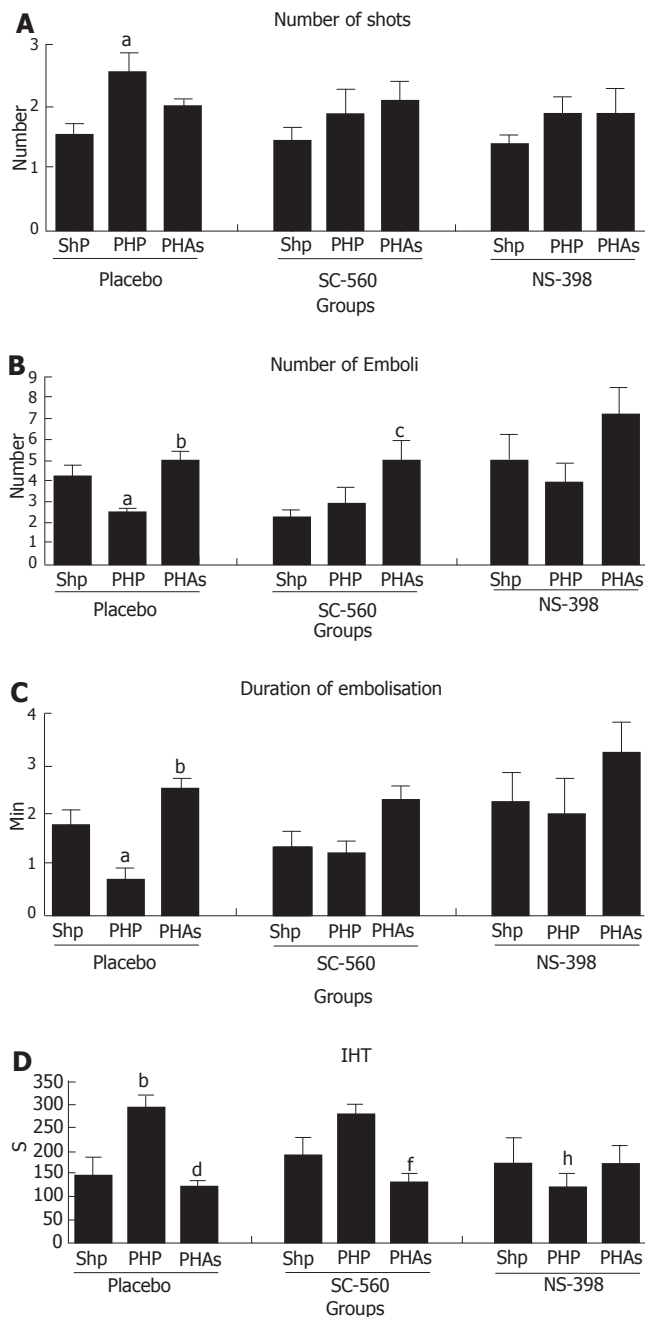


Figure 1 A: Study of laser induced thrombosis parameters; Number of shots (expressed in number): ^a $P < 0.05$ vs ShP (Placebo); B: Study of laser induced thrombosis parameters; Number of Emboli (expressed in number): ^a $P < 0.05$ vs ShP (placebo); ^b $P < 0.01$ vs PHP (placebo); ^c $P < 0.05$ vs ShP (SC-560); C: Study of laser induced thrombosis parameters; Duration of Embolisation (expressed in minutes): ^a $P < 0.05$ vs ShP (placebo); ^b $P < 0.001$ vs PHP (placebo); D: Study of Induced Hemorrhagic Time (expressed in seconds): ^a $P < 0.01$ vs ShP (placebo); ^a $P < 0.001$ vs PHP (placebo); ^f $P < 0.01$ vs PHP (SC-560); ^h $P < 0.001$ vs PHP (placebo).

have contradictory effects. Despite the antithrombotic effect of Indomethacin, the *in vivo* prothrombotic effect of ULDA (increasing number of emboli and duration of embolization) was increased, and its beneficial effect of reducing IHT in portal hypertensive rats was blocked. The present experiment was done to verify the hypothesis that these contradictory modifications produced by Indomethacin over ULDA's prothrombotic effect were the result of different ways in which COX 1 and 2 could affect

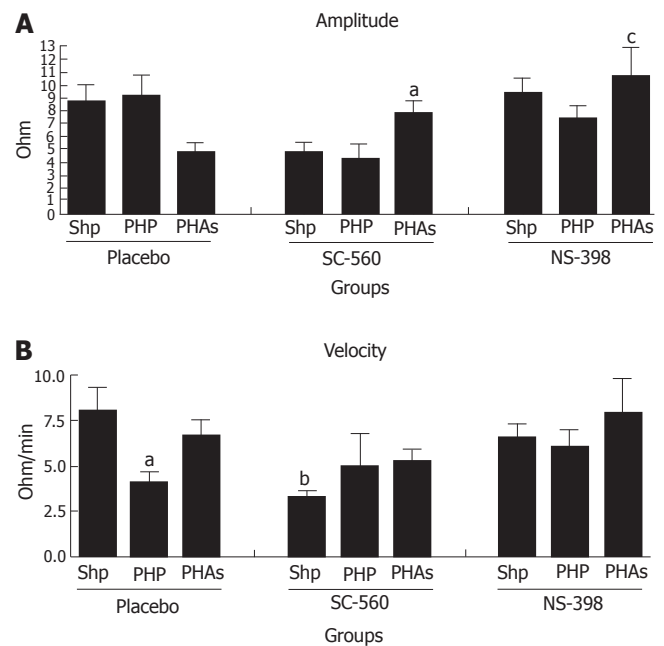


Figure 2 A: Study of Platelet aggregation induced by ADP, Amplitude (expressed in Ohm): ^a $P < 0.05$ vs PHP (SC-560); ^c $P < 0.05$ vs PHAs (placebo); B: Study of Platelet aggregation induced by ADP, Velocity (expressed in Ohm/min): ^a $P < 0.05$ vs ShP (placebo); ^b $P < 0.01$ vs ShP (placebo).

platelet-endothelial cell interaction.

The results found in this study corroborate the previously described effects of ULDA in portal hypertensive rats, normalizing number of emboli, duration of embolisation and the IHT^[6,9].

In rats with portal hypertension, an increase in 6-keto $\text{PGF}_{1\alpha}$ was observed, probably due to the known increased PGI_2 production described for the mesenteric vascular bed in this animal model^[3]. The addition of ULDA normalised this effect. As reported in an *in vitro* model with a vascular fragment, ULDA was active only in vascular fragments with an elevated PGI_2 production^[8]. This last observation is similar to our present results since the ULDA effect of decreasing 6-keto $\text{PGF}_{1\alpha}$ is observed only in the portal hypertensive group.

The administration of SC-560, had a slightly antithrombotic effect in sham operated rats, decreasing non significantly the Number of Emboli. This could be explained by a decrease in platelet synthesis of TXA_2 ^[13]. There was a decrease in 6-keto $\text{PGF}_{1\alpha}$ in the portal hypertensive group with COX 1 inhibition, and this effect is probably due to the inhibition of the increased production of PGI_2 ^[14,15] and COX 1 over-expression observed in this model of portal vein ligation^[16-18]. It is interesting to note that COX 1 inhibition had almost not modified the prothrombotic effect of ULDA in portal hypertensive animals and observed as an increase in number of emboli ($P < 0.05$) and a decrease in IHT ($P < 0.01$).

The administration of NS-398 had not modified the *in vivo* parameters (NE, DE and IHT) in the Sham operated group. In the portal hypertensive group, a non significant tendency to increase NE and DE was observed, as well as a statistically significant shortened IHT. After pre-treatment

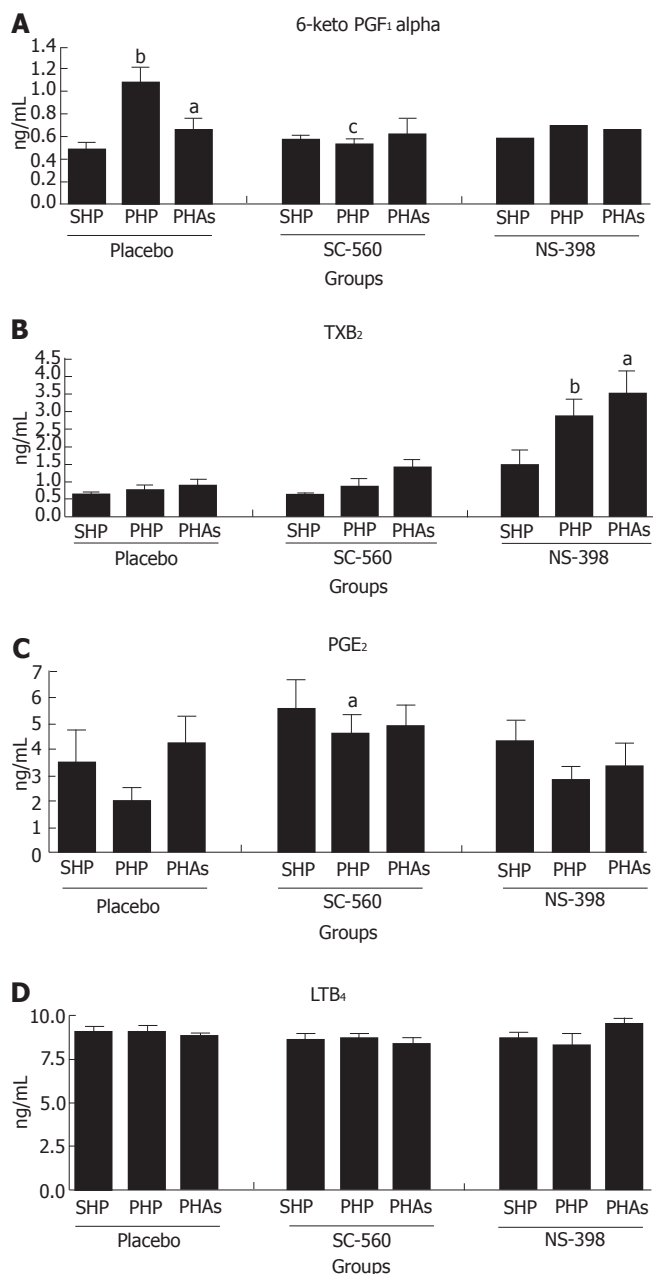


Figure 3 A: Plasmatic 6-keto PGF₁α concentrations (expressed in ng/mL): ^b*P* < 0.01 vs SHP (placebo); ^a*P* < 0.05 vs PHP (placebo); ^c*P* < 0.05 vs PHP (placebo); B: Plasmatic TXB₂ concentrations (expressed in ng/mL): ^b*P* < 0.001 vs PHP (placebo); ^a*P* < 0.05 vs SHP (NS-398) and *P* < 0.001 vs PHAs (placebo); C: Plasmatic PGE₂ concentrations (expressed in ng/mL): ^a*P* < 0.05 vs PHP (placebo); D: Plasmatic LTB₄ concentrations (expressed in ng/mL).

with the selective COX 2 inhibitor, NS-398, ULDA induced no further effect. There is an increase in TXB₂ in the portal hypertensive group with COX 2 inhibition, in which a decreased IHT was observed. Factors like trauma-hemorrhage, shear stress and pressure variations or lipopolysaccharide can modify TXA₂ synthesis in the liver or in endothelial cells^[19-21]. The increased TXB₂ was not modified by treatment with ULDA. It is interesting to have, in the portal hypertensive group, an increased PGE₂ after COX 1 inhibition and an increased TXB₂ after COX 2 inhibition, as if upon COX selective inhibition, the cell switched to a prostanoid produced by the non inhibited COX enzyme.

The effect of ULDA was confirmed in this study with a prehepatic portal hypertension with a normal liver. Further research will clarify if this potentially beneficial effect is produced in rats with cirrhosis and ascities. Other complex interactions can not be evaluated by this study. For example, a recent publication has pointed out that chronic COX inhibition with Indomethacin enhances the collateral vascular responsiveness to Arginin-Vasopressin, which is also able to activate platelets^[22,23].

In conclusion, despite that COX 1 inhibition caused a mild antithrombotic effect in sham operated rats, the prothrombotic effect of ULDA was not modified. COX 2 inhibition induced a mild prothrombotic effect over portal hypertensive rats, similar to that observed with ULDA alone confirming data of literature^[24] and the addition of ULDA in this group produced no further changes. ULDA induced a decrease in PGI₂ in portal hypertensive animals, without modifying TXA₂ levels. These results suggested a predominant COX 2 inhibition by ULDA, opposite to the predominant inhibition of COX 1 commonly observed with ASA in usual doses.

COMMENTS

Background

Ultra Low Dose Aspirin (ULDA) has shown prothrombotic properties capable of normalizing altered platelet function found in portal hypertension. This effect is clearly the opposite of the actual main use of Aspirin as an antithrombotic drug.

Research frontiers

The mechanism of this effect is unknown, but previous publications show changes in this effect after pretreatment with Indomethacin, a widely used non-selective COX inhibitor.

Innovation and breakthroughs

Only inhibition of NO synthesis, and perhaps Vasopressin has shown this property of modifying platelet alterations in portal hypertension.

Applications

ULDA could be useful in patients with prehepatic portal hypertension to control or decrease bleeding complications.

Peer review

Aspirin is widely known as a more powerful inhibitor of COX 1 than COX 2. This is the first paper showing a positive effect of Aspirin in portal hypertension, based in a not yet explained inhibition of COX 2, rather than COX 1.

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Induction of ischemic tolerance in rat liver *via* reduced nicotinamide adenine dinucleotide phosphate oxidase in Kupffer cells

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Abstract

AIM: To elucidate the mechanisms of hepatocyte preconditioning by H₂O₂ to better understand the pathophysiology of ischemic preconditioning.

METHODS: The *in vitro* effect of H₂O₂ pretreatment was investigated in rat isolated hepatocytes subjected to anoxia/reoxygenation. Cell viability was assessed with propidium iodide fluorometry. In other experiments, rat livers were excised and subjected to warm ischemia/reperfusion in an isolated perfused liver system to determine leakage of liver enzymes. Preconditioning was performed by H₂O₂ perfusion, or by stopping the perfusion for 10 min followed by 10 min of reperfusion. To inhibit Kupffer cell function or reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, gadolinium chloride was injected prior to liver excision, or diphenyleneiodonium, an inhibitor of NADPH oxidase, was added to the perfusate, respectively. Histological detection of oxygen radical formation in Kupffer cells was performed by perfusion with nitro blue tetrazolium.

RESULTS: Anoxia/reoxygenation decreased hepatocyte viability compared to the controls. Pretreatment with H₂O₂ did not improve such hepatocyte injury. In liver perfusion experiments, however, H₂O₂ preconditioning reduced warm ischemia/reperfusion injury, which was

reversed by inhibition of Kupffer cell function or NADPH oxidase. Histological examination revealed that H₂O₂ preconditioning induced oxygen radical formation in Kupffer cells. NADPH oxidase inhibition also reversed hepatoprotection by ischemic preconditioning.

CONCLUSION: H₂O₂ preconditioning protects hepatocytes against warm ischemia/reperfusion injury *via* NADPH oxidase in Kupffer cells, and not directly. NADPH oxidase also mediates hepatoprotection by ischemic preconditioning.

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Key words: Diphenyleneiodonium chloride; Ischemia/reperfusion injury; Ischemic preconditioning; Liver transplantation; Oxygen radicals

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INTRODUCTION

The interruption of hepatic blood flow followed by reperfusion, called ischemia/reperfusion, causes liver injury in a number of clinical interventions such as liver transplantation, hepatic resection, abdominal surgery with hepatic vascular occlusion, and hypovolemic shock^[1-4]. Especially in liver transplantation, ischemia/reperfusion injury can lead to the primary dysfunction of liver allografts; still the most feared complication after liver transplantation because of the associated high mortality, and the fact that there is no treatment other than retransplantation^[5-7]. The pathophysiology and substantial mechanisms of hepatic ischemia/reperfusion injury are increasingly well understood, and recent studies have drawn attention to ischemic preconditioning as one therapeutic strategy to decrease any injury^[4,8-10]. Ischemic preconditioning is a phenomenon by which brief periods of ischemia followed by reperfusion render tissues

resistant to subsequent prolonged ischemia/reperfusion. This phenomenon was originally characterized in a canine model of myocardial ischemia^[11], and has since been recognized in hepatic injury after ischemia/reperfusion in animal models^[12-14], as well as in humans^[15-18].

We have recently reported that hepatoprotection of ischemic preconditioning in rats is mediated by reactive oxygen species (ROS) produced by Kupffer cells. Moreover, we have shown that hepatic pretreatment with a sublethal dose of H₂O₂ mimics the hepatoprotective effect of ischemic preconditioning^[19]. Since H₂O₂ is produced as a consequence of ROS metabolism, it may be possible that H₂O₂ resulting from ROS production in Kupffer cells directly affects hepatocytes, and induces their adaptive response to ischemia/reperfusion injury. Therefore, in this study we sought to elucidate the mechanisms of H₂O₂ preconditioning to better understand the pathophysiology of ischemic preconditioning. We report here that H₂O₂ preconditioning did not protect hepatocytes directly, but via Kupffer cells, as in ischemic preconditioning. We also noted that the activation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in Kupffer cells may play an important role in hepatoprotection of H₂O₂, as well as in ischemic preconditioning.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 180-240 g were maintained on a commercial pellet diet and water *ad libitum*, in a room under normal lighting conditions. All animals received humane care in compliance with our institutional and National Institutes of Health guidelines.

In vitro experiments

Isolation of hepatocytes: Rat hepatocytes were isolated and cultured as previously described^[20]. Rat livers were minced after perfusion with 0.8 mg/mL collagenase (type I; Worthington Biochemical Corporation, Lakewood, NJ, USA) through the portal vein. Hepatocytes were separated from non-parenchymal cells by centrifugation at $50 \times g$ for 2 min at 4°C. The viability of the collected hepatocytes was $\geq 95\%$, as determined by the trypan blue exclusion test. Hepatocytes were resuspended in William's E medium containing 100 mL/L fetal calf serum, 27 mmol/L NaHCO₃, 100 nmol/L insulin, and 10 nmol/L dexamethasone at pH 7.4. Hepatocytes were then plated on type I collagen-coated 96-multiwell plates (Asahi Techno Glass Corporation, Tokyo, Japan) at a density of 4×10^4 cells/well and incubated overnight in an atmosphere of 95% air/5% CO₂ at 37°C.

Treatment of isolated hepatocytes: Isolated hepatocytes were treated by incubation for 10 min at 37°C with 100 μ L Krebs-Ringer N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (KRH) at pH 7.4 containing various concentrations of H₂O₂, and washed twice with KRH. We set doses of H₂O₂ up to 1 mmol/L in accordance with earlier liver perfusion experiments^[19]. Hepatocytes were then incubated in an anaerobic

chamber under anoxic conditions using an AnaeroPack (Mitsubishi Gas Chemical Corporation, Tokyo, Japan), and subsequently subjected to reoxygenation at 37°C. The oxygen concentration in the chamber decreased to $< 0.005\%$ within 30 min after insertion of AnaeroPack. Control hepatocytes were cultured under normoxic conditions for the same length of time at 37°C.

Cell viability assay: Cell viability was determined by propidium iodide fluorometry as previously described, with some modification^[21]. Hepatocytes were incubated in 100 μ L KRH containing 30 μ mol/L propidium iodide, after H₂O₂ treatment. Fluorescence from each well was measured immediately before anoxic incubation and then at given times after reoxygenation. Cell viability after reoxygenation was calculated, with final fluorescence corresponding to the 100% cell death obtained by addition of 350 μ mol/L digitonin. We used a Cytofluor S4000 fluorescence reader (Applied Biosystems, Stafford, TX, USA), employing 530 nm excitation and 645 nm emission filters.

Liver perfusion experiments

Preconditioning before warm ischemia/reperfusion injury: Rats were anesthetized intraperitoneally with 50 mg/kg pentobarbital sodium, and the abdomen was opened. Livers were perfused through the portal vein with Krebs-Henseleit bicarbonate buffer (KHB) saturated with 95% O₂ and 5% CO₂ at 30 mL/min for 10 min at 37°C. For H₂O₂ preconditioning, livers were perfused with KHB containing 1 mmol/L H₂O₂ for 10 min, and then perfused with KHB for 2 min to wash out H₂O₂, as previously described^[19]. For ischemic preconditioning, the perfusion was stopped for 10 min, followed by reperfusion for 10 min. In control rats, livers were manipulated similarly except for adding H₂O₂ or stopping the perfusion, respectively.

Drug treatments: In some rats, 20 mg/kg gadolinium chloride (GdCl₃; Wako Pure Chemical Industries, Tokyo, Japan) was injected intravenously at 24 h before H₂O₂ preconditioning, under light ether anesthesia to inhibit Kupffer cell function^[22,23]. GdCl₃ was dissolved in saline, and control rats were injected with the appropriate vehicle solution. The efficacy of GdCl₃ injection was confirmed by immunohistochemical staining of liver sections with anti-ED2 antibody in the preliminary experiments. In other rats, diphenyleneiodonium chloride (DPI; Sigma-Aldrich, St. Louis, MO, USA), an inhibitor of NADPH oxidase, at a final concentration of 10 μ mol/L, was added to the perfusate^[24] during H₂O₂ preconditioning, or the reperfusion period after ischemic preconditioning. Livers were then perfused with KHB for 10 min to wash out DPI. DPI was dissolved in dimethyl sulfoxide (DMSO) at 20 mmol/L. Livers of vehicle-treated rats were perfused with KHB containing 500 μ L/L DMSO during each preconditioning.

Warm ischemia/reperfusion: After H₂O₂ preconditioning or ischemic preconditioning, livers were stored in KHB

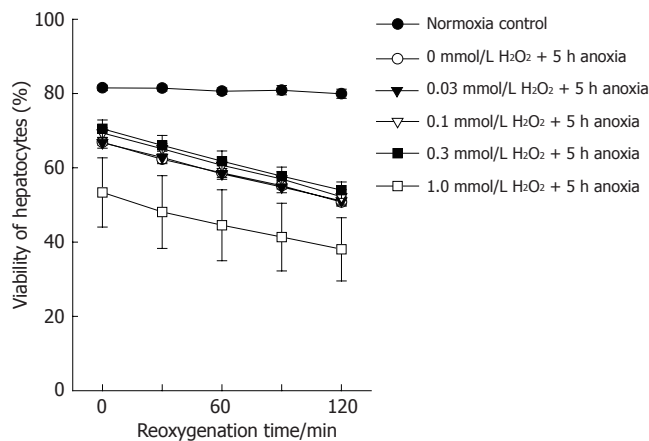


Figure 1 Viability of hepatocytes after anoxia/reoxygenation. (●): Viability of hepatocytes cultured under normoxia consistently. Viability of hepatocytes, pretreated with (○): 0 mmol/L; (▼): 0.03 mmol/L; (▽): 0.1 mmol/L; (■): 0.3 mmol/L; or (□): 1.0 mmol/L of H₂O₂ for 10 min before anoxia/reoxygenation.

at 37°C as previously described^[19]. After 40 min storage, livers were reperused in a recirculating system with 200 mL KHB saturated with 95% O₂ and 5% CO₂ at 37°C. The perfusate was collected after 60 min of reperfusion for determination of alanine aminotransferase (ALT) and lactate dehydrogenase (LDH) activity.

Biochemical assays: Commercial kits (Wako Pure Chemical Industries) were used to determine ALT and LDH activity in the perfusates.

Determination of oxygen radical formation in Kupffer cells after H₂O₂ preconditioning

Livers were perfused through the portal vein with KHB for 10 min, and then perfused with KHB containing 1 mmol/L H₂O₂ for 10 min. Subsequently, the livers were perfused with KHB containing 500 mg/L nitro blue tetrazolium (NBT; Sigma-Aldrich) for 10 min. Livers were then fixed by infusion of 10% formalin, embedded in paraffin, sectioned and stained with nuclear fast red. As formazan deposition is formed by the reaction of NBT with oxygen radicals in Kupffer cells, the number of formazan-positive cells was determined as the mean in 10 different areas of each section, observed by light microscopy at × 400 magnification (high power field).

Statistical analysis

All values are expressed as means ± SE. The difference between the means was analyzed with Student's *t* test, after confirming that the data passed normal distribution and equal-variance tests. *P* < 0.05 was considered significant.

RESULTS

Effect of H₂O₂ preconditioning on anoxia/reoxygenation injury of isolated hepatocytes

In the preliminary experiments, the viability of hepatocytes exposed to anoxia for > 3–4 h decreased after reoxygenation, compared to cells consistently cultured under normoxic conditions. Anoxic culture for

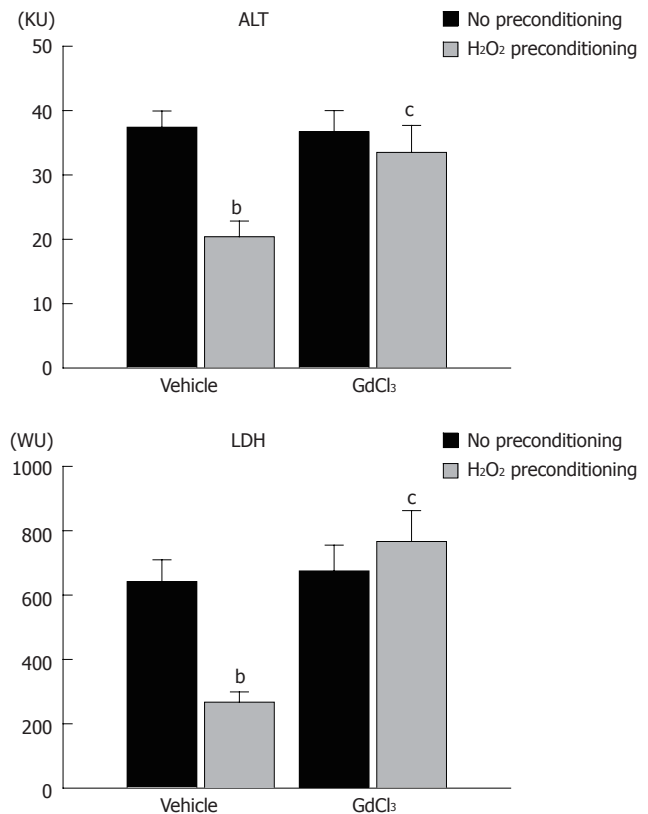


Figure 2 ALT and LDH activities in perfusate after ischemia/reperfusion in an isolated perfused liver system from no preconditioning control, H₂O₂ preconditioning, GdCl₃-no preconditioning, and GdCl₃/H₂O₂ preconditioning rats. Columns and bars represent means ± SE from six rats in each group. ^b*P* < 0.01 vs no preconditioning, ^c*P* < 0.05 vs vehicle treatment and H₂O₂ preconditioning. KU: Karmen unit; WU: Wróblewski unit.

6 h resulted in a marked and rapid loss of cell viability after reoxygenation. Accordingly, we set the anoxic time to 5 h, and determined cell viability serially for 2 h after reoxygenation. As shown in Figure 1, anoxia/reoxygenation decreased hepatocyte viability linearly, though the viability of normoxic controls was not affected during the corresponding period. Preincubation with 0–1 mmol/L H₂O₂ did not improve such anoxia/reoxygenation injury, and the highest concentration of H₂O₂ somewhat worsened cell viability.

Contribution of Kupffer cells to hepatocyte protection by H₂O₂ preconditioning

The results of the *in vitro* experiments suggested that H₂O₂ did not directly protect hepatocytes. We determined the contribution of Kupffer cells in H₂O₂ preconditioning, using an isolated perfused liver system. In untreated controls, which were perfused for the corresponding period without ischemia/reperfusion, ALT and LDH activity in the perfusate was 4.4 ± 0.6 and 35.1 ± 7.9, respectively (data not shown). As shown in Figure 2, warm ischemia/reperfusion markedly increased ALT and LDH activity in the perfusate to 37.4 ± 2.5 and 642 ± 68, respectively, with H₂O₂ preconditioning significantly reducing these values. GdCl₃ pretreatment itself did not change ALT and LDH activity after warm ischemia/reperfusion. In the H₂O₂-preconditioned group, however,

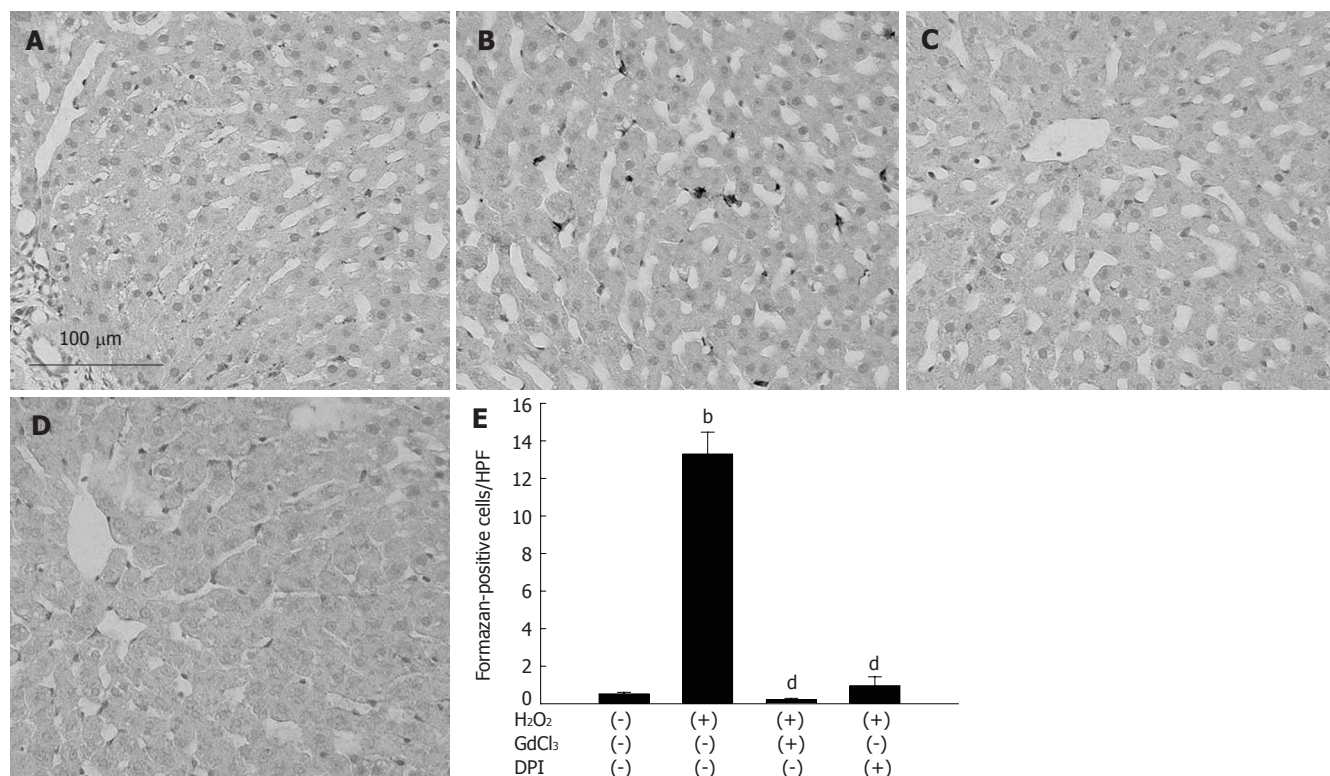


Figure 3 Oxygen radical formation in Kupffer cells after H₂O₂ preconditioning. **A:** Non-preconditioned livers; **B:** Livers after 10 min of H₂O₂ perfusion; **C:** Pretreatment with GdCl₃ and H₂O₂ perfusion; **D:** Treatment with DPI and H₂O₂ perfusion; **E:** The number of formazan-positive cells. Columns and bars represent means \pm SE from 3 rats in each group. ^b $P < 0.01$ vs non-preconditioned control; ^d $P < 0.01$ vs H₂O₂ perfusion.

GdCl₃ pretreatment reversed the decrease in ALT and LDH activity by H₂O₂ preconditioning.

Production of oxygen radicals in Kupffer cells after H₂O₂ preconditioning

To elucidate the mechanisms of the contribution of Kupffer cells in H₂O₂ preconditioning, we determined oxygen radical production in Kupffer cells. Livers were perfused with H₂O₂ for 10 min and then perfused with NBT. When non-preconditioned livers were perfused with NBT, formazan deposition did not develop (Figure 3A). As shown in Figure 3B, H₂O₂ perfusion induced formazan deposition in non-parenchymal cells. When rats were pretreated with GdCl₃ injection, H₂O₂ perfusion did not induce such dense deposition (Figure 3C). Moreover, formazan deposition did not develop after H₂O₂ perfusion in livers in which DPI was added to the perfusate during H₂O₂ perfusion (Figure 3D). The number of formazan-positive cells from three rats in each group is shown in Figure 3E; treatment with GdCl₃ or DPI significantly reduced the number of formazan-positive cells in H₂O₂-perfused livers to the non-preconditioned control level.

Contribution of NADPH oxidase to hepatoprotection by H₂O₂ preconditioning

To determine the contribution of NADPH oxidase to the effect of H₂O₂ preconditioning, DPI was added to the perfusate during H₂O₂ preconditioning, and livers were then subjected to warm ischemia/reperfusion. We confirmed that H₂O₂ preconditioning decreased ALT and LDH activity after warm ischemia/reperfusion in

a reproducible fashion. DPI treatment reversed this decrease in ALT and LDH activity induced by H₂O₂ preconditioning, although without H₂O₂ preconditioning, DPI alone did not change these values (Figure 4).

Contribution of NADPH oxidase to hepatoprotection by ischemic preconditioning

Finally, we determined whether DPI treatment could also reverse the protective effect of ischemic preconditioning. Ischemic preconditioning was performed with buffer containing DPI and livers were subjected to warm ischemia/reperfusion. As shown in Figure 5, ischemic preconditioning significantly reduced the increase in ALT and LDH activity after warm ischemia/reperfusion, with DPI reversing the hepatoprotection induced by ischemic preconditioning.

DISCUSSION

We have previously reported that brief liver perfusion with a buffer containing a low dose of H₂O₂ reduces warm ischemia/reperfusion injury similar to that with ischemic preconditioning^[19]. However, the mechanisms of such H₂O₂ preconditioning have not been clarified. Since H₂O₂ is produced as a consequence of ROS metabolism, and is now considered to be involved in not only pathological, but also physiological mechanisms^[25-27], it may be that H₂O₂ derived from Kupffer cells induces adaptation of hepatocytes to ischemia/reperfusion injury. Accordingly, in the present study, we sought to determine whether pretreatment with H₂O₂ directly protects

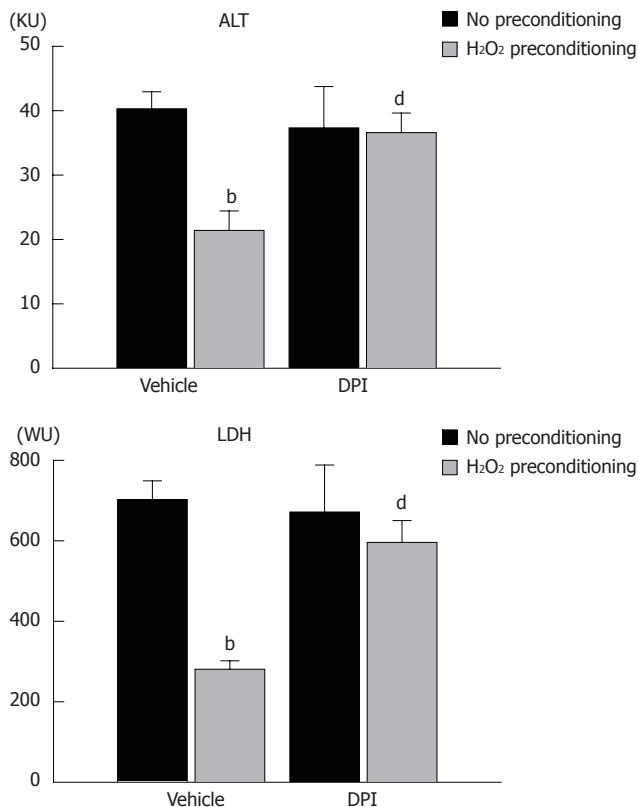


Figure 4 ALT and LDH activities in perfusate after ischemia/reperfusion in an isolated perfused liver system from no preconditioning control, H₂O₂ preconditioning, DPI-no preconditioning, and DPI/H₂O₂ preconditioning rats. Columns and bars represent means \pm SE from eight rats in each group. ^b*P* < 0.01 vs no preconditioning; ^d*P* < 0.01 vs vehicle treatment and H₂O₂ preconditioning.

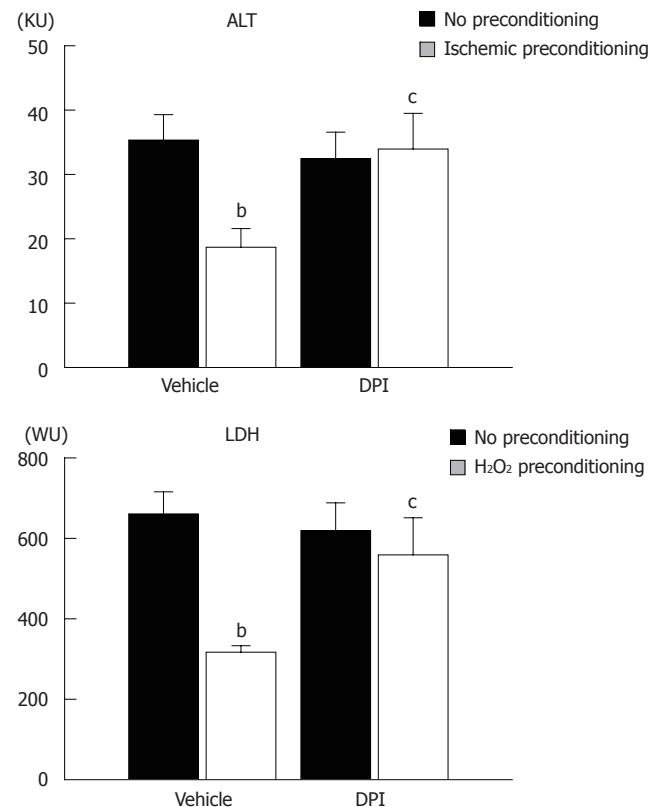


Figure 5 ALT and LDH activity in perfusate after ischemia/reperfusion in an isolated perfused liver system from no preconditioning control, ischemic preconditioning, DPI-no preconditioning, and DPI-ischemic preconditioning rats. Columns and bars represent means \pm SE from six rats in each group. ^b*P* < 0.01 vs no preconditioning; ^c*P* < 0.05 vs vehicle treatment and ischemic preconditioning.

isolated hepatocytes against subsequent warm ischemia/reperfusion injury.

To simulate ischemia/reperfusion injury *in vitro*, isolated rat hepatocytes were incubated under anoxic conditions using an anaerobic chamber, and subsequently subjected to reoxygenation, following a widely used model^[28,29]. Cell viability after reoxygenation was assessed with propidium iodide fluorometry. Propidium iodide binds to the double-stranded nucleic acids of permeable, non-viable cells^[21], and the fluorescence is linearly related to LDH release^[30]. Therefore, the changes in fluorescent intensity after anoxia/reoxygenation possibly represent *in vitro* ischemia/reperfusion injury. As shown in Figure 1, the viability of hepatocytes decreased slowly during anoxia and then rapidly after reoxygenation, compared with cells consistently maintained under normoxic conditions. These results were similar to those previously reported that showed necrotic hepatocytes after anoxia/reoxygenation^[29]. We pretreated cells with H₂O₂ before such anoxia/reoxygenation injury; however, the viability of hepatocytes was not improved with any of the concentrations of H₂O₂ examined. Pretreatment with 1 mmol/L H₂O₂ for 10 min even worsened hepatocyte viability, although liver perfusion with the same concentration of H₂O₂ for 10 min reduced warm ischemia/reperfusion injury in our earlier study^[19]. As some reports have indicated that isolated hepatocytes have lower tolerance to H₂O₂ than those *in vivo*, because of the absence of a sinusoidal structure^[31-33], the

present results might suggest that the effective dose of H₂O₂ in *in vitro* conditions is different from that during perfusion experiments. However, another interpretation is that sublethal H₂O₂ concentrations did not directly protect hepatocytes against warm ischemia/reperfusion injury.

Based on the hypothesis that Kupffer cells mediate hepatoprotection by H₂O₂ preconditioning, we performed liver perfusion experiments using rats in which Kupffer cell function was inhibited by GdCl₃ injection^[22,23]. As shown in Figure 2, GdCl₃ alone did not change ischemia/reperfusion injury, which showed that Kupffer cells were not involved in the development of warm ischemia/reperfusion injury in our model. Accordingly, it is suggested that hepatoprotection by H₂O₂ preconditioning cannot be ascribed to the suppression of the deleterious function of Kupffer cells. Moreover, GdCl₃ reversed the effect of H₂O₂ preconditioning. As previously recognized, inhibition of Kupffer cell function also reversed the effect of ischemic preconditioning^[19]. Therefore, we concluded that H₂O₂ preconditioning protected hepatocytes via Kupffer cells, as in ischemic preconditioning.

Kupffer cells are the principal source of oxygen radicals after prolonged hepatic ischemia/reperfusion^[34]. Moreover, even after a brief period of ischemia, Kupffer cells predominantly produce oxygen radicals^[19]. If H₂O₂ preconditioning protects hepatocytes via stimulation of Kupffer cells, as in ischemic preconditioning, a brief period of perfusion with H₂O₂ may induce oxygen radical

formation in Kupffer cells. As NBT reacts with oxygen radicals to form insoluble blue formazan, the NBT perfusion technique enables one to evaluate oxygen radical production *in situ*^[35,36]. As shown in Figure 3, histological analysis indicated that H₂O₂ perfusion stimulated Kupffer cells to produce oxygen radicals. It has been reported that structural changes in Kupffer cells, or transient increases in the phagocytosis of Kupffer cells, is induced by perfusion with 0.7 mmol/L H₂O₂ for 10 min or 1 mmol/L H₂O₂ for 5 min, respectively^[33,37]. These observations support our present findings; that stimulation of Kupffer cells with 1 mmol/L H₂O₂ for 10 min produced oxygen radicals.

Kupffer cells generate oxygen radicals by activating NADPH oxidase from a wide variety of stimulations^[38-40]. However, it has never been determined whether H₂O₂ activates NADPH oxidase in Kupffer cells. Therefore, we sought to determine the contribution of NADPH oxidase to oxygen radical production in Kupffer cells by brief H₂O₂ perfusion. As shown in Figure 3, oxygen radical formation induced by H₂O₂ perfusion was eliminated by inhibition of NADPH oxidase with DPI. Some of these histological findings suggested that even brief H₂O₂ perfusion induced oxygen radical formation via NADPH oxidase in Kupffer cells. The active NADPH oxidase complex, which is bound to the plasma membrane in Kupffer cells, primarily produces superoxide anion radicals by reducing extracellular oxygen with electrons from cytosolic NADPH^[40-42]. Since DPI acts on the cytosolic component of NADPH oxidase^[43] and H₂O₂ permeates the cell membrane^[41,44], an increase in intracellular H₂O₂ concentration by H₂O₂ treatment might act on NADPH oxidase in Kupffer cells. Subsequently, we examined the contribution of NADPH oxidase to the effect of H₂O₂ preconditioning against warm ischemia/reperfusion injury. As shown in Figure 4, inhibition of NADPH oxidase reversed the hepatoprotective effect of H₂O₂ preconditioning. Taken together with histological findings, the results indicate that NADPH oxidase in Kupffer cells mediates hepatocyte protection in H₂O₂ preconditioning.

Finally, we determined whether NADPH oxidase contributes to the effect of ischemic preconditioning. As shown in Figure 5, hepatocyte protection in ischemic preconditioning was also reversed by inhibition of NADPH oxidase. We concluded that H₂O₂ preconditioning, as well as ischemic preconditioning protects hepatocytes via NADPH oxidase in Kupffer cells. Moreover, the present results suggest that extracellular superoxide anion radicals produced by NADPH oxidase in Kupffer cells play a crucial role in hepatocyte protection by both H₂O₂ and ischemic preconditioning. Extracellular free radicals do not permeate the cell membrane^[41,44] and directly oxidize the lipid bilayer of the cell membrane^[45]. Thus, experiments to investigate the contribution of lipid peroxidation to hepatocyte preconditioning against ischemia/reperfusion injury are presently underway in our laboratory.

In conclusion, we report, to the best of our knowledge, a novel finding that hepatocyte protection against warm ischemia/reperfusion injury was achieved via NADPH oxidase in Kupffer cells. This was induced by preconditioning with a sublethal dose of H₂O₂

perfusion or brief ischemia/reperfusion. Based on the results of an earlier study, we assumed that a sublethal dose of H₂O₂ directly set off hepatocyte protection against ischemia/reperfusion injury. However, the present results reaffirmed the importance of Kupffer cells in induction of ischemic tolerance in liver. In general, Kupffer cells and their production of oxidative stress are considered to be a detrimental factor in hepatic ischemia/reperfusion injury^[1,3]. However, under sublethal conditions, they actually could play a principal role in hepatocyte preconditioning against such injury. According to this concept, to obtain hepatoprotection, Kupffer cell activation may have to be strictly controlled between a steady-state level and a stimulated injurious level. Investigations to elucidate how sublethal oxidative stress induces these protective effects on hepatocytes are needed to determine any clinical application.

ACKNOWLEDGMENTS

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COMMENTS

Background

As a therapeutic strategy against hepatic ischemia/reperfusion injury, which can occur in a number of clinical settings such as liver transplantation and hepatic resection, recent studies have drawn attention to ischemic preconditioning. Ischemic preconditioning is a phenomenon in which brief periods of ischemia followed by reperfusion render tissues resistant to subsequent prolonged ischemia/reperfusion. The authors have previously shown that reactive oxygen species derived from Kupffer cells mediate the hepatoprotection induced by ischemic preconditioning, and pretreatment with a sublethal dose of H₂O₂ mimics such hepatoprotection. However, the mechanism of H₂O₂ preconditioning or the role of H₂O₂ in the preconditioning phenomenon has not been determined.

Research frontiers

Clinical investigations of ischemic preconditioning in human liver resection and transplantation have recently been reviewed (*World J Gastroenterology* 2007; 13: 657-670). New findings about the beneficial role of reactive oxygen species in some experimental settings are increasingly being reported.

Innovations and breakthroughs

Cell-specific investigations of ischemic preconditioning have rarely been reported. The authors focused on Kupffer cells and their production of reactive oxygen species, which are in general regarded as detrimental factors in hepatic injury, and showed that, paradoxically, they mediated hepatoprotection induced by ischemic preconditioning. In the present article, we also demonstrated that superoxide anion radicals produced by NADPH oxidase in Kupffer cells were implicated in the hepatoprotective effects of ischemic and H₂O₂ preconditioning.

Applications

Investigations into the mechanisms of ischemic preconditioning will enable the establishment of a pharmacological preconditioning regime before liver transplantation or hepatic surgery, leading to a reduction in ischemia/reperfusion injury.

Terminology

Interruption of tissue blood flow followed by reperfusion causes tissue injury, called ischemia/reperfusion injury. Ischemic preconditioning is a phenomenon whereby brief periods of ischemia followed by reperfusion render tissues resistant to subsequent ischemia/reperfusion injury.

Peer review

This is an interesting study, well written and well designed. The methods are sound. The discussions are to the point and not overstated.

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Protective effects and mechanisms of Baicalin and octreotide on renal injury of rats with severe acute pancreatitis

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Abstract

AIM: To investigate the protective effects and mechanisms of Baicalin and octreotide on renal injury of rats with severe acute pancreatitis (SAP).

METHODS: One hundred and eighty SD rats were randomly assigned to the model group, Baicalin-treated group, octreotide-treated group and sham operation group. The mortality, plasma endotoxin level, contents of blood urea nitrogen (BUN), creatinine (CREA), phospholipase A₂ (PLA₂), nitrogen monoxide (NO), tumor necrosis factor (TNF)- α , IL-6 and endothelin-1 (ET-1) in serum, expression levels of renal Bax and Bcl-2 protein, apoptotic indexes and pathological changes of kidney were observed at 3, 6 and 12 h after operation.

RESULTS: The renal pathological changes were milder in

treated group than in model group. The survival at 12 h and renal apoptotic indexes at 6 h were significantly ($P < 0.05$) higher in treated group than in model group [66.67% *vs* 100%; 0.00 (0.02)% and 0.00 (0.04)% *vs* 0.00 (0.00)%, respectively]. The serum CREA content was markedly lower in octreotide-treated group than in model group at 3 h and 6 h ($P < 0.01$, 29.200 ± 5.710 $\mu\text{mol/L}$ *vs* 38.400 ± 11.344 $\mu\text{mol/L}$; $P < 0.05$, 33.533 ± 10.106 $\mu\text{mol/L}$ *vs* 45.154 ± 17.435 $\mu\text{mol/L}$, respectively). The expression level of renal Bax protein was not significantly different between model group and treated groups at all time points. The expression level of renal Bcl-2 protein was lower in Baicalin-treated group than in model group at 6 h [$P < 0.001$, 0.00 (0.00) grade score *vs* 3.00 (3.00) grade score]. The Bcl-2 expression level was lower in octreotide-treated group than in model group at 6 h and 12 h [$P < 0.05$, 0.00 (0.00) grade score *vs* 3.00 (3.00) grade score; 0.00 (0.00) grade score *vs* 0.00 (1.25) grade score, respectively]. The serum NO contents were lower in treated groups than in model group at 3 h and 12 h [$P < 0.05$, 57.50 (22.50) and 52.50 (15.00) $\mu\text{mol/L}$ *vs* 65.00 (7.50) $\mu\text{mol/L}$; $P < 0.01$, 57.50 (27.50) and 45.00 (12.50) $\mu\text{mol/L}$ *vs* 74.10 (26.15) $\mu\text{mol/L}$, respectively]. The plasma endotoxin content and serum BUN content (at 6 h and 12 h) were lower in treated groups than in model group. The contents of IL-6, ET-1, TNF- α (at 6 h) and PLA₂ (at 6 h and 12 h) were lower in treated groups than in model group [$P < 0.001$, 3.031 (0.870) and 2.646 (1.373) pg/mL *vs* 5.437 (1.025) pg/mL; 2.882 (1.392) and 3.076 (1.205) pg/mL *vs* 6.817 (0.810) pg/mL; 2.832 (0.597) and 2.462 (1.353) pg/mL *vs* 5.356 (0.747) pg/mL; 16.226 (3.174) and 14.855 (5.747) pg/mL *vs* 25.625 (7.973) pg/mL; 18.625 (5.780) and 15.185 (1.761) pg/mL *vs* 24.725 (3.759) pg/mL; 65.10 (27.51) and 47.60 (16.50) pg/mL *vs* 92.15 (23.12) pg/mL; 67.91 ± 20.61 and 66.86 ± 22.10 U/mL, 63.13 ± 26.31 and 53.63 ± 12.28 U/mL *vs* 101.46 ± 14.67 and 105.33 ± 18.10 U/mL, respectively].

CONCLUSION: Both Baicalin and octreotide can protect the kidney of rats with severe acute pancreatitis. The therapeutic mechanisms of Baicalin and octreotide might be related to their inhibition of inflammatory mediators and induction of apoptosis. Baicalin might be a promising therapeutic tool for severe acute pancreatitis.

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Key words: Severe acute pancreatitis; Baicalin; Octreotide; Renal injury; Rats; Tissue microarrays

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INTRODUCTION

Severe acute pancreatitis (SAP) is a fatal systemic disease featuring acute onset, serious conditions, high incidence of complications and 20%-30% of mortality mainly due to multiple organ failure at its early stage^[1-4]. Octreotide has been shown to exert its therapeutic effects on SAP mainly *via* inhibiting pancreatin secretion, release of inflammatory mediators and platelet aggregation, and reducing endotoxin generation^[5-8]. It is found to improve prognosis and lower mortality by enhancing the kidney protection during SAP. Baical skullcap root is an essential of "Qingyitang", a representative prescription of Traditional Chinese Medicine for SAP. Baicalin is its main effective ingredient (monomer). The *in vitro* experiment of Baicalin has confirmed it has anti-bacterial, antiviral and anti-inflammatory activities. It also can inhibit platelet aggregation and eliminate oxygen-free radicals. It was found in animal experiments that Baicalin could reduce the generation of endotoxin. In addition, Baicalein, which is the initial metabolite of Baicalin, has potent effect in inhibiting pancreatin. All these pharmacologic actions can inhibit SAP during its multiple stages^[9]. It is difficult to popularize octreotide especially in remote areas with poor economy since it features high price, short half-life and inconvenient administration, while Baicalin features low price, extensive routes of administration and preparation, multiple pharmacologic actions and precise therapeutic effects.

The idea of Baicalin treatment of pancreatitis was brought forward in 1999 and validated in 2000. At the beginning, the one-time Baicalin injection *via* vena dorsalis penis or vena femoralis injection was applied, which resulted in poor therapeutic effects. Later, it was found one-time injection was inappropriate due to the short half-life of Baicalin. The expected therapeutic effects could hardly be met with one-time injection. In 2001, the intravenous drip and large dosage were applied, which resulted in sound therapeutic effects. The idea was originated from the study of the principal on pancreatitis treated by Baicalein injection. Baicalin is hydrolyzed into Baicalein. The stability, solubility and therapeutic effects of Baicalin injection are all superior to those of Baicalein injection. In this experiment, the feasibility of Baicalin treatment for SAP has been studied by comparing the protective effects and mechanisms of Baicalin and octreotide on kidneys of rats with SAP.

MATERIALS AND METHODS

Experimental animals

Clean grade healthy male Sprague-Dawley (SD) rat, weighing 250-300 g, were purchased from the Experimental

Animal Center of Medical School of Zhejiang University, China.

Experimental reagents

Sodium taurocholate and sodium pentobarbital were purchased from USA Sigma Company. Octreotide was purchased from Swiss Pharmaceutical Company Novartis, and 5% Baicalin injection (China National Invention Patent Number ZL200310122673.6) was prepared by the first author at 305 mmol/L osmotic pressure. Plasma endotoxin tachypleus amebocyte lysate kit was purchased from Shanghai Yihua Medical Science and Technology Corporation (Institute of Medical Analysis, Shanghai, China); the calculation unit for content is EU/mL. Serum nitrogen monoxide (NO) was purchased from Nanjing Jiancheng Bioengineering Research Institute; the calculation units for content is $\mu\text{mol/L}$. TNF- α ELISA kit was purchased from Jingmei Bioengineering Corporation; the calculation unit for content is pg/mL (ng/L). IL-6 ELISA kit was purchased from Shanghai Shenxiong Biotech Company (China); the calculation unit for content is pg/mL (ng/L). Serum secretory phospholipase A₂ enzyme Assay ELA kit (PLA₂) was purchased from R&D system Ins; the calculation unit for content is U/mL. The serum endothelin-1 ELA kit (ET-1) was purchased from Cayman Chemical Company (Catalog Number: 583151), the calculation unit for content is ng/L (pg/mL). The Bax and Bcl-2 antibodies were purchased from Santa Cruz Company, USA. The main reagents for DNA *in situ* nick end-labeling (TUNEL) staining (Takara *In Situ* Apoptosis Detection Kit) was purchased from TaKaRa Biotechnology (Dalian) Co., Ltd. PK (protease K) was purchased from Sigma Company (USA). DAB (biphenyldiamine) was purchased from China Hua-meimei Company, China.

Preparation of animal models

The improved Aho's method^[10] was adopted to prepare 135 SAP rat models *via* retrograde injection of 35 g/L sodium taurocholate to the pancreatic duct through epidural catheter and duodenal papilla. The 135 SAP rat models were randomly assigned to the model group, Baicalin-treated group and octreotide-treated group, 45 rats in each group, while other 45 rats were assigned to the sham operation group (SO group). In sham operation group, only exploratory laparotomy (i.e., entering abdominal cavity, checking the pancreas and duodenum and then abdomen closure) was performed. Thereafter, the above-mentioned groups were randomly subdivided into 3-h, 6-h and 12-h groups, 15 rats in each group. The rats were observed at 3, 6 and 12 h after operation for: (1) Mortalities of rats in all groups followed by execution of rats and observation of gross pathological changes of kidney; (2) Kidney tissue samples were collected, fixed in accordance with relevant requirements and observed for the pathological score changes of kidney under HE staining; (3) Tissue microarray was applied to prepare the tissue microarray sections (2 mm in diameter) and immunostained using SP (streptavidin-peroxidase) method. Expressions of Bax and Bcl-2 protein in the kidney tissue were observed under light microscope, and grading was carried out based on the percentage of positive cells as follows: (-) = positive

cell count < 10%, (+) = positive cell count 10%-20%, (++) = positive cell count 20%-50%, and (+++) = positive cell count > 50%; (4) TUNEL staining technique was applied to detect apoptotic cells in the kidney and then apoptotic index was calculated as follows: Apoptotic index = apoptotic cell count/total cell count \times 100%; (5) The changes in blood urea nitrogen (BUN), creatinine (CREA), phospholipase A₂ (PLA₂), nitrogen monoxide (NO), tumor necrosis factor (TNF)- α , IL-6 and endothelin-1 (ET-1) contents in blood samples obtained from the heart were determined; and (6) The correlations among these indexes were analyzed.

Procedures

Fast but water restraint was imposed on all rat groups 12 h prior to the operation. The rats were anesthetized by intra-peritoneal injection of 20 g/L sodium pentobarbital (0.25 mL/100 g), laid and fixed on table, routinely shaved, disinfected and draped. After establishing the right external jugular vein transfusion passage by using the microinfusion pump for continuous transfusion (1 mL/h per 100 g), 35 g/L sodium taurocholate was administered to prepare SAP model. To establish model control group, through median epigastrium incision, the bile-pancreatic duct and hepatic hilus common hepatic duct were confirmed, the pancreas was disclosed, the duodenal papilla inside the duodenum duct wall was identified, and then a No. 5 needle was used to drill a hole in the avascular area of mesentery. After inserting a segmental epidural catheter into the duodenal cavity *via* the hole, the bile-pancreatic duct was inserted toward the direction of papilla in a retrograde way, a microvascular clamp was used to nip the duct end temporarily and meanwhile another microvascular clamp was used to temporarily occlude the common hepatic duct at the confluence of hepatic duct. After connecting the anesthetic tube end with the transfusion converter, 35 g/L sodium taurocholate (0.1 mL/100 g) was transfused by retrograde transfusion using the micro-injection pump (made by Zhejiang University) at a speed of 0.2 mL/min. After 4 to 5 min post-injection, the micro-vascular clamp and epidural catheter were removed. After checking for bile leakage, the hole in the lateral wall of duodenum was sutured. The anesthetic in the abdominal cavity was absorbed up by disinfected cotton ball and then the abdomen was closed. Sham operation group received laparotomy *via* upper midline incision, turning over of the pancreas and duodenum and finally closure of the abdomen.

Dosage

In Baicalin-treated group, the animal experiments of 5% Baicalin injection have been completed including the acute toxicity test and SAP rat treatment by small, middle and large dose. The large dose (10 mg/h per 100 g) can achieve the best therapeutic effect and the dosage referred to the result of the previous preliminary experiment^[10]. Ten minutes after successful modeling, Baicalin-treated group was first injected 5% Baicalin injection (10 mg/100 g) *via* the external jugular vein, followed by continuous intravenous administration (10 mg/h per 100 g) by microinfusion pump. Octreotide-treated group was

first injected octreotide (0.2 μ g/100 g) *via* the external jugular vein, followed by continuous intravenous transfusion (10 mg/h per 100 g) by microinfusion pump at a transfusion speed of 0.2 μ g/h per 100 g. All above-mentioned dosages have been proved as effective dosages in the previous preliminary experiment^[10]. Both the sham operation group and model control group were injected normal saline of equivalent volume at the corresponding time points after operation. The diameter of the drilling needle is 2.0 mm.

Statistical analysis

The values were presented as mean \pm SD for normal distribution variables or median and quartile range for highly skewed variables. The significance of differences among the four groups was analyzed using Kruskal-Wallis test for highly skewed data and analysis of variance (ANOVA) for normal distribution data. Multiple comparisons were subjected to Bonferroni correction test. Chi-square test was used to evaluate equality of frequencies for discrete variables. Correlations were tested using Spearman rank correlation coefficients. A *P* value less than or equal to 0.05 was considered statistically significant. All statistical analyses were conducted using SPSS version 11.5 for windows.

RESULTS

Survival rate

The mortalities of model group were 0% (0/15), 13.33% (2/15) and 33.33% (5/15) at 3, 6 and 12 h, respectively, while those of Baicalin-treated group and octreotide-treated group were 0% at different time points, indicating a marked difference at 12 h (*P* < 0.05). The whole sham operation group survived at different time points.

Serum BUN content

Serum BUN content was markedly higher in model group and treated groups than in sham operation group at all time points (*P* < 0.001). However, the content was not significantly different between Baicalin- and octreotide-treated groups at all time points. The content was lower in Baicalin-treated group than in model group at 3 and 12 h (*P* < 0.05). The content was not different between octreotide-treated group and model group at 3 h. The content was lower in Baicalin-treated group than in model group at 6 h (*P* = 0.001), lower in octreotide-treated group than in model group (*P* < 0.05), and lower in octreotide-treated group than in model group at 12 h (*P* < 0.01) (Table 1).

Serum CREA content

The CREA content was significantly higher in model group and treated groups than in sham operation group at all time points (*P* < 0.001). However, no significant difference was found between Baicalin-treated group and model group at all time points. The content was lower in octreotide-treated group than in Baicalin-treated group at 3 h and 12 h (*P* < 0.01), and also lower in octreotide-treated group than in model group at 3 h (*P* < 0.01) and 6 h (*P* < 0.05). But no marked difference was observed between Baicalin-

Table 1 Comparison of different indexes level in blood [*M (Q_n)*]

		Sham operation group	Model group	Baicalin treated group	Octreotide treated group
Endotoxin (EU/mL)	3 h	0.016 (0.005)	0.053 (0.029)	0.027 (0.005)	0.033 (0.006)
	6 h	0.016 (0.010)	0.059 (0.037)	0.039 (0.019)	0.031 (0.010)
	12 h	0.014 (0.015)	0.060 (0.022)	0.034 (0.015)	0.042 (0.014)
BUN (mmol/L)	3 h	5.310 (0.940)	12.050 (4.030)	10.530 (3.625)	9.850 (3.020)
	6 h	5.500 (2.200)	17.390 (3.850)	12.220 (4.530)	13.930 (5.500)
	12 h	4.860 (1.590)	22.270 (11.375)	13.720 (4.380)	13.520 (9.810)
NO (μmol/L)	3 h	7.500 (5.000)	65.000 (7.50)	57.500 (22.50)	52.500 (15.00)
	6 h	7.500 (5.000)	62.500 (38.75)	47.500 (37.50)	57.500 (15.00)
	12 h		74.100 (26.15)	57.500 (27.50)	45.000 (12.50)
TNF-α (pg/mL)	3 h	3.900 (3.200)	41.440 (37.72)	44.930 (45.84)	39.300 (30.60)
	6 h	4.000 (1.700)	92.150 (23.12)	65.100 (27.51)	47.600 (16.50)
	12 h	5.3000 (3.000)	65.020 (26.81)	47.650 (25.52)	54.500 (41.40)
IL-6 (pg/mL)	3 h	1.846 (0.346)	5.437 (1.025)	3.031 (0.870)	2.646 (1.373)
	6 h	1.743 (0.838)	6.817 (0.810)	2.882 (1.392)	3.076 (1.205)
	12 h	2.036 (0.818)	5.356 (0.747)	2.832 (0.597)	2.462 (1.353)
ET-1 (pg/mL)	3 h	15.293 (4.231)	24.745 (1.011)	19.635 (6.065)	16.827 (3.775)
	6 h	16.275 (3.180)	25.625 (7.973)	16.226 (3.174)	14.855 (5.747)
	12 h	14.173 (2.556)	24.725 (3.759)	18.625 (5.780)	15.185 (1.761)

Table 2 Comparison of serum CREA content (mean ± SD, μmol/L)

Groups	3 h	6 h	12 h
Sham operation group	17.867 ± 2.890	21.467 ± 3.044	19.733 ± 3.150
Model group	38.400 ± 11.344	45.154 ± 17.435	41.500 ± 12.122
Baicalin-treated group	37.615 ± 9.483	39.867 ± 13.648	50.733 ± 29.310
Octreotide-treated group	29.200 ± 5.710	33.533 ± 10.106	33.933 ± 9.145

and octreotide-treated groups. Moreover, the content was not different between octreotide-treated group and model group at 12 h (Table 2).

Gross changes and light microscopic changes of kidney

Sham operation group: Macroscopically, the morphous of kidney was normal without swelling, with no bleeding points on surface of renal cortex. Microscopically, there were normal structure of renal glomerulus, tubule and interstitium in most rats without visible pathological change; however, swelling and blurry boundary of renal tubular epithelial cells, and stenosis of lumens were found in very few rats.

Model group: Macroscopically, there was no gross change in the kidney at 3 h; but were kidney swelling, tension of renal envelope, scattered bleeding points on surface of renal envelope, and slightly hemorrhagic urine in pelvis in severe cases at 6 h and 12 h. Microscopically, there were capillary congestion of renal glomerulus, swelling, scattered necrosis and blurry boundary of renal tubule epithelial cell, stenosis or atresia of lumens, visible protein cast (Figure 1A), interstitial edema and inflammatory cell infiltration at 3 h; and capillary congestion of renal glomerulus, swelling and scattered necrosis of epithelial cell of renal tubule (Figure 1B), interstitial edema (Figure 1C) and inflammatory cell infiltration at 6 and 12 h. The floss and red cell with eosinophilic stainings were found in renal glomerulus and homogenous or red cell cast with eosinophilic staining in renal tubule (Figure 1D).

There was lamellar necrosis of epithelial cell of renal tubule in few rats.

Baicalin- and octreotide-treated groups: Macroscopically, the gross renal pathological changes were milder in Baicalin- and octreotide-treated group than in model group at 6 h and 12 h. Microscopically, there were less capillary congestion of renal glomerulus, swelling of renal tubular epithelial cell, floss and red cell with eosinophilic staining in renal capsule and inflammatory cell infiltration in treated group than in model group. Mild red cell cast was found occasionally in renal tubule of treated group. There were also renal interstitial edema and scattered necrosis of renal tubular epithelial cell in few cases. There was no visible difference between Baicalin- and octreotide-treated groups. Better therapeutic effects were achieved in octreotide-treated group.

Changes of pathological score of kidney in all groups

Pathological grading of kidney: The pathological grading of kidney was used (Table 3) and two pathologists performed the evaluation of degree of pathological changes in pancreatic tissue in double-blind fashion.

Comparison of pathological score of kidney: The score was significantly higher in model group, Baicalin- and octreotide-treated groups than in sham operation group at different time points ($P < 0.001$). However, the score was lower in Baicalin- and octreotide-treated groups than in model group at 6 h ($P < 0.05$). The score was lower in octreotide-treated group than in model group at 12 h ($P < 0.05$). There was not significant different between Baicalin- and octreotide-treated groups at different time points (Table 4).

Expression of Bax protein in renal tissue

Bax-positive staining was located in the kytoplasm of renal tubular epithelial cell (Table 5 and Figure 2A-E). The expression level was not different among all groups at 12 h. The level was higher in model group and Baicalin-treated group than in sham operation group at 3 h and

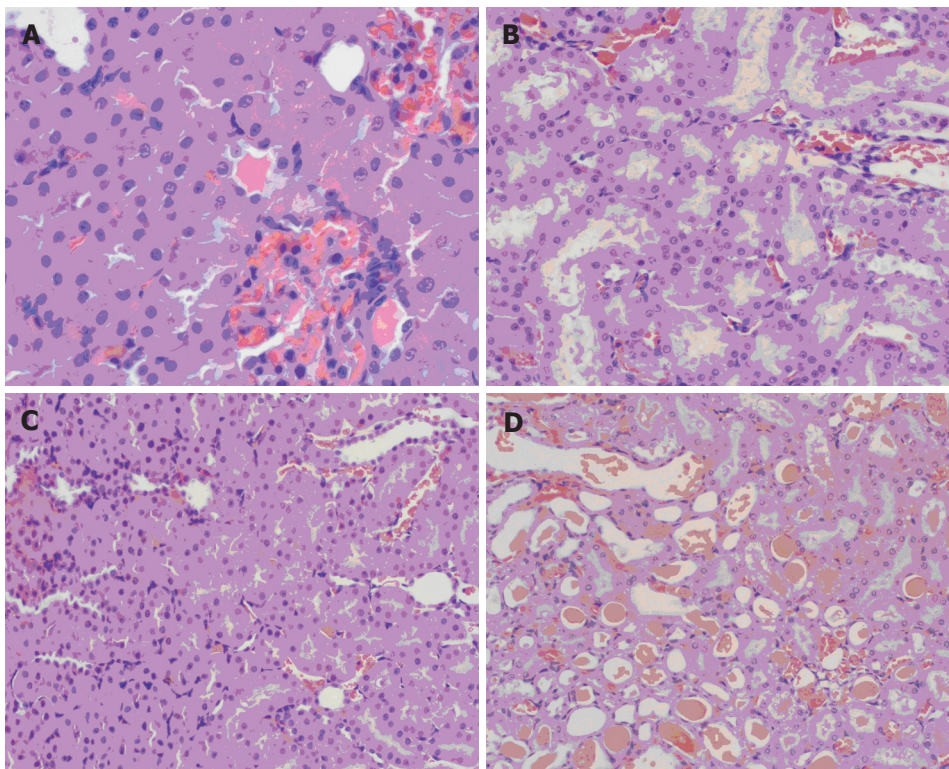


Figure 1 Light microscopic changes of kidney (HE, ×400). **A:** 3-h model group showing protein cast in renal tubule; **B** and **C:** 6-h model group showing scattered degenerative necrosis in renal tubular epithelial cells, and renal interstitial edema, respectively; **D:** 12-h model group showing visible red cell cast.

Table 3 Pathological score standard of kidney

Grade	Observation indexes
I	No cellular proliferation or fibrosis in renal glomerulus; no capillary congestion or microthrombus; swelling and blurry boundary of renal tubular epithelial cell; stenosis or atresia of lumens; protein cast and renal interstitial edema
II	Glomerular capillary congestion, scattered necrosis in renal tubular epithelial cell, interstitial edema and inflammatory cell infiltration
III	II + lamellar necrosis of renal tubular epithelial cell

Table 4 Comparison of pathological score of kidney in all groups [$M(Q_R)$]

Groups	3 h	6 h	12 h
Sham operation group	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Model group	2.00 (1.00)	2.00 (1.00)	2.00 (1.25)
Baicalin treated group	1.00 (1.00)	1.00 (1.00)	2.00 (1.00)
Octreotide-treated group	1.00 (1.00)	1.00 (1.00)	1.00 (1.00)

6 h ($P < 0.05$), and also higher in octreotide-treated group than in sham operation group at 6 h ($P < 0.05$). The expression level was not different between model group and Baicalin-treated group at all time points. Similarly, the expression level was not different between Baicalin- and octreotide-treated groups. The level was lower in octreotide-treated group than in model group at 3 h ($P < 0.05$) (Table 6).

Expression of Bcl-2 protein in renal tissue

Bcl-2-positive staining was located in the cytoplasm of renal tubular epithelial cells. (Table 7 and Figure 3A-C). The

Table 5 Expression of Bax protein in kidney

Groups	<i>n</i>	Pathologic grade			
		-	+	++	+++
Sham operation group	(3 h) 15	15			
	(6 h) 15	15			
	(12 h) 15	15			
Model group	(3 h) 15	7	4	4	
	(6 h) 13	10	1	2	
	(12 h) 10	7	1	1	1
Baicalin treated group	(3 h) 15	11	3	1	
	(6 h) 15	10	4	1	
	(12 h) 15	12	3		
Octreotide treated group	(3 h) 15	12	3		
	(6 h) 15	11	2	2	
	(12 h) 15	10	4	1	

level was higher in model group than in sham operation group at all time points ($P < 0.05$). The level was higher in Baicalin-treated group than in sham operation group at 12 h ($P < 0.05$), lower in octreotide-treated group than in Baicalin-treated group ($P < 0.05$), lower in Baicalin-treated group than in model group at 6 h ($P < 0.001$), and also lower in octreotide-treated group than in model group at 6 and 12 h ($P < 0.05$) (Table 8).

Comparison of renal apoptotic index

The apoptotic cells were renal tubular epithelial cells. The index was not different between model group and sham operation group at different time points. Moreover, the index was not different among all groups at 3 and 12 h. The index was higher in Baicalin- and octreotide-treated groups than in sham operation group and model group at 6 h ($P < 0.05$). The index was not different between Baicalin- and octreotide-treated groups at all time points (Table 9 and Figure 4A-D).

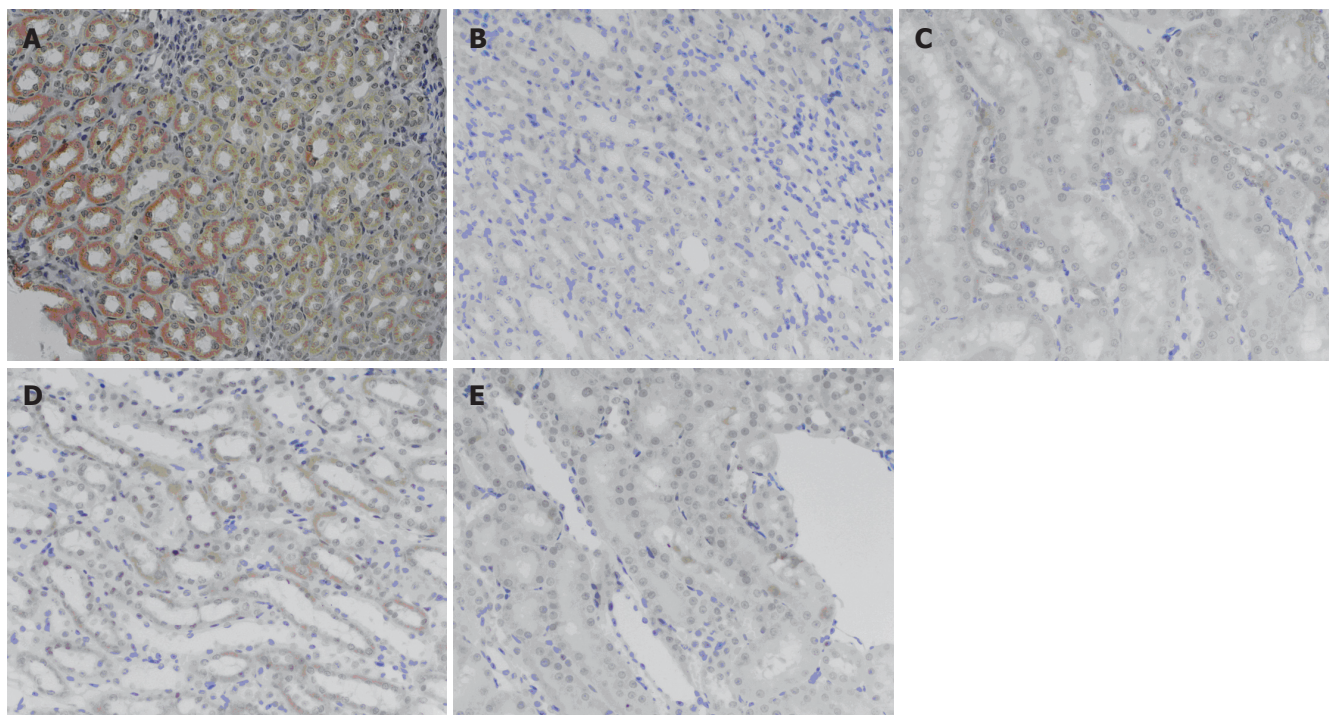


Figure 2 Bax expression in different groups (×200). **A:** 12-h model group showing high (+++) Bax expression; **B:** 3-h Baicalin-treated with negative (-) Bax expression; **C:** 12-h model group showing moderate (++) Bax expression; **D and E:** 6-h octreotide-treated group showing mild (+) Bax expression.

Table 6 Comparison of Bax protein in kidney [*M (Q₂)*] grade score

Groups	3 h	6 h	12 h
Sham operation group	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Model group	1.00 (2.00)	0.00 (0.50)	0.00 (1.25)
Baicalin-treated group	0.00 (0.50)	0.00 (1.00)	0.00 (0.00)
Octreotide-treated group	0.00 (0.00)	0.00 (1.00)	0.00 (1.00)

Table 7 Expression of Bcl-2 protein in kidney

Groups		<i>n</i>	Pathologic grade			
			-	+	++	+++
Sham operation group	(3 h)	15	15			
	(6 h)	15	15			
	(12 h)	15	15			
Model group	(3 h)	15	10	1	2	2
	(6 h)	13	4		1	8
	(12 h)	10	7	1	2	
Baicalin-treated group	(3 h)	15	14	1		
	(6 h)	15	15			
	(12 h)	15	11	4		
Octreotide-treated group	(3 h)	15	13	2		
	(6 h)	15	15			
	(12 h)	15	15			

Comparison of plasma endotoxin content

The content was higher in model group and treated group than in sham operation group at all time points ($P < 0.001$). The content was not different between Baicalin- and octreotide-treated groups at 6 and 12 h. The content was lower in Baicalin- and octreotide-treated groups than in model group at 3 h ($P < 0.001$), lower in Baicalin-treated

group than in octreotide-treated group at 3 h ($P < 0.01$), lower in Baicalin-treated group than in model group at 6 h ($P < 0.05$), lower in octreotide-treated group than in model group at 6 h ($P = 0.001$), lower in Baicalin-treated group than in model group at 12 h ($P < 0.001$), and also lower in octreotide-treated group than in model group at 12 h ($P < 0.01$) (Table 1).

Comparison of serum PLA₂ content

Serum PLA₂ content in model group and treated groups significantly exceeded sham operation group at different time points ($P < 0.001$). At 3 h, PLA₂ content in Baicalin-treated group was significantly less than model group and octreotide-treated group ($P < 0.01$), but no marked difference was observed between octreotide-treated group and model group. At 6 h and 12 h, PLA₂ content in Baicalin- and octreotide-treated groups was significantly less than model group ($P < 0.001$). There was no marked difference between Baicalin-treated group and octreotide-treated group at 6 h, while octreotide-treated group had significantly less PLA₂ content than Baicalin-treated group at 12 h ($P < 0.001$) (Table 10).

Comparison of serum NO content

Serum NO content in model group, Baicalin-treated group and octreotide-treated group significantly exceeded sham operation group at different time points ($P < 0.001$). At 3 h and 12 h, Baicalin-treated and octreotide-treated groups had significantly less serum NO content than model group ($P < 0.05$). There was no marked difference in serum NO content between Baicalin-treated group and octreotide-treated group at different time points (Table 1).

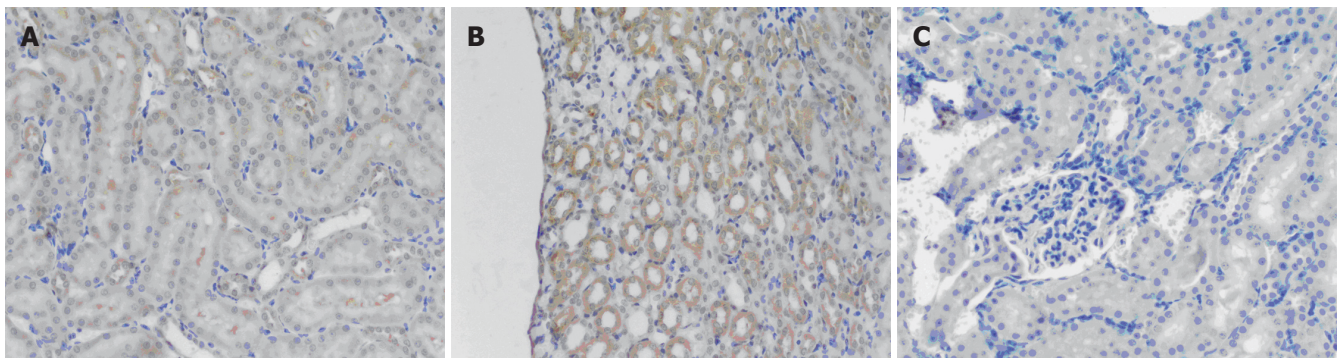


Figure 3 Bcl-2 expression in different groups (× 200). **A** and **B**: 6-h model group showing high (+++) Bcl-2 expression; **C**: 6-h octreotide-treated group with negative (-) Bcl-2 expression.

Table 8 Comparison of Bcl-2 protein in kidney [$M(Q_R)$] grade score

Groups	3 h	6 h	12 h
Sham operation group	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Model group	0.00 (2.00)	3.00 (3.00)	0.00 (1.25)
Baicalin-treated group	0.00 (0.00)	0.00 (0.00)	0.00 (1.00)
Octreotide-treated group	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)

Table 9 Apoptotic index of kidney [$M(Q_R)$] (%)

Groups	3 h	6 h	12 h
Sham operation group	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Model group	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
Baicalin-treated group	0.00 (0.01)	0.00 (0.02)	0.00 (0.00)
Octreotide-treated group	0.00 (0.00)	0.00 (0.04)	0.00 (0.00)

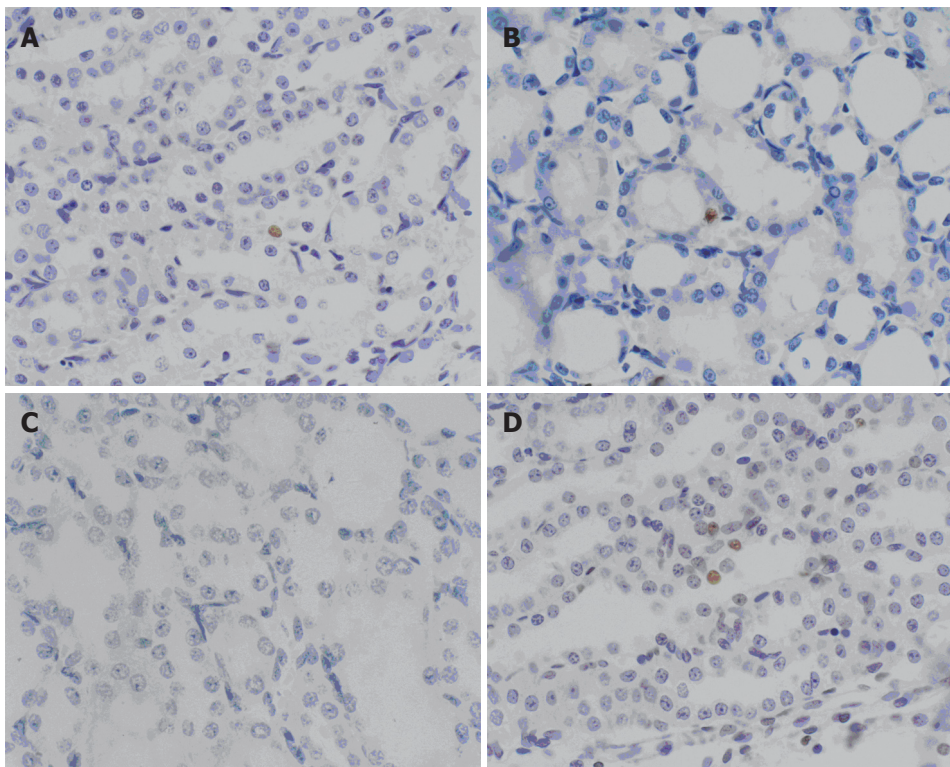


Figure 4 Apoptosis in different groups (TUNEL, × 400). **A**: 6-h octreotide-treated group showing apoptosis of renal tubular epithelial cells; **B**: 6-h Baicalin-treated group showing apoptosis of renal tubular epithelial cells; **C**: 6-h model group with no (-) apoptosis; **D**: 6-h octreotide-treated group showing apoptosis of renal tubular epithelial cells.

Comparison of serum TNF- α content

Serum TNF- α content in model group and treated groups significantly exceeded sham operation group at different time points ($P < 0.001$). There was no significant difference among model group, Baicalin-treated group and octreotide-treated group at 3 h and 12 h. At 6 h, serum TNF- α contents in Baicalin-treated group and octreotide-treated group were significantly less than model control group ($P < 0.001$); and octreotide-treated group had signif-

icantly less serum TNF- α content compared to Baicalin-treated group ($P < 0.01$) (Table 1).

Comparison of serum IL-6 content

Serum IL-6 contents at 3 h and 6 h were significantly higher in model control group and treated groups than in sham operation group ($P < 0.001$). Baicalin-treated group and octreotide-treated group had no significant difference in serum IL-6 content at all time points. Baicalin-

Table 10 Comparison of serum PLA₂ content (mean \pm SD, U/mL)

Groups	3 h	6 h	12 h
Sham operation group	14.62 \pm 3.02	17.49 \pm 3.82	19.02 \pm 5.07
Model group	76.10 \pm 16.70	101.46 \pm 14.67	105.33 \pm 18.10
Baicalin-treated group	56.25 \pm 22.43	67.91 \pm 20.61	66.86 \pm 22.10
Octreotide-treated group	74.37 \pm 19.94	63.13 \pm 26.31	53.63 \pm 12.28

and octreotide-treated groups had significantly lower serum IL-6 content compared to model control group at all time points ($P < 0.001$). The model control group had significantly higher serum IL-6 content than sham operation group at 12 h ($P < 0.001$), and so was Baicalin-treated group ($P < 0.01$), but no significant difference was found between octreotide-treated group and sham operation group (Table 1).

Comparison of serum ET-1 contents

Serum ET-1 content in model group was significantly higher than in sham operation group at all time points ($P < 0.001$). At all time points, Baicalin- and octreotide-treated groups had significantly lower serum ET-1 content than model group ($P < 0.001$). Octreotide-treated group had significantly lower ET-1 content compared to Baicalin-treated group at 3 h and 12 h ($P < 0.01$). At 3 h, Baicalin-treated group had significantly higher ET-1 content than sham operation group ($P < 0.01$), but no significant difference was found between octreotide-treated group and sham operation group. At 6 h, there was no marked difference between Baicalin-treated group or octreotide-treated group and sham operation group, or between Baicalin-treated group and octreotide-treated group. At 12 h, octreotide-treated group and sham operation group had no marked difference, and Baicalin-treated group had significantly higher ET-1 content than sham operation group ($P < 0.001$) (Table 1).

Comparison of correlations among various indexes

Correlation between apoptotic indexes and Bax and Bcl-2 expression in the kidney: The apoptotic index of Baicalin-treated group at 3 h and that of octreotide-treated group at 3 h and 6 h was positively correlated with Bax expression ($P < 0.001$). However, there was no correlation between apoptotic index and Bcl-2 expression.

Correlation between pathological score change and BUN and CREA of kidney: The pathological score of sham operation group at 12 h was positively correlated with BUN ($P < 0.01$) and CREA levels ($P < 0.05$). The pathological score of model group was positively correlated with CREA at 3 h ($P = 0.01$) and 6 h ($P < 0.001$). The score of Baicalin-treated group at 3 h and 6 h was positively correlated with CREA ($P < 0.05$). The pathological score of Baicalin-treated group was positively correlated with BUN at 6 h ($P < 0.05$) and 12 h ($P < 0.01$). The pathological score of octreotide-treated group at 3 h was positively correlated with BUN and CREA ($P < 0.05$), and that at 6 h and 12 h was positively correlated with CREA ($P < 0.01$) and BUN ($P < 0.01$), respectively.

Correlation analysis among inflammatory mediators: PLA₂ content at 12 h was positively correlated with TNF- α in model group ($P < 0.05$), and TNF- α was positively correlated with PLA₂ content ($P < 0.01$) at 3 h.

DISCUSSION

This study demonstrated that there were milder renal pathological changes and lower serum BUN content in treated groups as compared with model group. The survival rate was higher in treated groups compared to model group. All these indicate the potent therapeutic effect of Baicalin and octreotide on rats with severe acute pancreatitis. Baicalin showed superiority over octreotide in decreasing plasma endotoxin and PLA₂ content in SAP rats at 3 h, while octreotide was found to be superior to Baicalin in alleviating renal pathological changes and decreasing CREA, Bcl-2, TNF- α , PLA₂ (at 12 h) and ET-1 contents.

Regarding the mechanisms *via* which these two drugs improve renal pathological changes, we mainly hypothesized inhibition of inflammatory mediators and induction of apoptosis.

The endotoxin^[11] in plasma, and PLA₂^[12,13], NO^[14-18], TNF- α ^[19-21], IL-6^[22] and ET-1^[23,24] in serum are all important inflammatory mediators during SAP complicated with multiple organ injury. They are important indexes of severity and prognosis of acute pancreatitis and have two important features in common: (1) Dual effects: These inflammatory mediators, especially NO and ET-1, will protect body in low concentration and injure body in high concentration; and (2) There are interactions among different inflammatory mediators. This experiment confirmed a positive correlation between PLA₂ and TNF- α . According to many studies as well as our experiment, the concentrations of these inflammatory mediators increase during SAP^[25-31]. Our experiment demonstrated that almost all indexes of inflammatory mediators were lower in treated groups than in model group, while the indexes were not different between Baicalin- and octreotide-treated groups, thereby indicating that both drugs, with similar effects, could lower the concentration of inflammatory mediators, inhibit them and protect kidney.

Both necrosis and apoptosis are ways of death of injured cells^[32]. In contrast to necrosis, apoptosis does not cause intense inflammatory reaction^[33], while necrosis will cause systemic inflammatory response syndrome^[34]. At present, a consensus has been reached on apoptosis of pancreas during SAP^[35,36]. When necrosis and apoptosis coexist in pancreas and necrosis prevails, induction of pancreatic apoptosis will result in a protective effect. We believe this conclusion is also applicable to renal apoptosis, which has been demonstrated by this experiment.

This experiment clearly showed that the renal pathological changes were milder in treated group than in model group. The renal apoptotic indexes at 6 h were markedly higher in treated group than in model group. All these indicate the renal pathological changes have been alleviated after apoptosis of renal tubular epithelial cells. In addition, the renal apoptotic indexes were not different between model group and sham operation group, possibly

because apoptosis had not occurred in model group or its incidence was too low to be detected. The occurrence of apoptosis of renal cells in treated groups, however, demonstrated that both Baicalin and octreotide could induce apoptosis. But some researchers believe the pathological changes would be aggravated by renal apoptosis during SAP^[37,38], which is different from our view and therefore worth discussing.

Bax and Bcl-2 are two important apoptosis-regulating factors. The homo- or heterodimerization between anti-apoptotic Bcl-2 and proapoptotic Bax plays an important role in the apoptosis regulating function of the Bcl-2-related proteins. Interestingly, in an excess of Bax, Bax/Bax homodimers predominate, which promote apoptosis, whereas an excess of Bcl-2 leads to the formation of Bcl-2/Bax heterodimers, which inhibit apoptosis. Thus, the ratio of Bcl-2 to Bax appears to be a critical determinant of a cell's threshold for undergoing apoptosis. No expression of Bcl-2 gene has been found normal pancreatic tissue^[39]. In addition, there has been no report on expression of Bcl-2 gene in normal renal tissue. It was found in this experiment that the expression levels of both Bax and Bcl-2 protein had increased during SAP, possibly because the apoptosis-inducing and -inhibiting factors had been enhanced simultaneously. As a result of the conflict of the two factors, the apoptotic indexes were not different between model group and sham operation group. However, the apoptotic indexes at 6 h were higher in treated group than in sham operation group and model group, indicating that the apoptosis of renal tubular epithelial cells occurred because the apoptosis-inducing factor prevailed in treated group. It was also found that the apoptotic indexes at 3 h were positively correlated with Bax in Baicalin-treated group. The apoptotic indexes at 3 h and 6 h were positively correlated with Bax in octreotide-treated group. But there was no correlation between apoptotic indexes and Bcl-2. Thus these data indicate that Bax might have participated in the apoptosis of renal tubular epithelial cells. Compared to model group, the expression level of Bcl-2 protein was lower in Baicalin-treated group at 6 h, and in octreotide-treated group at 6 h and 12 h, thereby indicating that both Baicalin and octreotide can lower the expression level of Bcl-2 protein, enhance the apoptosis-promoting effect of Bax dimmer and thus protect kidney and alleviate its pathological changes.

The traditional histopathological section technique that has been surpassed by tissue microarray (TMA) features single sample and low efficiency^[40]. The TMA we adopted has advantages such as high throughput and reliable results and great potential in oncopathological study^[41,42]. Current studies are also mainly focused in this field^[43-50]. To our knowledge, this is the first report on the application of TMA to the pathological examination of pancreatitis around the world.

In conclusion, both Baicalin and octreotide can alleviate the renal pathological changes and improve the survival of SAP rats by inhibiting inflammatory mediators, decreasing the expression level of Bcl-2 protein and enhancing the apoptosis-promoting effect of Bax dimmer to induce the apoptosis of renal tubular epithelial cells. Tissue microarray is time- and energy-saving, highly efficient and

well representative in the pathological examination of pancreatitis. We believe Baicalin, a low-priced new drug with precise therapeutic effects, is hopeful to play certain role in SAP treatment in future.

COMMENTS

Background

Up to now, severe acute pancreatitis (SAP) is still an acute clinical disease featuring multiple complications, high mortality and difficult treatment. Recent studies found octreotide, a somatostatin analogue, could effectively treat SAP. However, octreotide is expensive, which has hindered its clinical application. Therefore, an important direction of the current study is to find other cheap and effective drugs. Baicalin injection (China National Invention Patent Number ZL200310122673.6) prepared by the first author would be one of the best choice to treat SAP. In this experiment, the feasibility of Baicalin treatment of SAP has been studied by comparing the protecting effects and mechanisms of Baicalin and octreotide on kidneys of rats with SAP.

Research frontiers

Both Baicalin and octreotide can alleviate inflammatory reactions by inhibiting the generation of inflammatory mediators and inducing renal cell apoptosis, and thereby exert therapeutic effects on SAP. The mechanism of Baicalin- and octreotide-induced renal cell apoptosis may be related to regulation of Bax and Bcl-2 protein expressions. The therapeutic effects and mechanism of Baicalin on SAP rats are similar to those of octreotide.

Innovations and breakthroughs

As a cheap medicine with extensive pharmacological actions, few side effects and convenient administration, Baicalin can hopefully become a new drug for treating SAP. The application of tissue microarrays in pathological examination of SAP has several advantages, including time- and energy-saving, high efficiency and good representativeness, and therefore is worth popularizing.

Applications

Both Baicalin and octreotide can alleviate the renal pathological changes and improve the survival of SAP rats by inhibiting inflammatory mediators, regulating the expression level of Bax and Bcl-2 protein and inducing the apoptosis of renal tubular epithelial cell. Tissue microarray is time- and energy-saving, highly efficient and well representative in the pathological examination of pancreatitis. We believe Baicalin, a low-priced new drug with precise therapeutic effects, is hopeful to play certain role in future SAP treatment.

Terminology

Baicalin is an important monomer of Baical skullcap root. Severe acute pancreatitis (SAP) is a fatal systemic disease featuring acute onset, serious conditions, high incidence of complications and 20%-30% of mortality.

Peer review

The authors analyzed the protecting effects and mechanism of Baical skullcap root in the treatment of SAP in rats. The authors showed that Baicalin seems to be equally effective as octreotide in terms of reduction of renal pathological alterations. This study is well performed and the results merit further investigation.

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CLINICAL RESEARCH

Modified physiological and operative score for the enumeration of mortality and morbidity risk assessment model in general surgery

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CONCLUSION: M-POSSUM correlates well with postoperative complications and mortality, and is more accurate than POSSUM.

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Key words: Physiological and operative severity score for the enumeration of mortality and morbidity; Postoperative morbidity; Mortality; Preoperative assessment; General surgery; Critical illness

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Abstract

AIM: To establish a scoring system for predicting the incidence of postoperative complications and mortality in general surgery based on the physiological and operative severity score for the enumeration of mortality and morbidity (POSSUM), and to evaluate its efficacy.

METHODS: Eighty-four patients with postoperative complications or death and 172 patients without postoperative complications, who underwent surgery in our department during the previous 2 years, were retrospectively analyzed by logistic regression. Fifteen indexes were investigated including age, cardiovascular function, respiratory function, blood test results, endocrine function, central nervous system function, hepatic function, renal function, nutritional status, extent of operative trauma, and course of anesthesia. Modified POSSUM (M-POSSUM) was developed using significant risk factors with its efficacy evaluated.

RESULTS: The significant risk factors were found to be age, cardiovascular function, respiratory function, hepatic function, renal function, blood test results, endocrine function, nutritional status, duration of operation, intraoperative blood loss, and course of anesthesia. These factors were all included in the scoring system. There were significant differences in the scores between the patients with and without postoperative complications, between the patients died and survived with complications, and between the patients died and survived without complications. The receiver operating characteristic curves showed that the M-POSSUM could accurately predict postoperative complications and mortality.

INTRODUCTION

With the progress in medical sciences, operative indications for many medical conditions are being expanded, the range of operations is growing, with operative complications increased accordingly. Assessment of the functional condition of organs and systems assists in quantifying the operative risks. Assessment methods allow us to judge the state of an illness and carry out the appropriate preventive treatment immediately, thereby decreasing operative complications and mortality. Numerous scoring systems are currently available, including the Glasgow coma scale (GCS), acute physiology, age and cHealth evaluation II (APACHE II)^[1], physiological and operative severity score for the enumeration of mortality and morbidity (POSSUM)^[2] and others^[3-5].

These scoring systems are important tools in deciding the course of treatment. The best known and most widely used scoring systems, APACHE II and POSSUM, have limitations when applied to high-risk general surgical patients. APACHE II is best suited to intensive care patients, but requires 24 h of observation, and weighing tables for individual disease state. POSSUM is limited by its somewhat subjective nature and incomplete evaluation of cardiac signs. We propose the modified POSSUM (M-POSSUM) as a reasonable, practical and objective scoring system that can be used across a broad disease spectrum in general surgery.

MATERIALS AND METHODS

Patients

The study group included 84 patients with postoperative complications who underwent surgery in our department from May 2002 to May 2004, 16 of them died. The control group included 172 patients without postoperative complications who were randomly selected during the same period of time. General clinical characteristics of the patients including age and gender are illustrated in Table 1. Diagnoses in the malignancy group included colorectal cancer, gastric carcinoma, pancreatic carcinoma, ampullary carcinoma and breast cancer. Diagnoses in the benign group included gastric ulcer, bowel obstruction, cholelithiasis and hyperthyroidism. Concomitant diseases included hypertension, coronary heart disease, pneumopathy, diabetes mellitus, hepatitis and hepatic cirrhosis, cholelithiasis, anemia, cerebrovascular disease and others.

The complications and deaths were caused by common illnesses. In this study, we defined complications as outlined in the book "Clinical General Surgery-Diagnostic Analysis and Treatment Gist". Patients were divided into a group of patients with complications and a group of patients without complications. The group of patients with complications was further divided into a group of patients who survived and a group of patients who died.

Methods

We recorded independent variables such as age, sex, function of all organs and systems, results of laboratory tests and special investigations, duration of operation and volume of intraoperative blood loss. The dependent variables were "complications or not" and "death or not" designated as "0" and "1." We used logistic regression analysis to determine significant risk factors and relative risk (RR).

The indices used were determined by our study results and the prescribed targets and standards of APACHE II and POSSUM. The remaining factors were assigned a point value score (0, 1, 2, 3, or 4). Thus a 15-factor, five-grade scoring system was developed. We compared the difference in M-POSSUM values between various groups, plotted receiver operating characteristic (ROC) curves and calculated the area under the curves to determine the accuracy of M-POSSUM in predicting perioperative and postoperative complication and mortality rates.

Statistical analysis

All information was stored in a computer database. The general clinical data were analyzed by chi-square test. Risk factors were analyzed by logistic regression analysis. Differences in quantitative data were analyzed by *t*-test. A ROC curve was used to evaluate the ability of M-POSSUM to predict postoperative complications and mortality. All these analyses were performed using the Statistical Package for Social Sciences (SPSS) version 11.5.

RESULTS

High risk factor analysis

The RR and *P* were analyzed by logistic regression (Table 2). Eleven significant variables determined were as follows: age, cardiovascular function, respiratory function, blood

Table 1 General clinical profile of patients studied

Clinic data	Postoperative complications	Postoperative death	Postoperative complications
No.	84	16	172
Age (yr)	50-79	50-79	50-79
Average age	68.67 ± 12.47	59.64 ± 13.48	69.82 ± 7.98
Gender (Male/Female)	55/29	11/5	109/63
Diagnosis, <i>n</i> (%)			
Malignancy	76 (90.5)	12 (75.0)	162 (94.2)
Benign	8 (9.5)	4 (25.0)	10 (5.8)
Concomitant diseases, <i>n</i> (%)			
Yes	14 (16.7)	1 (6.3)	68 (39.5)
No	70 (83.3)	15 (93.7)	104 (60.5)

Table 2 Relative risk of all factors

Factors	Suffered complications		Died	
	RR	<i>P</i>	RR	<i>P</i>
Age (yr) > 60	0.681	0.356	0.211	0.008
> 70	2.181	0.005	0.391	0.091
> 80	1.469	0.356	0.549	0.569
Abnormal circulation system	2.074	0.014	1.092	0.869
ECG mild change	0.893	0.274	0.374	0.933
ECG ST-T change	3.817	0.031	1.928	0.418
Abnormal respiratory system	3.581	0.000	1.723	0.311
Liver function	3.438	0.000	16.007	0.000
Blood system	2.610	0.000	2.735	0.070
Renal function	4.333	0.042	15.667	0.000
Gastrointestinal diseases	15.545	0.011	5.571	0.046
Endocrine system	2.374	0.002	1.615	0.359
Nutrition	4.938	0.000	6.000	0.003
Central nervous system	1.492	0.506	3.286	0.148
Operation time > 2 h	1.097	0.096	0.028	0.112
Operation time > 4 h	3.541	0.013	2.549	0.093
Operative hemorrhage > 300 mL	0.783	0.306	0.481	0.216
Operative hemorrhage > 500 mL	2.347	0.007	3.392	0.027
Palliative excision of malignant tumor	0.693	0.047	2.014	0.082
Malignant tumor can't excise	7.139	0.000	5.175	0.000
BP < 90/60 mmHg during operation	5.429	0.000	13.105	0.000
Steadyperioperative ECG monitoring	7.781	0.000	16.001	0.000

test results, gastrointestinal function, endocrine function, nutritional status, hepatic function, renal function, type of incision and course of anesthesia.

Development of M-POSSUM

Using our study results as well as the prescribed targets and standards of APACHE II and POSSUM, we were able to overcome the shortcomings of APACHE II and POSSUM, eliminate some non-significant variables, and determine the indices to be used. The remaining factors were assigned a point value score (0, 1, 2, 3 or 4). Thus, a 15-factor, five-grade scoring system was developed (Table 3).

Evaluation of M-POSSUM

We compared the M-POSSUM scores of the groups (Table 4), which were significantly different in all groups. The frequency distributional graph of M-POSSUM is shown in Figure 1. The ROC curves and areas under them are shown in Figures 2A and 2B, and Table 5. We were able to use them to predict the morbidity and mortality rates in postoperative patients and determine the accuracy of M-POSSUM in predicting morbidity and mortality.

Table 3 M-POSSUM system

Index	0	1	2	3	4
Age (yr)	< 60	60-69	70-79	≥ 80	
Circulatory system	Normal Car Fun, BP, ECG	Car fun grade I, mild HP and abnormal ECG, sinoatrial bradycardia/tachycardia, low voltage of limb lead, BBB	Car fun grade II, Mod. HP, well controlled by med, occasional atrial premature beats	Car fun grade III, myocardial infarct < 3 mon, mod HP by med, ectopic, arythm, ST-T change, atrial fibrillation	Serious car, insuf, AHF, mal HP
Respiratory system	Normal	Long history of smoking, CB, asthma, URI, thick pulmonary markings	Mild COPD, mild PF change, mild pneumo	Mod COPD, Mod to serious abnormal PF	Respiratory failure
Liver function	Normal	History of hepatitis/cirrhosis of liver, TB < 34.2 μmol/L	TB 34.2-51.3 μmol/L	TB > 51.3 μmol/L	
Renal function	Normal	BUN ≤ 10.1 mmol/L, Cr ≤ 170 μmol/L	BUN 10.1-15 mmol/L, Cr 170-300 μmol/L	Renal failure need dialysis	
Gastrointestinal tract	Normal	History of chronic gastroenteritis, controlled peptic ulcer	Active gastrointestinal diseases(hemorrhage/perforation of ulcers, active Crohn's disease	Percutaneous intestinal fistula	Short bowel syndrome, transplantation of small bowels
Blood system	Normal	PLT/WBC decreased mildly, Hb (rectified) > 85 g/L	Hematopathy as stable leukemia, WBC ≥ 14.5 × 10 ⁹ /L	Aplastic anemia, hypersplenism syndrome, leukemia etc.	
Endocrine system	Normal	Mild increased BG, UGLu (-), treated hyperthyroidism, hypothyroidism, acromegaly, gout, rheumatoid disease	Mild increased BG, UGLu (+ - + +), and controlled DM, by oral medicine, hormone therapy, active gout	DM, astable with oral medicine	Diabetic nephropathy
Nutritional Status ²	Normal	Slight malnutrition (albumin 30-35 g/L, weight decrease < 2.5 Kg/m)	Moderate malnutrition (albumin < 30 g/L, mass decrease 2.5-5 kg/m); Radiotherapy/chemotherapy	Cachexia	
GCS	15	12-14	9-11	≤ 8	
Operative wound		Mino (OPT < 2 h/hemorrhage volume < 300 mL)	Mod (OPT 2-4 h/hemorrhage volume 300-500 mL)	Major (OPT > 4 h/hemorrhage volume > 500 mL)/Palliative excision of Mal tumor	M Major ⁺⁺ (excision > 3 organs) /Mal tumor can't excise
Anesthesia course				Arrhythmia/low BP < 1/2 h	continual low BP/ cardio-pulmonary resuscitation

Nor: Normal; Car: Cardiac; Fun: Function; BP: Blood pressure; HP: Hypertension; BBB: Bundle branch block; ECG: Electrocardiogram; Insuf: Insufficiency; AHF: Acute heart failure; Mod: Moderate; Med: Medicine; Mon: Month; CB: Chronic bronchitis; URI: Upper respiratory infection; PF: Pulmonary function; TB: Total bilirubin; BG: Blood glucose; UGLu: Urine glucose; DM: Diabetes mellitus; OPT: Operation time; Hm: Hemorrhage; Mal: Malignant; GCS: Glasgow coma score.

¹Liver function consul bilirubin in Child-Pough; ²Nutritional status consider albumin.

Table 4 M-POSSUM in all groups of patients

Group	n	M-POSSUM	t-test	
			t	P
No complication	172	6.51 ± 2.22	-13.723	0.000
Complication	84	11.04 ± 2.95		
Alive	240	7.58 ± 2.83	-9.096	0.000
Dead	16	14.25 ± 3.02		
Alive with complication	68	10.28 ± 2.38	-5.693	0.000

Predictive formula

Logistic regression analysis yielded statistically significant equations for both morbidity and mortality. The morbidity equation was $\ln R/1-R = -7.287 + 0.765M-POSSUM$ ($P = 0.000$), and the mortality equation was $\ln R/1-R = -10.000 + 0.681M-POSSUM$ ($P = 0.000$). The predictive accuracy of morbidity equation and mortality equation was 83.6% and 94.1%, respectively.

DISCUSSION

In general surgery, postoperative morbidity rate ranges

15%-40%. Among patients at the age of 80 years or more undergoing abdominal surgery, the morbidity is higher and the mortality is about 4%^[6,7]. While the range and types of operation continue to expand and the treatment guidelines for carcinoma change (progressively emphasizing radical treatment, safety and function), postoperative morbidity and mortality are increasing. Scoring systems are used to objectively assess and quantify the severity of illness, determine prognosis, guide patient monitoring and treatment, and avoid preventable deaths. Ideally, an effective scoring system should also be able to provide useful comparisons between surgeons, surgical units, hospitals and regions.

In general surgery, patients at a higher risk are mostly the elderly, who often suffer from concurrent diseases. Most routine blood biochemistry tests cannot exactly reflect any organ's physiological status. Tests and special investigations cannot replace detailed case histories and overall somatoscopy. Baseline health status, previous complications and concurrent diseases can all be found in case history. The frequently used scoring systems, APACHE II and POSSUM, have some limitations^[8-13]. They lay stress on physiological criteria, but not on hepatic function,

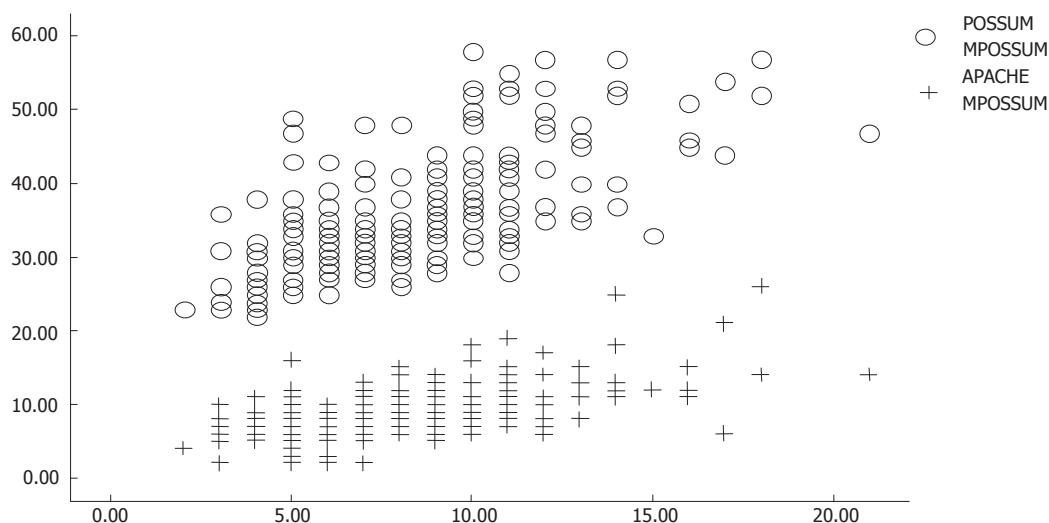


Figure 1 Relative spot chart of M-POSSUM and POSSUM with APACHE II.

Table 5 Area under ROC curves predicting morbidity and mortality

Variety	Area	SD	P
M-POSSUM (predicting for morbidity)	0.901	0.020	0.000
M-POSSUM (predicting for mortality)	0.955	0.016	0.000

blood glucose level and nutritional status, all of which were found to be independent factors affecting morbidity and mortality in our study. The POSSUM has been modified to form P-POSSUM, which is considered more accurate in predicting clinical results^[5,14]. However, no other reports support it^[15,16].

It has been accepted that age and morbidity is correlated with mortality. Elderly patients with concurrent diseases are more likely to develop perioperative and postoperative complications. In our study, a significant difference was found in the number of concurrent diseases between the groups of patients with and without complications. The concurrent diseases associated with a higher morbidity included high blood pressure, coronary disease, chronic lung disease, diabetes and hepatic cirrhosis. This is identical with the results in previous reports. Baue^[17] reported that the main causes of death after major operations are acute respiratory distress syndrome (ARDS), stress ulcer, renal failure, intra-abdominal abscess, multiple organ dysfunction syndrome (MODS) and systemic inflammatory response syndrome (SIRS). In our study, the complications in 16 patients who died were infection, fistula, and hemorrhage, leading to MODS and shock. We found that the factors with the highest correlation with death were blood pressure, electrocardiographic activity during anesthesia, hepatic function, renal function, nutritional status, gastrointestinal function and volume of intraoperative blood loss. Surgeons should therefore improve or correct such abnormalities before operation, to prevent complications, and should promptly identify and manage perioperative and postoperative complications.

A metabolically active organism is in a state of constant dynamic balance, and a static physiological index cannot reflect the dynamic changes. In our study, the significant independent factors were determined by linear multivariate

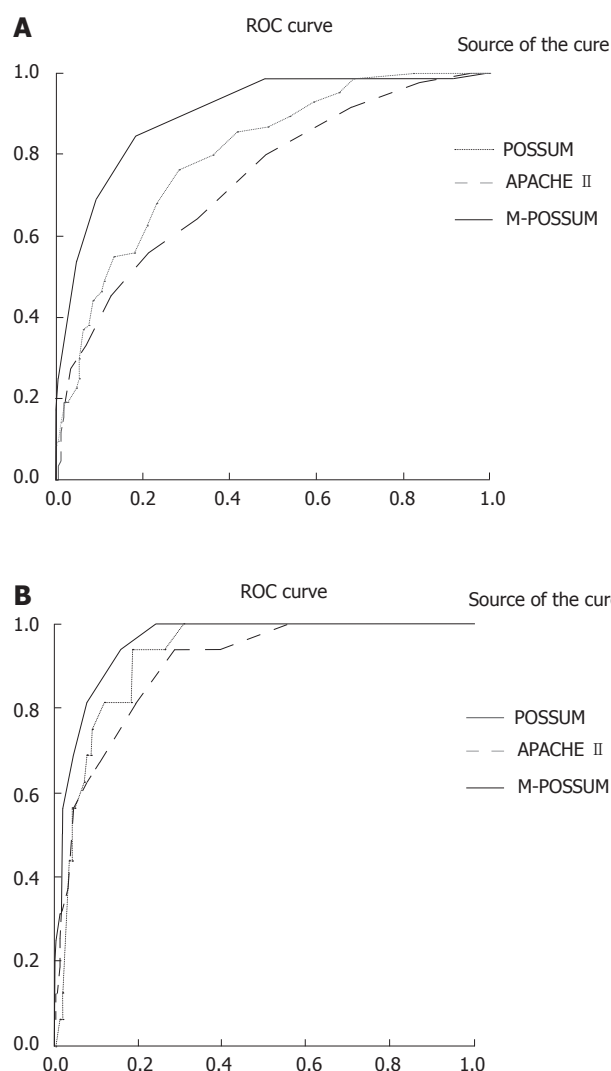


Figure 2 ROC curve of postoperative complications (A) and mortality (B). The red curve (M-POSSUM) covers the biggest area ($\times 100\%$) and illustrates the accuracy of M-POSSUM predicting the morbidity of post-operation before operation.

discriminant analysis.

In the present study, the main factors influencing morbidity were gastrointestinal function, course of anesthesia, nutritional status, renal function, respiratory function, he-

patic function, cardiovascular function and a non-excisable malignant tumor. The main factors influencing mortality were hepatic function, renal function, nutritional status, digestive tract function and a non-excisable malignant tumor. These results can guide surgeons to maintain stable blood pressure and monitor electrocardiographic activity perioperatively, and to be cautious about operating on late-stage malignant tumors, in order to decrease morbidity and mortality.

In contrast to APACHE II and POSSUM, the present scoring system is based on GCS, APACHE II and POSSUM, supplemented with the following indices: hepatic function (bilirubin), blood glucose level, gastrointestinal function and nutritional status (albumin). However, body temperature, heart rate, sodium and potassium, which are always normal preoperatively in general surgery, are not considered. To avoid repetition, duration of operation, volume of intraoperative blood loss and surgery are considered an operative wound index for a malignant tumor. M-POSSUM also takes blood pressure and electrocardiographic activity into consideration during anesthesia.

The area under the ROC curve reflects the accuracy of prediction. In general, the accuracy is low when the area is in the range of 0.5-0.7, intermediate when the area is between 0.7 and 0.9, and high when the area is greater than 0.9. The ROC curves for morbidity and mortality demonstrate that M-POSSUM is a more accurate predictor than POSSUM. The predictive accuracy for morbidity and mortality is 83.6% and 94.1%, respectively.

Using M-POSSUM, we can obtain a numerical estimate of the health status of an individual patient prior to operation, enabling us to adjust the type and duration of operation and determine reasonably individualized postoperative monitoring and treatment so as to decrease morbidity and mortality in general surgery, especially in aged patients.

In conclusion, M-POSSUM is more accurate than POSSUM and APACHE II in predicting postoperative morbidity and mortality, and therefore seems to be a better model for risk assessment.

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COMMENTS

Background

With the development in surgical techniques, the surgical domain is enlarging and more diseases are managed with surgery. The incidence of post-operation complications and mortality is increasing because of the increasing number of elderly and tumor patients. Surgeons should be able to evaluate patients effectively preoperatively, by quantitating the operative risk, in order to decrease the incidence of postoperative complications and mortality. An attempt was made to achieve this in the present study.

Research frontiers

Modern surgeons are faced with many problems, such as an aging population

and complicated critical trauma. There is a significant increase in the number of patients suffering from advanced tumors and critical organ disease or dysfunction. Therefore, it is important to increase curative effects and decrease failure rates of therapy. These issues can be solved with the development of scoring systems such as APACHE I, II, III and POSSUM.

Innovations and breakthroughs

Three indices of POSSUM which are poorly correlated with postoperative outcome (body temperature, serum electrolytes and type of surgery) were excluded from M-POSSUM. Six indices which are better correlated with past history, course of anesthesia, hepatic function, gastrointestinal function, endocrine function and nutritional status were included. M-POSSUM correlates well with APACHE II and POSSUM, and is superior to both of them.

Applications

M-POSSUM can decrease postoperative complications after targeted preoperative therapeutic measures in intermediate and high risk patients.

Terminology

Scoring systems such as those defined by APACHE I, II, III and POSSUM, or the American Society of Anesthesiologists (ASA) are used to quantify the severity of an illness before treatment or surgery.

Peer review

This study is interesting and scientific and readable.

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RAPID COMMUNICATION

Risk factors associated with pancreatic fistula after distal pancreatectomy, which technique of pancreatic stump closure is more beneficial?

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Abstract

AIM: To identify risk factors related to pancreatic fistula in patients undergoing distal pancreatectomy (DP) and to determine the effectiveness of using a stapled and a sutured closed of pancreatic stump.

METHODS: Sixty-four patients underwent DP during a 10-year period. Information regarding diagnosis, operative details, and perioperative morbidity or mortality was collected. Eight risk factors were examined.

RESULTS: Indications for DP included primary pancreatic disease ($n = 38$, 59%) and non-pancreatic malignancy ($n = 26$, 41%). Postoperative mortality and morbidity rates were 1.5% and 37% respectively; one patient died due to sepsis and two patients required a reoperation due to postoperative bleeding. Pancreatic fistula was developed in 14 patients (22%); 4 of fistulas were classified as Grade A, 9 as Grade B and only 1 as Grade C. Incidence of pancreatic fistula rate was significantly associated with four risk factors: pathology, use of prophylactic octreotide therapy, concomitant splenectomy, and texture of pancreatic parenchyma. The role that technique (either stapler or suture) of pancreatic stump closure plays in the development of pancreatic leak remains unclear.

CONCLUSION: The pancreatic fistula rate after DP is 22%. This is reduced for patients with non-pancreatic malignancy, fibrotic pancreatic tissue, postoperative prophylactic octreotide therapy and concomitant splenectomy.

INTRODUCTION

Distal pancreatectomy (DP), first performed by Billroth in 1884, is defined as the resection of pancreatic tissue to the left of the superior mesenteric vessels. This procedure was infrequently performed in the past because the tumours of the pancreatic tail and body were often irresectable at the time of diagnosis and due to the general dissatisfaction with this procedure for the management of chronic pancreatitis. However, the advent and development of imaging and diagnostic techniques has increased the frequency of DP. Recently, the indications for DP include malignant and benign pancreatic diseases, non-pancreatic malignancies, chronic pancreatitis, and trauma.

Pancreatic fistula is the most common major complication after DP, ranging from 5% to 40%. Pancreatic leakage often leads to further complications, such as fluid collections or intra-abdominal abscesses, wound infections, respiratory complications, and sepsis^[1]. The appropriate technique of closure the pancreatic stump is still under controversial^[1,2]. The most common used techniques for closure of the transected pancreatic parenchyma and for prevention of pancreatic fluid extravasations from the residual pancreatic tissue are the hand-sewn parenchymal closure and the stapled closure.

The risk factors in the development of pancreatic fistula are also unclear. The method of pancreatic stump closure, malignancy, trauma, patient's age, concomitant splenectomy, or obesity are implicated as potentially important^[3,4].

The purpose of this study was to determine possible risk factors that may be associated with the onset of pancreatic fistula after DP and to identify if the method of pancreatic stump closure plays a significant role in the development of pancreatic fistula.

Table 1 Main parameters for postoperative pancreatic fistula grading^[5]

Grade	A	B	C
Clinical conditions	Well	Often well	Ill appearing/bad
Specific treatment ¹	No	Yes/No	Yes
US/CT	Negative	Negative/Positive	Positive
Persistent drainage (after 3 wk)	No	Usually yes	Yes
Reoperation	No	No	Yes
Death related to fistula	No	No	Possibly yes
Signs of infection	No	Yes	Yes
Sepsis	No	No	Yes
Readmission	No	Yes/No	Yes/No

¹Partial (peripheral) or total parenteral nutrition, antibiotics, enteral nutrition, somatostatin analogue and/or minimal invasive drainage.

MATERIALS AND METHODS

A retrospective review of 64 patients who underwent DP from January 1996 to December 2005 at the Department of Digestive Surgery, Gemelli University Hospital of Rome, was conducted.

Patients' age, sex, indications for surgery, concomitant splenectomy, additional procedures, methods of pancreatic stump closure, and postoperative complications with a specific focus on pancreatic leaks, mortality, and duration of postoperative hospital stay were recorded. Operative details also included operating time and the texture of pancreatic parenchyma. No patient was excluded from the series.

The indications for DP included either primary pancreatic diseases or non-pancreatic malignancies. All the operations were performed by same surgical team. Division of the pancreatic parenchyma was by knife or linear stapler (GIA), while the pancreatic remnant was either closed by a linear stapler or by hand running absorbable monofilament 3-0 sutures. One open drain was positioned near the transected pancreas and it was removed when the daily fluid output was lower than 10 mL or when amylase concentration in the fluid drain was unremarkable in cases of pancreatic leaks (< 300 IU).

We used prophylactic octreotide in the last 34 patients (53%), postoperatively for 7 d, while in cases with fistula the octreotide was prolonged until recovery. The dose of octreotide was 0.5 mg three times a day.

Postoperative mortality and morbidity were registered for 30 d or during the total hospitalisation time, if longer. Postoperative pancreatic leaks were classified according to the international accepted definition reported by Bassi *et al.*^[5] for the international Study Group on Pancreatic Fistula (Table 1). Suspicion and diagnosis of fistula based on biochemical criteria included drainage of more than 10 mL of fluid in 24 h, with an amylase content of more than 3 times the serum amylase activity (> 300 IU) for more than 10 d after surgery. The amount of fluid amylase collected from the drainage tubes, as well as the serum amylase level were evaluated daily until postoperative d 10, and longer in cases with pancreatic fistula. The clinical criteria included the presence of clinical symptomatology such as fever greater than 38°C, leucocytosis with peripheral white blood

cells amount more than 10 000 cells/mm³, intra-abdominal pain or abscess, and the need of percutaneous drainage or reoperation. Intra-abdominal collections were detected by computed tomography (CT).

Oral feeding was generally started on second postoperative day in patients with insignificant amylase concentrations in the abdominal fluid collected by drainage or in patients with Grade A fistula. Conversely, patients with pancreatic fistula Grades B or C were kept with nothing by mouth and were supported with partial or total parenteral or enteral nutrition.

The following eight risk factors were analysed: age (patients older or younger than the age of 70 years old), gender, pancreatic disease or non-pancreatic malignancy, technique of pancreatic stump closure, splenic preservation, texture of the pancreatic parenchyma (soft or fibrotic tissue), additional procedures, and postoperative use of octreotide. The texture of the pancreatic parenchyma was adequately defined by histopathology examination.

Statistical analysis

All data were reported as the mean \pm standard deviation (SD) and/or median. The data were analyzed by means of SPSS 12.01 statistical package for Windows. Mann-Whitney *U* test and Chi-square test was used for group comparison and Students' *t* test to analyze normally distributed quantitative data. *P* < 0.05 was considered statistically significant.

RESULTS

All patients underwent DP for elective benign or malignant, pancreatic or non-pancreatic diseases. There were 30 males (47%) and 34 females (53%). The patients' age ranged from 42 to 84 years (median age, 72.3 years).

The indications for surgery included 38 patients (59%) with pancreatic disease and 26 patients (41%) with non-pancreatic malignancy. Forty-three patients (67%) underwent DP with splenectomy and one or more additional procedures due to primary malignancy: 17 of these patients (40%) had primary pancreatic malignancy infiltrated surrounding organs and 26 patients (60%) had non-pancreatic malignancy infiltrated pancreas. The overall number of additional procedures was 57. Spleen preserving DP was performed in 8 patients (13%); they all had benign or borderline diseases, while DP with splenectomy was performed in 13 patients (20%). The patients' demographics, indications for surgery, operative and technical factors are summarized in Table 2.

The median postoperative length of hospital stay, in patients without fistula, was 11 d (range, 6-15 d); while in patients with pancreatic fistula the median hospitalization was prolonged: 23 d (range, 16-28 d).

The postoperative mortality rate was 1.5% (one patient died due to sepsis), while the morbidity rate was 37% (*n* = 24). The patient with sepsis had both pancreatic and oesophago-jejunal fistula after total gastrectomy for cardiac cancer. The last 7 years no mortality rate occurred. Fourteen patients (22%) developed a pancreatic fistula; 4 of fistulas (28.6%) were classified as Grade A, 9 (64.3%)

Table 2 Patients' demographics, indications for surgery, operative and technical factors

	No. of patients (%)
Sex	
Male	30 (47)
Female	34 (53)
Indications for surgery	
Pancreatic	
Cystadenoma	19 (30)
Adenocarcinoma	11 (17)
Neuroendocrine tumour	3 (5)
Cystadenocarcinoma	3 (5)
Chronic pancreatitis	1 (1.5)
Lymphangioma	1 (1.5)
Non-pancreatic	
Gastric adenocarcinoma	16 (25)
Retroperitoneal sarcoma	4 (6)
Colonic adenocarcinoma	2 (3)
Renal carcinoma	2 (3)
Adrenal grand carcinoma	1 (1.5)
Gastrointestinal stromal tumour	1 (1.5)
Operations	
DP + splenectomy	13 (20)
Spleen preserving DP	8 (13)
DP + splenectomy + additional procedure	43 (67)
Additional procedures	
Gastrectomy	26 (46)
Colon resection	12 (21)
Adrenalectomy	10 (17)
Small intestine resection	5 (9)
Nephrectomy	4 (7)
Closure of pancreatic stump	
Stapler	29 (45)
Suture	35 (55)

Table 3 Postoperative results

	No. of patients (%)
Death	1 (1.5)
Reoperation	2 (3)
Complications	
No	40 (63)
Yes	24 (37)
Pancreatic fistula	14 (22)
Grade A	4 (28.6)
Grade B	9 (64.3)
Grade C	1 (7.1)
Intra-abdominal hemorrhage	2 (3)
Intra-abdominal abscess	3 (5)
Pulmonary	5 (8)

as Grade B and only 1 as Grade C (7.1%). Two patients (3%) required a second operation due to postoperative intra-abdominal bleeding. None of patients required a reoperation because of intra-abdominal abscess or fluid collection; these patients were treated by percutaneous drainage. Postoperative results are showed in Table 3.

Pancreatic fistula was significantly more common in patients who underwent DP for primary malignant or benign pancreatic diseases ($P = 0.04$) and in patients who did not receive postoperative prophylactic octreotide therapy ($P = 0.01$). Of 26 patients who were operated for non-pancreatic malignant disease, only 3 (11%) experienced a leak, compared with 11 (29%) of the 38

Table 4 Incidence of pancreatic fistula after distal pancreatectomy according to examined risk factors

	Patients (<i>n</i> = 64)	No. (<i>n</i> = 50)	Fistula Yes (<i>n</i> = 14)	<i>P</i> value
Age (yr)				NS
< 70	23 (36)	17 (34)	6 (43)	
> 70	41 (64)	33 (66)	8 (57)	
Sex				NS
Male	30 (47)	23 (46)	7 (50)	
Female	34 (53)	27 (53)	7 (50)	
Pancreatic stump closure				NS
Stapler	29 (45)	22 (44)	7 (50)	
Suture	35 (55)	28 (56)	7 (50)	
Pathology				0.04
Pancreatic disease	38 (59)	27 (54)	11 (79)	
Non-pancreatic malignancy	26 (41)	23 (46)	3 (21)	
Octreotide therapy				0.01
Yes	34 (53)	30 (60)	4 (28)	
No	30 (47)	20 (40)	10 (72)	
Texture of pancreatic parenchyma				0.006
Soft	27 (42)	15 (30)	12 (86)	
Fibrotic	37 (58)	35 (70)	2 (14)	
Concomitant splenectomy				0.002
Yes	56 (87)	46 (92)	10 (71)	
No	8 (13)	4 (8)	4 (29)	
Procedures				NS
Pancreatic resection only	21 (33)	14 (28)	7 (50)	
Additional procedures	43 (67)	36 (72)	7 (50)	

NS: No significant. Values in parentheses are percentages.

patients who were operated for pancreatic disease only. Four (12%) of 34 patients who received octreotide developed a pancreatic leak, compared with 10 (33%) of 30 patients who did not.

The potential relationship between the texture of the pancreatic parenchyma and pancreatic leak was also evaluated, considering the remnant stump including resection margin: only 2 (5%) of the 37 patients with fibrotic pancreatic tissue experienced a leak, compared with 12 (44%) of the 27 patients with soft tissue ($P = 0.006$). Finally, we observed significant statistical difference comparing the patients who underwent concomitant splenectomy with them who did not: 10 (18%) of 56 patients with concomitant splenectomy experienced a pancreatic fistula, while 4 (50%) of 8 patients with spleen preserving procedure developed a pancreatic leakage.

The other factors such as age, gender, technique of pancreatic stump closure, and type of surgical procedures were not significantly associated with pancreatic fistula formation. The incidence of pancreatic fistula after DP according to the eight examined risk factors is summarized in Table 4.

DISCUSSION

In this report, we describe our 10-year experience with DP for pancreatic and non-pancreatic benign diseases and malignancies. Our findings identify the importance of four risk factors in the development of pancreatic fistula: fibrotic pancreatic parenchyma, non-pancreatic disease, concomitant splenectomy, and postoperative prophylactic

octreotide therapy were found to result in a statistically significant reduction in the rate of postoperative pancreatic leakage. No significant differences were found regarding to the onset of pancreatic fistula according to the rest of examined factors such as age, gender, technique of pancreatic stump closure, and additional procedures.

Mortality and morbidity after DP have significantly decreased the last decades^[6]. In this study, we support that DP can be performed with low perioperative mortality (1.5%), while the incidence of pancreatic fistula, which was the most common postoperative complication, was 22%. These results are in accordance with the most authors' conclusions^[3,4,7-9].

In the past, in most of published studies there was no definition of pancreatic fistula and the diagnostic criteria for this had not yet been completely established. Balcom *et al*^[10] defined fistula as the drainage of more than 30 mL of amylase-rich fluid, while Balzano *et al*^[4] used a strict definition of fistula: > 5 mL for five days after the fifth postoperative day. In this study, pancreatic leaks were classified according to the International Study Group on Pancreatic Fistula definition^[5]. Grade A of pancreatic fistula, called transient fistula, has no clinical impact, Grade B requires a change in management or adjustment in the clinical pathway, while in Grade C, a major change in clinical management or deviation from the normal clinical pathway occurs. Our results showed that the majority of pancreatic fistulas were belonged to Grade B. These patients were supported with parenteral nutrition, the drains were maintained in place, some of them were covered by antibiotics and all were leaded to a delay in discharge. The only patient with Grade C fistula died postoperatively due to sepsis.

The appropriate closure of the pancreatic stump after DP in order to reduce postoperative complications, especially the incidence of pancreatic fistula, remains an unsolved surgical problem. A great number of surgical techniques and instruments for treating the resected pancreatic surface and preventing pancreatic fistula after DP has been proposed: hand-sewn suture^[2,11], stapler^[1,2,7,11], a combination of stapler and suture^[2,7,11], the use of pledgetted suture^[7], fibrin-glue sealing^[12,13], and prolamine injection^[14]. The most commonly used techniques of pancreatic remnant management are the stapler and suture closure. Stapler division of the pancreatic parenchyma has already been found that is a simple, quick, and secure method of pancreatic stump closure^[1,6,15]. The study by Bassi *et al*^[16] is the only randomized controlled trial that compared the two techniques. They observed that using the stapler technique had better results in comparison with the suture closure (stapler 14% *vs* hand suture 33%). Takeuchi *et al*^[1] described a statistically significant reduction in fistula rate after stapler closure, Fahy *et al*^[9] described suture closure as a risk factor for pancreatic fistula, while Sheehan *et al*^[2] found that the suture closure of the pancreatic remnant was superior compared with the stapler closure (25% *vs* 14% respectively). Finally, Kajiyama *et al*^[15] and Bilimoria *et al*^[7] found no differences between the two techniques. In our study, the authors' technical approach involved stapler and suture closure of the resected pancreatic parenchyma; in 45% of the patients

the pancreatic parenchyma was closed using a linear stapler (GIA), while in 55% of the patients an absorbable monofilament 3-0 suture was used. We were unable to determine the advantage of one method of pancreatic remnant closure on the development of pancreatic fistula: onset of fistula was observed in 24% of patients in stapler group, and in 20% of patients in hand suture group.

Several studies have shown that fistula formation was not related to the main pancreatic duct ligation^[3,17], while others reported the opposite^[6]. We ligated and closed the main pancreatic duct with sutures in all patients after DP thinking that may be helpful in reducing possible pancreatic leaks. In almost all of them, we had no difficulty to find out the pancreatic duct. However, there was no significant difference according to the incidence of pancreatic fistula in our study in comparison with the literature studies^[3,4,7-9].

Many authors state that the texture of the pancreatic parenchyma seems generally to be one of the most important risk factors responsible for the increased rate of pancreatic fistula^[2,9]. The fibrotic pancreatic tissue is believed to be less likely to pancreatic leakage. In accordance with the reported studies, we observed that the patients with soft pancreatic tissue had higher incidence of pancreatic leakage compared with them who had fibrotic pancreatic parenchyma (44% *vs* 5%, $P = 0.006$).

We used postoperative prophylactic octreotide treatment in 53% of the patients and 12% of them developed a pancreatic leak, compared with 33% of patients who did not receive octreotide. However, the role of this treatment still remains unclear. Two randomized trials by Lowy *et al*^[18] and Yeo *et al*^[19] failed to identify a decrease in the pancreatic leakage in patients underwent pancreaticoduodenectomy, while Gouillat *et al*^[20] demonstrated a decreased leak rate in a randomized trial of patients who underwent pancreaticoduodenectomy. Buchler *et al*^[21] reported that the use of octreotide could prevent pancreatic fistula following pancreatic resection. They showed that the incidence of pancreatic fistula was 18% in the patients received perioperative octreotide treatment and 37% in patients received placebo after pancreatic resection. Suc *et al*^[22] also described the advantage of using octreotide after pancreaticoduodenectomy.

The present report also highlights the importance of the primary disease in development of pancreatic fistula after DP: the patients underwent DP for pancreatic disease only had a higher incidence of pancreatic fistula compared with them who underwent DP for non-pancreatic malignancies. There is no doubt that it represents a quite strange observation because in patients with DP due to extrapancreatic malignancies the pancreatic tissue is generally softer than them who undergo DP for pancreatic diseases and we have no scientific explanation. However, new and larger studies are needed to determine if the extrapancreatic malignancies are associated with lower risk of pancreatic fistula after DP or not. Interestingly, there are no studies in the literature, at our knowledge, that examine the primary disease as significant risk factor in the onset of pancreatic fistula. In published series, only the underlying pancreatic disease have examined

as a responsible factor for the occurrence of pancreatic leakage^[2,9]. This is the first study to show that primary (pancreatic and non-pancreatic) disease is a risk factor for postoperative morbidity after DP, especially for pancreatic leakage ($P = 0.04$).

In this study, postoperative comparison suggests that the patients underwent DP with splenectomy had a significantly lower incidence of postoperative pancreatic leakage compared with patients who underwent DP with spleen preserving. In contrast, Balzano *et al*^[4] reported that their patients with spleen preservation had less pancreatic leakage compared to patients with splenectomy (20% *vs* 38%, $P = 0.15$), while Lillemoe *et al*^[23] reported that the patients who underwent a DP with splenectomy had a similar complication rate (30% *vs* 29%) compared to patients who underwent spleen preserving DP, but with no specific focus on pancreatic leak.

Finally, we did not find a relation between the onset of pancreatic fistula and demographic factors (age, gender) nor with the technique of pancreatic closure (there has not been any correlation between pancreatic texture and the dissection technique used) and the additional procedures. In our series, fistula occurred similarly in patients who underwent pancreatic resection only and in patients who underwent one or more additional procedures.

In conclusion, pancreatic fistula after DP affects 22% of patients. None of the two techniques we used seemed useful to significantly reduce this incidence. Based on these findings, we support that the role that technique (either stapler or suture) of pancreatic stump closure plays in the development of pancreatic leak is unclear. Both techniques are regarded as simple, quick and secure although the fistula rate remains high. However, there is a clearly determined relationship between the primary pathology, the octreotide therapy, the texture of the pancreatic parenchyma, the concomitant splenectomy and the postoperative pancreatic fistula formation.

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Electrogastrography: Poor correlation with antro-duodenal manometry and doubtful clinical usefulness in adults

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show a spatial correlation. The diagnostic value of EGG is poor, but EGG may have some value for the identification of patients with STC.

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Abstract

AIM: To investigate if there is a correlation between electrical activity measured by electrogastrography (EGG) and contractile activity of the stomach as measured by antroduodenal manometry (ADM). We also studied whether the underlying motility disorder could be predicted from EGG parameters.

METHODS: We compared 21 parameters measured from EGG with 8 parameters measured from ADM. The ability of EGG to identify the underlying diagnosis was tested by comparing EGG parameters for each diagnosis group against other patients. The study comprised recordings from 148 patients and 125 females. Their median age was 45 (range 17-76) years.

RESULTS: We found few and weak correlations between EGG and ADM. Specifically the correlation between parameters reflecting the response to meal was poor ($r = -0.07$, $P = 0.39$). The discriminatory power of EGG for underlying motility disorder was also low. Patients with slow transit constipation (STC) showed a lower postprandial power in normogastric (3.7 ± 0.5 vs 4.0 ± 0.5) and tachygastric (3.5 ± 0.4 vs 3.7 ± 0.4) regions, a lower percentage of time with normogastria [87.2 (56.5-100)% vs 95.7 (0-100)%], and a higher percentage of time with tachygastria [9.3 (0-33)% vs 3.5 (0-100)%] and bradygastria [1.8 (0-20)% vs 0 (0-17.1)%]. Patients with irritable bowel syndrome had a higher percentage of time with normogastria [96.5 (62.5-100)% vs 93.3 (0-100)%] and a less unstable dominant frequency as measured by the instability coefficient [15 (3-77) vs 24 (2-72)].

CONCLUSION: EGG and ADM seem to measure different aspects of gastric motor activity but cannot

INTRODUCTION

Electrogastrography (EGG) is a technique for recording gastric myoelectric activity using cutaneous electrodes placed on the abdominal wall overlying the stomach. EGG detects rhythm and power (amplitude) of gastric myoelectricity^[1]. Studies have shown a good correlation between cutaneous EGG recordings and myoelectric signals recorded from gastric serosal leads^[2]. However, there are major concerns regarding the clinical usefulness of this non-invasive procedure^[3].

EGG has been advocated as a diagnostic test in the clinical evaluation of patients with unexplained nausea, vomiting and other dyspeptic symptoms to gain insight into the mechanisms of symptom generation. Conflicting results have been obtained in previous studies. In some studies, subsets of patients with upper abdominal symptoms have depicted prominent EGG abnormalities whereas healthy volunteers rarely have exhibited EGG rhythm or power disturbances^[4,5]. Other studies have found no difference in EGG parameters between dyspeptic patients and healthy volunteers^[6,7].

Patients with systemic diseases such as Parkinson's disease^[8], myotonic dystrophy^[9], and diabetes mellitus^[10] show abnormal EGG findings. However, in patients with progressive systemic sclerosis (which is usually associated with poor gut motility), EGG shows unchanged parameters^[11]. Similarly, EGG shows a greater variability compared to normal volunteers but no typical EGG pattern could be identified for different surgical procedures in surgical patients after cholecystectomy, Nissen fundoplication, subtotal gastrectomy or vagotomy and gastric pull-up operations^[12].

Antroduodenal manometry (ADM) records lumen-occluding contractions of the gastric antrum^[13]. Under physiological conditions there is a temporal correlation between myoelectric slow waves and contractile activity of the antrum. It is less clear if there is a spatial correlation between electrical and contractile activity. A small study in healthy females showed an unstable EGG peak power during fasting and an increase in EGG peak power after a solid test meal as indicative of a correlation between electrical activity and gastric motor activity^[14]. It is conceivable that there should be a correlation between electrical activity measured by EGG and contractile activity measured by ADM. It would be of interest to know if EGG can predict gastric contractile activity. Surprisingly, a direct head to head comparison between EGG and ADM has not been done previously.

The aim of the present study was to compare measures of electrical activity determined by EGG and antral contractile activity from antroduodenal manometry. We also investigated whether EGG could differentiate between different underlying diagnoses of motility disorders.

MATERIALS AND METHODS

Subjects

This was a retrospective analysis of data collected during 1994-2001. Adult patients suffering from various functional and motility disorders of the gastrointestinal tract were included in our study. All of them were subjected to a combined EGG and ADM study as part of their clinical work-up for suspected gastrointestinal motility disorders. The two measurements were done simultaneously using two different systems, thus synchronous analysis was not possible in this study.

Electrogastrography

This procedure has been described elsewhere^[6]. Briefly, a single bipolar channel was used. The EGG signal was recorded using a specially designed digital recorder (Digitrapper-EGG, Medtronic Synectics, Stockholm, Sweden) and sampled at a rate of 1 Hz. EGG recordings were examined visually for artefacts and then categorized into excellent (no artefacts), good (< 25% of recording time excluded because of artefacts), fair (< 50% excluded) or poor quality (\geq 50% excluded). After exclusion of artefacts, the remaining parts of the recordings were analysed with a software provided by the vendor (Polygram version 6.40, Medtronic Synectics, Stockholm, Sweden). This program could analyse the EGG using fast Fourier transformations of 256-s runs with an overlap of 196 s, so-called running spectral analysis, and can also make a Fourier transformation of an entire period in order to obtain a single power spectrum for that period. The parameters derived from the automated analysis of EGG are shown in Table 1. Dominant frequency instability coefficient (DFIC) and dominant power instability coefficient (DPIC) are measures of the variability of the EGG signal, which were computed as the standard deviations divided by the mean value of the dominant frequency and dominant power, respectively, from the running spectral analysis of each period.

Table 1 Electrogastrography (EGG) and antroduodenal manometry (ADM) variables

1	EGG variables
(1)	Preprandial (fasting) and postprandial percentage of time with dominant frequency in:
	Bradygastria (0.5-2 cycles per minute)
	Normogastria (2-4 cycles per minute) (0.5-2 cycles per minute)
	Tachygastria (4-10 cycles per minute)
(2)	Pre- and postprandial power attributed to the three frequency bands (bradygastria, tachygastria and normogastria)
(3)	Pre- and postprandial dominant power instability co-efficient (DPIC)
(4)	Pre- and postprandial dominant frequency instability co-efficient (DFIC)
(5)	Pre- and postprandial dominant frequency (DF)
(6)	Pre and postprandial period dominant power (PDP)
(7)	Power ratio (ratio of postprandial to preprandial power of dominant frequency)
2	ADM variables
(1)	Pre- and postprandial motility index (MI)
(2)	Pre- and postprandial contractile frequency
(3)	Pre- and postprandial median amplitude
(4)	MI ratio (ratio between postprandial and preprandial motility index)
(5)	Amplitude ratio (ratio between postprandial and preprandial amplitude)

Antroduodenal manometry

ADM was performed using a flexible catheter with either 6 or 8 water-perfused channels. The 6-channel catheter has 3 channels spaced 2 cm apart for the recording of antral motor activity whereas the 8-channel catheter has 5 channels spaced 1 cm apart for the same purpose. We used a pneumo-hydraulic pump (Arndorfer Medical Specialties, Greendale, WI, USA) with catheters connected to external pressure transducers. Data logging was done with a Polygraph 12HR and the Polygram (Medtronic Synectics, Stockholm, Sweden). The protocol included a 3-hour fasting period followed by a test meal (500 kcal standardized mixed meal) and 2-hour post-prandial recording^[15]. Numerical analysis was done on data from the most distal recording site in the gastric antrum. Data were extracted using the Polygram that was set to detect contractions with an amplitude > 9 mmHg. We determined the mean frequency and amplitude of contractions and motility index during the last hour before and the first hour after intake of the test meal. The motility index was calculated as the area under the curve above baseline per time unit. We compared 8 variables from ADM with 21 variables from EGG (Table 1).

Statistical analysis

Normally distributed and lognormal data were expressed as mean \pm SD and data with a non-normal distribution were given as a median and full range. We used Student's *t*-test and Mann-Whitney *U*-test for assessing differences between the groups. Correlations were studied using Pearson's product moment correlation. Logistic regression analysis was done to test EGG parameters for their discriminatory value in predicting underlying diagnoses. *P* < 0.05 was considered statistically significant.

Table 2 Correlation matrix (*r*-values) for parameters from antroduodenal manometry and electrogastrography (*n* = 144)

Parameters	Pre MI ¹	Post MI ¹	MI ratio [§]	Pre Freq	Post Freq	Pre Amp ¹	Post Amp ¹	Amp Ratio ¹
Pre-Brady power ¹	0.15	0.01	-0.10	0.10	0.01	0.08	0.06	-0.01
Pre-Normo power ¹	0.06	0.03	-0.01	0.10	0.05	-0.01	-0.01	0.00
Pre-Tachy power ¹	0.10	0.05	-0.03	0.13	0.07	0.01	0.00	-0.01
Pre-Brady DF%	0.06	-0.01	-0.05	0.05	0.01	-0.09	0.08	0.12
Pre-Normo DF%	0.05	0.09	0.06	0.08	0.08	0.06	0.14	0.06
Pre-Tachy DF%	-0.03	-0.13	-0.11	-0.07	-0.12	-0.02	-0.18 ^a	-0.11
Pre-DFIC	-0.04	0.04	0.06	-0.12	0.02	0.07	0.03	-0.04
Pre-DPIC	-0.01	-0.13	-0.12	-0.12	-0.13	0.09	-0.10	-0.14
Pre-DF	-0.08	-0.11	-0.05	-0.10	-0.10	-0.04	-0.17 ^a	-0.09
Pre-PDP ¹	-0.09	0.01	0.08	0.00	0.03	-0.02	-0.04	-0.02
Post-Brady power ¹	0.04	-0.04	-0.07	0.13	-0.04	-0.10	0.02	0.09
Post-Normo power ¹	0.09	-0.01	-0.07	0.13	0.04	-0.03	-0.04	-0.01
Post-Tachy power ¹	0.08	0.01	-0.05	0.15	0.03	-0.06	-0.02	0.03
Post-Brady DF%	0.00	0.01	0.01	-0.02	-0.07	-0.06	0.01	0.05
Post-Normo DF%	-0.03	-0.03	-0.01	-0.06	-0.04	0.06	0.06	0.00
Post-Tachy DF%	0.02	-0.04	-0.05	0.05	-0.02	-0.05	-0.09	-0.02
Post-DFIC	0.04	0.03	-0.01	0.11	0.08	-0.09	-0.11	-0.01
Post-DPIC	-0.06	-0.1 ^a	-0.14	-0.09	-0.17 ^a	0.02	-0.18 ^a	-0.15
Post-DF	0.04	-0.01	-0.04	0.06	0.00	-0.05	0.00	0.04
Post-PDP ¹	0.06	-0.01	-0.04	0.08	0.04	0.01	-0.02	-0.03
Power ratio ¹	0.04	-0.04	-0.07	0.04	-0.01	-0.02	-0.04	-0.01

¹Log values, ^a*P* < 0.05.

RESULTS

A total of 185 patients were eligible for the study but 37 were excluded either because of previous gastric resection (*n* = 6), poor quality of the EGG recording (*n* = 23), or because of a technical failure (*n* = 8) in the EGG system. Another 4 patients were excluded from the comparison between EEG and ADM because ADM recordings did not show a recording from the antrum during the fasting or the fed period or both. Thus, the study population finally included 148 patients for comparing EGG parameters among various diagnosis groups and 144 patients for assessing the correlation between EGG and ADM parameters. The median age of the patients was 45 (range 17-76) years and 122 (82%) were females. There were 52 patients (35%) with IBS, 22 (15%) with enteric dysmotility (ED), i.e. abnormal small bowel motor activity but no bowel dilatation^[16], 26 (18%) with slow transit constipation (STC), 11 (7%) with chronic intestinal pseudo-obstruction (CIP), 13 (9%) with functional dyspepsia with or without gastroparesis and 24 (16%) with other diagnoses, including various systemic and neurological diseases.

Comparison of electrogastrography and antral manometry

Few correlations were found between EGG and ADM. The correlation matrix was analyzed between 21 EGG parameters and 8 ADM parameters in this study (Brady = bradygastria, Normo = normogastria, Tachy = tachygastria, DF = dominant frequency, DFIC = dominant frequency instability coefficient, DPIC = dominant power instability coefficient, PDP = period dominant power, Amp = amplitude, MI = motility index, Freq = frequency, Pre = preprandial, Post = postprandial) (Table 2).

Postprandial contractile activity (amplitude, frequency and motility index) showed weak, negative correlations with the postprandial dominant power instability coefficient. Preprandial tachygastria (reflected by the percent-

age of time with a dominant frequency in the tachygastria region and dominant frequency) was also negatively correlated with postprandial contractile amplitude. The comparison between EGG response to meal ingestion (ratio between postprandial and fasting power) and ADM response to ingestion of a 500 kcal test meal (ratio between postprandial and fasting motility index) revealed no correlation (*r* = -0.07, *P* = 0.44, Figure 1).

Electrogastrography and underlying diagnosis

The ability of EGG to identify diagnostic groups was tested by comparing EGG parameters for each group against all other patients included. Table 3 shows the discriminatory power of EGG for the larger patient groups. Variables with a normal or log-normal distribution were expressed as mean ± SD, else as median and full range. Patients with other diagnoses (*n* = 24) were not listed but included in the calculation of differences. Significant *P*-values were expressed as ^a*P* < 0.05, ^b*P* < 0.01, and ^d*P* < 0.001 (Brady: bradygastria, Tachy: tachygastria, Normo = normogastria, DFIC: dominant frequency instability coefficient, DPIC: dominant power instability coefficient, DF: dominant frequency, PDP: period dominant power).

In general, the discriminatory power of EGG was low. Only patients with STC in one diagnostic group, showed a reasonable number of differences in postprandial EGG parameters compared to those in all other diagnosis groups. STC patients had a lower postprandial power in the normogastric region (3.7 ± 0.5 vs 4.0 ± 0.5 , *P* < 0.01) and in the tachygastria region (3.5 ± 0.4 vs 3.7 ± 0.4 , *P* < 0.05). Similarly, the postprandial percentage of time with a dominant frequency was higher in bradygastric and tachygastria frequency bands and lower in the normogastric frequency band of STC patients (Table 3). Patients with STC also demonstrated a significantly lower postprandial dominant power (2.1 ± 0.6 vs 2.4 ± 0.5) compared to other patients.

In patients with CIP, EGG during fasting showed a sig-

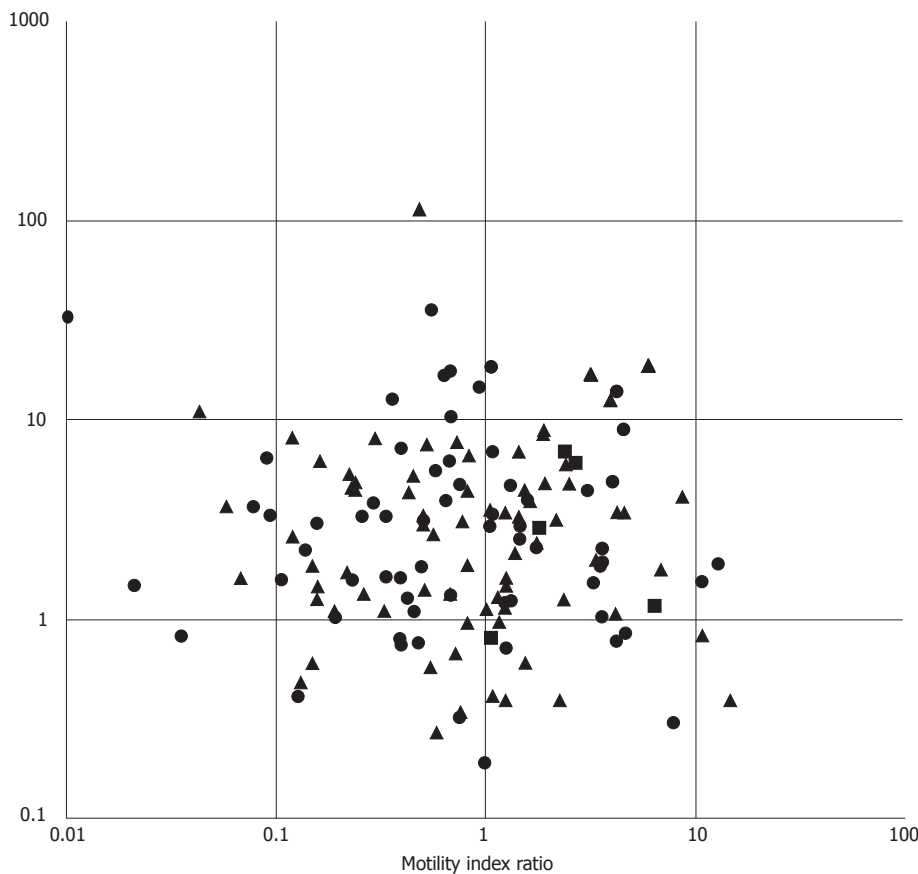


Figure 1 Scatterplot of motility index ratio and power ratio in 144 patients (logarithmic scales). Symbols indicate different qualities of the EGG record: ■ = excellent; ● = good; and ▲ = fair quality.

Table 3 Electrogastrography parameters among various diagnostic groups

EGG parameter	Chronic intestinal pseudo-obstruction (<i>n</i> = 11)	Enteric dysmotility (<i>n</i> = 22)	Irritable bowel syndrome (<i>n</i> = 52)	Functional dyspepsia/gastroparesis (<i>n</i> = 13)	Slow transit constipation (<i>n</i> = 26)
Preprandial					
Log Brady power	2.9 ± 0.4	2.8 ± 0.5	2.7 ± 0.5	2.7 ± 0.4	2.6 ± 0.4
Log Normo power	3.8 ± 0.5 ^a	3.7 ± 0.5	3.6 ± 0.5	3.6 ± 0.5	3.5 ± 0.5
Log Tachy power	3.7 ± 0.4 ^a	3.5 ± 0.5	3.3 ± 0.4	3.4 ± 0.4	3.3 ± 0.4
Brady DF%	2.7 (0-16.2)	1.8 (0-17.5)	0 (0-15.2)	0 (0-10.3)	2 (0-14.3)
Normo DF%	89.7 (0-100)	88.2 (36.2-100)	92.0 (38.6-100)	89.8 (59-100)	87.7 (30.8-100)
Tachy DF%	6.0 (0-100)	8.8 (0-61.7)	5.1 (0-61.4)	6.5 (0-73.7)	6.1 (0-69.2)
DFIC	22 (3-51)	32 (5-68)	23 (3-75) ^a	31 (1-70)	30 (3-69)
DPIC	77 (40-165)	67 (33-169)	64 (39-145)	65 (31-101)	60 (47-159)
DF	3.05 (2.58-11.25)	3.05 (2.34-3.98)	3.05 (2.11-6.05)	3.05 (2.58-3.75)	3.05 (2.11-8.91)
Log PDP	2.4 ± 0.6 ^a	2.0 ± 0.5	2.1 ± 0.6	2.1 ± 0.5	1.9 ± 0.5
Postprandial					
Log Brady power	3.0 ± 0.4	3.0 ± 0.5	2.9 ± 0.4	2.9 ± 0.5	2.8 ± 0.5
Log Normo power	4.1 ± 0.3	4.1 ± 0.5	4.0 ± 0.5	4.1 ± 0.7	3.7 ± 0.5 ^b
Log Tachy power	3.9 ± 0.4	3.8 ± 0.5 ^a	3.6 ± 0.4	3.8 ± 0.6	3.5 ± 0.4 ^a
Brady DF%	0 (0-5.8)	0 (0-5.0)	0 (0-14.5)	0 (0-17.1)	1.8 (0-20) ^b
Normo DF%	92.3 (0-100)	96.1 (32.1-100)	96.5 (62.5-100) ^a	95.5 (32-100)	87.2 (56.5-100) ^d
Tachy DF%	5.8 (0-100)	1.8 (0-66)	3.1 (0-18.8)	1.9 (0-21.1)	9.3 (0-33) ^b
DFIC	18 (4-63)	16 (2-62)	15 (3-77) ^a	27 (5-56)	27 (8-72) ^b
DPIC	67 (59-81)	56 (35-110)	70 (34-129)	61 (30-82)	64 (39-108)
DF	3.05 (2.58-9.14)	3.28 (2.34-9.61)	3.05 (2.58-3.75)	3.28 (2.11-10.08)	3.05 (2.34-3.75)
Log PDP	2.5 ± 0.5	2.5 ± 0.5	2.4 ± 0.5	2.5 ± 0.8	2.1 ± 0.6 ^b
Log Power ratio	0.2 ± 0.5	0.5 ± 0.4	0.4 ± 0.4	0.5 ± 0.6	0.2 ± 0.5

nificantly higher power in the tachygastric region (3.7 ± 0.4 vs 3.3 ± 0.4 , $P < 0.05$) and a higher period dominant power (2.4 ± 0.7 vs 2.0 ± 0.5) compared to patients without CIP. In patients with IBS there was a higher percentage of time with a normogastric dominant frequency in the postprandial period and a more stable dominant frequency mea-

sured by DFIC during both preprandial and postprandial periods (Table 3).

Twenty patients underwent gastric emptying studies. Five of them had delayed gastric emptying. No significant differences were found in EGG parameters among patients with or without delayed gastric emptying.

DISCUSSION

To the best of our knowledge, this is the largest series of simultaneous recordings of EGG and ADM in patients with motility disorders of the gut. In the present study, we studied the relationship between EGG parameters and antral manometric findings. We also tried to evaluate the role of EGG in discriminating between various motility disorders of the gastrointestinal tract. EGG is not capable to record spike activity, hence the major electrical indicator of contractile activity is absent in its recordings. However, an increase in wave amplitude on EGG (referred to as power from the spectral analysis) can be asserted as a reflection of contractile activity. It has been observed that EGG power increases after certain drugs or meals that stimulate motility and decreases after drugs or meals that inhibit motility^[12]. Therefore, to measure the significance of EGG power, it is best to compare EGG changes before and after the test meal given during EGG recording^[14]. We found few and weak correlations between ADM findings and EGG parameters, especially no correlation between the motility index ratio and the power ratio. This is contrary to the findings of another study^[17], where the EGG power ratio was significantly but weakly correlated with the motility index ratio. The number of patients in that study was, however, only 16. A partial correlation has also been found in children between the postprandial increase in amplitude of gastric electrical activity and antral motor activity^[18]. In the present study, a more stable dominant power, i.e. lower DPIC, was associated with a higher contractile activity and tachygastria during the preprandial period was associated with lower amplitudes of contraction during the postprandial period, but the correlations were weak in both cases.

One important reason for a poor correlation between EGG-recorded electrical activity and ADM-recorded motor activity of the stomach could be that EGG recordings are sensitive to the degree of gastric wall distension and its approximation to the anterior abdominal wall. An underlying motility disorder of the gut might cause the gastric wall to distend inappropriately in response to the test meal and therefore lead to an inadequate EGG recording of gastric myoelectric activity. In light of the weak correlation between ADM and EGG found in other studies^[17,18] and our observations, we think that EGG and ADM can measure two different aspects of gastric motor activity and a spatial relation cannot be fully determined between EGG and ADM.

Surprisingly, STC in the only diagnostic group stood out as clearly different from the other diagnostic groups on the basis of EGG parameters. Patients with STC exhibited differences mainly in the postprandial EGG parameters. Many studies have demonstrated a decrease in the density of interstitial cells of Cajal (ICC) in patients with slow transit constipation^[19-21]. It is possible that observed postprandial changes in EGG parameters might be the reflection of a more generalized gastrointestinal myoelectrical dysfunction in STC patients secondary to depletion of ICC and other neuroenteric abnormalities. Further studies are needed to validate the EGG findings in patients with STC in the present study.

It was reported that EGG is diagnostically useful in adult patients with CIP^[22]. Moreover, a comparison be-

tween normal subjects and children with CIP could reveal a significant increase of preprandial values for tachygastria and period dominant frequency and a decrease in normogastria and an increase in total tachygastria values in postprandial period^[23]. In our study, the CIP patients had a significantly higher power on EGG in the tachygastria region and a higher PDP during fasting.

Another field of interest among researchers interested in evaluating the clinical usefulness of EGG is functional dyspepsia with or without gastroparesis. Studies have shown abnormal EGG patterns such as abnormal slow wave propagation and coupling^[24], lower power ratio, lower postprandial level of increment in dominant power and a higher instability coefficient^[25,26] in patients with functional dyspepsia. However, abnormal parameters are not the same in different studies. Interestingly, significantly more gastric arrhythmias have been found in oesophageal reflux patients with dyspeptic symptoms than in those with no dyspeptic symptoms^[27].

Contradictory findings can be found when capability of EGG is assessed to predict delayed gastric emptying. It was reported that patients with impaired gastric emptying show a significantly lower percentage of normal gastric slow waves and a lower postprandial increment in dominant power^[28]. On the other hand, another study conducted in patients with systemic sclerosis has not found any correlation between delayed gastric emptying and EGG abnormalities^[11]. In our study, no difference in EGG parameters was found between dyspeptic patients with delayed gastric emptying and those with normal gastric emptying, possibly due to a small number of patients with gastric emptying.

Our study has certain limitations. One of them was the retrospective design of the present series, which is unlikely to have influenced the results of the present study concerning the correlation between EGG and ADM. On the other hand, the comparison of EGG parameters between different diagnostic groups was hampered by a small number of patients in certain groups. Another limitation was the absence of a control group to compare with patients with motility disorders. In the present series, however, comparison of both EGG and ADM comparison was available in each patient. In our opinion, the assessment of correlation between EGG and ADM is not affected by the absence of a control group. We used a single channel EGG instead of a multiple channel EGG in our study. Novel multi-channel EGG can study the propagation and coupling of slow waves in the stomach, and is perhaps more helpful than single channel EGG in the diagnosis of gastric dysfunction^[24]. It is possible that registrations from multiple channels would have a better chance to detect correlations with contractile activity, not least because sampling error from multiple channels would be smaller than those from a single channel. However, no data are available from comparisons between ADM and multi-channel EGG.

A high exclusion rate of patients in our series was due to EGG recordings that were not interpretable. Motion artefacts were common despite the stationary condition of the patients. Care was taken in preparing the skin so that the impedance between any two electrodes was less than

10 ohms. Nevertheless, many recordings exhibited low frequency aberrations and shifts of baseline. Moreover, various technical problems, such as faulty electrode cables or malfunction due to static electricity, also resulted in exclusion of a significant number of patients from analysis. We have not come across any account for such failures of EGG recording in previously published studies.

In short, no consistent relationship was observed between EGG and ADM in this series. No evidence was found in this study to favour a spatial correlation between the parameters measured by EGG and ADM. Moreover, the ability of EGG to identify motility disorders was generally poor in this study.

In conclusion, EGG findings in STC patients are interesting but further studies are required to validate the role of EGG in STC patients.

COMMENTS

Background

Electrogastrography is to measure the electrical activity of the stomach from electrodes attached to the abdominal surface. The usefulness of this technique for the diagnosis of gastrointestinal diseases is yet unknown. However, since electrical activity is pivotal for motor function, it is believed that measurement of electrical activity should reveal important diagnostic information in patients with motility disorders.

Research frontiers

The correlation between measures derived from electrogastronomy and other techniques such as manometry has received little attention. Such correlations are important for understanding the relation between different measurement techniques.

Innovations and breakthroughs

This is a head-to-head comparison of electrogastronomy and antroduodenal manometry, i.e. measurement of contractile activity of the stomach done at the same time from a large number of patients with gastrointestinal motility disorders.

Applications

This study could not show any correlation between antroduodenal manometry and electrogastronomy, suggesting that the two techniques can measure different aspects on gastric motor function. The study also showed that the ability of electrogastronomy to identify patients with different diagnoses was poor. To the surprise of the authors, patients with slow transit constipation, a severe form of intractable constipation, were the only group of patients identified by electrogastronomy. The reason for this is not clear, but it is known from previous research that patients with slow transit constipation have a lack of pacemaker cells in the gut, which could explain why they also had disturbed electrical rhythm in the stomach.

Terminology

Correlation is a measure of the relative correspondence of two sets of data. Manometry is the measurement of (in this case, intraluminal) pressure over time. Motility disorders comprise a large number of disorders that affect gastrointestinal motor activity. The most common disorder is irritable bowel syndrome.

Peer review

This is a well written paper on a subject with a considerable debate. Whether electrogastronomy and antroduodenal manometry can measure different aspects of gastric motor activity needs to be further studied.

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RAPID COMMUNICATION

24-hour esophageal pH-monitoring in children suspected of gastroesophageal reflux disease: Analysis of intraesophageal pH monitoring values recorded in distal and proximal channel at diagnosis

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Abstract

AIM: To assess values of 24-h esophageal pH-monitoring parameters with dual-channel probe (distal and proximal channel) in children suspected of gastroesophageal reflux disease (GERD).

METHODS: 264 children suspected of gastroesophageal reflux (GER) were enrolled in a study (mean age $\chi = 20.78 \pm 17.23$ mo). The outcomes of this study, immunoallergological tests and positive result of oral food challenge test with a potentially noxious nutrient, enabled to qualify children into particular study groups.

RESULTS: 32 (12.1%) infants (group 1) had physiological GER diagnosed. Pathological acid GER was confirmed in 138 (52.3%) children. Primary GER was diagnosed in 76 (28.8%) children (group 2) and GER secondary to allergy to cow milk protein and/or other food (CMA/FA) in 62 (23.5%) children (group 3). 32 (12.1%) of them had CMA/FA (group 4-reference group), and in remaining 62 (23.5%) children neither GER nor CMA/FA was confirmed (group 5). Mean values of pH monitoring parameters measured in distal and proximal channel were analyzed in individual groups. This analysis showed statistically significant differentiation of mean values in the case of: number of episodes of acid GER, episodes of acid GER lasting > 5 min, duration of the longest episode of acid GER in both channels, acid GER index total and supine in proximal channel. Statistically significant differences of mean values among examined groups, especially between group 2 and 3 in the case of total acid GER index (only distal channel) were confirmed.

CONCLUSION: 24-h esophageal pH monitoring

confirmed pathological acid GER in 52.3% of children with typical and atypical symptoms of GERD. The similar pH-monitoring values obtained in group 2 and 3 confirm the necessity of implementation of differential diagnosis for primary vs secondary cause of GER.

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Key words: Children; Gastroesophageal reflux disease; 24-h esophageal pH-monitoring; 2-channel probe; Gastroesophageal reflux; Primary and secondary; CMA/FA; Oral food challenge test

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INTRODUCTION

Acid gastroesophageal reflux (GER) in children at any age, could be the reason of various clinical manifestation (typical and atypical) of variable intensity, dependent on the range of reflux (high reflux, low reflux)^[1-9]. On the basis of reflux symptoms it is hardly possible to differentiate primary GER from GER secondary to allergy to cow milk protein and/or other food (CMA/FA)^[10-13]. In children suspected of gastroesophageal reflux disease (GERD), 24-h esophageal pH-monitoring is a diagnostic procedure that enables to confirm or exclude pathological reflux of acid gastric contents into esophagus i.e. acid GER^[14-20]. Diagnostic procedure, including food allergy contribution in GER, is necessary to distinguish primary GER from secondary GER. Such procedure includes immunoallergological tests i.e. Prick tests, cIgE, sIgE^[2,12,21-25]. Positive result of food oral challenge test with potentially noxious nutrient (biological trial) confirmed contribution of food allergy in triggering off reflux symptoms^[25,26]. Comparative analysis of 24-h intraesophageal pH monitoring with dual-channel probe (distal and proximal channel) in children suspected of gastroesophageal reflux disease (GERD).

MATERIALS AND METHODS

264 children suspected of GERD, of both sexes (140 boys-53.0% and 124 girls-47.0%) were enrolled in a study. Patients were 1.5 mo to 102 mo old, mean age $\bar{x} = 20.78 \pm 17.23$ mo. Information gathered at interview revealed that various gastrointestinal diseases appeared in family histories of all patients.

24-h esophageal pH monitoring

24-h intraesophageal pH-monitoring was performed with antimony dual-channel pH monitoring probe (distal channel/distal and proximal channel) and a device recording the values Digitraper MK III, Synectics Medical. Antimony electrode was calibrated with buffer solutions of pH = 7.0 and pH = 1.0. pH-monitoring probe, 2.1 diameter, was positioned in esophagus through one of the nostrils and pharynx with distal channel (2) at the height of 3-5 cm, and proximal channel (1) at 10, 15 or 20 cm above the upper edge of lower esophageal sphincter (LES). To localize the probe Strobel's mathematical mode, radiological examination, and manometry of LES were carried out^[27-29].

The type of dual-channel probe (sensors spacing 5, 10 or 15 cm) and its positioning in esophagus depended on the age of a patient (various lengths of esophagus) and clinical manifestation of GER (typical or atypical symptoms). The analysis of the type of reflux (acid/non-acid) was based on the pH monitoring values recorded in distal channel (2).

Recording from proximal channel (1) of the probe above LES (at various height) enabled the assessment of the range of reflux (high/low reflux) and the control of the proper positioning of the probe. It was also possible to compare the number of acid and non-acid refluxes in both positions of pH-monitoring. Esophageal pH monitoring always began in the morning, after fasting all night. The study lasted 24 h and comprised night sleep.

Patients discontinued particular medicines 3 d before the test. Among these medicines were: antacids, gastrokinetics, medications affecting tension of LES. Proton pump inhibitors, H₂-receptor antagonists were discontinued 7 d before the test^[30].

Computer calculations of measurements obtained from both channels concerned the following pH-monitoring parameters: number of episodes of acid GER (intraesophageal pH below 4.0), number of episodes of acid GER lasting more than 5 min (so-called "long episodes"), reflux index i.e. the percentage of time that the pH is below 4.0. ESPGHAN diagnostic criteria were implemented in diagnosis of acid GER in examined children^[14,15].

In children below 2 years of age the values of intraesophageal pH-monitoring were juxtaposed against normative values (borderline values) of Vandenplas *et al*^[16,17] and another authors^[18,19].

In older children (above 2 years of age) the borderline values at quantitative and qualitative assessment of pathological GER in both channels were^[14,15,31-33]: total number of acid GER episodes (pH < 4.0/24 h) = 50; number of episodes of acid GER lasting more than 5 min

≤ 2; the percentage of time with pH below 4.0 (%) - total acid GER index = 5.0%; the percentage of time with pH below 4.0 (%) - acid GER index supine = 2.5%.

Differentiation of pathological acid GER

In order to differentiate pathological GER into primary (idiopathic) and secondary - triggered off or aggravated by CMA/FA, the own diagnostic and therapeutic algorithm was administered in examined children. This algorithm comprises selected immunoallergological tests^[10,23-25,34].

Skin tests (Prick): (1) with 12 native food allergens i.e. fresh (cow's milk, soya, of hen's egg white, hen's egg yolk, chicken's meat, beef, wheat flour, peanuts, bananas, fish, orange, white sesame); (2) with 9 commercial inhalant allergens (SmithKline Beecham-USA) (house dust mites, grass, trees, bushes and weeds pollens, dog's fur, cat's fur, mixed feathers, wool).

71 out of 138 children, of different age, with pathological acid GER and 32 children with CMA/FA exclusively, underwent these test once in order to confirm or exclude the ability of early IgE-dependent hypersensitivity to food allergens and/or inhalant allergens (atopic factor influence and or cross reactions) to trigger off symptoms observed. Results of control tests were the point of reference in assessment of reaction to allergens. The diameter of blister ≥ 3 mm assessed after 15-20 min of allergen placement was concerned a positive result of skin Prick tests, compared to negative result of negative control.

Eosinophilia: One-time assessment of relative eosinophilia in full blood count and its analysis were performed in 138 children with pathological acid GER and in 32 children with CMA/FA exclusively. Improper percentage value of eosinophilia, determined in full blood count, was > 5%.

Total IgE concentration (c IgE) in serum-assessed with Fluoro-Fast method (3M Diagnostic Systems, USA): One-time assessment of serum IgE concentration was done in 170 children-138 with acid GER and 32 with CMA/FA exclusively. Serum c IgE concentration > 50 IU/mL was considered as elevated in examined children. Taking into consideration restricted specificity of one-time measurement of total IgE in diagnosis of atopy, this test was performed together with determination of specific Ig in this particular class for selected food allergens.

Qualitative and quantitative assessment of specific IgE against food allergens (a-s IgE) and inhalant allergens (i-s IgE) with Fluoro-Fast method (3M Diagnostic Systems, USA): Assay of allergen specific Ig concentration in examined children enabled confirmation of IgE-dependent pathomechanism of food allergy and determination of food allergens. These tests appeared to be helpful in cases where tests cannot be performed or their results are doubtful, due to various reasons. 103 patients suspected of allergy, with positive Prick tests results (food allergens and/or inhalant allergens and

increased total serum IgE concentration) underwent qualitative and quantitative assessment of a-s IgE and i-s IgE. Positive results of specific IgE were: a/a-s IgE against cow milk proteins, hen's egg white, hen's egg yolk, soy, fish, orange b/i-s IgE against grass, trees, bushes and weeds pollens, house dust mites and cat's fur, assayed in serum-presence supported in class ≥ 2 -5.

Oral food challenge test^[25,26]: Open or blind oral food challenge test (depending on the age of patient) was carried out in order to establish causative relationship between food and clinical symptoms, regardless of pathogenetic mechanisms of allergy (IgE-dependent or IgE-non-dependent)^[25]. The first stage of diagnostic procedure preceding the beginning of oral food challenge tests was eliminatory diet implementation, lasting 4 wk in 138 children with acid GER. Diet depended on elimination of the most common food allergens, suspected of triggering off symptoms in examined children. Eliminatory diet was determined on the basis of information gathered from medical history of past nutrition and the results of additional tests (skin Prick tests, s IgE)^[25,26]. At the time of study, patients didn't receive or had maximally reduced antiallergic and/or antihistaminic medications. 138 children at various age, with pathological acid GER, after eliminatory diet implementation (milk-free and/or hypoallergic diet) underwent 204 biological oral food challenge tests; 138 (67.6%) with cow's milk and 66 (32.4%) with other food. In order to establish primary diagnosis, open food challenge test was performed in the 104 youngest children (below 3 years of age) and blind food challenge test in 34 children (above 3 years of age) with mainly cow's milk (low-lactose Bebilon, Ovita Nutricia) or with other potentially noxious nutrients^[25,26]. Every time child spent 1-3 d at hospital (Laboratory of Allergy Diagnostics, of III Department of Pediatrics). Time of appearance of biological reaction in examined child was counted from the last food challenge up to 48-72 h after intake of specific nutrient in native, blind form. Every patient examined received every day observation chart for reporting intensity of clinical manifestation. In case of cow's milk allergy or soy milk allergy and/or other food allergy the time of challenge test lasted 4 wk. Positive result of food challenge test and/or positive results of immunoallergological tests enabled to qualify a selected 62 children into group 2-children with GER secondary to FA.

In order to exclude the cause of secondary GER other than food allergy, the results of additional examinations performed in patients were analyzed. Among these examinations were: chest and upper gastrointestinal tract X-ray with barium swallow, X-ray or computed tomography of sinuses (in school children). On the basis of the aforementioned examinations, the type, localization and character of coexisting ailments were assessed. In order to confirm or exclude infectious cause of the symptoms presented, the following tests were taken into consideration: full blood count, erythrocyte sedimentation rate, CRP, ASO, protein fraction pattern, concentration of IgA, IgM, IgG, IgG antibodies against *Helicobacter pylori* and

Table 1 Qualification of 264 children suspected of GERD into study groups (at diagnosis)

Groups of examined children	Sex	Examined children with reflux symptoms							
		Age range (mo)							
		Number		>1.5-4		>4-16		>16-102	
		n	%	n	%	n	%	n	%
Group 1 physiological GER n = 32	Boys	17	6.4	17	6.4	-	-	-	-
	Girls	15	5.7	15	5.7	-	-	-	-
Group 2	Boys	39	14.8	-	-	23	8.7	16	6.1
Primary GER n = 76	Girls	37	14	-	-	21	7.9	16	6.1
Group 3	Boys	33	12.5	-	-	16	6.1	17	6.4
GER + CMA/FA n = 62	Girls	29	11	-	-	14	5.3	15	5.7
Group 4 reference group	Boys	19	7.2	-	-	7	2.6	12	4.5
CMA/ FA n = 32	Girls	13	4.9	-	-	5	1.9	8	3
Group 5 GER (-) + CMA/	Boys	32	12.1	-	-	8	3	24	9.1
FA (-) n = 62	Girls	30	11.4	-	-	10	3.8	20	7.6
Total		264	100	32	12.1	104	39.4	128	48.5

iron level. Moreover, bacteriological examinations were performed in some children (tests of blood, urine, faeces, bile, pharyngeal and nasal excretion). Pilocarpine test (chlorine concentration in perspiration) was performed to exclude cystic fibrosis. Moreover, metabolic screening was done by assaying lactic acid, ammonia, acid-base balance parameters in blood^[2,9,13,34].

Assignment of children into study groups

264 children were assigned into specific study groups (Table 1) after consideration of the results of 24-h esophageal pH monitoring, complex differential diagnosis, oral food challenge test with noxious nutrient, eliminatory diet, and nutrition analysis.

Acid GER was diagnosed in 170 children. Out of 170 patients (64.4%) with acid GER, of both sexes i.e. 89 boys and 81 girls, 32 (12.1) infants with physiological GER (group 1) were selected. This selected group consisted of 17 boys (6.4%) and 15 girls (5.7%), aged 1.5-4 mo (mean age $\chi = 2.2 \pm 0.48$ mo).

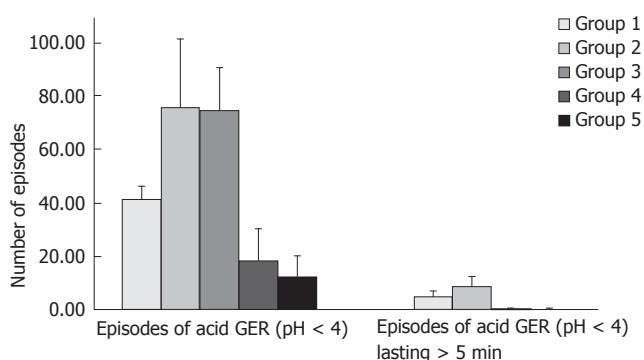
The diagnosis was put forward on the basis of the number of reflux episodes exclusively, recorded during pH monitoring. The results of remaining parameters were within the normative reference values (age-related normative values). Due to physiological character of reflux (not complicated), typical for the youngest patients, these infants were not the subject of prospective clinical observation and further clinical analysis.

In 138 children (52.3%) pathological acid GER was diagnosed and classified into primary and secondary GER. These children were assigned into group 2 and 3. Group 2 constituted 76 patients (28.8%) with pathological primary acid GER, of both sexes (39 boys-14.8%, 37 girls-14.0%), aged 4-102 mo (mean age $\chi = 25.2 \pm 27.28$ mo). In group 3 were 62 patients (23.5%), of both sexes (33 boys-12.5%; 29 girls-11.0%), aged 74 mo (mean age $\chi = 21.53 \pm 17.79$ mo) with pathological GER secondary to CMA/FA.

Acid GER was not confirmed in 94 (35.6%) out of 264 patients with symptoms suggesting GERD. These children were qualified into groups 4 and 5. Group 4-the reference group constituted 32 patients (12.1%), of both sexes (19 boys-7.2%; 13 girls-4.9%), aged 7-69 mo (mean age $\chi = 23.7$

Table 2 Statistically analysis of selected parameters of 24-h pH-monitoring in 264 children suspected of GERD; pH-monitoring with 1- or 2- channel probe

Groups of examined children with specific ailment <i>n</i> = 264	pH-monitoring parameters-distal channel range of values; mean; standard deviation (\pm SD); median; statistical significance (<i>P</i>)				
	Number of episodes of acid GER (pH < 4)	Number of episodes of acid GER (pH < 4), lasting > 5 min	Duration of the longest episode of acid GER (min)	Total acid GER index (%)	Acid GER index (supine position) (%)
Statistical significance between the groups (<i>P</i>)					
Group 2 and 3				<i>P</i> = 0.0001	NS
Group 2 and 4				<i>P</i> = 0.0001	<i>P</i> = 0.0001
Group 2 and 5				<i>P</i> = 0.0001	<i>P</i> = 0.0001
Group 3 and 4	<i>P</i> = 0.0001	<i>P</i> = 0.0001	<i>P</i> = 0.0001	<i>P</i> = 0.0001	<i>P</i> = 0.0001
Group 3 and 5				<i>P</i> = 0.0001	<i>P</i> = 0.0001
Group 4 and 5				NS	NS

**Figure 1** Summary analysis of number of episodes of acid GER (pH < 4) and episodes of acid GER (pH < 4) lasting > 5 min of 24-h pH-monitoring in 264 children suspected of GERD; pH-monitoring with 1st- or 2-channel probe. ¹Values of pH-monitoring parameters in group 1 did not undergo comparative analysis with corresponding values in remaining groups.

± 12.63 mo) with symptoms typical for cow milk allergy and/or other food allergy (CMA/FA). Group 5 constituted 62 patients (23.5%) (32 boys-12.1%; 30 girls-11.4%), aged 4-102 mo (mean age $\chi = 31.3 \pm 27.98$ mo).

Neither reflux cause nor allergic cause of the symptoms were confirmed in these children, and hence they were not the subject of prospective observation and further clinical analysis.

The study was approved by local Bioethical Committee of the Medical University of Bialystok and informed parental consent was obtained from parents of examined children.

Statistical analysis

The statistical analysis of the results comprised arithmetical mean, standard deviation, minimal and maximal values and median-for measurable features and quantitative percentage distribution for qualitative features. To compare the groups, features compatible with normal distribution, assessed with Kolomogorov compatibility test, were assessed together with the post hoc Bonferroni one-way analysis of variance. Features non-compatible with the distribution underwent Kruskal-Wallis test and if the differences were statistically significant, Mann-Whitney test was applied. Statistical significance was *P* < 0.05. Calculations were performed with the help of statistical package SPSS[®]12.0 PL.

RESULTS

24-h pH monitoring

Preliminary comparative analysis of selected values of 24-h intraesophageal pH monitoring parameters with single-channel probe (distal channel) was carried out in 32 children (12.1%) (group 1) and with dual-channel (distal and proximal channel) in 232 children (87.9%) (groups 2, 3, 4 and 5). The results of the analysis are presented in Tables 2, 3 and Figures 1-6 Results of pH esophageal monitoring in group 1 did not undergo comparative analysis with respective values obtained in children from remaining groups. Pathological acid GER was diagnosed in 138 children (52.3%), in 76 children primary GER (group 2) and in 62 children GER secondary to CMA/FA (group 3). Pathological acid GER was not confirmed in 94 children (35.6%), out of which 32 had CMA/FA diagnosed (group 4-reference group). In remaining 62 children neither GER nor CMA/FA were confirmed (group 5). The values of esophageal pH monitoring parameters obtained in individual groups: 2, 3, 4, and 5 were compared against each other. The analysis of mean values of pH monitoring parameters measured in children from individual groups is presented as follows.

According to the number of episodes of acid GER (pH < 4)

Distal channel (Table 2, Figure 1): In children with primary GER (group 2) the mean value of the parameter ($\chi = 75.68 \pm 25.61$) was similar to mean value ($\chi = 74.6 \pm 16.02$) in children with GER and CMA/FA (group 3). These values are higher than mean number of episodes of acid GER, constituting $\chi = 18.56 \pm 11.74$ and $\chi = 12.6 \pm 7.74$ in group 4 and 5, respectively.

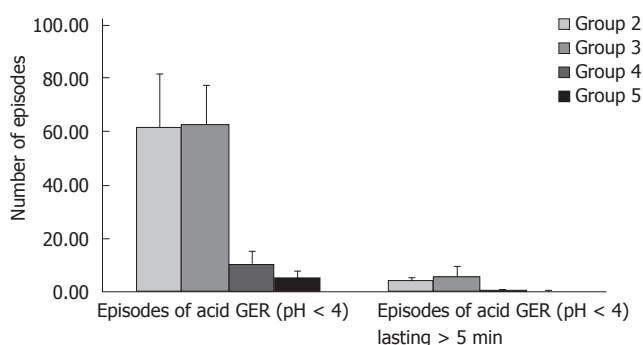
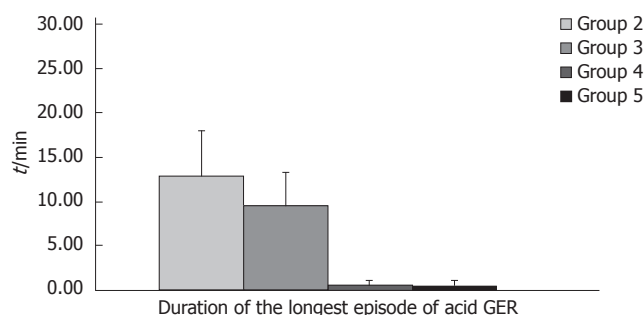
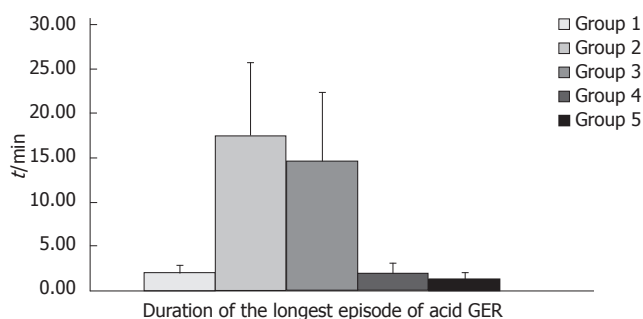
Proximal channel (Table 3, Figure 2): In children from group 2 mean value of the examined parameter ($\chi = 61.45 \pm 20.43$) was similar to mean value ($\chi = 62.48 \pm 14.67$) in children from group 3. These values are higher than mean number of episodes of acid GER $\chi = 10.5 \pm 4.5$ and $\chi = 5.36 \pm 2.09$ in group 4 and group 5, respectively.

According to number of episodes of acid GER lasting > 5 min

Distal channel (Table 2, Figure 1): In children from group 2 mean value of this parameter ($\chi = 5.17 \pm 1.95$) was lower than mean value ($\chi = 8.87 \pm 3.65$) in children from group 3. At the same time these values are higher

Table 3 Summary analysis of selected parameters of 24-h pH-monitoring in 232 children suspected of GERD; pH-monitoring with 2-channel probe

Groups of examined children with specific ailment <i>n</i> = 232	pH-monitoring parameters-proximal channel range of values; mean; standard deviation (\pm SD); median; statistical significance (<i>P</i>)				
	Number of episodes of acid GER (pH < 4)	Number of episodes of acid GER (pH < 4), lasting > 5 min	Duration of the longest episode of acid GER (min)	Total acid GER index (%)	Acid GER index (supine position) (%)
Group 2 and 3	Statistical significance between the groups (<i>P</i>)				
Group 2 and 4					
Group 2 and 5					
Group 3 and 4					
Group 3 and 5					
Group 4 and 5					
	<i>P</i> = 0.0001	<i>P</i> = 0.0001	<i>P</i> = 0.0001	<i>P</i> = 0.0001	<i>P</i> = 0.0001

**Figure 2** Summary analysis of number of episodes of acid GER (pH < 4) and episodes of acid GER (pH < 4) lasting > 5 min of 24-h pH-monitoring in 232 children suspected of GERD; pH-monitoring with 2-channel probe.**Figure 4** Summary analysis of duration of the longest episode of acid GER of 24-h pH-monitoring in 232 children suspected of GERD; pH-monitoring with 2-channel probe.**Figure 3** Summary analysis of duration of the longest episode of acid GER of 24-h pH-monitoring in 264 children suspected of GERD; pH-monitoring with 1st or 2-channel probe. ^aValues of pH-monitoring parameters in group 1 did not undergo comparative analysis with corresponding values in remaining groups.

than mean values of parameters $\chi = 0.33 \pm 0.49$ and $\chi = 0.15 \pm 0.36$ measured in group 4 and 5, respectively.

Proximal channel (Table 3, Figure 2): In children from group 2 mean value of parameter measured ($\chi = 3.96 \pm 1.37$) was lower than mean value in group 3 ($\chi = 5.87 \pm 3.64$). At the same time these values are higher than mean values of episodes of acid GER lasting more than 5 min: $\chi = 0.28 \pm 0.46$ and $\chi = 0.11 \pm 0.32$ in group 4 and 5, respectively.

According to the duration of the longest episode of acid GER (minutes)

Distal channel (Table 2, Figure 3): In children from

group 2 mean value of this parameter ($\chi = 17.45 \pm 8.21$) was higher than mean value ($\chi = 14.61 \pm 7.68$) in children from group 3. These values were higher than mean time of the longest episode of acid GER: $\chi = 2.08 \pm 1.05$ and $\chi = 1.4 \pm 0.66$ in group 4 and 5, respectively.

Proximal channel (Table 3, Figure 4): In children from group 2 mean value of parameter measured ($\chi = 12.91 \pm 5.14$) was higher than mean value ($\chi = 9.51 \pm 3.78$) in children from group 3. At the same time these values are higher than mean time of the longest episode of acid GER: $\chi = 0.67 \pm 0.49$ and $\chi = 0.44 \pm 0.62$ in group 4 and 5, respectively.

According to total acid GER index (%)

Distal channel (Table 2, Figure 5): In children from group 2 mean value of this parameter ($\chi = 13.42 \pm 5.52$) was lower than mean value ($\chi = 17.17 \pm 6.96$) in children from group 3. These values are higher than mean values of total acid GER index: $\chi = 2.69 \pm 1.07$ and $\chi = 3.1 \pm 0.78$ in groups 4 and 5, respectively.

Proximal channel (Table 3, Figure 6): In children from group 2 mean value of this parameter ($\chi = 11.26 \pm 4.18$) was higher than mean value ($\chi = 10.47 \pm 3.8$) in children from group 3. At the same time these values were higher than mean values of total acid GER index: $\chi = 0.91 \pm 0.68$ and $\chi = 0.37 \pm 0.16$ in group 4 and 5, respectively.

According to acid GER index, supine position (%)

Distal channel (Table 2, Figure 5): In children from

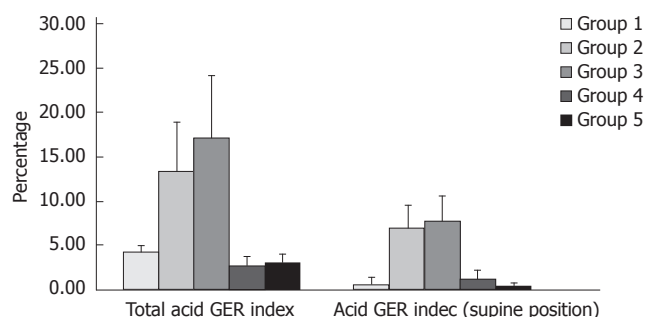


Figure 5 Summary analysis of total acid GER index and acid GER index (supine position) of 24-h pH-monitoring in 264 children suspected of GERD; pH-monitoring with 1st- or 2-channel probe. ¹Values of pH-monitoring parameters in group 1 did not undergo comparative analysis with corresponding values in remaining groups.

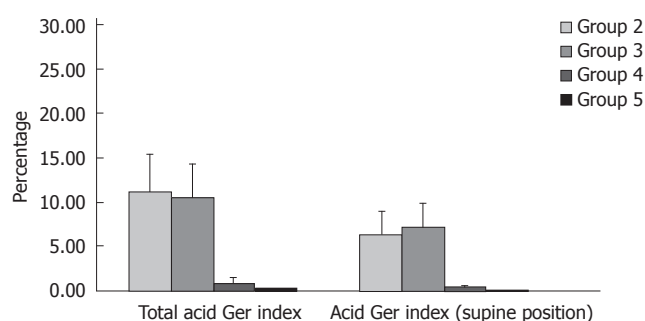


Figure 6 Summary analysis of total acid GER index and acid GER index (supine position) of 24-h pH-monitoring in 232 children suspected of GERD; pH-monitoring with 2-channel probe.

group 2 mean value of this parameter ($\chi = 6.96 \pm 2.64$) was lower than mean value ($\chi = 7.67 \pm 2.87$) in children from group 3. These values were higher than mean values of supine acid GER index: $\chi = 1.09 \pm 1.06$ and $\chi = 0.39 \pm 0.31$ in groups 4 and 5, respectively.

Proximal channel (Table 3, Figure 6): In children from group 2 mean value mean value of parameter measured ($\chi = 6.41 \pm 2.64$) was lower than mean value ($\chi = 7.16 \pm 2.76$) in children from group 3. These values were higher than mean value of supine acid GER index: $\chi = 0.43 \pm 0.22$ and $\chi = 0.09 \pm 0.11$ in groups 4 and 5, respectively.

The comparative analysis carried out between group 2 and 3 (children with GER) and between group 4 and 5 (children without GER) proved statistically significant differentiation ($P < 0.05$) of mean values (abnormal distribution) in the case of: number of episodes of acid GER, number of episodes of acid GER lasting more than 5 min and duration of the longest episode of acid GER in both channels (distal and proximal) as well as acid GER index: total and supine, in proximal channel exclusively.

This analysis also confirmed statistically significant differences ($P < 0.05$) of mean values (normal distribution) in the case of total acid GER index, in distal channel exclusively, among individual groups, especially between group 2 (children with primary GER) and group 3 (children with GER secondary to CMA/FA). However, in the case of supine acid GER, in distal channel, no statistical significance of its mean values between group 2 and group

Table 4 Type of graphic recording of intraesophageal pH in 264 examined children suspected of GERD at diagnosis

Groups of examined children	24-h intraesophageal pH-monitoring - preliminary study							
	Pathological recording				Regular recording (physiological)			
	Phasic		Non phasic		Phasic		Non phasic	
	n	%	n	%	n	%	n	%
Group 1 ¹								
Physiological GER	-	-	-	-	-	-	32	100
n = 32 (100.0%)								
Group 2								
Primary GER	-	-	76	100	-	-	-	-
n = 76 (100.0%)								
Group 3								
GER + CMA/FA	9	14.5	53	85.5	-	-	-	-
n = 62 (100.0%)								
Group 4								
reference group	-	-	-	-	3	9.4	29	90.6
n = 32 (100.0%)								
Group 5 ¹								
GER (-) + CMA/FA (-)	-	-	-	-	-	-	62	100
n = 62 (100.0%)								

¹Children excluded from further prospective clinical studies.

3 was confirmed.

On the basis of pH monitoring recording at preliminary examination in children of individual groups (Table 4), phasic recording of intraesophageal pH monitoring values was registered in 9 children (14.5%) with pathological GER secondary to CMA/FA (group 3) and in 3 children (9.4%) with CMA/FA exclusively (group 4-reference group).

DISCUSSION

Of 264 examined children, acid GER was confirmed in 170 (64.4%) of them on the basis of 24-h esophageal pH monitoring. In 138 of these children (52.3%) GERD was confirmed.

Age of examined children did not reveal statistically significant differences among individual groups. Among 138 children, GERD was more often attributed to acid GER in boys (72-52.2%) in comparison to girls (66%-47.8%).

On the basis of the results of immunoallergological tests and verifying positive oral food challenge test^[25,26], pathological acid GER in 138 children was divided into primary (28.8% of children, group 2) and secondary to CMA/FA (23.5%, group 3). At the same time, on the basis of complex differential diagnosis other causes of acid GER were excluded.

Among 264 children with family history of gastrointestinal tract diseases, acid GER was not confirmed in 94 of them (35.6%).

The assumption of esophageal pH monitoring was that the higher the electrode sensor was positioned, the less number of short-term reflux episodes was recorded, and the total time of reflux is shortened, which is consequent upon better efficiency of neutralizing mechanisms and the ability to self-purification of this part of esophagus^[18,20,32,33].

The results of our studies do not support the stated hypothesis completely because the mean values of analyzed

pH monitoring parameters in proximal channel were not lower (not statistically significant) than in distal part of esophagus in children with GERD in groups 2 and 3.

Percentage values of the number of episodes of acid GER recorded in proximal channel accounted for 81%, in group 2 and 84% in distal channel, in group 3.

The number of episodes of acid GER lasting more than 5 min recorded in proximal part of esophagus accounted for 76% in group 2, whereas in distal part of esophagus made 66%, group 3.

The duration of the longest episode of acid GER recorded in proximal part of esophagus constituted 74%, group 2, whereas in distal part of esophagus it accounted for 65%, group 3. Total acid GER index recorded in proximal part of esophagus made 84%, group 2 and in distal part of esophagus 61%, group 3. Supine acid GER index in proximal part of esophagus accounted for 92%, group 2 and in distal part of esophagus it made 93%, group 3.

In children with primary GER (group 2) and secondary GER (group 3), the mean values of individual pH monitoring parameters did not reveal significant difference between both channels. On the basis of the results obtained, it appears that there was no significant quantitative difference in the episodes of acid GER, reaching both distal and proximal channel, regardless of the age of examined children.

It was shown that reflux episodes in proximal channel were similar in number only in patients with primary GER (group 2) and those with GER secondary to CMA/FA (group 3). It may be assumed that high gastroesophageal reflux reaching proximal channel is particularly meaningful in children of both groups, but with atypical symptoms, especially of respiratory tract (silent reflux), therefore suggesting the possibility of microaspiration of gastric content into the bronchial tree^[2,18-20].

Silent reflux in children below 3 years of age, with recurrent infections of respiratory tract in past history was confirmed on the basis of pH monitoring with single-channel probe in 56% and 57% of children's gastroenterological centers in Poland^[35,36].

According to these data the diagnostic value (sensitivity) of 24-h esophageal pH monitoring in detecting pathological GER accounted for 89% in all patients examined with this type of probe but made only 84% in patients with atypical symptoms.

On the basis of 24-h esophageal pH monitoring with dual-channel probe in children with symptoms out of gastrointestinal tract, within the same age group, in own studies the higher percentage of high gastroesophageal reflux was reported, which accounted for 77.4% and 88.3% in both groups, respectively. The results of own studies give information on the intensity of acid GER reaching distal and proximal part of esophagus, and mean values of examined pH monitoring parameters in distal and proximal part of esophagus reveal statistical significance between the groups. The comparable results of supine acid GER index in distal channel constitute an exception. This differentiation of pH monitoring parameters between the groups appears to be helpful in predicting, who of the examined children is at risk of primary GER, and which

symptoms are consequent upon GER secondary to CMA/FA. The result of preliminary study is also important, in which the increasing number of reflux episodes reflecting the higher value of reflux index, and it was comparable in distal and proximal channel, in both study groups.

Italian authors reported the interpretation of graphic recording of intraesophageal pH monitoring, in which they showed a phasic decrease of values after milk meal and their rapid increase after the following meal in children with GER and/or CMA^[37].

Quantitative characteristics of patients of individual groups proved that this phasic recording of pH was a common feature in 9 children (14.5%) with GER and CMA/FA (group 3) and in 3 children (9.4%) with CMA/FA (group 4-reference group), which accounted for 12 out of 94 children with cow milk allergy. Interestingly enough, this type of recording of pH monitoring was not observed in any of the children with GER but without allergy.

In conclusion, pH monitoring performed in children with typical and atypical GERD symptoms enabled to diagnose acid GER in 52.3% of all examined patients. In order to define the extent of reflux i.e. the dynamics of each reflux episode, dual-channel probe should be used to intraesophageal pH recording in distal and proximal channel. Finally, similar pH monitoring results obtained in both study groups with GERD confirm that pH monitoring is clinically important for diagnosing of acid GER but not for differential diagnosis for between primary and secondary GER patients. This support the necessity of early implementation of complex differential diagnosis that enables to distinguish the causes of primary GER from the causes of GER secondary to FA. This causal differentiation leads to necessity of defining proper treatment strategy. It also influences the efficacy of treatment and natural history of gastroesophageal reflux disease in children and the young.

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RAPID COMMUNICATION

Crohn's disease incidence evolution in North-western Greece is not associated with alteration of NOD2/CARD15 variants

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Abstract

AIM: To assess the trends in the incidence of inflammatory bowel disease (IBD) over 23 years in the same area and to identify genetic factors related to incidence evolution.

METHODS: Patients with IBD arising from North-western Greece were systematically recorded through the 1983-2005 period. Trends in disease incidence and genetic patterns related to CARD15 variants were documented and correlated.

RESULTS: A total of 447 patients with IBD were recorded (23.5% Crohn's disease, 72.7% Ulcerative colitis and 3.8% indeterminate colitis). Mean annual incidence rates of CD and UC were 0.9/100 000 (95% CI 0.1-1.7) and 2.7/100 000 (95% CI 1.7-4.1) inhabitants, respectively. There was a statistically significant increase of CD incidence ($P < 0.01$) during the study period, in contrast to the UC incidence. There were no statistical differences in CARD15 variants over the study period.

CONCLUSION: The incidence of CD in North-western Greece has risen disproportionately to that of UC in the 21st century. This is not related to alterations of genetic background though.

INTRODUCTION

Inflammatory bowel disease (IBD) encompasses two distinct clinicopathological entities, Crohn's disease (CD) and ulcerative colitis (UC), possessing different etiological and epidemiological correlations. Both are considered of unknown aetiology, and various endogenous and exogenous factors have been etiologically implicated, leading to the concept of a multifactorial pathogenetic process involving interplay of environmental risk factors and immunologically triggered alterations^[1]. Environmental factors have for long been incriminated in the pathogenesis of both forms of IBD, usually based on data of geographic or temporal variation of disease incidence. A global north-south variation in the incidence of IBD has been reported^[2]. In Europe, the EC-IBD population based study showed a higher incident of UC (11.8/100 000 *vs* 8.7/100 000) and CD (7.0/100 000 and 3.9/100 000, respectively) in Northern compared to Southern Europe^[3]. Such variations do exist in the overall prevalence of each form, with variable data on the incidence trends of CD in recent years^[4-6], but an invariable increase in CD incidence in Southern European regions^[7,8]. Evaluation of the factors triggering this evolving incidence may allow for identifying major endogenous or exogenous factors related to the pathogenesis of the disease.

In North-western Greece, an area traditionally categorized to the poorest of Europe, previous studies have shown rarity of CD and common appearance of UC during the previous decades^[9,10]. This study assesses current evolution of incidence of CD and correlates these trends with genetic (CARD15 mutations) factors.

MATERIALS AND METHODS

Study area

The study area, North-western Greece, represents a population of 506 142 inhabitants according to the

National Census of 2001, including four Districts situated on the mainland (Districts of Ioannina, Arta, Preveza and Thesprotia) and two Districts situated in islands (Districts of Corfu and Lefkada). Urban residents represented about 40% of the total population, residing mainly in the District capitals.

Case identification and diagnostic criteria

Cases have been recorded in the frame of a systematic recording system for IBD, using multiple sources of retrieval, developed in this defined area of North-western Greece between January 1983 and December 2005. The health care system in our area includes both a National Health Service and a private sector. All gastroenterologists are members of North-western Greece Gastroenterology Group and were updated about the methods and aims of this study both through newsletters and meetings. The system records cases from the following sources: (1) in- and outpatients referred to the Hepato-gastroenterology Unit of the Ioannina University Hospital; (2) patients referred to outpatient Gastroenterology clinics in Districts hospitals and (3) patients referred to the private gastroenterologists practicing inside the area. A review of collected data was performed in the University Hospital of Ioannina, by two investigators (ME and EVT). Diagnosis of CD and UC was based on typical clinical, radiological, endoscopic and histological criteria and the diagnosis of intermediate colitis (IC) was only set when a distinct diagnosis of UC or CD could not be established. CD was classified according to the Vienna System^[11] and UC according to Lennard-Jones^[12]. Perianal disease was defined by the presence of perianal abscesses, fistulae or ulcers, but not by the presence of skin tags. A final diagnosis of CD, UC, or ulcerative proctitis (UP) though was made by two expert gastroenterologists and recorded as definite, probable, or possible following criteria previously published^[9]. Phenotypic details were obtained on 2 occasions by retrospective case-note review between January and December 2005 by 2 investigators (ME and EVT). Duration of follow-up was defined as the interval between diagnosis and latest case-note review. Details regarding other demographic characteristics were further supplemented by a patient interview or completed postal questionnaire. During the last year blood was collected from all CD patients. An incidence case was defined as any IBD patient, diagnosed during the study period, resident in the study area for at least one year before the diagnosis. All patients first diagnosed during the study period 1983-2005 were included in the study. All patients included in the study were evaluated at least twice during the study period.

The study protocol conformed to the ethical guidelines of the Declaration of Helsinki.

Genotyping

Three NOD2/CARD15 variants previously identified as being independently associated with Crohn's disease, R702W, G908R, L1007fsinsC^[13-15] were genotyped. Each individual reaction contained primers to amplify a nonpolymorphic genomic control sequence. A single thermocycling program was used for all reactions. The

Table 1 Demographic characteristics of IBD patients diagnosed during the period 1983-2005, in northwest Greece

	Crohn's disease	Ulcerative colitis
Total numbers of patients	105	325
Region of origin		
Ioannina	59	140
Arta	5	10
Preveza	2	36
Thesprotia	5	21
Corfu	33	113
Lefkada	1	5
Male: female (ratio)	66:39 (1.69)	208:117 (1.77)
Age at diagnosis (yr) (mean \pm SD)	33.2 \pm 12.9	45.7 \pm 16.1
(range)	(18-70)	(18-93)
Men	33.4 \pm 12.9	47.3 \pm 15.1
Women	33.1 \pm 13.2	42.9 \pm 17.3
Follow up (mo) (mean \pm SD)	92.9 \pm 72.3	119.6 \pm 73.8
(range)	(4-264)	(5-276)

products were electrophoresed on 1% agarose gels with ethidium bromide and viewed under ultraviolet light. An image was recorded digitally. Healthy controls match for age and gender with CD patients were randomly selected from Blood Donors in University Hospital of Ioannina and District Hospital of Corfu.

Statistical analysis

Incidence rates were calculated as the number of cases per 100 000 inhabitants, and 95% Confidence Intervals were estimated using the normal distribution. Population data were based on databases of the National Statistical Service (National Censuses 1981, 1991, and 2001). The significance of time trends of incidence rates was tested using the χ^2 -trends in proportion test. Frequency and susceptibilities of NOD2/CARD15 variants among CD patients, UC patients and controls was compared with the chi-square test. Odds ratios (OR) were calculated with the chi-squared distribution test and 95% confidence intervals (95% CI). The Fisher exact test was used for comparing differences between allele frequencies in patients and controls. The *P* values obtained were two-tailed and statistical significance was assumed below 0.01. Inference was aided by GraphPad InStat (version 3.00; GraphPad Software).

RESULTS

During the study period, a total of 447 IBD cases were recorded in the study area. One hundred and five patients were diagnosed with CD (23.5%), 325 with UC (72.7%), and 17 with Indeterminate Colitis (3.8%). The majority of the patients were derived from the regions of Ioannina and Corfu, accounting for 92% of the total CD cases and 78% of the total UC cases.

The main demographic and clinical characteristics of CD and UC patients are presented in Table 1.

A familial history of IBD was present in 11 patients (2.5%). Terminal ileal localization of the disease was present in 35 (33.3%), ileocolonic in 28 (26.7%) and colonic in 42 (40%) of the CD patients; stenotic disease

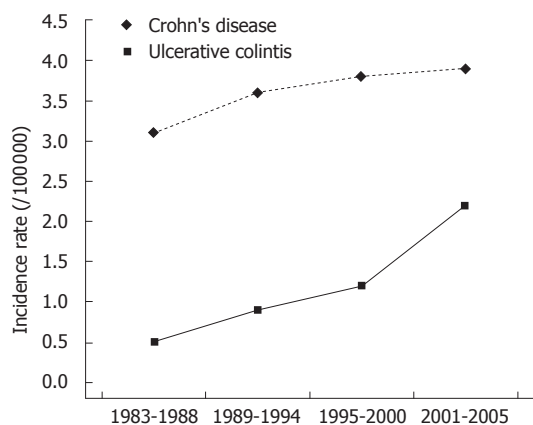


Figure 1 Trends in standardised incidence of Crohn's disease (CD) and Ulcerative colitis (UC) (Northwestern Greece 1983-2005).

Table 2 Evolution of genetic, demographic and clinical correlations of Crohn's disease through the study period

Period	Any CARD15 mutation	M:F ratio	Mean age (yr)	CD:UC ratio, cumulative	CD:UC ratio, b region of Ioannina	CD:UC ratio, region of Corfu	Perianal
Pre-1994	46.10%	1.4	30.4	0.23	0.26	0.2	13.80%
1995-1999	61.90%	3.4	35.4	0.32	0.46	0.3	27.20%
2000-2005	46.90%	1.55	33.1	0.61	> 1	0.5	31.30%
Total	51%	1.83	32.8	0.36	0.38	0.32	25.50%

was recorded in 23 (21.9%), fistulating in 25 (23.8%) and perianal disease in 26 (24.8%) CD patients. Of the UC patients, 71 (21.8%) had pancolitis, 99 (30.5%) had proctitis, and 155 (47.7%) had left-sided UC.

Mean annual crude incidence rates of CD and UC were 0.9 and 2.9/100 000 inhabitants, respectively. Age-adjusted incidence rates were 0.9/100 000 (95% CI 0.1-1.7) and 2.7 (95% CI 1.7-4.1), respectively. The age-adjusted mean annual incidence rate of IC was 0.2/100 000 (95% CI 0.1-0.4).

The incidence of CD was higher in men than in women. The highest incidence rate was found in the age group 18-44 years for men and women. UC incidence was higher in men also. The highest incidence rate was found in the age group 45-64 for both sexes.

There was a statistically significant increase of CD incidence ($P < 0.01$) during the study period, while the incidence of UC increased slightly and not in a statistically significant manner (Figure 1). As a consequence, the ratio of CD to UC practically doubled, surpassing 1 in the district of Ioannina, the largest population district and centre of the study, in the year 2002, and remaining above 1 for the following years (Table 2).

When demographic and clinical parameters were evaluated in correlation with the increase in CD incidence, age, gender, and regional patient distribution did not exhibit any statistically significant trends. There were also no differences in the percentages of patients with terminal ileal or colonic localization, and stenotic or fistulating disease during the pre-1995 period, compared

Table 3 CARD15 variant allele frequencies

Samples	Genotype			Mutant allele frequency (%)	P; OR (95% CI)
	-/-	-/+	+/+		
R702W					
CD	85	8	3	7.29	0.015; 3.85 (1.24-11.93)
UC	171	9	0	2.5	0.78; 1.26 (0.38-4.13)
Control	96	4	0	2	
G908R					
CD	83	13	0	6.77	0.70; 0.83 (0.39-1.78)
UC	156	24	0	6.66	0.61; 0.82 (0.42-1.58)
Control	84	16	0	8	
Leu1007fsincC					
CD	52	36	8	27.08	< 0.0001; 7.06 (3.46-14.37)
UC	166	9	5	5.27	1; 1.06 (0.48-2.32)
Control	90	10	0	5	

-/-, homozygous wild-type; +/-, heterozygous; +/+, homozygous mutant. CD: Crohn's disease; UC: Ulcerative colitis; OR: Odds Ratio; CI: Confidence Interval. The CARD15 gene variants determined in 96 CD patients (from 102 living CD patients out of 105, from our cohort 6 refused genetic test), 180 out of 300 living UC patients and 100 randomly chosen healthy blood donors.

to the 1995-1999 and the 2000-2005 periods. A statistically significant increase in the percentage of patients with perianal disease was observed in the latter two periods compared to the pre-1995 period (Table 2).

The association of CD with mutations in the three casual CARD15 variants is shown in Table 3.

There were no statistically significant differences on any mutations over time (Table 2).

DISCUSSION

North-western Greece is comprised of the mainland district of Epirus and the islands Corfu and Lefkada. Due to geographical reasons and relatively underdeveloped transport facilities to the rest of Greece the region can be considered secluded, and thus an ideal frame for epidemiological analysis of certain diseases. We have shown in the past that UC was a common entity in this region, while CD on the contrary was all too rare, with the CD:UC ratio in the 1980 s and early 1990 s being near 0.1^[9,10]. Herein, the results of this 23-year period study, clearly show a rapid and sustained increase in CD incidence from 2000 on in North-western Greece, an increase that overturned the CD:UC ratio in the largest province of the study, the region of Ioannina, by 2002.

These findings are in accordance with formerly published studies showing an increasing trend for CD^[16-22]. In these studies the increasing trend was often related to increasing age at diagnosis and could thus be related to improvements in average survival and surveillance (global increase^[20,21] or a higher proportion of older patients^[5,18,22]).

This is not the case in our study in which the majority of CD patients are young, similar to other studies^[6,19] and the average patient age has not altered significantly over the years. Furthermore this increase could not be

attributed to enhanced surveillance and patient reporting; characteristically, the increase in incidence is predominantly expressed in the region of Ioannina, which, hosting the study centre for the past 25 years, guarantees total patient reporting and absence of bias. Moreover, a similar increase was not observed for UC during this period. This rising incidence suggests that unknown triggering factors continue to work in North-western Greece. To explain this increase in CD incidence throughout the period 1983-2005 we hypothesized that certain etiologic factors exist, and we evaluated the potential role of genetic susceptibility changing slowly over time in this incidence trend.

Although there are differences in ethnic, racial and geographic distribution^[23], the genetic association of CD with CARD15 is undoubtedly replicated widely at present^[24,25]. There were no significant differences in the prevalence of CARD15 variants during the study period in our study, indicating an absence of alterations in the genetic background. The prevalence of the three CARD15 variants is similar with that found in cohorts in central Europe and much higher than in northern Europe^[25]. Yet these results are different from two other Greek studies: the first, based on the island of Crete outlined the rarity of SNP 13^[26], an inconsistency that may be attributed to the fact that Crete is an isolated geographic region where this mutation does not seem to predispose to the disease, or to the relatively small size of the examined sample. The relatively higher frequencies in all three SNPs in the second study may be attributed to patient selection (hospitalized patients in a period of time, high proportion of ileocolonic disease)^[27]. Our study, being population based, may be more representative of prevalence of CARD15 variants in Greece.

One could speculate that the increased incidence of CD recorded in recent years may actually reflect increase in detection, but not incidence of colonic disease, more easily recognized through increased endoscopy implementation. Although the total number of endoscopies performed naturally increased over time, there was no difference in endoscopy success rates, furthermore significant trends in disease localization over time were not recorded, apart from the already mentioned perianal CD (data not shown).

A potential criticism of the recruitment methods used in this study would be that ascertainment might have been incomplete, missing patients with CD or those with mild ulcerative proctitis who might have never required gastroenterologic consultation or hospital admission. However in the Greek population, during the time period of this study, there was an almost universal admission policy for investigation of IBD. Although North-western Greece has a relatively small population and isolated geographic area, this has actually been a great advantage and has resulted in a comprehensive study of the entire population, excluding any case leaks to other centers or existence of not notified cases followed-up by general practitioners.

In conclusion, we have shown that a significant increase in CD incidence in a secluded geographical region of Greece where CD was previously rare is not related to any alterations in the genetic profile or typical demographical factors. The study being population based

outlines the need for further investigation of the interplay of environmental factors and disease.

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COMMENTS

Background

The incidence of Crohn's disease and ulcerative colitis varies greatly between specific geographic areas, and is supposed to be related to exogenous factors such as affluence and dietary habits, as well as endogenous factors such as genetic predisposition. North-western Greece was characterized by a low incidence of Crohn's disease (CD) although Ulcerative Colitis (UC) was not uncommon according to a previous study undertaken in the early 1990s.

Innovations and breakthroughs

The present study is one of the few to evaluate genetic predisposition to Crohn's disease and ulcerative colitis through time in a given area. The presence of absence of relation to incidence evolution allows for evaluation of the etiological role of genetic predisposition in disease.

Applications

Similar studies, when applied in larger areas, may be hampered by planning difficulties though.

Terminology

NOD2/CARD15 mutations are mutations of a specific genetic locus that have been shown to interfere with altered immune response and induce increased predisposition to the development of Crohn's disease.

Peer review

The author examined the relation between the change in the annual incidence rates of IBD patients and CARD15 variants in North-western Greece. Although the incidence of CD has significantly increased during the study period, there was no statistical differences in NOD2/CARD15 variants among the Groups. The data is interesting and certainly provides the new evidences in this research field.

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Lead exposure increases oxidative stress in the gastric mucosa of HCl/ethanol-exposed rats

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Abstract

AIM: To investigate the role of reactive oxygen species in the ulcer-aggravating effect of lead in albino rats.

METHODS: Albino Wistar rats were randomly divided into three groups and treated orally with 100 mg/L (low dose) or 5000 mg/L (high dose) of lead acetate for 15 wk. A third group received saline and served as control. At the end of wk 15, colorimetric assays were applied to determine the concentrations of total protein and nitrite, the activities of the oxidative enzymes catalase and superoxide dismutase, and lipid peroxidation in homogenized gastric mucosal samples.

RESULTS: Exposure of rats to lead significantly increased the gastric mucosal damage caused by acidified ethanol. Although the basal gastric acid secretory rate was not significantly altered, the maximal response of the stomach to histamine was significantly higher in the lead-exposed animals than in the unexposed control group. Exposure to low and high levels of lead significantly increased gastric lipid peroxidation to $183.2\% \pm 12.7\%$ and $226.1\% \pm 6.8\%$ of control values respectively ($P < 0.0$). On the other hand, lead exposure significantly decreased catalase and superoxide dismutase (SOD) activities and the amount of nitrite in gastric mucosal samples.

CONCLUSION: Lead increases the formation of gastric ulcers by interfering with the oxidative metabolism in the stomach.

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Key words: Lead; Ulcer; Lipid peroxidation; Catalase; Gastric acid

INTRODUCTION

Reactive oxygen species (ROS) have been shown to be involved in the etiology of many inflammatory disorders of the gastrointestinal system^[1,2]. This is evidenced by the increased oxidative stress by pro-ulcerative factors in the gut such as *H. pylori*^[3,4], use of non-steroidal anti-inflammatory drugs^[5], smoking^[6], psychological stress, corticosteroid use^[7], and loss of sleep^[8]. Lipid peroxidation (LPO), a result of the reaction of oxyradicals and polyunsaturated acids, has been suggested as an attack factor in the gastric mucosa^[9]. Also GSH, an endogenous sulfhydryl compound, is an important substance in the cellular defense system^[10].

Nitric oxide (NO) depletion also plays significant roles in the pathogenesis of gastric ulcers. NO along with superoxide (O_2^-) and the products of their interaction initiate a wide range of toxic oxidative reactions causing tissue injury^[11]. Large amounts of NO, generated primarily by iNOS, can be toxic and pro-inflammatory. Likewise, neutrophils too produce oxidants and release granular constituents comprising lytic enzymes performing an important role in inflammatory injury^[12].

Although lead acetate does not initiate any excess generation of reactive oxygen species in a cerebral synaptosomal suspension, it has a marked ability to enhance the pro-oxidant properties of ferrous iron in the same system^[13]. It also amplifies oxidative stress induced by l-glutamate^[14]. Recent studies suggest that accumulated lead exposure is related to several chronic disorders of aging including disorders that have been associated with oxidative stress^[15-17]. Sass^[19] first reported that lead poisoning in dogs is associated with perforating gastrointestinal ulcers. Bercowitz and Laufer^[19] observed that gastrointestinal ulcer patients had accumulated lead in their teeth, thus suggesting a relationship between lead and ulcer. In a previous study, we reported that long term exposure of rats to lead predisposes the stomach to higher ulcerogenic effects of indomethacin and acidified ethanol^[20]. However, the exact mechanism(s) by which lead

promotes gastric ulceration is not yet understood. The objective of the present study thus was to investigate the involvement of ROS in the aggravation of ulceration in the stomach of lead-exposed rats.

MATERIALS AND METHODS

Chemicals and animals

Lead acetate and urethane were obtained from BDH chemicals Ltd, Poole, England. Epinephrine, 5,5-dithio-bis-2-nitrobenzoic acid, hydrogen peroxide, sodium pentobarbitone, and thiobarbituric acid (TBA) were purchased from Sigma (St Louis, MO USA). All other reagents were of analytical grade and were obtained from the British Drug Houses, Poole, UK.

Young male albino Wistar rats (80-90 g) were obtained from the small animal house, College of Medicine, University of Ibadan, Nigeria. They were randomly divided into three groups with adequate matching of weight. They were kept in wire meshed cages and fed with commercial rat pellets (Ladokun Feeds Ltd, Ibadan, Nigeria) and allowed to take water ad libitum.

Lead treatment

Animals were exposed to lead as previously described^[20]. The high lead group (HiPb) was given 5000 mg/L lead acetate in drinking water daily while the low lead group (LoPb) received 100 mg/L lead acetate in drinking water. The control animals received only drinking water. At the end of a 15 wk exposure, blood samples were collected from the rats for analysis of the Packed Cell Volume (PCV), red and white blood cell counts, and neutrophil numbers by standard methods. Afterwards, ulcer was induced as follows.

Induction of experimental ulcer

Acidified ethanol was used to induce experimental gastric ulcers. Thirty six hours fasted rats were given 1.0 mL of HCl/ethanol mixture containing 0.15 mol/L HCl in 70% mL/L ethanol^[21]. Four hours after administering the ulcerogen and under sodium pentobarbitone anaesthesia (60 mg/kg, ip), the animals were sacrificed. The stomachs were removed, inflated with 10 mL of 2% formaldehyde for 10 min to fix the tissue walls and opened along the greater curvature. The hemorrhagic lesions were stretched out on a glass plate and their sizes were estimated using an underlying graph paper with a 1 mm² grid. Lesion areas were summed up per stomach and expressed as % of total mucosal area.

Measurement of gastric acid secretion

Gastric acid secretion was studied in the 3 groups using a modification of the method originally described by Ghosh^[22]. The animals were fasted for 24 h at the end of the 15 wk lead treatment. Under urethane anaesthesia [250 g/L urethane (6 mL/kg body, ip)], the animals were surgically prepared for in situ stomach perfusion. The pylorus was semi-transected at its junction with the duodenum and a pyloric cannula inserted and tied into place. An oesophageal cannula for infusion from a flow

meter (Watson-Marlow Inc., USA) was passed through the mouth to the stomach. Ten minute effluent samples (10.0 ± 0.1 mL each) were collected *via* a pyloric cannula and titrated against 0.01 mol/L NaOH using phenolphthalein as indicator. Acidity was expressed in mol/L/L per min life. After obtaining a consistent basal acid output, each animal was injected *via* a femoral vein cannula with either 9 g/L NaCl or histamine acid phosphate.

Biochemical analysis

At the end of chronic lead exposure, the animals were killed under deep ether anaesthesia and the stomachs were removed. Each stomach was cut open through the greater curvature, rinsed in normal saline, weighed, and homogenized in 10 mL KCl (pH = 7.4). The homogenate was subsequently used for total protein estimation. The protein content of the gastric mucosa was estimated by the method of Lowry *et al*^[23] using bovine serum albumin as a standard.

Determination of lipid peroxidation. Lipid peroxidation was assayed by measuring the thiobarbituric acid reactive (TBAR) products using the procedure of Walls *et al*^[24]. Briefly, the homogenate was supplemented with 0.75 g/L TBA in 0.1 mol/L HCl. The reactants were then supplemented with 5 mL n-butanol-pyridine mixture, shaken vigorously for 1 min and centrifuged for 10 min at 4000 r/min. Absorbance was then read at 532 nm and the results expressed as nmol TBA per 100 mg wet tissue.

Determination of catalase activity. Activity of catalase in gastric mucosa was determined according to the procedure of Sinha^[25]. This method is based on the reduction of dichromate in acetic acid to chromic acetate when heated in the presence of H₂O₂, with the formation of perchromic acid as an unstable intermediate. The chromic acetate so produced is measured calorimetrically at 530 nm.

Determination of SOD activity. For determination of SOD activity, samples of gastric mucosal homogenates were taken as described above. A method originally described by Misra and Fridovich^[26] as reported by Magwere *et al*^[27] was employed. This method is based on the ability of superoxide dismutase to inhibit the autoxidation of epinephrine caused by O₂ generated by xanthine oxidase reaction.

Determination of nitrite in gastric mucosa. The gastric mucosa was cooled in ice-cold distilled water before homogenization (100 g/L). The crude homogenate was centrifuged at 21,000 g for 20 min at 4°C. Aliquots of the supernatants were taken to determine nitrite levels. The amounts of nitrite were measured in the gastric mucosa by performing the Griess reaction. 100 mL of sample were incubated with 100 mL of Griess reagent (Sigma) at room temperature for 20 min. Nitrite level was determined by measuring the absorbance at 550 nm using a spectrophotometer (DU 640B, Beckman, Fullerton, California, USA)^[28,29].

Determination of gastric mucous. Adherent gastric glandular mucous was measured by the method of Corne *et al*^[30]. Excised gastric glandular portion of the stomachs were transferred for 2 h to 0.1% Alcian blue dissolved

Table 1 Effects of lead exposure on acidified ethanol-induced gastric injury (mean \pm SEM, $n = 8$)

Treatment	Mass gain (% of initial mass)	Mucosal damage score (% of total mucosal area)	Total protein (mg/g)	Gastric mucus (mg/g)
Control	43.3 \pm 4.4	6.94 \pm 0.42	9.80 \pm 0.01	31.0 \pm 0.31
Low lead	17.4 \pm 6.3 ^a	9.72 \pm 0.65 ^a	7.50 \pm 0.14	28.0 \pm 0.27 ^a
High lead	6.3 \pm 5.1 ^a	12.50 \pm 0.51 ^a	6.20 \pm 0.01	23.0 \pm 0.24 ^a

^a $P < 0.05$ vs control.

in buffer solution containing 0.1 mol/L sucrose and 0.05 mol/L sodium acetate (pH adjusted to 5.8 with hydrochloric acid). After washing the stomach twice in 0.25 mol/L sucrose (15 min and 45 min), the dye complexed with mucous was eluted by immersion in 10 mL aliquots of 0.5 mol/L $MgCl_2$ for 2 h. The resulting blue solution was shaken with equal volumes of diethyl ether and the optical density of the aqueous phase was measured spectrophotometrically at 605 nm.

Statistical analysis

All values presented in tables are expressed as mean \pm SEM. The appropriate comparisons between groups were made using Student's *t*-test. The difference between the groups is taken to be significant at $P < 0.05$.

RESULTS

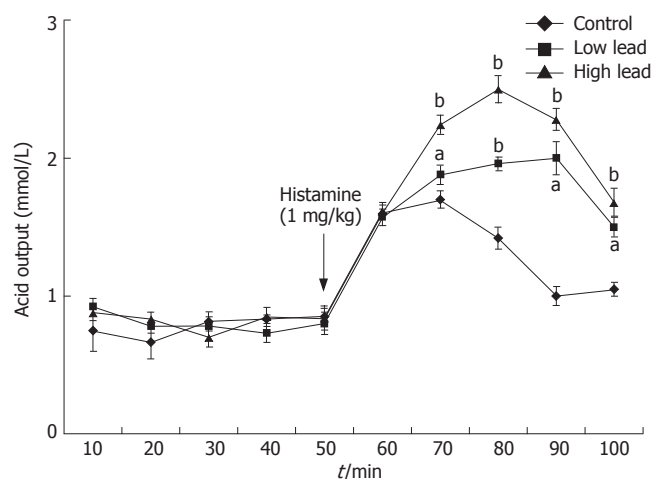
Development of gastric lesions

Administration of acidified ethanol caused severe damage to the rat stomachs under investigation. The site of the formation of lesions is the corpus mucosa. The mean ulcer score in the control animals was 6.94% \pm 0.42%. The severity of lesions was markedly increased by chronic exposure to both low and high lead concentrations (Table 1, $P < 0.05$). Also shown in Table 1 is the result of the total protein and adherent mucous content of the glandular stomach. Long term exposure to lead significantly decreased both gastric mucous and protein contents, the inhibition being greatest in the high lead treated group.

Basal rate of gastric acid secretion was not significantly affected by lead exposure. However, the maximal response of the stomach of lead-exposed animals to histamine was significantly higher than those of the unexposed control group (Figure 1).

Lipid peroxidation and gastric antioxidant enzymes

Lipid peroxidation (measured as the amount of TBA reactants in the gastric mucosa) in the unexposed control animals was 595 \pm 82 mmol/g tissue. Exposure of rats to low and high lead levels significantly increased gastric TBA reactant level to 183.2% \pm 12.7% and 226.1% \pm 6.8% of the control value, respectively (Figure 2A). On the other hand, SOD activity in the intact gastric mucosa (control group) averaged 334 \pm 22 nkat/g. Exposure of rats to low and high lead levels significantly decreased SOD activity to 78% \pm 3% and 66% \pm 6% of the control, respectively

**Figure 1** Gastric acid secretory output after administration of histamine (1 mg/kg) in rats (^a $P < 0.05$, ^b $P < 0.01$ vs control).

(Figure 2B). Similarly, lead exposure decreases gastric mucosal catalase activity to 88.22% \pm 3.21% and 55.29% \pm 2.15% of the control, respectively (Figure 2C).

Gastric mucosal nitrite concentration

The control gastric mucosal nitrite level was 62.4 \pm 4.2 nmol/g tissue (Figure 3). Chronic exposure of rats to lead for 15 wk depleted the gastric nitrite level to 51.6 \pm 3.1 nmol/g tissue ($P < 0.05$) and 49.7 \pm 2.5 nmol/g tissue ($P < 0.01$).

DISCUSSION

The present study investigated the role of oxidative stress in the ulcer-promoting action of prolonged lead exposure in rats. The doses and the duration of lead treatment used in our study have been shown to cause significant high blood lead levels in previous studies^[20,31]. Thus, there is no doubt that the animals had high blood lead levels. The acidified ethanol model has been used widely to produce gastric mucosal damage^[21]. We observed that long-term exposure of rats to lead given through drinking water significantly increased the incidence of gastric ulcer produced by the ulcerogen. This agrees with our previous report in which lead was shown to aggravate gastric ulcers induced by indomethacin and acidified ethanol^[20].

It is well established that gastric acid secretion plays a role in gastric ulcer. Moreover, many anti-ulcerogenic drugs act by reducing the acid secretion^[32]. The present result suggests that basal gastric acid secretory rate was not significantly affected by prolonged lead treatment. However, maximal stimulation by the established secretagogue, histamine, was markedly increased in the lead exposed rats. We had earlier reported that acid output from pylorus ligated stomach of lead exposed rats was increased by lead treatment. Further studies may therefore be required using different acid secretory models before a conclusion can be made on the exact effect of lead exposure on gastric acid secretion.

Lipid peroxidation has been implicated in the etiology

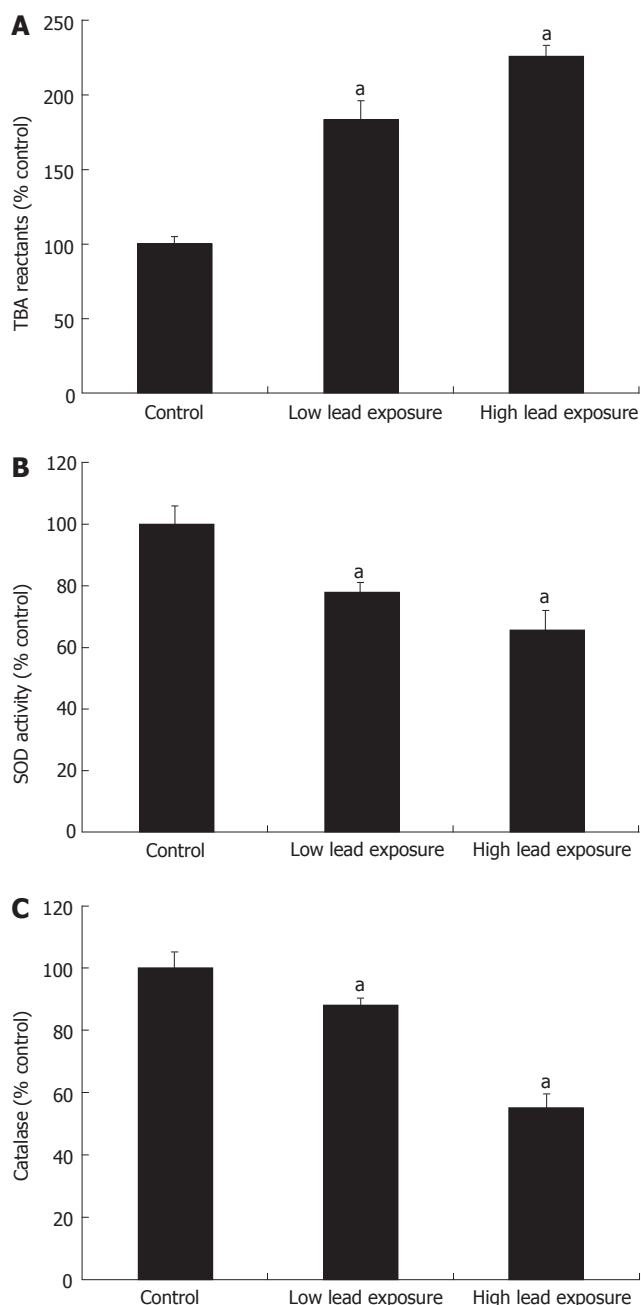


Figure 2 TBA reactants, superoxide dismutase and catalase activities in gastric mucosa of rats exposed to chronic lead treatments (mean \pm SEM, $n = 8$, ^a $P < 0.05$ vs control).

of damage to subcellular membranes and then injury in the cell. In the present study, lipid peroxidation, as measured by the amount of TBA reactants, was increased by lead exposure. The implication of this is that lead causes an increase in the formation of free radicals, which, if not mopped up by free radical scavengers as SOD, CAT, or glutathione, will expose the stomach to inflammation.

Wapnir *et al*^[33] reported that juvenile rats fed a diet containing 1% lead acetate for 7 wk suffered from malabsorption of certain amino acids, as the intestinal absorption of glycine, lysine, and phenylalanine were decreased. Furthermore, administration of HCl and ethanol has been shown to produce ulcerative lesions and

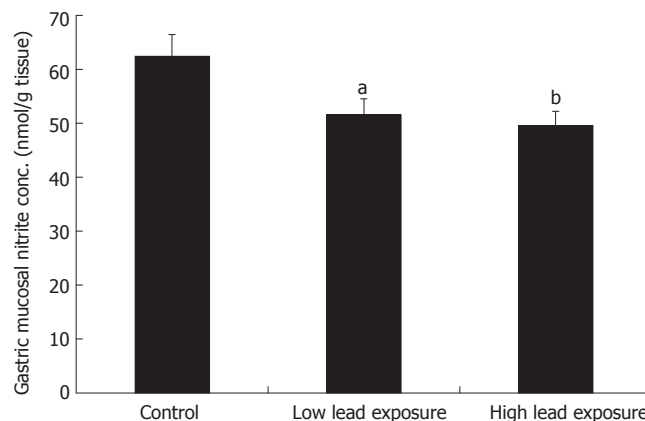


Figure 3 Gastric mucosal nitrite concentration in lead exposed rats (mean \pm SEM, $n = 8$ rats, ^a $P < 0.05$, ^b $P < 0.01$ vs control).

increased lipid peroxidation in the gastric mucosa with depletion of endogenous antioxidants^[21]. The result of this study showed a significant decrease in the protein glandular protein level of the rats given low and high doses of lead acetate. This may also explain the significant low levels of the antioxidizing enzymes SOD and CAT observed in the treated groups.

In gastroprotection, the first line of antioxidative enzyme is SOD which catalyses the dismutation of superoxide radical anion (O_2^-) into less noxious hydrogen peroxide (H_2O_2). H_2O_2 is then inactivated by the degradation into water by catalase or glutathione peroxidase^[35,36]. Depletion of these enzymes, as evident by the results of this study, therefore predisposes the stomach to a greater impact of the free radicals produced *via* increased lipid peroxidation, hence increased ulcer formation following lead exposure.

Apart from the free radical scavenging enzymes, another agent that has been widely believed to be involved in the regulation of various gastric functions and in the modulation of gastric mucosal integrity is nitric oxide (NO). This is evidenced by reports showing that suppression of NO production by NO synthase inhibitor worsens gastric lesions induced by ethanol in rats^[37,38]. NO has also been suggested to prevent infiltration by neutrophils, which are a source of superoxide radical anions^[39]. The reduction in the gastric tissue nitrite in the lead treated groups in the present study is therefore indicative for a reduced protective capacity of endogenous NO.

In summary, chronic exposure of rats to lead intensifies HCl/ethanol-induced gastric mucosal damage and this may be related to reduced gastric contents of endogenous NO and the antioxidative enzymes SOD and CAT.

COMMENTS

Background

A number of humans are occupationally exposed to high levels of lead through paints, car exhausts battery fumes, etc. Lead exposure also occurs through ingestion with food and via inhalation. Previous studies in dogs and rats have shown that long-term exposure to high level lead in drinking water predisposes the stomach to ulcer. The mechanism of this effect of lead is not understood.

Research frontiers

Oxidative stress has been shown to be a major causative factor for many diseases, including gastrointestinal ulcers. The hotspot of this study is to examine if the increased susceptibility of the stomach to ulcer after prolonged lead exposure can be explained (partly or totally) by an increased oxidative stress in the stomach.

Innovations and breakthroughs

The results of this study show that chronic exposure of rats to lead intensifies HCl/ethanol-induced gastric mucosal damage and this may be related to reduced gastric contents of endogenous nitric oxide and antioxidative enzymes.

Applications

The study suggests that prolonged exposure to lead may be a factor in the etiology of gastrointestinal ulcers.

Terminology

Oxidative stress: a condition of increased oxidant production in animal cells characterized by the release of free radicals and resulting in cellular degeneration. Free radicals damage components of the cells' membranes, proteins, or genetic material by "oxidizing" them—the same chemical reaction that causes iron to rust; Lead: a soft heavy toxic malleable metallic element; bluish white when freshly cut but tarnishes readily to dull grey; Lead poisoning: Lead can come into the body in a number of ways: through water that goes through lead pipes, through badly canned food and through small pieces of paint. Victims of lead poisoning may get headaches, dizziness, confusion, and problems seeing. They may also become slowly paralyzed, starting with the hands. In very serious cases, it can cause death.

Peer review

This is an interesting and generally well written paper with some new information.

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N-acetylcysteine attenuates oxidative stress and liver pathology in rats with non-alcoholic steatohepatitis

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Abstract

AIM: To evaluate attenuating properties of N-acetylcysteine (NAC) on oxidative stress and liver pathology in rats with non-alcoholic steatohepatitis (NASH).

METHODS: Male Sprague-Dawley rats were randomly divided into three groups. Group 1 (control, $n = 8$) was free accessed to regular dry rat chow (RC) for 6 wk. Group 2 (NASH, $n = 8$) was fed with 100% fat diet for 6 wk. Group 3 (NASH + NAC₂₀, $n = 9$) was fed with 100% fat diet plus 20 mg/kg per day of NAC orally for 6 wk. All rats were sacrificed to collect blood and liver samples at the end of the study.

RESULTS: The levels of total glutathione (GSH) and hepatic malondialdehyde (MDA) were increased significantly in the NASH group as compared with the control group (GSH; 2066.7 ± 93.2 vs 1337.5 ± 31.5 $\mu\text{mol/L}$ and MDA; 209.9 ± 43.9 vs 3.8 ± 1.7 $\mu\text{mol/g}$ protein, respectively, $P < 0.05$). Liver histopathology from group 2 showed moderate to severe macrovesicular steatosis, hepatocyte ballooning, and necroinflammation. NAC treatment improved the level of GSH (1394.8 ± 81.2 $\mu\text{mol/L}$, $P < 0.05$), it did not affect MDA (150.1 ± 27.0 $\mu\text{mol/g}$ protein), but led to a decrease in fat deposition and necroinflammation.

CONCLUSION: NAC treatment could attenuate oxidative stress and improve liver histology in rats with NASH.

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Key words: N-acetylcysteine; Oxidative stress; Non-alcoholic steatohepatitis

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INTRODUCTION

Non-alcoholic steatohepatitis (NASH) is a liver disease characterized by macrovesicular steatosis, hepatocyte necrosis, inflammation, Mallory bodies, and fibrosis^[1]. NASH is closely associated with the metabolic or insulin resistance syndrome^[2]. This is a cluster of disorders, such as obesity, diabetes mellitus, dyslipidemia, arteriosclerosis, and hypertension, with insulin resistance as a common feature^[3]. In initial phases, during which fat accumulates in the liver, no clinical symptoms are evident. In advanced stages, fibrosis is detectable, which might progress into cirrhosis in some patients^[4].

There are many models of NASH-like liver injuries in animals as the genetic model of *ob/ob* mice^[5], the methionine and choline deficient diet model^[6,7], and a model with high-fat liquid diet in which 71% of energy is derived from fat, 11% from carbohydrates, and 18% from protein^[8].

Oxidative stress is believed to play an important role in pathogenesis of NASH. It is likely involved in the progression of disease from steatosis to NASH and potentially cirrhosis. It has been shown that chronic oxidative stress, generated through the oxidation of cytotoxic free fatty acids, can lead to upregulation of cytokines^[9], induction of the liver cytochrome P450 enzyme 2E1 (CYP2E1), and depletion of hepatic antioxidant concentration^[6]. In addition, enhanced lipid peroxidation leads to the generation of byproducts, such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA), which have been shown to further stimulate cytokine production. They are involved in hepatic stellate cell activation^[10], fibrogenesis, and enhanced extracellular matrix protein deposition.

According to the concepts of pathogenesis of NASH, these might make a wise basis for the use of antioxidants or drugs that could protect hepatocytes from oxidative stress. N-acetylcysteine (NAC) is a glutathione precursor which increases glutathione levels in hepatocytes^[11]. Increased glutathione levels, in turn, limit the production of reactive oxygen species (ROS)

which cause hepatocellular injury^[12]. Oral NAC treatment (1 g/d) of 11 NASH patients for 3 mo was demonstrated to improve liver function test significantly at the end of treatment period^[11]. In a controlled study, NAC (600 mg/d) was administered to NASH patients for 4 wk, and a significant improvement in aminotransferase levels was found^[13]. Although NAC was shown to improve liver function test in NASH patients, the mechanism remained unclear. Treatment of NASH with diet or diet plus NAC could attenuate oxidative stress as well as improve biochemical parameters and liver histopathology. However, the result of addition of NAC is not better than diet treatment alone^[14]. Therefore, this study was conducted to determine the effects of NAC on oxidative stress and liver pathology in a rat model of 100% fat diet induced NASH^[15].

MATERIALS AND METHODS

Animal preparation

This study was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. Male Sprague-Dawley rats weighing 220-260 g from the National Laboratory Animal Center, Mahidol University, Salaya, Nakorn Pathom were used. The animals were allowed to rest for a week after arrival at the Animal Center, Department of Physiology, Faculty of Medicine, Chulalongkorn University. They were kept at a controlled temperature of $25 \pm 1^\circ\text{C}$ under standard conditions (12 h dark: 12 h light cycle), fed with regular dry rat chow ad libitum, and had freely access to drinking water.

Experimental protocols

Rats were randomly divided into three experimental groups. Group 1: Fed ad libitum with regular dry rat chow for 6 wk (control group, $n = 8$). Group 2: Fed ad libitum with 100% fat diet for 6 wk to induce NASH (NASH group, $n = 8$). Group 3: Fed ad libitum with 100% fat diet plus 20 mg/kg per day of NAC orally (NASH + NAC₂₀ group, $n = 9$) for 6 wk.

All rats were weighed weekly. They were sacrificed to collect blood, serum, and liver samples at the end of the study, 20 h after the last NAC treatment. The diagram of the experiment was shown as follow.

At the end of the study, all rats were anaesthetized using intraperitoneal injection of an overdose (45 mg/kg) of sodium pentobarbital, and the abdominal walls were opened. Blood was drawn by cardiac puncture for total glutathione assay and biochemical assay. The livers were excised quickly and cleaned in iced-cold NSS. One lobe of the liver was collected for MDA measurement, the remaining liver was fixed in 40 g/L formaldehyde solution for histological examination.

Total glutathione determination

Total glutathione levels were quantified using Cayman's GSH assay kit. This assay uses glutathione reductase for determination of glutathione. The sulfhydryl group of glutathione reacts with DTNB (5, 5'-dithiobis-2-

nitrobenzoic acid, Ellman's reagent) and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB (between glutathione and TNB) that is concomitantly produced, is reduced by glutathione reductase to recycle glutathione and to produce more TNB. The rate of TNB production is directly proportional to this recycling reaction which is in turn directly proportional to the concentration of glutathione in the sample. Measurement of the absorbance of TNB at 405 nm provides an accurate estimation of glutathione in the sample.

Hepatic malondialdehyde (MDA) determination

One lobe of the liver was removed and weighed. One gram of the tissue was placed in a test tube containing 2.25 mL homogenization buffer (11.5 g/L KCl) and homogenized in an ice box using a homogenizer at a rotational speed of 12000 r/min for 1 min. MDA was quantified by using the thiobarbituric acid reaction as described by Ohgawa *et al*^[16]. MDA levels in the samples were determined the linear regression equation from a standard curve. The content of lipid peroxide is expressed as nmol of MDA/g of wet weight, and the total protein was determined by the Lowry method^[17] to correct the MDA level which is expressed in terms of $\mu\text{mol/g}$ protein.

Histopathological examination

The remaining liver samples were fixed in 40 g/L formaldehyde solution at room temperature. They were processed by standard methods. Briefly, tissues were embedded in paraffin, sectioned at 5 μm , stained with HE, and then picked up on glass slides for light microscopy. An experienced pathologist blinded to the experiment evaluated all samples. All fields in each section were examined for grading of steatosis and necroinflammation according to the criteria described by Brunt *et al*^[18].

The severity of steatosis was scored on the basis of the extent of involved parenchyma as 1 if fewer than 33% of the hepatocytes were affected, as 2 if 33%-66% of the hepatocytes were affected, as 3 if more than 66% of the hepatocytes were affected, and as 0 if no hepatocytes were affected.

Hepatic necroinflammation was graded from 0 to 3; score 1 (mild) = sparse or mild focal zone 3 hepatocyte injury/inflammation, score 2 (moderate) = noticeable zone 3 hepatocyte injury/inflammation, score 3 (severe) = severe zone 3 hepatocyte injury/inflammation, and score 0 = no hepatocyte injury/inflammation.

Statistical analysis

The data were expressed as mean \pm SEM using the SPSS version 11.5 for Windows program. Statistical comparisons between groups were analyzed by ANOVA and post hoc comparisons were done with Bonferroni correction. $P < 0.05$ were considered significant.

RESULTS

Body mass and general condition

The body mass at 6 wk of the NASH group and NASH

Table 1 Body mass and serum biochemical parameters in all groups

Parameter (mean \pm SEM)	Control (n = 8)	NASH (n = 8)	NASH + NAC ₂₀ (n = 9)
Body mass (g) at the beginning	239.0 \pm 2.27	245.1 \pm 1.0	251.4 \pm 1.7
at 6 wk	438.4 \pm 9.7	197.0 \pm 8.1 ^a	207.8 \pm 6.9 ^a
AST (U/L)	86.8 \pm 4.3	53.6 \pm 9.3 ^a	65.6 \pm 8.7
ALT (U/L)	40.2 \pm 2.4	23.0 \pm 1.9 ^a	25.4 \pm 5.7 ^a
Cholesterol (g/L)	71.8 \pm 1.8	94.8 \pm 3.1 ^a	91.4 \pm 3.5 ^a
Triglycerides (g/L)	90.3 \pm 19.1	147.8 \pm 32.6	89.2 \pm 28.2

^a*P* < 0.05 vs control.**Table 2** Effects of NAC on liver histology in rats with NASH (scores)

Group	n	Steatosis				Necroinflammation			
		0	1	2	3	0	1	2	3
Control	8	8	-	-	-	8	-	-	-
NASH	8	-	-	5	3	-	5	2	1
NASH + NAC ₂₀	9	-	6	2	1	3	4	1	1

+ NAC₂₀ group were decreased compared to the control (197.0 \pm 8.1 g, 207.8 \pm 6.9 g *vs* 438.4 \pm 9.7 g, *P* < 0.05). Despite weight loss, the general condition of 100% fat diet-fed rats remained good throughout the observation period. After the first 6 wk, rats were fed with regular dry rat chow for additional 4 wk. The body mass was significantly increased in all groups (Table 1).

Serum biochemical parameters

Serum biochemical parameters in the control and the experimental groups are given in Table 1. Serum AST and ALT activities decreased significantly in the NASH group when compared to the control group (AST; 53.7 \pm 9.3 U/L *vs* 86.8 \pm 4.3 U/L, ALT; 23.0 \pm 1.9 U/L *vs* 40.1 \pm 2.4 U/L, *P* < 0.05). Serum ALT but not AST activity returned to control levels in the NASH + NAC₂₀ group (ALT 25.4 \pm 5.7 U/L; AST 65.6 \pm 8.7 U/L). Serum cholesterol was significantly higher in the NASH group and NASH + NAC₂₀ group than that in the control group (94.8 \pm 3.1 g/L, 91.4 \pm 3.5 g/L *vs* 71.8 \pm 1.8 g/L, *P* < 0.05), whereas there were no significant differences in serum triglycerides (Table 1).

Total glutathione level in whole blood

Whole blood total glutathione levels were significantly higher in the NASH group compared to the control group (2066.7 \pm 93.8 μ mol/L *vs* 1337.5 \pm 31.5 μ mol/L, *P* < 0.05). Glutathione in NASH + NAC₂₀ group was significantly lower than in the NASH group (1394.8 \pm 81.2 μ mol/L *vs* 2066.7 \pm 93.8 μ mol/L, *P* < 0.05).

Hepatic MDA content

MDA was elevated significantly in the NASH group when compared to the control group (209.9 \pm 43.8 μ mol/g protein *vs* 3.8 \pm 1.7 μ mol/g protein, *P* < 0.05). There was no statistical significant difference in MDA levels in NASH + NAC₂₀ group (150.1 \pm 27.0 μ mol/g protein).

Histopathological examination

Liver sections from rats fed with the regular dry rat chow had normal morphological appearance. In the NASH group, all animals developed moderate to severe macrovesicular steatosis, hepatocyte ballooning, mild to moderate inflammation, and regeneration of hepatocytes (Table 2). NAC treatment improved steatosis and necroinflammation scores in animals of the NASH + NAC₂₀ group when compared with the NASH group (Figure 1).

DISCUSSION

Histopathology of NASH is similar to that of ethanol-induced hepatitis with the presence of macrovesicular steatosis, hepatocyte ballooning, necroinflammation, Mallory bodies, and fibrosis^[1]. To study the pathogenesis of or therapeutic options for NASH, there are many models that can be used including a genetic model (obese rats), a model of methionine and choline deficient diet, a model of high fat liquid diet, and a 100% fat diet^[5-8,15]. In this study, 100% fat diet was chosen to induce NASH in Sprague-Dawley rats as this procedure is fast, easy, and provides a comparable pattern of pathological changes as in humans although this model represents malnutrition induced steatohepatitis.

By feeding rats with 100% fat diet, the hepatic lesions of NASH were apparent within 6 wk. Histopathological examination showed macrovesicular steatosis, hepatocyte ballooning, Mallory bodies, and mild to moderate inflammation. One hundred percent fat diet caused mobilization of free fatty acid (FFA) from adipose tissue and transport into hepatocytes. In this condition, the liver failed to synthesize apolipoprotein that is required for packaging and exporting fat from the liver, triglycerides (TG) thus accumulate in the liver^[19]. β -oxidation of FFA in hepatocytes produces reactive oxygen species (ROS) which activate lipid peroxidation^[20]. ROS and lipid peroxidation cause direct damage to hepatocytes by disrupting membranes, protein, and DNA^[21,22]. Hepatocyte damage and lipid peroxidation products induce an inflammatory response.

AST and ALT are useful screening tests for detecting liver injury^[23]. They are found in hepatocytes and can not diffuse out of the cells in the physiological condition. When the hepatocyte is injured, plasma membrane can be disrupted and the leakage through extracellular fluid of the enzyme occurs where they can be detected at abnormal levels in the serum^[24]. AST and ALT activities have been found to be increased in NASH rats^[10,25-28]. In contrast, AST and ALT activities decreased significantly with 6 wk of 100% fat diet in this study. The decreased serum transaminases may be due to poor nutrition or hepatocyte death. Rats fed with 100% fat diet derived main energy from fat, when there were low in vitamin and mineral contents. The decreased AST and ALT levels were probably due to nutritional deficiency of pyridoxal phosphate which is a cofactor for both AST and ALT to catalyze the transfer of the α amino group from aspartate or alanine to α -ketoglutarate with made the release of pyruvate, oxaloacetate, and glutamate^[23]. In addition,

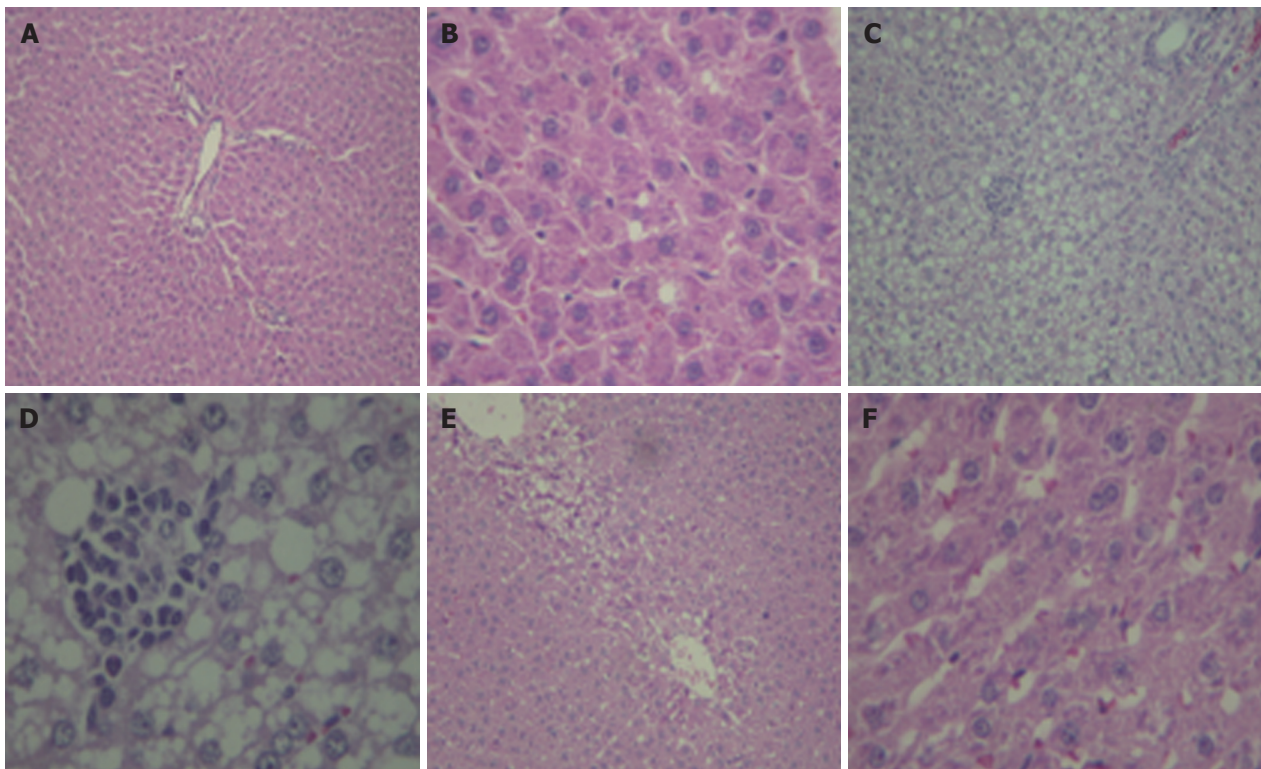


Figure 1 Hematoxylin and eosin staining of liver tissue. **A, B:** control; **C, D:** NASH, fed with 100% fat diet group showed macrovesicular steatosis, ballooning changes, Mallory bodies, hepatocyte necrosis, and infiltration of inflammatory cells; **E, F:** NASH + NAC₂₀, showed the improvement in steatosis and necroinflammation (**A, C, E:** x 10; **B, D, F:** x 40).

oxidative stress condition may be a cause of hepatocyte death, therefore, aminotransferases can not be produced.

In 100% fat diet-fed rats, body mass decreased significantly ($P < 0.05$) as compared to the control group. While serum cholesterol significantly increased, serum TG level was unchanged. Feeding with 100% fat diet for 6 wk caused a loss of body mass that may be due to a metabolic imbalance of carbohydrate, protein, and fat. Moreover, 100% fat diet contained highly saturated fat which may increase blood cholesterol concentration by 15% to 25%^[29]. This result was from an increase of fat deposition in the liver which then provides the increased quantities of acetyl CoA in the liver cell for production of cholesterol^[29]. The increased cholesterol was found in this experiment and had been observed in another study that used 10% lard oil and 2% cholesterol supplement adding into the standard diet^[29].

FFA causes oxidative stress that has the potential to induce NASH^[2]. FFA in the body is increased and this is associated with state of starvation^[2]. Stored FFA can be mobilized from adipose tissue through lipolysis^[2]. FFA metabolism increases the production of ROS which activated lipid peroxidation. Consequences are the disruption of membranes and the production of reactive metabolites such as MDA^[20]. This study found high hepatic MDA levels in 100% fat-diet fed rats in accordance with studies by others^[25-28]. Glutathione is the major intracellular non-protein antioxidant and plays a crucial role in the detoxification of free radicals^[30,31]. Serum level of glutathione was increased in patients with NASH^[32]. Similarly in this experiment, an increasing in total glutathione in whole blood with 100% fat diet

feeding could be explained by compensatory protection mechanism against oxidative stress.

NAC is a thiol compound that acts directly as free radical scavenger and as a precursor of reduced glutathione^[33]. Therefore, treatment with 20 mg/kg of NAC improved the total glutathione level to normal level in NASH + NAC₂₀ group and improved necroinflammation score. Because of some limitations of our study, such as dose of NAC, time for treatment, and the number of animals, the effect of NAC on reducing hepatic MDA level remained unclear. In our previous study, diet treatment alone and diet plus NAC groups, total glutathione, serum AST, ALT, cholesterol, TG, and hepatic MDA returned to normal levels as in the control group. In addition, the pathological changes of liver in these groups were improved^[14]. These results emphasized how crucial the nutritional composition of the diet is. Good proportion of nutrients (i.e., carbohydrate, lipid, and protein) is essential for growth and maintenance. These nutrients supply energy, promote growth, repair body tissues, and regulate metabolic processes^[34].

In conclusion, feeding with 100% fat diet for 6 wk induced macrovesicular steatosis, hepatocyte ballooning, and inflammation in rats similar to histopathology of NASH. Treatment with NAC in NASH could improve oxidative stress and liver histopathology.

COMMENTS

Background

Non-alcoholic steatohepatitis (NASH), in advanced stages, can cause liver fibrosis, eventually progressing to cirrhosis in some patients. Oxidative stress is believed

to play an important role in pathogenesis of NASH. N-acetylcysteine (NAC) is a glutathione precursor which increases glutathione levels in hepatocytes. Increased glutathione levels, in turn, limit the production of reactive oxygen species (ROS) which cause hepatocellular injury that could protect hepatocytes from oxidative stress.

Research frontiers

NAC is a thiol compound that acts directly as free radical scavenger. In the pathogenesis of NASH, prevention of oxidative stress could protect hepatocytes from injury. The hotspots of this study indicate that NAC treatment could attenuate oxidative stress and improve liver histology in rats with NASH.

Innovations and breakthroughs

According to a previous report, oral NAC treatment of NASH patients for several months was found to significantly improve aminotransferase levels. However, the mechanism remained unclear. This study is a novel and well conducted experimental study showing the efficacy of NAC on improvement of total glutathione level and hepatic MDA in rats with NASH. Furthermore, treatment with NAC showed improvement in steatosis and necroinflammation.

Applications

Our data indicate that NAC treatment could attenuate oxidative stress and improve liver histology in rats with NASH.

Terminology

NASH is a liver disease characterized by macrovesicular steatosis, hepatocyte necrosis, inflammation, Mallory bodies, and fibrosis. In initial phases, during which fat accumulates in the liver, no clinical symptoms are evident. In advanced stages, fibrosis is detectable, eventually progressing to cirrhosis. NAC is a glutathione precursor which increases glutathione levels in hepatocytes. Increased glutathione levels, in turn, limit the production of ROS which cause hepatocellular injury.

Peer review

This is an experimental work on a steatosis model in the rat, induced by 100% fat diet in which the co-administration of NAC protects against fat induced liver injury. This is a very interesting and well conducted experimental study showing the efficacy of NAC in preventing biochemical and histological alterations secondary to a fat rich diet.

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Risk factors for lymph node metastasis and evaluation of reasonable surgery for early gastric cancer

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Abstract

AIM: To give the evidence for rationalizing surgical therapy for early gastric cancer with different lymph node status.

METHODS: A series of 322 early gastric cancer patients who underwent gastrectomy with more than 15 lymph nodes retrieved were reviewed in this study. The rate of lymph node metastasis was calculated. Univariate and multivariate analyses were performed to evaluate the independent factors for predicting lymph node metastasis.

RESULTS: No metastasis was detected in No.5, 6 lymph nodes (LN) during proximal gastric cancer total gastrectomy, and in No.10, 11p, 11d during for combined resection of spleen and splenic artery and in No.15 LN during combined resection of transverse colon mesentery. No.11p, 12a, 14v LN were proved negative for metastasis. The global metastatic rate was 14.6% for LN, 5.9% for mucosa, and 22.4% for submucosa carcinoma, respectively. The metastasis in group II was almost limited in No.7, 8a LN. Multivariate analysis identified that the depth of invasion, histological type and lymphatic invasion were independent risk factors for LN metastasis. No metastasis from distal cancer (≤ 1.0 cm in diameter) was detected in group II LN. The metastasis rate increased significantly when the diameter exceeded 3.0 cm. All tumors (≤ 1.0 cm in diameter) with LN metastasis and mucosa invasion showed a depressed macroscopic type, and all protruded carcinomas were > 3.0 cm in diameter.

CONCLUSION: Segmental/subtotal gastrectomy plus D₁/D₁ + No.7 should be performed for carcinoma (≤ 1.0 cm in diameter, protruded type and mucosa invasion).

Subtotal gastrectomy plus D₂ or D₁ + No.7, 8a, 9 is the most rational operation, whereas No.11p, 12a, 14v lymphadenectomy should not be recommended routinely for poorly differentiated and depressed type of submucosa carcinoma (> 3.0 cm in diameter). Total gastrectomy should not be performed in proximal, so does combined resection or D₂⁺/D₃ lymphadenectomy.

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Key words: Lymph node; Metastasis; Surgery; Early gastric cancer

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INTRODUCTION

Gastric cancer is still one of the important causes of cancer-related death in China. Although early gastric cancer accounts for less than 10% of gastric cancers, excellent outcome of surgery has been reported, with a 5-year survival rate higher than 90%^[1-3]. In the past 20 years, most surgeons considered D₂ lymphadenectomy the standard and optimal surgical procedure for patients with early gastric cancer. Even total gastrectomy and D₃ lymphadenectomy with combined resection of other organs have been used to achieve curative (R₀) resection^[3-9]. The lymph node metastasis rate of early gastric cancer is reported to be 11%-18% and 70%-80% patients will undergo over-surgery with D₂ lymphadenectomy^[1,4,8,9]. Consequently, investigating the risk factors for lymph node metastasis is the key to rational surgery of early gastric cancer, which may improve the 5-year survival rate of patients and their quality of life. This study was to retrospectively analyze the location, frequency, degree of and risk factors for lymph node metastasis in 322 patients with early gastric cancer, in order to rationalize surgical therapy.

MATERIALS AND METHODS

Patients

Between February 1972 and August 2006, a consecutive series of 322 early gastric cancer patients underwent

Table 1 Site and frequency of lymph node metastasis of cancer in the lower and middle thirds of stomach

The lower third of the stomach			The middle third of the stomach		
Frequency of LNM			Frequency of LNM		
Possitive	%		Possitive	%	
Group 1			Group 1		
No.3	12	4.9	No.1	3	4.8
No.4	16	6.5	No.3	6	9.7
No.5	3	1.2	No.4	0	0.0
No.6	14	5.7	No.5	1	1.6
			No.6	1	1.6
Total	35	14.3	Total	8	12.9
Group 2			Group 2		
No.1	1	0.4	No.7	3	4.8
No.7	13	5.3	No.8a	1	1.6
No.8a	7	2.9	No.9	0	0.0
No.9	2	0.8	No.11p	0	0.0
No.11p	0	0.0	No.12a	0	0.0
No.12a	0	0.0			
No.14v	0	0.0			
Total	18	7.3	Total	4	6.5

LNM: Lymph node metastasis; No.1: Right paracardial LN; No.3: LN along the lesser curvature; No.4: LN along the greater curvature; No.5: suprapyloric LN; No.6: Infrapyloric LN; No.7: LN along the left gastric artery; No.8a: LN along the common hepatic artery (anterosuperior group); No.9: LN around the celiac artery; No.11p: LN along the proximal splenic artery; No.12a: LN in the hepatoduodenal ligament (along the hepatic artery); No.14v: LN along the superior mesenteric vein.

gastrectomy at the Department of Oncologic Surgery, First Affiliated Hospital, China Medical University. The patients (242 men and 80 women) ranged in age from 19 to 80 (53.8 ± 12.3) years participated in the study. Early gastric cancer was located in the lower third of stomach (L/LM) of 145 patients, in the middle third (M/ML/MU) of 14 patients, in the upper third (U/UM) of 11 patients, and in whole stomach (UML) of 4 patients. A total of 292 patients underwent distal gastrectomy, 10 proximal gastrectomy, 20 total gastrectomy, 2 combined resection of spleen, and 3 combined resection of transverse colon mesentery. D₁ lymphadenectomy was performed in 57 patients; D₁ + No.7 in 58 patients; D₁ + No.7, 8a, 9 in 63 patients; D₂ in 107 patients; and D₂⁺ or D₃ in 37 patients. The methods of pathology diagnosis, lymph node grouping and surgery have been described previously^[10].

Pathology

Serial section of specimens was performed for an accuracy pathological diagnosis. Mucosa carcinoma was diagnosed in 152 patients and submucosa carcinoma in 170 patients based on the depth of invasion. Protruded type was found in 23 patients, flat type in 38 patients, and depressed type in 263 patients, respectively in the light of macroscopic appearance. The tumor diameter ranged from 0.4 to 14.0 (3.2 ± 1.8) cm. Well or moderately differentiated tumor was found in 142 patients, and poorly differentiated tumor in 180 patients according to their histological type. Mass type was observed in 102 patients, nest type in 91 patients and diffused type in 129 patients, respectively, based on the histological growth pattern. Lymphatic vessel invasion occurred in 19 patients.

Statistical analysis

All data were analyzed by SPSS11.5. The correlation between clinicopathological factors and nodal involvement was evaluated by univariate analysis. Multivariate analysis was performed to evaluate the independent factors for predicting lymph node metastasis. $P < 0.05$ was considered statistically significant.

RESULTS

Evaluation of lymphadenectomy

Five patients underwent combined resection of other organs due to misdiagnosis as advanced gastric carcinoma. Depressed type of mucosa and submucosa carcinoma was diagnosed in 2 and 3 patients, respectively. Proximal gastric cancer without metastasis in No.10, 11p, 11d lymph nodes was diagnosed in 2 patients undergoing combined resection of spleen and splenic artery. Distal gastric cancer without metastasis in No.15 lymph node was diagnosed in 3 patients undergoing combined resection of transverse colon mesentery. No.5, 6 lymph nodes were negative in 20 patients with proximal gastric cancer after total gastrectomy.

Metastasis of distal gastric cancer was found in 2 out of the 37 patients undergoing extended lymphadenectomy ($> D_2$). Depressed type of mucosa and submucosa carcinoma was found in the 2 patients. Metastasis of group I lymph nodes was detected both in 285 patients with middle and distal gastric cancer who underwent $\leq D_2$ lymphadenectomy. The detection rate was 12.9% and 14.3%, respectively. A frequent metastasis of No.7, 8a lymph nodes (6.5%-8.2%) and an occasional metastasis of No.9 lymph nodes were also found. No metastasis was detected in No.11, 12a, 14v lymph nodes (Table 1).

Relationship between frequency of lymph node metastasis and location of focus

The number of retrieved lymph nodes in all patients was more than 15, ranging from 15 to 75 (median, 16). Lymph node metastasis was detected in 47 patients (14.6%), and the number of metastatic lymph nodes ranged from 1 to 16 (median, 2). Of the patients with lymph node metastasis, 33 were male (13.6%), 14 female (17.5%). Distal gastric cancer was diagnosed in 38 patients (15.5%), middle gastric cancer in 9 (14.5%), protruded type in 3 patients (14.3%), flat type in 4 patients (10.5%), and depressed type in 40 patients (15.2%). The diameter of tumor was ≤ 1.0 cm in 5 patients (19.5%), 1.1-2.0 cm in 13 patients (11.2%), 2.1-3.0 cm in 6 patients (10.0%), > 3.0 cm in 23 patients (19.2%). Mucosa carcinoma was found in 9 patients (2.9%), submucosa carcinoma in 38 patients (22.4%), poorly differentiated carcinoma in 35 patients (19.4%), well or moderately differentiated in 12 patients (8.5%), mass type in 15 patients (14.7%), nest type in 12 patients (13.2%), diffused type in 20 patients (15.5%), negative lymphatic invasion in 36 patients (57.9%), and positive lymphatic invasion in 11 patients (11.9%).

Of the 245 patients with distal gastric cancer, 35 had metastasis in group I lymph nodes (14.3%), and 18 in group II lymph nodes (7.3%). Metastasis was detected in all group

Table 2 Comparison of clinicopathological features between patients with and without lymph node metastasis

Factors	Node negative	Node positive	P value
Dissected nodes (mean \pm SD)	19.3 \pm 7.5	20.8 \pm 6.9	0.188
Age, yr (mean \pm SD)	53.9 \pm 12.1	52.9 \pm 13.2	0.606
Tumor maximum diameter (cm, mean \pm SD)	3.1 \pm 1.8	3.5 \pm 1.8	0.197
Gender			
Male	209	33	0.465
Female	66	14	
Tumor location			
Upper	11	0	0.435
Middle	53	9	
Lower	207	38	
Total	4	0	
Depth of invasion			
Mucosa	143	9	< 0.001
Submucosa	132	38	
Histological type			
Differentiated	130	12	0.007
Undifferentiated	145	35	
Macroscopic type			
Protruded	18	3	0.746
Flat	34	4	
Depressed	223	40	
Growth manner			
Mass	87	15	0.891
Nest	79	12	
Diffuse	109	20	
lymphatic invasion			
Negative	267	36	< 0.001
Positive	8	11	

Differentiated: Papillary and tubular adenocarcinoma; Undifferentiated: Poorly differentiated adenocarcinoma and signet-ring cell carcinoma; Protruded: I and II a; flat: II b; depressed: II c and III.

Table 3 Logistic regression analysis for variables associated with lymph node metastasis in EGC

Explanatory variables	Odds ratio	95% CI	P value
Depth of invasion	3.67	1.62-8.30	0.002
Histological type	3.39	1.39-8.27	0.007
Lymphatic invasion	8.41	2.86-24.74	< 0.001
Tumor maximum diameter	1.23	1.0-1.49	0.042
Gender	1.10	0.46-2.21	0.981
Age	0.99	0.97-1.03	0.726
Tumor location	0.60	0.29-1.23	0.161
Growth manner	0.58	0.26-1.30	0.186
Macroscopic type	1.14	0.56-2.34	0.715

CI: Confidence interval. Depth of invasion, histological type, lymphatic invasion and tumor maximum diameter are the independent risk factors correlated with lymph node involvement.

I lymph nodes with a frequency of 5.7% for No.6, 6.5% for No.4, 4.9% for No.3, and 1.2% for No.5, respectively. In group II lymph nodes, the most frequent metastasis was detected in No.7 lymph nodes (5.3%) and No.8a lymph nodes (2.9%), and less frequent metastasis in No.9 lymph nodes (0.8%), No.1 lymph nodes (0.4%). Metastasis of group III lymph nodes was detected in only 2 patients.

Of the 62 patients with middle gastric cancer, 8 had metastasis of group I lymph nodes (12.9%), and 4 had metastasis of group II lymph nodes (6.5%). The rate

of metastasis of group I lymph nodes was 9.7% for No.3, 4.8% for No.1, 1.6% for No.5, and 1.6% for No.6, respectively. Metastasis of No.7 (4.8%) and No.8a (1.6%) lymph nodes was detected in group II lymph nodes. No lymph node metastasis was detected in the 11 patients with proximal gastric cancer.

Risk factors correlated with lymph node involvement

In this series, the mean number of retrieved lymph nodes was 19.3 ± 7.5 in patients without lymph node metastasis, and 20.8 ± 6.9 in patients with lymph node metastasis. The difference was not significant ($F = 1.741$, $P = 0.188$). The univariate analysis showed that three variables were significantly indicative of lymph node metastasis: depth of invasion, histological type, and lymphatic invasion ($P < 0.001$). The lymph node metastasis rate of poorly differentiated submucosa carcinoma with positive lymphatic invasion was significantly higher than that of well differentiated mucosa carcinoma with negative lymphatic invasion (Table 2).

The multivariate analysis showed that all the variables (depth of invasion, histological type, and lymphatic invasion) remained significant, indicating that the independent risks were correlated with lymph node involvement. The lymph node metastasis of submucosa carcinoma was 3.7 times higher than that of mucosa carcinoma. The lymph node metastasis of poorly differentiated carcinoma was 3.4 times higher than that of well differentiated carcinoma. The positive lymphatic invasion was 8.4 times higher than that of negative lymphatic invasion. The maximum diameter of the tumor was also an important variable correlated with lymph node involvement (OR = 1.23, $P = 0.042$), the rate of lymph node metastasis was associated with the maximum tumor diameter (Table 3).

Relationship between lymph node metastasis and clinicopathological factors for distal gastric cancer

Metastasis of distal gastric cancer hardly went beyond group I lymph nodes when the maximum tumor diameter was ≤ 1.0 cm. Corresponding to the increased maximum diameter, the rate of metastasis in group II lymph nodes was increased, but often limited in No.7, 8a lymph nodes. Only when the maximum tumor diameter was > 3.0 cm, could metastasis of No.1 and 9 lymph nodes be detected. Compared with protruded type of carcinoma, in which metastasis could only be detected in group I lymph nodes, depressed type of carcinoma often had combined metastasis in both group I and II lymph nodes, and the number of metastasis lymph nodes would increase significantly.

Metastasis of mucosa carcinoma was detected in all group I lymph nodes. Metastasis of poorly differentiated and depressed types of mucosa carcinoma in No.1 lymph nodes was detected in only 1 patient, whose tumor diameter was > 3.0 cm. Metastasis of submucosa carcinoma was detected in both group I and II lymph nodes, and the rate of lymph node metastasis was significantly higher than that of mucosa carcinoma ($P < 0.01$). Metastasis of poorly and well differentiated carcinoma was detected in group I and II lymph nodes, but the metastasis rate of poorly differentiated

Table 4 Depth of invasion, histological type, macroscopic type, lymphatic penetration of lymph node metastasis and tumor maximum diameter in the lower third of stomach

Factors	Group 1 metastasis (person)						Group 2 metastasis (person)					
	No.3	No.4	No.5	No.6	Total	P value	No.1	No.7	No.8a	No.9	Total	P value
Tumor maximum diameter (cm)												
≤ 1.0	1	0	0	3	4		0	0	0	0	0	
1.1-2.0	4	4	2	4	11		0	3	3	0	5	
2.1-3.0	0	2	1	0	3		0	1	0	0	1	
> 3.0	7	10	0	7	17	0.143	1	9	4	2	12	0.018
Histological type												
Diff	4	4	0	4	8		0	2	0	1	2	
Undiff	8	12	3	10	27	0.025	1	11	7	1	16	0.006
Depth of invasion												
M	1	4	2	3	9		1	0	0	0	1	
Sm	11	12	1	11	26	0.010	0	13	7	2	17	< 0.001
Growth manner												
Mass	5	5	0	5	10		0	4	3	1	7	
Nest	3	3	2	2	8	0.669	0	3	2	1	4	0.689
Diffuse	4	8	1	7	17		1	6	2	0	7	
Macroscopic type												
Protruded	0	2	0	0	2		0	0	0	0	0	
Flat	1	2	0	1	3		0	2	0	0	2	
Depressed	11	12	3	13	30	0.819	1	11	7	2	16	0.501
Lymphatic invasion												
Negative	10	13	2	11	27		1	8	6	2	13	
Positive	2	3	1	3	8	< 0.001	0	5	1	0	5	< 0.001

Diff: Differentiated histological type; Undiff: Undifferentiated histological type; M: Mucosa; Sm: Submucosa.

carcinoma was significantly higher than that of well differentiated carcinoma ($P < 0.05$). The metastasis rate of positive lymphatic invasive carcinoma was also significantly higher than that of negative lymphatic invasive carcinoma ($P < 0.01$) (Table 4).

DISCUSSION

Since 1980's, D₂ lymphadenectomy has been widely accepted as the standard surgery for early gastric cancer, especially for submucosa carcinoma. However, it was reported that D₂ lymphadenectomy does not increase the long-term survival of patients, compared with D₁ or D₁⁺ lymphadenectomy^[7-9,11]. Since lymph node metastasis remains one of the most important predictors for survival, reduction in lymphadenectomy will probably result in residue of metastatic lymph nodes. Unnecessarily extended resection will induce a series of complications, which also result in a poor quality of life. Thus, it is important to standardize the optimal extent of lymph node dissection by investigating lymph node metastasis of early gastric cancer.

In the present study, total gastrectomy for carcinoma in the upper third of stomach was selected according to the status of No.5 and 6 lymph nodes. Metastasis was not detected in all proximal gastric cancer patients after total gastrectomy. Considering the complications after total gastrectomy, it was not used as a routine operation for proximal early gastric cancer. Furthermore, metastasis was not detected in No.10, 11p, 11d lymph nodes after combined resection of spleen or splenic artery and in No.15 lymph nodes after combined resection of transverse colon mesentery. Therefore, we conclude that combined resection of other organs should not be performed in early gastric cancer. In our study, most patients undergoing

combined resection were due to adhesion of serosa and transverse colon mesentery or due to inflammatory swelling of inflammatory splenic hilum lymph nodes. Thus, estimating the depth of invasion accurately is the key to optimal surgery. Endoscopic ultrasonography and three-dimensional spiral CT (3DCT) provide more accurate information on the depth of invasion. It was reported that 70%-80% of early gastric cancers can be diagnosed by endoscopic ultrasonography combined with three-dimensional CT^[12-14].

Whether D₂ lymphadenectomy for early gastric cancer should be performed remains controversial. Many researchers consider D₁ + No.7, 8a or D₁ + No.7, 8a, 9 lymphadenectomy as the standard operation for most of early gastric cancers^[15-17]. In our series, no metastasis of middle and distal gastric cancer was detected in No. 11p, 12a, 14v lymph nodes, suggesting that neither dissection of No. 11p, 12a, 14v lymph nodes nor D₂⁺/D₃ lymphadenectomy is necessary for distal early gastric cancer.

It was reported that the lymph node metastasis rate is 0%-3% for mucosa carcinoma and 20% for submucosa carcinoma. It has been shown that 9%-16% of metastatic lymph nodes are detected in group I, 4%-6% in group II, and 0.3%-1% in group III^[16-18]. In this study, the metastasis rate in group I and III lymph nodes is consistent with the reported data. The metastasis rate in group II was 7.3% for distal cancer and 6.5% for middle cancer, higher than that in former studies.

It has been widely accepted that the status of lymph node involvement is closely correlated with the depth of invasion, but the relationship between metastasis and focus diameter, histological type and macroscopic type is still controversial^[8,16,17,19-21]. Kunisaki *et al*^[17] reported that mucosa carcinoma (> 3.0 cm in diameter) has a

higher lymph node involvement, while there is no distinct correlation between the diameter, macroscopic type, histological type and metastasis of submucosa carcinoma. Shimoyama *et al*^[16] found that the metastasis rate for intestinal submucosa carcinoma (≤ 1.5 cm in diameter) and diffused submucosa carcinoma (≤ 1.0 cm in diameter) is very low (3%), and limited in group I lymph nodes. If the diameter went beyond the former cut-point, the rate of metastasis would increase significantly and No.7, 8a, 9 lymph nodes (2.3%) could be detected even in group II lymph nodes. Gotoda *et al*^[20] reported that lymph node metastasis of well differentiated mucosa carcinoma (≤ 3.0 cm in diameter) is seldom detected. Whereas lymph node metastasis of submucosa carcinoma (> 3.0 cm in diameter) with positive lymphatic invasion increase significantly.

In our study, no metastasis of distal gastric cancer (≤ 1.0 cm in diameter) with mucosa invasion was detected in group II lymph nodes. The rate of metastasis in group II lymph nodes significantly increased when the tumor diameter was > 3.0 cm. All the carcinomas (≤ 1.0 cm in diameter and/or mucosa invasion) with lymph node involvement showed depressed macroscopic type. The diameter of protruded carcinoma with lymph node involvement was > 3.0 cm. Multivariate analysis showed that the depth of invasion, histological type and lymphatic penetration were independent risk factors for lymph node metastasis and the maximum diameter was also an important factor. Our study also revealed the relationship between metastasis of distal gastric cancer in group II lymph nodes and the diameter, histological type, depth of invasion and lymphatic invasion of the tumor. The rate of involved lymph nodes significantly increased compared with poorly differentiated, sub-mucosa carcinoma and positive lymphatic invasion, suggesting that the following high risk factors for lymph node metastasis of distal gastric cancer in group II lymph nodes are tumor diameter > 3.0 cm, depressed type, poorly differentiated submucosa carcinoma and positive lymphatic invasion. Standard D₂ lymphadenectomy should be performed to achieve curative (R₀) resection in such patients.

In summary, selection of reasonable surgery for early gastric cancer should be based on the pathobiologic behaviour of the tumor. Segmental/subtotal gastrectomy plus D₁/D₁ + No.7 lymphadenectomy should be performed for carcinoma (≤ 1.0 cm in diameter, protruded type and mucosa invasion). Subtotal gastrectomy plus D₂ or D₁ + No.7, 8a, 9 lymphadenectomy is the most rational operation for poorly differentiated and depressed type of submucosa carcinoma (> 3.0 cm in diameter), whereas No.11p, 12a, 14v lymphadenectomy should not be performed routinely. Total gastrectomy should not be performed for proximal gastric cancer. Combined resection of other organs or D₂⁺/D₃ lymphadenectomy should always be avoided.

have been used to achieve curative (R₀) resection. However, it was reported that application of extended surgery does not reasonably increase the long-term survival of patients.

Research frontiers

It has been widely accepted that the status of lymph node involvement is closely correlated with the depth of invasion, but the relationship between metastasis and diameter of focus, histological type and macroscopic type is still controversial. Since lymph node metastasis remains one of the most important predictors for survival, reduction in lymphadenectomy will probably result in residue of metastatic lymph nodes. Unnecessarily extended resection will induce a series of complications, which also result in a poor quality of life.

Innovations and breakthroughs

The results of this study show that depth of invasion, histological type and lymphatic invasion are independent risk factors for lymph node metastasis. There is a relationship between the pathobiologic behavior of tumor and reasonable surgery for early gastric cancer.

Applications

With the knowledge of the risk factors and rules of lymph node metastasis, clinical doctors can select surgery for early gastric cancer more reasonably.

Terminology

D lymphadenectomy: Lymph node dissection according to the Japanese Gastric Cancer Association (JGCA) criteria. Lymph node dissection is divide into D₁ lymphadenectomy, D₂ lymphadenectomy and D₃ lymphadenectomy.

Peer review

The authors rationalized surgical therapy for early gastric cancer with different lymph node status and suggest that segmental/subtotal gastrectomy plus D₁/D₁ + No.7 should be performed for carcinoma (≤ 1.0 cm in diameter, protruded type and mucosa invasion). Subtotal gastrectomy plus D₂ or D₁ + No.7, 8a, 9 is the most rational operation for poorly differentiated and depressed type of submucosa carcinoma (> 3.0 cm in diameter), whereas No.11p, 12a, 14v lymphadenectomy should not be recommended routinely. Total gastrectomy or D₂⁺/D₃ lymphadenectomy should not be performed in proximal and combined resection.

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COMMENTS

Background

Since 1980's, D₂ lymphadenectomy has been widely accepted as the standard surgery for early gastric cancer, especially for submucosa carcinoma. Even total gastrectomy and D₃ lymphadenectomy with combined resection of other organs

- Extended lymphadenectomy (D2) in patients with early gastric cancer. *Eur J Surg Oncol* 2005; **31**: 875-881
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Pretreatment of cromolyn sodium prior to reperfusion attenuates early reperfusion injury after the small intestine ischemia in rats

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Abstract

AIM: To investigate the effects of Cromolyn Sodium (CS) pretreated prior to reperfusion on the activity of intestinal mucosal mast cells (IMMC) and mucous membrane of the small intestine in ischemia-reperfusion (IR) injury of rats.

METHODS: Thirty-two Sprague-Dawley (SD) rats were randomly divided into four groups: sham group (group S), model group (group M), high and low dosage of CS groups, (treated with CS 50 mg/kg or 25 mg/kg, group C1 and C2). Intestinal IR damage was induced by clamping the superior mesenteric artery for 45 min followed by reperfusion for 60 min. CS was intravenously administrated 15 min before reperfusion. Ultrastructure and counts of IMMC, intestinal structure, the expression of tryptase, levels of malondialdehyde (MDA), TNF- α , histamine and superoxide dismutase (SOD) activity of the small intestine were detected at the end of experiment.

RESULTS: The degranulation of IMMC was seen in group M and was attenuated by CS treatment. Chiu's score of group M was higher than the other groups. CS could attenuate the up-regulation of the Chiu's score, the levels of MDA, TNF- α , and expression of tryptase and the down-regulation of SOD activity and histamine concentration. The Chiu's score and MDA content were negatively correlated, while SOD activity was positively correlated to the histamine concentration respectively in the IR groups.

CONCLUSION: Pretreated of CS prior to reperfusion protects the small intestine mucous from ischemia-reperfusion damage, the mechanism is inhibited IMMC from degranulation.

INTRODUCTION

Mast cell degranulation is an important component of inflammatory tissue responses^[1]. Bortolotto proved that ischemia-reperfusion injury was depending upon the presence of mast cells in skeletal muscle^[2].

Intestinal mucosal mast cells (IMMCs) are particularly frequent in close proximity to epithelial surfaces where they are strategically located for optimal interaction with the environment and for their putative functions for host defense^[3].

Some studies on the role of IMMC in small intestine ischemia-reperfusion injury have been reported^[4-7]. Lindstrom and his colleagues^[4] found the eosinophils and mast cells in rat ileum gradually increased after intestine ischemia (60 min)/reperfusion (60 min), and reported that the epithelium permeability increased significantly after ischemia-reperfusion. The researches of Kanwar and Schramm^[5,6] were focused on mast cells stimulating neutrophil adherence, resupination, recruitment, and the intestinal mucosal injury. Boros^[7] reported that mast cell degranulation prior to ischemia may induce a potentially protective mechanism in the small bowel mucosa and decrease ischemia-reperfusion injury in the dog.

Cromolyn Sodium (CS) is a MC membrane stabilizer. MC-stabilization protocols were proved to reduce the leukocyte recruitment^[8]. Kimura^[9] reported that the intestine ischemia and reperfusion could induce a decrease in the mucosal histamine content, an increase in plasma histamine levels, and an significantly enhance in mucosal permeability. However, MAR-99, another mast cell stabilizer, can prevented these changes by pretreatment prior to ischemia. Kalia^[10] found that all ketotifen-pretreated animals (1 mg/kg orally twice daily for 3 d before ischemia)

survived after ischemia-reperfusion and ketotifen could abrogate the leukocyte adherence induced by ischemia-reperfusion within the villus mucosal capillaries and supplying arterioles and largely prevented pulmonary injury.

The above studies proved that IMMC are associated with the small intestine injury after ischemia-reperfusion, and MC membrane stabilizer pretreatment prior to ischemia can protect against the injury, such as CS and MAR-99. While the studies about the intestinal mucosal injury with CS pretreatment after the small intestine ischemia before reperfusion were few. Oxidative stress is one of the mechanism about the small intestine ischemia-reperfusion injury has been generally acknowledged. We hypothesized that CS have an influence on the oxidative stress during the small intestine ischemia-reperfusion, and the purpose of our present study was to investigate whether CS pretreatment prior to reperfusion could protect against early intestinal mucosal damage induced by ischemia-reperfusion through inhibition of IMMC degranulation or oxidative stress. To test our hypothesis, we showed in a rat IR gut injury model: (1) the ultrastructure and counts of IMMC in the early reperfusion; (2) mucosal damage with CS pretreatment; (3) expression of tryptase in the IMMC; and (4) the levels of malondialdehyde (MDA), TNF- α , histamine and superoxide dismutase (SOD) activity of the small intestine in rats.

MATERIALS AND METHODS

Acute ischemia-reperfusion injury of the intestinal mucosa in rats

Thirty-two healthy Sprague-Dawley rats (200-250 g, provided by Animal Center of Sun Yat-Sen University and approved by the University Animal Study Committee) were randomly divided into four groups each of which contained 8 rats. Laboratory temperature was kept at 25°C-27°C. Surgery was conducted under general anesthesia with intra-peritoneal sodium pentobarbital (45 mg/kg) after they were fasted for 18 h. Tracheotomy was performed for ventilation. The right femoral vein was cannulated for fluid infusion and drugs. The rat abdomen was opened and its superior mesenteric artery (SMA) was found and clamped for 45 min. Then the clamp was released and reperfusion of the splanchnic region was maintained for 60 min (in M group). In control group, SMA was found but not clamped and i.v. saline solution via the right femoral vein at 30th min after the start of experiment (sham group). In another two groups, the same operation was done and CS (50 mg/kg or 25 mg/kg, The dosage and method of CS pretreatment according to Cordeiro *et al*^[11] for Cromolyn sodium is poorly absorbed by oral.) was given via right femoral vein 15 min before the opening of the clamp (C1 and C2 groups).

Preparation of specimens and measurements

After ischemia-reperfusion, the rats were killed and bled rapidly. A segment of 0.5-1.0 cm intestine was cut from 5 cm to terminal ileum and fixed in 4% formaldehyde polymerisatum, then embedded in paraffin for section. Another segment of small intestine was washed with frozen saline and dried with suction paper and at -70°C.

The segment of small intestine was stained with hematoxylin-eosin. The damages of intestinal mucosa were evaluated by two different pathologist according to the criteria of Chiu's method^[12]. Criteria of Chiu grading system consists from 5 subdivisions according to the changes of villus and gland of intestinal mucosa: grade 0, normal mucosa; grade 1, development of subepithelial Gruenhagen's space at the tip of villus; grade 2, extension of the space with moderate epithelial lifting; grade 3, massive epithelial lifting with a few denuded villi; grade 4, denuded villi with exposed capillaries; and grade 5, disintegration of the lamina propria, ulceration and hemorrhage.

Transmission electron microscopy

Intestines were immersed and fixed in 2.5% glutaraldehyde overnight at 4°C and washed three times in PBS. Then they were postfixed in aqueous 1% OsO₄ and 1% K₃Fe (CN)₆ for 1 h. After three times of PBS washes, the tissue was dehydrated through a graded series of 30% to 100% ethanol and 100% propylene oxide and then infiltrated in 1:1 mixture of propylene oxide and Polybed 812 epoxy resin for 1 h. The infiltration solution was changed to 100% resin. After 24 h of infiltration, the tissue was embedded in molds and cured at 37°C overnight, followed by additional hardening at 65°C for 2 d. Ultrathin (70 nm) sections were collected on 200-mesh copper grids and stained with 2% uranyl acetate in 50% methanol for 10 min, followed by 1% lead citrate for 7 min. Sections were photographed using a Hitachi H-600 transmission electron microscope (TOSHI-BA, Japan) at 80 kV onto electron microscope film.

Detection of concentration of protein in intestine

Intestinal tissues were homogenized with normal saline. Intestinal protein quantitation was by the Bradford method^[13] with a BSA standard using kits were provided by Shenerg Biocolor BioScience & Technology Company, Shanghai, China.

Detection of content of MDA in the intestine

Intestinal tissues were homogenized with normal saline. MDA content was determined by the TBA method (Jiancheng Bioengineering Ltd, Nanjing, China). Homogenate (0.1 mL) was taken to detect MDA content. Briefly, 0.1 mL 8.1% SDS, 0.8 mL acetic acid buffer, 0.8 mL 0.8% TBA and 0.2 mL distilled water were added into the sample tubes and one standard tube (containing 0.1 mL tetrathoxypropane). All the tubes were then incubated at 100°C for 1 h. After cooled at -20°C for 5 min, 2 mL of n-butyl alcohol was added into the sample, which was then vibrated for 1 minute and centrifuged for 10 min at 3000 r/min. The supernatant of the samples were assayed to detect absorbance at 532 nm; and the results were expressed as nmol/mL. The content of MDA in intestine was calculated as millimicromole per milligram of protein.

Detection of activity of SOD in the intestine

Intestinal tissues were made into a homogenate with normal saline, frozen at -20°C for 5 min and centrifuged for 15 min at 4000 r/min. Supernatants were transferred into fresh tubes for evaluation of SOD activity. SOD activity was evaluated with an SOD detection kit according to the

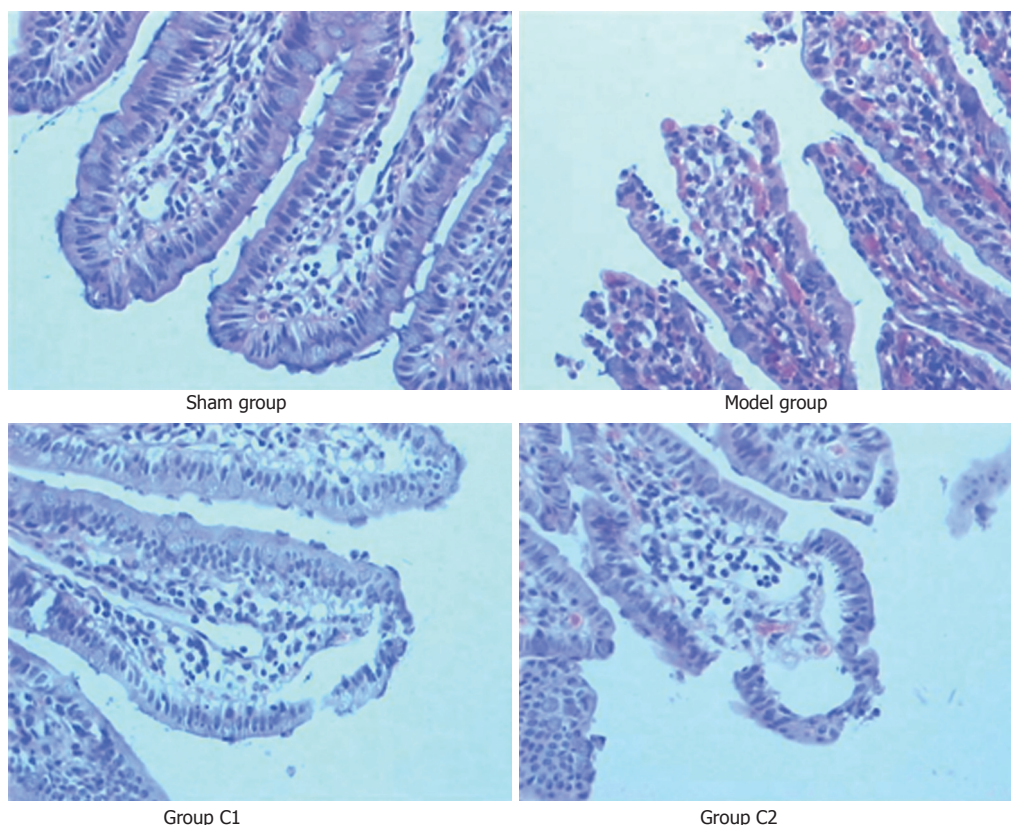


Figure 1 Microscopic appearance after hematoxylin and eosin staining ($\times 200$). In the model group, there are multiple erosions and bleeding in mucosal epithelial layer; the villus and glands were normal and no inflammatory cell infiltration was observed in mucosal epithelial layer in sham group; these mucosal changes are ameliorated by treatment with Cromolyn Sodium (group C1 and group C2).

manufacturer's instructions (Jiancheng Bioengineering Ltd, Nanjing, China). Results were expressed as nmol/mL. The activity of SOD in the intestine was calculated as U per milligram of protein.

Detection of the concentration of $TNF-\alpha$ in the intestine

Intestinal tissues were made into a homogenate with normal saline, frozen at -20°C for 5 min and centrifuged for 15 min at 4000 r/min. Supernatants were transferred into fresh tubes for evaluation of concentration of $TNF-\alpha$ (Biosource, USA) using a commercially available ELISA kit in accordance with the manufacturer's instructions, results were expressed as pg/mL. The concentration of $TNF-\alpha$ in the intestine was calculated as picogram per milligram of protein.

Detection of the concentration of histamine in the intestine

Intestinal tissues were made into a homogenate with normal saline, frozen at -20°C for 5 min and centrifuged for 15 min at 4000 r/min. Supernatants were transferred into fresh tubes for evaluation of concentration of histamine (RapidBio Lab, USA) using a commercially available ELISA kit in accordance with the manufacturer's instructions, results were expressed as ng/mL. The concentration of histamine in the intestine was calculated as nanogram per milligram of protein.

Immunohistochemical detection of tryptase in intestine

Five μm thick sections were prepared from paraffin-embedded tissue. After deparaffinization, endogenous peroxidase was quenched with 3% H_2O_2 in deionised water for 10 min. Nonspecific binding sites were blocked by incubating the sections in 10% normal rabbit serum for 1 h. The sections were then incubated with polyclonal rat anti-mast cell tryptase (dilution 1: 50) for 30 min at 37°C ,

followed by incubation with biotinylated goat-anti-rat IgG at room temperature for 10-15 min. After 3×5 min PBS rinses, the horseradish-peroxidase-conjugated streptavidin solution was added and incubated at room temperature for 10-15 min. The antibody binding sites were visualized by incubation with a diaminobenzidine- H_2O_2 solution. The sections incubated with PBS instead of the primary antibody were used as negative controls. Brown-yellow granules in the cytoplasm were recognized as positive staining for tryptase. We calculated the tryptase positive mast cells and their intensity in 5 representative areas at $\times 400$ magnification by Image-Pro Plus 5.0 (USA).

Statistical analysis

Data were expressed as mean \pm SD and analysis of variance was performed using SPSS 11.0 software. One-way analysis of variance was used for multiple comparison, least significant difference test (LSD-t) was used for intra-group comparison or Tamhane's T2 test was used if equal variances was not assumed. Pearson analysis was used for the correlation in the ischemia and reperfusion groups. Differences were considered significant when P was < 0.05 .

RESULTS

Changes of intestinal mucosa under light microscope

The villus and glands were normal and no inflammatory cell infiltration was observed in mucosal epithelial layer in sham group. Multiple erosions and bleeding were observed in model group. Light edema of mucosa villus and infiltration of few necrotic epithelial inflammatory cells neutrophil leukomonocyte were found in mucosa epithelial layer in C2 and C1 groups (Figure 1).

Table 1 Changes of IMMC counts, expression of tryptase, and Chiu's score in small intestine in various groups (mean \pm SD)

Group	n	IMMC (n/field)	Expression of tryptase	Chiu's score
S	8	10 \pm 2	126 \pm 4	0.4 \pm 0.5
M	8	25 \pm 8 ^b	173 \pm 4 ^b	4.9 \pm 0.4 ^b
C1	8	15 \pm 2 ^a	138 \pm 3 ^{b,d}	2.4 \pm 0.5 ^{b,d}
C2	8	17 \pm 2 ^a	155 \pm 8 ^{b,d,f}	2.9 \pm 0.4 ^{b,d,e}

^a $P < 0.05$, ^b $P < 0.01$ vs Group S; ^d $P < 0.01$ vs Group M; ^e $P < 0.05$, ^f $P < 0.01$ vs Group C1.

Chiu's score of small intestinal structure

The Chiu's score in sham group was the lowest, while in the model group it was the highest in the four groups ($P < 0.05$). The Chiu's score in C1 group was significantly lower than in C2 group after treated with CS ($P < 0.05$) (Table 1).

Changes of ultrastructure of small intestinal

The ultrastructure of small intestinal was normal in group S. There was seen the karyopyknosis of epithelial cell of small intestine in group M, the nuclear membrane was more irregularity, and the swelling microvillus became shorter and thicker, most of the microvillus were shedding. The nucleus of epithelial cell of small intestine in group C1 and C2 was deflated, the nuclear membrane was irregularity, and the light swelling microvillus became shorter (Figure 2).

Changes of ultrastructure of IMMC

The ultrastructure of IMMC was normal in sham group. There were abundant vacuolus with a reduction granulation in their endochylema in model group. There were few swollen granules with a reduction in IMMC homogeneity in C1 and C2 group (Figure 3).

Changes of MDA in small intestine

The content of MDA in intestine of model group was the highest in all experimental groups, it decreased significantly compared with the model group after treated with CS ($P < 0.05$) and there was no significant difference compared with the sham group ($P > 0.05$) (Table 2).

Changes of activity of SOD in small intestine

The activity of intestinal SOD decreased significantly in ischemia-reperfusion injury groups compared with the sham group ($P < 0.05$), treated with CS it increased significantly compared with the model group ($P < 0.05$), and there was no significant difference between group C1 and C2 ($P > 0.05$) (Table 2).

Changes of TNF- α in small intestine

The concentration of TNF- α of intestine in model group rats was higher than the other three groups ($P < 0.05$). There were no significant difference in sham group, C1 group and C2 group ($P > 0.05$) (Table 2). There was a positive correlation between the Chiu's score and the concentration of TNF- α in the ischemia and reperfusion groups ($r = 0.734$, $P < 0.05$).

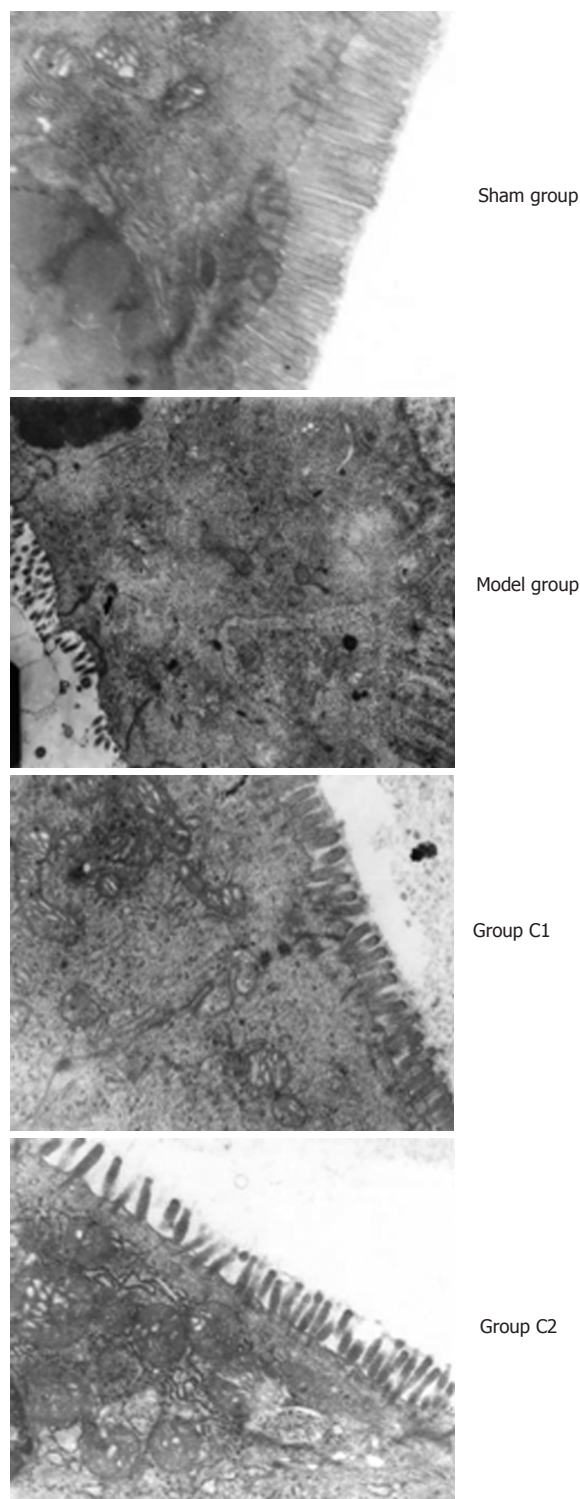


Figure 2 Changes of ultrastructure of small intestinal in each group ($\times 10000$). The ultrastructure of small intestinal was normal in group S. There were seen the karyopyknosis of epithelial cell of small intestine in group M, and the nuclear membrane was more irregularity, and the swelling microvillus became shorter and thicker, most of the microvillus were shedding. The nucleus of epithelial cell of small intestine in group C1 and C2 was deflated, the nuclear membrane was irregularity, and the microvillus became shorter and light swelling.

Changes of histamine in small intestine

The histamine concentration of intestine in the model and C2 groups decreased significantly compared with the sham group ($P < 0.05$), it increased significantly after pretreated with CS compared with the model group ($P < 0.05$). There

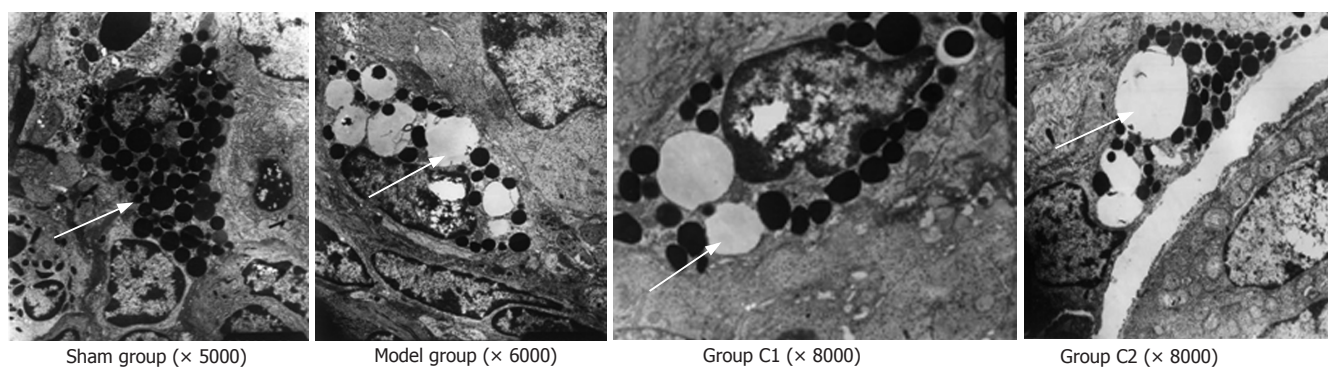


Figure 3 Ultrastructure of intestinal mucosal mast cells of rats in each group. There are abundant vacuolus with a reduction of granulation in their endochylema in model group; there are filled with granulation endochylema and there is no vacuolus in their endochylema in sham group, these changes of ultrastructure are ameliorated by treatment with Cromolyn Sodium (group C1 and C2).

Table 2 Changes of TNF- α , MDA content, SOD activity and histamine concentration in small intestine in various groups (mean \pm SD)

Group	n	TNF- α (pg/mg protein)	MDA (nmol/mg protein)	SOD (U/mg protein)	Histamine (ng/mg protein)
S	8	3.7 \pm 0.4	0.44 \pm 0.09	179.2 \pm 15.3	5.7 \pm 0.5
M	8	4.7 \pm 0.4 ^b	0.66 \pm 0.07 ^b	130.6 \pm 10.6 ^b	4.2 \pm 0.4 ^b
C1	8	3.8 \pm 0.4 ^d	0.45 \pm 0.06 ^d	147.9 \pm 12.4 ^{b,c}	5.3 \pm 0.6 ^d
C2	8	3.9 \pm 0.4 ^d	0.47 \pm 0.07 ^d	145.3 \pm 15.7 ^{b,c}	4.8 \pm 0.6 ^{a,c}

^a $P < 0.05$, ^b $P < 0.01$ vs Group S; ^c $P < 0.05$, ^d $P < 0.01$ vs Group M.

were no significant difference between C1 and C2 groups ($P > 0.05$) (Table 2). The Chiu's score and MDA content were negatively correlated to the histamine concentration respectively ($r = -0.676$, $P < 0.05$ or $r = -0.452$, $P < 0.05$), while the SOD activity was positively correlated to the concentration of histamine in the ischemia and reperfusion groups ($r = 0.579$, $P < 0.05$).

Counts and expression of tryptase of IMMC

Expression of tryptase in sham group was the lowest, while in the model group it was the highest in the four groups ($P < 0.05$), and the expression of tryptase in C1 group was significantly lower than in C2 group ($P < 0.05$). The number of IMMC increased significantly in ischemia-reperfusion injury groups compared with the sham group ($P < 0.05$), no difference was compared among the three groups ($P > 0.05$) (Table 1, Figure 4).

DISCUSSION

IMMC are located in close proximity to submucosal collecting venules, which are primary targets of leukocyte-endothelial interactions during ischemia-reperfusion injury. IMMC are particularly frequent in close proximity to epithelial surfaces where they are strategically located for optimal interaction with the environment and for their putative functions for host defense. They sense the foreign material invading the mucosa in an appropriate inflammatory response, and were considered as one of components of the fourth level of mucosal defense^[14]. Acute inflammation could lead to increase of IMMC counts and release of a multi-faceted spectrum of proinflammatory mediators by IMMC such as cytokines and chemokines, and MC

have the capacity to coordinate trafficking of leukocytes^[15]. Boros^[16] proved that intestinal ischemia induced the release of a variety of IMMC-derived inflammatory compounds and resulted in a spectrum of injury ranging from reversible permeability changes to structural mucosal damage.

The ischemia time of small intestine rats' model is from 30 min to 60 min^[17-19], here we used the median time (45 min). Cizova reported that the concentration of thio-barbituric acid reactive substances was increased at the end of the ischemia lasting from 30 to 90 min^[20], and CS plasma life *in vivo* is very short. Thus the reperfusion time in our study was watched in 60 min, it was the early reperfusion according to Hamar *et al*^[21]. All of previous studies were focused on the MC membrane stabilizer pretreatment prior to ischemia, and had proved that IMMC were associated with the damage to intestinal mucosal after the small intestine ischemia-reperfusion. While the main purpose of our study was to see whether pretreatment with CS prior to reperfusion also have the protective effects during early reperfusion after the small intestine ischemia.

Tryptase is one of the specificity markers of IMMC^[22]. We counted the IMMC counts through the expression of tryptase using immunohistochemical methods which is more accuracy than oluidine blue staining. Our study found that the expression of tryptase and IMMC counts increased significantly in 60 min reperfusion injury in model group. There were abundant vacuolus in IMMCs in the model group after they were degranulated by electron microscope. IMMC is the main source of histamine in intestine. The levels of intestinal histamine includes the concentration of histamine intra- and extro-IMMC. The level of intestinal histamine is mainly represent of the concentration of histamine intra-IMMC as the extracellular

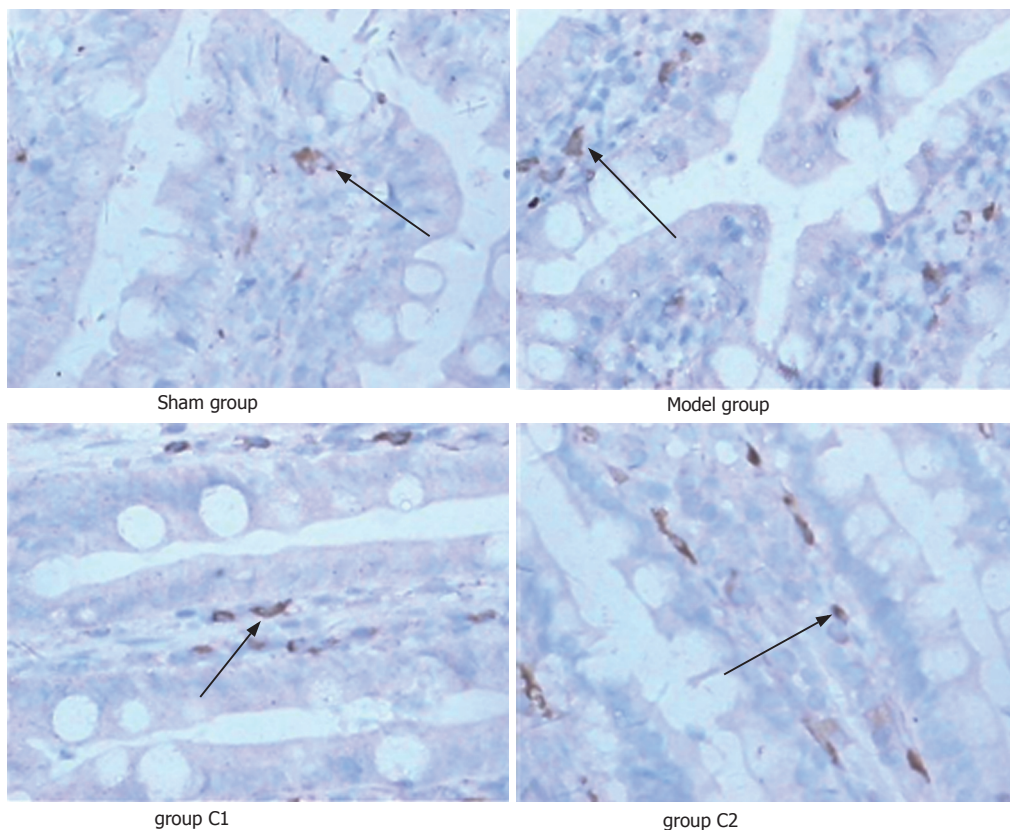


Figure 4 Immunohistochemical detection of tryptase in small intestinal of rats in each group ($\times 400$). Expression of tryptase and number of IMMC are increased in the model group; these changes were ameliorated by treatment with Cromolyn Sodium (group C1 and C2).

histamine released from IMMC in the gastrointestinal tract is rapidly cleared and degraded^[23] and the more IMMC degranulate, the lower concentration of histamine is found in intestine^[24]. Our study found the histamine level decreased significantly in model group while IMMC counts increased, suggesting that IMMC may degranulate and release histamine in 60 min reperfusion.

Histamine has many pathophysiological roles, and it is an important messenger in the gut^[25]. Akerstrom *et al*^[26] reported that anti-histaminergic pretreatment could decrease the trauma-induced leakage of albumin by mechanisms which may involve readjustments of pressures and flows in capillaries as well as a prevention of histamine effects on capillary permeability on a model of mechanical intra-abdominal trauma in rats. Our results found that the intestinal histamine concentration decreased after early ischemic-reperfusion, and there was a negative correlation between the small intestinal Chiu's score and the level of histamine in the intestine. This result suggested that histamine took part in the ischemia-reperfusion intestinal mucosal damage.

TNF- α is an inflammatory cytokine that may be an important mediator in the development of reperfusion-induced tissue injury and lethality^[27]. Grewal^[28] demonstrated that treatment of rats with anti-TNF antibodies could prevent neutrophil influx, tissue injury. Our study found that the intestinal TNF- α concentration increased after ischemic-reperfusion; and there was a positive correlation between the small intestinal Chiu's score and the level of TNF- α in the intestine. This result suggested that TNF- α also took part in the ischemia-reperfusion intestinal mucosal damage. Although the most intestinal TNF- α is considered by some one from the mast cells^[29], it has been

proved that many sorts of cells also release the TNF- α besides the mast cells. We believe the increase of the TNF- α is contributed by many factors.

CS is a stabilizing agent of mast cell which prevents histamine and TNF- α released from IMMC^[30]. Szabo *et al*^[31] reported that 30 min segmental ischemia and 120 min reperfusion induced significant tissue injury, elevated the segmental vascular resistance, and decreased intramucosal pH (pHi), and CS pretreatments prior to ischemia significantly inhibited the permeability changes, but did not influence the pHi and morphological alterations induced by ischemia-reperfusion, they conclude that intestinal mast cells and mast cell-induced reactions contribute to the mucosal permeability alterations during reperfusion, but play only a minor role in ischemia-reperfusion-induced structural injury. Pretreatment with CS protecting against degranulation, caused a significant impairment of plasma exudation at 30 min of inflammation corresponding to a significantly decreased level of histamine, one of the most potent vasoactive factors released from activated mast cells^[32].

The results in our study showed that the injury of small intestinal villus and microvillus was alleviated after CS pretreatment prior to reperfusion and that the ultrastructure of IMMC was basically normal. The expression of tryptase and TNF- α concentration were also alleviated by CS pretreatment prior to reperfusion, and the concentration of histamine in intestine was increased compared with the model group after CS pretreatment prior to reperfusion. The results indicated that CS decreased ischemia-reperfusion injury by prevention of IMMC degranulation, thus it decreased the release of histamine and TNF- α . This protection may be dose-dependent as high dose of CS with

more powerful effect.

There were many reports about intestinal ischemia and reperfusion resulted in the increase of MDA and decrease of SOD activity, toxic-free oxygen radicals are produced in the ischemic tissue^[33,34]. Our study also demonstrated that ischemia-reperfusion injury elevated the oxygen radicals and lipid radicals. Frossi^[35] reported that oxidative stress could induce a pro-type 2 inflammatory response and degranulation of mast cells. Fukuishi^[36] found the compound 48/80, a typical histamine liberator elicited superoxide anion generation in mast cells in a dose-dependent fashion. These studies indicated that degranulation of mast cells was able to induce oxidative stress injury and oxygen radicals could make mast cells to degranulate. In this study we found there were correlations among the MDA, SOD activity and the concentration of histamine, the other findings of our study were that MDA content increased and SOD activity decreased remarkably in the model group, while pretreatment by cromolyn sodium prior to reperfusion could attenuate the up-regulation of MDA content and the down-regulation of SOD activity. The results were indicating that IMMC degranulation and oxidative stress can affect each other, and the less IMMC degranulation can make less oxidative stress. Future studies are need to focus on the relationships *in vitro*.

In conclusion, pretreatment of Cromolyn Sodium prior to reperfusion could attenuate early reperfusion injury after the small intestine ischemia in rats. The mechanisms includes: inhibited IMMC from degranulation, decreased the release of histamine and TNF- α from IMMC, and decreased oxidative stress.

COMMENTS

Background

Intestinal mucosal mast cells (IMMCs) is associated with the mucosal damage. The aim of this study was to investigate the effects of Cromolyn Sodium (CS) pretreated prior to reperfusion on the activity of IMMC and mucous membrane of the small intestine in ischemia-reperfusion (IR) injury of rats.

Research frontiers

Previous studies proved that IMMC are associated with the small intestine injury after ischemia-reperfusion, and MC membrane stabilizer pretreatment prior to ischemia can protects against the injury, such as CS and MAR-99.

Innovations and breakthroughs

While the studies about the intestinal mucosal injury with CS pretreatment after the small intestine ischemia before reperfusion were few. Oxidative stress is one of the mechanism about the small intestine ischemia-reperfusion injury has been generally acknowledged. We hypothesized that CS have an influence on the oxidative stress during the small intestine ischemia-reperfusion, and the purpose of our present study was to to investigate whether CS pretreatment prior to reperfusion could protect against early intestinal mucosal damage induced by ischemia-reperfusion through inhibition of IMMC degranulation or oxidative stress.

Applications

Pretreated of CS prior to reperfusion protects the small intestine mucous from ischemia-reperfusion damage, the mechanism is inhibited IMMC from degranulation.

Terminology

Restoration of blood supply to tissue which is ischemic due to decrease in normal blood supply. The decrease may result from any source including atherosclerotic obstruction, narrowing of the artery, or surgical clamping. It is primarily a procedure

for treating infarction or other ischemia, by enabling viable ischemic tissue to recover, thus limiting further necrosis. However, it is thought that reperfusion can itself further damage the ischemic tissue, causing REPERFUSION INJURY.

Peer review

This paper reports an experimental study very well designed and performed and very elegant results and discussion. The final conclusions are nicely shown. Their english is of good quality. The purpose of this paper was to determine whether cromolyn sodium reduces or prevents injury of the small intestine of rats following ischemia-reperfusion. To this end the authors perform a number of biochemical measurements (MDA, TNF α , histamine, SOD) as well as ultrastructural studies of mast cells and microscopical investigations of the small intestine.

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Wilson disease: Identification of two novel mutations and clinical correlation in Eastern Chinese patients

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Abstract

AIM: To study mutations in the P-type ATPase (ATP7B) gene responsible for Wilson disease (WD) in the Eastern Chinese population, and the possible correlation of specific mutations with clinical characteristics.

METHODS: Mutations of the ATP7B gene were sought by means of direct sequencing in 50 Eastern Chinese WD patients of Han ethnic origin.

RESULTS: Two novel mutations, Asp96Gly and Asp196Glu, were first identified. We also compared the characterization of mutations in ATP7B with the clinical findings, and a significant correlation with hepatic manifestations between patients carrying the Arg778Leu mutation and those without was found.

CONCLUSION: Gene sequencing analysis was shown to have a high detection rate and accuracy. It may become the first priority in screening of WD patients.

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Key words: Wilson disease; ATP7B gene; Mutations; Polymorphisms

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INTRODUCTION

Wilson disease (WD, hepatolenticular degeneration) is an autosomal recessive disorder with an incidence of 1 in 35 000 to 100 000 live births^[1-3]. It is characterized by pathological copper accumulation in different tissues, especially in the liver and brain. As a result of defective putative copper-transporting ATPase in the liver, copper remains in the liver and causes hepatic dysfunction. The clinical presentation of WD consists of hypoceruloplasminemia, presence of Kayser-Fleischer (KF) rings, and hepatic, psychiatric and/or neurological disturbance^[4]. Treatment of WD has progressed from chelation therapy using D-penicillamine and trientine to the more recent use of zinc, and finally to liver transplantation for fulminant presentation^[5]. Timely diagnosis and treatment may protect patients from severe organ damage. The diagnosis of WD is determined by clinical presentation and laboratory testing for KF rings, hepatic injury and low serum ceruloplasmin. However, some asymptomatic patients do not receive effective treatment before irreversible injury is present. Genetic diagnosis may detect presymptomatic patients, in whom initiation of prophylactic therapy can effectively prevent the otherwise inevitable hepatic and neurological injury^[6,7].

Mutations in the P-type ATPase (ATP7B, MIM#277900) gene are responsible for WD. The ATP7B gene, which was identified in 1993, is located on chromosome 13q14.3, which spans a genomic region of ~80 kb. It comprises 21 exons and encodes for a P-type copper-transporting ATPase^[8-10]. Direct mutation analysis has been performed in many WD patients and > 200 different ATP7B mutations have been detected^[11-13]. H1069Q is the most common type of mutation, with an allelic frequency of 10%-40% in European patients^[14-16]. However, Arg778Leu in exon 8 is the most frequently observed mutational type in Chinese, Japanese and Korean patients^[17-22]. It has been reported that the Arg778Leu mutation may be correlated with hepatic manifestations in Chinese patients^[12]. However, the mutations in Chinese patients with WD, and the possible correlation of specific mutations with clinical characteristics, have not been addressed.

In the present study, mutations of the ATP7B gene were sought by means of direct sequencing in 50 Eastern Chinese WD patients of Han ethnic origin, who comprise 99% of the population of mainland China. We also

compared the characterization of mutations in ATP7B with the clinical findings, and report the preliminary results of the genotype/phenotype correlation.

MATERIALS AND METHODS

Subjects

A total of 50 unrelated Han ethnic subjects (24 female, 26 male) in mainland Eastern China were diagnosed as being affected by WD. They were mainly from Zhejiang, Anhui and Fujian Provinces. They were recruited when they came to the hospital for seeking assistance. The diagnosis was based on the presence of hepatic disturbance, typical neurological symptoms, KF rings, biochemical tests (low serum concentrations of ceruloplasmin and copper, and high urinary and hepatic copper content). Each of these patients had a score of at least 3 according to a scoring system based on clinical and biochemical parameters^[23]. DNA samples from 100 healthy Chinese individuals (50 female, 50 male) were screened to determine whether the missense/splicing mutations identified in this study were present in the normal population. The healthy subjects were mainly students in our teaching hospitals. Informed consent was obtained from all patients or their parents before inclusion in the study.

PCR and DNA sequencing

Anticoagulated blood samples were obtained from the WD patients. Genomic DNA was isolated from peripheral blood lymphocytes by a DNA extractor kit (Qiagen, Germany). All 21 exons of ATP7B were amplified by polymerase chain reaction (PCR). PCR was performed in a 50- μ L reaction volume containing 800 ng genomic DNA, 2.5 U Taq polymerase (Takara, Japan), 12.5 pmol each primer, 3 mmol/L MgCl₂, 200 μ mol/L each dNTP, and 1 \times PCR Buffer (Takara, Japan). The PCR reaction conditions were as follows: 15 min initial activation step at 94°C; 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 120 s; and a final extension at 72°C for 10 min. PCR products were visualized on 2% agarose gel containing ethidium bromide, and subsequently purified using QIAEX II (Qiagen, Germany). Direct sequencing was performed using an ABI 377 fluorescent sequencer (Applied Biosystems, Foster City, CA, USA).

Analysis of genotype-phenotype correlations

We analyzed the correlation between Arg778Leu genotype and WD phenotype, including age of onset and initial symptoms. Data were analyzed using SPSS 13.0. Age of onset of symptoms was compared using Student's *t* test. Categorical variables were compared between groups by the χ^2 test or Fisher's exact test. Differences were considered to be significant at the $P < 0.05$ level.

RESULTS

Mutation analysis

DNA from the 50 patients with WD was screened for mutations. Exons 1-21 were PCR-amplified and the products were sequenced bidirectionally. Mutations in

ATP7B were found in at least one of the alleles in all the patients with WD, distributed throughout the gene. In eight patients, only one allelic change was detected, which suggests that the other one might have been located in the introns or regulatory regions. We identified five different mutations (Table 1). Two different mutations found in exons 8 and 13 account for about 77% of all 100 WD alleles studied, thereby indicating that these exons are important regions for detecting mutations in Eastern Chinese patients with WD. The most frequent mutation, Arg778Leu, was found in 32 (64%) patients, in at least one allele. The frequency of the other most prevalent mutation Pro992Leu was 27%. In the copper-binding domain, we found two novel mutations: one non-conservative, which replaced a highly conserved acidic amino acid with a small non-polar amino acid (Asp96Gly); and the other conservative (Asp196Glu), but lying at a highly conserved position in Menkes disease and other copper-transporting ATPases. The two novel mutations were both missense mutations, and the nucleotide changes were not found in the 100 normal individuals. The novel features of these mutations were identified according to the database that is maintained by the University of Alberta (<http://www.medicalgenetics.med.ualberta.ca/wilson/index.php>).

DNA polymorphism

Seven DNA sequence polymorphisms were identified (Table 2). DNA sequence polymorphisms were those nucleotide changes that either did not modify the amino acid sequence of the polypeptide, resulted in conservative changes in the amino acid residues, or that were found in normal chromosomes or chromosomes with known disease-causing mutant alleles. In order to clarify these nucleotide and amino acid sequence changes were mutations or polymorphisms, we analyzed 100 normal individuals with direct sequencing of their PCR products. The nucleotide changes present in those 100 normal individuals were considered to be polymorphisms, and those which were not present, were considered to be mutations.

Genotype-phenotype correlations

A total of 50 patients with WD were analyzed. The mean age of patients at disease manifestation was 8.9 ± 1.9 years (median, 8 years; range, 4-13 years). Thirty patients (60%) had a primary hepatic manifestation, 13 showed a primary neurological manifestation, and five had combined hepatic and neurological manifestations. Two patients (4%) identified by family screening had no symptoms; they were classified as asymptomatic, and were not included in analysis of genotype-phenotype correlations.

We identified 18 homozygotes and 14 heterozygotes for Arg778Leu. In the 32 patients carrying Arg778Leu, 25 (78.1%) had hepatic manifestations (age of onset, 8.1 ± 1.7 years). There were a total of 18 patients (36%) without the Arg778Leu mutation, 10 (55.6%) with hepatic manifestations (age of onset, 9.2 ± 2.3 years). The Arg778Leu homozygous patients were not significantly younger at the time of symptom onset (8.5 ± 2.1 years), compared with compound heterozygotes (7.9 ± 1.8

Table 1 Mutations identified in WD chromosomes

Mutation	Nucleotide change	Exon	Predicted effect	Frequency of WD alleles (%)	Frequency of WD alleles in Caucasian patients (%)
Asp96Gly	287A>G	2	Disrupts Cu 1	8	novel
Asp196Glu	588C>A	2	Disrupts Cu 2	2	novel
Arg778Leu ¹	2333G>T	8	Disrupts TM4	50	2
Pro992Leu ²	2975C>T	13	Disrupts Ch/TM6	27	2
Val1216Met ³	3646G>A	17	Disrupts ATP binding	3	< 1

¹Mutation previously described^[5]. ²Mutation previously described^[1]. ³Mutation previously described^[1].

Table 2 Polymorphism identified at the ATP7B locus

Polymorphism	Nucleotide change	Exon	Frequency (%)	
			Healthy individuals (n = 100)	Patients (n = 50)
-75 A > C ¹		5'UTR	38	10
-123 del CGCCG ¹		5'UTR	10	25
S406A ¹	1216 T>G	2	35	30
V456L ¹	1366 G>C	3	36	22
L770L ²	2310 C>G	8	2	50
K832R ²	2495 G>A	10	27	30
S1166S ³	3498 C>T	16	19	20

¹Polymorphism previously described^[12]. ²Polymorphism previously described^[12]. ³Polymorphism previously described^[13].

years). However, the number of Eastern Chinese patients with hepatic manifestations among those carrying the Arg778Leu mutation was significantly greater than that among those without the mutation ($\chi^2 = 5.26$, $P < 0.05$).

DISCUSSION

Mutation analysis is important in the early diagnosis of patients with a family history of WD, as well as in prenatal diagnosis. Here, we report a group of 50 subjects affected with WD, analyzed by direct sequencing of the entire coding sequence of ATP7B gene. We found mutations in 50 patients. The detection rate of mutation was 92%. Seventy-seven percent of the mutations detected were lying in exons 8 and 13. According to this study, we recommend screening of exons 8 and 13 by sequence analysis. Mutations in exons 14 and 18, which have been found to have a high frequency among Caucasian patients^[24-27], were not detected in our study. As reported previously^[12], Arg778Leu was the most common WD chromosomal mutation detected in the present study (50%). However, Arg778Gln was not detected in our study, which is similar to the results of previous research in Shanghai^[12]. The unusual high frequency of codon 778 mutation may have been due to sampling disequilibrium or to the presence of a founder effect among the Eastern Chinese population. Pro992Leu was the second most frequent allele, with a frequency of 27%. The above-mentioned two mutations accounted for ~80% of all mutations detected in our study. In eight patients, only one allelic change was detected, which suggests that the other change might be located in the introns or regulatory regions. Incomplete assessment of intronic and regulatory sequences may have

accounted for the second unidentified mutation.

We could not find any significant difference between Arg778Leu homozygosity and heterozygosity with regard to the mean age of onset of symptoms, although Wu *et al.*^[7] have reported that the average age of onset in 18 Chinese homozygotes was significantly lower than that in 11 Chinese compound heterozygotes for Arg778Leu. However, the number of patients with hepatic manifestations among those carrying Arg778Leu mutation was significantly greater than that among patients without the mutation. Caca *et al.*^[28] have reported that symptoms in most His1069Gln homozygotes started between 16 and 25 years of age. In our study, symptoms in most Arg778Leu homozygotes (15/18) started before 10 years of age, which suggests that the age of onset in Arg778Leu homozygotes in Eastern Chinese patients is earlier than that in His1069Gln homozygotes in East German patients. His1069Gln is the most common WD mutation found in the European population^[29-31], but it was not detected in our study in Eastern Chinese patients.

Molecular diagnosis of pre-symptomatic WD patients is important in the control of disease progression and treatment. Mutation detection in WD is challenging because of the presence of a large number of mutations in a 4.4-kb coding region in 21 exons spread over 80 kb of genomic DNA. The mutation detection rate among the WD chromosome was > 90% in our study. This gene sequencing analysis method was shown to have a high detection rate and accuracy. It may become the first priority in the screening of WD patients.

COMMENTS

Background

Direct mutation analysis has been performed in many WD patients, and more than 200 different ATP7B mutations have been detected.

Research frontiers

The mutations in the Chinese population with WD, and the possible correlation of specific mutations with clinical characteristics have not been investigated. In the present study, mutations of the ATP7B gene were sought by means of direct sequencing in 50 Eastern Chinese patients of Han ethnic origin with WD.

Innovations and breakthroughs

Two novel mutations were identified in our normal individuals. The novel features of the mutations were identified according to a database that is maintained by the University of Alberta.

Peer review

It is an interesting manuscript. The first concern is on the study population. The

subjects were from mainland Eastern China, which covers a large area. More specific information on subject source, which clinic they attended, and conditions under which they were recruited would be helpful.

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Sirolimus-related pulmonary toxicity mimicking 'asthma like' symptoms

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Abstract

Sirolimus is an immunosuppressant with expanding use in pediatric organ transplantation, dermatology and rheumatology. We report two cases of children who developed asthma like symptoms and were diagnosed with interstitial lung disease, which responded to discontinuation of sirolimus. Pediatricians should be aware about the pulmonary side effects of sirolimus.

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Key words: Sirolimus; Pulmonary toxicity; Interstitial lung disease; Asthma; Small bowel transplantation; Intestinal transplantation; Organ transplantation

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INTRODUCTION

Sirolimus (rapamycin) is a macrolide immunosuppressant with increasing use in various pediatric subspecialties^[1,2]. Its dose-related side effects leading to thrombocytopenia and hypercholesterolemia are widely known. Pulmonary toxicity associated with sirolimus therapy has only been recently recognized as a potentially serious complication^[3]. Among the twenty-eight children (18 with liver transplant and 10 with small bowel transplant) treated with sirolimus in our center, two developed pulmonary complications which are described in this case report.

CASE REPORT

Case 1

A 2-year-old girl with microvillous inclusion disease underwent combined small bowel and liver transplantation (CSBLTx). She received tacrolimus and prednisolone according to our immunosuppression protocol. She developed severe rejection at the age of 2 years due to poor compliance with medications. Sirolimus (0.1 mg/kg per day) was added to her immunosuppression regime aiming at levels of 8-10 ng/mL for both tacrolimus and sirolimus.

Four months after starting sirolimus treatment, she developed a perforation near the anastomotic site of the intestinal allograft and sirolimus was stopped. She remained generally well in the next year, but in view of deteriorating renal function, and persistent hypomagnesaemia, the tacrolimus dose was halved (target level 3-5 ng/mL) and sirolimus was added (target level 8-10 ng/mL). Two months after commencing sirolimus she acquired adenovirus infection which required mechanical ventilation and reduction of immunosuppression (target level of tacrolimus and sirolimus 3-5 ng/mL). After the reduction of immunosuppression, she showed an uneventful recovery.

During the next 5 mo (now 4 years and 9 mo after transplantation), in which sirolimus was re-introduced, she developed a dry cough and increased respiratory rate with mild subcostal recession. Her clinical examination was otherwise unremarkable. Initially she had no response to bronchodilators for possible asthma. Pulmonary function tests were normal. Her chest X-ray showed some loss of volume in the left lower lobe and coarse interstitial shadowing throughout both lung fields. Immunofluorescence and viral culture of nasopharyngeal aspirates and broncho-alveolar lavage (BAL) including adenovirus were all negative. The BAL fluid grew haemophilus influenzae, which was treated with amoxicillin. Blood cultures for fungi and bacteria were negative, as was PCR for CMV and EBV. A high resolution CT scan of the chest demonstrated fine nodular changes in the interstitial air spaces. A lung biopsy was considered but deferred. Sirolimus-associated pulmonary toxicity was suspected and sirolimus was discontinued. Her respiratory symptoms of cough and dyspnea improved within 3 mo and her chest X-ray changes resolved within 6 mo.

Case 2

A 9-mo-old boy with Hirschsprung disease with



Figure 1 Chest X-ray showing bilateral interstitial shadowing and nodularity.

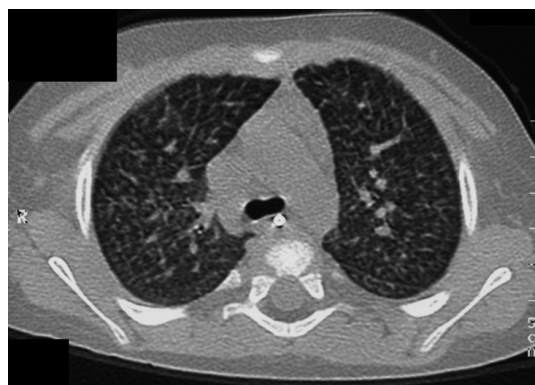


Figure 2 CT scan of the chest showing thickening of the interlobular septae with fine nodular changes.

total aganglionosis extending proximally to the pylorus underwent CSBLTx. He was commenced on tacrolimus and prednisolone according to our standard immunosuppression protocol. An episode of moderate acute rejection 10 d after transplant was treated with a single dose of steroids and two doses of basiliximab four days apart. Sirolimus (0.14 mg/kg) was commenced 6 wk after transplant. The aim was to maintain tacrolimus and sirolimus levels at 8-10 ng/mL and 6-8 ng/mL, respectively. Five months after commencing sirolimus, he showed features of bone marrow suppression with neutropenia and anemia. A bone marrow aspirate showed myeloid maturation arrest and responded well to weekly injection of granulocyte colony stimulating factor for 3 mo and supportive treatment.

In view of bone marrow suppression, the dose of sirolimus was reduced to maintain a level 4-6 ng/mL. Seven months after introduction of sirolimus, he presented with a persistent dry cough and breathlessness on exertion. Respiratory examination was normal. Chest X-ray showed bilateral interstitial shadowing and nodularity (Figure 1), but overnight pulse oximetry observation was normal. A comprehensive evaluation for post-transplant lymphoproliferative disease (PTLD), including biopsies from upper GI endoscopy, liver biopsy, and ileoscopy was negative. EBV PCR titer was low with a level of 1000 genome copies/mL. Investigations for respiratory infection, including BAL, revealed no infection and special staining for pneumocystis carinii pneumonia, acid-fast bacilli staining and immunochemistry for cytomegalovirus and Epstein-Barr virus were negative. A high resolution CT scan revealed thickening of the interlobular septae with fine nodular changes (Figure 2). A lung biopsy was considered and deferred. Following discontinuation of sirolimus, his symptoms resolved within 4 wk, but radiological changes were not resolved after 18 mo of follow-up.

DISCUSSION

The resolution of clinical symptoms and improvement in radiological changes after withdrawal of sirolimus in our patients, with the absence of other infectious factors, strongly implicates that sirolimus is the causative factor for the pulmonary changes.

Sirolimus-associated pulmonary toxicity is mostly described in the adult literature and actually represents a spectrum of clinico-pathologic syndromes characterized clinically by dyspnea, cough, fever, fatigue or haemoptysis, and histologically by the presence of organizing pneumonia, interstitial pneumonitis, focal fibrosis or by the presence of alveolar haemorrhage^[4]. In the largest report on sirolimus-associated pulmonary toxicity in adult kidney transplant recipients, features of pneumonitis were seen within 6-12 mo after commencing sirolimus therapy^[3]. Our patients showed a similar time course.

The children in this report exhibited 'asthma like' symptoms with recurrent episodes of dry cough and breathlessness on exertion. In children on immunosuppressive drugs, the diagnostic challenge is to rule out opportunistic infections. Lung biopsy looking for any histological changes was not performed due to the invasive nature of the procedure and resolution of the symptoms and radiological changes on cessation of sirolimus. A review of drug history did not identify any other medicines in the complex cases, which could give rise to the 'asthma' like symptoms.

The management consists of excluding other etiologies, especially opportunistic infections, and discontinuation or dose reduction of sirolimus^[3]. As in the previous reports, respiratory symptoms resolved within 2-4 wk after cessation of sirolimus therapy with improvement of CXR changes in 6-18 mo, which is consistent with other case reports^[3,5]. We opted to stop the treatment as the long-term outcome of interstitial lung changes in children is not known. It is possible that the degree of reversal of pulmonary symptoms depends on the extent and the chronicity of parenchymal and interstitial changes. The children reported were on low dose steroids as a part of their immunosuppression regime and developed interstitial lung disease despite being on steroids.

The exact pathogenic mechanism of sirolimus-induced pulmonary toxicity is not known. Possible mechanisms include idiosyncratic cell-mediated autoimmune response due to the exposure of cryptic antigens and T cell-mediated delayed type hypersensitivity reaction^[3,6]. Of the 28 children (10 with small bowel transplant, 18 with liver transplant) treated with sirolimus in the liver unit at BCH, two children with small bowel transplant developed the

changes, while none of the children with liver transplants developed this complication. It is entirely possible that the complication may be related to the higher intensity of immunosuppression used in the children with intestinal transplantation. However, a simple dose-dependant toxicity reaction seems less likely since other dose-dependent side effects such as thrombocytopenia or hypercholesterolemia were absent. Morath C *et al*^[7] have documented that an increase in sirolimus levels 3 wk prior to the onset of symptoms and an older age are the risk factors for developing interstitial lung disease. Similar observations could not be made from our two cases.

In conclusion, with the expanding use of sirolimus in children, the appearance of persistent respiratory symptoms, especially cough and dyspnea, should alert the pediatrician to the possibility of sirolimus-associated pulmonary toxicity and the drug may have to be discontinued.

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CASE REPORT

Upper gastrointestinal bleeding from duodenal vascular ectasia in a patient with cirrhosis

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Abstract

We report a cirrhotic patient with duodenal vascular ectasia and spontaneous bleeding. The bleeding was successfully controlled with argon plasma coagulation. Duodenal vascular ectasia may be a cause of upper gastrointestinal bleeding in patients with cirrhosis, and argon plasma coagulation may be effective and safe to achieve hemostasis of this lesion.

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Key words: Vascular ectasia; Duodenum; Cirrhosis; Gastrointestinal hemorrhage; Hemostasis

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INTRODUCTION

Vascular ectasia of the duodenum is rare^[1] and an uncommon source of upper gastroduodenal bleeding. It is associated with chronic illnesses such as aortic valve disease and end-stage renal disease when patients are under long-term hemodialysis^[2-4]. Gallagher *et al*^[5] reported a patient with small bowel capillary dilatation and cirrhosis, and suggested that small bowel capillary dilatation appears to be unique in patients with portal hypertension, and may play a role in causing gastrointestinal bleeding. However,

to our knowledge, only a few cases of duodenal vascular ectasia have been reported in patients with cirrhosis^[5-7]. We report a case of upper gastrointestinal bleeding in a patient with cirrhosis and duodenal vascular ectasia, which was successfully controlled by argon plasma coagulation (APC).

CASE REPORT

A 62-year-old man was admitted because of melena, dizziness, and anemia with a follow-up history for chronic hepatitis B infection, liver cirrhosis, and hepatocellular carcinoma. About a 2 cm-sized tumor mass was located in subcapsular portion of segment 7. After two sessions of transarterial chemoembolization for the tumor mass, no visible tumor was found. He visited the emergency room two years ago because of hematemesis, and an endoscopic examination revealed esophageal varices with bleeding, which were treated with endoscopic band ligation.

Physical examination revealed blood pressure of 100/80 mmHg, pulse rate of 72 beats per minute, respiratory rate of 14 breaths per minute, and body temperature of 36.8°C. He was alert and oriented to person, place, and time. His sclera was nonicteric and conjunctiva was pale. Chest and heart examinations were normal. Abdominal examination revealed a distended abdomen with prominent shifting dullness. Pitting edema was noted in both lower extremities.

The results of initial laboratory tests were as follows: white blood count (WBC) = 3700/mm³, hemoglobin = 7.3 g/dL, platelets = 34000/mm³, prothrombin time = 23.8 s, international normalized ratio = 2.05, C-reactive protein = 82.6 mg/dL, total bilirubin = 2.07 mg/dL, direct bilirubin = 0.96 mg/dL, protein = 5.23 g/dL, albumin = 2.83 g/dL, alanine aminotransferase = 67 IU/L, aspartate aminotransferase = 62 IU/L, gamma-glutamyl transferase = 38 IU/L, alkaline phosphatase = 87 IU/L, glucose = 142 mg/dL, blood urea nitrogen (BUN)/creatinine (Cr) = 19.57/0.75 mg/dL, and Na/K/Cl = 120/4.1/87 mEq/mL. Paracentesis revealed clear yellow-colored ascitic fluid, which on analysis showed 0/mm³ WBC, 2000/mm³ red blood cells, 242 mg/dL glucose, and 252 mg/dL protein. The serum α -fetoprotein concentration was 6.2 ng/mL. He was positive for hepatitis B surface antigen and negative for hepatitis B envelope antigen.

Bleeding from the esophageal varices was suspected and urgent endoscopic examination was performed. However, the urgent endoscopy showed small esophageal varices

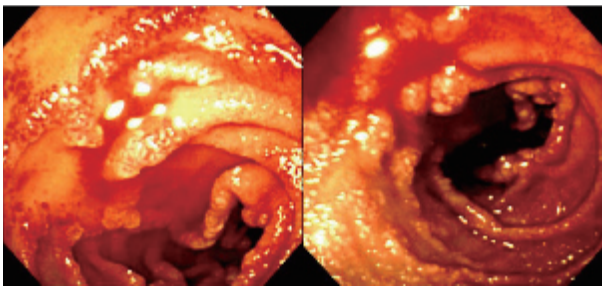


Figure 1 Endoscopy showing a vascular ectasia with active bleeding on the second portion of duodenum.

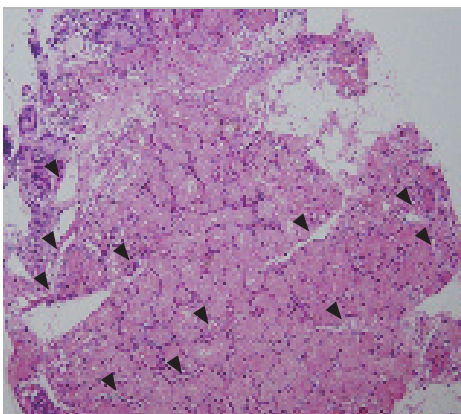


Figure 2 Biopsy from duodenal mucosa showing grossly dilated blood vessels (arrowheads) in the Brunner's glands (HE, $\times 100$).

with no evidence of bleeding, and unexpectedly, vascular ectasia with oozing was found on the duodenal bulb and second portion (Figure 1). Endoscopic biopsy was performed on the vascular ectatic mucosa and histological examination revealed grossly dilated blood vessels in the Brunner's glands at the mucosal layer (Figure 2). To achieve hemostasis, APC (Arco-2000, Soring, Germany) was performed (argon gas flow = 1.5 L/min, power = 50 W), which controlled the bleeding without any complications such as bowel perforation (Figure 3). Follow-up endoscopic examination after one week showed minimal blood oozing on the telangiectatic duodenal mucosa, and a second session of APC was performed (Figure 4A). After endoscopic treatment, his hemoglobin concentration did not decrease and his symptoms improved, and the patient was discharged.

Two months after discharge, endoscopic examination revealed a remnant vascular ectatic duodenal mucosa but no spontaneous bleeding (Figure 4B).

DISCUSSION

Upper gastrointestinal bleeding is one of the most frequent causes of morbidity and mortality during the clinical course of liver cirrhosis^[8-11] and has an overall mortality rate of 24% at six weeks and 40% at one year^[12]. A recent study showed that gastroesophageal varices (59.1%) comprise the most frequent bleeding lesion in patients with cirrhosis^[13]. Others have reported a similar frequency

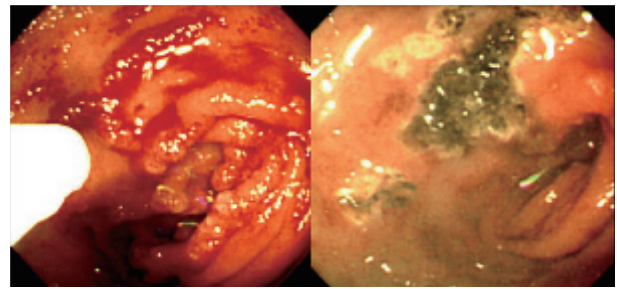


Figure 3 Induction of hemostasis by APC (Arco-2000, Soring, Germany) (left) with blood oozing controlled without complications (right).

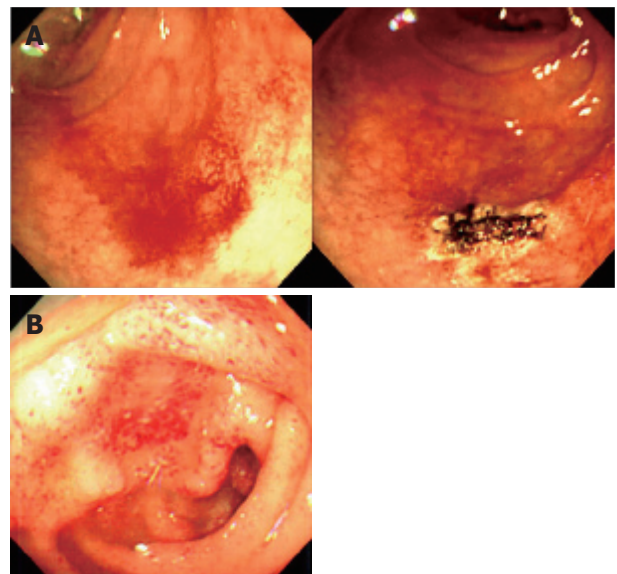


Figure 4 A one-week follow-up endoscopy showing the successfully controlled blood oozing from vascular ectasia on the second portion of the duodenum with APC (A) and a two-month follow-up endoscopy showing the ectatic vascular duodenal mucosa with no further spontaneous bleeding (B).

of esophageal varices bleeding, ranging 49%-72%^[12,14,15]. Peptic ulcer bleeding is the second most frequent type of bleeding lesion (15.7%)^[13].

Upper gastrointestinal vascular ectasia is recognized as an increasingly important source of gastrointestinal bleeding, and can present as that of either overt or of an obscure cause^[3,16-19]. Gastric antral vascular ectasia (GAVE) is a distinct vascular abnormality involving mainly the gastric antrum. Endoscopic examination shows linear, friable red streaks radiating from the pylorus. Angiodysplastic lesions are typically discrete, flat, or slightly raised bright-red lesions 2-10 mm in size, often with fern-like margins and a surrounding pale rim^[20]. The clinical presentation is either chronic, low-grade bleeding, which often leads to iron-deficiency anemia, or acute bleeding with hematemesis or melena. Patients may have recurrent episodes of bleeding which require multiple transfusions, and become transfusion dependent^[20]. A recent study showed that 29% of patients with vascular ectasia also have chronic liver diseases^[21].

By definition, GAVE occurs only in the antrum. However, ectopic ectasia has been reported in the cardiac region^[22], gastric corpus^[23], duodenum^[22,24], and possibly in

the colon^[22]. **Astaldi and Strosseli**^[25] suggested that small bowel biopsies from patients with hepatic cirrhosis might show vascular stasis arising from portal stasis. **Bank et al**^[26] have noted a 'striking increase in vascularity of the villi' in a small bowel biopsy of a patient with cirrhosis and esophageal varices. **Gallagher et al**^[5] suggested that capillary dilatation is probably caused by increased pressure in the portal system.

To our knowledge, only a few cases of vascular ectasia of the duodenum have been reported in patients with cirrhosis^[5-7]. In one report, **histology of the endoscopic duodenal biopsy** revealed grossly dilated capillaries in the villi of the duodenum and jejunum, the patient received a portacaval shunt, but died postoperatively^[5]. **Arendse et al**^[6] reported a fatal case of vascular ectasia of the duodenum in a patient with cirrhosis, which was revealed by autopsy. **Cales et al**^[7] reported an ectasia in a patient with cirrhosis, which was revealed by autopsy. The patient was treated with endoscopic sclerotherapy and a portosystemic shunt to control the bleeding, although neither was successful. Our study seems to be the first to report successful control of bleeding from vascular ectasia of the duodenum by APC in a patient with cirrhosis.

We attempted to treat bleeding from duodenal vascular ectasia by APC and found a certain improvement on endoscopic examination of the duodenum. However, we did not eradicate the duodenal vascular ectasia. **Pavey et al**^[20] reported that patients with GAVE require more sessions to fully control the bleeding and more transfusion than patients with angiodysplasia. Our patient also seemed to need more sessions of APC to completely eradicate ectasia, although this was not possible because he did not visit our hospital after the last endoscopic examination.

Before the widespread availability of therapeutic endoscopy, vascular ectasia was treated with surgical resection^[27,28]. More recently, upper gastrointestinal vascular ectasias have been treated with various endoscopic techniques including heater probe coagulation^[29], bipolar electrocautery^[30] and Nd: YAG laser^[31-33], and APC^[20]. APC is a no-touch electrocoagulation technique in which a high-frequency monopolar alternating current is delivered to the tissue through ionized argon gas^[34,35]. The advantages of this method include the technical ease in treating large areas and the ability to achieve superficial coagulation with a controllable depth of injury. The treated areas heal faster and the endpoint of therapy can be reached sooner^[34,35]. In our patient, to reduce the risk of bowel perforation, we applied irradiation for 4-6 s with a current of 50 W, and were able to successfully control the bleeding without any serious complications.

In conclusion, duodenal vascular ectasia may be a cause of upper gastrointestinal bleeding in patients with cirrhosis. APC can be used as an effective and safe therapeutic tool to achieve hemostasis of this lesion.

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CASE REPORT

Endoscopic ultrasound-guided fine-needle aspiration cytology diagnosis of solid pseudopapillary tumor of the pancreas: A case report and literature review

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Abstract

We describe the clinical, imaging and cytopathological features of solid pseudopapillary tumor of the pancreas (SPTP) diagnosed by endoscopic ultrasound-guided (EUS-guided) fine-needle aspiration (FNA). A 17-year-old woman was admitted to our hospital with complaints of an unexplained episodic abdominal pain for 2 mo and a short history of hypertension in the endocrinology clinic. Clinical laboratory examinations revealed polycystic ovary syndrome, splenomegaly and low serum amylase and carcinoembryonic antigen (CEA) levels. Computed tomography (CT) analysis revealed a mass of the pancreatic tail with solid and cystic consistency. EUS confirmed the mass, both in body and tail of the pancreas, with distinct borders, which caused dilation of the peripheral part of the pancreatic duct (major diameter 3.7 mm). The patient underwent EUS-FNA. EUS-FNA cytology specimens consisted of single cells and aggregates of uniform malignant cells, forming microadenoid structures, branching, papillary clusters with delicate fibrovascular cores and nuclear overlapping. Naked capillaries were also seen. The nuclei of malignant cells were round or oval, eccentric with fine granular chromatin, small nucleoli and nuclear grooves in some of them. The malignant cells were periodic acid Schiff (PAS)-Alcian blue (+) and immunocytochemically they were vimentin (+), CA 19.9 (+), synaptophysin (+), chromogranin (-), neuro-specific enolase (-), α 1-antitrypsin and α 1-antichymotrypsin focal positive. Cytologic findings were strongly suggestive of SPTP. Biopsy confirmed the above cytologic diagnosis. EUS-guided FNA diagnosis of SPTP is accurate. EUS findings,

cytomorphologic features and immunostains of cell block help distinguish SPTP from pancreatic endocrine tumors, acinar cell carcinoma and papillary mucinous carcinoma.

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Key words: Endosonography; Fine-needle aspiration; Solid pseudopapillary tumor; Pancreas; Cytology

Salla C, Chatzipantelis P, Konstantinou P, Karoumpalis I, Pantazopoulou A, Dappola V. Endoscopic ultrasound-guided fine-needle aspiration cytology diagnosis of solid pseudopapillary tumor of the pancreas: A case report and literature review. *World J Gastroenterol* 2007; 13(38): 5158-5163

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INTRODUCTION

Solid pseudopapillary tumor of the pancreas (SPTP) is a rare neoplasm with a reported frequency of between 0.17% and 2.7% of all nonendocrine tumors of the pancreas^[1], and 6.5% of all pancreatic tumors and tumor-like lesions resected in one large institute^[2]. This tumor seems to preferentially occur in young females with a reported mean age of 25 to 30 years, ranging 11-73 years^[2,3]. In the elderly population it has been suggested that SPTP tend to be malignant^[4,5]. It was first described by Frantz in 1959^[6]. Multiple descriptive names have been used for this tumor, including papillary epithelial neoplasm, papillary/cystic neoplasm, solid-and-papillary epithelial neoplasm, papillary-cystic carcinoma, solid-and papillary neoplasm, low-grade papillary neoplasm, and the Frantz tumor^[7]. The WHO pancreatic tumor working group recently recommended the use of the term solid-pseudopapillary neoplasm^[8], a term that has been widely used and accepted by pathologists and clinicians in daily practice^[9,10].

Almost 610 SPTPs, including more than 57 diagnosed by percutaneous fine-needle aspiration (FNA), have been reported since the initial description in 1959^[6]. In recent years, image-guided FNA has been increasingly performed for pancreatic lesions. Endoscopic ultrasound (EUS)-guided FNA can have an important role and provide an

accurate preoperative diagnosis particularly when the EUS findings of the mass are inconclusive. EUS-guided FNA can differentiate SPTP from other pancreatic neoplasms of similar radiologic and cytologic appearance but with different biologic behaviour and treatment, such as pancreatic endocrine tumors, acinar cell carcinoma, and papillary mucinous carcinoma^[11-17]. The cytomorphology of this tumor is highly characteristic, with features that are distinctive from those of other cystic and solid tumors of the pancreas. It is important that this tumor is accurately diagnosed because management protocols differ from other tumor types originating in the pancreas.

In this paper, we describe the clinical, imaging, cytomorphologic features and differential diagnosis of a new case of SPTP diagnosed by EUS-guided FNA with a review of the literature.

CASE REPORT

A 17-year-old woman was admitted with complaints of an unexplained episodic pain for 2 mo and a short history of hypertension in the endocrinology clinic of our hospital (Athens General Hospital, Greece). Clinical laboratory examinations revealed polycystic ovary syndrome (PCOs), splenomegaly and low serum amylase and carcinoembryonic antigen (CEA) levels. CT-scan revealed a mass of the pancreatic tail with solid and cystic consistency.

EUS-guided FNA was performed using 22-gauge needles *via* a transgastric approach. Smears were made at the bedside in the endoscopy suite. The aspirated material was smeared onto glass slides, air-dried, and immediately stained with rapid Hemo-color stain for specimen adequacy assessment and preliminary diagnostic interpretation. Other smears also were fixed immediately in 95% alcohol for subsequent Papanicolaou staining. Additional aspirated material was fixed in formalin, embedded in paraffin, and processed for routine histologic examination using standard techniques. Staining with periodic acid Schiff (PAS) and Alcian-blue (AB) stains was performed. Immunohistochemical stains for vimentin (Dako; 1:100), neuron-specific enolase (NSE) (Dako; 1:100), synaptophysin (NovoCastra, Newcastle UK), chromogranin (Dako; 1:800), CA 19.9 (Dako), α 1-antitrypsin (Dako; 1:3200), α 1-antichymotrypsin (Dako; 1:800), were also performed. Avidin-biotin peroxidase complex technique was used.

Smears and cell block sections were examined with an emphasis on the evaluation of cytomorphologic features and immunohistochemical results.

EUS confirmed a mass, both in body and tail of the pancreas, with distinct borders, which caused dilation of the peripheral part of the pancreatic duct [major diameter (m.d.) 3.7 mm]. More specifically the tumor mass was solid and cystic, hypoechoic and heterogenous with a size measuring 65.4 mm \times 54.2 mm (Figure 1). The EUS differential diagnosis included serous cystadenoma, mucinous cystadenoma, mucinous cystadenocarcinoma, adenocarcinoma with cystic degeneration, endocrine tumor and SPTP.

Smears were hypercellular and characteristically showed branching papillary arrangements composed of delicate fibrovascular cores and microadenoid structures with at-

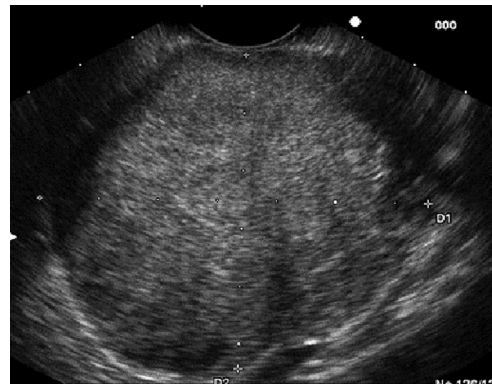


Figure 1 Endoscopic ultrasound image (EUS) showing a mass in body and tail of the pancreas.

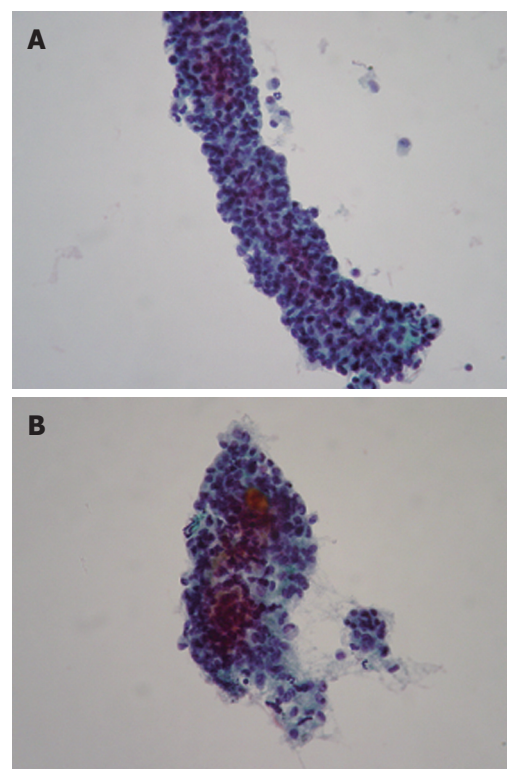


Figure 2 Papillary arrangement composed of delicate fibrovascular core with attached monotonous cuboidal neoplastic cells (A) and adenoid structures composed of cuboidal neoplastic cells (B) (PAP, \times 400).

tached monotonous cuboidal neoplastic cells (Figure 2A-B). The nuclei of malignant cells were round or oval, eccentric with finely granular chromatin, small nucleoli and in some of them nuclear grooves. Cytoplasm was granular or finely vacuolated with wispy borders. No mitotic activity or significant atypia was observed. The architectural features were more evident in the cell block sections. Histochemically, tumor cells revealed PAS Alcian-blue (+) (Figure 3A-B). Immunostains performed on the cell block yielded vimentin positivity (Figure 4A), CA 19.9 positivity (Figure 4B), synaptophysin, α 1-antitrypsin and α 1-antichymotrypsin focal positivity whereas NSE and chromogranin were negative. These findings were strongly suggestive of SPTP.

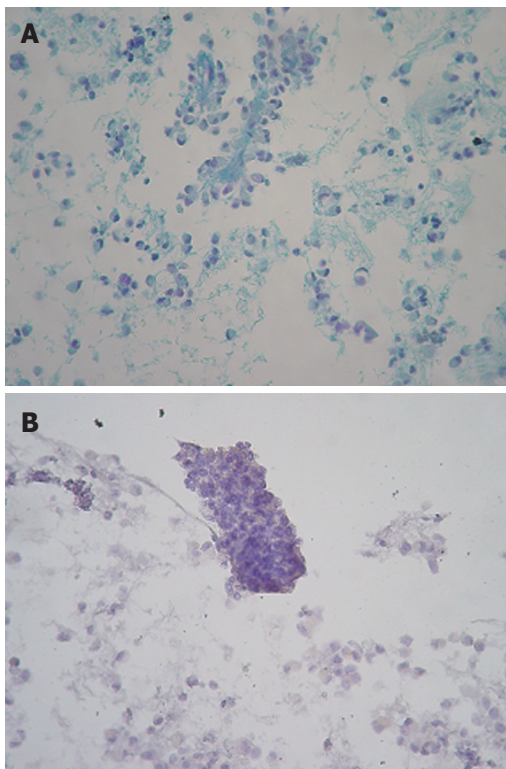


Figure 3 Staining with periodic acid Schiff (PAS) (A) and Alcian-blue (AB) (B) (x 400).

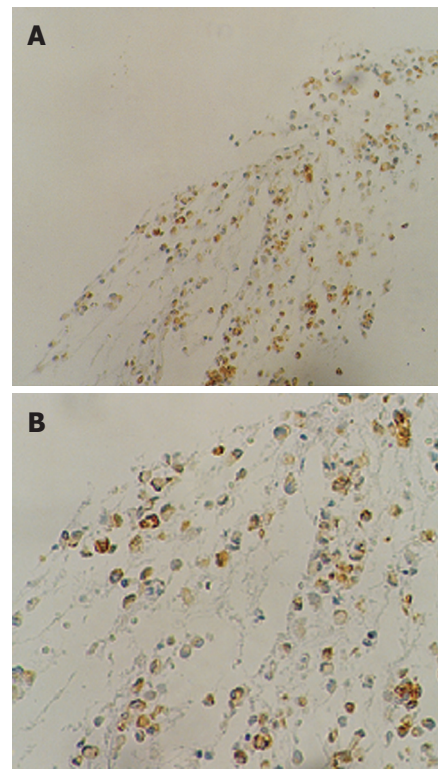


Figure 4 Immunostain for vimentin (A) and CA 19.9 (B) (x 400).

DISCUSSION

Solid-pseudopapillary neoplasm is an exceedingly rare pancreatic tumor with a reported frequency of less than 1% of all pancreatic diseases. Most of these reports are in the form of small case series with only few reports with greater than 50 cases identified in the literature.

It predominantly occurs in adolescent girls and young women with a reported frequency of 87% to 90% (mean age of 25 to 35 years)^[2,3]. Such a striking predilection of SPTP occurring in women has suggested a hormonal influence on the pathogenesis of this tumor^[18,19]. Cases occurring at the first decade of life are rare and less than 10% of SPTP cases have been reported in patients older than 40 years^[2,20]. Occurrence of SPTP in men is rare, accounting for 7% of cases^[21].

Clinically, SPTP may present as an abdominal mass with discomfort or pain, or it may be an incidental finding in work up for unrelated conditions. These tumors are generally large with a mean diameter of 10.3 cm and approximately 72% arise in the body and tail of the pancreas and less frequently in the head^[2]. Unusual presentations of SPTP include multicentricity^[22,23], occurrence in extra-pancreatic sites such as mesocolon^[24], retroperitoneum^[25], omentum^[26], liver^[27] and duodenum^[28]. In our case, the sex (female), age (17 years old) and tumor localization (body and tail of the pancreas) were typical of SPTP.

In most patients, the tumor follows an indolent clinical course and complete resection is often curative^[20,29]. Up to 15% of cases have shown aggressive behaviour consisting of extension into adjacent blood vessels and organs, local recurrence and distant metastasis^[20,28,30,31]. However, even metastatic SPTPs are growing slowly and have excellent prognosis. Only one death, due to metastatic spread of

SPTP has been reported^[32]. Also, spontaneously regressing tumors have been reported^[33].

Benign-appearing SPTP might contrast with cellular anaplasia present in the metastatic deposits^[34]. Thus, there are no histological features that can predict aggressive clinical behaviour. Proposed pathologic features related to aggressive behaviour or metastatic potential include diffuse growth pattern, venous invasion, nuclear pleomorphism, mitotic rate, necrosis and areas of dedifferentiation^[35]. Most SPTPs display diploid DNA content^[1]. However, DNA aneuploidy is more prominent in malignant SPTPs^[34,36]. Chromosomal abnormalities, including double loss of X chromosomes and trisomy for chromosome 3, and unbalanced translocation between chromosomes 13 and 17 have been found in SPTP associated with aggressive behaviour and might be indicators of possible metastatic potential^[37,38]. Thus, since the malignant potential of SPTP is difficult to determine, this tumor is considered a low malignant or borderline malignant potential.

The histogenesis of SPTP has been, and to date remains, elusive. Multiple theories have been proposed, including origin from small duct epithelium, derivation from acinar cells, endocrine pancreas, totipotent stem cells, or primitive cells capable of differentiating exocrine and endocrine^[4,39-41], along with genital ridge-related cells that are incorporated in the pancreas during early embryogenesis^[42]. Another recent report showed that SPTP is positive for melanocytic markers (S-100 protein, human melanoma black 45 (HMB45) and melanoma antigen recognized by T-cells 1 (MART-1) by immunohistochemistry and demonstrated the presence of premelanosomes and melanosome granules in the tumor cells by transmission electron microscopy, suggesting that this tumor may be of neural

crest origin^[43]. Also, predilection in young women suggests that sex hormones play a role in the histogenesis or progression of this tumor. However, the ER and PR expression are not consistent in the literature. Using a binding assay, Ladanyi *et al*^[18] demonstrated the expression of ER in SPTP and proposed that this tumor is hormone sensitive.

Diagnosis of SPTP is important for the clinical management of patients with this tumor. Diagnostic modalities including CT and magnetic resonance imaging can only suggest a diagnosis of SPTP. CT findings include an encapsulated lesion with well-defined borders and variable central areas with cystic degeneration, necrosis, or hemorrhage. Calcifications may occasionally be seen. Magnetic resonance imaging is helpful for identifying the characteristic internal signal intensities of blood products, which help distinguish this tumor from other cystic pancreatic tumors^[44].

In recent years, advances in technology have permitted the performance of fine-needle aspiration biopsy under EUS guidance^[45,46]. The overall accuracy of EUS is superior to CT scan and magnetic resonance imaging for detecting pancreatic lesions. It has been shown that EUS alone (94%) is more sensitive than CT scan (69%) and magnetic resonance imaging (83%) for detecting lesions, especially when they are smaller than 3.0 cm^[47]. EUS permits a better evaluation of SPTPs, but the findings also are not specific. A small SPTP, often a monocystic tumor most frequent in males, might have the EUS appearance of a solid endocrine neoplasm, and it might be difficult to distinguish between the two^[48].

An accurate preoperative diagnosis is highly desirable, since local surgical excision is usually curative and this is possible by EUS-guided FNA cytology. Bondeson *et al*^[49] were the first to correctly diagnose SPTP by preoperative FNA.

Since then, to our knowledge, cytologic findings of percutaneous FNA-diagnosed SPTP have been described in 57 cases^[9,21,22,31,37,49-53]. However, only few cases have been diagnosed by EUS-guided FNA^[9,54]. FNA cytomorphologic features are highly characteristic and distinct from those of other cystic or solid tumors of the pancreas. On aspirated materials, the most frequent features are the presence of marked cellularity with pseudopapillary fragments composed of fibrovascular stalks lined with one to several layers of tumor cells intermingled with discohesive neoplastic cells^[1,21,22,49,51]. Careful evaluation of smears for mitotic activity is recommended, since this parameter has recently been considered one predictive criterion for biologic aggressiveness^[55]. Inter or intracellular pink hyaline globules, mucus-like globules, as in our case, surrounded by stromal cells and cellular debris are also frequent features.

Immunohistochemically, most SPTPs are immunoreactive for vimentin (vim), a1-antitrypsin (AAT), a1-antichymotrypsin (AACT)^[56], occasionally positive for neuron-specific enolase and synaptophysin (syn)^[9], and nonreactive for S-100, CA 19.9 and chromogranin A. In our case, SPTP was vim (+), AAT (+), AACT (+), CA 19.9 (+) and syn (+). The above-mentioned cytologic and immunohistochemical features are strongly suggestive of SPTP.

The differential diagnosis of SPTP includes pancreatic

neuroendocrine tumor, acinar-cell carcinoma, papillary mucinous carcinoma and intraductal papillary mucinous tumor. Pancreatic endocrine tumors predominantly occur in older patients and can be associated with a variety of clinical syndromes. These tumors share some cytological features with SPTP. The presence of rosettes without papillary structures and typical "salt and pepper" chromatin indicates a pancreatic endocrine tumor^[20,16]. Additionally, these tumors express neuroendocrine markers such as chromogranin, neuron-specific enolase, and synaptophysin which are usually nonreactive in cases of SPTP. Acinar-cell carcinoma occurring in a wide range of age, is more commonly observed in males, and shows no predilection for head, body or tail location in the pancreas. Aspirates are cellular and composed only of acinar neoplastic cells containing enlarged nuclei with irregular membranes and distinct nucleoli. Patients with papillary mucinous carcinoma often have a single, large unilocular cystic mass and the neoplastic cells are columnar with cytoplasmic vacuoles, variable nuclear anaplasia, prominent nucleoli and mucinous background that should not be mistaken for the myxoid stroma^[22,57]. Also, the thick, glistening and viscid mucus almost always present in intraductal papillary mucinous tumor is an important feature that distinguishes this neoplasm from SPTP^[58].

In conclusion, we believe that EUS-FNA provides an excellent cellular yield and an overall sensitivity for the diagnosis of SPTP. Clinical correlation, radiological findings and cytomorphologic features from EUS-guided FNA achieve the accurate diagnosis of SPTP.

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Meeting Canadian Digestive Diseases Week (CDDW)
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RNA interference and antiviral therapy

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Abstract

RNA interference (RNAi) is an evolutionally conserved gene silencing mechanism present in a variety of eukaryotic species. RNAi uses short double-stranded RNA (dsRNA) to trigger degradation or translation repression of homologous RNA targets in a sequence-specific manner. This system can be induced effectively *in vitro* and *in vivo* by direct application of small interfering RNAs (siRNAs), or by expression of short hairpin RNA (shRNA) with non-viral and viral vectors. To date, RNAi has been extensively used as a novel and effective tool for functional genomic studies, and has displayed great potential in treating human diseases, including human genetic and acquired disorders such as cancer and viral infections. In the present review, we focus on the recent development in the use of RNAi in the prevention and treatment of viral infections. The mechanisms, strategies, hurdles and prospects of employing RNAi in the pharmaceutical industry are also discussed.

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Key words: RNA interference; Short hairpin RNA; Micro RNA; Antiviral therapy; Viral infection; Human immunodeficiency virus; Hepatitis C virus; Hepatitis B virus; SARS-coronavirus

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INTRODUCTION

RNA interference (RNAi), a highly conserved gene silencing mechanism plays an important role in the

regulation of gene expression. This system was examined in a broad variety of species including plants, fungi, yeasts, nematodes, flies and mammals. In fact, RNAi serves as a safeguard for the preservation of genomic integrity. It protects the host from viral infections and invasion by mobile genetic elements by degrading the exogenous genomic material (e.g., viral RNAs).

RNAi is triggered by small double-stranded RNA (dsRNA) and functions at all levels, including transcription^[1], post-transcription^[2] and translation^[3]. The first reports on RNA-induced post-transcriptional gene silencing (PTGS) phenomena were published in the early 90s, when Napoli^[4] and Van der Krol^[5] described the co-suppression of both viral transgenes and their homologous endogenous genes in transgenic plants. Similar inactivation of gene expression called "Quelling" was observed in *Neurospora crassa* by transformation with homologous sequences^[6]. In 1995, sense RNA was demonstrated to be as effective as antisense RNA in disrupting the expression of *par-1* in *Caenorhabditis elegans*^[7]. The mechanism of action remained enigmatic until 1998, when Fire and Mello discovered that dsRNA, instead of the single-stranded sense or antisense RNA, mediated gene silencing by degrading endogenous mRNAs in a sequence-specific manner^[8]. They also challenged a previous report published in 1995 claiming it to be an artificial effect of dsRNA contamination. Further studies have revealed that RNAi can occur at both the transcription and post-transcription levels. Transcriptional gene silencing involves histone H3 methylation and the formation of heterochromatin^[9-11]. Post-transcriptional gene silencing includes small interfering RNA (siRNA) that mediates sequence-specific target RNA degradation, and micro RNA (miRNA) which promotes blockage of protein translation at the 3'-untranslated region (3'UTR)^[12].

In recent years, RNAi has become a powerful tool to probe gene functions and to rationalize drug design. It has been employed as a prophylactic and therapeutic agent for combating a wide range of disorders, including infectious diseases, tumors and metabolic disorders. Several lethal viruses, including human immunodeficiency virus (HIV), the hepatitis C and B viruses (HCV & HBV), coronavirus, influenza A virus (IAV), human papillomavirus (HPV), have been shown to be inhibited or eliminated by RNAi. These findings have emphasized the potential of RNAi in clinical applications. In the present review, we discuss the mechanism of RNAi, and its role in the prevention and the treatment of viral infections.

Mechanisms of RNAi

Biochemical and genetic studies have revealed the detailed

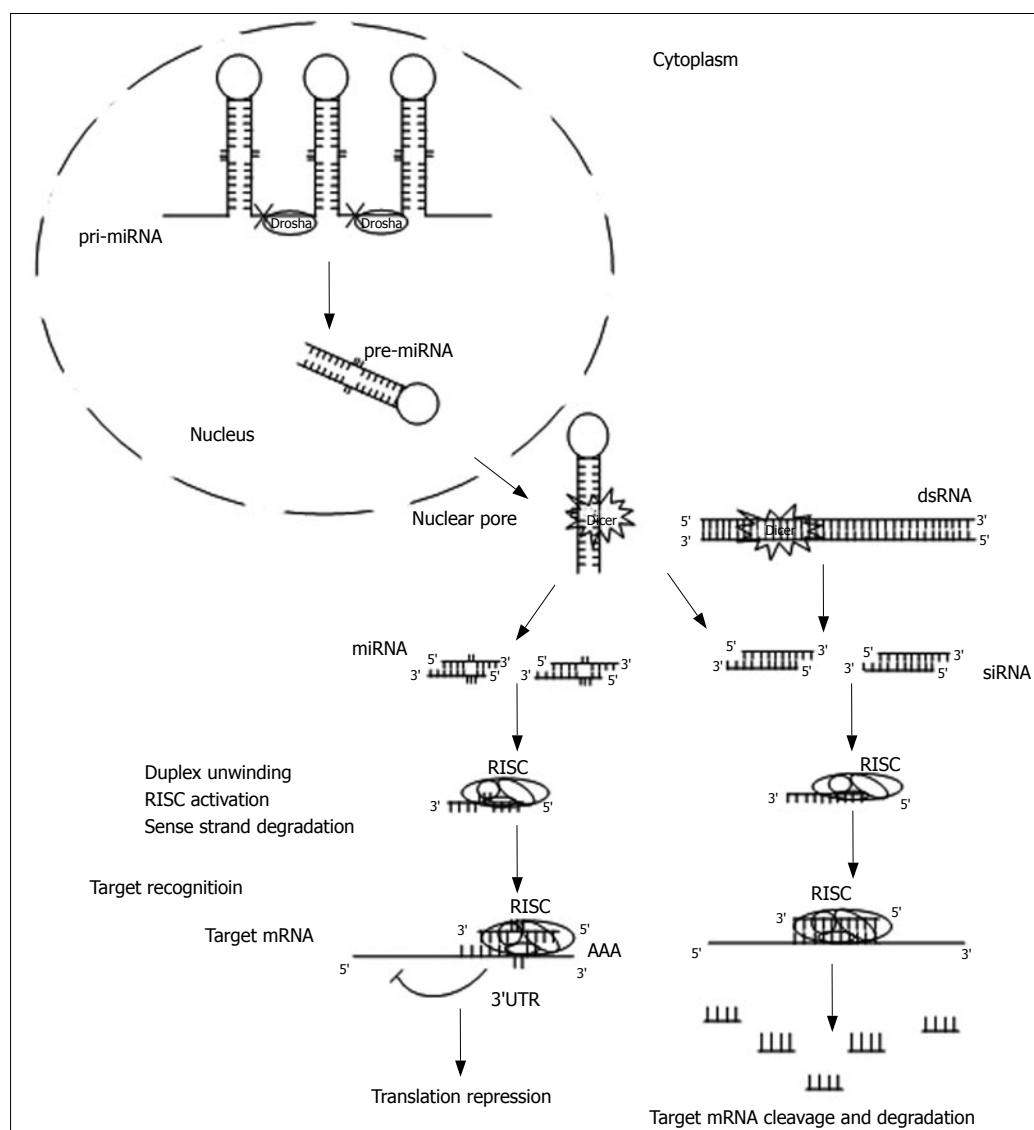


Figure 1 The RNA interference pathways.

mechanism by which dsRNA-mediated gene silencing takes place. In general, the mechanism includes two major steps: the initiator step and the effector step (Figure 1).

In the initiator step, long dsRNAs, which are produced by endogenous genes, invading viruses, transposons or experimental transgenes, are initially recognized by a dsRNA-binding protein, RDE-4/R2D2^[13,14]. They are then submitted to and cleaved by the RNase III-like nuclease Dicer^[15], which generates 21-23 nucleotide duplex RNAs with overhanging 3' ends^[16], called small interfering RNAs (siRNAs). The presence of highly conserved Dicer in yeast^[11], plants^[17,18], *C. elegans*^[19], *Drosophila*^[15], mice^[20] and humans^[21,22], suggests that the RNAi pathways share similar basic mechanisms in these organisms.

In the effector step, siRNAs are incorporated into a multicomponent nuclease complex, the RNA-induced silencing complex (RISC)^[23]. The antisense strand of the duplex directs RISC to recognize and to cleave cognate target RNAs, which undergoes specific base pairing and endonucleolytic cleavage. This leads to the degradation of the unprotected and single-stranded target RNA. To date,

several components of the RISC have been identified, including some conserved argonaute proteins that share the PAZ domain with Dicer family proteins^[24].

The Dicer also cleaves the 60-70nt long precursor miRNA (pre-miRNAs) into miRNAs, which are of similar size as siRNAs. This pathway is referred to as miRNA-dependent gene silencing. The pre-miRNAs, whose structures are imperfectly complementary to each strand, are generated from endogenous stem loop precursors or hairpins, named primary-miRNA (pri-miRNA). The pri-miRNAs are first cleaved by Droscha RNase III in the nucleus^[25]. The resulting pre-miRNAs are then exported into the cytoplasm for further processing by Dicer. The complex of the activated RISC and miRNA binds the 3'UTR of specific mRNAs, which triggers cleavage by perfect base-pairing, or translational repression by partial base-pairing recognition^[26-29].

Strategies for RNA interference

In order to study the functional genomics and biology of RNA interference, much effort has gone into the study of

artificial RNAi-inducing gene silencing. Strategies for delivery of RNAi reagents into mammalian cells can be divided into two types, the transient RNAi and the stable/inducible RNAi.

The methods commonly used in producing siRNA extraneously include chemical synthesis, *in vitro* transcription, and recombinant human Dicer/*E. coli* RNase III digestion of long dsRNAs. These siRNAs can be transiently transfected into target cells. Alternatively, the short hairpin RNAs (shRNAs) are expressed endogenously from plasmids and viral vectors. The shRNA expression cassettes can be stably integrated into the genome of target cells, transcribed intranuclearly and processed into siRNAs by Dicer in the cytosol. In general, RNA Pol III promoters (i.e., U6, H1 and tRNA promoters) are commonly used to drive shRNA expression in the RNAi studies. The viral vectors including retro-^[30-33], lenti-^[34-36], adeno- and adeno-associated viral vectors^[37-39] have been demonstrated to feature high-efficiency gene delivery and can overcome the obstacles of cell-type-dependent transfection. The development of an inducible RNAi system has certainly enhanced our understanding of candidate genes' functions, as it provides an invaluable genetic switcher that allows the inducible and reversible control of specific gene's expression *in vitro*^[40-42] and *in vivo*^[43-45].

RNAi applications to combat viral infection

Viral infection is a serious public health, social and economic problem. More effective approaches are urgently needed to prevent viral propagation. Several studies have shown that RNAi technology has potential advantages over traditional measures such as the use of anti-viral drugs and vaccines, because of its ease of use, rapidity of action, high efficiency and specificity of activity when applied to the different stages of virus-host interactions^[46]. In this section we will focus on the prospective use of RNAi in several common human pathogens such as HIV, HCV, HBV, SARS-coronavirus and influenza virus.

Human immunodeficiency virus

Human immunodeficiency virus type 1 (HIV-1) is the first primate virus shown to be inhibited by RNAi. HIV is a retrovirus that has been categorized into the subgroup of lentiviruses. Upon infection, the positive strand of the HIV's RNA genome is reversely transcribed into a linear dsDNA soon after the virus enters the host cells by receptor recognition and cell adhesion. The linear dsDNA becomes circularized, is then transported into the nucleus and integrated into the host chromosome as a provirus. By utilizing the host enzymes, HIV provirus converts viral genes into mRNAs, which are used as blueprints for the subsequent expression of viral structure proteins and enzymes. It has been suggested that the genomic RNA or the newly transcribed viral mRNAs are good targets for siRNAs intervention.

It is unclear whether RNAi can target RNA genome of HIV-1 infectious particles. Jacques reported siRNA-mediated inhibition of the early and late steps of HIV-1 replication, by targeting various regions of the HIV-1 genome and by preventing the formation of viral complementary-DNA intermediates^[47]. Other workers

have suggested that the incoming HIV-1 RNA genome may not be accessible to siRNAs^[48,49]. To date, several viral target sequences have been identified. These include the structure proteins, Gag^[48,50-52] and Env^[52,53]; the reverse transcriptase Pol^[48]; the regulatory proteins, Tat^[54,55] and Rev^[54,56], and the two accessory proteins Nef^[47,57,58] and Vif^[47] (Table 1). The long terminal repeats that the integrase employs to insert HIV's DNA genome into host DNA, have also been targeted^[47,51].

Several studies have demonstrated that HIV may be able to escape RNAi target by mutations^[58-60]. To overcome this problem, lentiviral vectors incorporated with different shRNA-expressing-cassettes, which can simultaneously target multiple sequences including conserved sequences of the HIV genome, have been constructed^[61,62]. Another proposed strategy using RNAi application is the targeting of host genes. Some host genes are essential for viral replication but have a much slower mutation rate than the viral genes. These genes have been targeted by RNAi, and the results are very encouraging^[63-69] (Table 1). Down-regulation of the cell surface CD4 receptor and/or one of the co-receptors CCR5 and CXCR4 by RNAi has led to dramatic reduction of viral entry into cells^[34,70,71]. Compared with CD4 and CXCR4, CCR5 has been found to be a preferential target, since no immune defects or host mortality was observed on its deletion^[72,73]. Therefore, careful selection of host immutable co-factors that are important for viral replication, but not for host survival, is of prime importance in the development of anti-HIV strategies. Furthermore, simultaneous targeting by RNAi of both the virus and host factors^[50,74] has been shown to be more effective in inhibiting HIV-1 replication than the targeting of either virus or host factors alone.

Hepatitis C virus

Hepatitis C virus infection is a major cause of chronic liver diseases, including liver cirrhosis and hepatocellular carcinoma (HCC). The estimated number of infected individuals are about 170 million worldwide^[75], which accounts for nearly 3% of the world's population. The World Health Organization (WHO) has recognized HCV infection as a global health problem.

HCV is a small, enveloped RNA virus that belongs to the Flaviviridae family. The cytoplasmic replicating virus contains a 9.6 kb RNA genome that functions as the messenger RNA and replication template. The development of anti-HCV drugs has accelerated since the replicon-based culture system was established a few years ago^[76,77]. Several regions of the HCV's RNA genome, including 5'UTR and the coding sequences of Core, NS3, NS4B and NS5B, are sensitive to the action of siRNA^[78-83] (Table 2). The therapeutic potential of RNAi was further emphasized by *in vivo* studies^[84,85]. The administration of siRNA and shRNA to target cell surface receptor FAS^[86], caspase 8^[87] and NS5B^[84], has resulted in the destruction of cognate mRNAs and protection of mice from liver failure. The use of multiple siRNAs against highly conserved HCV sequences with and without host cell cofactors may limit the emergence of resistant viruses as has been demonstrated in several studies^[88-92] (Table 2).

Table 1 Strategies designed to inhibit HIV replication *via* RNA interference

Target gene	RNAi inducer (length)	Promoter	Vector	Cell type	Delivery methods	Inhib. of virus prod. (fold)	Reference
Viral Gene							
LTR, Vif, Nef	siRNA (21 bp)/shRNA (19 bp) ¹	T7	Plasmid	Magi, PBLs	Transfection	> 20	[47]
Gag, Pol	siRNA (21 bp)	-	-	HOS.T4.CXCR4	Transfection	> 10	[48]
Gag, LTR	siRNA (23 bp)/dsRNA (21nt) ²	-	-	U87-CD4 ⁺ -CCR5 ⁺ /CXCR4 ⁺ , PBMC	Transfection	4	[51]
Gag, Env	dsRNA (441-531nt) ³	-	-	COS, Hela-CD4 ⁺ , PBMC, ACH2	Transfection	70	[52]
Tat + Rev	siRNA (21 bp)	-	-	293T, Jurkat, PBMC	Transfection	> 15	[54]
Rev (Tat)	siRNA (21 bp) ⁵	Dual U6	Plasmid	293/EcR	Transfection	10 000	[56]
Nef	dsRNA (556nt) ³	-	-	MT4-T, U937	Transfection	2.5	[57]
Env	siRNA (20 bp)/shRNA (20 bp) ⁴	U6	Plasmid, Lentivirus	COS, MT-4	Transfection / Transduction	> 10	[53]
Nef	shRNA (21 bp)	H1	Retrovirus	SupT1	Transduction	> 10	[58]
Gag, Pol, Int, Vpu	shRNA (21 bp)	H1	Lentivirus	293T, Magi, GHOST hi5, CEM-A, Molt-4, PBMC	Transduction	> 20	[61]
Cellular gene							
Tsg101	siRNA (21 bp)	-	-	293T	Transfection	10-20	[63]
LEDGF/p75	siRNA (21 bp)	-	-	Hela	Transfection	NR	[64]
P-TEFb (CDK9/CyclinT1)	siRNA (21 bp)	-	-	Hela, Magi	Transfection	3-5	[65]
hRIP	siRNA (21 bp)	-	-	Hela, Jurkat, Macrophages	Transfection	-100	[66]
Emerin	siRNA (21 bp)	-	-	Hela, Macrophages	Transfection	> 10	[67]
LEDGF/p75, HRP2	siRNA (21 bp)	-	-	Hela-P4	Transfection	2-3	[68]
CXCR4	siRNA (21 bp)	-	-	HOS-CD4 ⁺ , HOS-CD4 ⁺ -CXCR4 ⁺ /CCR5 ⁺	Transfection	3-5	[70]
Importin 7	siRNA (21 bp)	-	-	Hela, Macrophages	Transfection	-10	[69]
CXCR4 ⁺ CD4 ⁺	shRNA (19/21 bp) ⁶	-	-	Magi-CXCR4/CCR5, PBMC	Transfection	> 15	[71]
CCR5	shRNA (19 bp)	U6	Lentivirus	Magi-CCR5, PBLs	Transduction	3-7	[34]
Combination of viral and cellular genes							
Gag, CD4	siRNA (21 bp)	-	-	Magi-CCR5, Hela-CD4	Transfection	4-25	[50]
Tat, RT, NF-κB (p65)	siRNA (21 bp)	-	-	Magi, Jurkat	Transfection	5-500	[74]

The fold inhibition of virus production refers to the results obtained with the most potent siRNA/shRNA tested in a specific cell model. All siRNAs were prepared by chemical synthesis unless indicated otherwise. LTR: Long terminal repeat; PBLs: Peripheral blood lymphocytes; PBMC: Peripheral blood mononuclear cell; Pol: Polymerase; Env: Envelope; Tsg101: Tumor susceptibility gene 101; LEDGF/P75: Lens epithelium-derived growth factor/transcription co-activator p75; NR: Not reported; P-TEF: Positive transcription elongation factors; hRIP: Human Rev-interacting protein; HRP2: Hepatoma-derived growth factor related protein 2; RT: Reverse transcriptase; NF-κB: Nuclear factor-NF-κB. ¹shRNA expressed from a transfected plasmid under the control of a T7 promoter. ²dsRNA produced by *in vitro* T7 promoter-mediated transcription. ³dsRNA produced by *in vitro* SP6/T7 promoter-mediated transcription. ⁴shRNA and siRNA expressed from transfected plasmids under the control of one and two U6 promoters respectively, shRNA further stably expressed from a recombinant lentiviral vector driven by a U6 promoter. ⁵siRNA expressed from a transfected plasmid under the control of two U6 tandem promoters that drive the synthesis of each of the siRNA strand. ⁶shRNA produced by *in vitro* T7 promoter-mediated transcription.

Hepatitis B virus

Hepatitis B virus infection is a major public health problem. It is estimated that, approximately 2 billion people are infected with HBV worldwide, and about 400 million are HBV chronic carriers^[93]. HBV infection is highly prevalent in Asia and South Africa and results in over one million deaths worldwide annually.

Although the clinical symptoms caused by HBV and HCV infection are very similar, the viruses are completely unrelated^[94]. HBV, the prototypical member of the Hepadnaviridae family, is one of the smallest DNA viruses (-3.2 kb), which can undergo reverse-transcription for viral replication. The HBV genome contains four overlapping open reading frames: P (polymerase-reverse transcriptase), C (core structure protein), S (surface glycoprotein) and X (HBx protein). After the uncoated nucleocapsids enter the nucleus, the HBV genome is repaired to form a covalently closed circular DNA (cccDNA), which is a template for messenger RNA transcription. The RNA

intermediates-pregenomic and subgenomic RNAs, coding for viral multifunctional proteins, are transported into the cytoplasm where translation is initiated. After the pregenomic transcript is packaged into virion core particle, it is reversely transcribed by viral reverse transcriptase, thus producing a single stranded (-) DNA. Based on the structure of the (-) stranded DNA, a complementary (+) DNA strand is synthesized. Due to the lack of proofreading function of its polymerase, HBV undergoes rapid mutagenesis, with the creation of a large number of drug-resistant variants. These drug-resistant variants are further amplified under selective pressure during antiviral treatment, resulting in the elimination of the anti-viral effect and virus rebound during treatment. In severe cases, this can lead to death, even after cessation of treatment. Because of this challenge, new drugs with different targets or drug metabolism mechanisms are urgently required for better treatment outcome.

Several sites of the HBV genome including the *P*, *Pre*

Table 2 Strategies designed to inhibit HCV replication *via* RNA interference

Target gene	RNAi inducer (length)	Promoter	Vector	Model	Delivery methods	Inhib. of virus prod. (fold)	Reference
<i>In vitro</i> studies							
Viral gene							
5'-UTR	siRNA (21 bp)	-	-	5-2 cells (Huh-7)	Transfection	-6	[79]
	siRNA (21 bp)/shRNA(19 bp) ¹	U6	Plasmid	293T, Huh 7	Transfection	> 10	[78]
NS4B	siRNA (23 bp)	-	-	Huh-7.5	Transfection	-80	[80]
NS3, NS4B, NS5A, NS5B	siRNA (21 bp)	-	-	S1179I (Huh-7)	Transfection	-23	[81]
IRES, NS3, NS5B	siRNA (23bp)/shRNA (21 bp) ²	Dual H1	Plasmid	Huh-7	Transfection	> 9	[82]
5'-UTR, C, NS4B, NS5A, NS5B	esiRNA (15-40 bp) ³ /shRNA (19bp)	H1	Mo-MuLV	Huh-7	Transfection / Transduction	-100	[88]
5'-UTR, C, NS3, NS5B	siRNA (21 bp)/shRNA (19 bp) ⁴	U6	Plasmid/Lentivirus	Huh-7	Transfection / Transduction	-7	[83]
Cellular gene							
L α , PTB, eIF2B γ , hVAP33	shRNA (19 bp)	U6	Plasmid/Adenovirus	Huh-7	Transfection / Transduction	-13	[91]
Cyp-A,B,C	shRNA (NR)	U6	Plasmid/Retrovirus	Huh-7	Transfection / Transduction	-10	[92]
Combination of viral and cellular genes							
5'-UTR, 3'-UTR, PSMA7, HuR	shRNA (19-21 bp)	U6	Plasmid/Retrovirus	Huh-7	Transfection / Transduction	> 2	[89]
CD81, IRES, NS5B	shRNA (19-21 bp)	H1	Lentivirus	Huh-7	Transduction	> 32	[90]
<i>In vivo</i> studies							
NS5B	siRNA (23 bp)	-	-	Mice	Hydrodynamic transfection	3	[84]
IRES	shRNA (19-25 bp) ⁵	-	-	Mice	Hydrodynamic transfection	-50	[85]

The fold inhibition of virus production represents the most potent effect caused by a specific siRNA or combinatorial siRNAs. All siRNAs were prepared by chemical synthesis unless indicated otherwise. UTR: Untranslated region; NS: Non-structural; IRES: Internal ribosomal entry site; C: Core protein; esiRNA: Endoribonuclease-prepared siRNA; Mo-MuLV: Moloney murine leukemia virus; PTB: Polypyrimidine tract-binding protein; eIF2B γ : Subunit gamma of human eukaryotic initiation factors 2B; hVAP-33: Human VAMP-associated protein of 33 kDa; Cyp: Cyclophilin; PSMA7: Proteasome α -subunit 7; HuR: Hu antigen R; N.R: not reported. ¹stem-loop- and tandem-type siRNA expressed from DNA-based vectors driven by one and two U6 promoters respectively. ²shRNA expressed from a transfected plasmid under the control of two H1 tandem promoters that drive the synthesis of each of the siRNA strand. ³esiRNA generated by *in vitro* T3/T7 promoter-mediated transcription. ⁴shRNA expressed from a transfected plasmid or a lentivirus vector respectively under the control of a U6 promoter. ⁵ shRNA generated by *in vitro* T7 promoter-mediated transcription.

C/C, PreS/S, X gene, have been employed as targets to examine the *in vitro* efficacy of RNAi^[95-99] (Table 3). Some sites have also been tested in hydrodynamic HBV model and transgenic HBV model^[100-104] (Table 3). Our group has successfully designed multiple shRNAs that target DR elements and regions that code for core, polymerase, PreS, S, and X proteins. These shRNA were found to potently inhibit HBV replication and showed synergistic antiviral effects with the commonly used antiviral drug, lamivudine^[105]. In a recent study, we showed that simultaneous delivery of two shRNAs that target different regions, exhibited strong synergistic antiviral effects in a hydrodynamic transgenic mice model. In this study, both S and e antigens were reduced to undetectable levels, and the viral load was reduced by greater than one hundred-fold (He *et al* unpublished observations). These results clearly demonstrate the potential of RNAi application in anti-HBV therapy.

SARS-coronavirus

Severe acute respiratory syndrome (SARS) outbreak affected nearly 30 countries during the years 2002-2003. This epidemic was caused by a novel SARS-associated coronavirus (SARS-CoV)^[106-108]. SARS-CoV is a large (-30 kb), enveloped, positive-stranded RNA virus and its genome is composed of replicase (*rep*), spike (*S*), envelope

(*E*), membrane (*M*), and nucleocapsid (*N*) genes. The prophylactic and therapeutic efficacies of siRNAs were tested because of the absence of any effective drugs or vaccines against SARS-CoV infection. Both *in vitro* and *in vivo* applications proved satisfactory, using synthetic siRNAs as well as vector-based shRNAs against leader sequence^[109,110], 3'-UTR^[110], non-structural^[111] and structural genes^[110,112-115] of SARS-CoV (Table 4). Another recent report revealed that the siRNA-mediated depletion of the host cellular clathrin heavy chain gene, reduced the SARS-CoV infectivity^[116]. Locked nucleic acid (LNA)-modified siRNAs, an RNA-like high affinity nucleotide analogue, has been found to improve the performance of gene silencing via enhancement of siRNA biostability and specialty. The improvement was clearly apparent when siRNA was transfected into Vero cells prior to a lethal SARS-CoV attack^[117].

It is worth mentioning that our group was the first to demonstrate in 2003 the remarkable inhibition and replication of SARS-CoV infection by siRNAs against *rep* gene^[118]. Subsequently, we designed siRNAs that could target both *rep* and structural genes. We also evaluated the antiviral effect, dose response, duration and viral kinetics of siRNAs in foetal rhesus kidney (FRhK-4) cells^[119,120]. Two of the siRNAs were further evaluated for safety and antiviral efficacy in a rhesus macaque SARS model^[119].

Table 3 Strategies designed to inhibit HBV replication *via* RNA interference

Target gene	RNAi inducer (length)	Promoter	Vector	Model	Delivery methods	Inhib. of virus prod. (fold)	Reference
<i>In vitro</i> studies							
C	siRNA (21 bp)	-	-	Huh-7, HepG2	Transfection	-4-5	[95]
	siRNA (19 bp)	-	-	HepAD38, HepAD79	Transfection	-50	[96]
C, X	shRNA (19 bp)	hH1	Plasmid	Huh-7, HepG2.2.15	Transfection	2-20	[97]
C, S, P, X, DR	shRNA (21-24 bp)	mU6	Plasmid	HepG2.2.15	Transfection	-2	[98]
	shRNA (21 bp)	hU6	Plasmid	HepG2	Transfection	> 30	[105]
S	shRNA (19 bp)	hH1	PFV, AAV	293T.HBs, HepG2.2.15	Transduction	4-9	[99]
<i>In vivo</i> studies							
C, S, P, X	shRNA (25 bp)	hU6	Plasmid	Immunocompetent C57BL/6J mice, Immunocompromised NOD/SCID mice	Hydrodynamic transfection ¹	3-12	[100]
C, S	siRNA (21 bp)	-	-	Male NMRI mice	High-volume injection <i>via</i> tail vein ¹	-4	[101]
S	shRNA (19 bp)	hH1, hU6	Plasmid	BALB/c mice, HBsAg-transgenic FVB/N mice	Hydrodynamic transfection ²	-9	[102]
P, S, X	shRNA (20 bp)	hH1	Plasmid	C57BL/6 HBV-transgenic mice	Hydrodynamic transfection ¹	19-99	[103]
P, S, X	shRNA (NR)	mU6	Adenovirus	HBV-transgenic mice	Hydrodynamic transfection	> 9	[104]

The fold inhibition of virus production refers to the results obtained with the most potent siRNA/shRNA. All siRNAs were prepared by chemical synthesis unless indicated otherwise. C: Core antigen; S: Surface antigen; P: Polymerase; X: X protein; DR: Direct repeat element; PFV: Prototype foamy virus; AAV: Adeno-associated virus; mU6: Mouse U6; hU6: Human U6; hH1: Human H1. ¹shRNA expression plasmid/naked siRNA coinjected with the pHBV construct. ²shRNA expression plasmid simultaneously or subsequently injected with the pHBV/pSAg construct in BALB/c mice.

Table 4 Strategies designed to inhibit SARS-CoV replication *via* RNA interference

Target gene	RNAi inducer (length)	Promoter	Vector	Model	Delivery methods	Inhib. of virus prod. (fold)	Reference
<i>In vitro</i> studies							
Viral gene							
Leader, TRS, 3'-UTR, S	siRNA (21 bp)	-	-	Vero E6	Transfection	9	[110]
N	shRNA (20 bp)	U6	Plasmid	293	Transfection	NR	[112]
E, M, N	siRNA (21 bp)	-	-	Vero E6	Transfection	> 4	[113]
P	shRNA (19 bp)	H1	Plasmid	Vero	Transfection	> 100	[114]
S	shRNA (22 bp)	U6	Plasmid	Vero E6, 293T	Transfection	-6	[115]
Rep	siRNA (21 bp)	-	-	FRhk-4	Transfection	> 12	[118]
Cellular gene							
CHC	siRNA (25 bp)	-	-	HepG2, COS7	Transfection	-1	[116]
<i>In vivo</i> studies							
S, NSP12	siRNA (21 bp)	-	-	BALB/C mouse, Rhesus macaque (<i>Macaca mulatta</i>)	<i>i.t.</i> ¹ and <i>i.n.</i> ² administration	3	[119]

The fold inhibition of virus production refers to the results obtained with the most potent siRNA/shRNA. All siRNAs were prepared by chemical synthesis unless indicated otherwise. TRS: Transcription-regulating sequence; UTR: Untranslated region; S: Spike protein; N: Nucleocapsid protein; NR: Not reported; E: Envelope protein; M: Membrane protein; P: RNA polymerase; Rep: Replicase; FRhk-4: Fatal Rhesus monkey kidney cells; CHC: Clathrin heavy chain; NSP: Non-structural protein; *i.t.*: Intratracheal; *i.n.*: Intranasal. ¹siRNA and target-sequence containing reporter plasmid co-administered intratracheally into mouse lungs in D5W or Infasurf solution; ²siRNA instilled intranasally to monkey in D5W solution with different dosing regimens.

These siRNAs relieved SARS-like symptoms, and were safe for prophylaxis and therapeutic treatment. These findings greatly encouraged the clinical testing of siRNAs as an anti-SARS therapy.

Influenza virus

Influenza virus is one of the public health scourges worldwide. Three influenza epidemics have occurred in

the last century and have caused tens of millions of deaths globally. Recent outbreaks of highly pathogenic avian influenza in Asia and Europe have greatly increased public awareness, and accelerated the development of measures for the prophylaxis and therapy of this infection.

Influenza viruses are enveloped, single-stranded, segmented (7-8) RNA viruses which belong to the Orthomyxoviridae family^[121]. They are classified into

influenza virus types A, B, and C, based on their nucleoproteins and matrix proteins. Influenza A virus (IAV) is the most prevalent respiratory pathogen worldwide.

Since it is an RNA virus, IAV has the ability for rapid genetic changes through antigen drift^[122] or antigen shift^[123]. This involves the accumulation of minor mutations within the viral genome, or reassortment of RNA segments between different viruses, which results in the emergence of new viral strains. Ge *et al.*^[124,125] and Tompkins *et al.*^[126] verified the efficacy of siRNAs which specifically target the conserved regions of the influenza virus genome (nucleocapsid and acid polymerase). They confirmed that siRNAs were potent inhibitors of the influenza virus both *in vitro* and *in vivo*, and could be administered both prior to and subsequent to a lethal IAV challenge. Moreover, Ge developed an unconventional delivery system, administering small volumes of siRNAs or DNA vectors encoding shRNA in complex with polyethyleneimine (PEI) by slow intravenous infusion^[127]. This system was effective in reducing virus production in infected mice and provided helpful suggestions for future clinical application of siRNAs.

Progress of RNAi for clinical application

Since RNAi was found to have antiviral activity in transgenic plants, much evidence has emerged with regard to its pivotal role in antiviral therapeutic applications. Numerous investigations have reported successful inhibition of viral replication in cultured cells and in murine/nonhuman primate models using both transient transfection of synthetic siRNA and stable expression of shRNA. To harness the full potential of RNAi for therapeutic applications, pharmaceutical companies are actively engaged in clinical trials. In 2004, Acuity Pharmaceuticals initiated a clinical trial using RNAi in the treatment of macular degeneration; encouraging results have been obtained in the Phase I / II studies^[128]. In 2006, Alnylam Pharmaceuticals launched a Phase I clinical trial in the U.S. of an inhaled formulation of ALN-RSVO1 (an RNAi-based drug) to combat respiratory syncytial virus (RSV) infection^[129]. Other potential indications for RNAi use include asthma, Huntington's disease, spinocerebellar ataxia, and HIV, HAV, HBV and influenza virus infections, and clinical trials are under consideration in many of these conditions^[130].

Challenges and perspectives

Despite the rapid progress in RNAi use, its clinical application still poses several challenges. These include target specificity, biostability, biosafety, and delivery efficacy of the RNAi system in various diseases. Recent studies have indicated off-target effects associated with the use of siRNA^[131-133]. In order to improve the power of gene silencing and to avoid undesirable adverse effects induced by siRNAs, such as nonspecific gene silencing and immunoactivation^[134,135], great effort has been made to improve siRNA design, including its sequence^[136], size^[137] and structure^[138]. However, the poor pharmacokinetic properties of siRNAs have added another hurdle in the development of RNAi-based therapies. Multiple chemical modifications at different positions of the siRNA duplexes,

including sugars^[117,139-141], backbones^[142,143], and bases of oligonucleotides^[144,145] have been found to prolong siRNA half-life in serum. Conjugation of one or both strands of siRNAs with lipids^[146,147] and peptides^[148,149], has been shown to enhance nuclease stability and improve cellular uptake.

The systematic and site-specific deliveries of siRNA also need to be addressed. Non-viral vectors, such as cationic lipids^[150-152] and polymers^[153-156], have been widely used for *in vitro* and *in vivo* siRNA delivery. It has been reported that siRNAs encapsulated into stable nucleic acid lipid particles (SNALPs) improve the potency, lengthen the half-life, lower the effective dose and reduce the dosing frequency. This was observed in a study comparing unformulated siRNAs in rodents challenged with replicating virus^[157,158] and non-human primates^[159]. Besides, Song *et al.* designed a protamine-antibody fusion protein to deliver siRNA to HIV-infected or envelope-transfected cells. This study established a systemic, cell-type specific, antibody-mediated *in vivo* delivery system of siRNAs *via* cell surface receptors^[160]. The current advances have brought siRNA close to the era of clinical trials and real-life therapeutic applications in infected human subjects.

However, before RNAi-based clinical trials can be carried out, the toxicity and side-effects of RNAi, and the harmful potential of viral vectors need careful attention. It has been shown that over-expression of shRNA by double-stranded AAV8 viral vectors resulted in severe hepatic toxicity and even death. Moreover, it has been observed that over-expressed shRNA can saturate the miRNA pathway^[161]. Our studies have shown that simultaneous delivery of two shRNAs using a weaker expressing viral vector (AAV2) did not produce any obvious liver toxicity or side-effects (He *et al.* unpublished). Therefore, it is essential to use safer vectors and in this respect we believe that inducible viral vectors may be good candidates for future clinical studies.

Scientists in different fields, including geneticists, biochemists, pharmacologists, chemists and materials scientists, have supported the use of RNAi in clinical applications. As a part of the research force, our team while being cautious, is optimistic regarding the use of RNAi in human diseases.

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LIVER CANCER

Hepatic steatosis as a possible risk factor for the development of hepatocellular carcinoma after eradication of hepatitis C virus with antiviral therapy in patients with chronic hepatitis C

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Abstract

AIM: To elucidate risk factors contributing to the development of hepatocellular carcinoma (HCC) among patients with sustained viral response (SVR) after interferon (IFN) treatment and to examine whether HCV-RNA still remained in the liver of SVR patients who developed HCC.

METHODS: Two-hundred and sixty-six patients, who achieved SVR, were enrolled in this study. We retrospectively reviewed clinical, viral and histological features of the patients, and examined whether the development of HCC depends on several clinical variables using Kaplan-Meier Method. RT-PCR was used to seek HCV-RNA in 3 out of 7 patients in whom liver tissue was available for molecular analysis.

RESULTS: Among the enrolled 266 patients with SVR, HCC developed in 7 patients (7/266; 2.6%). We failed to detect HCV-RNA both in cancer and non-cancerous liver tissue in all three patients. The cumulative incidence for HCC was significantly different depending on hepatic fibrosis (F3-4) ($P = 0.0028$), hepatic steatosis (Grade 2-3) ($P = 0.0002$) and age (≥ 55) ($P = 0.021$) at the pre-interferon treatment.

CONCLUSION: The current study demonstrated that age, hepatic fibrosis, and hepatic steatosis at pre-interferon treatment might be risk factors for developing HCC after SVR.

INTRODUCTION

Continuous eradication of hepatitis C virus (HCV) with interferon (IFN) therapy significantly inhibits development of hepatocellular carcinoma (HCC) in patients with chronic hepatitis C^[1-3]. However, HCC sporadically developed even after achievement of sustained viral response (SVR) with IFN therapy, especially reported from Japan^[4-14], and therefore it is of clinical importance to identify patients who are at high risk for HCC after achievement of SVR.

Several investigators have made efforts to elucidate risk factors for occurrence of HCC in patients with SVR^[6,15-18], and so far several factors including age^[6,15-18], sex^[6,15], alcohol consumption^[16,17], staging of fibrosis^[15-17], platelet counts^[18], and AST levels^[18] at baseline were regarded as candidate risks. However, these results seem to be controversial, and there may be other crucial contributing factors. In addition, reappearance of HCV in serum after achievement of SVR has also been reported^[19-22]. In some cases, HCV-RNA is still present in liver of patients without detectable HCV-RNA in serum^[19-22]. Therefore, the remaining HCV-RNA in the liver may contribute to the occurrence of HCC in patients with SVR^[23]. Nevertheless, the studies^[6,15-18] describing risk factors for the occurrence of HCC lack any evaluation for the presence of HCV-RNA in the liver.

Furthermore, chronic HCV infection frequently results in hepatic steatosis^[24]. It has been reported that HCV genotype 3 infection is likely to cause steatosis possibly due to direct effect of HCV, whereas steatosis in patients infected with HCV genotype 1 seems to be associated

with co-existing risk factors including an increased body mass index (BMI) or presence of insulin resistance, thus resembling to those non-alcoholic steatohepatitis (NASH)^[25-29]. Apparently hepatic steatosis is not altered in patients infected with HCV genotype 1, even after continuous eradication of HCV with antiviral therapy^[30]. In addition, obesity-related cryptogenic cirrhosis has been recently paid attention as an alternative etiology for HCC^[31,32]. Taken together, it may be possible that hepatic steatosis, still present after achievement of eradication of HCV genotype 1, results in progression of fibrosis as well as occurrence of HCC. The investigators describing risk factors for HCC in patients with SVR^[6,15-18] have not mentioned whether hepatic steatosis is observed in pre-treatment liver histology.

Therefore, in the current study, we aimed to elucidate contributing factors for occurrence of HCC in patients in whom serum HCV-RNA has been eradicated with IFN therapy. First, we examined whether HCV-RNA still remained in the liver at the development of HCC using sensitive reverse-transcriptase polymerase chain reaction (RT-PCR). In addition, we evaluated clinical features and histopathological fibrosis and steatosis of the patients at the pre-IFN treatment, and examined whether the occurrence of HCC depends on these factors using Kaplan-Meier model.

MATERIALS AND METHODS

Patients

This single-center study was conducted at the Department of Medicine, Teikyo University School of Medicine. Since the introduction of IFN therapy for patients with chronic hepatitis C in 1986, 1101 patients were treated with IFN alone or IFN and ribavirin combination therapy. Among them, 266 patients, who achieved SVR, defined as absence of serum HCV-RNA at 6 mo after termination of IFN therapy, and did not fulfill the exclusion criteria described below, were enrolled. We verified that anti-HCV antibody as well as HCV-RNA was detected in sera before IFN treatment in all enrolled patients. Serum anti-HCV antibody was examined using third-generation antibody to HCV (Abbott Japan, Tokyo, Japan). Serum HCV-RNA was sought with Amplicor HCV v2.0 (Nippon Roche, Tokyo, Japan), employing RT-PCR. Also, liver biopsy was performed in all cases as long as 3 mo before beginning of IFN therapy for assuring the presence of chronic hepatitis. Exclusion criteria for this study were hepatitis B virus (HBV) infection determined by seropositivity for HBsAg and/or HBcAb, autoimmune hepatitis, alcoholic abuse (daily alcohol consumption > 60 g), and presence of HCC detected by abdominal ultrasound (US) and/or computed tomography (CT).

Follow-up and diagnosis of hepatocellular carcinoma

After termination of IFN therapy, patients were regularly checked up at the out-patient clinic at every 2-3 mo. The average period of follow-up for the enrolled patients in this study was 9.9 ± 4.1 years. Blood chemistries as well as tumor markers were examined. Additionally, imaging

studies, abdominal US and/or CT, were performed 1-2 times per year. The diagnosis of HCC was made if both abdominal US and CT demonstrated the presence of HCC, and was further confirmed by histopathological studies after surgical resection. In cases without resection, abdominal angiography was used to verify the diagnosis of HCC.

Variables

We selected several pre-IFN treatment valuables of patients to assess risk factors for development of HCC, including the age at pre-IFN treatment and sex of the patient, the stage of liver fibrosis and the grade of hepatic steatosis, HCV genotype and serum ALT level. The stage of liver fibrosis was determined by two independent pathologists according to the classification of the Metavir group^[33] as follows: F0 (no fibrosis); F1 (portal fibrosis); F2 (few bridges); F3 (many bridges); and F4 (cirrhosis). Hepatic steatosis was also graded by two independent pathologists according to Brunt *et al.*^[34]. In short, steatosis observed in up to 33%, 33%-66% and more than 66% of the liver histology was determined as grade 1, 2 and 3, respectively. Hepatic steatosis, if not observed, was graded as grade 0. When two pathologists estimated the classification of fibrosis and the grading of steatosis differently, the mean value of the two was applied for statistical analysis. Genotyping of HCV-RNA was performed with HCV coregenotyping (Nippon Roche).

Detection of HCV-RNA in liver tissue

HCV-RNA in liver tissue was detected by RT-PCR. Formalin-embedded liver biopsy specimens obtained prior to IFN treatment and snap-frozen liver tissue taken at the development of HCC were used as starting materials. Total RNA was extracted using Isogen (Nippon gene, Tokyo, Japan) and was converted into cDNA using Superscript™ II RNase H⁻ Reverse Transcriptase (Invitrogen, Carlsbad, CA). The primer specific to HCV-RNA 5' UTR region (HC1R: ACTCGCAAGCACCCTATCA, nt 293-312) was used for cDNA synthesis. Thereafter HCV-RNA was sought using "semi-nested" PCR consisting of 1st, 2nd and 3rd amplification, using one 5' forward primer (HC1F: GAGCCATAGTGGTCTGCGGA, nt 135-154) and three sets of reverse primers (HC1R, HC2R: ACTCGGCTAGCAGTCTTGCG, nt 240-259; and 2H-AS: GTTTATCCAAGAAAGGACCC, nt 188-207). The 1st PCR consisted of initial denaturing at 95°C for 5 min, followed by 50 amplification cycles at 95°C for 15 s for denaturing and at 62°C for 1 min for primer annealing and extension, and a final extension at 72°C for 7 min. The 2nd PCR was done for 35 cycles by the identical program, but annealing and extension at 63°C. The 3rd PCR consisted of initial denaturing at 95°C for 5 min, followed by 30 amplification cycles at 95°C for 15 s, at 58°C for 30 s for primer annealing and at 72°C for 1 min for extension, and a final extension at 72°C for 7 min. The PCR products of 178, 125 and 73 bp after 1st, 2nd and 3rd PCR, respectively, were electrophoresed on 15 g/L agarose gel, and visualized by ethidium bromide staining. In addition, to ensure the quality of extracted RNA, we also amplified β -actin as positive controls using the identical liver-derived total

Table 1 Viral, clinical and histopathological profiles of patients with SVR who developed HCC

	Age/sex	HCV genotype	Pre-IFN treatment				Occurrence of HCC			Duration until HCC (yr)
			ALT (IU/L)	Fibrosis	Steatosis	BMI	Fibrosis	Steatosis	BMI	
Case 1	67/M	1b	138	F3	Grade 2	28.4	F2	Grade 1	27.4	5.4
Case 2	58/M	2a	39	F1	Grade 1	23.5	F2	Grade 0	22.5	9.5
Case 3	68/M	n.t.	111	F4	Grade 2	24.2	F2	Grade 2	25.0	9.6
Case 4	53/M	1b	82	F2	Grade 1	19.6	NT	NT	20.3	8.4
Case 5	58/M	1b	21	F3	Grade 1	23.5	NT	NT	24.8	3.3
Case 6	65/F	2a	145	F3	Grade 1	25.6	F2	Grade 1	26.7	2.9
Case 7	54/M	1b	85	F2	Grade 2	26.1	F2	Grade 1	22.4	4.9

NT: Not tested; BMI: Body mass index. In cases 4 and 5, the liver tissues with the occurrence of HCC were not obtained because HCC was not treated with surgical operation.

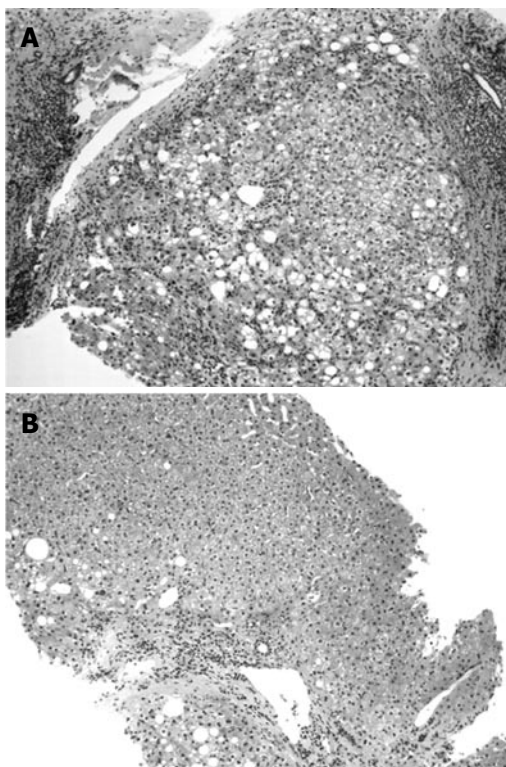


Figure 1 Liver histology of case 1 (HE staining). **A:** Pre-IFN treatment liver histology showing F3 fibrosis grade 2 steatosis; **B:** Liver histology at the occurrence of HCC showing F2 fibrosis and grade 1 steatosis.

RNA. Random primer was used for synthesis of cDNA as template for amplification of β -actin.

Statistical analysis

Statistical analysis was performed using SAS software version 9.1 (SAS Institute, Cary, NC). The kappa value was calculated to evaluate the degree of agreement in two pathologists for histological evaluation. For determining risk factors contributing the occurrence of HCC, continuous variables such as age and pre-treatment ALT value were classified into two categories: age ≥ 55 and age < 55 ; ALT > 80 IU/L and ALT ≤ 80 IU/L. Cumulative incidence for development of HCC was calculated using Kaplan-Meier method, and the differences between groups were analyzed using the log-rank test. *P* value less than 0.05 was considered statistically significant.

RESULTS

Patients with SVR who developed HCC

Of 266 patients, HCC was detected in 7 patients (6 males and 1 female) during follow-up for the patients with SVR after termination of IFN therapy. Clinical and histopathological profiles of these patients are mentioned in Table 1. The age and mean ALT value at the beginning of IFN therapy was 60.4 ± 6.2 (range, 53-68) years, and 89 ± 47 (range, 21-145) IU, respectively. The duration between IFN therapy and development of HCC was 6.3 ± 2.9 (range, 2.9-9.6) years. Undetectable serum HCV-RNA and normal ALT had been continuously maintained since IFN therapy in all patients. All patients denied excess consumption of alcoholic beverage (> 20 g/d), and were seronegative for HBsAg as well as HBcAb. The staging of liver fibrosis on pre-IFN liver biopsy was F1 in one patient, F2 in two, F3 in three and F4 in one. Hepatic steatosis was observed in all patients, grade 1 in four patients and grade 2 in three.

The comparison of liver histological findings at baseline and at the development of HCC is shown in Table 1. Overall, the grading of fibrosis was improved in cases 1, 3 and 6, unchanged in case 7, and worsened in case 2. Steatosis was decreased in cases 1, 2 and 7, and unchanged in cases 3 and 6. In cases 4 and 5, we failed to obtain liver tissue at the development of HCC since these patients were treated by transcatheter arterial embolization, instead of surgical resection of the tumor. Liver histology of case 1 before IFN therapy and at the occurrence of HCC as a representative example is shown in Figure 1.

Failure to detect HCV-RNA in HCC

In 3 patients (cases 1, 2 and 4), we tried to detect HCV-RNA in liver tissue before IFN treatment as well as at the development of HCC to elucidate whether HCV-RNA, which could remain in the liver tissue even long after successful IFN treatment^[22], might be involved in hepatocellular carcinogenesis. The tumors were surgically resected in cases 1 and 2, and the liver specimens were obtained from both the cancerous as well as non-cancerous lesion and snap-frozen at -80°C until use. In case 4, as described above, the HCC was treated by transcatheter arterial embolization, and thus the liver specimens at occurrence of HCC were not available. Instead, biopsied specimen from the liver obtained after

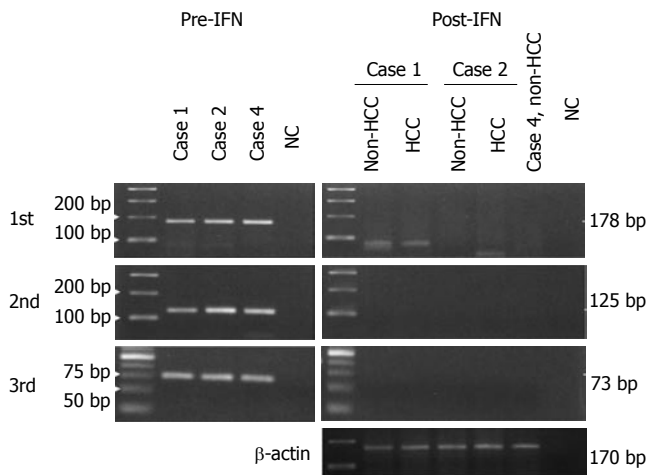


Figure 2 Amplification of HCV-RNA in liver tissues in cases 1, 2 and 4. HCV was detected in all liver tissues at the pre-IFN therapy. NC: Without template cDNA, gave no distinct band. By contrast, HCV was demonstrated in neither HCC nor non-HCC tissue in cases 1 and 2 even after 3 cycles of RT-PCR. In case 4, in whom liver tissue was obtained after IFN therapy with percutaneous biopsy, HCV was not found either. β -actin was well amplified in these post-IFN specimens, demonstrating that total RNA had not been degraded. NC: Negative control.

IFN therapy was used. HCV-RNA was successfully amplified in all specimens before IFN treatment. However, we failed to detect HCV-RNA in any sample, irrespective of cancerous and non-cancerous tissue (Figure 2). β -actin was successfully amplified in all liver tissue after IFN therapy and at HCC development (Figure 2). Therefore, we concluded that failure to detect HCV-RNA in liver tissue at development of HCC was not due to degradation of RNA, but resulted from disappearance of HCV-RNA in the liver with successful IFN treatment.

Cumulative incidence of HCC during follow-up

We demonstrated viral, clinical and histopathological profiles in all enrolled patients, the patients with and without HCC (Table 2). The cumulative incidence of HCC was analyzed for 4 variables which had been reported as risk factors before (Figure 3A-D), and pre-treatment hepatic steatosis (Figure 3E). As for the histopathological evaluation, there was an excellent agreement in the histopathological evaluation by two independent pathologists, since the kappa value was 0.70 in fibrosis and 0.78 in steatosis. The age of patients with/without HCC at baseline was 60.4 ± 6.2 and 44.8 ± 13.3 (Table 2), respectively and the patients with age ≥ 55 were at significantly higher risk for the development of HCC ($P = 0.021$, Figure 3A). The staging of pre-treatment hepatic fibrosis was available in 238 of 266 patients. The number of patients with F1, F2, F3 and F4 was 1, 2, 3, 1 in patients with HCC and 102, 97, 25, 7 in patients without HCC, respectively (Table 2). Log-rank test analysis revealed that advanced hepatic fibrosis (F3-4) was a risk factor for HCC as well ($P = 0.0028$, Figure 3C). Pre-IFN hepatic steatosis was evaluated in 231 of 266 patients, and the number of patients in grade 0, 1, 2 and 3 was 116, 95, 19 and 1, respectively. It is notable that no patient in grade 0 (no steatosis) at the IFN pre-treatment developed HCC, whereas 3 of 19 patients with grade 2 steatosis

developed HCC in the follow-up (4.9, 5.4 and 9.6 years after IFN therapy, respectively) (Table 2). Indeed, log-rank test analysis demonstrated that there was a significant difference in the grade of steatosis for cumulative incidence of HCC ($P = 0.0002$, Figure 3E). By contrast, we failed to demonstrate that sex and pre-treatment ALT value were risk factors for HCC ($P = 0.389$ and 0.251 , respectively, Figure 3C and D).

DISCUSSION

Among 266 cases, who achieved SVR with IFN therapy, 7 patients developed HCC in the follow-up period. Statistical analysis using log-rank test demonstrated that the cumulative incidence of development of HCC after SVR was significantly different depending on age and hepatic fibrosis, as previously suggested, and also on hepatic steatosis in these patients.

In the previous investigations, age^[6,15-18], sex^[6,15], alcohol consumption^[16,17], staging of fibrosis^[15-17], platelet counts^[18], and AST levels^[18] at baseline were regarded as risk factors for occurrence of HCC in patients with SVR. In the current study, we selected age, sex, ALT and staging of fibrosis as candidate contributing variables. Excess consumption of alcohol beverage was not noted in any patient with HCC and therefore was very unlikely to be a risk factor in the enrolled population. Since it is assumed that platelet counts are equivalent to staging of fibrosis and that serum ALT levels are more specific to hepatocellular injury, we adapted fibrosis and ALT values, instead of platelet counts and AST levels.

The exact determination of stage of hepatic fibrosis as well as the grade of hepatic steatosis at pre-IFN treatment is a vital premise in the current study. In this regard, grading of fibrosis and grading of steatosis were performed by two independent pathologists. The kappa values were 0.70 in fibrosis and 0.78 in steatosis, and there was a good agreement in the decision of the two. The results appeared comparable to previous reports^[18]. For instance, the proportion of the patients graded in F1, F2, F3 and F4 was 43%, 42%, 12% and 3% in the current study, and 41%, 30%, 17% and 10% in the report by Ikeda *et al.*^[18] who included 1056 patients with SVR. In addition, hepatic steatosis in our study was observed in 115 of 231 (50%) patients evaluated, and it is generally accepted that steatosis is present in 40%-60% of the patients with chronic hepatitis C^[24]. Therefore, it is conceivable to conclude that hepatic fibrosis as well as steatosis were adequately assessed in this study.

In the current study, the incidence of the patients who developed HCC after SVR was only 2.6% (7/266). Therefore, in the current study, we performed statistical analysis with only Kaplan-Meier method, and thus the effect of confounding factors among each valuable could not be excluded. In this regard, we should be reluctant to conclude that age, hepatic fibrosis and hepatic steatosis, demonstrated in this study as statistically significant, would be the only determinants of risk for developing HCC. Nevertheless, hepatic steatosis, which has never been investigated as a risk factor for developing HCC after SVR, is identified as a statistically significant factor, and

Table 2 Pre-IFN viral, clinical and histopathological profiles of all patients with SVR

	Age (mean \pm SD)	Sex (M/F)	ALT (IU) (mean \pm SD)	Genotype (1/2/NT)	Hepatic fibrosis (F1/F2/F3/F4/NT)	Hepatic steatosis (Grade 0/1/2/3/NT)
All (<i>n</i> = 266)	46.3 \pm 14.3	184/82	112 \pm 99	102/142/20	103/99/28/8/28	116/95/19/1/35
HCC (<i>n</i> = 7)	60.4 \pm 6.2	6/1	89 \pm 47	4/2/1	1/2/3/1/0	0/4/3/0/0
No HCC (<i>n</i> = 259)	44.8 \pm 13.3	178/81	113 \pm 100	98/140/19	102/97/25/7/28	116/91/16/1/35

NT: Not tested.

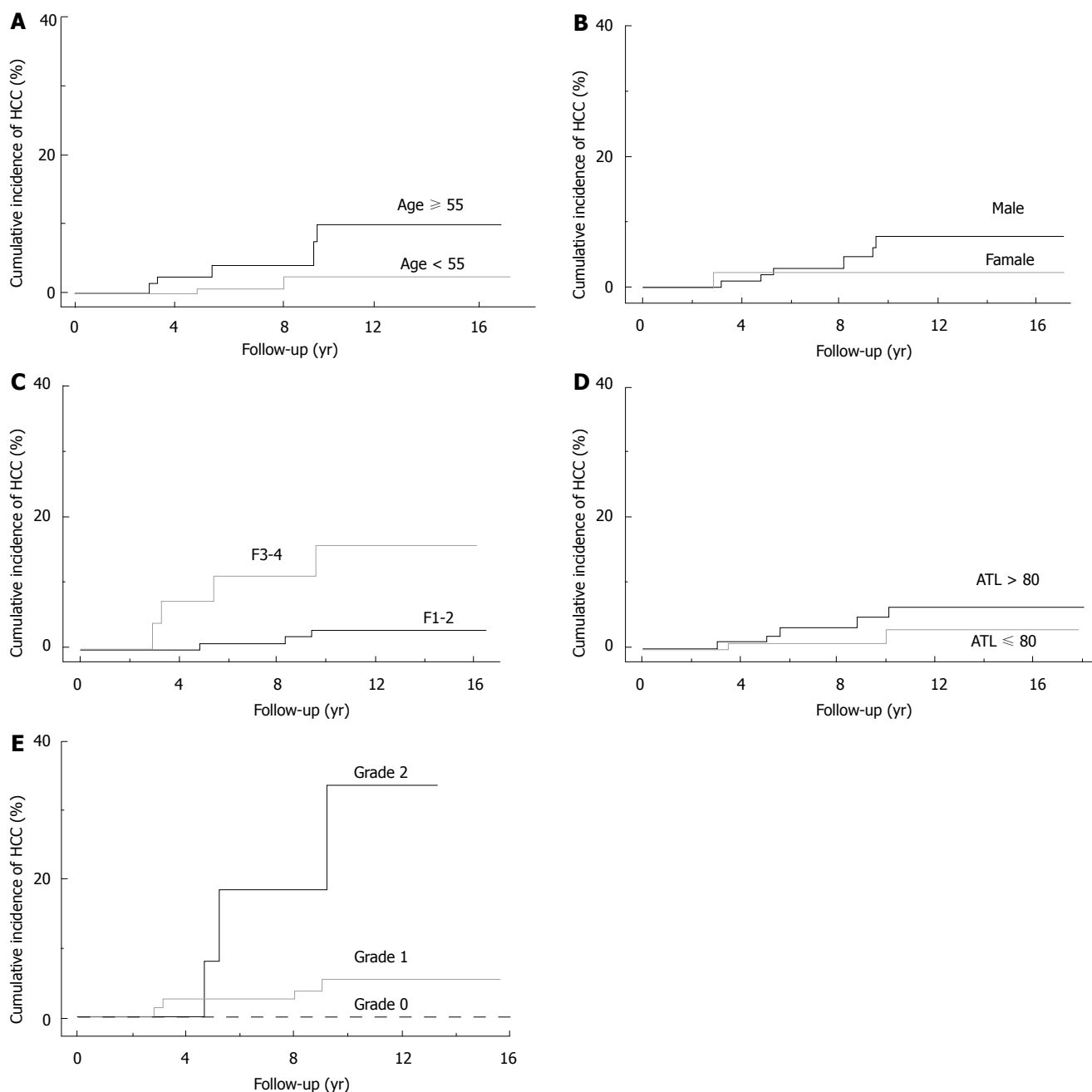


Figure 3 Cumulative incidence of HCC in SVR patients using the log-rank test. **A:** Patients with age ≥ 55 (black line) and < 55 (gray line) years ($P = 0.021$); **B:** Male (black line) and female (gray line) patients ($P = 0.389$); **C:** patients with grading of fibrosis, F3-4 (black line) and F1-2 (gray line) ($P = 0.0028$); **D:** Patients with pre-treatment ALT levels, ALT > 80 IU (black line) and ≤ 80 IU (gray line) ($P = 0.251$); **E:** Patients with the grading of hepatic steatosis, grade 2 (black line), grade 1 (gray line), and grade 0 (gray dotted line) ($P = 0.0002$ between grade 2 and grade 1 or grade 0).

we believe that it is worth evaluating its potential role for developing HCC after eradication of HCV.

Hepatic steatosis is known to be decreased after eradication of HCV in HCV genotype 3 infection.

However, HCV genotype 3 is very rare in Japan, and indeed we found no patient infected with genotype 3 among the 266 subjects enrolled in the current study. By contrast, it has been reported that hepatic steatosis with HCV genotype 1

infection is not altered after successful antiviral treatment^[30]. In the current study, among 6 patients who developed HCC after SVR and in whom HCV genotype was examined, 4 and 2 were infected with genotype 1 and 2, respectively (Table 1). Disappearance of hepatic steatosis (grade 0) after successful IFN therapy was noted only in 1 patient (case 2), infected with HCV genotype 2a; steatosis still remained in 4 of 5 patients whose histologies on the occurrence of HCC were investigated (Table 1). Thus, hepatic steatosis at baseline continuously remained after eradication of HCV, and might play a role in development of HCC, along with other factors, such as age and fibrosis in the liver.

It has been repeatedly reported that NASH caused by hepatic steatosis eventually could result in development of HCC^[31,32,35-39], and indeed NASH is regarded as an alternative etiology of HCC worldwide^[36,40,41]. It is believed that cryptogenic cirrhosis, occasionally producing HCC, may be a late complication of NASH, even though steatosis is not observed in the end-stage cirrhotic liver^[31,32]. In the current study, however, liver histology on the onset of HCC was not coincident with cryptogenic cirrhosis. Rather, the staging of fibrosis was F2 in 5 of 7 patients who developed HCC, relatively at early stage. Moreover, although hepatic steatosis was noted at the pre-IFN treatment, histological hallmarks of NASH, such as perisinusoidal/pericellular fibrosis or infiltration of polymorphonuclear cells^[42,43], were not observed in our patients. Therefore, hepatic steatosis in these patients should be regarded as non-alcoholic fatty liver diseases (NAFLD), instead of NASH. Taken together, the role of hepatic steatosis in the hepatocarcinogenesis after SVR seems to be different from those in NASH liver. Rather, the results in the current study suggest the possibility that steatosis and HCV infection, even if eradicated, would cooperatively operate for development of HCC.

We should be cautious, however, in concluding that hepatic steatosis would be a risk factor for developing HCC after SVR. First of all, multivariate analysis was not performed in this study and therefore the effect of confounding factors could not be excluded, as described above. Second, it is still controversial whether hepatic steatosis facilitates hepatocarcinogenesis in untreated patients with infection of HCV^[44,45], and prospective well-controlled studies are required to conclude the association between hepatic steatosis and development of HCC. Third, it is also notable that HCC developed within 10 years after successful eradication of HCV in the presented cases, even though almost half of the enrolled 266 patients with SVR had been followed up more than 10 years. Since hepatic steatosis would remain in a similar manner after eradication of HCV, HCC might develop in patients after more than 10 years as well. Finally, very low amount of HCV, even undetectable by RT-PCR, had still remained in the liver after SVR and might play a crucial role in hepatocarcinogenesis. Radkowski *et al.*^[22] recently demonstrated that HCV-RNA was detected in 3 of 11 patients with SVR after treatment, even though previous reports describing the occurrence of HCC in patients with SVR have repeatedly shown the absence of HCV-RNA in the tumor and non-tumor liver tissue^[8,9,12].

In conclusion, the current study demonstrates that development of HCC in patients after SVR depends on age,

hepatic fibrosis, and hepatic steatosis at pre-IFN treatment. As discussed, the remote effect of HCV infection could be more important for hepatocarcinogenesis, and a large-scale, multi-center cooperative study is required to conclude whether hepatic steatosis at baseline is a contributing factor for development of HCC. Nevertheless, it should be kept in mind that patients with hepatic steatosis at pre-IFN treatment may be at a high risk for developing HCC, and therefore should be closely monitored after SVR.

COMMENTS

Background

Continuous eradication of hepatitis C virus (HCV), i.e., sustained viral response (SVR) with interferon (IFN) therapy greatly reduces the risk of developing hepatocellular carcinoma (HCC). However, HCC was sporadically found even in patients with SVR, and it is of clinical importance to elucidate which patients with SVR are at higher risk for HCC.

Research frontiers

In the current study, we retrospectively examined 266 patients with SVR and detected 7 patients among them (2.6%) who developed HCC after SVR. RT-PCR failed to detect HCV-RNA in the liver. The cumulative incidence for HCC was significantly different depending on hepatic fibrosis ($P = 0.0028$), hepatic steatosis ($P = 0.0002$) and higher age ($P = 0.021$) at the pre-interferon treatment.

Innovations and breakthroughs

Although age and fibrosis were previously reported as risk factors, hepatic steatosis was firstly demonstrated as a possible risk in the current study.

Applications

Further large-scale study is warranted to confirm the contribution of hepatic steatosis for developing HCC after SVR. For the moment, patients with high hepatic steatosis should be closely monitored for HCC, even after SVR with successful antiviral treatment.

Peer review

This is a very interesting study. There are few papers with such histological and follow up studies in SVR and the article is well written.

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VIRAL HEPATITIS

Low-dose intermittent interferon-alpha therapy for HCV-related liver cirrhosis after curative treatment of hepatocellular carcinoma

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HCC was also higher in the non-IFN group than IFN group (6.7% and 27% vs 0 and 0% at 1- and 3-year, $P = 0.048$, respectively).

CONCLUSION: Low-dose intermittent IFN-alpha therapy for patients with HCV-related compensated cirrhosis after curative HCC treatment was effective by making patients tolerant to medical or surgical treatment for recurrent HCC in the later period of observation.

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Key words: Hepatitis C virus; Hepatocellular carcinoma; Interferon therapy; Liver cirrhosis; Liver function; Recurrence; Survival

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Abstract

AIM: To assess the efficacy of low-dose intermittent interferon (IFN) therapy in patients with hepatitis C virus (HCV)-related compensated cirrhosis who had received curative treatment for primary hepatocellular carcinoma (HCC).

METHODS: We performed a prospective case controlled study. Sixteen patients received 3 MIU of natural IFN-alpha intramuscularly 3 times weekly for at least 48 wk (IFN group). They were compared with 16 matched historical controls (non-IFN group).

RESULTS: The cumulative rate of first recurrence of HCC was not significantly different between the IFN group and the non-IFN group (0% vs 6.7% and 68.6% vs 80% at 1- and 3-year, $P = 0.157$, respectively). The cumulative rate of second recurrence was not also significantly different between the IFN group and the non-IFN group (0% vs 6.7% and 35.9% vs 67% at 1- and 3-year, $P = 0.056$, respectively). Although the difference in the Child-Pugh classification score between the groups at initial treatment of HCC was not significant, the score was significantly worse at the time of data analysis in the non-IFN group than IFN group (7.19 ± 1.42 vs 5.81 ± 0.75 , $P = 0.0008$). The cumulative rate of deviation from objects of any treatment for recurrent

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant neoplasms worldwide. Approximately 80% of Japanese patients with HCC have a history of hepatitis C virus (HCV) infection, and most such patients have liver cirrhosis^[1-3]. Although recent advances in imaging techniques and treatment of HCC have improved prognosis of patients with HCV-related HCC, the outcome is still unsatisfactory; the 5-year survival rate is only 50% to 70% even after curative treatment such as hepatic resection and local ablation^[4]. The reasons for this unfavorable prognosis is considered to include high intrahepatic tumor recurrence rates and biochemical deterioration by sustained hepatic damage, both resulting from persistent HCV infection^[5]. Even after curative hepatic resection for HCV-related HCC, the rate of intrahepatic tumor recurrence within 1 year is 20% to 40%, rising to about

80% by 5 years^[4,6-8]. Intrahepatic recurrences of HCC may result from intrahepatic metastasis originating from the primary HCC or from ongoing multicentric carcinogenesis related to chronic HCV infection. In addition, sustained underlying HCV-related hepatic damage may compromise hepatic functional reserve, worsening clinical outcome. Thus, prevention of HCC recurrence and preservation of liver function are both highly important priorities in improving prognosis of patients with HCV-related HCC.

Interferon (IFN) therapy for patients with HCV infection is effective as evident by reduction of serum alanine transaminase (ALT) activity and eradication of HCV. Accordingly, IFN is valuable in minimizing hepatic necrosis, inflammation, and fibrosis, as well as reducing the likelihood of hepatocarcinogenesis^[9-16]. The primary goal of treatment of patients with HCV infection is elimination of the virus. Several studies have reported recently that IFN therapy provided after curative treatment for HCV-related HCC prevents HCC recurrences and improves survival^[17-23]. Such improvement of prognosis is more predominant when IFN therapy results in elimination of HCV RNA^[24]. However, most patients with HCV-related HCC also have liver cirrhosis. Many centers do not advocate IFN therapy of patients with compensated cirrhosis, mainly because of the disappointing sustained virological response (SVR) rates in such patients^[25]. Several studies indicated that the response of cirrhotic patients to antiviral therapy is low^[26-28]. The reasons for the low SVR rate in such patients include inability to administer IFN at recommended doses due to adverse effects and dose-limiting cytopenia. On the other hand, several investigators suggested that the use of low-dose IFN therapy for viral elimination was as effective in the treatment of cirrhotic patients with HCV as it is in non-cirrhotic patients^[29,30]. Furthermore, they indicated that the same therapy could improve the underlying liver histology. There is evidence to suggest that low-dose IFN therapy might be beneficial in HCV-related cirrhosis, not only because it prevents the progression of liver disease, but also because it reduces the risk of hepatocarcinogenesis^[31,32]. In this regard, low-dose IFN therapy seems to be tolerable without significant life-threatening adverse effects than the standard dose of IFN.

However, it is not known whether low-dose IFN after curative treatment of primary HCC could slow disease progression or reduce the rate of clinical decompensation in cirrhotic patients, in addition to prevention of HCC recurrence. Several studies used the standard dose of IFN after HCC treatment^[17,23,33], and studies using low-dose IFN therapy for HCV-related cirrhosis after HCC treatment also reported that such regimen may reduce late recurrence of HCC^[34].

In this prospective case controlled trial, we assessed the efficacy of low-dose intermittent IFN therapy on HCV-related liver cirrhosis after curative treatment of primary HCC in terms of overall survival, HCC recurrence, and liver function.

MATERIALS AND METHODS

Patients

A total of 176 consecutive patients received their initial

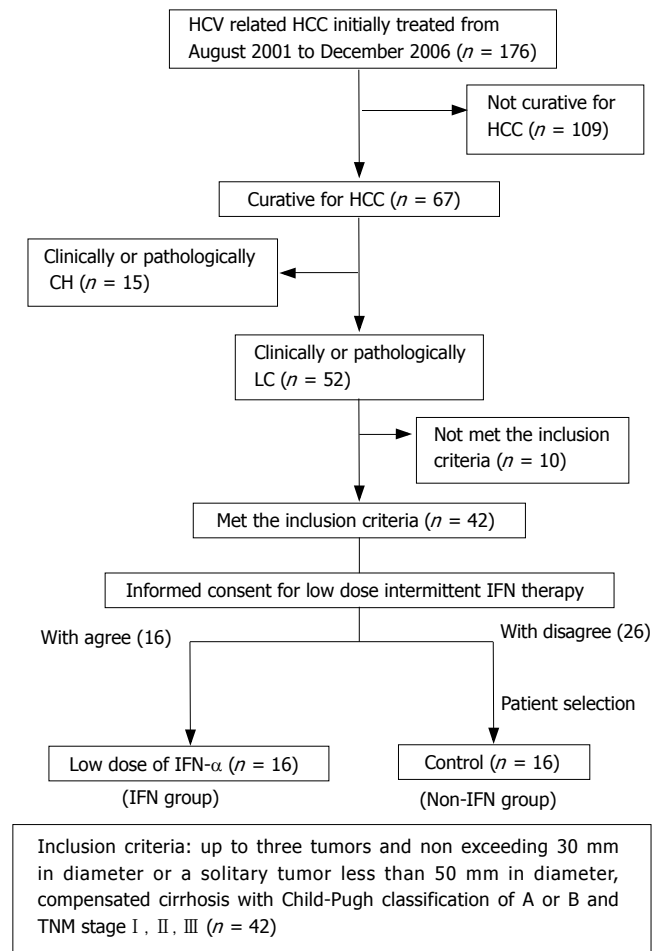


Figure 1 Schematic flow chart of enrolled patients.

treatment for HCV-related primary HCC at Hiroshima University Hospital between August 2001 and December 2006. Of these, 67 patients with HCC underwent first medical or surgical therapeutic intervention with curative intent (defined as complete tumor eradication with no visible residual tumor in computed tomographic images, or resection of all evident tumor tissue). Medical treatments included percutaneous radiofrequency (RF) ablation and ethanol injection, while surgical procedures included hepatic resection and RF ablation under laparotomy. Among these 67 patients, 52 patients with liver cirrhosis (LC), which was diagnosed clinically and pathologically, were considered for this prospective study. Figure 1 shows our study flow. Among these 52 patients with HCV-related LC, we assessed 42 patients who met the following inclusion criteria: (1) the presence of up to three tumors with none exceeding 30 mm in diameter or a solitary tumor less than 50 mm in diameter; (2) tumor-node-metastasis (TNM) stage of I, II or III; (3) detectable serum HCV RNA; (4) all seronegativity for hepatitis B marker including hepatitis B surface antigen, hepatitis B anti-core antibody and hepatitis B surface antibody; (5) compensated cirrhosis with a Child-Pugh class A or B; (6) platelet count $\geq 40000/\mu\text{L}$; and (7) absence of local recurrence during the follow-up period and of any ectopic intrahepatic recurrence within 12 wk after treatment for primary HCC. We used the TNM classification system

Table 1 Characteristics of participating patients

	Interferon group	Non-interferon group	P value
No. of patients	16	16	
Age in years (range)	68.5 ¹ (53-73)	67.5 ¹ (58-75)	NS
Gender (Male/Female)	10/6	11/5	NS
Albumin (g/dL)	3.7 ¹ (3.0-4.8)	3.7 ¹ (3.0-4.5)	NS
Platelet count ($\times 10^4$ /L)	8.0 ¹ (4.5-14.2)	8.4 ¹ (4.6-14.3)	NS
ICG R-15 (%)	17.3 ¹ (6.1-40.8)	18.2 ¹ (5-45)	NS
Alanine aminotransferase (IU/L)	59 ¹ (35-99)	58 ¹ (21-143)	NS
Alpha fetoprotein (ng/mL)	54 ¹ (5.3-293.6)	38 ¹ (5.0-1217)	NS
Child-Pugh score (A/B)	13/3	13/3	NS
Main tumor size (mm)	15 ¹ (10-50)	18 ¹ (10-40)	NS
No. of HCC tumors (single/multiple)	9/7	10/6	NS
Stage (I / II / III)	8/3/5	7/5/4	NS
Treatment (medical/surgical)	8/8	9/7	NS
HCV genotype (1/2)	12/4	14/2	NS
Viral loads (low/high)	6/10	5/11	NS

ICG-R15: Indocyanine green retention at 15 min; Low viral loads: HCV RNA < 100 KIU/mL, high viral loads: HCV RNA \geq 100 KIU/mL. ¹median.

of the Liver Cancer Study Group of Japan as a staging system for HCC^[35]. The underlying liver condition leading to LC was identified by histopathological examination of resected tissue samples. When this was not available, laboratory tests were performed including serum albumin, platelet, prothrombin time and indocyanine green retention at 15 min (ICG-R15), and radiological examination such as ultrasonography and computed tomography.

Of the 42 patients with LC who met the above eligibility criteria, 16 patients received low-dose IFN therapy after signing a written informed consent (IFN group). Of the remaining 26 patients who rejected IFN therapy, we selected 16 patients as the control (non-IFN group). These 16 patients, who met the eligibility criteria mentioned above, were matched by age, gender, tumor size, number of tumors, TNM stage of HCC, serum albumin level, platelet counts, ICG-R15 and Child-Pugh class with patients of the IFN group. Thus, a total of 32 patients (16 in the IFN group and 16 in the non-IFN group) were enrolled in this study. All agreed to participate in the research protocol, which was approved by the hospital research ethics board. Table 1 shows the baseline characteristics of patients of the two groups. The data indicates no significant differences between the groups for age, gender, liver function, tumor characteristics, and therapeutic methods used against primary HCC.

IFN therapy

In the IFN group, patients received 3 MIU of natural IFN- α (human lymphoblastoid IFN; Sumiferon, Daiinippon Sumitomo Pharmaceuticals, Osaka, Japan) intramuscularly three times weekly for at least 48 wk as long as possible. IFN therapy commenced within 12 wk after initial treatment for HCC. Patients received post-treatment IFN therapy up to the detection of HCC recurrence, and then patients who could have curative treatment for recurrent HCC restarted IFN therapy when possible. However, patients who had advanced liver dysfunction or untreatable progressive HCC did not receive IFN therapy. In the control group, none of the patients received IFN therapy after curative treatment of HCC; instead, they

were on ursodeoxycholic acid (UDCA) and stronger neomycinophagen C (SNMC).

Follow-up

After curative treatment for primary HCC, all patients underwent liver function tests, serum tumor marker assays such as alpha-fetoprotein (AFP) and protein induced by vitamin K absence or antagonist (PIVKA)-II, every month, abdominal ultrasonography every 3 mo, and dynamic computed tomography (CT) every 6 mo. If recurrences of HCC were suspected, additional examinations including CT during arteriography or tumor biopsy were performed. Recurrence of HCC was defined as any new nodules appearing as hyperattenuation by CT during hepatic arteriography or as hypoattenuation in CT performed during arteriography. Hypovascular HCC was confirmed histopathologically by fine-needle aspiration biopsy. Patients with recurrent HCC were treated medically or surgically, with curative intent if possible. Patients without curative treatment of recurrent HCC then received transcatheter chemoembolization. After repeated transcatheter chemoembolization, patients were finally unable to receive any treatment for recurrent HCC.

End points

We analyzed the outcome of this prospective study in December 2006. We compared the rate of HCC recurrence and the survival rate between IFN group and control group. We assessed whether low-dose of IFN therapy was effective in inhibiting recurrence of HCC, preserving liver function and prolonging survival. In addition, we also assessed the cumulative rate of deviation from objective of any treatment against recurrent HCC due to progression of HCC and/or underlying liver dysfunction.

Statistical analysis

The Chi-square and Fisher exact tests were used for categorical variables, while Student's *t*-test and the Mann-Whitney *U* test were used for continuous and ordinal variables, as appropriate. The Kaplan-Meier method used to assess cumulative survival and recurrence rates calculated from the date of diagnosis to the date of

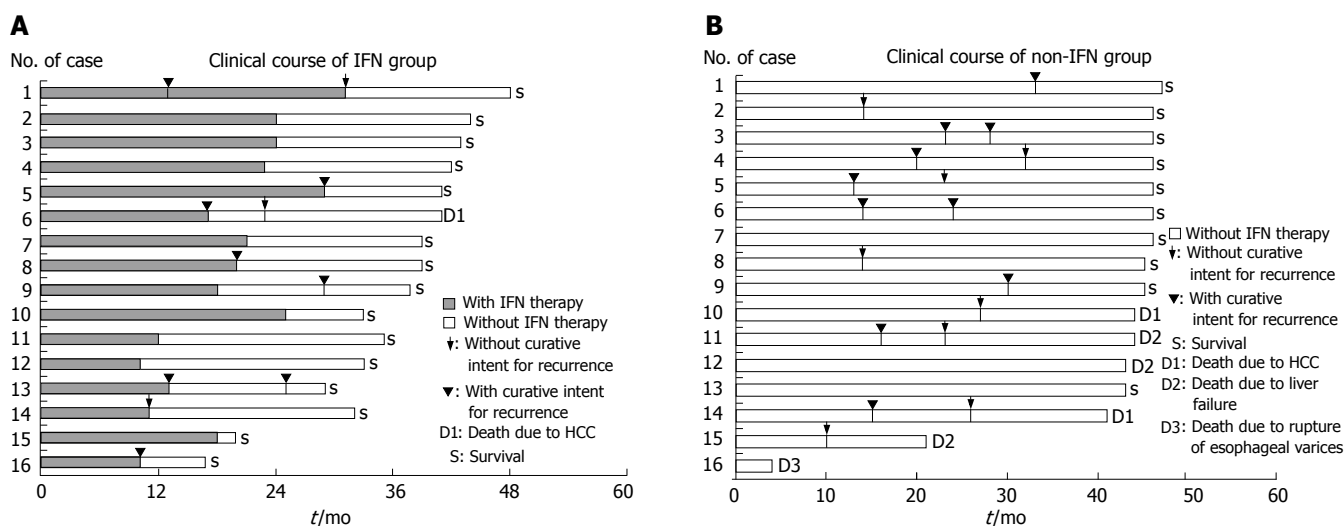


Figure 2 A: Clinical course of the interferon group. Patients who had a curative treatment for primary HCC received 3 MIU of natural interferon- α three times weekly for at least 48 wk as long as possible except Cases 12, 14 and 16. Recurrent HCCs were treated with or without curative treatment; B: Clinical course of the non-interferon group. Patients who had a curative treatment of primary HCC did not receive IFN therapy. Recurrent HCCs were also treated with or without curative treatment.

disease recurrence or death. Surviving patients and patients who died of causes unrelated to the liver were defined as censored cases, while patients who died of causes related to the liver were defined as noncensored cases. The log-rank test was used to compare survival and recurrence curves. *P* values below 0.05 were considered to indicate statistical significance. The JMP version 5.1 statistical software package (SAS Institute, Cary, NC) was used for analysis of data.

RESULTS

Clinical course of IFN group

Figure 2A shows the clinical course of 16 patients of the IFN group from the initial treatment of primary HCC to the date of data analysis. The duration of low-dose IFN therapy ranged from a minimum of 10 mo to a maximum of 25 mo (median 16 mo). Although 8 patients did not have HCC recurrence, HCC recurred in 8 patients after initial treatment of HCC during a median follow-up period of 37 mo. Of the recurred patients, 7 developed HCC recurrence during IFN therapy (Cases 1, 5, 6, 8, 13, 14 and 16) except 1 patient (Case 9) who had HCC recurrence after discontinuation of IFN therapy. Of the 8 patients with HCC recurrence, 4 were treated with surgical resection therapy (Cases 5, 9, 13 and 16), 3 patients with percutaneous RF ablation therapy (Cases 1, 6 and 8) and 1 patient transcatheter chemoembolization (Case 14). Of these patients, a patient with transcatheter chemoembolization (Case 14) could not have curative treatment and repeated transcatheter chemoembolization. He was excluded from the study concerning the next recurrence. Of the 7 patients with curative treatment for HCC recurrence, 2 restarted IFN therapy, one continued IFN therapy until next recurrence (Case 1), which was not curative, and the other continued until intolerant generalized fatigue (Case 8). The remaining 5 patients (Cases 5, 6, 13, 14 and 16) were followed without IFN therapy because of rejection of

IFN therapy. Although one of these 5 patients was not curative for first recurrence (Case 14), he was tolerant to repeated transcatheter chemoembolization and was still alive at the date of data analysis. Two patients without curative treatment at the second recurrence (Cases 1 and 6) were also relatively tolerant to the repeated medical treatment such as transcatheter chemoembolization. Of these patients, one died of progression of HCC in spite of repeated transcatheter chemoembolization and hepatic arterial infusion (Case 6), another was alive at the date of data analysis (Case 1). Of 3 patients without curative treatment of HCC, two survivors' status of HCC were not progressive (stage II and stage III) and underlying liver function could be tolerant to the treatment such as transcatheter chemoembolization because of relatively preserved function (Cases 1 and 14).

The 16 patients who received IFN therapy included 2 patients with virological response (Cases 2 and 3) and 14 patients who did not get SVR [3 transient responders (Cases 8, 9 and 11), and 11 non-responders (Cases 1, 4, 5, 6, 7, 10, 12, 13, 14, 15 and 16)]. Among the 14 patients who did not show SVR, 8 were biochemical responders with normalized ALT (Cases 1, 4, 5, 7, 9, 10, 13 and 16), including 4 transient responders and 4 non-responders. Two sustained virological responders who received IFN therapy for 96 wk have viral characteristics of genotype 1 and low viral load. Among the patients who did not show SVR, 7 discontinued IFN treatment because of recurrence of HCC, while 2 patients restarted IFN therapy after the curative treatment of recurrent HCC. None of the patients who received IFN therapy developed life-threatening side effects.

Clinical course of non-IFN group

Among the non-IFN group, the first recurrence of HCC occurred in 13 patients during a median follow-up period of 45 mo (Figure 2B). HCC recurred in 6 of the 7 non-IFN patients who had a sustained normalized ALT. Of the 13 patients with recurrent HCC among the non-IFN group, 4 were treated with hepatic resection (Cases 1, 4, 9 and 11),

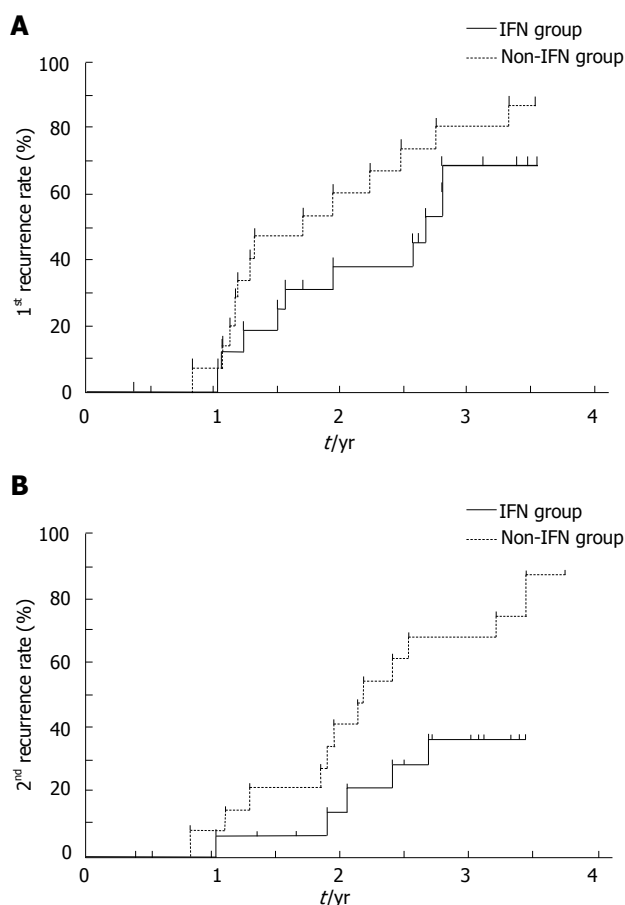


Figure 3 A: Cumulative rate of first recurrence. Rates of first recurrence for the IFN and non-IFN groups. The rate of first recurrence of HCC in the IFN group was not significantly different from that of the non-IFN group ($P = 0.157$); B: Cumulative rate of second recurrence. Rates of second recurrence for the IFN and non-IFN group. The rate of second recurrence of HCC in the IFN group was not significantly different from that of the non-IFN group ($P = 0.056$).

6 with local ablation including percutaneous RF ablation or ethanol injection (Cases 3, 5, 6, 7, 10 and 14) and 3 with transcatheter chemoembolization (Cases 2, 8 and 15). Of the 13 recurrent patients, 5 patients (2 received ethanol injection and 3 transcatheter chemoembolization) could not be treated curatively and was excluded from the study concerning the next recurrence. These 5 patients were treated repeatedly with transarterial chemoembolization after first recurrence. Among the remaining 8 patients who were treated curatively for first recurrence, 7 developed a second recurrence (Cases 3, 4, 5, 6, 9, 11 and 14). Among these 7 patients with second recurrence, 2 were treated curatively for HCC [1 with RF ablation (Case 3) and 1 with hepatic resection (Case 6)], while the remaining 5 patients were not (4 patients due to uncontrolled multiple HCC and one patient due to underlying liver dysfunction). The latter group of 5 patients received transarterial chemoembolization repeatedly after second recurrence.

Comparison of the first and second recurrence rates of HCC

We compared the overall cumulative rates for first and second recurrence between IFN and non-IFN groups (Figure 3). The 1-, 2- and 3- year rates of first recurrence

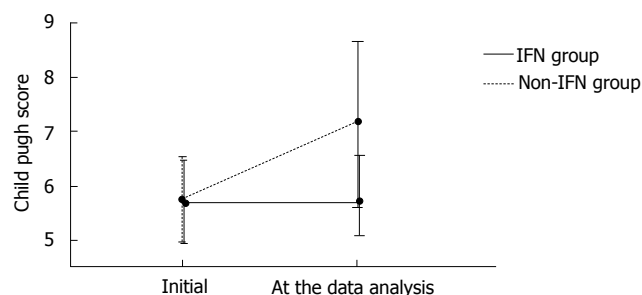


Figure 4 Effect of IFN therapy after curative treatment of HCC on Child-Pugh scores. IFN-treated patients were less likely to show deterioration of hepatic function. The average scores of Child-Pugh of the IFN group were significantly better preserved than the non-IFN group ($P = 0.0008$).

of HCC in the IFN and non-IFN group were not different (0% vs 6.7%, 38.1% vs 60% and 68.6% vs 80%, respectively, Figure 3A, $P = 0.156$). The 1-, 2- and 3-year rates of second recurrence in the IFN and non-IFN groups were 0% vs 6.7%, 13.5% vs 33.3% and 35.9% vs 67%, respectively (Figure 3B, $P = 0.056$).

Liver function

Patients of the IFN group were less likely to develop worsening of hepatic dysfunction compared with the non-IFN group. We compared the average score determined for Child-Pugh classification at initial treatment of HCC with that at the time of data analysis (Figure 4). Although the difference in the Child-Pugh classification score between the two groups at initial treatment of HCC was not significant, the score was significantly worse at the time of data analysis in the non-IFN group than IFN group ($P = 0.0008$).

Deviation from objects of any treatments for recurrent HCC

At the date of data analysis, patients who developed recurrent HCC were treated repeatedly, as possible, for the purpose of curative treatment including surgical resection and ablative therapy such as RF ablation and ethanol injection. Patients who were difficult to treat with curative intent received transcatheter chemoembolization or hepatic arterial infusion. Although patients with recurrent HCC received repeated treatments, some patients finally could not be treated because of excessive progression of HCC or liver dysfunction. Figure 5 shows that the cumulative rate of deviation from objects of any treatment for recurrent HCC between the IFN group and non-IFN group. In the IFN group, one patient could not receive treatment due to progressively advanced HCC in later period. On the other hand, 8 patients in the non-IFN group could not receive treatment because of underlying liver dysfunction ($n = 2$) and progressively advanced HCC ($n = 6$). The 1-, 2- and 3- year rates of deviation from objects of any treatment for recurrent HCC in the IFN and non-IFN group were 0% vs 6.7%, 0% vs 20% and 0% vs 27%, respectively ($P = 0.048$). Thus, the IFN group tended to be treatable for recurrent HCC compared with the non-IFN group.

Survival of patients

At the date of data analysis, 1 patient among the IFN

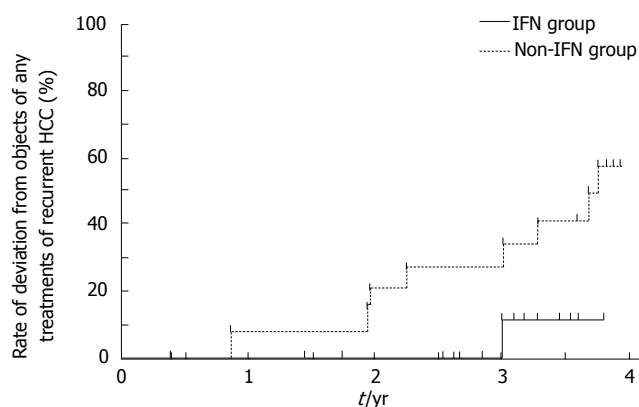


Figure 5 Cumulative rate of deviation from objects of any treatment of recurrent HCC. Recurrent HCC tended to be treatable later in the IFN group than non-IFN group ($P = 0.048$).

group and 6 patients among the non-IFN group had died of liver disease. Of the 8 recurrence patients among the IFN group, 1 died of advanced multiple HCC and none died of liver failure. On the other hand, of the 13 recurrence patients among the non-IFN group, 2 died of advanced HCC and 2 died of liver failure in spite of the relatively early stage of HCC. Among the 3 patients without recurrent HCC of the non-IFN group, 1 died of liver dysfunction and 1 died of ruptured esophageal varices.

With regard to the cumulative survival rates of the IFN and non-IFN groups (Figure 6), the respective rates of survival were 100% *vs* 93.7% at 1 year, 100% *vs* 87.5% at 2 years, 100% *vs* 87.5% at 3 years and 83.3% *vs* 61.4% at 4 years. Thus, the cumulative survival rate was not significantly different between the two groups for first 4 years after curative treatment of HCC ($P = 0.45$). The median survival time following the first treatment of HCC was 37 mo (range, 17 to 45) for the IFN group and 45 mo (range, 4 to 47) for the non-IFN group.

DISCUSSION

HCC recurrence is still a risk even if HCV-related HCC is treated with curative intent. Most of such patients with HCC have underlying liver cirrhosis, and deterioration of underlying hepatic function may be a hindrance to treatment of recurrent HCC and be associated with prognosis. The present prospective case controlled study of cirrhotic patients shows that low-dose intermittent IFN therapy after curative treatment of HCC could preserve liver function and increase the chance of treatment for recurrent tumor.

Previous studies indicated that IFN therapy after curative treatment of HCC was effective in inhibiting or delaying the development of recurrent HCC^[17-23,34,36]. Although several recent studies have reported the efficacy of chemoprevention with IFN therapy after treatment of HCV-related HCC, the basis of the benefit was not clear. Shiratori *et al.*^[23,33] and Ikeda *et al.*^[17] reported that IFN therapy in cirrhotic patients reduced recurrence of HCC and improved prognosis. Although they used standard IFN dosage per time, there are no other reports on the effect of

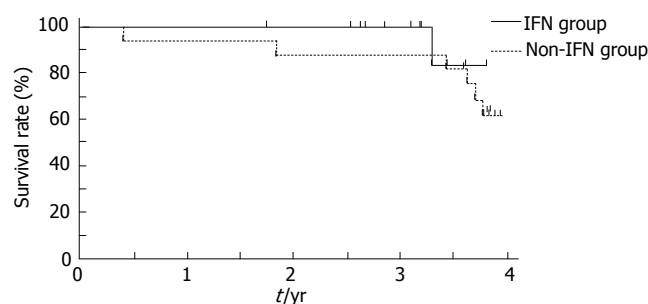


Figure 6 Cumulative survival rate. Comparison of the cumulative survival rates of the IFN and non-IFN groups. The cumulative survival rate was not significantly different between the two groups ($P = 0.45$).

low-dose IFN therapy after curative treatment of primary HCC in cirrhotic patients. Sakaguchi *et al.*^[21] reported that low-dose, long-term, intermittent IFN therapy in patients who had curative HCV-related HCC suppressed recurrence of HCC and improved survival, though it was not clear whether their patients had underlying liver cirrhosis or not. On the other hand, Mazzaferro *et al.*^[34] indicated that low-dose intermittent IFN therapy seemed to reduce late recurrence in patients with HCV-related cirrhosis after resection of HCC. Considered together, these results suggest that low-dose IFN therapy is potentially useful for cirrhotic patients when used as long as possible. However, our results of low-dose intermittent IFN therapy showed no significant difference in recurrence between those who received IFN therapy and those who did not. Unfortunately, since the difference in treatment outcome between the above three studies might be due to the use of different IFN regimens (e.g., dosage and frequency), and background characteristics of cirrhotic patients (e.g., performance status), the results varied and no standard IFN regimen to pursue after curative treatment of HCV-related HCC could be advocated.

The design of the present study was not randomized controlled type, and differed in details of the IFN protocol and characters of patients from the other studies. Although there was no significant difference in the recurrence rate between the IFN and non-IFN groups, the recurrence rate in the later period of observation including second recurrence appeared to be lower in patients with IFN therapy. Furthermore, the recurrent HCC in patients on IFN therapy did not seem to be aggressive compared with that in patients without IFN therapy, probably because they could be treated with curative intent during the observation period. Thus, low-dose intermittent IFN therapy seemed to have delayed or reduced the chance of development of recurrent HCC in the later period of observation, although IFN did not completely inhibit HCC recurrence in our cirrhotic patients.

Most cirrhotic patients cannot receive a standard full-dose IFN regimen due to underlying liver dysfunction and unfavorable complication such as cytopenia. Hence, it could be difficult to achieve SVR in most cirrhotic patients on low-dose intermittent IFN therapy. Valla *et al.*^[37] performed a randomized, controlled trial of IFN- α 2b but the results showed a lack of any benefits in terms of sustained biochemical response, liver function test

results, histology, occurrence of decompensation or HCC, or prolongation of survival. On the other hand, Everson and coworkers^[29,30] suggested that the use of low-dose IFN therapy for viral elimination was as effective in the treatment of cirrhotic patients with HCV as it is in non-cirrhotic patients. Several recent studies have reported that IFN therapy following HCC treatment improved liver function of patients with HCV-related HCC, although it is not clear which specific IFN action is important for these benefits. We also demonstrated that preservation of liver function was significantly better in the IFN group than in the non-IFN group even when HCV was not completely eradicated. Thus, hepatic functional preservation increases the chance of treatment for recurrent. Therefore, the cumulative rate of deviation from objects of any treatment for recurrent HCC might be lower in patients with IFN therapy than in patients without IFN therapy as we showed that low-dose IFN resulted in less advanced recurrence and hepatic functional preservation. Although the survival rates were not significantly different between the two groups in our observation period, we need a longer observation to determine differences in survival rates. Although we also assessed the correlation between the observed beneficial effects of the low-dose intermittent IFN therapy and HCV genotype, we could not reach the clear conclusion due to small sample size. In the future, the study with large sample size may be needed to conclude.

In our study, only about 12.5% (2/16) of patients who received IFN therapy had sustained viral elimination. And there were no significant difference in population of patients with normalized ALT between the IFN and non-IFN group ($n = 10$, $n = 7$, respectively). In spite of these results, patients treated with low-dose intermittent IFN therapy have a hepatic functional preservation greater than IFN untreated patients who received continuous medication with UDCA or SNMC after curative treatment of HCC. Although the mechanism of this reason is not well known, we suggested that the anti-inflammatory activity by low-dose intermittent IFN therapy may be stronger than medication with UDCA or SNMC and induce regression or retardation of underlying hepatic fibrosis, and finally, inhibits the progression of hepatic dysfunction.

Adverse effects such as reduction in blood counts by low-dose of IFN- α were not observed in our study, although neutropenia and/or thrombocytopenia were identified before IFN therapy. Furthermore, none of the patients required dose reduction in our study. Although 4 patients discontinued IFN therapy because of generalized fatigue, 2 of these patients restarted IFN therapy after that. Therefore, low-dose intermittent IFN- α therapy can be used relatively safely for cirrhotic patients with thrombocytopenia. However, patients who can not receive even low-doses of IFN also exist due to severe cytopenia or advanced liver cirrhosis. Medication with UDCA or SNMC or phlebotomy may be useful in decreasing ALT level for those patients.

Most cirrhotic patients who had received curative treatment for primary HCC have a limited hepatic reserve or thrombocytopenia. Therefore, low-dose intermittent IFN therapy might be effective for better prognosis. However, further studies of larger samples followed-up for

longer periods should be conducted to establish a definite conclusion about the effect of low-dose IFN therapy for the prevention of progressive liver disease and effect of treatment for recurrent HCC.

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H. pylori

A novel phenol-bound pectic polysaccharide from *Decalepis hamiltonii* with multi-step ulcer preventive activity

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Abstract

AIM: To investigate H^+ , K^+ -ATPase inhibition, anti-*H. pylori*, antioxidant, and the *in vivo* antiulcer potential of a pectic polysaccharide from Swallow root (*Decalepis hamiltonii*; SRPP).

METHODS: SRPP, with known sugar composition [rhamnose: arabinose: xylose: galactose in the ratio of 16:50:2:32 (w/w), with 141 mg/g of uronic acid] was examined for anti-ulcer potency *in vivo* against swim/ethanol stress-induction in animal models. Ulcer index, antioxidant/antioxidant enzymes, H^+ , K^+ -ATPase and gastric mucin levels were determined to assess the anti-ulcer potency. Anti-*H. pylori* activity was also determined by viable colony count and electron microscopic studies.

RESULTS: SRPP, containing phenolics at 0.12 g GAE/g, prevented stress-induced gastric ulcers in animal models by 80%-85%. Down regulation of gastric mucin 2-3 fold, antioxidant/antioxidant enzymes and upregulation of 3 fold of H^+ , K^+ -ATPase in ulcerous animals were normalized upon treatment with SRPP. Histopathological analysis revealed protection to the disrupted gastric mucosal layer and epithelial glands. SRPP also inhibited H^+ , K^+ -ATPase *in vitro*, at an IC_{50} of 77 μ g/mL as opposed to that of 19.3 μ g/mL of Lansoprazole and *H. pylori* growth at Minimum Inhibitory Concentration (MIC) of 150 μ g/mL. In addition, free radical scavenging (IC_{50} -40 μ g/mL) and reducing power (3200 U/g) activities were also observed.

CONCLUSION: SRPP, with defined sugar composition and phenolics, exhibited multi-potent free radical scavenging, antioxidant, anti-*H. pylori*, inhibition of H^+ , K^+ -ATPase and gastric mucosal protective activities. In addition, SRPP is non-toxic as opposed to other known anti-ulcer drugs, and therefore may be employed as a potential alternative for ulcer management.

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INTRODUCTION

Ulcer is a common global problem with increasing incidence and prevalence. Worldwide 14.5 million people have ulcers with a mortality of 4.08 million (<http://digestive.nidk.nih.gov/statistics/statistics.htm>/peptic ulcer prevalence). The increasing incidence and prevalence of ulcers have been attributed to several factors encountered during day-to-day life, such as stress^[1], exposure to bacterial infection^[2], and use of non-steroidal anti-inflammatory drugs (NSAIDs)^[3]. Indeed, NSAIDs are used daily by approximately 30 million people world wide, constituting a world market in excess of \$2 billion. Associated serious side effects are ulceration and gastric bleeding, which are due to inhibiting cyclooxygenase-1 activity that is required for mucosal protection^[4]. Gastric lesions develop due to loss of the delicate balance between gastro-protective and aggressive factors. Reduction in gastroprotective factors, such as mucus, bicarbonate secretion and gastric mucosa-blood flow, and enhancement of aggressive factors, such as increase of acid/pepsin secretion and *H. pylori* infection, results in gastric ulceration^[1,2]. Mucosal damage, an initial step in ulcer development, has been known to be due to oxidative stress (OS) by Reactive Oxygen Species (ROS), hypersecretion of HCl through H^+ , K^+ -ATPase action^[5], harboring of *H. pylori* on the damaged mucin layer^[6], and the blockade of the cyclooxygenase enzyme system by NSAIDs^[4], as depicted in Figure 1.

A modest approach to control ulceration, therefore, is *via* stimulation of gastric mucin synthesis, enhancement of antioxidant levels in the stomach, scavenging of ROS, inhibition of H^+ , K^+ -ATPase and *H. pylori* growth^[7]. Although the antisecretory drugs, such as H^+ , K^+ -ATPase pump inhibitors-omeprazole, lansoprazole; histamine H_2 -receptor blockers-ranitidine, famotidine, are being used to control acid secretion and acid related disorders; however, they are not the drugs of choice since they produce

potential adverse effects on human health^[8].

In light of the above, it is pertinent to study natural products from food/plants as potential anti-ulcer compounds. Due to the lack of side effects compared to synthetic drugs, approximately 60% of the world's population relies entirely on such natural medications. In Indian traditional medicine, several plants have been employed to treat gastrointestinal disorders, including gastric ulcers^[9]. Antiulcer properties have been attributed generally to phenolics^[10,11] and occasionally to polysaccharides^[12-14] of plant extracts.

In this paper we report a pectic polysaccharide from *Decalepis hamiltonii* (Swallow root) containing a sulfonamide group and phenolics as an effective antiulcer compound *in vivo*. We envisage a multi-potent role for this phenolic-polysaccharide in the upregulation of mucin, antioxidant levels, modulation of oxidative status, inhibition of H⁺, K⁺-ATPase activity against swim and ethanol stress-induced ulcers in experimental animal models, in addition to its ability to inhibit *H. pylori*. This paper reveals the potency and multi-step action of phenolic polysaccharide in preventing gastric ulceration.

MATERIALS AND METHODS

Chemicals

Monoclonal anti-gastric mucin from Sigma Chemicals (St. Louis, MO, USA), Ham's F-12 media from HiMedia (Mumbai, India), Alkaline phosphatase conjugated-rabbit anti mouse IgG secondary antibody from GENEI (Bangalore, India). All other reagents were of analytical grade purchased from Qualigens fine chemicals (Mumbai, India).

Plant

Decalepis hamiltonii Wight & Arn. (*Asclepiadaceae*) roots (Batch No. 6, 2005) were procured from a local vendor at Devaraja market, Mysore, India, originally collected from the Gumballi forest range located between 11-13 N and 77-78 E, South-East corner of Mysore district in July 2005 and identified by a taxonomist in the herbarium of Vivekananda Girijana Kalyana Kendra, B.R. Hills, Chamaraja Nagar, Karnataka, India, where a voucher specimen is deposited.

Isolation of pectic polysaccharide from swallow root

Pectic polysaccharide was isolated from defatted powder of swallow root as described previously^[15]. Briefly, 10 g of defatted powder were depleted with proteins, amylose and amylopectins by specific enzymatic (protease, termamylase and glucoamylase) digestions at their optimum reaction conditions and centrifuged. Further, the residue was extracted with 200 mL of 0.25% (w/v) ammonium oxalate solution and filtered; the filtrate was precipitated by ethanol at 4°C. The precipitate was resuspended in 10 mL of water and lyophilized to obtain pectic polysaccharide. Total sugar content was estimated by a Phenol-sulphuric acid method. A total yield of 6% was obtained as carbohydrate and this pectic polysaccharide of swallow root is designated as SRPP. Sugar composition analysis revealed the presence of rhamnose: arabinose: xylose: galactose in the ratio 16:50:02:32, in addition to 141 mg uronic acid/g of SRPP.

Determination of the phenolic content and antioxidant activity of SRPP

Since phenolics are generally found to be associated with polysaccharides, we evaluated the phenolic content in SRPP using a Folin-Ciocalteu reagent as described previously^[16]. Gallic acid was used as a standard for the generation of a calibration curve. Total phenolic content is expressed as Gallic Acid Equivalents (GAE) in mg/g of SRPP.

The reducing power and free radical scavenging activity of SRPP was determined according to the method described previously^[16]. SRPP at 10-100 µg was employed for determining the reducing power and free radical scavenging activity. Capability to scavenge the DPPH radical was calculated using the following equation.

Scavenging effect (%) = (Absorbance of control at 517 nm - Absorbance of sample at 517 nm) / Absorbance of control at 517 nm × 100

Characterization of SRPP by Fourier transform infra-red spectroscopy (FTIR)

Pectic polysaccharides, particularly with sulfur, also have been shown to exhibit antioxidant activity and SRPP was subjected to FTIR study. The samples were prepared in the form of pellets by mixing with dry KBr. Potassium bromide discs containing 1% (w/w) of film material were scanned at 4 mm/s with a resolution of 4/cm over 400-4000/cm, averaging over 128 scans for each type of film and determined the presence of sulphur group.

Inhibition of H⁺, K⁺-ATPase in vitro

Fresh sheep stomach was obtained from a local slaughterhouse at Mysore and an enzyme extract was prepared^[17]. The enzyme extract was incubated with different fractions of swallow root polysaccharide, in a reaction mixture containing 16 mmol/L Tris buffer (pH 6.5). The reaction was initiated by adding substrate (2 mmol/L ATP, 2 mmol/L MgCl₂ and 10 mmol/L KCl) and after 30 min of incubation at 37°C, the reaction was stopped by the addition of an assay mixture containing 4.5% ammonium molybdate and 60% perchloric acid. Inorganic phosphate formed was measured spectrophotometrically at 400 nm. Enzyme activity was calculated as micromoles of Pi released per hour at various doses of SRPP.

Toxicity studies

Toxicity studies were carried out in Albino Wistar rats that were orally fed with SRPP at 1 mg/kg b.w. for 15 d. Analysis showed biochemical changes as described previously^[17].

Assessment of antiulcer potential of SRPP against swim/ethanol stress induced ulcers

Wistar albino rats, weighing about 180-220 g and maintained under standard conditions of temperature, humidity and light, were provided with standard rodent pellet diet (Amruth feeds, Bangalore, India) and water *ad libitum*. The study was approved by the institutional ethical committee, which follows the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals, Reg. No. 49, 1999), Government of India, New Delhi, India.

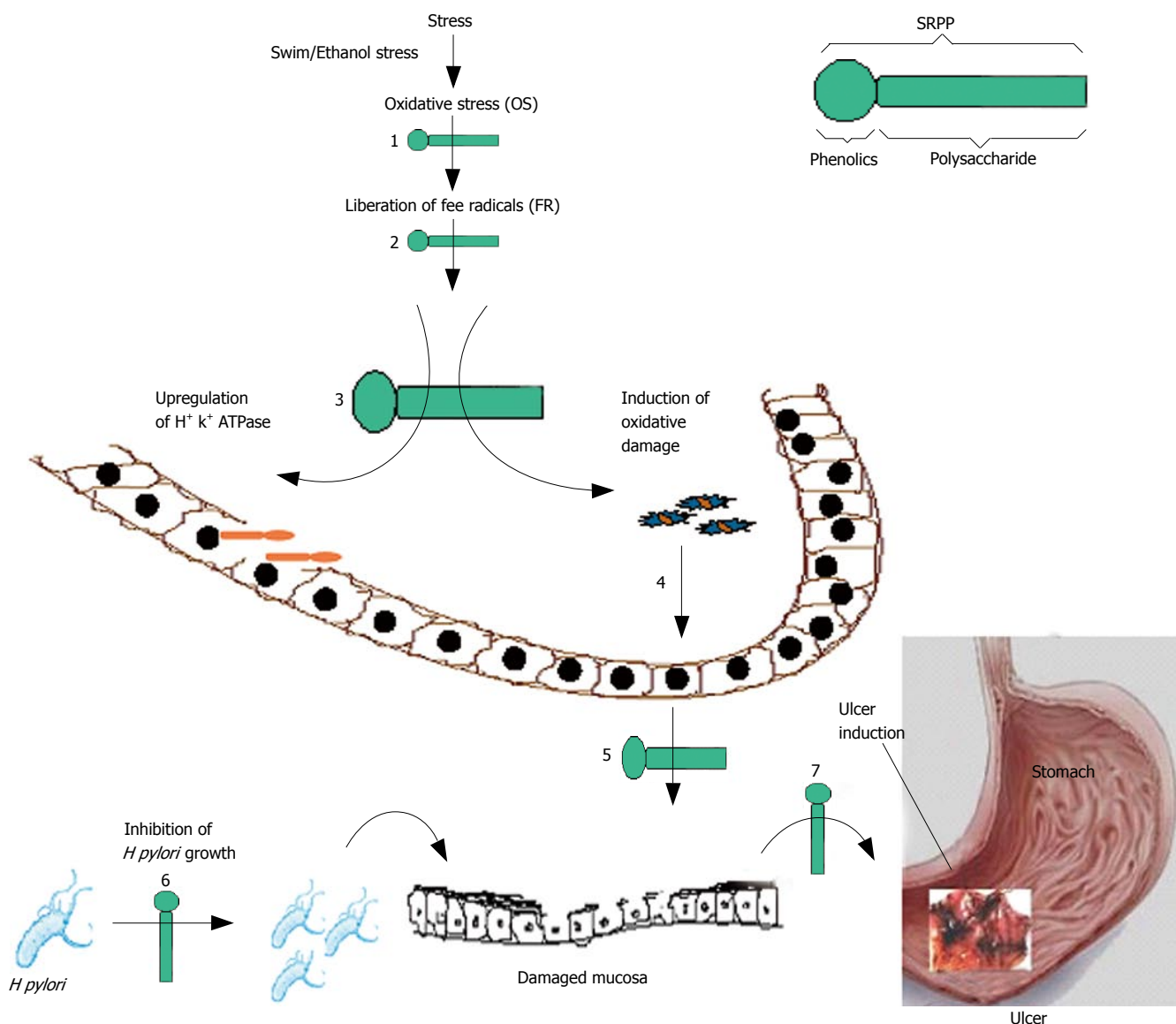


Figure 1 Scheme representing various steps of ulcer pathogenicity and multi-step anti-ulcer action by SRPP (*); (*) and (-) represents phenolic and polysaccharide portions of SRPP respectively. Swim/Ethanol stress leading to OS (1) and liberation of FR (2). FR upregulated H⁺, K⁺-ATPase (3) and induced oxidative damage to mucosa (4) leading to mucosal damage (5). *H. pylori* may invade on to damaged mucosa and together may cause ulcers (7). SRPP has ability to inhibit steps 1-7 including the growth of *H. pylori* in vitro (6).

All the animals were categorized into two sets of five groups with 6 animals in each group ($n = 6$). SRPP and ranitidine at indicated doses were administered orally twice daily for 14 d. At the end of 14 d, animals were fasted for 18 h and then subjected to the ulcer inducing treatment. In the first set, ulcers were induced by forced swim stress per a published protocol^[18], in which rats were briefly subjected to forced swim stress by making them swim in a jar of 30 cm height and 10 cm diameter containing water up to 15 cm height for 3 h. In the second set, ulcers were induced in rats by administering 100% ethanol (5 mL/kg b.w.) for 1 h^[19]. Animals were sacrificed under deep ether anesthesia and stomachs were examined for mucosal integrity and occurrence of ulcers. Lower to higher gradings were assigned to milder to severe symptoms, respectively. The following are descriptions of ulcer scores: 0.5-red coloration, 1.0-spot ulcers, 1.5-hemorrhagic streaks, 2.0-ulcers more than 3 mm and less than 5 mm, 3.0-ulcers more than 5 mm. Mean ulcer scores for each experimental

group were calculated and expressed as the ulcer index (UI)^[20]. Stomach/liver tissues were used for enzyme assays. Serum was collected from the blood of all animals and analyzed for various biochemical parameters.

Preparation of extracts from tissues for biochemical analysis

The stomach and liver tissues were collected, weighed and homogenized in chilled Tris-buffer (10 mmol/L, pH 7.4) at a concentration of 5% (w/v). The homogenates were centrifuged at $1000 \times g$ at 4°C for 20 min using a high speed cooling centrifuge (REMI C 24, Mumbai, India). The clear supernatant was used to analyse biochemical parameters^[21].

Assessment of gastric mucin and H⁺, K⁺-ATPase

Gastric mucin was isolated from the glandular segments of stomach and quantitated employing a monoclonal anti-human gastric mucin antibody (MAb-GM) by ELISA^[5], as well as by Alcian blue dye binding methods^[22]. Histological

Table 1 Toxicity studies with Swallow root pectic polysaccharide ($n = 6$) mean \pm SD

Parameters	Control	SRPP treated
Total protein	348 ^a \pm 32.21	361.81 ^a \pm 28.10
SGOT (U/mg protein)	18.34 ^a \pm 1.55	16.22 ^a \pm 1.34
SGPT (U/mg protein)	21.31 ^a \pm 2.70	23.21 ^a \pm 2.29
ALP (U/mg protein)	35.52 ^a \pm 3.879	33.62 ^a \pm 2.95
TBARS (n moles/mg protein)	0.166 ^a \pm 0.08	0.186 ^a \pm 0.11

SGPT: Serum glutamate pyruvate transaminase; SGOT: Serum glutamate oxaloacetate transaminase; ALP: Alkaline phosphatase; TBARS: Thiobarbituric acid reactive substances. ^a $P < 0.05$ between control and SRPP treated groups.

and immunohistological evaluation was done as described previously^[17]. Equal weight of gastric tissue from animals of each group was homogenized using Tris-HCl buffer pH 7.4. The gastric membrane vesicles enriched in H⁺, K⁺-ATPase were prepared and the activity was assessed as described above.

Assessment of oxidant/antioxidant and antioxidant enzymes

Lipid peroxidation products of serum, stomach and liver homogenates were determined as TBARS and the malondialdehyde (MDA) that formed was quantitated using the molar extinction coefficient of the MDA molecule^[21].

Glutathione (GSH) content was determined as described by Das and Banerjee^[21]. The activity of superoxide dismutase (SOD) was assayed by measuring the reduction in the NBT in the presence of SOD^[23] and catalase (CAT) was assayed by decomposition of H₂O₂ in the presence of catalase at 240 nm^[24]. Glutathione peroxidase (GPx) was estimated based on the degradation of H₂O₂ in the presence of GSH and the decrease in absorbance was read at 340 nm^[25]. SOD and CAT activity was expressed as units per milligram protein per min. The activity of GPx was expressed as nanomoles of NADPH oxidized per min per milligram of protein. The protein content of the homogenate was determined by Lowry's method^[26].

Determination of anti-*Helicobacter pylori* activity of SRPP

H. pylori is a major ulcerogen, and anti-*H. pylori* activity was therefore determined. *H. pylori* was obtained by endoscopic samples of gastric ulcer patients from KCDC (Karnataka Cardio Diagnostic Centre, Mysore, India) and cultured on Ham's F-12 agar medium supplemented with 5% FBS at 37°C for 2-3 d in a microaerophilic condition^[27]. *H. pylori* culture was characterized by specific tests as described by Siddaraju and Shylaja^[16].

Anti-*H. pylori* activity of an aqueous solution of SRPP (200 µg/mL) was determined by a viable colony count method^[28]. 100 µL of SRPP treated *H. pylori* were also processed for scanning electron microscopy (SEM)^[29] and examined by SEM (Model No. LEO 425 VP, Electron microscopy LTD, Cambridge, UK) with an acceleration voltage of 20 KV. Multiple fields of visions were viewed. The MIC value was determined by a conventional broth dilution method^[16]. MIC was defined as the lowest

concentration to restrict the growth to less than 0.05 absorbance units at 625 nm.

Statistical analysis

All values are expressed as mean \pm SE. Significance was calculated with student's *t* test (parametric). When several groups were compared, significance was calculated using an ANOVA. Enzyme estimations were carried out as described and results were tabulated. After calculating means and standard deviations, Dennett's test was performed to obtain the significance between the treated groups and the control groups. A value of $P < 0.05$ was considered to indicate a significant difference.

RESULTS

Toxicity studies

Toxicity studies with aqueous solution of SRPP were carried out in rats for safety evaluation. These studies indicated no lethal effect up to 1 g/kg b.w. when orally fed for 14 d. There were no significant differences in total protein, TBARS levels, SGPT, SGOT and ALP between normal and SRPP treated rats (Table 1), indicating no adverse effect on the major organs. After the above treatment schedules, animals remained as healthy as control animals with normal food and water intake, body weight gain and behavior.

SRPP prevented swim stress/ethanol induced gastric ulcer

Healthy rats showed no lesions in their stomachs (Figure 2A), while rats treated with forced swim stress for 3 h or ethanol stress showed damage in the gastric wall with a hemorrhagic form of lesions and intraluminal bleeding (Figure 2B and C). Rats treated with only SRPP (Figure 2D) also showed no lesions, which is similar to the controls. Oral treatment of SRPP at 100 and 200 mg/kg b.w., as well as Ranitidine at 30 mg/kg b.w., showed protection in a dose dependent manner with no intraluminal bleeding and an insignificant number of gastric lesions (Figure 2E-I). Ranitidine protected both ethanol/swim stress-induced ulcers up to 66%-87% at 30 mg/kg b.w., while SRPP protected up to 80%-85%, respectively, indicating an ulcer preventive effect. Quantitative reduction in the ulcer index (%) in treated rats, compared to either ulcer induced or healthy, is depicted in Figure 3.

SRPP prevents gastric mucosal damage; Alcian blue binding/ELISA/histo-and Immunohistological studies

Gastric wall mucus is damaged during ulcer development and becomes the first target of stress-induced reactive oxygen species. Mucin oxidation or degradation takes place and subsequently loses the protective effect. In the current study, we evaluated the effect of *in vivo* ingestion of SRPP on protection of gastric wall mucus during ulceration induced by swim/ethanol stress. Since Alcian blue binds to carboxylated mucopolysaccharides as well as sulfated and carboxylated glycoproteins, any disruption results in reduction in the dye binding, which can be quantitated. The gastric mucin of stomach tissue was decreased to 17 and 16 mg/g in swim stress/ethanol stress-induced ulcerous rats, respectively, when compared to that of controls (45

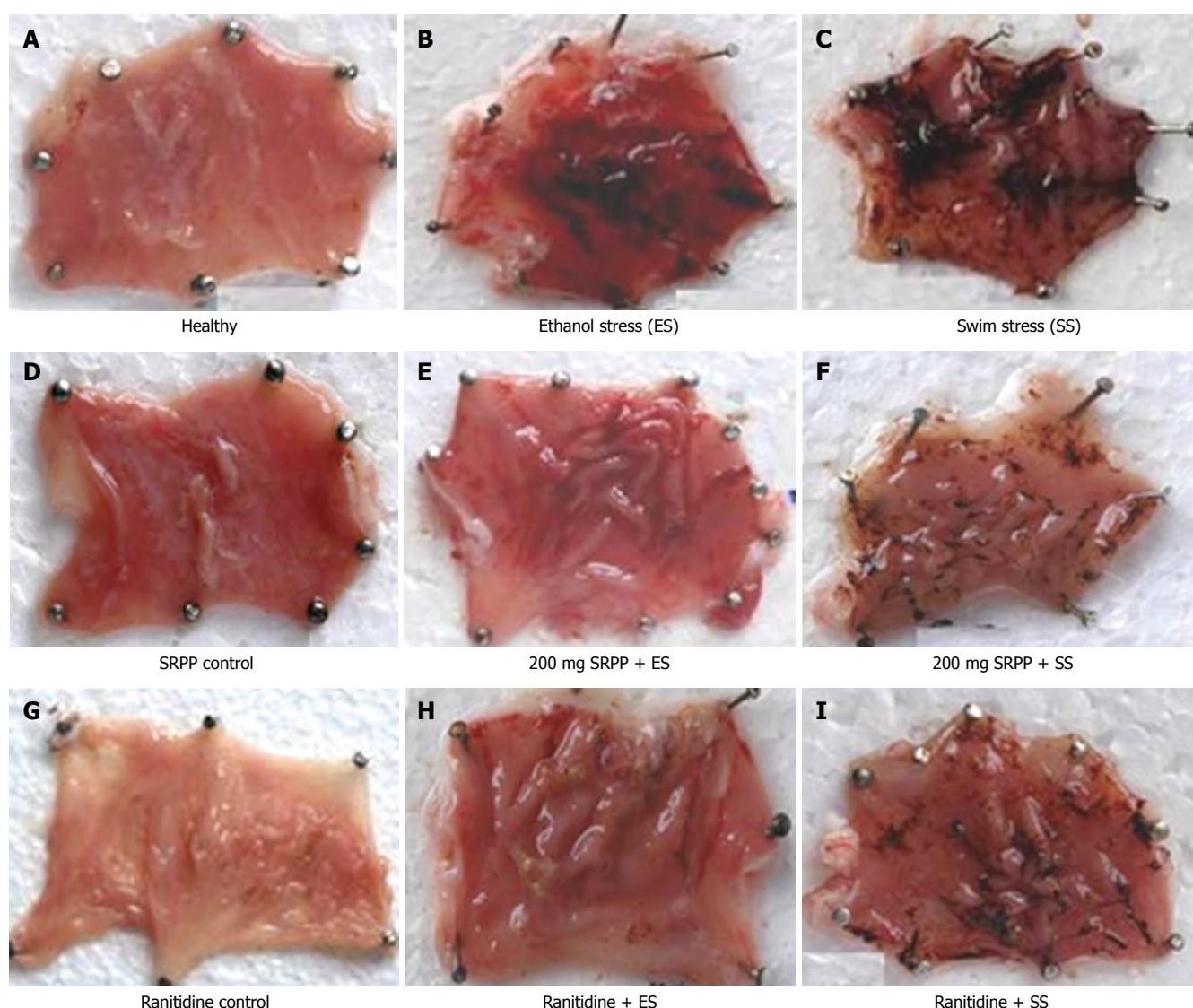


Figure 2 Macroscopic observation of Ulcers in ulcer induced/protected stomachs in swim stress/ethanol stress induced ulcer models; Ulcer was induced in animals by either swim stress (SS) or ethanol stress (ES) in group of pretreated/untreated animals at indicated concentrations. In healthy (A), SRPP control (D), Ranitidine control (G)-no ulcer lesions or damage in the stomach tissue were observed. In ethanol stress (B) and swim stress (C) induced animals ulcers score were very high. SRPP (E and F) and ranitidine (H and I) treated animals showed reduced stomach lesions.

mg/g) (Table 2). Results were substantiated by observing increased gastric mucin content by monoclonal antibody-based immuno histological studies (Figure 4). Hematoxylin and eosin staining of stomach tissue sections in control animals indicated intact structures (Figure 4A). Ulcer induction showed damage in the mucosal epithelium, destruction of regular glandular organization, very high inflammatory exudates, proliferated fibroblasts, infiltration of leucocytes and cellular debris (Figure 4B). SRPP and ranitidine treated rats showed recovery in the mucosal epithelium, regained glandular structure and mucosal regeneration (Figure 4C and D). Immunohistological analysis clearly revealed the intact mucosal epithelium in the control group (Figure 4E) and complete loss or eradication in ulcer-induced tissue sections (Figure 4F). Complete recovery was observed upon treatment with SRPP (Figure 4G) and partial recovery is depicted during ranitidine treatment (Figure 4H).

Evaluation of SRPP potential on oxidant and antioxidant status in ulcerous and treated animals

Tables 3 and 4 indicate antioxidant, antioxidant enzymes and TBARS levels in stomach/liver homogenate and the serum of swim/ethanol stress models. SOD and GPx levels increased in stomach (2 fold) and CAT and GSH decreased (1.8 fold) during stress-induced ulcerous conditions and were normalized upon treatment with SRPP in a dose dependent manner. An approximately 4 fold increase in TBARS levels depicts lipid peroxidation or damage of stomach tissue in ulcerous animals and was recovered up to 80% upon treatment with SRPP. Ranitidine, although showing protection against ulcer, showed no significant improvement in GSH or antioxidant enzyme levels. Similar changes in antioxidant enzymes except catalase was also observed in serum and liver homogenates. A 2 fold increase in TBARS levels was shown in the ulcer condition and, SRPP treatment at 200 mg/kg b.w. showed up to 90% recovery.

Inhibition of *H pylori*

Initially, anti-*H pylori* activity was assayed by a viable colony

Groups of animals (<i>n</i> = 6)	Control	Ulcer induced	SRPP 100 mg/kg b.w.	SRPP 200 mg/kg b.w.	Ranitidine 50 mg/kg b.w.
Swim stress mean ulcer index \pm SE	000.0 \pm 0.00	86.0 \pm 6.8	52.2 ^a \pm 5.1	13.3 ^b \pm 1.4	11.2 ^b \pm 1.5
Protection (%)	-	0	40	85	87
Ethanol stress mean ulcer index \pm SE	000.0 \pm 0.00	67.4 \pm 3.2	28.2 ^a \pm 2.5	13.4 ^b \pm 1.8	22.4 ^b \pm 1.2
Protection (%)	-	0	58	80	66

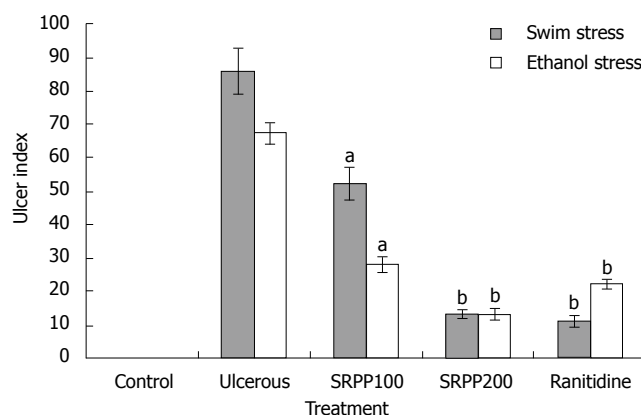


Figure 3 Effect of SRPP on gastric lesions in swim/ethanol stress induced ulcer models; Ulcers were scored as described under the methods and expressed as ulcer index. Maximum ulcer index observed during stress induction was controlled in a concentration dependent manner. Reduction in ulcer index and percent protection is depicted. ^a*P* < 0.05 and ^b*P* < 0.01 between ulcerated and treated groups.

Table 2 Gastric mucin and H⁺, K⁺-ATPase levels in healthy, ulcerated and protected rats (*n* = 6) mean \pm SD

Group, <i>n</i> = 6	Mucin content (mg/g)	H ⁺ , K ⁺ -ATPase (μ moles Pi released/mg/h)
Healthy	45.04 ^d \pm 4.128	0.807 ^d \pm 0.072
Swim stress induced ulcer model		
Swim stress induced	17.78 ^a \pm 2.557	2.209 ^a \pm 0.152
SRPP 100 mg/kg b.w.	27.13 ^b \pm 4.082	1.771 ^b \pm 0.081
SRPP 200 mg/kg b.w.	35.35 ^c \pm 3.221	1.601 ^b \pm 0.091
Ranitidine 50 mg/kg b.w.	31.42 ^{bc} \pm 2.327	1.621 ^b \pm 0.092
Ethanol stress induced ulcer model		
Ethanol stress induced	16.32 ^a \pm 3.821	2.621 ^a \pm 0.211
SRPP 100 mg/kg b.w.	32.13 ^b \pm 3.457	2.123 ^b \pm 0.241
SRPP 200 mg/kg b.w.	39.53 ^{bc} \pm 3.082	1.512 ^c \pm 0.121
Ranitidine 50 mg/kg b.w.	37.13 ^b \pm 1.507	1.485 ^c \pm 0.124

Different letters a to d in the column represents that values are significantly different when compared between ulcer induced with healthy control and SRPP/Ranitidine treated groups. Range was provided by Duncan multiple test at *P* < 0.05. a: Less significant; b: Moderately significant; c: Very significant and d: Most significant.

count method. SRPP showed up to 95% inhibition at a 200 μ g/mL concentration, which is equivalent to that of a susceptible antibiotic amoxicillin at 10 μ g/mL. MIC, determined by a broth dilution method, indicated significant anti-*H. pylori* activity at 55 μ g/mL (*P* = 0.003) (Figure 5A).

SEM observations

Normal *H. pylori* shows uniform rod shaped cells (Figure 5B), whereas the cells treated with SRPP (200 μ g/mL) changed from a helical form to coccoid and became necrotic (showed in arrows in Figure 5C). A similar coccoid form was observed with *H. pylori* treated with amoxicillin (Figure 5D) and this form has been known to result in a loss of infectivity^[30]. A coccoid form with blebs in the bacterial surface,

appearance of vacuoles, granules and an area of low electron density in the cytoplasm (shown in arrow marks) were observed in SRPP treated samples indicating the lysis of *H. pylori*. Substantiating this viable colony test indicates the loss of more than 95% viability upon treatment with SRPP, supporting an antimicrobial nature of SRPP.

Effect of SRPP on H⁺, K⁺-ATPase activity

An approximately 3 fold increase in H⁺, K⁺-ATPase activity in ulcer-induced stomach homogenate was brought to normal levels in a concentration dependent manner by SRPP at 100 and 200 mg/kg b.w. Approximately 58% and 62% (1.5 fold) were reduced at 200 mg/kg b.w. in both ethanol and swim stress-induced ulcer models (Table 2).

To further validate the inhibition of H⁺, K⁺-ATPase enzyme by SRPP, sheep stomach parietal cells were used. Inhibition of H⁺, K⁺-ATPase *in vitro* was examined with different polysaccharide fractions of swallow root including SRPP. Only SRPP inhibited H⁺, K⁺-ATPase activity, with an IC₅₀ of 77 μ g as opposed to that of Lansoprazole (19.3 μ g), whereas other polysaccharide fractions did not show inhibitory activity (Figure 6A).

Characterization of SRPP, an antiulcer compound from swallow root and its relation to antiulcer activity

FTIR spectra obtained using a FTIR spectrometer (Perkin-Elmer 2000 spectrophotometer) equipped with TGS detector with solid samples at a concentration of 1-10 mg provides a signal at 1329 and 1145 cm⁻¹ indicating the presence of sulfonamides where sulfate may be found attached to aminosugars of pectic polysaccharide (Figure 6B).

We evaluated its phenolic content and subsequently its antioxidant property. 0.12 g GAE/g of SRPP yielding 12% of phenolics in SRPP is intriguing since this is the first report of pectic polysaccharides containing such a high level of phenolics. This could be due to the presence

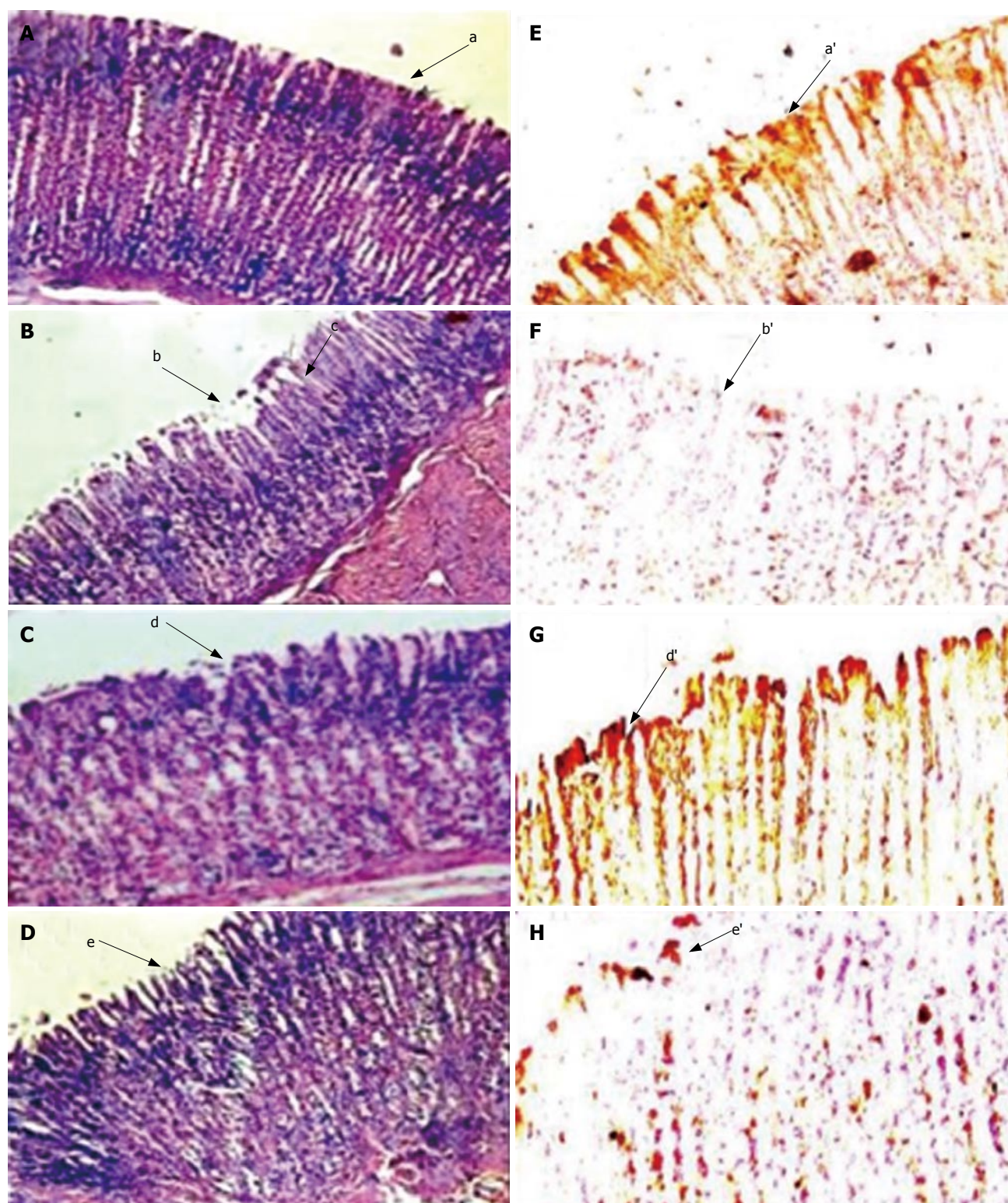


Figure 4 Histopathologic/Immunohistopathologic observation of stomach from ulcer induced/SRPP and Ranitidine treated animals; **A-D** indicates HE staining sections ($\times 40$), while **E-H** reveal anti-gastric mucin stained sections ($\times 40$, and magnified the selected portion in computer photoshop). Control (**A, E**) shows intact mucosal epithelium with organized glandular structure (**a**) and intense brown staining for gastric mucin by antibody (**a'**). Ulcer induction (**B, F**) showed damaged mucosal epithelium (**b**) and disrupted glandular structure (**c**), loss of brown staining (**b'**) in figure **F** indicate the loss of gastric mucin. Complete recovery of mucosal damage (**d** and **d'** of **C, G**) by SRPP and partial recovery by ranitidine (**e** and **e'** of **D, H**) treatments were observed.

of higher levels of phenolics (34 mg/g) in swallow root per se. Presence of higher levels of phenolics was substantiated by expression of potent reducing power ability with 3200 absorbance Units/g of SRPP (Figure 6C). In addition, dose dependent free radical scavenging activity was also found (Figure 6D) with an IC_{50} of 40 $\mu\text{g/mL}$.

DISCUSSION

Recently, phytomedicines from medicinal plants and nutraceuticals from food sources have become attractive sources of new and natural drugs. However, the active

Table 3 Antioxidant/antioxidant enzymes and TBARS levels in swim stress induced ulcer model ($n = 6$) mean \pm SD

Parameters	Protein (mg/g)	SOD (U/mg)	Catalase (U/mg)	Glutathione Peroxidase (η moles/g)	GSH (nmoles/mg)	TBARS η moles
Stomach						
Healthy	2.23 ^c \pm 0.21	9.86 ^a \pm 1.1	829.2 ^c \pm 41.6	0.21 ^a \pm 0.009	224 ^c \pm 10.0	0.31 ^a \pm 0.01
Ulcerated	1.95 ^a \pm 0.13	19.10 ^c \pm 1.8	462.4 ^a \pm 30.2	0.49 ^d \pm 0.01	121 ^a \pm 18.9	1.12 ^c \pm 0.20
SRPP 100 mg/kg	1.90 ^a \pm 0.09	16.32 ^{b,c} \pm 2.1	488.1 ^{a,b} \pm 32.8	0.34 ^b \pm 0.02	174 ^b \pm 22.1	0.94 ^c \pm 0.10
SRPP 200 mg/kg	2.10 ^b \pm 0.19	13.06 ^b \pm 2.6	679.6 ^b \pm 9.9	0.22 ^a \pm 0.01	208 ^c \pm 16.5	0.55 ^{a,b} \pm 0.00
Ranitidine	2.16 ^b \pm 0.22	15.22 ^b \pm 1.2	505.5 ^{a,b} \pm 35.5	0.39 ^c \pm 0.01	136 ^a \pm 12.1	0.92 ^b \pm 0.10
Serum						
Healthy	6.62 ^a \pm 0.51	112.3 ^a \pm 28	44.20 ^c \pm 4.9	0.221 ^a \pm 0.004	23.6 ^c \pm 3.0	0.165 ^a \pm 0.01
Ulcerated	6.84 ^a \pm 0.53	264.6 ^d \pm 32	22.90 ^a \pm 3.1	0.286 ^c \pm 0.02	11.1 ^a \pm 1.8	0.326 ^d \pm 0.02
SRPP 100 mg/kg	6.35 ^a \pm 0.59	201.1 ^c \pm 36	28.63 ^b \pm 2.3	0.298 ^d \pm 0.03	16.5 ^b \pm 2.1	0.261 ^c \pm 0.03
SRPP 200 mg/kg	6.95 ^a \pm 0.48	168.2 ^b \pm 21	40.12 ^c \pm 3.8	0.268 ^b \pm 0.03	19.8 ^{b,c} \pm 12.9	0.162 ^a \pm 0.01
Ranitidine	6.35 ^a \pm 0.63	196.3 ^{b,c} \pm 23	30.82 ^b \pm 2.9	0.226 ^a \pm 0.02	12.8 ^a \pm 2.6	0.186 ^b \pm 0.01
Liver						
Healthy	24.2 ^c \pm 0.31	261.5 ^b \pm 41	28.42 ^d \pm 3.1	0.32 ^a \pm 0.02	414 ^c \pm 51	0.98 ^a \pm 0.13
Ulcerated	21.9 ^a \pm 0.23	142.4 ^a \pm 18	22.18 ^{b,c} \pm 2.6	0.58 ^c \pm 0.05	221 ^a \pm 26	2.41 ^d \pm 0.23
SRPP 100 mg/kg	23.1 ^b \pm 0.28	164.2 ^a \pm 13	19.63 ^{b,c} \pm 2.4	0.36 ^{a,b} \pm 0.03	315 ^b \pm 36	1.84 ^c \pm 0.16
SRPP 200 mg/kg	23.9 ^b \pm 0.28	361.5 ^d \pm 39	15.54 ^a \pm 2.1	0.28 ^a \pm 0.02	214 ^a \pm 24	1.26 ^b \pm 0.11
Ranitidine	23.6 ^b \pm 0.26	314.4 ^{c,d} \pm 36	17.34 ^a \pm 1.9	0.32 ^a \pm 0.02	254 ^a \pm 28	1.41 ^b \pm 0.12

SOD: Superoxide dismutase; GSH: Glutathione; TBARS: Thiobarbituric acid reactive substances. Different letters a to d in the column represents that values are significantly different when compared between ulcer induced with healthy control and SRPP/Ranitidine treated groups.

Table 4 Antioxidant/antioxidant enzymes and TBARS levels in ethanol induced ulcer model ($n = 6$) mean \pm SD

Parameters	Protein (mg/g)	SOD (U/mg)	Catalase (U/mg)	Glutathione Peroxidase (η moles/g)	GSH (U/mg)	TBARS η moles
Stomach						
Healthy	2.23 ^a \pm 0.21	09.86 ^a \pm 1.1	829.2 ^c \pm 41.6	0.21 ^a \pm 0.009	224 ^d \pm 23.2	0.31 ^a \pm 0.1
Ulcerated	2.32 ^a \pm 0.09	17.86 ^c \pm 2.4	201.5 ^a \pm 18.9	0.30 ^c \pm 0.01	102 ^a \pm 12.6	1.26 ^d \pm 0.3
SRPP 100 mg/kg	2.16 ^a \pm 0.16	16.21 ^c \pm 1.0	193.3 ^a \pm 62.5	0.26 ^b \pm 0.01	162 ^b \pm 15.5	0.92 ^c \pm 0.1
SRPP 200 mg/kg	2.41 ^a \pm 0.20	11.09 ^b \pm 1.0	540.5 ^b \pm 40.2	0.33 ^c \pm 0.02	196 ^c \pm 16.4	0.54 ^b \pm 0.1
Ranitidine	2.42 ^a \pm 0.19	12.42 ^b \pm 1.4	468.6 ^c \pm 31.6	0.22 ^a \pm 0.03	152 ^b \pm 16.3	0.96 ^c \pm 0.2
Serum						
Healthy	6.62 ^a \pm 0.51	112.3 ^a \pm 28	44.20 ^c \pm 4.9	0.221 ^a \pm 0.04	23.6 ^d \pm 3.0	0.165 ^a \pm 0.01
Ulcerated	6.52 ^a \pm 0.69	282.3 ^d \pm 26	28.36 ^a \pm 3.2	0.315 ^c \pm 0.03	09.6 ^a \pm 1.2	0.465 ^d \pm 0.03
SRPP 100 mg/kg	6.35 ^a \pm 0.70	228.4 ^c \pm 32	34.25 ^{a,b} \pm 3.3	0.286 ^b \pm 0.03	18.6 ^c \pm 2.2	0.321 ^c \pm 0.04
SRPP 200 mg/kg	6.24 ^a \pm 0.56	172.3 ^b \pm 2	39.60 ^b \pm 4.51	0.243 ^b \pm 0.02	18.2 ^c \pm 1.9	0.181 ^a \pm 0.02
Ranitidine	6.32 ^a \pm 0.69	210.7 ^c \pm 28	34.12 ^{a,b} \pm 4.6	0.252 ^b \pm 0.03	14.6 ^b \pm 1.6	0.214 ^{a,b} \pm 0.02
Liver						
Healthy	24.2 ^a \pm 0.31	261.5 ^b \pm 1.1	28.42 ^c \pm 3.1	0.32 ^b \pm 0.02	414 ^c \pm 51	0.98 ^a \pm 0.13
Ulcerated	24.3 ^a \pm 0.31	118.1 ^a \pm 16	19.64 ^b \pm 2.2	0.48 ^{b,c} \pm 0.03	392 ^{b,c} \pm 41	2.98 ^d \pm 0.31
SRPP 100 mg/kg	23.5 ^a \pm 0.21	121.8 ^a \pm 15	18.32 ^b \pm 1.6	0.39 ^b \pm 0.03	268 ^b \pm 25	2.15 ^c \pm 0.22
SRPP 200 mg/kg	26.4 ^a \pm 0.41	325.4 ^d \pm 34	13.17 ^a \pm 1.6	0.29 ^a \pm 0.02	241 ^{a,b} \pm 28	1.65 ^b \pm 0.14
Ranitidine	26.8 ^a \pm 0.29	254.5 ^b \pm 26	14.24 ^a \pm 1.8	0.31 ^a \pm 0.03	211 ^a \pm 28	1.61 ^b \pm 0.16

Range was provided by Duncan multiple test at $P < 0.05$. a: Less significant; b: Moderately significant; c: Very significant and d: most significant. Different letters a to d in the column represents that values are significantly different when compared between ulcer induced with healthy control and SRPP/Ranitidine treated groups.

ingredients and mode of action have been rarely established, which is very crucial for understanding the long-term potency of these antiulcer sources. Among the majority of identified sources, flavonoids^[11,31], and occasionally polysaccharides, have frequently been implicated as antiulcer agents^[12-14]. We previously reported on a non-toxic, edible antioxidant source^[17] - *Decalepis hamiltonii*, a significant antiulcerogenic properties *in vitro* and *in vivo*. High levels of antioxidant properties, probably just little less than that found in green tea, with multiple compounds^[32] may play a critical role in inhibiting oxidative induced mucosal damage in ulcers^[33]. In the current paper, we report the antiulcerogenic potential of a combinational

molecule, which is a pectic polysaccharide with bound phenolics from swallow root. In human nutrition, pectic polysaccharides play a key role as low energy foods and break down products have been known to have health beneficial properties.

Gastric ulcers have multiple etiopathogeneses. Stress ulcers are due to both physiological and psychological factors, which affect gastrointestinal defense and increased accumulation of acid due to influx of H^+ into the lumen of the stomach by parietal cell plasma membrane bound H^+ , K^+ -ATPase leading to autodigestion of the gastric mucosa^[34], and generation of free radicals. Ethanol stress, on the other hand, is known to act on the gastric mucin

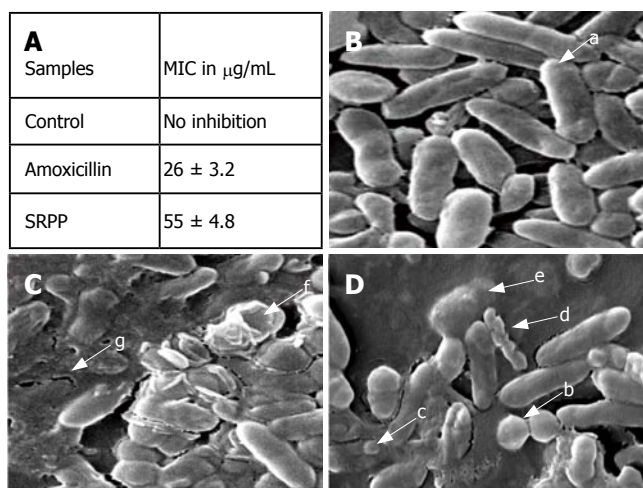


Figure 5 Effect of SRPP on *H. pylori*; Minimum Inhibitory Concentration (MIC) (A) was established by serial dilution technique; B-D indicate the scanning electron microscopic pictures at 15 k magnification of control (B), SRPP (C) and amoxicillin (D) treated *H. pylori*. Untreated control cultures indicate uniform rod shaped (a) *H. pylori* cells. Amoxicillin treatment showed coccoid form (b), blebbing (c), fragmented (d) and lysed (e) cells. SRPP treatment in addition indicates cavity formation (f) with disrupted structures (g).

directly, affecting mucosal defense. Nevertheless, in both cases the causes of severe ulcerations are depicted in the current study in addition to observations from other investigators^[35,1]. Our earlier studies indicated that phenolic antioxidants were efficient in inhibiting upregulated H^+ , K^+ -ATPase and recovering the depleted levels of antioxidant and antioxidant enzymes^[17].

The effect of SRPP on gastric ulcers induced by swim and ethanol stress was investigated in *in vivo* rat models. Oral administration of 100 and 200 mg/kg b.w. reduced gastric lesions. It is evident from our data (Figures 2-4 and Tables 2-4) that swim stress and ethanol stress induced gastrointestinal effects, such as gastric erosions, gastric or duodenal ulcerations, gastrointestinal hemorrhages and perforations. These effects were modulated by the inhibition of upregulated H^+ , K^+ -ATPase, and enhancement of down regulated gastric mucin, antioxidant and antioxidant enzyme levels. Histological studies indicated that characteristic ulcerogenic pathogenicity, with a distinct ulcer margin formed by the adjacent non-necrotic mucosa, the epithelial component, and granulation tissue at the ulcer base, was normalized upon treatment with SRPP. Current data, together with the results of our previous paper^[17], indicate clearly that phenolic antioxidants of SRPP may contribute to H^+ , K^+ -ATPase inhibition, rather than the polysaccharide per se since swallow root antioxidants inhibited H^+ , K^+ -ATPase at 36 $\mu\text{g/mL}$ as apposed to that of SRPP (77 $\mu\text{g/mL}$).

Ethanol induced gastric lesions are thought to arise as a result of direct damage to gastric mucosal cells, resulting in the development of free radicals and hyperoxidation of lipids. Recently, it was discovered that *Solanum nigrum* extract provides significant antioxidant activity as one of the possible gastroprotective mechanisms against ethanol-induced gastric ulceration^[19]. SRPP may also act similarly in reducing ulcerations in stomach since it showed potent antioxidant properties.

In addition, SRPP is a safer source since toxicity studies indicated no lethal effect up to an oral dose of 1 g/kg b.w. for 14 d. To understand the potential role of SRPP in gastric mucosal protection, it is important to know that mucin is an insoluble adherent mucus gel, which is quite stable and has significant buffering capacity for neutralization of luminal acid in the presence of bicarbonate. SRPP showed 2 fold upregulation of gastric mucin as revealed by immunohistological/biochemical and ELISA methods, indicating the stabilization of the mucosal layer.

Further, SRPP possessed H^+ , K^+ -ATPase inhibitory activity, although not as potent as that of phenolic fractions. Phenolics present in SRPP together with those reported in the literature revealed that phenolic antioxidants are potent H^+ , K^+ -ATPase blockers^[11]. The significant levels of phenolics present in SRPP may also contribute towards inhibition of H^+ , K^+ -ATPase activity, which plays a tremendous role in reducing an acidic condition in the gastric lumen.

Results are intriguing that SRPP also showed potential anti-*H. pylori* activity. The results are in accordance with the observation made by Lee *et al.*^[36], where inhibition of *H. pylori* growth by pectic polysaccharide was reported. However the mechanism still needs to be established. Several mechanisms may be proposed for potential inhibition of *H. pylori* by SRPP. SRPP phenolics may inhibit microbial activity as phenolics were thought to exert their antimicrobial effect by causing (1) hyper acidification at the plasma membrane interface of the micro organism, or (2) intracellular acidification, resulting in the disruption of H^+ , K^+ -ATPase required for ATP synthesis of microbes, or (3) may be related to inactivation of cellular enzymes causing membrane permeability changes^[10,37]. The rate of inactivation of microbial cellular enzymes is dependent on the rate of penetration of phenolic antioxidants into the cell. In the case of *H. pylori*, phenolics may be inactivating the urease enzyme, which is specifically expressed at its surface to neutralize hyperacidification to survive in the gastric environment of the stomach^[38]. It is thus clear that SRPP is creating a cavity in the organism (Figure 5C) with the loss of cellular contact resulting in loss of viability of *H. pylori*.

There are several schools of thought that indicate the ulcer healing component must be proliferative, amplify cell migration, and enhance angiogenesis in order to enhance re-epithelialization in the ulcer healing process. However, antiulcer compounds with proliferative ability, and the ability to enhance angiogenesis, may be carcinogenic also. This statement is also substantiated by observation of induction of cancer upon the usage of antiulcer drugs on a long-term basis^[8]. In this context, SRPP, although found to be antiulcerogenic, has been shown to be anticancerous (Unpublished observation, 2006). Hence the treatment of ulcer by SRPP even for longer periods of time may not pose side effects.

Generally antioxidants have been known to be antimicrobial by binding to the microbial membrane leading to disruption^[39]. SRPP, by virtue of phenolics, may be antimicrobial. In addition, SRPP may also participate in enhancement of gastric mucin. The enhancement of gastric mucin contents, as measured by ELISA and Alcian blue binding, may suggest that enhancement is most probably

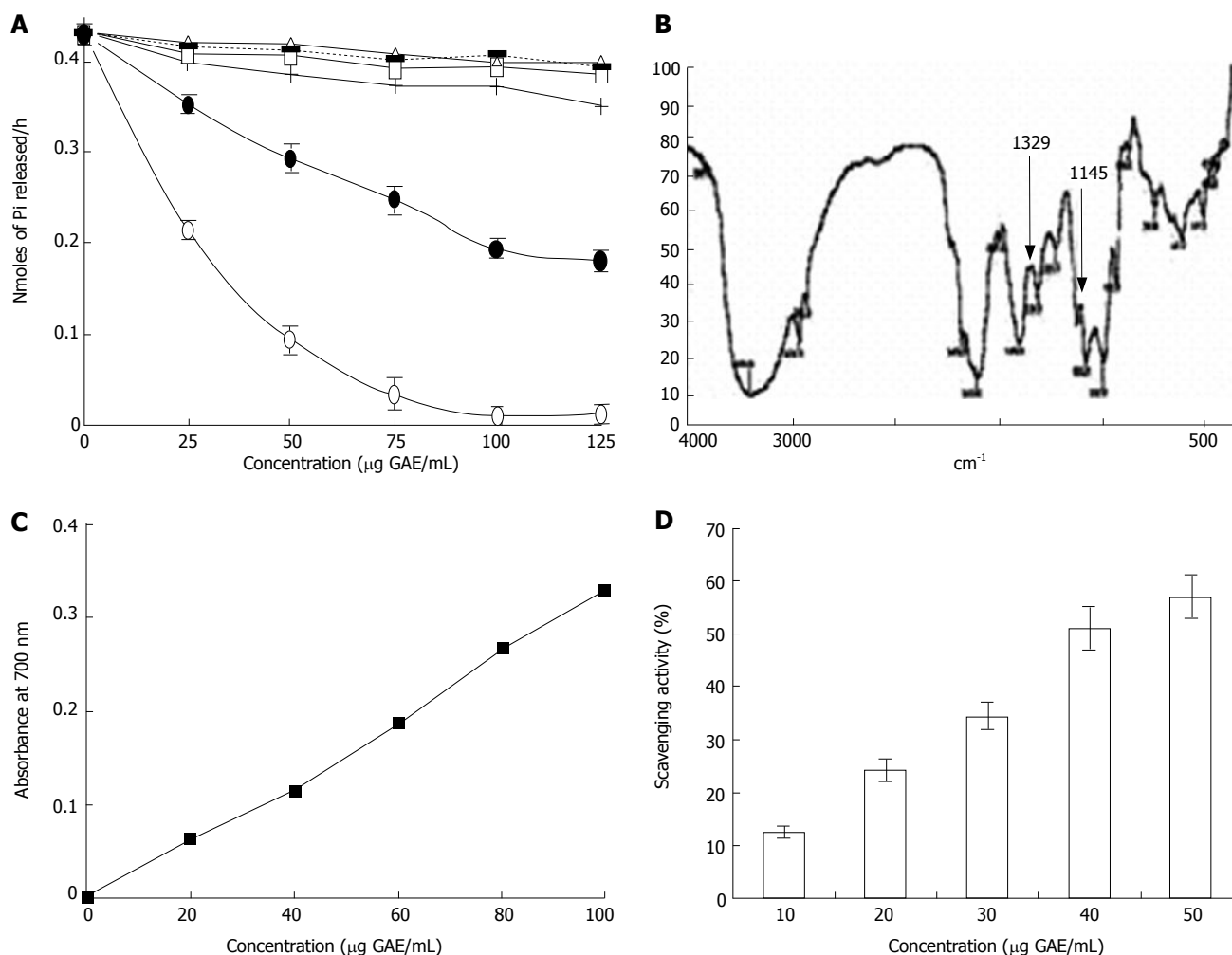


Figure 6 H⁺, K⁺-ATPase (A), Fourier Transform Infra-Red Spectroscopy (B), Reducing power (C) and Free radical scavenging activity (D) of SRPP. A: inhibition of H⁺, K⁺-ATPase only by SRPP (●) and not by other polysaccharides of Swallow root; SR water soluble polysaccharide (◆), SR Hemicellulose A (◊), Hemicellulose B (◻), SR alkali insoluble residue (○) and inhibition by lansoprazole (◐) a known blocker is also depicted in the figure; B: arrow at 1329 and 1145 cm⁻¹ indicate the presence of sulfonamide group in FTIR spectrum. Dose dependent antioxidant activity evaluated as reducing power ability (C) and free radical scavenging ability (D) indicates potential antioxidant activity by phenolics of SRPP.

due to prevention/protection of mucosal injury during ulceration rather than direct increase in synthesis. This is supported by no upregulation of gastric mucin in SRPP controls where animals were fed with SRPP without inducing ulcers. However, regulated synthesis might occur, which may be evaluated by tracer techniques. Antioxidant potency may also be contributed by both phenolics and sulfonamide groups containing polysaccharides^[40,41]. Further, it is also possible that SRPP, by virtue of its anionic nature, may bind effectively to positively charged amino acid residues of gastric mucin as well as sucralfate and other polysaccharides^[42]. This binding may avoid gastric mucin damage and subsequent ulceration. SRPP thus can be a safe and promising multi-step ulcer blocker (Figure 1).

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causative factors being unavoidable such as stress, use of non-steroidal anti-inflammatory drugs; and the side effects of available antiulcer drugs; alternatives that are safer, but effective in ulcer prevention must be envisaged.

Research frontiers

Frontiers of research on development of antiulcer drugs must emphasize on (a) detection and diagnosis of *H. pylori*-a major ulcerogen; (b) identification of ulcerogens and antiulcerogens from diet since some components of food are ulcerogens; (c) antiulcer drugs with less or no side effects, so that it can be used by subjects who are using NSAIDs and also alcohol.

Innovations and breakthroughs

Herbal/dietary sources are the challenging alternatives for potential ulcer management. Since herbal medicines include extracts from either edible or non-edible source, side effect has been a threat. Dietary sources are therefore

optional. We report a novel potent multi-step ulcer blocker which inhibits acid secretion/growth and invasion of *H. pylori* and enhances mucosal defense.

Applications

The antiulcer component identified is inexpensive, effective and nontoxic; hence can be directly applied to human health. Single compound with multi-potency implies a potential reduction in the drug load during ulcer treatment.

Terminology

Ulcer, H⁺, K⁺-ATPase, pectic polysaccharide, *H. pylori*, antioxidant, gastric mucin, mucosal injury, non-steroidal anti-inflammatory drugs.

Peer review

This is a nicely done description of the effects of SRPP. Through *in vivo* studies, the authors concluded that SRPP with defined sugar composition and phenolics exhibited multi-potent free radical scavenging, antioxidant, anti-*H. pylori*, inhibition of H⁺, K⁺-ATPase and gastric mucosal protective activities.

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BASIC RESEARCH

CTGF, intestinal stellate cells and carcinoid fibrogenesis

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fibrotic GI carcinoids (< 15 ng/mL).

CONCLUSION: SI carcinoid tumor fibrosis is a CTGF/TGF β 1-mediated stellate cell-driven fibrotic response. The delineation of the biology of fibrosis will facilitate diagnosis and enable development of agents to obviate its local and systemic complications.

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Key words: Carcinoid; Connective tissue growth factor; fibrosis; Small intestine; Stellate cell; TGF β

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Abstract

AIM: To investigate the role of small intestinal carcinoid tumor-derived fibrotic mediators, TGF β 1 and CTGF, in the mediation of fibrosis *via* activation of an "intestinal" stellate cell.

METHODS: GI carcinoid tumors were collected for Q RT-PCR analysis of CTGF and TGF β 1. Markers of stellate cell desmoplasia were identified in peritoneal fibrosis by immunohistochemistry and stellate cells cultured from fresh resected fibrotic tissue. CTGF and TGF β 1 were evaluated using quantitative tissue array profiling (AQUA analysis) in a GI carcinoid tissue microarray (TMA) with immunostaining and correlated with clinical and histologically documented fibrosis. Serum CTGF was analyzed using a sandwich ELISA assay.

RESULTS: Message levels of both CTGF and TGF β 1 in SI carcinoid tumors were significantly increased (> 2-fold, $P < 0.05$) versus normal mucosa and gastric (non-fibrotic) carcinoids. Activated stellate cells and markers of stellate cell-mediated fibrosis (vimentin, desmin) were identified in histological fibrosis. An intestinal stellate cell was immunocytochemically and biochemically characterized and its TGF β 1 (10-7M) initiated CTGF transcription response (> 3-fold, $P < 0.05$) demonstrated. In SI carcinoid tumor patients with documented fibrosis, TMA analysis demonstrated higher CTGF immunostaining (AQUA Score: 92 ± 8 ; $P < 0.05$), as well as elevated TGF β 1 (90.6 ± 4.4 , $P < 0.05$). Plasma CTGF (normal 12.5 ± 2.6 ng/mL) was increased in SI carcinoid tumor patients (31 ± 10 ng/mL, $P < 0.05$) compared to non-

INTRODUCTION

Carcinoid (neuroendocrine) tumors are enigmatic, generally slow growing malignancies that occur most frequently (67%) in the GI tract^[1]. They are not rare lesions, arising in 1.68 of every 100 000 people^[1]. The commonest gut tumor is the SI carcinoid tumor^[1,2], which is derived from neuroendocrine enterochromaffin (EC) cells. SI carcinoid tumors are usually identified based on their characteristic paroxysmal symptomatology of flushing, sweating and diarrhea. They are often, however, detected at surgery for unexplained bowel obstruction^[3], as a consequence of the fibrosis that they engender^[4]. The etiology of this desmoplastic response is unknown but is a consequence of conversion of the normally filmy and flexible mesentery into a contracted fibrous adhesive mass with bands and even retroperitoneal desmoplasia^[5,6]. These events are due both to tumor invasion and the ability of secretory products of the EC cell to initiate fibrosis by activating local cells to produce a desmoplastic response^[7]. SI carcinoid tumor patients also develop distant (cardiac) fibrosis suggesting that the bioactive agents involved in the process have both a paracrine and a systemic effect^[6]. In contrast, neither gastric carcinoids (derived from the neuroendocrine EC-like (ECL) cell) nor pulmonary carcinoids are associated with extensive local or systemic desmoplastic responses^[8].

The mechanism whereby such fibrosis occurs is unknown although serotonin has previously been suggested as a mediator^[6]. TGF β 1 and CTGF are well-characterized fibrotic factors^[9-12]. TGF β 1 is a profibrotic mediator that

induces CTGF expression^[10]. Together, these factors stimulate over-production of collagen synthesis^[13,14]. The target cells of TGFβ1 and CTGF are activated myofibroblasts, also known as stellate cells^[15,16]. In the pancreas, TGFβ1 activates pancreatic stellate cells (PSCs) in both experimental and human pancreatic fibrosis; these cells are the main cellular source of collagen in chronic pancreatitis^[17-19]. SI neuroendocrine tumors express TGFβ1 and its receptors, while stromal cellular elements around tumor nests express the TGFβ receptor^[20]. This suggests a mechanism by which tumor cells can interact with and alter the character of the surrounding stroma.

We hypothesized that tumor TGFβ1 and CTGF produced by EC cells is involved in the mechanism of SI carcinoid tumor fibrosis via activation of an “intestinal” stellate cell. The aims of this study were to: (1) quantify CTGF and TGFβ1 message in carcinoid tumor tissue; (2) examine protein expression levels of CTGF and TGFβ1 and matrix proteins using immunohistochemistry in SI carcinoid tumors and intestinal fibrosis; (3) isolate and characterize the “intestinal” stellate cell; (4) examine the effects of TGFβ1 on this cell type; (5) quantitatively analyze CTGF and TGFβ1 protein levels on a GI carcinoid tissue microarray by AQUA analysis; and 6) determine whether serum CTGF discriminated SI carcinoid tumor patients with fibrosis from other non-fibrotic GI carcinoids.

MATERIALS AND METHODS

These studies were approved by the Human Investigations Committee at the Yale University School of Medicine.

Tissue specimens

Tissue for molecular analysis: Tumor tissue from ten GI carcinoid patients (M:F = 6:4; median age [range] = 60 years [40-78]) diagnosed with either SI EC cell carcinoid tumors (*n* = 5) or gastric ECL cell carcinoids (*n* = 5) were collected for this study (Table 1). None of the patients had received therapy (surgery or somatostatin analogues) prior to tissue procurement. Paired normal tissue samples were also obtained from adjacent, macroscopically normal, non-tumor mucosa in nine cases from these patients.

Tissue for cell culture analysis: Tumor tissue and mesenteric fibrotic tissue was obtained from a patient with a fibrotic SI carcinoid tumor (male, 43 years; sample #6) operated on at Yale University (by IMM). This patient had not received medical therapy (somatostatin analogues) prior to surgery and was a de novo case of SI fibrosis.

GI Carcinoid TMA: Formalin-fixed paraffin-embedded tissue blocks containing GI carcinoids (stomach: *n* = 7; and SI: *n* = 36) diagnosed between 1965 and 2001 at the Yale University School of Medicine Department of Pathology were retrieved. Follow-up information was available (median follow-up: 110 mo, range: 24-456 mo) for all patients. The TMA consisted of primary GI carcinoids, matched normal mucosa and peritoneal fibrotic material and was represented by 2 cores/case. Complete clinical details including fibrosis were known for all patients.

Table 1 Clinical details of carcinoid tumors used for mRNA analysis

No	Sex	Age ²	Race	Tumor site	Lymph node involvement	Liver involvement	Fibrosis ³
1 ¹	M	71	H	G	N	N	N
2 ¹	M	45	W	G	N	N	N
3 ¹	F	74	W	G	N	N	N
4 ¹	M	78	W	G	N	N	N
5	F	40	W	G	N	N	N
6 ⁴	M	43	W	SI	N	Y	Y
7 ¹	F	60	W	SI	16/22	N	Y
8 ¹	M	59	W	SI	N	Y	N
9 ¹	M	73	W	SI	1/9	Y	N
10 ¹	F	53	W	SI	1/12	N	N

¹Normal tissue available, ²Age at time of procedure, ³Identified at surgery;

⁴Used to isolate and culture intestinal stellate cell. H: Hispanic; W: White; G: Gastric ECL carcinoid; SI: SI EC cell carcinoid tumor.

Clinically significant fibrosis was determined at surgery, and all samples were examined by a pathologist (RLC) to histologically confirm fibrosis.

Serum: Twenty-nine subjects (median age [range] = 42 years [20-83]; M:F = 17:12) attending the Neuroendocrine Referral, Oncology and Surgery outpatient clinics at Yale University School of Medicine were recruited for serum analysis. These included 29 patients with GI carcinoids: SI EC cell carcinoid tumors (*n* = 16), gastric ECL cell carcinoids (*n* = 7), and six other GI carcinoids [rectal: *n* = 2, parotid: *n* = 1, appendiceal: *n* = 2, duodenal: *n* = 1]. Serum samples from ten age-, sex-matched control subjects were also collected.

Tissue techniques

Quantitative RT-PCR: Total RNA was isolated from frozen carcinoid tumor tissue (*n* = 10) and normal mucosa (*n* = 9) with TRIzol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's guidelines. RNA was dissolved in DEPC water, measured spectrophotometrically and an aliquot analyzed on a denaturing gel using electrophoresis to check the quality of RNA isolated.

CTGF and TGFβ1 message were quantitatively measured in the ten tumor and nine control samples as described^[21,22]. Briefly, Q RT-PCR was performed using the ABI 7900 Sequence Detection System. Total RNA from each sample was subjected to reverse transcription using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). 2 μg of total RNA in 50 μL of water was mixed with 50 μL of 2X RT mix containing Reverse Transcription Buffer, dNTPs, random primers and Multiscribe Reverse Transcriptase. RT reaction was carried out in a thermal cycler for 10 min at 25°C followed by 120 min at 37°C. Real time PCR analysis was performed in triplicate^[21,22]. cDNA in 7.2 μL of water was mixed with 0.8 μL of 20 × Assays-on-Demand primer (CTGF = Hs00170014, TGFβ1 = Hs00171257, GAPDH = Hs99999905) and probe mix, 8 μL of 2 × TaqMan Universal Master mix in a 384 well optical reaction plate. The following PCR conditions were used: 50°C for 2 min, then 95°C for 10 min, followed by 40 cycles at 95°C/0.15

min and 60°C/1 min. A standard curve was generated for each gene using cDNA obtained by pooling equal amounts from each sample ($n = 19$). The expression level of target genes was normalized to internal GAPDH. Data was analyzed using Microsoft Excel and calculated using the relative standard curve method (ABI, User Bulletin #2).

Immunohistochemistry: Serial sections (5 μm) encompassing SI carcinoid tumors or fibrotic tissues were deparaffinized in xylene and rehydrated in graded alcohols. For antigen retrieval purposes, sections were immersed in citrate buffer (10 mM sodium citrate, pH 6.0), and subjected to 1×10 min high temperature-high pressure treatment followed by treatment with 0.3% H_2O_2 in methanol for 30 min at 37°C to inactivate endogenous peroxidase. In some studies, sections were incubated with goat antiserum to CTGF (1:250) or TGF β 1 (1:1000) (both from Santa Cruz Biotechnology, Santa Cruz, CA) diluted in Tris-buffered saline containing BSA and a monoclonal antibody against CgA (0.5 $\mu\text{g}/\text{mL}$) or serotonin (2 $\mu\text{g}/\text{mL}$) (both from DAKO, Carpinteria CA) for 24 hr at 4°C and then with Alexa 488-labeled anti-mouse IgG (1:100 dilution) for 1 hr at RT. Donkey anti-goat antibody conjugated to a horseradish peroxidase-decorated dextran polymer backbone (Envision; DAKO Corp, Carpinteria, CA) was used as a secondary reagent. HRP-amplification was performed. CTGF or TGF β 1 was visualized with a fluorescent chromogen (Cy-5-tyramide; NEN Life Science Products, Boston, MA). Dual-positive cells (CTGF + serotonin or CTGF + CgA) were counted in a minimum of 5-well orientated sections and expressed as a percentage. In other studies, fibrotic areas from the peritoneum of patients with SI carcinoid tumors were stained with mouse anti- α -smooth muscle actin (1:1000) or desmin (1:1000, both DAKO), goat anti-vimentin (1:1000), collagen III (1:1000) or CTGF (1:250). Stromal (myofibroblast) cells were separable from tumor cells that were identified by the use of a fluorescently tagged anticytokeratin antibody cocktail (AE1/AE3; DAKO Corp). Nuclei were visualized by 4', 6-diamidino-2-phenylindole (DAPI 10 mg/mL). Localization of expression of products was used to determine whether stromal (non-cytokeratin staining) or tumor cells expressed these products.

Intestinal stellate cell culture and analysis: Stellate cells were isolated using a modification of the method by Bachem *et al*^[15]. Briefly, cells were isolated from the fibrotic tumor specimen (hand dissected, digested in collagenase (0.25 mg/mL)/DNase (100 U/mL) solution for 60 min at 37°C under constant aeration) and were cultured on 10 cm^2 uncoated culture wells in 10% fetal calf serum in a 1:1 (vol/vol) mixture of DMEM and Ham's F12 medium supplemented with 2% L-glutamine, 100 IU/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, and 1% amphotericin. Twenty-four hours after seeding, the culture medium was changed and the myofibroblasts remained attached to the plastic. After reaching confluence, cells were subcultured by trypsinization using a 0.025% trypsin solution containing 0.01% EDTA in PBS. For immunofluorescence microscopy, cells were seeded on 1 cm^2 glass coverslips in six-well (10 cm^2/well ; 2 mL medium) plates (2-3 glass coverslips per well). Phase-contrast microscopy was used

to identify the translucent fat droplets in the cytoplasm and stellate-like morphology that typifies stellate cells^[15]. These studies were undertaken within the first 3-d as culturing cells results in a transdifferentiation from a vitamin A-storing phenotype to a myofibroblastic phenotype^[15]. For immunocytological characterization, cells cultured on uncoated glass coverslips were fixed for 30 min in -20°C acetone and air-dried. Coverslips were preincubated for 15 min in TBS (pH 7.4) with 3% bovine serum albumin and 0.3% hydrogen peroxide. Incubations with the primary antibody (mouse monoclonal: α -smooth muscle actin 1:1000) was performed at room temperature in a humidified chamber for 1 h. Non-specific staining was controlled by omitting the primary antibody and including mouse, non-immune serum at the same dilution as used for the specific primary antibody. After rinsing (three times for 5 min with TBS/Tween-0.5%), the second antibody (HRP goat anti-mouse, diluted 1:100) was added and incubated for 1 h at room temperature. Cy5-labelled tyramide (TSA; NEN Life Science Products, Boston, MA) was used with DAPI (10 mg/mL) to stain nuclei and cells observed with a fluorescence microscope. For RNA studies, cultured cells were stimulated with TGF β 1 (10^{-7} M) for 24 h. Thereafter, RNA was isolated and Q RT-PCR performed as described above to quantitatively measure TGF β 1-stimulated CTGF message.

AQUA Analysis of CTGF and TGF β 1 in the carcinoid TMA: Tissue microarray slides were stained as described^[21,23]. Antigen retrieval and immunostaining for CTGF, TGF β 1 (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), cytokeratin and nuclei were as above. Protein expression (CTGF or TGF β 1) was determined using an automated tissue microarray reader. Automated image acquisition and analysis using AQUA has been described previously^[21,23]. In brief, monochromatic, high-resolution (1024×1024 pixel; 0.5- μm) images were obtained of each histospot. Areas of tumor separate from stromal elements were distinguished by creating a mask from the cytokeratin signal. Coalescence of cytokeratin at the cell surface localized the cell membranes, and DAPI was used to identify nuclei. The Cy-5 signal from the membrane area of tumor cells was scored on a scale of 0-255 and expressed as signal intensity divided by the membrane area. Histospots containing < 10% tumor, as assessed by mask area (automated), were excluded from further analysis. Previous studies have demonstrated that the staining from a single histospot provides a sufficiently representative sample for analysis^[24].

Serum techniques

CTGF serum ELISA: Serum CTGF-W (whole molecule) and CTGF-N (N-terminal fragment) were assayed by two separate sandwich enzyme-linked immunosorbent assays (ELISA). The CTGF-W ELISA uses a capture mAb reactive to the amino terminus of CTGF, and detects the bound CTGF-W with an alkaline phosphatase labeled mAb reactive to the carboxyl-terminal region of CTGF. A second ELISA uses two non-cross blocking monoclonal antibodies reacting to distinct NH₂-terminal epitopes of CTGF. This assay detected both CTGF-W and CTGF N fragment, so-called CTGF N + W, as described previ-

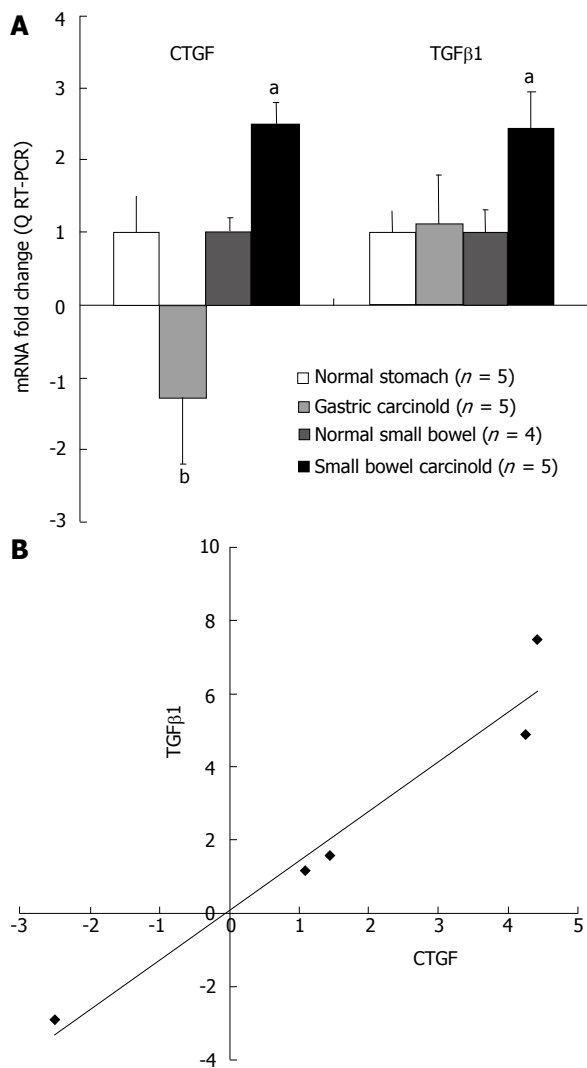


Figure 1 A: Message levels of both CTGF and TGFβ1 determined by Q RT-PCR. Levels were corrected against expression of the housekeeping gene, GAPDH, compared to similarly corrected gene levels in normal mucosa, and represented as fold increase over normal (1.0). TGFβ1 was significantly over-expressed (about 2.5-fold) in SI carcinoid tumor samples compared to normal mucosa ($^aP < 0.05$) but not the gastric carcinoids. CTGF was significantly over-expressed (about 2.5-fold) in SI carcinoid tumor samples compared to normal mucosa ($^bP < 0.05$) while gastric carcinoids had significantly decreased CTGF compared to SI carcinoid tumors ($^bP < 0.01$). Mean \pm SE; B: Correlation analysis of QRT-PCR results in SI EC cell carcinoid tumors. There was a good correlation between CTGF and TGFβ1 transcript levels in tumor samples ($R^2 = 0.9445$, $P < 0.01$, $n = 5$).

ously^[25]. CTGF-N is a value calculated by subtracting CTGF-W from the CTGF N + W level measured by the second assay. Standards for both assays were made from purified full-length CTGF and expressed in nanograms per milliliter. The intra- and interassay coefficient of variation was 5 and 15%, respectively, for both ELISA tests. Data on CTGF-W is presented in this study.

Statistical analysis

Results are expressed as mean \pm SE; n indicates the number of patients in each study group. Statistical significance was calculated by the Student's test for unpaired values or non-parametric statistics as appropriate. On the TMA, the unpaired 2-tailed Student's t -test was used to identify statistically significant differences in fibrotic protein expression

between different patient groups (patients with clinical evidence of fibrosis versus non-fibrosis, fibrosis versus gastric carcinoid).

RESULTS

Quantitative RT-PCR

Q RT-PCR analysis was undertaken using Assays on Demand (Applied Biosystems) on the RNA isolated from SI EC cell carcinoid tumors (fibrosis associated) ($n = 5$); gastric ECL cell tumors (little fibrosis) ($n = 5$); normal SI mucosal samples ($n = 4$) and normal gastric mucosa ($n = 5$) to quantitatively measure the levels of CTGF and TGFβ1 mRNA expression in these two different tumor types. Transcript levels of both CTGF and TGFβ1 were significantly elevated in the five SI carcinoid tumor samples ($P < 0.05$ vs normal mucosa) (Figure 1A). In contrast, TGFβ1 message was not different (+ 1.13-fold) in gastric carcinoid tumor samples compared to normal, and message levels of CTGF were significantly decreased (-1.3-fold; $P < 0.01$) compared to SI carcinoid tumors (Figure 1A). There was a good correlation ($R^2 = 0.95$) between CTGF and TGFβ1 message levels in the SI carcinoid tumor samples demonstrating that transcription of these growth factors was tightly associated in this tumor tissue (Figure 1B). No relationship was noted between TGFβ1 mRNA levels and CTGF mRNA levels in gastric carcinoids ($R^2 = 0.01$). These results demonstrate while both gastric and SI carcinoid tumors express mRNA for TGFβ1, CTGF mRNA is over-expressed only in SI carcinoid tumors. CTGF and TGFβ1 transcript levels are associated in SI carcinoid tumors.

Immunohistochemistry

CTGF and TGFβ1 in tumor samples: CTGF was localized in the cytoplasm of SI carcinoid tumor cells (Figure 2). Co-staining with anti-CgA (Figure 2A) or anti-serotonin (Figure 2B) antibodies demonstrated a significant co-localization with CTGF and either antibody ($80 \pm 12\%$ and $93 \pm 6\%$ respectively) in tumor mucosa. Like CTGF, TGFβ1 was cytoplasmic and was present in $> 75\%$ of tumor cells (Figure 2C). These results demonstrate that TGFβ1 and CTGF expression are characteristic features of SI EC cell carcinoid tumors.

Matrix production in fibrosis: α -smooth muscle actin-positive cells were identified interspersed with carcinoid tumor cells in areas of fibrosis (Figure 3A). α -smooth muscle actin is a marker for activated myofibroblasts (or stellate cells) and indicates that, as for the pancreas, stellate cells are present in peritoneal fibrotic material associated with SI carcinoid tumor mesenteric invasion^[15]. Vimentin, desmin and collagen-III positivity was identified with stellate cells (Figure 3B-D). These are markers of a TGFβ1-mediated stellate-cell driven fibrosis^[15,19,26], and indicate that this response occurs in SI carcinoid tumors. CTGF was present in both tumor cells and stellate cells (Figure 3E and F), consistent with the expression of this fibrotic mediator in both cell types.

Intestinal stellate cell isolation and culture

Myofibroblasts from SI carcinoid tumor fibrotic surgical

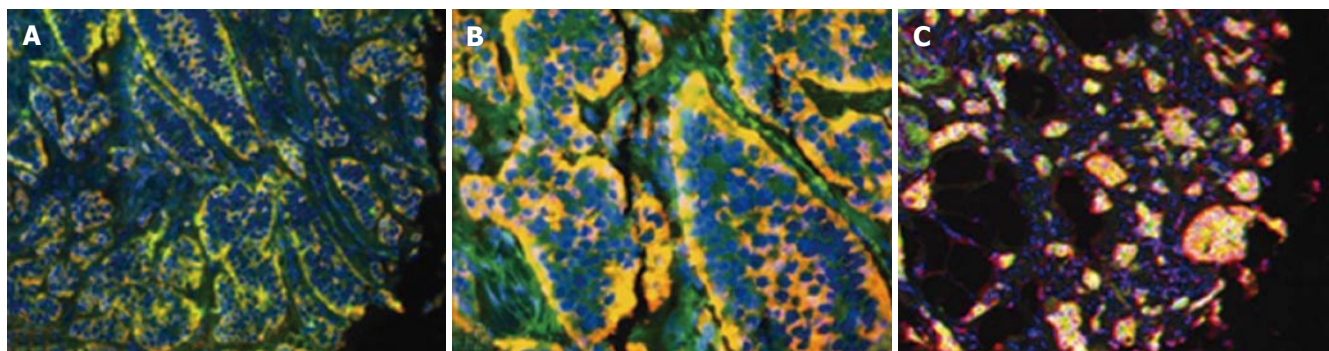


Figure 2 **A:** Triple color staining of nuclei (blue-DAPI), CgA (green-Alexa 488) and CTGF (red-Cy5) in a SI carcinoid tumor from the carcinoid TMA. Staining for both CgA and CTGF was cytoplasmic. Dual-stained (CgA + CTGF) cells are yellow. A majority of CgA cells (about 80%) were also CTGF positive (x 400); **B:** Triple color staining of nuclei, serotonin (green-Alexa 488) and CTGF in a carcinoid tumor from the TMA. Staining for both Serotonin and CTGF was cytoplasmic. Dual-stained (Serotonin + CTGF) cells are yellow. A majority of the serotonin cells (about 95%) were also CTGF positive. (x 600); **C:** Triple color staining of nuclei (blue-DAPI), cytokeratin (green - Alexa 488) and TGF β 1 (red-Cy5) in a carcinoid tumor from the TMA. Staining for TGF β 1 was cytoplasmic. A majority of the carcinoid tumor cells (cytokeratin-positive) (about 85%) were also TGF β 1 positive (x 200).

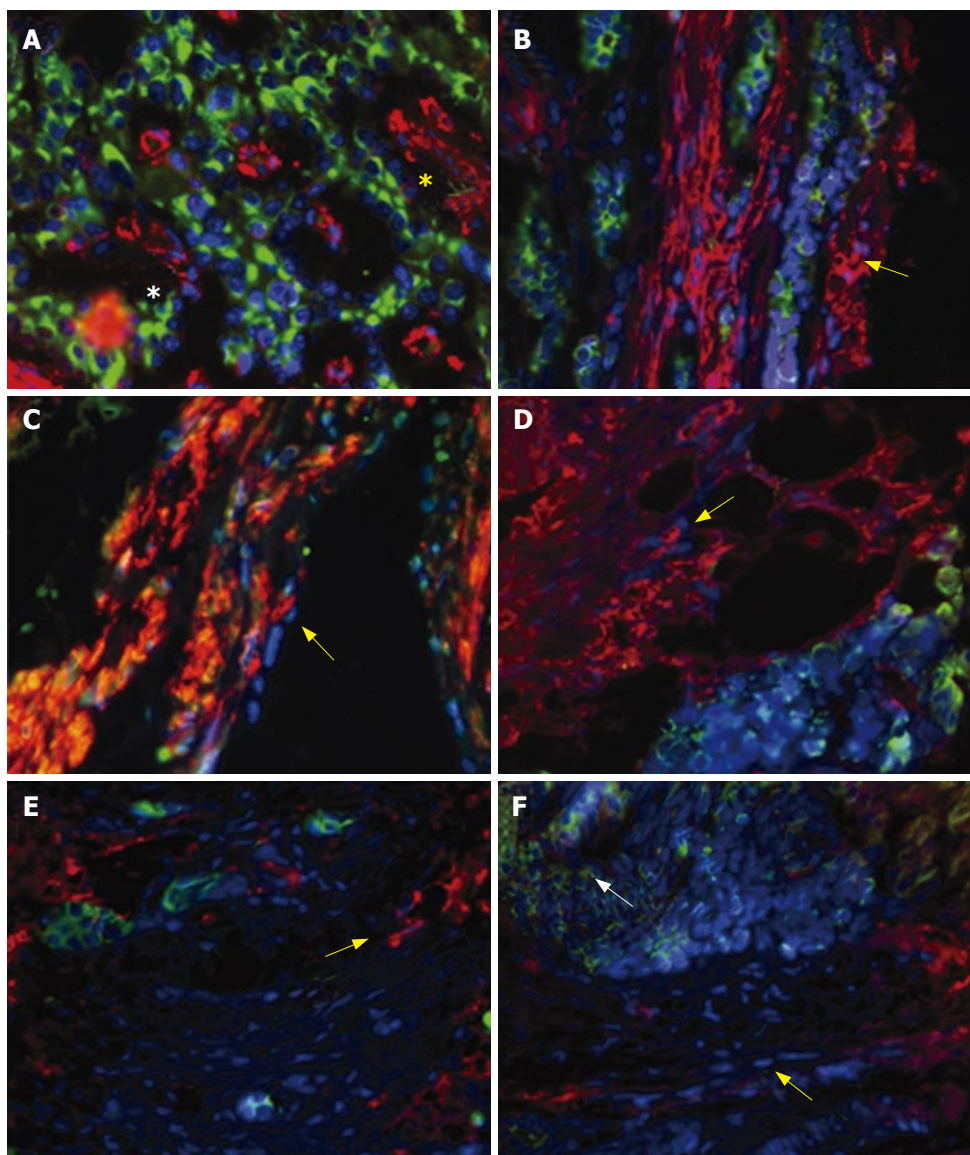


Figure 3: Immunostaining of areas of SI carcinoid tumor fibrosis with α -smooth muscle actin (**A**), vimentin (**B**), desmin (**C**), collagen III (**D**) and CTGF (**E/F**). Triple color staining of nuclei (blue-DAPI), cytokeratin-carcinoid tumor cells (green-Alexa 488) and the protein of interest (red-Cy5). (**A**) Discrete α -smooth muscle actin-positive cells (yellow star) were noted interspersed with tumor cells (white star) in areas of fibrosis. Cells consistent with myofibroblasts were associated with vimentin (**B**), desmin (**C**), collagen-III (**D**) and CTGF (**E/F**) production (yellow arrows). Within the fibrosis, carcinoid tumor cells were also CTGF-positive (**F**) (white arrow) (400 \times magnification).

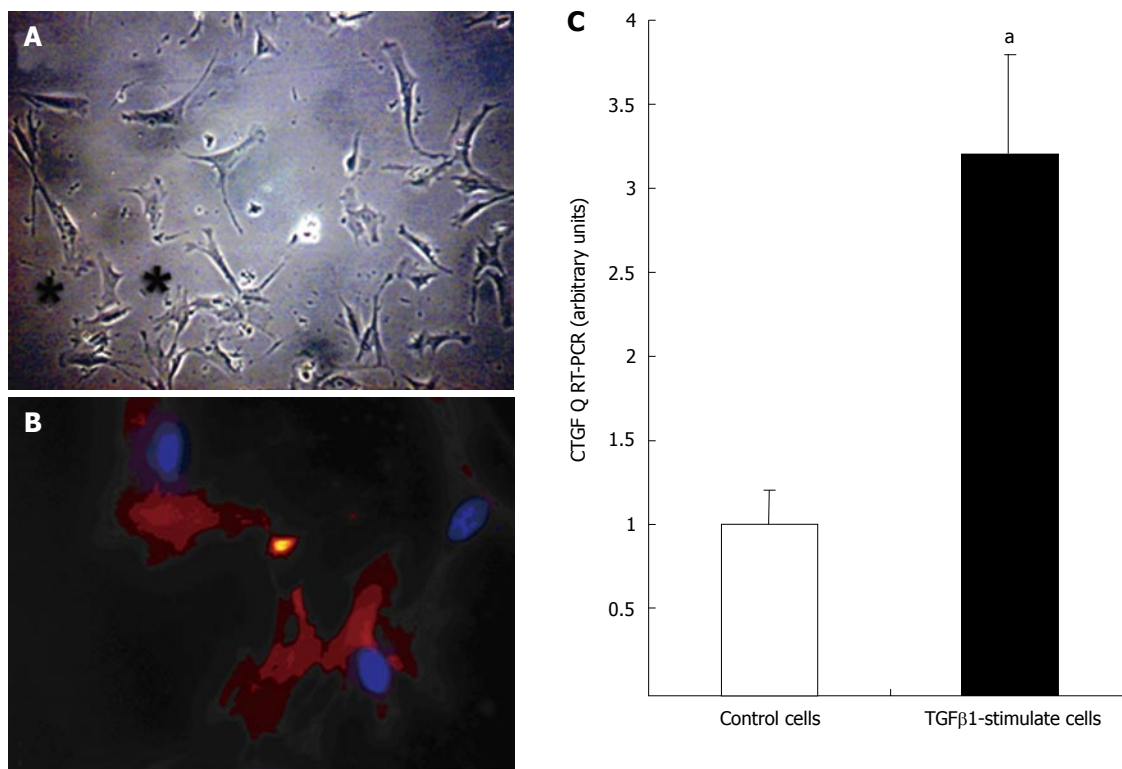


Figure 4 Micrographs of primary cultured human myofibroblasts isolated from human fibrotic material (SI carcinoid tumor). **A:** Light microscopy identified the typical stellate shape (black stars) in 5-day cultured cells (200 × magnification); **B:** Immunostaining with α -smooth muscle actin (Cy-5-red stain; nuclei are blue-DAPI) in same cells after 7-d culture (x 600); **C:** Message levels of CTGF determined by Q RT-PCR in primary cultured human myofibroblasts. CTGF was significantly over-expressed (about 3-fold) in TGFβ1 (10^{-7} mol/L, 24 h) stimulated cells compared to control (un-stimulated) cells ($^aP < 0.05$), mean \pm SE, $n = 3$.

tissue were cultured on plastic as described. Cells in primary cultures flattened and developed long, cytoplasmic extensions. During the 5-7 d in culture, cells developed the typical stellate shape (Figure 4A) and became positive (100%) for α -smooth muscle actin- α marker of myofibroblasts (Figure 4B). This is the classical stellate cell (myofibroblast) activation pathway^[15,19]. Stimulating the cells with TGFβ1 (10^{-7} mol/L) for 24 h significantly increased CTGF mRNA expression (3.2 ± 0.7 , $P < 0.05$ *vs* un-stimulated cells) (Figure 4C).

AQUA Analysis of CTGF and TGFβ1

An examination of the CTGF-stained histospots from the 36 patients with SI carcinoid tumors demonstrated that CTGF expression levels ranged from: AQUA score: 49.7-186.3. Higher levels of CTGF staining (AQUA score: 92.5 ± 8.2 ; $P = 0.017$) were identified in the fifteen SI carcinoid tumor patients with clinical (surgical) and histologically documented evidence of peritoneal fibrosis compared to the twenty-one patients (AQUA score: 72.7 ± 3.2) with no evidence of fibrotic disease (Figure 5). CTGF levels in non-tumor, non-fibrotic normal SI mucosal tissue were significantly lower (59 ± 4 , $P < 0.005$) than in patients with clinically and histologically documented fibrotic disease.

An examination of the CTGF-stained histospots from the seven patients with gastric carcinoids assessed by AQUA demonstrated that expression levels were not elevated in these patients compared to normal matched gastric mucosa (64 ± 3 *vs* 72 ± 3) but were significantly lower than in SI carcinoid tumors associated with fibrosis ($P < 0.03$).

An examination of the TGFβ1-stained histospots from patients with SI carcinoid tumors demonstrated that although TGFβ1 expression levels were elevated in patients with documented fibrosis (AQUA score: 90.6 ± 4.4) compared to the patients with no evidence of fibrotic disease (AQUA score: 82.7 ± 4.0) this did not reach statistical significance ($P = 0.08$). TGFβ1 levels were, however, lower in the matched normal SI mucosal samples (65 ± 4 , $P < 0.05$ versus fibrotic tumor samples). In the gastric mucosa, expression levels were not elevated in patients with gastric carcinoids compared to normal matched mucosa (61 ± 5 *vs* 64 ± 3) but, as for CTGF, values in these non-fibrotic samples were significantly lower than in SI carcinoid tumors associated with fibrosis ($P < 0.03$).

CTGF serum ELISA

Serum levels of CTGF ranged from 7.2-171 ng/mL. Significantly higher serum CTGF levels were found in patients with SI carcinoid tumors (31.0 ± 10) than in patients with ECL cell carcinoids (12.5 ± 4.9 , $P < 0.03$), other GI carcinoids (12.9 ± 0.6 , $P < 0.04$) and control patients (12.4 ± 4 , $P < 0.02$) (Figure 6). A comparison of serum CTGF levels with tissue levels of CTGF (AQUA scores) (where available) identified a strong correlation between these two measurements ($R^2 = 0.91$, $P < 0.005$, $n = 9$).

DISCUSSION

In the current study, we present data in support of our hypothesis that fibrosis is associated with invasion of

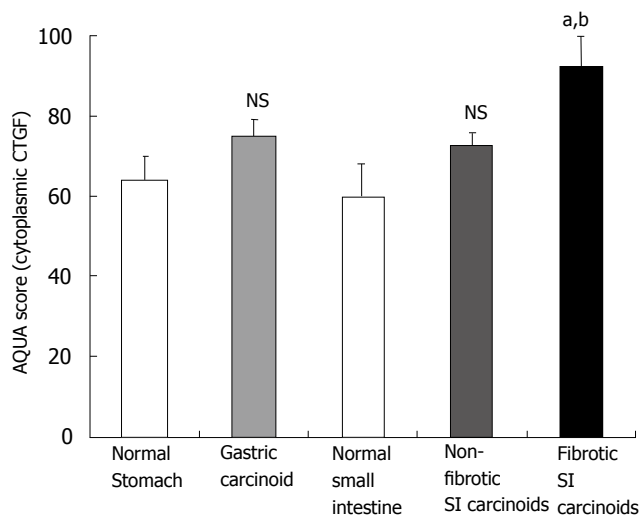


Figure 5 AQUA scores for CTGF protein expression in the TMA. Levels in tumors from carcinoid patients with clinically or histologically documented fibrosis (fibrotic SI carcinoid tumors) were significantly higher than tumors from patients with no evidence of fibrosis (non-fibrotic SI carcinoid tumors and gastric carcinoids) and normal mucosa. No differences in expression were noted between either non-fibrotic SI carcinoid tumors or gastric carcinoids and normal mucosa respectively. (^a $P < 0.05$ vs non-fibrotic SI carcinoid tumors, ^b $P < 0.01$ vs normal SI mucosa). NS = not significant. mean \pm SE.

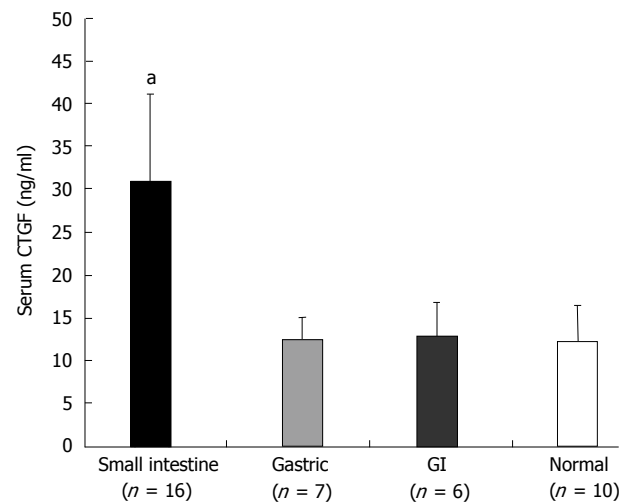


Figure 6 Serum levels of CTGF in patients with SI EC cell carcinoid tumors, gastric ECL cell carcinoids, other GI carcinoids [hepatic, rectal or appendiceal] and normal controls. Levels (ng/mL) were significantly elevated (> 2 -fold versus all other patient groups) in patients with SI EC cell carcinoid tumors compared to the other GI carcinoid tumors. ^a $P < 0.05$ vs all other samples. mean \pm SE.

the mesentery by SI carcinoid tumor cells and is a consequence of the secretory activity of these cells. In addition we have demonstrated that the mechanism may be due to CTGF production, and TGF β related events that activate an intestinal stellate (myofibroblastic) cell resulting in a local desmoplastic response. The latter is responsible for the clinical consequences of mesenteric fibrosis and adhesive obstruction noted in SI carcinoid tumors.

In our studies, Q RT-PCR demonstrated that all samples from patients with SI carcinoid tumors had elevated CTGF message levels (+ 1.1 to + 4.4-fold). In contrast, non-fibrotic gastric ECL cell carcinoids had significantly decreased CTGF levels. This analysis demonstrates that CTGF was quantitatively over-expressed in SI tumors. Message levels for TGF β 1 were elevated in SI carcinoid tumor samples but not in gastric samples. These results indicate that CTGF and TGF β 1 are potentially functionally related in the tumor EC cell but not in the ECL cell. We have previously reported that type I gastric (ECL cell) carcinoids (with no evidence of fibrosis) failed to express detectable levels of CTGF message by standard RT-PCR^[27]. These results suggest that CTGF message produced by a transformed neuroendocrine cell (the SI EC cell) is associated with fibrosis.

Immunohistochemistry demonstrated that the majority ($> 75\%$) of SI carcinoid tumor cells expressed CTGF. In normal mucosa, CTGF immunostaining was restricted to the basal third of the SI crypts with either CgA or serotonin-positive cells. Approximately one-third of serotonin-expressing (EC) cells were CTGF-positive (data not shown). It is likely that the remainder of the CTGF-staining cells are myofibroblasts in the crypts. CTGF-positive myofibroblasts have previously been demonstrated in the rectum^[28].

Carcinoid tumor cells also express TGF β 1, and pre-

sumably this growth factor is secreted by these cells during mesenteric invasion. This was previously noted by Chaudhry *et al*^[20] who reported that stromal cells expressed the TGF β receptor. This suggests a mechanism by which tumor cells can interact with stromal cells and influence their function. Our immunohistochemical analysis demonstrated that stromal cells in areas of mesenteric fibrosis were α -smooth muscle actin positive. α -smooth muscle actin is a marker for activated myofibroblasts (or stellate cells^[15,19]) and indicates that fibrosis-induction in the small intestine is associated with a stellate cell phenotype. This is a typical phenotype of both pancreatic- and hepatic-associated fibrosis^[17,19], and suggests this may be an archetypal GI fibrotic phenomenon. This postulate is supported by evidence that vimentin, desmin and collagen-III, all markers of a stellate-cell driven fibrosis, were present in SI fibrosis.

In order to confirm whether stellate cells were present in this tissue and played a role in fibrosis, we isolated and characterized a cell type from a patient with SI carcinoid tumor fibrosis that exhibited the hallmarks of a stellate cell^[15]. During primary culture, this cell flattened, initially developed long, cytoplasmic extensions, and subsequently, the typical stellate shape of activated myofibroblasts. The presence of α -smooth muscle actin staining confirmed the stellate cell phenotype. Addition of TGF β 1 resulted in activation of CTGF message and demonstrated the cell type to be functionally responsive to this growth factor. These functional data, together with the immunohistochemical evidence of activated intestinal stellate cells in situ, strongly suggest that carcinoid-induced fibrosis is a stellate-cell induced phenomenon. It is possible that the "intestinal stellate" cell could be derived from precursor cells in blood stream and there is some evidence that bone marrow-derived cells can migrate into the SI^[29]. A study of hepatic stellate cells, however, conclusively identified that these cells were not derived from bone marrow derived fibrocytes^[30]. The latter did not stain for α -smooth muscle

actin or desmin and were considered a separate population within the liver. This, as well as our immunohistochemical results strongly suggests the presence of an endogenous intestinal stellate cell population.

Having established that mesenteric fibrosis was associated with elevated CTGF and TGF β 1 in SI carcinoid tumors and identified a mesenteric target cell (intestinal stellate cell), we next used TMA analysis to both quantitate the protein expression as well as the cellular source of CTGF and TGF β 1 and statistically determine whether these proteins were related to clinically and histologically documented evidence of fibrosis. Our results demonstrated that TGF β 1 levels were elevated in patients with fibrosis, and were significantly increased compared to normal SI mucosa and to gastric carcinoids. The difference in protein expression between fibrotic SI carcinoid tumors and non-fibrotic gastric carcinoid samples identified on the TMA further supports a role for TGF β 1 in the etiology of this fibrosis. The role of CTGF was confirmed by the unambiguous relationship between increased expression of CTGF protein in primary SI carcinoid tumors and fibrosis. It is of interest to note that five patients who initially had exhibited elevated CTGF AQUA scores (87 ± 5) on the TMA subsequently developed fibrosis.

In order to identify a clinically useful tool to recognize patients at risk for fibrosis, we sought to measure CTGF in serum. Secreted CTGF protein could be identified in patient serum and was elevated in patients with SI carcinoid tumors compared to patients with gastric ECL cell carcinoids. Serum levels of CTGF from the latter patient group were similar to values in control subjects as might be predicted given that the gastric carcinoids are not associated with carcinoid fibrosis. The highest levels of serum CTGF in this study were identified in two patients with SI carcinoid tumors who also had the typical carcinoid "flushing" symptoms consistent with disseminated disease. This suggests this protein is identifiable in serum and can discriminate SI from gastric carcinoids. Prospective longitudinal studies in patients with and without fibrosis are needed to determine whether plasma levels have clinical significance in the detection, or prediction of peritoneal or cardiac fibrosis.

In conclusion, SI carcinoid tumors over-express CTGF and TGF β 1 mRNA and synthesize CTGF and TGF β 1 protein which are significantly elevated in patients with clinically documented fibrosis. In addition, SI carcinoid tumors secrete CTGF, which is readily detectable in the serum. We have also immunohistochemically identified and biochemically characterized intestinal stellate cells from mesenteric fibrosis. These cells respond to TGF β 1 with CTGF mRNA transcription. In addition, matrix production in SI carcinoid tumor fibrosis was similar to that identified in other stellate cell-driven reactions (e.g., liver or pancreas)^[15,17,19]. We postulate that intestinal stellate cells are the target cells that are activated by profibrotic mediators (TGF β 1 and CTGF) synthesized and secreted by invasive SI carcinoid tumor cells. Furthermore, once activated, these stellate cells may auto-regulate the fibrotic phenotype (by production of CTGF). The detection of blood levels of CTGF may ultimately provide a diagnostic opportunity to predict the development of fibrosis and pre-empt its local and systemic complications.

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Exogenous sphingomyelinase causes impaired intestinal epithelial barrier function

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Abstract

AIM: To test the hypothesis that hydrolysis of sphingomyelin to ceramide changes the composition of tight junctions (TJs) with increasing permeability of the intestinal epithelium.

METHODS: Monolayers of Caco-2 cells were used as an *in vitro* model for the intestinal barrier. Permeability was determined by quantification of transepithelial flux and transepithelial resistance. Sphingolipid-rich membrane microdomains were isolated by a discontinuous sucrose gradient and characterized by Western-blot. Lipid content of microdomains was analysed by tandem mass spectrometry. Ceramide was subcellularly localized by immunofluorescent staining.

RESULTS: Exogenous sphingomyelinase increased transepithelial permeability and decreased transepithelial resistance at concentrations as low as 0.01 U/mL. Lipid analysis showed rapid accumulation of ceramide in the membrane fractions containing occludin and claudin-4, representing TJs. In these fractions we observed a concomitant decrease of sphingomyelin and cholesterol with increasing concentrations of ceramide. Immunofluorescent staining confirmed clustering of ceramide at the sites of cell-cell contacts. Neutralization of surface ceramide prevented the permeability-increase induced by platelet activating factor.

CONCLUSION: Our findings indicate that changes in lipid composition of TJs impair epithelial barrier functions. Generation of ceramide by sphingomyelinases might contribute to disturbed barrier function seen in diseases such as inflammatory, infectious, toxic or radiogenic bowel disease.

INTRODUCTION

Abnormal mucosal permeability is a hallmark of inflammatory bowel disease (IBD)^[1,2]. The epithelial barrier function and its relevance in IBD pathophysiology has gained increasing attention in recent years^[2] as it may contribute to increased bacterial translocation and subsequent inflammatory responses in the mucosa.

Many signaling molecules involved in the pathogenesis of IBD such as TNF- α or IFN- γ may cause an alteration of the lipid composition in the cell membrane by activation of sphingomyelinases (SMases)^[3,4]. SMases are characterized by a specific optimal pH and accordingly are divided into acid, neutral and basic sphingomyelinase species. The acid sphingomyelinase (ASM) contributes to lysosomal sphingomyelin turnover and is also secreted upon cellular treatment with inflammatory stimuli^[5]. In contrast, neutral SMase is membrane-bound, alkaline SMase is found in the bile. Activation of SMases is followed by rapid hydrolysis of plasma membrane sphingomyelin to the second messenger ceramide^[6]. The sphingolipid ceramide is an important messenger involved in many signaling pathways with influences on cell differentiation, growth suppression and apoptosis. It is generated upon ligation of receptors like cluster of differentiation (CD) 40, CD95, IL-1 or TNF receptor^[7-10], as response to ionizing radiation^[11-13], ischemia-reperfusion injury^[14] and infections with bacteria like *Neisseria gonorrhoeae* and *Pseudomonas aeruginosa*^[15-17] or viruses like Sindbis- or Rhinovirus^[18,19]. Ceramide alters the composition of cholesterol- and sphingolipid-enriched membrane microdomains^[20] and thereby promotes transmembrane signaling^[7-9]. It also has the capacity to restructure the membrane to allow the release of vesicles^[21].

Grassme *et al*^[7,8] have demonstrated that cellular stimulation triggers a translocation of the acid sphingomyelinase from intracellular stores onto the extracellular leaflet of the cell membrane. Surface ASM initiates a release of ceramide which mediates clustering of sphingolipid-rich membrane domains, termed “lipid rafts”. Lipid rafts are also described as detergent insoluble cholesterol- and glycosphingolipid-enriched membrane microdomains (DIGs) because the tight packing of the lipids renders rafts resistant to solubilization by non-ionic detergents at low temperatures^[22,23].

Intestinal permeability is influenced by the lipid content of epithelial cells. Dietary fatty acids are known to affect barrier function of the mucosa^[24,25]. Clinical studies showed that omega 3-fatty acids may be of beneficial effect on the course of Crohns disease^[26,27]. Paracellular permeability is controlled by a junctional complex of proteins and lipids which form different strands, commonly described as adherens-junction and tight-junction (TJ). TJs have been identified as microdomains in the plasma membrane with similar characteristics as DIGs^[28,29]. Depletion of cholesterol from Caco-2 cell layers increases permeability^[30], suggesting that cholesterol is critical in maintaining the barrier function.

The proinflammatory cytokines TNF- α and IFN- γ , known to induce ceramide-generation, have recently been shown to disrupt the barrier function of epithelial cells independent from their apoptosis-inducing property^[31]. With regard to the finding that ceramide displaces cholesterol from sphingolipid-enriched microdomains^[20], we hypothesized that formation of ceramide may be an initial event leading to structural lipid-rearrangements of TJs with impaired barrier integrity.

To test this hypothesis we used the intestinal epithelial cell line Caco-2 to assess the effect of exogenous SMase on barrier function and the accompanying lipid composition of TJs.

MATERIALS AND METHODS

Cells and reagents

The human intestinal epithelial cell line Caco-2 was obtained from American Type Culture collection (HTB 37). Cells were maintained in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% non-essential amino acids 1% sodium pyruvate in an atmosphere containing 10% CO₂ at 37°C. Sphingomyelinase from staphylococcus aureus, platelet activating factor (PAF) and deoxycholic acid were purchased from Sigma-Aldrich, Germany.

Antibodies

The following antibodies were used for Western-blot analysis: Goat polyclonal anti-occludin (C-19) and goat polyclonal anti-claudin-4 (C-18) from Santa Cruz, CA, USA; mouse anti-E-cadherin (clone HECD-1) from Calbiochem, CA, USA; rabbit polyclonal anti p38 mitogen activated protein kinase (MAPK) from New England Biolabs, Beverly, MA, USA. The rabbit polyclonal anti-toll like receptor 4 (TLR4) Ab was a kind gift from Dr. Werner

Falk, Regensburg, Germany. For immunohistochemical staining of tight junctions we used the rabbit polyclonal anti-ZO-1 Ab from Zymed lab. Inc., CA, USA. For visualization and neutralization of ceramide, the monoclonal mouse IgM anti-Ceramide Ab (MID 15B4) from Alexis, Germany was used.

Measurement of transepithelial permeability and transepithelial resistance

For permeability assays, Caco-2 cells were seeded in 12 well plates with a growth area of 1.0 cm² and a pore size of 3 μ m (Transwell[®] permeable supports by Corning Incorporated, MA, USA) at a density of 2×10^5 cells/cm². Media was replaced every 3 or 4 d. Experiments were performed 13-15 d after cells reached confluency with a transepithelial electrical resistance (TEER) between 500-750 Ω ·cm². Permeability was quantified by measuring the transepithelial flux of fluorescein-sulfonic acid (Molecular Probes Inc., Germany). After treatment of Caco-2 monolayers with the indicated substances, fluorescein-sulfonic acid was added to the apical side of the monolayers at a final concentration of 100 μ g/mL. After incubation for 4 h 100 μ L aliquots of medium were removed from the basolateral chambers, and fluorescence signal by fluorescein was measured using a fluorescence microplate reader. TEER of the Caco-2 monolayers was measured using a Millicell[®]-ERS epithelial volttohmmeter by Millipore with a pair of chopstick electrodes. Untreated monolayers were used as negative controls. All measurements were performed in duplicate.

Caspase-3/7 activity assay

Caco-2 cells were incubated with the indicated substances 6 h before measurement of caspase-activity. As a positive control for induction of apoptosis, cells were treated with deoxycholic acid (DCA) for 1 h. DCA was sonicated at 40°C for 20 min prior to the experiments. The colorimetric caspase-3/7-activity assay Apo-ONE[™] (Promega, WI, USA) was used according to the manufacturer's recommendations. Activity was quantified using a fluorescence microplate reader with appropriate wavelengths for excitation (485 nm) and emission (530 nm).

Fluorescence microscopy

For fluorescence studies Caco-2 cells were grown on chamber slides (Nunc, Germany) and stimulated with 0.25 U/mL SMase for 10 min. After stimulation the cells were washed with phosphate buffered saline (PBS) at 4°C and fixed for 15 min in 1 % (w/v) paraformaldehyde in PBS at room temperature. Then cells were washed three times with PBS and blocked with Tween 20 0.05% in PBS. Cells were washed again and then incubated with anti-ceramide mAb 15B4 (1/50 dilution) and anti ZO-1 Ab for 30 min in washing buffer (PBS with 2% fetal calf serum, 0.01% NaN₃) to block sites of non-specific binding. The anti-ceramide Ab was visualized with a PE-labeled anti-mouse IgM, the anti-ZO-1 Ab with a fluorescein-isothiocyanate (FITC)-anti rabbit Ab. Fluorescence staining was viewed with an Axiovert 100 fluorescence microscope (Zeiss, Germany).

Isolation of detergent resistant microdomains

Confluent layers of Caco-2 cells were stimulated in 75 cm² flasks in 5 mL of culture media. Stimulation was terminated by washing the cells with 5 mL ice-cold TNE (25 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, and 5 mmol/L EDTA) on ice. Cells were washed twice with TNE at 4°C and lysed for 20 min in 1.5 mL of ice-cold TNE containing 1% of Triton X-100 and protease inhibitors (10 µg of aprotinin and leupeptin and 200 µmol/L phenylmethylsulfonyl fluoride (PMSF)). Cells were then further homogenized with 20 strokes in a Wheaton loose fitting Dounce-homogenizer. Nuclei and cellular debris were pelleted by centrifugation at 600 g for 5 min, 4°C. To isolate low-density, Triton X-100-insoluble complexes, the supernatant was adjusted to 40% sucrose, transferred to an ultracentrifugation tube, and overlaid with a 35% and 5% discontinuous sucrose gradient. Ultracentrifugation was performed at 29000 r/min (110000 × *g*) at 4°C for 16 h in a Beckman SW41 rotor. Gradient fractions (1 mL) were either collected from the top of the tube or from the 35%/5%-interface to compare the effect of cell stimulation on raft fractions. For mass spectrometry, fractions were prepared as described below. For Western blotting, proteins were precipitated using 400 µL of 10% trichloroacetic acid, neutralized and separated by 10% SDS-PAGE.

Immunoblotting

Proteins were separated by 10% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane (Hybond). Blots were blocked with 5% nonfat dry milk powder and incubated overnight at 4°C with the indicated primary antibodies. All antibodies were diluted 1:750 in Tris-buffered saline supplemented with 0.1% Tween 20. The membranes were further incubated with peroxidase-conjugated secondary antibodies, and protein bands were visualized using a commercial chemiluminescence detection kit (ECL plus, Amersham Biosciences).

Tandem mass spectrometry

Lipids were quantified by electrospray ionization tandem mass spectrometry (ESI-MS/MS) in positive ion mode as described previously^[32-35]. Samples were quantified by direct flow injection analysis using the analytical setup and the data analysis algorithms described by Liebisch *et al.*^[34]. A parent ion scan of *m/z* 184 specific for phosphocholine-containing lipids was used for phosphatidylcholine, sphingomyelin^[33] and lysophosphatidylcholine^[33]. Neutral loss scans of *m/z* 141 and *m/z* 185 were used for phosphatidylethanolamine and phosphatidylserine, respectively. Ceramide was analyzed similar to a previously described method^[35] using N-heptanoyl-sphingosine as internal standard. Free cholesterol and cholesteryl esters were quantified using a fragment ion of *m/z* 369 after selective derivatization of free cholesterol^[36]. Quantification was achieved by calibration lines generated by addition of naturally occurring lipid species to cell homogenates^[32-36].

Statistical analysis

Data are shown using vertical scatter plots with Box-

Whisker plots (25% and 75% values), generated in the basic module of the program SigmaPlot. Statistical analysis was performed by Mann-Whitney *U*-test, with *P* < 0.05 considered statistically significant. Data are given as means ± SE (SD in case of lipid analysis).

RESULTS

Exogenous sphingomyelinase enhances permeability in Caco-2 epithelial cell layers

To study a potential regulation of intestinal permeability by sphingomyelinases, Caco-2 cell layers were exposed to different concentrations of exogenous SMase. Transepithelial permeability was determined by measurement of transepithelial flux of fluorescein-sulfonic acid across a monolayer grown on permeable supports. Incubation with SMase to the apical chamber induced a concentration-dependent increase of permeability which could be detected at concentrations as low as 0.01 U/mL SMase (181.6% ± 16.7%, *P* < 0.01) (Figure 1A). Using 0.05 U/mL SMase, permeability was increased by 201.1% ± 15.8% (*P* < 0.01) and by 224.0% ± 18.0% (*P* < 0.01) when 0.125 U/mL SMase were used. Increase of SMase-concentration to 0.25 U/mL did not further increase transepithelial flux (192.0% ± 15.3%, *P* < 0.01) (Figure 1A). In a different set of experiments with the same experimental conditions, PAF was used as a positive control. At a concentration of 5 µmol/L, PAF increased permeability by 162.8% ± 13.0% (Figure 2).

To gain insight into the mechanisms of ceramide-mediated permeability we measured the transepithelial electrical resistance (TEER). Exogenous SMase produced a significant decline in TEER at concentrations as low as 0.01 U/mL (17.5% ± 6.2%, *P* < 0.05) (Figure 1B). The fall in TEER with 0.05 U/mL was much higher (38.1% ± 6.0%, *P* < 0.01). Using 0.125 U/mL SMase or 0.25 U/mL SMase did not further decrease TEER (32.2% ± 7.3%, *P* < 0.01 and 33.2% ± 6.4%, *P* < 0.01, respectively).

To exclude apoptotic or necrotic cell death caused by SMase within the time frame of our experiments, caspase-3/7-activity and LDH release assays were performed. As shown in Figure 1C, 0.25 U/mL SMase induced no activation of caspase-3/7 within 6 h. Deoxycholic acid (500 µmol/L for 1 h) was used as a positive control. Release of LDH from Caco-2 monolayers by SMase was also not detectable (data not shown).

Neutralization of surface ceramide prevents permeability-increase induced by PAF

Next, we investigated whether the increased permeability induced by PAF might be linked to rearrangement of tight-junctional lipids. Incubation of the monolayers with 5 µmol/L PAF increased permeability by 162.8% ± 13.0% (Figure 2). To examine the role of ceramide in PAF-mediated permeability we co-incubated Caco-2 cell layers with ceramide-antiserum (1/100 dilution). Co-incubation of the Caco-2-monolayer with ceramide-antiserum prevented the increase of permeability induced by 5 µmol/L PAF (111.6% ± 9.86%, *P* < 0.05) (Figure 2), indicating a stabilization of tight-junctional complexes by the IgM-anti-ceramide Abs.

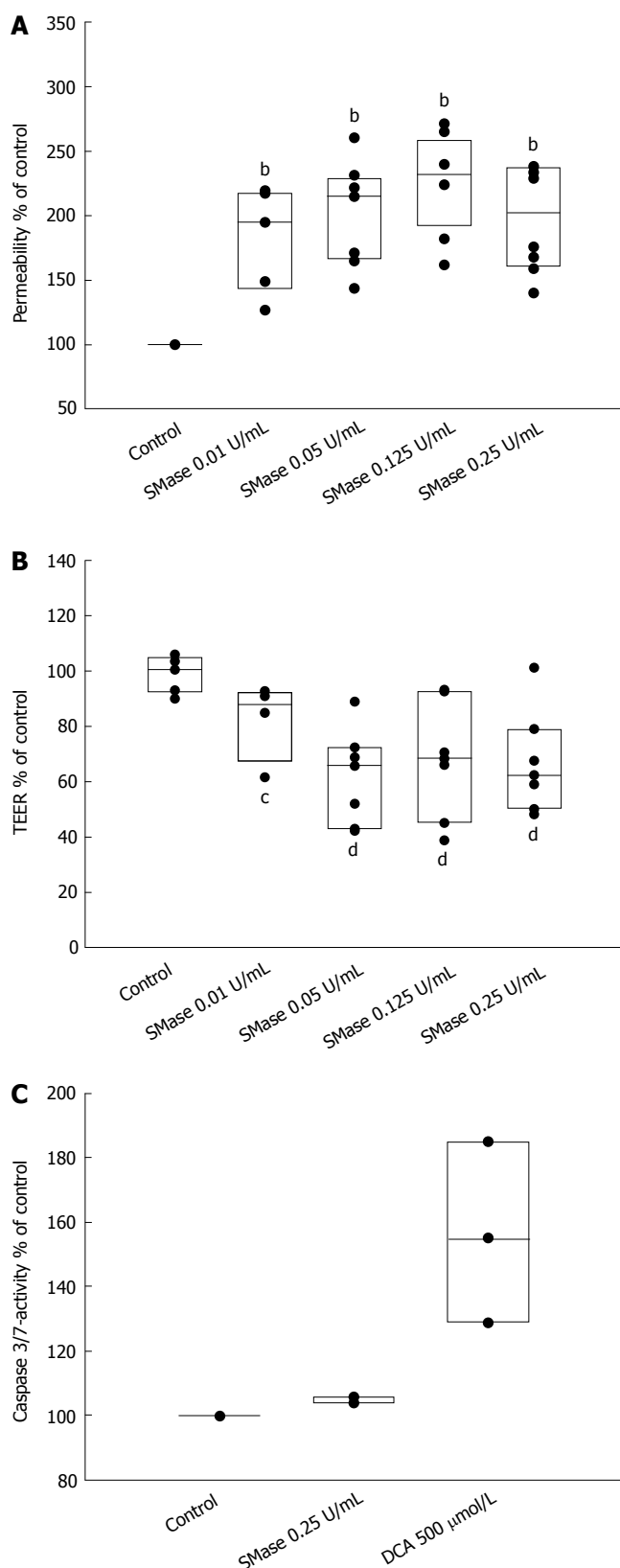


Figure 1 Exogenous sphingomyelinase increases permeability of Caco-2 epithelial monolayers. **A:** Caco-2 monolayers were incubated with different concentrations of exogenous SMase. ^b $P < 0.01$ between control and treated samples; **B:** Transepithelial electrical resistance (TEER) was measured across the cell monolayers 4 h after treatment of the monolayers with exogenous SMase. The data were compared with TEER before treatment and are expressed as % of untreated. ^c $P < 0.05$, ^d $P < 0.01$; **C:** Epithelial apoptosis was monitored 6 h after incubation with 0.25 U/mL SMase by measurement of caspase-3/7-activity with colorimetric assays. As a positive control, deoxycholic acid (DCA) was used for 1 h.

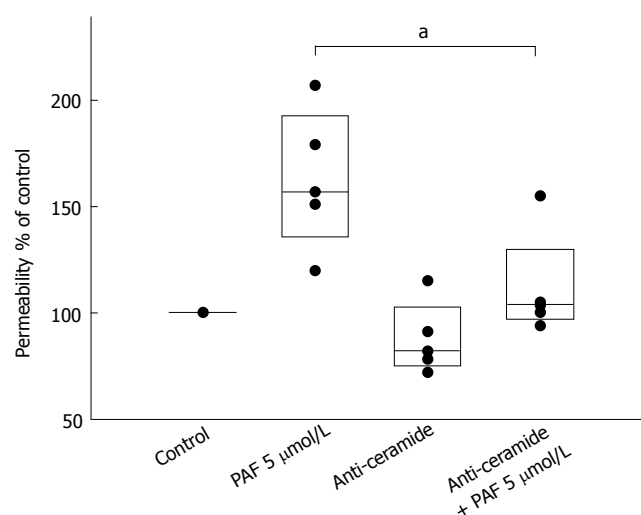


Figure 2 Neutralization of surface-ceramide prevents PAF-mediated increase of permeability. Caco-2 cell layers were incubated with the IgM ceramide-antiserum (15B4) 30 min prior to stimulation with PAF. Permeability was determined by measurement of transepithelial flux of fluorescein sulfonic acid from the upper chamber of the Transwell insert to the lower chamber across the Caco-2-monolayer after 4 h. The data are expressed as % of control. ^a $P < 0.05$ between PAF-treated samples.

Detergent insensitive glycosphingolipid-enriched domains (DIGs) contain major pools of tight junction proteins like occludin and claudin 4

To further test our hypothesis, DIGs were isolated using sucrose gradient techniques and analysed for their lipid- and protein-composition. Analysis of the lipid composition by tandem mass spectrometry revealed the presence of high amounts of sphingomyelin ($12.5\% \pm 1.4\%$), cholesterol ($44.9\% \pm 4.9\%$) and ceramide ($0.74\% \pm 0.18\%$) in DIGs with only $3.8\% \pm 0.6\%$, $19.2\% \pm 3.9\%$ and $0.19\% \pm 0.03\%$ in the total cell lysate (including DIGs), respectively (Figure 3A, values are given as % of analysed lipids \pm SD). This indicated a good separation of DIGs from other membrane components. To ascertain isolation of tight junctions within the Triton X-100 insoluble preparations, Western-blot experiments were performed to prove the presence of tight-junctional proteins. As shown in Figure 3B, major pools of the tight-junctional proteins occludin and claudin-4 were present in Triton X-100 insoluble preparations and to a lesser extent in cell lysate. The basolateral membrane protein E-cadherin was also present in DIGs but was not as prominent. To exclude contamination with proteins not associated with DIGs, Western-blot of TLR4 and p38MAPK were performed which could not be detected in preparations of DIGs (Figure 3B).

Ceramide accumulates in DIGs, whereas sphingomyelin and cholesterol decrease

Tandem mass spectrometry was used to analyze the effect of SMase on the lipid content of DIGs which overlap with tight junctions. Incubation of Caco-2 monolayers with 0.25 U/mL SMase resulted in rapid increase of ceramide in DIGs by $5.0\% \pm 2.4\%$ after 10 min ($P < 0.01$) and $7.3\% \pm 2.6\%$ after 30 min ($P < 0.01$), whereas the increase was

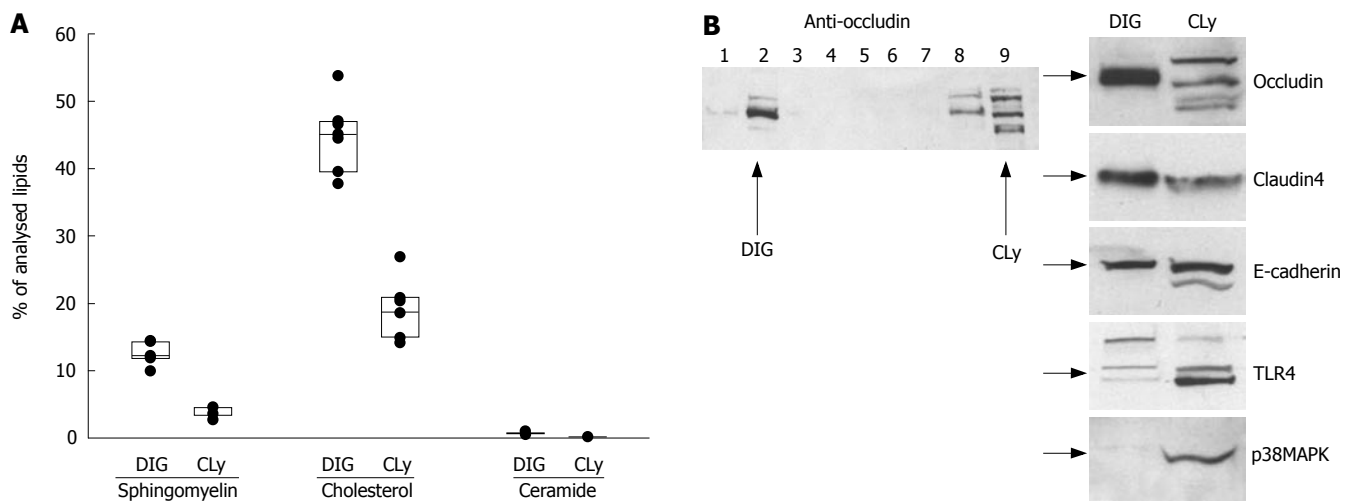


Figure 3 Preparations of detergent insensitive glycosphingolipid-enriched domains (DIGs) contain TJ-proteins. **A:** Lipid analysis of DIGs from non-stimulated Caco-2 cells by tandem mass spectrometry revealed the presence of high amounts of sphingomyelin, cholesterol and ceramide when compared to the total cell lysate; **B:** Gradient fractions were separated by 10% SDS-PAGE and analyzed by Western blot. Fraction No. 2 represents the raft-containing fraction at the 35%/5% interface (DIG); No. 9 represents the soluble fraction (cell lysate = CLy). The presence of high amounts of TJ-proteins occludin and claudin-4 and, to a lesser extent, E-cadherin show a clear association of TJ-proteins with DIGs. The simultaneous absence of other proteins like TLR4 and p38MAPK excludes significant contamination with non-raft fractions.

only $0.84\% \pm 0.14\%$ and $1.25\% \pm 0.23\%$ in the whole cell lysate (including DIGs), respectively (Figure 4A, data shown as % of analysed lipids). In the same preparations of DIGs the concentration of sphingomyelin decreased by $4.73\% \pm 1.4\%$ after 10 min ($P < 0.01$) and $7.6\% \pm 2.2\%$ after 30 min ($P < 0.01$) (Figure 4B). The concentration of cholesterol declined by $5.0\% \pm 3.2\%$ after 10 min and $4.8\% \pm 2.3\%$ after 30 min (Figure 4C). This reflects a decrease of the percentage of cholesterol by $10.9\% \pm 7.3\%$ after 10 min ($P < 0.05$) and $10.6\% \pm 4.8\%$ after 30 min ($P < 0.01$).

Clusters of ceramide colocalize with tight junctions

Fluorescence microscopy was performed to demonstrate the localization of ceramide accumulation. We induced generation of ceramide by incubation of Caco-2 cells with 0.25 U/mL SMase for 10 min. After stimulation, staining of the cells with anti-ceramide 15B4 antibodies revealed the formation of ceramide-clusters (Figure 5A and B) that were frequently located at the sites of cell-cell-contact. Costaining for the tight-junction protein ZO-1 confirmed colocalization of ceramide-clusters with the junctional complexes (Figure 5B).

These data indicate that the generation of ceramide by SMase leads to localized accumulation of ceramide at the sites of cell/cell-contact with increased intestinal permeability and suggest a central role of ceramide in the regulation of barrier integrity.

DISCUSSION

Our data indicate that hydrolysis of sphingomyelin to ceramide increases intestinal epithelial cell permeability in a well-established model of Caco-2 cell monolayers^[37]. To obtain naturally occurring long chain ceramides, formation of ceramide was induced by addition of bacterial sphingomyelinase (SMase). This approach was used because neutral as well as acid SMase are capable of generating ceramide in the outer leaflet of

the cell membrane by rapid hydrolysis of membrane sphingomyelin. Previous studies suggested that, upon cellular stimulation, the acid sphingomyelinase (ASM) translocates onto the outer surface of sphingolipid-rich membrane microdomains^[7,8]. The fact that ASM activity is also increased in the serum of mice treated with endotoxin^[38] or PAF^[39], indicates a possible role of extracellularly located ASM. Another advantage of using exogenous SMase is the enzymatic cleavage of sphingomyelin at the sites of sphingomyelin-enriched domains in the cell membrane, whereas the addition of long chain ceramides might lead to lateral assembly of ceramide to pre-existing DIGs (and/or TJs) without affecting the structure inside these microdomains. Apart from that, exogenous ceramides are likely to be rapidly metabolised, thus having a rather transient effect on lipid composition. Exogenous SMase was effective in terms of increased permeability and decrease of TEER, indicating a reduction of the paracellular barrier by hydrolysis of sphingomyelin. Other possible effects of SMase include an increase of transcellular transport of vesicles. Therefore, we measured intracellular fluorescence after exposure of the cells with SMase and fluorescein-sulfonic acid with colorimetric assays of the cell lysates and performed transepithelial flux experiments with simultaneous colchicine-treatment (data not shown). In both cases we did not observe any differences, making transcellular transport unlikely. To exclude damage of monolayers as a possible cause for increased permeability, activity of caspase-3/7 in the cell lysate and lactate dehydrogenase (LDH) in the supernatant were measured without significant results.

Lipid analysis of Triton X-100 insoluble preparations clearly demonstrated the separation of membrane portions which were enriched in cholesterol, sphingomyelin and ceramide. Western-blot experiments of DIGs revealed that these preparations contained major pools of the TJ-proteins occludin and claudin-4. Upon stimulation with

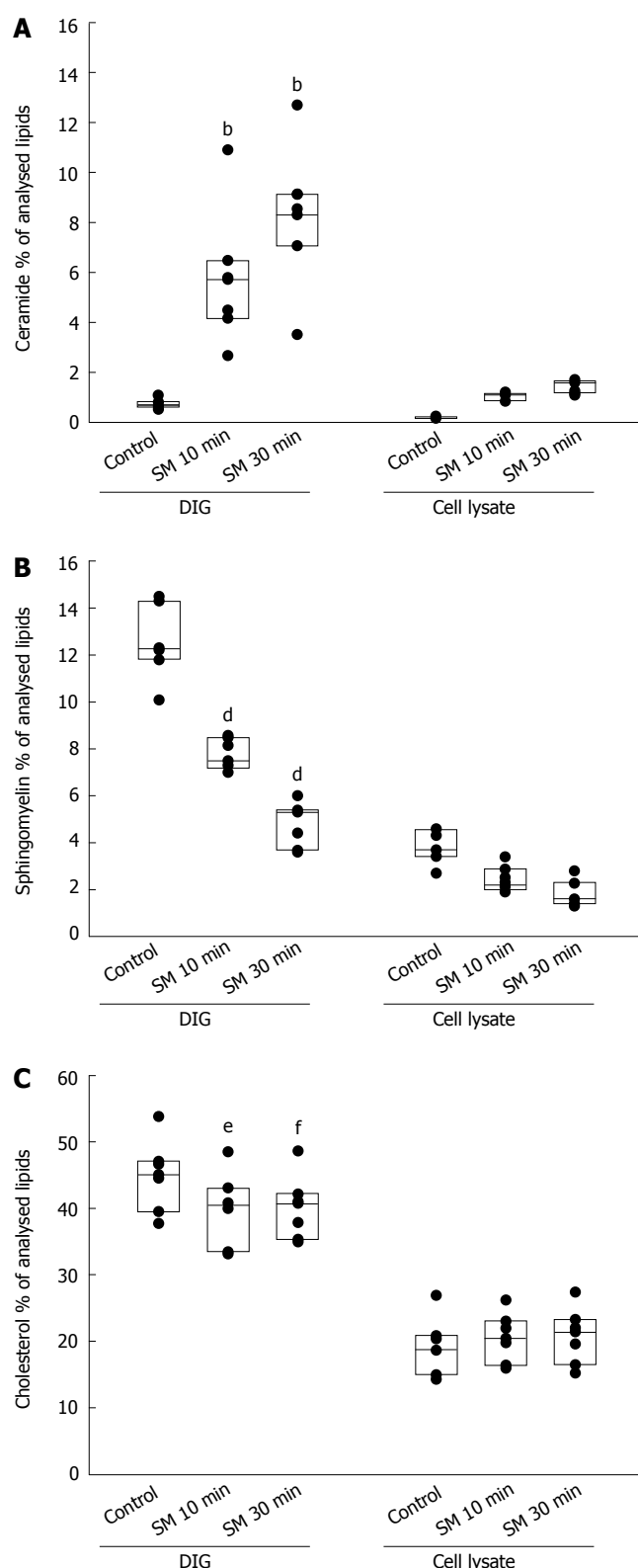


Figure 4 A: Lipid analysis by tandem mass spectrometry revealed a rapid accumulation of ceramide in preparations of DIGs after incubation with 0.25 U/mL SMase for 10 or 30 min whereas the increase of ceramide in the cell lysate was only small in comparison ($^bP < 0.01$); B: The content of sphingomyelin in DIGs decreased at the same time with a relative reduction comparable to the generation of ceramide ($^dP < 0.01$); C: Cholesterol also decreased in DIGs whereas the concentration in the cell lysate remained constant ($^eP < 0.05$ and $^fP < 0.01$).

SMase we detected a rapid accumulation of ceramide in the same membrane fractions, indicating selective

accumulation of ceramide in sphingolipid-enriched membrane microdomains, including junctional complexes.

Previous studies suggested displacement of cholesterol from DIGs by ceramide^[20,40,41]. To our knowledge, there is no data in the available literature about the modulation of ceramide and cholesterol in TJs by sphingomyelinases. Therefore we compared the content of cholesterol in DIGs which overlap with TJs. In accordance with these studies we also observed a decrease of cholesterol in these microdomains with increasing concentrations of ceramide, suggesting a rearrangement of tight junctional lipids by ceramide with impaired barrier function.

Lambert *et al*^[30] showed a decrease of TEER by 80%-90% after extraction of 40%-45% of cholesterol from Caco-2 monolayers. Compared to these results the decrease of tight-junctional cholesterol by ceramide is moderate. Nevertheless, these changes could explain early disturbances of tight-junctional integrity upon cellular stress, deriving from toxic, infectious or immunologic challenges of the intestinal epithelium. Therefore, we propose the following pathophysiologic model: Cellular stimulation leads to localized accumulation of ceramide with concomitant decrease of sphingomyelin and cholesterol leading to destabilization of tight junctional strands and loss of barrier integrity.

Long chain ceramides also have the unique property of fusing membrane domains and tend to form cluster, which was also observed in our studies. Mechanisms of ceramide to modify the structure of bilayers and their effect in promoting efflux and release have been studied in several models^[21,42,43]. Montes *et al*^[21] demonstrated release of molecules with a molecular mass up to 20 kDa upon treatment of unilamellar vesicles with sphingomyelinase. A possible mechanism for this release was suggested by Siskind and Colombini who detected formation of large stable channels by ceramide in planar bilayers through electrophysiological methods^[43]. The influence of ceramide on the architecture of TJs also seems to be important in the PAF-mediated increase of permeability. Pretreatment of Caco-2 cells with a monoclonal IgM ceramide-antiserum prevented the loss of barrier function induced by 5 μ mol/L PAF.

Together, these findings provide evidence for a new aspect of cellular ceramide generation. Lipid rearrangements triggered by enzymatically catalysed formation of ceramide upon cellular stimulation may be a frequent process for localized exposure of intraluminal antigens to the immune system. Repeated stimulation or extension of the stimulated area may lead to an imbalance of the lipid geometry producing a stronger leakiness of the affected junctional complexes resulting in initiation of an inflammatory process which may further perpetuate itself. Better knowledge of the relevant lipids which control this "lipid-barrier" and their modifying enzymes would be helpful to develop treatment strategies to strengthen this barrier. Lipid-enriched diets or inhibition of relevant lipid-modifying enzymes could be possible treatment modalities to control chronic inflammation of the intestine as seen in IBD^[44].

In summary, our data demonstrate that hydrolysis

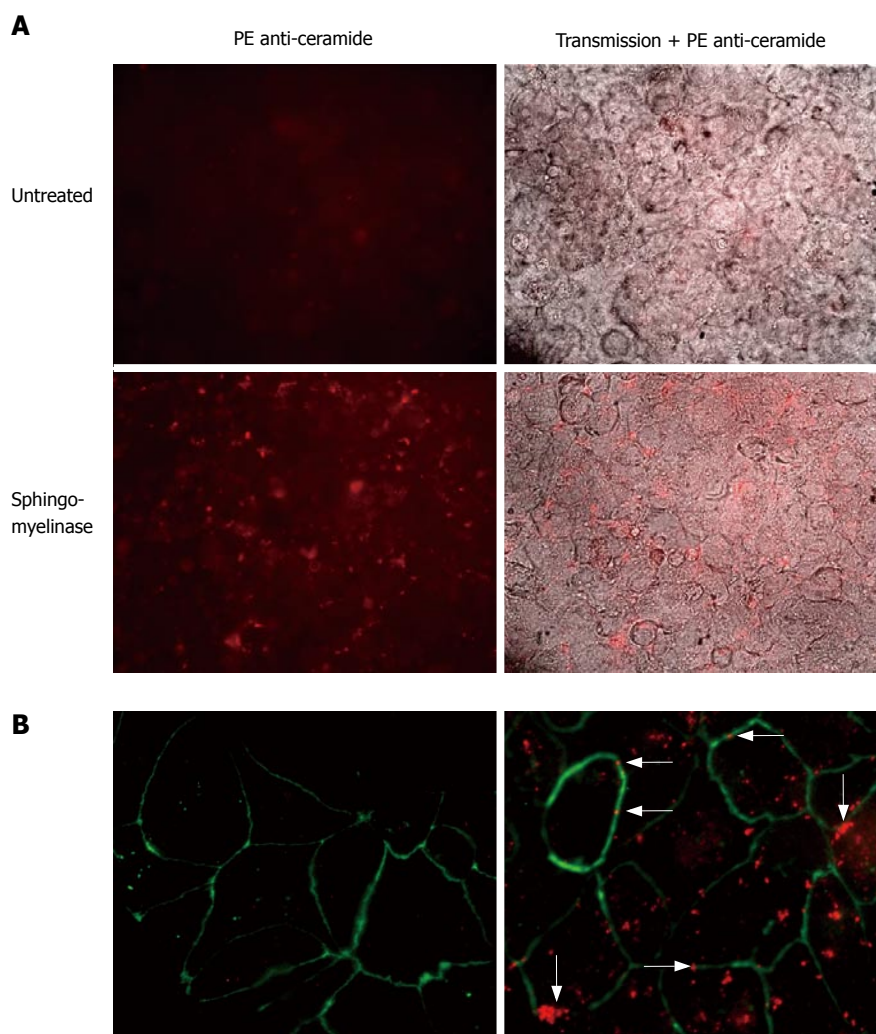


Figure 5 Ceramide clusters at the sites of cell-cell contacts. Caco-2 cell layers were incubated with 0.25 U/mL SMase for 10 min or left untreated. After fixation of the cells with paraformaldehyde, ceramide was visualized by staining of the cells with the monoclonal anti-ceramide 15B4 antibody and PE-coupled anti-mouse antibodies. **A:** Top, left: Non-stimulated cells displayed a certain level of background staining that was used to determine the time of exposure. Top, right: Overlay of fluorescence and transmission. Bottom, left: Incubation with SMase resulted in formation of multiple ceramide-clusters. Bottom, right: Overlay of fluorescence and transmission. Close inspection of the clusters revealed a frequent localization of the clusters at the sites of cell/cell-contact; **B:** Costaining of tight junctions with FITC-labeled anti ZO-1 Abs. Left: non-stimulated. Right: Predominant colocalization of ceramide-clusters with tight junctions upon stimulation with SMase (arrows indicate ceramide-clusters at the sites of cell-cell contact).

of sphingomyelin to ceramide affects transepithelial permeability and resistance. Long chain ceramides, generated by SMase, accumulate in cholesterol- and sphingolipid-enriched membrane microdomains which include TJs. These microdomains fuse to large, ceramide-enriched clusters, located on the surface of the cells and at the sites of cell-cell contact, explaining the effect of long-chain ceramides on barrier integrity. Finally, the effect of PAF on paracellular permeability is inhibited by neutralization of surface ceramide, suggesting a regulation of the paracellular permeability by the arrangement of membrane ceramide.

COMMENTS

Background

The sphingolipid ceramide, generated by signal-activated sphingomyelinases, has emerged as a second messenger of stimuli as diverse as ligation of various receptors, ionizing radiation, chemotherapy or infection with some bacteria and viruses. Upon stimulation of sphingomyelinases, ceramide is generated in distinct sphingolipid-enriched membrane microdomains of the cell membrane, termed "lipid rafts". Tight junctions (TJs) are structurally related to lipid rafts, and thus, we hypothesized that hydrolysis of sphingomyelin to ceramide changes the composition of TJs with increasing permeability of the intestinal epithelium.

Research frontiers

Intestinal permeability is influenced by the lipid content of epithelial cells and some lipids may be beneficial for the course of inflammatory bowel disease. Modification of the lipid

content of TJs may be a possible strategy to improve intestinal barrier functions.

Innovations and breakthroughs

Our data indicate that hydrolysis of sphingomyelin to ceramide by sphingomyelinase (SMase) increases intestinal epithelial cell permeability. Lipid analysis after stimulation with SMase demonstrated rapid accumulation of ceramide in the membrane fractions which contain the TJ-proteins occludin and claudin-4, while sphingomyelin and cholesterol decrease. Pretreatment of cells with a monoclonal IgM ceramide-antiserum prevented the loss of barrier function induced by platelet activating factor.

Applications

Better knowledge of the relevant lipids and their modifying enzymes that control this "lipid-barrier" of the intestine would be helpful to develop treatment strategies to strengthen this barrier. Lipid-enriched diets or inhibition of relevant lipid-modifying enzymes could be possible treatment modalities to control chronic inflammation of the intestine as seen in inflammatory bowel disease.

Terminology

Sphingomyelinase is an enzyme which hydrolyses sphingomyelin to ceramide upon various cellular stimuli. Paracellular permeability is controlled by a junctional complex of proteins and lipids which form different strands, commonly described as adherens-junction and tight-junction (TJ). Platelet activating factor is a lipid messenger which increases paracellular permeability in intestinal epithelial cells.

Peer review

The authors set out to test the hypothesis that hydrolysis of sphingomyelin to ceramide changes the composition of tight junctions (TJs) with increasing permeability of the intestinal epithelium. Their findings suggested that changes in lipid composition of TJs impair epithelial barrier functions. Generation of ceramide

by sphingomyelinases might contribute to disturbed barrier function seen in diseases such as inflammatory, infectious, toxic or radiogenic bowel disease.

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BASIC RESEARCH

Propolis reduces bacterial translocation and intestinal villus atrophy in experimental obstructive jaundice

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effect on ileal mucosa and reduced bacterial translocation in the experimental obstructive jaundice model. Further studies should be carried out to explain the mechanisms of these effects.

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Key words: Obstructive jaundice; Bacterial translocation; Ileal morphology

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Abstract

AIM: To investigate the effects of propolis on bacterial translocation and ultrastructure of intestinal morphology in experimental obstructive jaundice.

METHODS: Thirty Wistar-Albino male rats were randomly divided into three groups, each including 10 animals: group I, sham-operated; group II, ligation and division of the common bile duct (BDL); group III, BDL followed by oral supplementation of propolis 100 mg/kg per day. Liver, blood, spleen, mesenteric lymph nodes, and ileal samples were taken for microbiological, light and transmission electron microscopic examination on postoperative 7th d after sacrifice.

RESULTS: The mean number of villi per centimeter and mean mucosal height of the propolis group were significantly different in the BDL group ($P = 0.001$ and 0.012 , respectively). The electron microscopic changes were also different between these groups. Sham and BDL + propolis groups had similar incidence of bacterial translocation (BT). The BDL group had significantly higher rates of BT as compared with sham and BDL + propolis groups. BT was predominantly detected in MLNs and the most commonly isolated bacteria was *Escherichia coli*.

CONCLUSION: Propolis showed a significant protective

INTRODUCTION

The cytotoxicity of bile salts and the toxicity of high levels of intracellular bilirubin are suspected as mediators of some of the systemic consequences of obstructive jaundice. Recent data suggest that more complex mechanisms involving changes in gut flora, mucosal integrity, and macrophage-immune system interactions may be responsible for the complications of obstructive jaundice^[1].

The gastrointestinal tract is not only a passive organ of nutrient absorption, but it additionally displays important endocrine, immunologic, metabolic, and barrier functions^[2]. Bacterial translocation is the migration of bacteria or bacterial products from the intestinal lumen to mesenteric lymph nodes or other extraintestinal organs and sites^[3]. Obstructive jaundice impairs intestinal barrier function leading to bacterial and endotoxin translocation in experimental and clinical studies. It affects the three levels of gut barrier globally; the immune barrier, the biological barrier, and the mechanical barrier^[2]. Moreover, biliary obstruction in the rats results in a significant depression of the reticuloendothelial system (RES) phagocytic function, which may cause impaired systemic bacterial clearance and is associated with decreased survival following *E. coli* endotoxemia^[4].

Propolis is a natural product collected by honey bees from various plant sources. It has antibacterial,

antifungal, scolicidal, antiinflammatory, antioxidant, immunomodulatory, antiviral and anticarcinogenic properties^[5-13].

According to these properties, we planned to use propolis for determining the effects on bacterial translocation and the ultrastructure of intestinal morphology in experimental obstructive jaundice.

MATERIALS AND METHODS

Animals

Thirty Wistar-Albino male rats, weighing 250 ± 25 g, were housed under constant temperature ($21^\circ\text{C} \pm 2^\circ\text{C}$) individually in wire cages with a 12 h light-dark cycle. Twelve hours before anesthesia, animals were deprived of food, but had free access to water two hours before anesthesia. No enteral or parenteral antibiotics were administered at any time. The rats that died during the experiment were excluded from the experiment and no new rat was included. The procedures in this experimental study were performed in accordance with the National Guidelines for The Use and Care of Laboratory Animals and approved by Animal Ethics Committee of Ankara Research and Training Hospital.

Study groups

Rats were randomly divided into three groups, each including 10 animals: group I, sham-operated; group II, ligation and division of the common bile duct (BDL); group III, BDL followed by oral supplementation of propolis 100 mg/kg per day (Balparmak LTD, Istanbul, Turkey) with a nasogastric tube that was inserted daily and taken off after propolis supplementation. The first dose of propolis was given on postoperative d 1 and continued until the rats were sacrificed. Animals were sacrificed on postoperative d 7 by high-dose diethyl ether inhalation. Liver, blood, spleen, mesenteric lymph nodes, and ileal samples were taken for microbiological, light and transmission electron microscopic examination.

There is no standard dose for propolis based on previous experimental studies. In previous studies the dose ranged between 30-200 mg/kg per day^[14,15]. We gave propolis at a dose of 100 mg/kg per day to each rat.

Operative procedure

Animals were anesthetized by intramuscular injection of 30 mg/kg ketamine hydrochloride (Ketalar®; Parke-Davis, Istanbul, Turkey) and 5 mg/kg xylazine (Rompun®, Bayer, Istanbul, Turkey). Midline laparotomy was performed under sterile conditions. In the sham-operated group (group I) the common bile duct (CBD) was freed from the surrounding soft tissue and was manipulated without ligation and transection. In group II and III, CBDs of the rats were identified, double ligated with 5-0 silk, and divided between the ligatures. The same surgeon performed all procedures. The abdominal incisions were closed in two layers with continuous 3-0 silk sutures. Animals were allowed to feed after the operation.

Microbiological examination

The mesenteric lymph nodes, spleen and liver were

chopped with sterile instruments under aseptic conditions. Then the tissue samples were weighed and placed in tubes containing 1.5 mL broth (thioglycollate, Oxoid, UK) and homogenized. Subsequently, 0.01 mL tissue samples were inoculated on blood agar (Oxoid, UK) and Levine Eosine Methylene Blue (EMB) agar (Oxoid, UK). Plates were incubated at 37°C for examination of bacterial growth. The growth of bacteria in quantitative culture was observed at 24 and 48 h.

One mL blood samples taken from inferior vena cava of rats were inoculated on the media of aerobic and anaerobic blood cultures. The aerobic and anaerobic blood cultures were observed by incubation in BACTEC 9240 blood culture system (Becton Dickinson, USA) at 37°C for seven days. Samples taken from the blood culture bottle, which gives a positive signal, was cultured by inoculating on blood agar and EMB agar. The subcultures were inoculated at 37°C under aerobic and anaerobic conditions and examined at 24 and 48 h.

Histopathological examination

For light microscope analyses, tissue samples from the terminal ileum were obtained from all animals. In order to avoid mucosal suffering, the intestinal lumen was carefully cannulated and gently washed with normal saline solution before the sampling. The ileal samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at $5\text{ }\mu\text{m}$ by Leica RM 2125 RT, and stained with hematoxylin and eosin (HE) for routine light microscopic examination. Histopathological examinations were performed by a pathologist who was blinded to the study design and photographs were taken with Nikon Eclipse E 600. The number of villi per centimeter (V/cm) and the total mucosal thickness (measured from the tip of the villus to the muscularis mucosa) were assessed in all groups. The mucosal thickness was measured in a minimum of 20 well-preserved villi in each randomly selected sample from each tissue block.

For transmission electron microscopic (TEM) analyses, samples were fixed with phosphate buffered (pH: 7.3) 2.5% glutaraldehyde and 2% PFA mixture solution for 2 h at room temperature. They were washed with phosphate-buffered saline solution (PBS) (pH: 7.3) and were fixed with 1% osmium tetroxide for 2 h as secondary fixation. After washing, they were embedded in Araldite 6005 and were cut with Leica EM FCS (Vien-Austria) ultramicrotome. $1\text{ }\mu\text{m}$ semi-thin sections were stained with Toluidin blue-Azur II to select the region of interest for the following procedures. 60-70 nm thin sections were stained with uranyl acetate and lead citrate. They were examined and photographed using a LEO 906 E TEM (80 kV-Oberkochen-Germany).

Statistical analysis

Differences between the numbers of positive cultures from the groups were evaluated by Chi-square test and *P* values of less than 0.05 were considered to be significant. Scores of total mucosal thickness and number of villi per centimeter were presented as mean \pm SD and compared by One-Way ANOVA or Kruskal-Wallis variance analysis. If the *P* values of the variance analyses were statistically

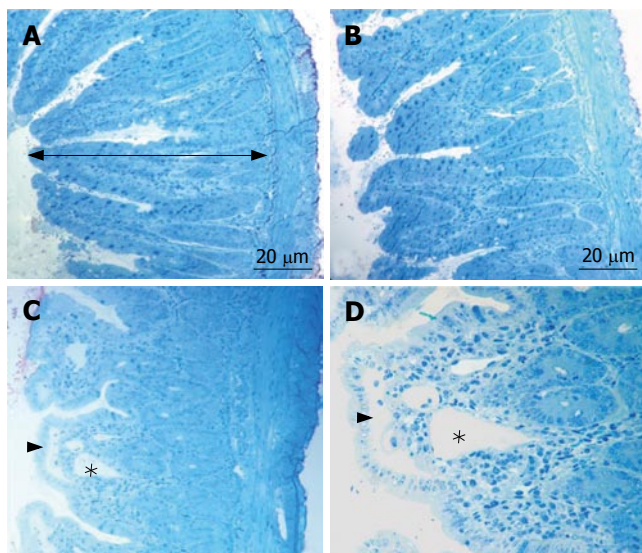


Figure 1 The micrographs of light microscope stained with toluidin blue. **A:** Typical structure of villi and the total mucosal thickness (arrow) in the group I ; **B:** The normal villous architecture in group III ; **C, D:** Blunting of the villi, the subepithelial edema (arrow head) and the dilated the lacteal (*) in group II .

Table 1 Mean number of villi per cm

Groups	n	Mean number of villi per cm	P values
Sham (Group I)	10	84.40 ± 3.75	< 0.001 ^b
BDL (Group II)	8	73.01 ± 2.83	0.001 ^d
BDL + Propolis (Group III)	9	80.89 ± 3.87	

^b*P* < 0.001 group I vs II ; ^d*P* = 0.001 group II vs III. BDL: Bile duct ligation.

significant, differences between groups were analysed with the Mann-Whitney *U* test. Statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS), version 13.0 for Windows (SPSS Inc., Chicago, USA). *P* < 0.05 was considered to be statistically significant.

RESULTS

All rats were sacrificed on postoperative d 7. Two rats from group II (BDL group) and one from group III (BDL + propolis group) died in the early postoperative period probably due to anesthesia.

Intestinal morphology

In all specimens of the sham group, the histologic features showed regular appearance of ileal tissue. When we evaluated the specimens systematically, including assessment of villous architecture, surface and crypt epithelia, lamina propria constituents and submucosal structures, no alteration was found in the sham group (Figure 1A). Non specific morphological abnormalities were evident in the intestinal mucosa of the BDL group. The specimens of the BDL group presented villous blunting associated with reduced mucosal thickness. We indicated subepithelial edema mostly located at the tip of the villi, but also extended throughout the villus, with the

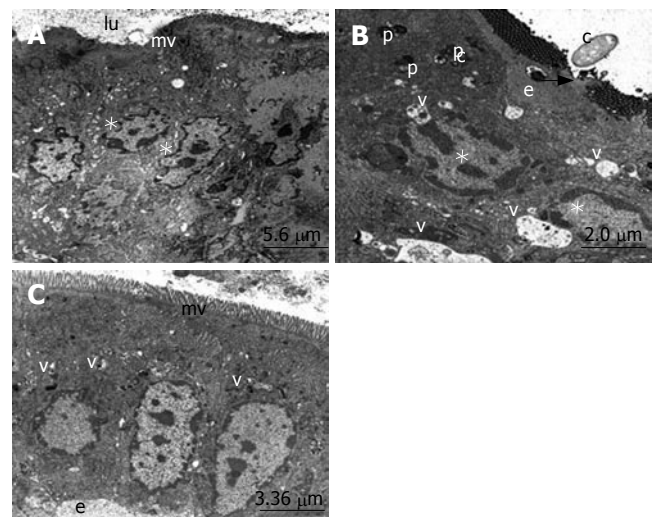


Figure 2 These transmission electron microscope (TEM) micrographs illustrate the main ultrastructural features of enterocytes, and the absorptive cells of the ileum. **A:** The regular structure of microvilli (mv), lumen (lu) and the nuclei of the enterocytes (*). **B:** The subepithelial edema (e), phagosomes (p), vacuoles (v), desquamation of epithelial tissue (arrow), nuclei of enterocytes (*) and candida (c). **C:** The regular structure of microvilli (mv), small vacuoles (v) and subepithelial edema (e).

Table 2 Mean height of mucosa (μm)

Groups	n	Mean height of mucosa	P values
Sham (Group I)	10	640.02 ± 43.72	0.002 ^b
BDL (Group II)	8	567.50 ± 34.54	0.012 ^a
BDL + Propolis (Group III)	9	612.78 ± 29.69	

^b*P* = 0.002 < 0.01 group I vs II ; ^a*P* = 0.012 < 0.05 group II vs III. BDL: Bile duct ligation.

epithelial layer moderately lifted from the lamina propria. We observed that the crypts were generally preserved. The number of villi per centimeter (V/cm) (villus density) was decreased in the BDL group (Figure 1B). In group III, the subepithelial edema still existed, but villous blunting was not evident. Further, the crypts generally appeared to be preserved (Figure 1C). Although the number of villi per centimeter and the height of the mucosa were higher in the sham group, there was no statistically significant difference between sham and propolis groups (*P* > 0.05). On the other hand, there was a statistically significant difference between the BDL group and other groups (*P* < 0.05). The mean number of villi per centimeter and mean mucosal height of the groups are given in Table 1 and Table 2.

The ultrastructure of the ileum was observed by electron microscopy. The intestinal surface epithelium showed regular architecture with a large number of microvilli exposed to the lumen and the enterocytes, which are tightly bound by junctional complexes, showed the regular architecture of the small intestine surface epithelium in the sham group (Figure 2A). When we evaluated the micrographs of group II, we observed the desquamation of the epithelial tissue, cytoplasmic vacuoles with the inclusion of lipid droplets and phagosomes.

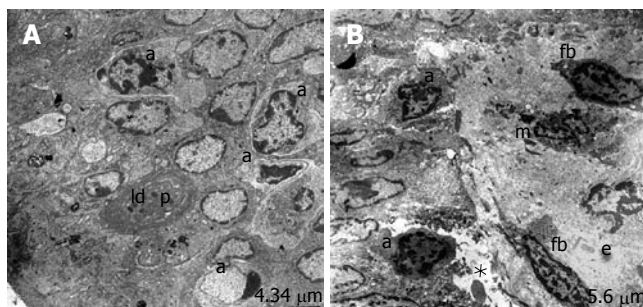


Figure 3 A: The apoptotic cell nuclei (a) and phagosomes (p) with lipid droplets (ld). B: The the apoptotic cell nuclei (a), fibrocytes (fb), macrophage (m) and subepithelial edema (e) with the separation of the basal membrane (*).

The nucleus of the enterocytes were flattened possibly due to both apical surface and subepithelial edema. Ultrastructural findings indicating the invasion of candida, especially located in the desquamated epithelial, were detected in many areas (Figure 2B). The number of apoptotic cells, characterized by cytoplasmic condensation and nuclear fragmentation, was qualitatively increased. The phagosomes, including lipid droplets, were observed in the cytoplasm of the epithelial cells. The infiltration of macrophages and fibrocytes were evident. Basal membranes were separated due to subepithelial edema (Figure 3). Structure of the enterocytes and microvilli were regular in the propolis group. The junctional complexes were in common appearance and adjoining outer membranes of surface absorptive cells were close to each other. The cytoplasmic vacuoles were present but they were smaller than the vacuoles in the BDL group. The nuclei of surface absorptive epithelial cells were regular, in accordance with the regression of the apical surface and subepithelial edema. Findings supporting the invasion of candida were not evident (Figure 2C).

Bacterial translocation

The rates of bacterial translocation (BT) to liver, spleen, MLNs, and blood for all groups are summarized in Table 3. Sham and BDL + propolis groups had similar incidence of BT. The BDL group had significantly higher rates of BT as compared with sham and BDL + propolis groups. Only BT rates to spleen were not statistically different between the BDL and BDL + propolis groups. BT was predominantly detected in MLNs and the most commonly isolated bacteria was *E. coli*.

DISCUSSION

Propolis is a resinous material collected by bees from various plants. Once collected, this material is enriched with salivary and enzymatic secretions of bees. Propolis is used by bees to cover hive walls, fill cracks or gaps and embalm killed invader insects. Hundreds of chemical compounds have been identified from propolis. Propolis contains a variety of flavonoids, phenols, alcohols, terpenes, sterols, vitamins and amino acids. Antimicrobial properties of propolis seem attributable mainly to the flavonoids, pinocembrin, galangin, and pinobanksin.

Table 3 Bacterial translocation rates of the groups

Groups	Liver	Spleen	MLNs	Blood
Sham (Group I)	0/10 (0%)	0/10 (0%)	1/10 (10.0%)	0/10 (0%)
BDL (Group II)	6/8 (75.0%)	4/8 (50.0%)	7/8 (87.5%)	4/8 (50%)
BDL + Propolis (Group III)	2/9 (22.2%)	2/9 (22.2%)	3/9 (33.3%)	0/9 (%)
<i>P</i> values				
I vs II	0.002	0.023	0.002	0.023
II vs III	0.044	> 0.05	0.036	0.029
I vs III	> 0.05	> 0.05	> 0.05	> 0.05

BDL: Bile duct ligation; MLNs: Mesenteric lymph nodes.

Pinocembrin also exhibits antifungal properties. Flavonoids are well-known plant compounds that have antioxidant, antibacterial, antifungal, antiviral, and antiinflammatory properties^[16-18].

Bacterial translocation is defined as the phenomenon by which bacteria, their products, or both bacteria and products, cross the intestinal barrier. The first site encountered by the microorganisms or products undergoing translocation is the mesenteric lymph node. Subsequently, extension to the liver, spleen, and systemic circulation may occur. The mechanisms which are proposed to promote bacterial translocation are small bowel bacterial overgrowth, immune deficiency states, and physical damage to the intestinal mucosa and vasculature that causes increased permeability. Certain enteric organisms such as *E. coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, seem to undergo translocation more easily. When any of the three mechanisms that promote translocation becomes severe, prolonged, or combined with other mechanisms, bacterial translocation may lead to sepsis and death in experimental animals^[19].

Obstructive jaundice is a common clinical entity complicated by intestinal failure and endotoxemia, leading to high morbidity and mortality rates^[2]. Kuzu *et al*^[20] collected peritoneal swab, mesenteric lymph node, portal venous blood, liver wedge biopsy, and bile samples for culture in patients with obstructive jaundice, and they demonstrated translocation of enteric bacteria despite common use of preoperative antibiotics. Welsh *et al*^[21] demonstrated a reversible impairment in gut barrier function in patients with cholestatic jaundice by using the lactulose/mannitol permeability test and concluded that these data might directly identify an important underlying mechanism contributing to the high risk of sepsis in jaundiced patients.

Obstructive jaundice globally affects three levels of gut barrier: the immune barrier, the biological barrier, and the mechanical barrier. Obstructive jaundice depresses Kupffer cell clearance capacity and natural killer cell activity, reduces T cells in intestinal epithelium, alters intestinal mucosal immunity and deprives the gut from biliary secretory IgA and from other specific and nonspecific antibodies contained in bile that inhibit adhesion of enteric bacteria on the intestinal wall. Bile salts exert bacteriostatic properties, and therefore their absence from the intestinal lumen results in quantitative and qualitative disruption of the indigenous microflora. Absence of intraluminal

bile also deprives the gut from their trophic effect resulting in intestinal atrophy^[2]. The cellular alterations of the mechanical barrier are associated with significant disturbances of intestinal oxidative status, with increased lipid peroxidation, protein oxidation and oxidation of non-protein and protein thiols. These biochemical changes are indicative of high oxidative stress in the intestine after biliary obstruction and represent another significant parameter of intestinal injury leading to barrier failure^[22].

Propolis has antibacterial, antioxidant and anti-inflammatory properties. There are a number of studies documenting the antimicrobial functions of propolis, its extracts, and constituents. This is a broad spectrum activity against Gram-positive (*S. aureus*, *S. pyogenes*, *S. viridens*, *D. pneumoniae*, and *C. diphtheria*) and Gram-negative (*E. coli*, *K. pneumoniae*, *P. vulgaris*, *P. aeruginosa*, *S. typhi*, *S. paratyphi-A*, *S. paratyphi-B*, and *S. flexneri*) rods and cocci, *Helicobacter pylori*, *Trypanosoma cruzi*, *Toxoplasma gondii*, *Trichomonas vaginalis*, *Candida*, *Saccharomyces*, *Cryptococcus*, *Mycobacteria*, as well as viruses (HIV, Herpes viruses, influenza viruses, adenovirus, poliovirus type 2)^[5]. Propolis also possesses potent antimicrobial activity, providing an alternative therapy against infections caused by resistant strains such as methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium*^[23]. Although the chemical composition of propolis varies depending on the site of its collection, antimicrobial properties seem attributable mainly to the flavonoids pinocembrin, galangin, and pinobanksin. Pinocembrin also exhibits antifungal properties. Other active compounds are ester of coumaric acid and caffeic acids. Prenylated *p*-coumaric and diterpenic acids possess antibacterial and cytotoxic activities. Caffeoylquinic acid derivatives show immunomodulatory and hepatoprotective actions and furofuran lignans inhibit the growth of some bacteria^[24].

Propolis has been used in the treatment of cutaneous lesions such as burns, wounds, and ulcers. Morales *et al*^[25] used a hypoallergic formula of propolis and obtained a very satisfactory evolution and cicatrization in cases of wounds with and without infection. A fast cure, shorter treatment period, and less septic complications were also obtained. The cicatrization was evident between the 4th and 5th d by the early formation of granulation tissue. The antimicrobial capacity was evident with a fast regression of the septic component of the supurated wounds. Propolis exerts a wound healing effect by minimizing acute inflammatory exudate, stimulating macrophage activity, promoting collagen production, and stimulating epithelialization. In our present study, the mean number of villi per centimeter and mean mucosal height of the propolis group were significantly different from the BDL group ($P = 0.001$ and 0.012 , respectively). The electron microscopic changes were also different between these groups (Figures 1-3). We conclude that enhancement of wound healing capacity by the use of propolis might be the reason for decreased atrophy of intestinal mucosal villi.

Propolis also has immunomodulatory activity. In an experimental study, a water-soluble derivative of propolis (WSDP) and its components stimulated macrophages thus influencing specific and nonspecific immune defence mechanisms^[13,26]. In another study, it was shown that

WSDP treatment induced extensive proliferation of nucleated cells in the spleen and bone marrow, which are mainly macrophages and hematopoietic cells^[17].

Ara *et al*^[27] found that intraperitoneal administration of CAPE reduced tissue levels of malondialdehyde and myeloperoxidase, but increased levels of glutathione in the ileum after bile duct ligation. Additionally, CAPE decreased interleukin-1 α , interleukin-6, tumor necrosis factor- α , and intestinal mucosal injury, but the effect of CAPE on bacterial translocation was not revealed. In this study, although bacterial translocation in CAPE-treated rats was lower than in the control group, the difference was not significant.

In our study, bacterial translocation was reduced significantly. The BDL group had significantly higher rates of BT as compared with sham and BDL + propolis groups ($P < 0.05$). Propolis may reduce bacterial translocation by enhancing mucosal barrier function, supporting generalized immune function, and reducing bacterial overgrowth. As we mentioned before, these three mechanisms are the main mechanisms responsible for bacterial translocation.

In conclusion, propolis showed a significant protective effect on ileal mucosa and reduced bacterial translocation in an experimental obstructive jaundice model. Further studies should be carried out to explain the mechanisms of these effects.

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BASIC RESEARCH

***In vitro* pancreas duodenal homeobox-1 enhances the differentiation of pancreatic ductal epithelial cells into insulin-producing cells**

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ductal epithelial cells into insulin-producing cells *in vitro*. *In vitro* PDX-1 transfection is a valuable strategy for increasing the source of insulin-producing cells.

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Key words: Pancreatic ductal epithelial cells; Islet; Differentiation; Pancreas duodenal homeobox-1

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Abstract

AIM: To observe whether pancreatic and duodenal homeobox factor-1 enhances the differentiation of pancreatic ductal epithelial cells into insulin-producing cells *in vitro*.

METHODS: Rat pancreatic tissue was submitted to digestion by collagenase, ductal epithelial cells were separated by density gradient centrifugation and then cultured in RPMI1640 medium with 10% fetal bovine serum. After 3-5 passages, the cells were incubated in a six-well plate for 24 h before transfection of recombination plasmid XIHbox8VP16. Lightcycler quantitative real-time RT-PCR was used to detect the expression of PDX-1 and insulin mRNA in pancreatic epithelial cells. The expression of PDX-1 and insulin protein was analyzed by Western blotting. Insulin secretion was detected by radioimmunoassay. Insulin-producing cells were detected by dithizone-staining.

RESULTS: XIHbox8 mRNA was expressed in pancreatic ductal epithelial cells. PDX-1 and insulin mRNA as well as PDX-1 and insulin protein were significantly increased in the transfected group. The production and insulin secretion of insulin-producing cells differentiated from pancreatic ductal epithelial cells were higher than those of the untransfected cells *in vitro* with a significant difference (1.32 ± 0.43 vs 3.48 ± 0.81 , $P < 0.01$ at 5.6 mmol/L; 4.86 ± 1.15 vs 10.25 ± 1.32 , $P < 0.01$ at 16.7 mmol/L).

CONCLUSION: PDX-1 can differentiate rat pancreatic

INTRODUCTION

Diabetes mellitus is a common endocrine and metabolic disease in the world. At present, insulin injection therapy is used in the treatment of type 1 and some type 2 diabetes. However, the results are not satisfactory. Transplantation of islets is one of the promising therapies for type 1 diabetes and can effectively prevent diabetic nephropathy, retinopathy and other complications^[1]. Since it is limited by the shortage of islets, expanding the sources of islet cells (especially beta cells) has become a hot field of research in pancreas transplantation.

In recent years, great efforts have been made to differentiate embryonic stem cells, pancreatic ductal epithelial multipotent progenitor cells and bone marrow stem cells into islet cells^[2-4]. However, it was reported that the most promising way is to differentiate pancreatic stem cells into islet cells, because the process of cell differentiation and growth is shorter than that of bone marrow stem cells and embryonic stem cells, and the induction method *in vitro* is relatively simple^[5]. The amount of islets differentiated from pancreatic stem cells *in vitro* is minimal and insulin released by islets is insufficient to meet the clinical needs^[6]. How to enhance the efficiency of differentiation *in vitro* and increase the output of insulin-producing cells and insulin-release needs to be studied.

The pancreatic and duodenal homeobox factor-1 (PDX-1), also known as islet/duodenum homeobox-1/somatostatin-DE transactivating factor 1/insulin promoter

factor DE 1, a homeodomain containing transcription factor, is homologous to a *Xenopus* endoderm-specific homeodomain protein, XIHbox 8. It plays a central role in regulating pancreatic development and insulin gene transcription^[7]. Transfection of PDX-1 gene into rat intestinal epithelial cell line IEC-6 can produce insulin^[8]. Ferber *et al.*^[9] showed that PDX-1 can endow some cells in the liver with pancreatic beta-cell characteristics *in vivo* using recombinant adenovirus-mediated gene delivery. Bonner-Weir *et al.*^[10] reported that PDX-1 protein can permeate pancreatic duct and islet cells due to an Antennapedia-like protein transduction domain sequence in its structure and transduced PDX-1 functions similarly to endogenous PDX-1. PDX-1 protein transduction is a safe and valuable strategy for enhancing insulin gene transcription and facilitating differentiation of ductal progenitor cells into insulin-producing cells without gene transfer technology. Recently, Yamada *et al.*^[11] showed that mature liver cells can also be induced into insulin-producing cells by *in vitro* PDX-1 gene transfection. These studies have highlighted the potential usefulness of PDX-1 as a reprogramming factor of non-beta-cells toward beta-cell-like cells that can be used in diabetes cell/gene therapy.

Due to the high homology of PDX-1 sequence in different species^[12], we investigated the role of exogenous PDX-1 in pancreatic ductal epithelial cells in adult rats by transfecting exogenous PDX-1 (XIHXbox8) into pancreatic ductal epithelial cells *in vitro*. The results demonstrate that we can enhance the differentiation of pancreatic ductal epithelial cells into insulin-producing cells and insulin-release via PDX-1 (XIHXbox8-VP16) transfection.

MATERIALS AND METHODS

Isolation of cells and culture conditions

Rat (adult male S-D rats, weighing 250-300 g) pancreatic tissue was digested in 1 g/L type V collagenase (Sigma) and incubated at 37°C for 40 min with intermittent shaking and then terminated by Hank's solution, followed by centrifugation at 1000 r/min for 5 min. After purification on a Ficoll gradient, 50%-95% islets were found on the top interface (1.062/1.096 densities) with varying amounts of duct and degranulated acinar tissues, and 1%-15% islets were found on the middle interface (1.096/1.11 densities). Duct, degranulated acini and pellet were composed of well-granulated acinar tissue with less than 1% islets. In the top and middle layers, there were sheets of ductal epithelium from larger ducts whereas the clumps of exocrine cells found in all layers consisted of small intercalated ducts continuing into the acini. The epithelial cells were washed 3 times with Hank's solution containing 5% fetal bovine serum (FBS, Gibco), then put into RPMI 1640 culture medium (Hyclone) containing 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cell suspensions were put into non-treated T-75 flasks and incubated at 37°C in an atmosphere containing 5% CO₂. After 48 h, the nonadherent tissue (both viable and dead) was removed. The medium was changed. The adherent or residual cells were expanded for up to 1 wk with media changed every 2-3 d. The adherent or residual cells were continuously cultured in an atmosphere containing 5%

CO₂ and 95% humidity. After 3-5 passages, the ductal epithelial cells were used in experiments.

Transfection of plasmids

Plasmid pCS2-TTR-XIHXbox8VP16 was kindly provided by Professor J. M.W. Slack (Centre for Regenerative Medicine, Department of Biology and Biochemistry, University of Bath, Bath)^[13]. Plasmids were transfected into pancreatic ductal epithelial cells with Lipofectamine 2000 (Invitrogen) transfection reagent according to the manufacturer's instructions. The medium was replaced with a fresh medium after 24 h of transfection. The cells were cultured for a further 7 d, and analyzed by reverse transcriptase polymerase chain reaction (RT-PCR) or Western blotting at variable time points.

In vitro induction of differentiation

To induce differentiation of pancreatic ductal epithelial cells to insulin-producing cells, forty-eight hours after transfection, pancreatic ductal epithelial cells were transferred to a medium supplemented with 200 µg/mL G418, 10 mmol/L nicotinamide, and insulin/transferrin/selenium (ITS, Sigma). G418-resistant colonies were found about 4 wk after transfection. The resulting clusters were cultured for 1-5 d in RPMI 1640 supplemented with 10% FBS, 10 mmol/L nicotinamide, 200 µg/mL G418, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, and 5.6 mmol/L glucose^[2].

Lightcycler quantitative real-time PCR

Fifty mg pancreatic tissues and 1 mL TRIzol were mixed with 0.3 mL chloroform added, and then centrifuged. Isopropanol (0.5 mL) was added to supernatant and centrifuged with supernatant discarded. Deposit was washed with 70% ethanol, and dissolved in DEPC-treated water to obtain total mRNA. In RT-PCR, 4 µL of mRNA and 0.5 µL of Oligo (dt) were added to 6.5 µL of distilled water. After annealing at 70°C for 5 min and immediate cooling on ice, 4.0 µmol/L of 5 × first strand buffer, 2.0 µL of 10 mmol/L dNTP, 0.5 µL of RNasin and 0.5 µL RTase were added to get a total reaction volume of 20 µL. The reaction was allowed to proceed at 37°C for 60 min, followed by at 95°C for 5 min to inactivate the enzyme. RT-PCR assay was performed three times for each cDNA sample. The total PCR volume consisted of 1 µL of cDNA, 1 µL of SYBRGreen PCR I, 5 µL 10 × buffer, 1.6 µL of primers (rat PDX-1: sense 5'-CTTGGGTATGGATCTGTGG-3' and antisense 5'-CGGACTCATCGTACTCCTGCTT-3'; *Xenopus* PDX-1 homologue XIHXbox8: sense 5'-TGCCAACTTCATCCCAGCCC-3' and antisense 5'-GGCAGATGAAGAGGGCTC-3'; insulin: sense 5'-GCTACAATCATAGACCATC-3' and antisense 5'-GGCGGGGAGTGGTGGACTC-3'; beta-actin: sense 5'-CTTGGGTATGGAATCCTGTGG-3' and antisense 5'-CGGACTCATCGTACTCCTGCTT-3'), 7 µL of MgCl₂, 0.5 µL of Taq DNA polymerase, 1 µL of dNTP and 33 µL of distilled water. After denaturation of the enzyme at 94°C for 2 min, 45 cycles of PCR assay were carried out, with denaturation at 94°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 30 s. Fluorometric PCR was

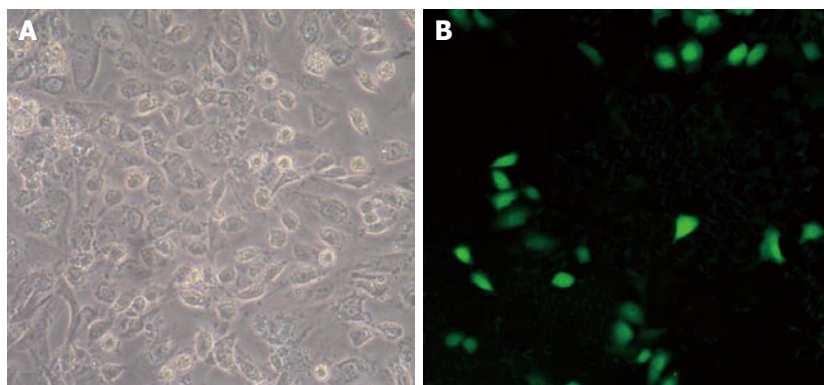


Figure 1 Transfection of recombination plasmids into cultured (A) and non-cultured (B) pancreatic ductal epithelial cells.

performed with the FTC-2000 system. The expression of each transporter protein gene determined was relative to the β -actin RNA gene.

Western blotting

Total protein was obtained as previously described^[14] at various time points (d 0, 1, 3, 5, 7, 14, 21, 28 after transfection). Proteins in samples were separated by polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and blocked overnight in a blocking solution (5% dry milk in PBS). The membrane was incubated at 37°C for 1 h with goat anti PDX-1 antibody (1:500, Santa Cruz) and goat anti insulin antibody (1:500, Santa Cruz), washed with PBS and incubated at 37°C for 1 h with horseradish peroxidase-labeled secondary antibody (1:2500). Finally, proteins were visualized on a film with the ECL method.

Dithizone staining

A dithizone (DTZ; Sigma) stock solution was prepared with 50 mg of DTZ in 5 mL of dimethylsulfoxide (DMSO) and stored at -15°C^[15]. *In vitro* DTZ staining was performed by adding 10 μ L of the stock solution to 1 mL of culture medium. The staining solution was filtered through a 0.2 μ m filter and used as the DTZ working solution which was added to culture dishes and incubated at 37°C for 15 min. Clusters were examined under a phase contrast microscope.

Secreted insulin measurement

Differentiation of cells transfected with or without TTR-XIHbox8VP16 was induced with either normal dose glucose (5.5 mmol/L) or high dose glucose (16.7 mmol/L). Insulin concentration was measured using an insulin RIA kit (Linco).

Statistical analysis

The data were expressed as mean \pm SD. Individual treatment was compared using Student's *t*-test and ANOVA. *P* < 0.05 was considered statistically significant.

RESULTS

Transfection of XIHbox8 plasmids into cells

Green fluorescence could be seen in pancreatic ductal epithelial cells transfected with XIHbox8. The distribution

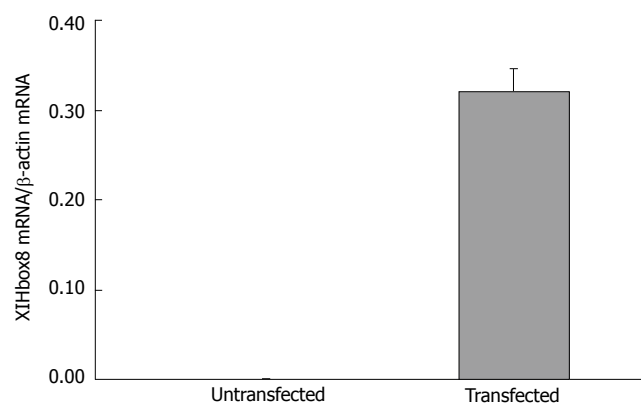


Figure 2 XIHbox8 mRNA expression 24 h after transfection of recombination plasmids.

of green fluorescence in nuclei and cytoplasm indicated the characteristics of location of cytokines in these cells. Twenty-four hours after transfection, the expression of GFP fluorescence was observed under fluorescence microscope (Figure 1). Green fluorescent cells were calculated. The transfection efficiency of pancreatic ductal epithelial cells was 3%-5%. These results suggest that exogenous XIHbox8 could be transfected into nuclei and cytoplasm of pancreatic ductal epithelial cells.

Expression of PDX-1 and insulin in transfected cells

To investigate the change in gene expression patterns caused by exogenous PDX-1 (XIHbox8) expression in pancreatic ductal epithelial cells, we performed RT-PCR analysis. As shown in Figure 2, the cells transfected with TTR-XIHbox8VP16 showed the expression of XIHbox8 mRNA, which was not detectable in the untransfected cells. In addition, the results also showed that the expression of PDX-1 and insulin mRNA increased between the 1st and 7th d after transfection (Figure 3), which is consistent with the reported results^[13]. In contrast, the expression of PDX-1 and insulin mRNA was not detectable in the untransfected cells.

Western blotting showed that PDX-1 and insulin protein were expressed in the transfected cells but not in the untransfected cells. After induction, the untransfected cells also began to express PDX-1 and insulin protein. Moreover, the expression increased between the 14th and 28th d in the transfected cells. The expression of PDX-1

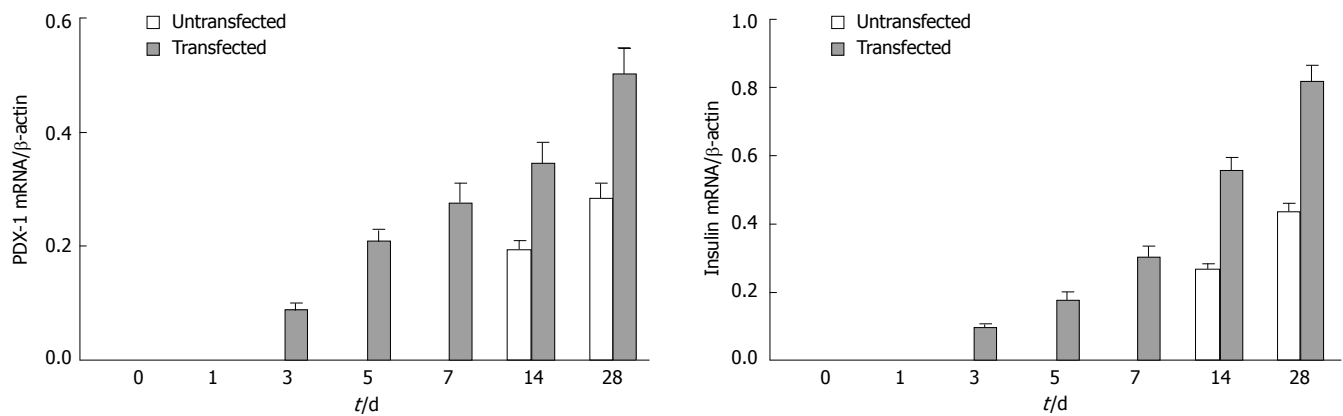


Figure 3 PDX-1 and insulin mRNA expression after transfection of recombination plasmids.

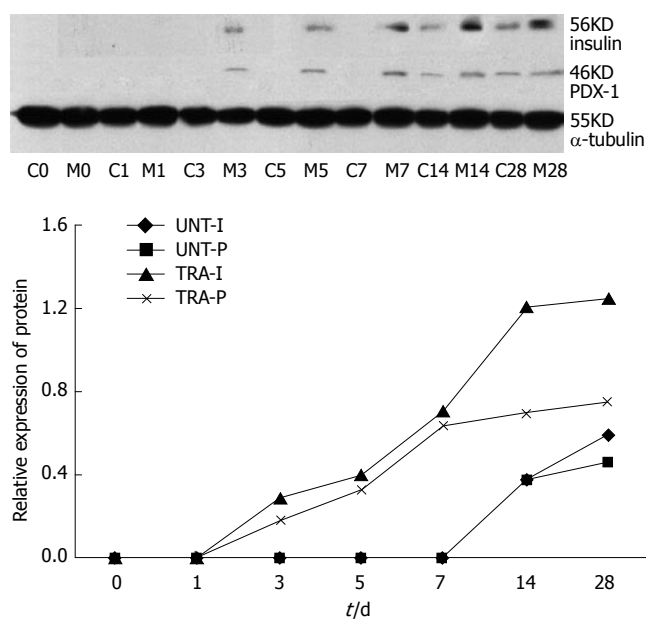


Figure 4 Western blot analysis of PDX-1 and insulin protein showing the increased expression of PDX-1 ($P < 0.01$) and insulin ($P < 0.01$) protein in the transfected cells. C0-C28: Untransfected group on d 0-28; M0-M28: Transfected group on d 0-28; UNT-I: Insulin expression in untransfected group; UNT-P: PDX-1 expression in untransfected group; TRA-I: Insulin expression in transfected group; TRA-P: PDX-1 expression in transfected group.

and insulin protein in the transfected cells increased more obviously (Figure 4).

These results suggest that once XIHbox8 was transfected into pancreatic ductal epithelial cells, endogenous PDX-1 gene transcription was amplified by this XIHbox8 and might enhance differentiation of pancreatic ductal epithelial cells into insulin-producing cells.

Induction of XIHbox8-expressing pancreatic ductal epithelial cells to insulin-producing cells

Pancreatic ductal epithelial cells were transfected with XIHbox8 expression plasmid, and stable transfectants were selected according to G418 resistance. Further differentiation of pancreatic ductal epithelial cells was induced in serum-free medium supplemented with ITS, keratinocyte growth factor (KGF, Sigma) and basic fibroblast growth factor (bFGF, Sigma). During

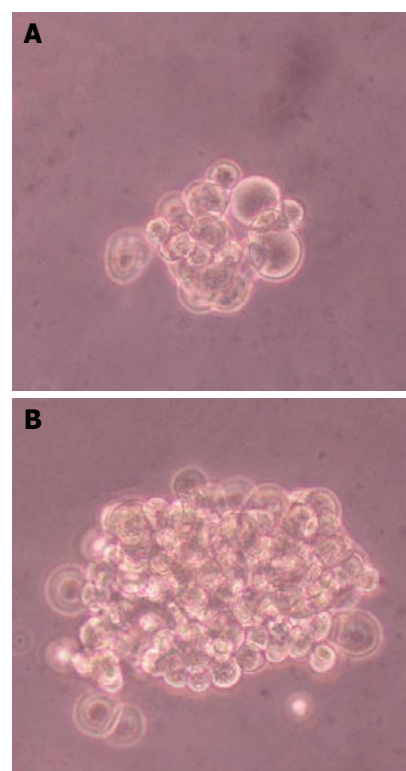


Figure 5 Dithizone staining of insulin-producing cells in untransfected group (A) and transfected group (B).

induction, most insulin-producing cells could be seen under microscope. Dithizone staining showed more insulin-producing cells in the transfected group than in the untransfected group (Figure 5).

Response of differentiated insulin-producing cells to physiological stimuli

The most important and characteristic property of insulin-producing cells is their ability to secrete insulin in response to an elevated external glucose concentration. We examined the insulin concentration in the medium of differentiated cells after challenged with 5.6 and 16.7 mmol/L glucose, using a RIA kit (Figure 6). The results showed that insulin secretion was significantly increased in the transfected group compared with the untransfected group.

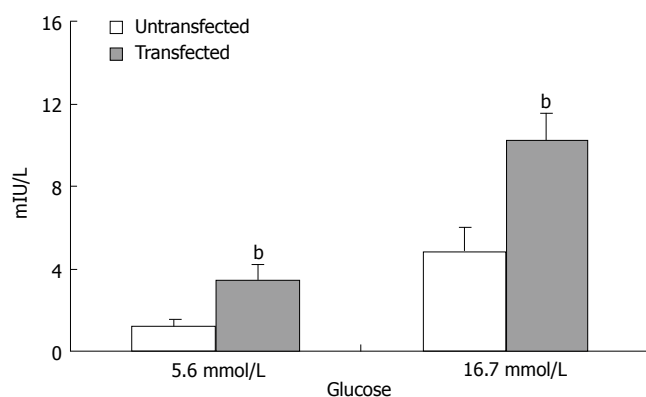


Figure 6 Response of insulin secretion to physiological stimuli. Insulin secretion was significantly increased in the transfected group compared with the untransfected group (^b $P < 0.01$).

DISCUSSION

Pancreas duodenum homeobox-1 (PDX-1) is also known as islet/duodenal homeobox-1 (IDX-1), insulin promoter factor-1 (IPF-1), insulin upstream factor-1 (IUF-1), somatostatin transactivating factor-1 (STF-1) and glucose-sensitive factor (GSF)^[7]. PDX-1 is a transcription factor encoded by Hox-like homeodomain gene and also one of the specific signals of pancreatic stem cells. Human PDX-1 gene consists of two exons and spans a region of about 6 kb in humans and 282 amino acids^[16]. In mice lacking PDX-1, pancreas development is blocked at its very early stage, and there are no or diminished endocrine cells in the rostral duodenum and stomach^[17,18], suggesting that PDX-1 plays a very important role in the development and maintenance of normal pancreatic beta cell function, which also has a potential value in gene therapy for diabetes. Amplifying and inducing differentiation of non-islet cells into insulin-producing cells by PDX-1 has become a hot field of study. Miyazaki *et al*^[19] reported that islet-like cellular clusters are significantly increased and insulin-producing cells can release more insulin by transfecting exogenous PDX-1 into embryonic stem cells. Further investigation found that PDX-1 can enhance the expression of insulin 2, somatostatin and HNF6 genes in the differentiated cells^[19]. XIHbox8 is the homologous of PDX-1. Horb *et al*^[20] transfected XIHbox8-VP16 into liver cells and found that some liver cells could be trans-differentiated into pancreatic cells.

Bonner-Weir *et al*^[3] reported human pancreatic ductal epithelial cells can expand and differentiate into islet cells *in vitro*. In this study, most of early adherent cells and a few fibroblasts were found to be pancreatic ductal epithelial cells. PDX-1 may express in the whole pancreas of an early embryo. However, in pancreas of adult rats, PDX-1 expresses primarily in beta and delta cells, but not in pancreatic ductal epithelium. PDX-1 can also express in activated pancreatic ductal epithelial cells^[12,14]. When pancreatic ductal epithelial cells are transfected with exogenous homologous of PDX-1 and XIHbox8, endogenous PDX-1 gene can be activated, increasing insulin mRNA and protein expression^[9], suggesting that once XIHbox8 is transfected into pancreatic ductal

epithelial cells, endogenous PDX-1 gene transcription is activated by XIHbox8 and insulin gene, thus enhancing its expression. In our study, the expression of insulin mRNA and protein was enhanced after treatment with exogenous XIHbox8, indicating that XIHbox8 may promote differentiation of pancreatic ductal epithelial cells into insulin-producing cells. When pancreatic ductal epithelial cells were transfected with exogenous PDX-1, endogenous PDX-1 gene transcription was amplified, promoting differentiation of pancreatic ductal epithelial cells into insulin-producing cells, which is in agreement with the reported results^[10]. In the present study, after XIHbox8-VP16 was transfected into pancreatic ductal epithelial cells, differentiation of pancreatic ductal epithelial cells to insulin-producing cells was significantly increased, suggesting that this method can produce more insulin-producing cells. It was reported that unmodified PDX-1 is insufficient to activate transcription in the absence of other cofactors, such as Pbx and Meis^[20]. Beta cells are able to secrete insulin in response to an elevated external glucose concentration. In this study, the results of RIA show that the treated cells can produce more insulin than the untreated cells. However, whether differentiated insulin-producing cells have their physiological function *in vivo* still needs to be further tested *in vivo*. Moreover, the low transfection efficiency also limits the production of insulin-producing cells.

In conclusion, PDX-1 (XIHbox8) can significantly promote *in vitro* differentiation of pancreatic ductal epithelial cells into insulin-producing cells. This new method can be used in the treatment of diabetes.

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COMMENTS

Background

Diabetes mellitus is a common endocrine and metabolic disease in the world. Although transplantation of islets is one of the promising therapies for type 1 diabetes, its application is limited due to the shortage of islets.

Research frontiers

Expanding the sources of islet cells (especially beta cell) is a hot field of research in pancreas transplantation. Great efforts have made to differentiate embryonic stem cells, pancreatic ductal epithelial progenitor cells and bone marrow stem cells into islet cells. The most promising method is to differentiate pancreatic stem cells into islet cells, but the amount of islets obtained through differentiation of pancreatic ductal cells *in vitro* is minimal and insulin released by islets is insufficient.

Innovations and breakthroughs

The pancreatic and duodenal homeobox factor-1 (PDX-1) plays a central role in regulating pancreatic development and insulin gene transcription. In this study, we transfected PDX-1 (XIHbox8VP16) plasmid into human pancreatic ductal cells.

Applications

PDX-1 (XIHbox8) can significantly promote *in vitro* differentiation of pancreatic ductal epithelial cells into insulin-producing cells. This new method may be used in the treatment of diabetes.

Peer review

This manuscript provides direct evidence for the enhanced differentiation of pancreatic ductal cells into insulin-producing cells. The study is well designed and the results are convincing.

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CLINICAL RESEARCH

Maintenance of remission with infliximab in inflammatory bowel disease: Efficacy and safety long-term follow-up

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Abstract

AIM: To evaluate the safety and efficacy of a long-term therapy with infliximab in Crohn's disease (CD) and ulcerative colitis (UC) patients retrospectively.

METHODS: The medical charts of 50 patients (40 CD and 10 UC), who received after a loading dose of 3 infliximab infusions scheduled re-treatments every 8 wk as a maintenance protocol, were reviewed.

RESULTS: Median (range) duration of treatment was 27 (4-64) mo in CD patients and 24.5 (6-46) mo in UC patients. Overall, 32 (80%) CD and 9 (90%) UC patients showed a sustained clinical response or remission throughout the maintenance period. Three CD patients shortened the interval between infusions. Eight (20%) CD patients and 1 UC patient underwent surgery for flare up of disease. Nine out of 29 CD and 4 out of 9 UC patients, who discontinued infliximab scheduled treatment, are still relapse-free after a median of 16 (5-30) and 6.5 (4-16) mo following the last infusion, respectively. Ten CD patients (25%) and 1 UC patient required concomitant steroid therapy during maintenance period, compared to 30 (75%) and 9 (90%) patients at enrolment. Of the 50 patients, 16 (32%) experienced at least 1 adverse event and 3 patients (6%) were diagnosed with cancer during maintenance treatment.

CONCLUSION: Scheduled infliximab strategy is effective in maintaining long-term clinical remission both in CD and UC and determines a marked steroid sparing effect. Long-lasting remission was observed following infliximab withdrawal.

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Key words: Inflammatory bowel disease; Crohn's disease; Ulcerative colitis; Infliximab therapy; Steroid sparing

INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) are chronic-relapsing diseases the clinical course of which is characterized by periods of remission and periods of acute flare up. The clinical symptoms have a strong impact on the quality of life of patients with inflammatory bowel disease (IBD)^[1-3]. Although many drugs have been used in the treatment of IBD, none has, so far, been shown to modify the natural history of the diseases or to maintain a stable remission over time^[4,5]. For many years, corticosteroids have represented the cornerstone of therapy for induction of remission in IBD, having demonstrated efficacy in inducing a rapid clinical response, in CD as well as in UC; however, long-lasting remission was not achieved and the side-effects emerging with long-term use exceeded the clinical benefits^[6]. Immunomodulators have been demonstrated to be efficacious as adjunctive therapy and as steroid-sparing agents; but their slow onset of action precludes their use in the active clinical setting as a sole therapy^[7].

The introduction of biological agents in the therapeutic armamentarium for CD and UC has significantly changed the treatment strategies and clinical outcomes. Infliximab (IFX) is a chimeric monoclonal IgG1 antibody directed against tumor necrosis factor- α (TNF- α) able to almost completely neutralize its biological activity^[8]. Several placebo-controlled trials have demonstrated the efficacy of IFX treatment in active luminal and fistulizing CD^[9-13]. Recently, IFX has become an alternative choice also in the treatment of UC. Indeed, following the conflicting conclusions of 2 placebo-controlled studies performed in patients with moderately severe steroid-resistant UC^[14,15], showing opposite clinical effects, another two randomized controlled trials have since then been published, demonstrating the clinical efficacy of IFX therapy in patients with moderate-to-severe UC^[16,17].

Some safety issues are associated with IFX use, mostly related to the development of adverse events (e.g., opportunistic infections, autoimmune disorders, and infusion reactions)^[18-20]. Major concerns are related to the reactivation

of latent tuberculosis and development of malignancy, even if there is no clear evidence that the use of IFX increases the incidence of solid cancers^[21,22]. Although the efficacy of a therapeutic strategy consisting of a loading dose of 3 IFX infusions (5 mg/kg) at 0, 2, and 6 wk and, thereafter, every 8 wk is supported by several placebo-controlled studies, very few data are available on the use of IFX for > 12 mo or > 8 doses, either in active CD or in active UC^[23,24].

The aim of the present single-centre study was to retrospectively analyze the prospectively collected data on the safety and efficacy of long-term therapy with IFX in CD and UC patients treated with a scheduled regimen.

MATERIALS AND METHODS

Subjects

The medical charts of 79 patients affected by IBD (59 CD and 20 UC patients, mean age 47.7 years) and treated with IFX (Remicade; Centocor Inc., Malvern, PA, USA) between January 1999 and September 2005 at the Department of Digestive Diseases of the Campus Bio-Medico University Hospital were retrospectively reviewed (Table 1). Diagnosis of IBD was based on the standard combination of clinical, endoscopic, histological, and radiological criteria. Patients were classified into one of 3 groups of treatment, according to IFX infusion administration: (1) episodic therapy only in 10 patients (5 CD, 5 UC), (2) episodic followed by on-demand maintenance in 19 patients (14 CD, 5 UC), and (3) induction therapy followed by scheduled maintenance therapy every 8 wk in 50 patients (40 CD, 10 UC).

Patients presented with mild to moderate IBD, as defined by a score of 150-350 (or less in steroid-dependent patients) on the Crohn's Disease Activity Index (CDAI) for CD patients^[25], and a score of > 10 (or less in steroid-dependent patients) on the Clinical Activity Index (CAI) for UC patients^[26]. Indications for IFX treatment were disease severity, steroid-dependent disease (unable to reduce prednisolone < 10 mg/d), steroid-refractory disease (active disease with prednisolone up to 0.7 mg/kg per day), or contraindication to steroids (e.g., diabetes, osteoporosis, acne, mood disturbance and severe hypertension). Exclusion criteria were cancer or history of cancer, pregnancy, chronic heart failure, previous tuberculosis, or presence of symptomatic intestinal strictures or abscesses.

All patients were allowed to continue concomitant therapies such as 5-aminosalicylates, immunosuppressive agents [azathioprine (AZA), 6-mercaptopurine (6-MP), methotrexate], and antibiotics; patients on steroids discontinued treatment using a tapering regimen of 10 mg weekly starting at the first IFX infusion. The study protocol was approved by the University Ethic Committee.

Pretreatment protocol

Clinical and instrumental assessment was performed within the 3 wk before IFX induction treatment. Blood samples were collected, after overnight fasting, prior to IFX infusion for routine laboratory tests, including inflammatory [C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR)] and biochemical parameters. A chest X ray

Table 1 Demographic and clinical characteristics of the study population

Clinical characteristics	IBD (CD + UC)	Crohn's disease	Ulcerative colitis
<i>n</i>	50	40	10
Gender (M/F)	23/27	18/22	4/6
Age (yr) ¹	47.7 ± 15.6	45.4 ± 17.9	52.7 ± 8.9
Median disease duration (yr) (range)	10.8 (0.3-22.2)	9.2 (0.3-22.2)	9.3 (2.1-15.1)
Disease activity (T0) ¹	N/A	245.0 ± 99.3 (CDAI) ²	12.9 ± 2.8 (CAI)
Site of disease <i>n</i> (%):			
Small bowel/pouch		13 (32.5)	
Large bowel		4 (10)	10
Ileum-colon		23 (57.5)	
Steroid therapy at enrolment (%) (≥ 0.7 mg/kg per day)	39 (78)	30 (75)	9 (90)
Concomitant medications <i>n</i> (%):			
Salicylates	29	21 (52.5)	8 (80)
6-MP/azathioprine	31	28 (70)	3 (30)
Antibiotics	25	24 (60)	1 (10)

¹Mean ± SD; ²5 steroid-dependent patients had baseline Crohn's disease activity index (CDAI) < 150; CAI: Clinical activity index; T0: Baseline time; N/A: Not applicable.

evaluation was performed before the first IFX infusion in order to exclude previous or latent tuberculosis infection. Patients' symptoms were routinely recorded before each infusion, in order to calculate the CDAI and CAI.

Infliximab administration

All 50 IBD patients were treated with an induction regimen consisting of 3 intravenous (iv) infusions of IFX at a dose of 5 mg/kg for induction of remission (wk 0, 2, 6 for CD; wk 0, 4, 8 for UC). Eight weeks after the third infusion, clinical assessment was repeated in all patients to evaluate treatment efficacy: in CD patients, the clinical response was defined as a ≥ 70 point reduction in the CDAI score, and clinical remission was defined as a CDAI score ≤ 150; in UC patients, clinical response was defined as a CAI score ≤ 10 points, and inactive disease as a CAI score ≤ 4. Thereafter, all responders (both CD and UC patients) received IFX maintenance infusions at 8-wk intervals. Written patient's consent was obtained before every IFX infusion.

All adverse events were recorded by means of direct questioning of patients. Infusion reactions to IFX were classified as either acute or delayed. Any adverse reaction, during or within 24 h of an initial or subsequent IFX infusion, was considered as an acute infusion reaction. A delayed infusion reaction refers to any adverse reaction occurring from 24 h to 14 d after re-treatment with IFX. Acute and delayed reactions were further defined as mild, moderate, or severe, according to the severity of signs and symptoms reported by the patient. Serum sickness-like reactions included any IFX-related event that occurred 1 to 14 d after infusion and consisted of a cluster of symptoms such as myalgias and/or arthralgias with fever and/or rash. Severe infection, cancer, and death were considered as serious adverse events if potentially related to IFX treatment.

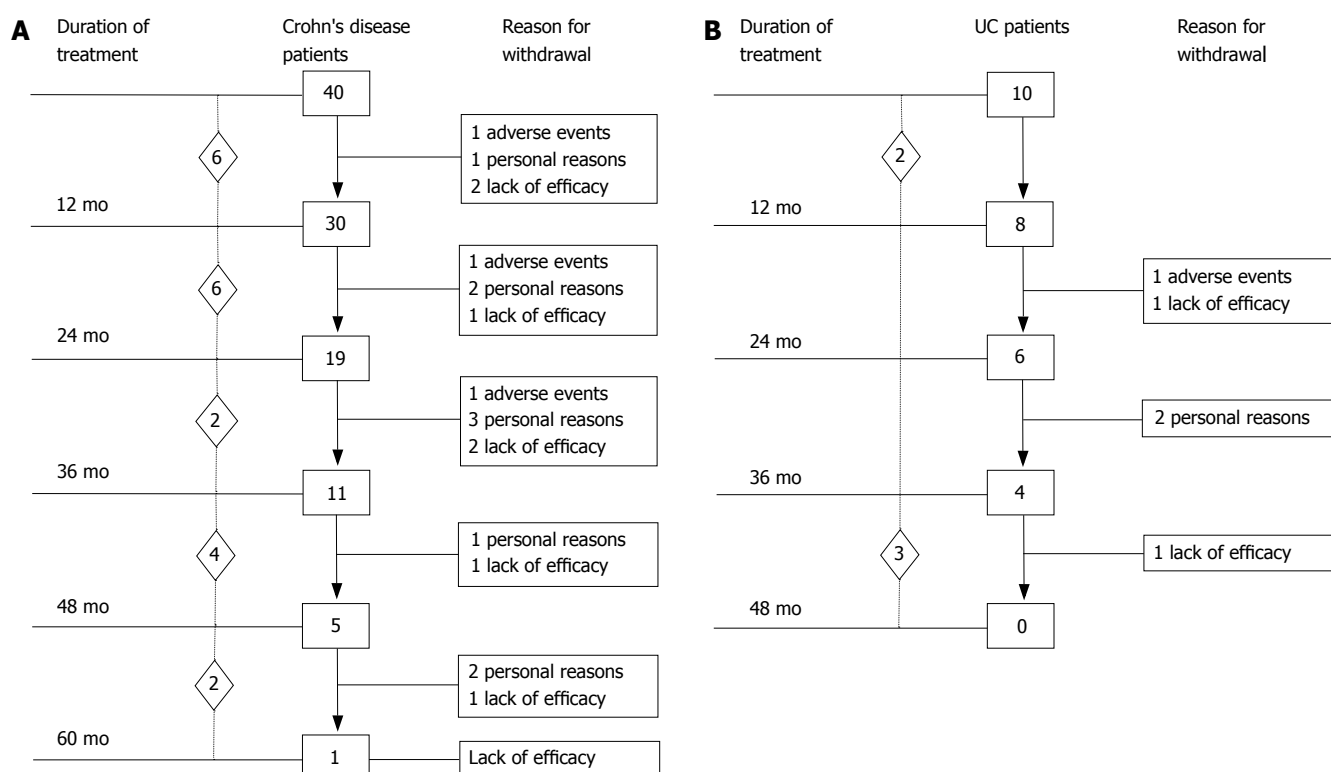


Figure 1 Patients observed at 12-mo intervals (endpoints) during IFX maintenance therapy. **A:** CD; **B:** UC. Numbers in boxes refer to patients reaching each endpoint. Numbers in rhombics refer to patients not reaching 12-mo endpoints.

Table 2 Efficacy profile of maintenance protocol

	IBD (CD + UC)	Crohn's disease	Ulcerative colitis
IFX infusions total number	637	493	144
Median number (range)	15 (4-25)	15 (4-25)	14.5 (6-22)
Median duration (mo) of IFX therapy (range)	27 (4-64)	27 (4-64)	24.5 (6-46)
No. patients maintaining remission with IFX (%)	41/50 82	32/40 80	9/10 90
Median duration (mo) of remission during IFX treatment (range)	25 (4-59)	25.5 (4-59)	25 (6-46)
No. patients discontinuing therapy during clinical remission (%)	13/38 34	9/29 31	4/9 44
Median duration (mo) of remission after treatment discontinuation (range)	15.5 (4-30)	16 (5-30)	6.5 (4-16)
No. patients on corticosteroids ¹	1/50	10/40	1/10
during maintenance with IFX (%)	2	25	10

¹Median corticosteroid dose \leq 0.25 mg/kg per day.

Statistical analysis

Results related to continuous data are expressed as median (range). The cumulative probability of relapse was calculated using the Kaplan-Meier method.

RESULTS

Baseline demographic and clinical data of the 50 patients (40 CD, 10 UC) who underwent induction therapy followed by scheduled maintenance therapy every 8 wk are outlined in Table 1. The summary of IBD patients' outcome observed at 12-mo intervals (endpoints) during IFX

maintenance therapy is shown in Figure 1. A total of 637 IFX infusions were administered (493 in CD, 144 in UC). The median duration of scheduled IFX treatment in the whole group of IBD patients was 27 (range, 4-64) mo.

Crohn's disease patients

The 40 CD patients undergoing the maintenance treatment strategy had a median disease duration of 9.2 years (range, 0.3-22.2). At baseline, all patients presented luminal disease; 6 patients had also draining fistulas (4 perianal and 2 recto-vaginal fistulas). Median number of IFX infusions was 15 (range, 4-25). The median duration of IFX treatment was 27 (range, 4-64) mo. Of the 40 CD patients, 35 (87.5%) underwent scheduled infusion strategy every 8 wk. In 3 patients (7.5%), showing a progressive loss of response (i.e., increase in CDAI and serum CRP level), the dose intervals were reduced to 6 wk; in 2 patients (5%), on account of stable clinical remission, the infusion intervals were prolonged to 12 wk. In 29 out of 37 CD patients (78%) the initial clinical improvement remained unchanged during IFX scheduled treatment, with CDAI below the remission level (< 150) throughout the maintenance period (Table 2). The median duration of remission during IFX treatment was 25.5 (range, 4-59) mo. Of the 29 CD patients, the 9 (31%) who chose to discontinue IFX scheduled treatment, were still relapse-free at a median of 16 (range, 5-30) mo after the last infusion; all these patients were on concomitant immunomodulatory treatment; 2 CD patients were lost at follow-up. Twenty CD patients are currently on IFX treatment with a median duration of therapy of 20 (range, 5-57) mo. Of the 40 CD patients, 8 patients (20%) who showed worsening of clinical symp-

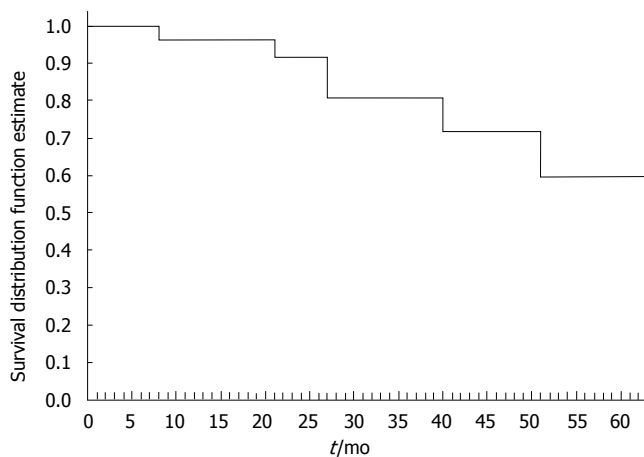


Figure 2 Kaplan-Meier survival curve for CD patients ($n = 40$). The cumulative probability of being free of relapse in CD patients with complete response was: 97.2% (CI: 91.8%-100%) at 12 mo, 90.3% (CI: 79.7%-100%) at 24 mo, 81.7% (CI: 66.9%-96.5%) at 36 mo, 73.5% (CI: 53.3%-93.7%) at 48 mo, and 61.3% (CI: 33.6%-88.9%) at 51 mo.

toms underwent surgery for intestinal strictures and/or abdominal abscesses. These patients presented with higher baseline values of acute phase reactants - in terms of serum ESR and CRP levels - compared to the patients not undergoing surgery. The difference was not significant. The cumulative probability curve of maintaining the initial response in those patients with clinical benefit at the third infusion is shown in Figure 2. The cumulative probability of being free of relapse in CD patients with complete response was: 97.2% (CI: 91.8-100%) at 12 mo, 90.3% (CI: 79.7-100) at 24 mo, 81.7% (CI: 66.9-96.5%) at 36 mo, 73.5% (CI: 53.3-93.7%) at 48 mo, and 61.3% (CI: 33.6-88.9%) at 51 mo. In this study, fistula closure was not considered as a goal of the clinical outcome.

Ulcerative colitis patients

Ten UC patients who underwent scheduled re-treatment every 8 wk as maintenance protocol had a median disease duration of 9.3 (range, 2-15) years (Table 2). The median number of IFX infusions was 14.5 (range, 6-22). Median duration of IFX treatment was 24.5 (range, 6-46) mo. In 9/10 (90%) UC patients, the CAI score remained below the remission level (< 4) throughout the maintenance period. The median duration of remission during IFX treatment was 25 (range, 6-46) mo. Of the 9 UC patients, 4 withdrew from scheduled treatment due to non compliance ($n = 2$) and to adverse events ($n = 2$) after a median time of 24.5 (range, 21-46) mo and were still relapse-free after a median time of 6.5 (range, 4-16) mo after the last IFX infusion; 3 of these patients were on concomitant immunomodulatory treatment. One UC patient, who showed a gradual loss of benefit, underwent surgery for flare up of the disease 24 mo after starting scheduled treatment. Five UC patients are currently on IFX therapy with a median duration of therapy of 36 (range, 6-39) mo. In patients who discontinued IFX treatment, endoscopic evaluation performed before withdrawal revealed complete mucosal healing in all cases.

Steroid-sparing effect

At baseline, 39 (78%) IBD patients were on steroid thera-

py; namely, 30/40 (75%) CD patients and 9 out of 10 (90%) UC patients required a median corticosteroid dose of 0.7 mg/kg per day. All patients were able to discontinue corticosteroids following IFX induction therapy, but 10/40 (25%) CD patients and 1/10 (10%) UC patients required reintroduction of steroid therapy during IFX scheduled treatment. Moreover, in patients who required concomitant steroid therapy, the median daily corticosteroid dose decreased to 0.25 mg/kg per day. The 10 CD and 4 UC patients, who discontinued scheduled treatment and remained relapse-free, did not require steroid therapy after the last IFX infusion.

Safety and infusion reactions

Of the 50 IBD patients, 16 (32%) experienced at least 1 adverse event: number and type of adverse events are listed in Table 3. Of the 50 IBD patients, 3 (6%) experienced moderate acute infusion reactions characterized by headache, dizziness, nausea, flushing, chest pain, dyspnoea, and pruritus, which developed after the second infusion. All 3 patients were successfully reinfused following intravenous hydrocortisone (200 mg) premedication before any subsequent IFX infusion. One patient presented serum sickness-like symptoms within 10 d of the third IFX infusion, developing arthralgias, myalgias, and fever; laboratory tests showed non-viral acute liver failure. The patient was treated with a high dose of iv corticosteroids and fully recovered after prolonged hospitalization. IFX treatment was discontinued in this patient.

Infections

Of the 50 IBD patients, 9 (18%) presented opportunistic infections during maintenance therapy. Six cases of viral infections occurred (5 herpes simplex virus and 1 varicella-zoster virus) in 5 CD patients and 1 UC patient had herpes simplex virus. Atypical pneumonia developed in 2 CD patients; full recovery was achieved in both patients following antibiotic therapy. All these patients were on concomitant immunomodulatory treatment which was withdrawn upon diagnosis. All infections developed within the first year of treatment and, in 3 patients, viral infections recurred during IFX maintenance treatment.

Malignant disorders

Three patients (6%) developed neoplasia during maintenance treatment (Table 4). Median time interval between the first infusion and diagnosis of neoplasia was 21 (range, 14-24) mo. Median number of IFX infusions was 10 (range, 6-13). Two patients with neoplasia were on concomitant immunomodulatory treatment (azathioprine). The histological types of neoplasia were gastric adenocarcinoma (T2aN0M0), which subsequently developed into peritoneal carcinomatosis and Krukenberg tumor, and endometrioid carcinoma (T1cN0M0), both in CD patients; breast cancer (T2mN3M0) developed in one UC patient.

Deaths

One CD patient, aged 68 years, who withdrew from the maintenance treatment protocol (9 infusions within 13 mo) because of complete disease remission, died of acute myocardial infarction 10 mo after the last IFX infusion. The patient did not present with any cardiovascular risk factors,

Table 3 Summary of IFX related adverse events

Adverse event	IBD (CD + UC)	Crohn's disease	Ulcerative colitis
Acute infusion reaction (%) (Mild, moderate, severe)	3/50 (6)	3/40 (7.5)	0
Delayed infusion reaction (%) (Serum sickness-like)	1/50 (2)	1/40 (2.5)	0
Opportunistic infection (%)	9/50 (18)	8/40 (20) 5 HSV 1 VZV 2 atypical pneumoniae	1/10 (10) 1 HSV
Lymphoproliferative diseases	0	0	0
Malignant disorder (%)	3/50 (6)	2/40 (5)	1/10 (10)

HSV: Herpes simplex virus; VZV: Varicella-zoster virus.

except for mild chronic renal failure, not requiring medical therapy and/or dialysis. The event was considered unrelated to IFX as it occurred 10 mo after the last infusion.

DISCUSSION

Despite the large amount of literature demonstrating the efficacy of IFX for the induction of remission in moderate to severe IBD, few data are available regarding the use of IFX for more than 12 mo or for more than 8 doses in IBD patients. Results of this retrospective study confirm current data on the efficacy of IFX in inducing a rapid clinical response in CD and support the finding, emerging from uncontrolled study data, of prolonged clinical efficacy in maintaining long-lasting remission beyond 1 year of treatment. While the efficacy of IFX therapy in the treatment of CD it is well established, only few studies have been performed on the use of IFX in UC. Järnerot *et al*^[16] found IFX to be an effective rescue therapy in patients experiencing an acute flare up of UC, leading to a significant reduction in the emergency colectomy rate. Moreover, Rutgeerts *et al*^[17] were the first to demonstrate that IFX can induce and maintain a clinical response and remission up to 54 wk of scheduled treatment in patients with moderately to severely active UC showing an inadequate response to conventional therapy. No data are yet available concerning the efficacy and safety of IFX treatment lasting for more than 1 year in UC. In our study population the median duration of IFX treatment was 27 mo in CD patients and 24.5 mo in UC patients, during which most of the patients (80 % of CD patients and 90% of UC patients) remained relapse free, following the clinical response or remission obtained with the induction treatment phase. It is worthwhile stressing that at enrolment, all these patients had active disease despite conventional therapy (or had a steroid-dependent disease). At baseline, considering all IBD patients, 58.5% were receiving 5-aminosalicylates, 78% corticosteroids, and 75.6% immunosuppressants. Moreover, compared to the 78% of patients on corticosteroids at baseline, only 25% needed concomitant corticosteroid therapy during IFX maintenance treatment, with a decreased median daily corticosteroid dose (0.7 mg/kg per day *vs* 0.25 mg/kg per day). The steroid-sparing effect of IFX was another important finding emerging from our study, which confirmed

Table 4 Clinical characteristics of patients developing malignant disorders during IFX treatment

Patients	1	2	3
Gender	F	F	F
Disease	Ulcerative colitis	Crohn's disease	Crohn's disease
Disease duration (yr)	22	3	3
Site of disease	Left colon	Ileum-colon	Ileum-colon
Type of neoplasia (TNM)	Breast cancer (T2mN3M0)	Gastric cancer (T2aN0M0)	Endometrial cancer (T1cN0M0)
Concomitant medication	Azathioprine	Azathioprine/ cyprofloxacin/ metronidazole	Azathioprine/ cyprofloxacin/ metronidazole
N. IFX infusions	13	6	10
Months since first IFX infusion	21	24	14

TNM: Tumour, nodes, metastasis.

the efficacy of a scheduled treatment regimen in avoiding the well-known morbidity associated with long-term corticosteroid therapy^[4,7].

Interestingly, long-term IFX therapy in IBD has been demonstrated to potentially modify the course of the disease. Indeed, 9 out of the 29 CD and 4 out of the 9 UC patients, who discontinued IFX scheduled treatment were still relapse-free after a median of 16 (range, 5-30) and 6.5 (range, 4-16) mo since the last IFX infusion, respectively. All these patients were off steroids and 12 were on immunosuppressants. Before stopping therapy, endoscopic evaluation was performed only in the subgroup of UC patients, showing complete mucosal healing in all. This result could account for the sustained clinical benefit maintained after withdrawal of treatment.

Long-term safety is an emerging and important issue as IFX use and indications are rapidly increasing worldwide. Recently, large retrospective studies have evaluated the incidence of serious adverse events and onset of neoplasia in CD patients treated with IFX^[18-24,27]. Colombel *et al*^[27], who studied 500 CD patients for a median of 17 (range, 0-48) mo with a median of 3 IFX infusions, reported the presence of 8.6% serious adverse events, 6.0% of which were considered possibly related to IFX therapy. In our study, 32% of IBD patients experienced at least 1 adverse event; 8% of the patients had an infusion reaction (3 patients experienced an acute infusion reaction and 1 a serum sickness-like disease). Moreover, in our study population, the incidence of opportunistic infections was 18%, which is higher than that observed in the study by Colombel *et al*^[27] (8.2%). This finding could be due both to the longer scheduled treatment protocol and the concomitant use of immunomodulatory treatment (AZA or 6-MP). Albeit, the long duration of scheduled treatment associated with the concomitant use of immunomodulators may give rise to the risk of opportunistic infections; regular maintenance therapy has been proven to reduce the number of infusion reactions with respect to episodic dosing, which is more immunogenic, as demonstrated by the results of the AC-CENT I trial^[11]. In fact, in the study by Hanauer *et al*^[11], the antibodies to IFX (ATI), which may be associated with decreased clinical response and increased risk of infusion reactions, were detected at higher rate in the episodic-

treated group (30%) compared to the maintenance strategy protocol (8%). Comparable results emerged from the studies by Baert *et al*^[19] and Farrell *et al*^[28], in which IFX was used episodically, thus demonstrating that the formation and concentrations of ATI were correlated with lower post-infusion serum IFX concentrations and with the need to shorten infusion intervals. Moreover, in patients who underwent bowel surgery after IFX withdrawal, no increase in the number of severe infections or surgical complications was observed in the perioperative period (data not shown).

An interesting finding emerging from our study, for which further investigation is necessary, concerns the relatively large number of malignancies (6%) observed in our IBD patients treated with IFX. In fact, the high incidence of solid tumors is somewhat inconsistent with the results reported so far. Biancone *et al*^[21], in their multi-centre case-controlled study, evaluated the risk of developing neoplasia in IFX-treated CD patients: the incidence of newly diagnosed neoplasia was comparable in the 2 groups of CD patients, treated (2.2%) or not treated (1.73%) with IFX. Colombel *et al*^[27], in a retrospective study found that 3 out of 500 of the CD patients treated with IFX had a malignant disorder, possibly related to biologic therapy. The fact that our study did not include a control group and consisted of a rather heterogeneous and relatively small population makes it difficult to establish a direct cause-effect relationship between anti-TNF- α therapy and the increased risk of developing malignancies. However, it should be pointed out that a slight difference was observed in terms of mean number of IFX infusions between patients who developed malignancies and those who did not (9.66 *vs* 14.6, respectively). This result would appear to suggest that there is no linear dose-dependent increase in risk.

Another important issue concerns the risk of hematologic malignancies related to the use of biologic therapies in IBD. It is well known that patients with long-standing IBD treated with immunomodulatory drugs may be more susceptible to developing lymphoproliferative disease^[29-32]. Even though the use of TNF- α blocking agents has been associated with an increased risk of developing lymphoma^[32], a finding, however, not confirmed by the data of a large US-based CD registry (TREAT)^[22], we did not observe any cases of hematologic malignancy in our study population.

The results from this study need to be interpreted with an understanding of both the strengths and limitations of retrospective analysis of prospectively collected data. Although it allowed us to have a long term follow-up, the patients could have not been monitored as rigorously as prospective, randomized controlled studies. In any event, these limitations are not relevant to the analysis of efficacy of IFX treatment in IBD and of serious infections reported in this setting.

In conclusion, IFX scheduled treatment has proven to be an effective strategy in our IBD patient population for long-term maintenance of clinical remission. The scrupulous selection of patients to be started on IFX therapy is a fundamental issue, not only to obtain maximum efficacy, but also to avoid serious adverse events. A note of caution is mandatory when considering the possible risk of malignancy associated with the use of anti-TNF- α therapy. Fur-

ther studies on larger series are needed to further clarify these important aspects.

COMMENTS

Background

For many years, corticosteroids have represented the cornerstone of therapy for induction of remission in inflammatory bowel disease (IBD); however, long-lasting remission was not achieved and the side-effects emerging with long-term use exceeded the clinical benefits. Immunomodulators have been demonstrated to be efficacious as adjunctive therapy and as steroid-sparing agents. The introduction of biological agents in the therapeutic armamentarium for Crohn's disease (CD) and ulcerative colitis (UC) has significantly changed the treatment strategies and outcomes of these patients.

Research frontiers

Despite the large amount of literature demonstrating the efficacy of infliximab (IFX) for the induction of remission in moderate to severe IBD, few data are available regarding the use of IFX for more than 12 mo or for more than 8 doses in IBD patients. Some safety issues are associated with IFX use, mostly related to the development of adverse events (e.g., opportunistic infections, autoimmune disorders, and infusion reactions). Major concerns are related to the reactivation of latent tuberculosis and development of malignancy, even if there is no clear evidence that the use of IFX increases the incidence of solid cancers.

Related publications

Colombel *et al*^[27] studied 500 CD patients for a median of 17 mo (range, 0-48) with a median of 3 IFX infusions and reported the presence of 8.6% serious adverse events, 6.0% of which were considered possibly related to IFX therapy. Hanauer *et al*^[11] found that the antibodies to IFX, which may be associated with decreased clinical response and increased risk of infusion reactions, were detected at higher rate in the episodic-treated group (30%) compared to the maintenance strategy protocol (8%). Comparable results emerged from the studies by Baert *et al*^[19] and Farrell. Biancone *et al*^[21], in their multi-centre case-controlled study, evaluated the risk of developing neoplasia in IFX-treated CD patients: the incidence of newly diagnosed neoplasia was comparable in the 2 groups of CD patients, treated (2.2%) or not treated (1.73%) with IFX.

Innovations and breakthroughs

IFX scheduled treatment has proven to be an effective strategy in our IBD patient population for long-term maintenance of clinical remission. The scrupulous selection of patients to be started on IFX therapy is a fundamental issue, not only to obtain maximum efficacy, but also to avoid serious adverse events.

Terminology

Infliximab is a chimeric monoclonal IgG1 antibody directed against TNF- α able to almost completely neutralize its biological activity. Crohn's Disease Activity Index was used to monitor Crohn's disease activity and the Clinical Activity Index was used for assessing UC activity.

Peer review

This article contains valuable information that would be useful for practicing clinicians.

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Transcutaneous cervical esophagus ultrasound in adults: Relation with ambulatory 24-h pH-monitoring and esophageal manometry

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Abstract

AIM: To determine the gastroesophageal refluxate in the cervical esophagus (CE) and measure transcutaneous cervical esophageal ultrasound (TCEUS) findings [anterior wall thickness (WT) of CE, esophageal luminal diameter (ELD), esophageal diameter (ED)]; to compare TCEUS findings in the patient subgroups divided according to 24-h esophageal pH monitoring and manometry; and to investigate possible cut-off values according to the TCEUS findings as a predictor of gastroesophageal reflux (GER).

METHODS: In 45/500 patients, refluxate was visualized in TCEUS. 38/45 patients underwent esophagogastroduodenoscopy (EGD), 24-h pH monitoring and manometry.

RESULTS: The 38 patients were grouped according to 24-h pH monitoring as follows: Group A: GER-positive ($n = 20$) [Includes Group B: isolated proximal reflux (PR) ($n = 6$), Group C: isolated distal reflux (DR) ($n = 6$), and Group D: both PR/DR ($n = 8$); Group E: no reflux ($n = 13$); and Group F: hypersensitive esophagus (HSE) ($n = 5$). Groups B + D indicated total PR patients ($n = 14$), Groups E + F reflux-negatives with HSE ($n = 18$), and Groups A + F reflux-positives with HSE ($n = 25$). When the 38 patients were grouped according to manometry findings, 24 had normal esophageal manometry; 7 had hypotensive and 2 had hypertensive lower esophageal sphincter (LES); and 5 had ineffective esophageal motility disorder (IEM). The ELD measurement was greater in group A + F than group E ($P = 0.023$, 5.0 ± 1.3 vs 3.9 ± 1.4 mm). In 27/38 patients, there was at least one pathologic acid reflux and/or pathologic manometry finding. The cut-off value for ELD of 4.83 mm had 79% sensitivity and 61% specificity in predicting the PR

between Groups B + D and E (AUC = 0.775, $P = 0.015$).

CONCLUSION: Visualizing refluxate in TCEUS was useful as a pre-diagnostic tool for estimating GER or manometric pathology in 71.1% of adults in our study, but it was not diagnostic for CE WT.

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Key words: Ambulatory 24-h pH monitoring; Cervical esophageal ultrasound; Gastroesophageal reflux; Esophageal manometry; Esophageal refluxate

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INTRODUCTION

Esophageal ultrasonography (US) is a non-invasive, readily available, repeatable, cheap, fast and highly sensitive technique^[1-4] in the diagnosis of gastroesophageal reflux (GER) in infants and children^[5-8]. The esophageal US studies in GER have mainly focused on the evaluation of the gastroesophageal junction (GEJ)^[9-11] and esophageal motility^[12-14]. These studies were performed by transabdominal or endoluminal routes. Although cervical US is a part of neck US, it has not been routinely used in infants and adults for diagnosis of GER^[9]. There are only a few studies about the transcutaneous cervical esophagus ultrasonography (TCEUS), but these were in normal^[15,16] and pathologic conditions^[17,18].

Intraluminal refluxate can be recognized by US images. Sonographic GER diagnosis was made by backward movement of gastric content into the esophagus and the visualization of the clearance of refluxate material^[15,19,20]. The visualization of GER episodes or gastroesophageal reflux disease (GERD) estimation in the GEJ region in US provided the background for our study. The aims of this study were to evaluate the possible pathologies in 24-h (h) pH monitoring and esophageal manometry in patients with refluxate in the lumen of the cervical esophagus

(CE) during TCEUS; to compare TCEUS findings in the patient subgroups divided according to 24-h esophageal monitoring and manometry; and to investigate possible cut-off values according to the TCEUS findings as a predictor of GER.

MATERIALS AND METHODS

Study design

Patient features: Five hundred patients (45.82 ± 14.15 years, 163 M/337 F) who were admitted to the outpatient clinic between the years from January 2006 to January 2007 with complaints other than of the gastrointestinal system underwent TCEUS. Refluxate material was found in the esophageal lumen in 45 (9%) of the 500 patients during TCEUS. Forty-five patients were questioned regarding GERD symptoms, and all had reflux symptoms.

Thirty-eight of the 45 patients underwent esophagogastroduodenoscopy (EGD), 24-h pH monitoring and esophageal manometry [7 patients were excluded as follows: pH monitoring not accepted ($n = 5$), nasal cannulation could not be performed due to nasal operation history ($n = 1$), inability to continue the 24-h pH monitoring/pH catheter extracted ($n = 1$)]. The period between the TCEUS and the pH monitoring was 1-3 d.

Patients had no history of weight loss, gastrointestinal bleeding, gastrointestinal motility disorder, pneumonic dilatation, collagen vascular disease, any operation around the cervical region, or gastrointestinal operation. None of the patients was taking medications known to affect esophageal motor function, including promotility agents, antacids, H₂ receptor antagonist, or proton-pump inhibitors (PPI); 3 patients had been taking PPI but they had been discontinued for two weeks before manometric investigation and 24-h pH monitoring.

Test techniques

Questionnaire for GERD: All patients were evaluated for typical (acid regurgitation and heartburn) and extraesophageal (hoarseness, asthma-like clinical presentation, nocturnal cough, and nocturnal wake-up) GERD symptoms.

Esophagogastroduodenoscopy (EGD): The procedure was performed by Pentax EG 2940 with 2% xylocaine topical anesthesia after a 12-h overnight fast. Reflux esophagitis was evaluated according to Los Angeles classification^[21]. The presence of hiatal hernia and the distance between GEJ and diaphragmatic impression were recorded.

TCEUS: Each patient was given an 800 kcal standard meal (15% protein, 50% carbohydrate, and 35% fat) and TCEUS was performed at postprandial 1-2 h with patient in supine position (Hitachi EUB, 6-13 MHz linear probe). TCEUS was performed as defined by Zhu and Mateen^[15,16]. The esophagus was demonstrated at thyroid cartilage level with the guidance of thyroid gland acoustic window up to the supraclavicular level to thoracic inlet (manubrium sterni) by linear probe in transverse and longitudinal sections without a pillow under the neck. CE was evaluated

by using a slightly flexed neck position with head turned 45° to the opposite side by left and right lateral approaches over 15 min to determine the presence of refluxate (the luminal anechoic fluid and/or linear bright stratifying small lines indicating gas in refluxate) and its to-and-fro movement, with the patients not swallowing^[15,20,22]. Then all patients were required to swallow and the clearance of refluxate was observed. The presence of comet-tail artifact (during swallowing, the presence of saliva mixed with air and downward movement of refluxate generated a strong echogenic appearance^[15,20,22]) was observed in patients. After the clearance of refluxate was observed, a few patients had backward flow of refluxate into the esophagus, which can perhaps be considered by the terminology "re-reflux"^[23].

Anterior wall thickness (WT) of the esophagus (distance between adventitia and the mucosa, with 5-7 esophageal wall layers), esophageal diameter (ED) [distance between the adventitia (outer to outer)], and esophageal luminal diameter (ELD) with or without refluxed material [distance between the mucosa (inner to inner)] were measured in longitudinal section at left lateral cervical approach. The GEJ was not evaluated during US in this study. The TCEUS appearance with or without refluxate is given in Figure 1.

Ambulatory 24-h pH monitoring: pH monitoring was performed using Synetics Digtrapper MHIII, and double-channel, 15 cm antimony catheter. The esophageal pH catheter was placed 5 cm above the upper border of the lower esophageal sphincter (LES). Findings were evaluated by Microsoft esophagram version 2.04. The pathologic measurements were evaluated as follows: Proximal reflux (PR): The upper esophageal sphincter (UES) localization was determined by manometry and PR was determined by the proximal probe localization and UES. If proximal probe was localized in the UES or above it, a single acid reflux synchronously occurring with distal probe was accepted as pathologic acid reflux; if the probe was localized under the UES, acid contact time above 1% of total time was accepted as pathologic in PR. De Meester score > 14.72 and acid contact greater than 4.0% of total time below pH 4 were accepted as pathologic in distal reflux (DR). Hypersensitive esophagus (HSE) was defined if symptom index (SI) for distal measurements (SI = number of symptoms in pH < 4/total number of symptoms) was $\geq 50\%$ while there was no measurable DR or PR^[23-25].

Esophageal manometry: Esophageal manometry was performed using MMS (ver. 8.4i Beta) and eight-channel Dent-sleeve catheter. After calibration, catheter was sent through the nose to the stomach and advanced 65 cm by swallowing. When all channels were in stomach, with patient in supine position, UES and LES were determined as the catheter was slowly withdrawn back into the esophagus. LES pressure (LESP), relaxation, esophageal body pressure, body contractions, contraction amplitudes and duration, peristalsis and upper esophageal contractions were recorded. Manometric findings were grouped as: normal, spastic (hypertensive LES, if LESP > 45 mmHg),

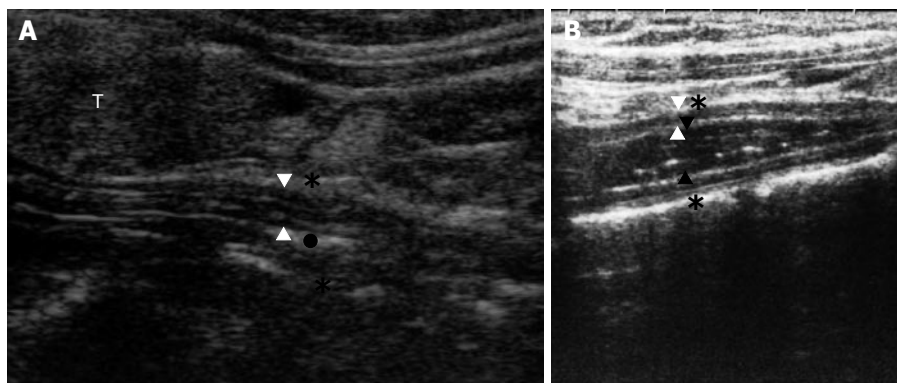


Figure 1 The TCEUS appearance with and without refluxate. **A:** A pattern showing no anechoic fluid in the lumen. Note anterior esophageal wall (distance between the open arrowheads), esophageal lumen without fluid inside and hyperechogenic bands representing collapsed lumen and mucosa (dark dot). T: thyroid; **B:** A pattern showing anechoic fluid in the lumen. Note esophageal wall (distance between the open arrowheads), refluxate in the esophageal lumen (distance between the dark arrowheads) and esophageal diameter (distance between the asterisks).

non-spastic [hypotensive LES, if $LESP < 10$ mmHg] or ineffective esophageal motor contractions (IEM), if contraction amplitude was < 25 mmHg in $> 30\%$ of wet swallows]^[26].

Statistical analysis

Descriptive and comparative statistical analyses were performed using statistical software system (SPSS v11.0). Where appropriate, average data were presented as mean \pm SD. Comparison between groups was performed by Kruskal Wallis analysis. All possible pair-wise comparisons were done by Mann-Whitney *U* test with Bonferroni correction. Fisher-Freeman-Halton test generalized at Fisher's exact test to $m \times n$ tables was used for categorical variables. The cut-off values were determined using receiver operating characteristic (ROC) curve for TCEUS parameters between all possible patient group pairs according to pH-metry and manometry to determine reflux or any pathologic manometry finding. The sensitivity and the specificity were determined according to the measured cut-off values. The significance of the area under curve (AUC) was tested ($P < 0.05$).

Investigators interpreting sonography, 24-h pH monitoring and esophageal manometry were blinded to the patients' features. None of the patients was sedated during EGD. All patients provided written informed consent and the study conformed to the guidelines of the Helsinki Declaration.

RESULTS

Forty-five (9%) of 500 patients who underwent TCEUS were found to have anechoic fluid and/or air echogenicities of refluxate in the cervical esophageal lumen. CE was not visualized clearly in 1 (0.2%) of the 500 patients due to neck anatomy.

Esophagitis (all grade A), hiatal hernia, antral gastritis, and grade 1 bulbitis were diagnosed in 10.7%, 10%, 14%, and 3%, respectively, in EGD. None of the patients had malignancy, or gastric or duodenal ulcer disease.

Thirty-eight patients were grouped according to 24-h pH monitoring as follows: Group A: Acid reflux-positive ($n = 20$, 52.7%) [includes Group B + Group C + Group D] [Group B: Isolated PR but no DR ($n = 6$, 15.8%); Group C: Isolated DR but no PR ($n = 6$, 15.8%); Group D: Both PR and DR ($n = 8$, 21.1%); Group E: No acid reflux ($n = 13$, 34.2%); and Group F: patients with hypersensitive

esophagus (HSE) ($n = 5$, 13.1%). Group B + D indicated total patients with PR ($n = 14$, 36.9%) and Group E + F: acid reflux-negatives with HSE ($n = 18$, 47.3%) and Group A + F: Acid reflux-positives with HSE ($n = 25$, 65.8%). The demographic and TCEUS findings of subjects grouped according to 24-h pH monitoring are given in Table 1.

When the 38 patients were grouped according to manometry findings, 24 (63.2%) patients had normal esophageal manometry; 7 (18.4%) had hypotensive and 2 (5.3%) had hypertensive LES; 5 (13.1%) had ineffective esophageal motility disorder (IEM). Demographic and TCEUS findings of subjects grouped according to manometric results are given in Table 2.

Patient symptoms are given in Table 3. None of the patients had complaints of dysphagia or asthma-like dyspnea.

Statistical analysis was performed between (1) 24-h pH monitoring subgroups, (2) esophageal manometry subgroups, and (3) categorized groups according to combined 24-h pH monitoring and esophageal manometry findings as acid reflux/abnormal, acid reflux/normal, no acid reflux/abnormal, and no acid reflux/normal with the following parameters: Age, sex, body mass index (BMI), LES localization defined during manometry, and TCEUS findings (WT, ED, ELD).

There were no significant differences in BMI, LES localization, and typical and extraesophageal symptoms between subjects grouped according to 24-h pH monitoring and according to esophageal manometric findings. There was no correlation between the TCEUS findings and sex or presence of hiatal hernia or esophagitis.

When 24-h pH monitoring subgroups were compared according to TCEUS findings, the ELD measurement was greater in group A + F than group E ($P = 0.023$). No significant differences were determined between the other subgroups when compared regarding TCEUS findings.

There was a positive significant correlation between ED and ELD ($r = 0.889$, $P = 0.000$) and ED and WT ($r = 0.499$, $P = 0.001$) (Pearson correlation analysis).

There were 2 patients with hypertensive LES. Excluding this group, when the nonspastic esophageal motor disorder group (hypotensive LES, IEM) was compared with the normal manometric group, there was no significant difference with regard to TCEUS findings and the LES localization between subgroups.

There was no significant difference according

Table 1 Demographic and TCEUS findings in groups divided according to pH monitoring (mean \pm SD)

	Group A (n = 20)	Group B (n = 6)	Group C (n = 6)	Group D (n = 8)	Group E (n = 13)	Group F (n = 5)	Group B + D (n = 14)	Group E + F (n = 18)	Group A + F (n = 25)	P
Age (yr)	43.4 \pm 12.0 (24-72)	40.0 \pm 11.4 (24-58)	47.8 \pm 12.7 (38-72)	42.6 \pm 12.5 (25-62)	43.17 \pm 9.6 (25-65)	37.2 \pm 18.8 (15-64)	41.5 \pm 11.7 (24-629)	40.5 \pm 10.7 (16-65)	42.3 \pm 13.7 (15-72)	NS
Sex (F/M)	8/12 (40/60)	3/3 (50/50)	1/5 (16.7/83.3)	4/4 (50/50)	11/2 (84.6/5.4)	5/0 (100/0)	7/7 (50/50)	2/16 (88.9/11.1)	12/13 (52/48)	NS
BMI (kg/m ²)	27.4 \pm 4.5 (19.6-37.4)	27.4 \pm 4.4 (19.6-32)	27.4 \pm 4.4 (19.6-32)	27.9 \pm 5.6 (20.6-37.5)	27.7 \pm 4.6 (17.6 \pm 35.0)	24.9 \pm 2.1 (22.7-27.4)	27.7 \pm 4.9 (19.6-37.4)	26.9 \pm 4.24 (17.6-35.0)	26.9 \pm 4.3 (19.6-37.5)	NS
ED (mm)	9.4 \pm 1.4 (6.1-12.1)	9.7 \pm 0.9 (8.2-10.8)	9.1 \pm 1.6 (8.0-12.0)	9.6 \pm 1.71 (6.1-11.4)	8.54 \pm 1.82 (5.5-11.1)	10.6 \pm 1.3 (8.8-12.4)	9.6 \pm 1.4 (6.2-11.4)	9.1 \pm 1.9 (5.5-12.4)	9.7 \pm 1.5 (6.1-12.4)	NS
ELD (mm)	5.0 \pm 1.2 (3.0-7.7)	5.1 \pm 1.1 (3.00-6.00)	4.6 \pm 1.6 (3.4-7.7)	5.2 \pm 1.18 (3.0-6.4)	3.9 \pm 1.4 (1.5-6.2)	5.3 \pm 1.7 (3.4-7.9)	5.16 \pm 1.10 (3.0-6.4)	4.3 \pm 1.6 (1.5-7.9)	5.0 \pm 1.3 (3.0-7.9)	0.023 ^a
Esophageal WT (mm)	2.2 \pm 0.2 (1.5-2.6)	2.3 \pm 1.2 (2.2-2.6)	2.2 \pm 0.1 (2.0-2.3)	2.1 \pm 0.3 (1.5-2.5)	2.3 \pm 0.3 (2.0-3.3)	2.6 \pm 0.7 (2.2-4.0)	2.2 \pm 0.3 (1.6-2.6)	2.4 \pm 0.50 (2.0-4.0)	2.3 \pm 0.4 (1.6-4.0)	NS
DeMeester score	19.5 \pm 12.1 (1.7-46.2)	7.9 \pm 3.1 (4.6-13.2)	24.3 \pm 14.4 (1.7-46.2)	24.5 \pm 9.1 (14.4-38.9)	6.4 \pm 4.7 (0.8-14.7)	7.5 \pm 4.9 (1.4-17.3)	17.4 \pm 10.9 (4.6-38.9)	6.7 \pm 4.7 (0.8-14.7)	17.7 \pm 11.8 (1.4-46.2)	NS

TCEUS: Transcutaneous cervical esophagus ultrasonography; ED: Esophageal diameter; ELD: Esophageal luminal diameter; Esophageal WT: Esophageal wall thickness; BMI: Body mass index. ^a*P* < 0.05 between group A + F and group E. NS: Non-significant.

Table 2 Demographic and TCEUS findings of the groups divided according to esophageal manometry (mean \pm SD)

	Normal (n = 24)	IEM (n = 5)	Hypo LES (n = 7)	Hyper LES (n = 2)	Total patients (n = 38)	P
Age (yr)	40.1 \pm 11.4 (15-64)	50.6 \pm 11.9 (39-65)	48.9 \pm 11.3 (38-72)	29.0 \pm 4.3 (26-32)	42.5 \pm 12.2 (15.0-72.0)	NS
Sex (F/M), n (%)	8/16 (66.7%/33.3%)	4/1 (80%/20%)	2/5 (28.6%/71.4%)	2/0 (100%/0%)	24/14 (63.2%/36.8%)	NS
BMI (kg/m ²)	26.7 \pm 4.6 (17.6-37.5)	26.7 \pm 4.1 (20.6-31.1)	29.58 \pm 3.93 (23.1-35.0)	25.4 \pm 2.7 (23.4-27.3)	27.2 \pm 4.3 (17.6-37.4)	NS
ED (mm)	9.1 \pm 1.59 (6.0-12.4)	9.5 \pm 2.3 (5.5-11.4)	9.8 \pm 1.7 (7.5-12.1)	9.6 \pm 1.1 (8.9-10.4)	9.3 \pm 1.7 (5.5-12.4)	NS
ELD (mm)	4.5 \pm 1.3 (2.0-7.9)	4.7 \pm 1.9 (1.5-6.4)	5.1 \pm 1.7 (3.1-7.7)	4.7 \pm 0.9 (4.1-5.4)	4.68 \pm 1.43 (1.5-7.9)	NS
Esophageal WT (mm)	2.2 \pm 0.4 (1.6-4.0)	2.4 \pm 2.2 (2.0-2.6)	2.3 \pm 0.4 (2.0-3.3)	2.35 \pm 0.2 (2.2-2.5)	2.3 \pm 0.4 (1.6-4.0)	NS

IEM: Ineffective esophageal motility; Hypo LES: Hypotensive lower esophageal sphincter; Hyper LES: Hypertensive lower esophageal sphincter; ED: Esophageal diameter; ELD: Esophageal luminal diameter; Esophageal WT: Esophageal wall thickness; BMI: body mass index. NS: Non-significant.

Table 3 Symptoms of patients according to esophageal monitoring n (%)

	Group A (n = 20)	Group E (n = 13)	Group B + D (n = 14)	Group E + F (n = 18)
Extra-esophageal symptom	12 (60.0)	7 (53.8)	9 (64.3)	12 (66.6)
Cough	6 (30.0)	6 (46.2)	4 (28.5)	8 (44.4)
Hoarseness	5 (25)	3 (23.1)	4 (28.6)	4 (22.2)
Nocturnal wake-up with reflux	10 (50)	5 (38.5)	8 (57.1)	9 (50.0)
Typical symptom	20 (100)	12 (92.3)	14 (100.0)	17 (94.4)
Heartburn	18 (90)	12 (92.3)	12 (85.7)	17 (94.4)
Regurgitation	15 (75)	10 (76.9)	12 (85.7)	13 (72.2)

to TCEUS findings when 24-h pH monitoring and esophageal manometry subgroups were evaluated together (Table 4). Table 5 shows the detailed 24-h pH monitoring and manometric findings. In 27/38 (71.1%) patients, there was at least an acidic reflux and/or pathologic manometry finding. 11/38 (28.9%) with refluxate in CE had no acid reflux and normal esophageal manometric findings.

We tried to find cut-off values in order to differentiate total GER, PR or the other reflux subgroups from the reflux-negatives and to differentiate each manometry subgroup according to TCEUS parameters. The groups which had significant cut-off values (AUC, *P* < 0.05) are

Table 4 Distribution of patients according to 24-h pH monitoring and manometric findings n (%)

	Manometric findings (abnormal)	Manometric findings (normal)
24-h pH monitoring (Acid reflux)	7 (18.4)	13 (34.3)
24-h pH monitoring (No acid reflux)	7 (18.4)	11 (28.9)

given in Table 6 with their sensitivity and specificity rates for ELD in determining reflux. The ROC curve is given in Figure 2 according to TCEUS findings in patients with total PR (group B + D) (*n* = 14) and in patients without reflux (group E) (*n* = 13).

DISCUSSION

The esophagus is a 23-24 cm muscular channel. The longitudinal scan of the esophagus shows a tubular structure with hypoechogenic muscular layer on the wall and one or two echogenic inner layer(s) representing the mucosa and the collapsed lumen of the esophagus^[3,10,15,16,20,22].

US evaluation is performed at four sites of the esophagus: GEJ^[4,9,27,28], thoracic esophagus^[22], CE^[15,16], and upper esophageal sphincter^[29].

Table 5 24-h pH monitoring and esophageal manometry results

24-h pH monitoring	IEM (n = 5)	Hypo LES (n = 7)	Hyper LES (n = 2)	Normal manometry (n = 24)
PR + DR	2	2		4
PR				6
DR		3		3
Reflux negatives	3	2	1	7
HSE			1	4

IEM: Ineffective esophageal motility; Hypo LES: Hypotensive lower esophageal sphincter; Hyper LES: Hypertensive lower esophageal sphincter; PR: Proximal reflux; DR: Distal reflux; HSE: Hypersensitive esophagus.

Table 6 Cut-off values for esophageal luminal diameter (ELD) to determine acid reflux

Between groups	Cut off (mm)	AUC	P	Sensitivity (%)	Specificity (%)
Group B + D (total PR) (n = 14)/Group E (reflux negative) (n = 13)	4.83	0.775	0.015 ^a	79	61
Group B + D (total PR) (n = 14)/Group E + F (reflux negative with HSE) (n = 18)	4.95	0.708	0.046	71	77
Group A (n = 20)/Group E (reflux negative) (n = 13)	4.95	0.721	0.034	60	77

HSE: Hypersensitive esophagus; PR: Proximal reflux; AUC: Area under the curve. P values show significance of AUC (^aP < 0.05).

GERD arises from increased exposure and/or sensitivity of the esophageal mucosa to gastric contents^[30,31], and affects 5%-40% of the population^[32,33]. The content of refluxate can be isolated liquid (acid or non-acid nature), isolated gas, or gas/liquid mixture. 24-h pH monitoring and multichannel intraluminal impedance (MII) are the gold standard techniques to evaluate GER^[19,34,35].

US has been used in GERD since 1984^[36]. The GEJ was first described by Westra^[6] and Gomes^[1] during US by transabdominal route. The first-line use of esophageal GEJ US in GERD for infants and children was established by multiple studies^[2,4,6,37]. Sonographic sensitivity was 81%-94%^[3,4]. US provides a morphologic and functional approach. In infants and children, 24-h pH monitoring and esophageal US are the complementary techniques of choice^[2,4].

Zhu points out the importance of conventional US to evaluate the GEJ, but the use of the CE was defined to be restricted. Zhu defined the normal sonographic parameters of the CE (7.5-12 MHz transducer) transcutaneously^[15]. Mateen *et al* used a modified technique which differed from the normal neck US to evaluate the CE. Visualization failure of the right lateral two-thirds CE was decreased from 36% to 2% using this modified technique^[16]. In our study, use of this modified technique resulted in failed visualization in only 1 (0.2%) of 500 patients due to the deformed anatomy of the patient.

GERD was diagnosed in 26% of a healthy population of infants and children according to US^[3]. In our study, postprandial refluxate was seen in 45 of 500 (9%) adults.

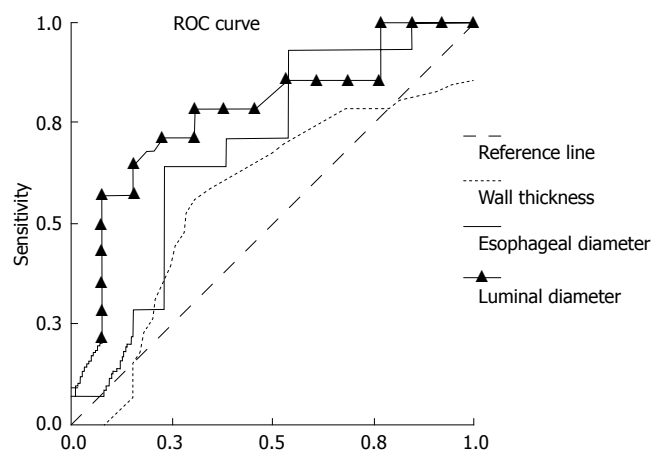


Figure 2 The ROC curve showing the relation between TCEUS findings according to total PR group (B + D) (n = 14) and reflux-negative group (E) (n = 13) (AUC = 0.775, P = 0.015 for ELD). PR: Isolated proximal reflux; ELD: Esophageal luminal diameter.

Furthermore, 20/38 (52.63%) of the patients who admitted to the hospital for other than gastrointestinal symptoms had refluxate in TCEUS and acidic reflux according to the 24-h pH monitoring. Since non-acid reflux was not evaluated, the 18 other patients were not evaluated in this respect.

Cool *et al*^[38] showed that respiratory and ear, nose and throat symptoms were especially related with gas reflux with weak acidity and not abnormal proximal acid reflux. We did not find any correlation in our study between proximal acid reflux, GER and any symptoms.

During the first hour after a meal, 20% more reflux episodes reach a higher proximal extent than during the fasting period and the late postprandial period (after 1 h). Acid reflux can reach 15 cm above the LES in approximately 6.8-21 s^[39,40]. We performed the sonography at the postprandial 1st-2nd h.

The content of liquid refluxate, whether acidic or not, did not affect the sonographic appearance^[41]. The new studies have pointed out that gas reflux with weak acidity is quite often determined in PR studies performed by pharyngeal impedance-pH recordings. Mixed reflux of gas and fluid is more frequent than pure fluid reflux^[39].

In the study of Mittal performed simultaneously with high frequency endoscopic US (HFEUS) and pH-metry, five US refluxate patterns were identified, as fluid, gas, first gas later fluid transition, first liquid later gas transition, or no luminal opening^[19]. In our study, we observed these patterns as fluid with or without gas and its to-and-fro movement during a period patients did not swallow. During swallowing, the comet-tail artifact was detectable in 42.8% of the cases^[15,19]. We observed it in approximately half of the patients. A few patients had reverse movement of refluxate to the esophagus after swallowing. This could be US documentation of a new terminology, "re-reflux"^[23].

Jang *et al* found no correlation between reflux number in 15 min counted during US and the reflux index in the 24-h pH monitoring. Sonographic reflux number was not considered as a specific indicator of disease severity^[2]. We did not count the reflux episodes in our study. Only one reflux episode in CE during US was included. Dickmann *et*

et al^[42] had shown that the acid reflux period below pH 4 was significantly lower when the distal pH probe was located 16 cm above the LES than 1, 6 or 11 cm above the LES in non-erosive reflux disease (NERD).

Transcutaneous CE WT has been reported as 2.3 ± 0.3 (1.3-4.1) mm in healthy adults^[15]. Mateen reported right-side thickness as 2.8 ± 0.4 mm (upper limit of normal, 3.6 mm) and left-side thickness as 2.9 ± 0.2 mm (upper limit of normal, 3.3 mm). We determined the left anterior WT as 2.29 ± 0.38 mm (1.57-4.0) in TCEUS (Table 1).

Dogan *et al*^[43] reported an increase in distal esophageal WT in conjunction with increasing age (1.56 ± 0.32 mm vs 1.29 ± 0.24 mm). In our study, we found no correlation between age, sex and TCEUS findings in subjects with refluxate.

Endosonographically, GEJ and 10 cm above thicknesses were given as 2.43 ± 0.16 and 2.28 ± 0.21 mm in healthy adults, respectively. The distal esophagus wall was thicker than the proximal^[28]. In reflux esophagitis, total esophageal WT and the smooth muscle layer were observed to be thicker than in normal subjects^[19,28,32]. Changchien measured normal GEJ WT as 3.8 ± 1.2 (2-5) mm using real time US. During acute severe inflammation in reflux esophagitis, the GEJ wall was observed as 7.6 ± 2.1 (5-10) mm, which was significantly thicker than normal^[9]. The submucosal healing due to lansoprazole in GERD was evaluated by US and the WT had decreased significantly in the GEJ region^[32,44]. We did not determine any significant difference in WT between patients with or without reflux according to 24-h pH monitoring. The distal esophageal WT increased with reflux according to the literature as described above, but we could not confirm this observation for the proximal esophagus in GER. Although HSE is a new terminology in the GERD spectrum, there was no significant difference between the HSE subgroup and the other subgroups with regard to CE WT.

Zhu reported normal transverse ED as 11.1 ± 1.6 (7.1-13.9) mm and anteroposterior diameter as 7.5 ± 1.2 mm (4.9-10.1)^[15]. Mateen measured the transverse diameter as 6.8 ± 2.7 mm (max 12.2)/ 10.7 ± 4.0 mm (18.7) and anteroposterior diameter as 6.5 ± 1.1 (max 8.7) mm/ 7.4 ± 1.5 (10.4) mm with right and left approaches, respectively, using the modified technique^[16]. We measured the cervical anteroposterior ED as 5.5-12.4 mm, and the ELD with refluxate was 1.5-7.9 mm in patients longitudinally (Tables 1 and 2). No significant differences were determined between proximal and total reflux patient groups and the other subgroups with regard to ED. ELD measurements with refluxate were statistically greater in group A + F than group E ($P = 0.023$). There was no difference between the other groups regarding ELD. Peak ED was given as 22 mm during physiologic swallows with 15 mL water^[19]. PR volume has not been accurately diagnosed to date, though esophageal continuous aspiration and scintigraphic studies have been used in an effort to obtain results about the reflux volume^[39]. In our study, the ED and ELD measurements may be an indirect indicator of reflux amount.

The distal esophageal distension and the cross-sectional area (CSA) are known to be wider than the proximal esophagus^[19]. Mittal reported that healthy asymptomatic

individuals had comparable esophageal diameter and CSA measurements according to spontaneous fluid GER and 5 mL swallow. It is difficult to differentiate between the ingested fluid and the refluxate of esophageal content. Mittal made the differentiation by looking at transient LES relaxations synchronously. In our study, our patients did not drink water during the TCEUS measurements. We measured the esophageal refluxate during a non-swallowing period. Our ED measurements (Table 1) were compatible with the 5 mL water intake in Mittal's studies^[19].

Mittal *et al* observed many reflux episodes determined by pH probe but not concomitant sonographic reflux by HFEUS. Similar observations were also made using impedance techniques. They concluded the gas-dominant or mixed reflux episodes could be the contributory factor^[19]. In our study, we did not perform TCEUS and the 24-h pH monitoring concurrently. This finding points out that US in GERD has some shortcomings. In contrast to this finding is the short reflux period which was determined by color Doppler (CD) US but not by pH monitoring^[45].

There was a positive significant correlation between ED and ELD ($r = 0.889$, $P = 0.000$) and ED and WT ($r = 0.499$, $P = 0.001$) (Pearson correlation analysis). The positive relation could be explained by presence of refluxate in the esophageal lumen. Although there was positive correlation between ED and WT, no significant difference was found between groups. This could be explained by the small patient groups.

Esophageal motor disorders can cause abnormal fluid or viscous bolus transit^[37,46]. Esophageal dysmotility can cause reflux esophagitis and reflux can cause esophageal dysmotility^[12,37]. Patients with normal esophageal motility, diffuse esophageal spasm (DES) and achalasia had 35%, 67%, and 100% abnormal fluid or viscous bolus transit, respectively^[37]. The possible manometric disorders that could be responsible for the PR were also evaluated in our study. We did not observe any patients with achalasia, DES or nutcracker esophagus. The manometric abnormality prevalence in patients with cervical refluxate during TCEUS was 36.84% (14/38 patients) [5 (13.16%) IEM, 7 (18.4%) hypotensive LES, 2 (5.4%) hypertensive LES] (Table 2).

There is a gradual increase in muscle thickness, thickening of the muscularis propria and increase in CSA from the proximal to distal esophagus in primary spastic esophageal motor disorders like achalasia, DES, nutcracker esophagus, hypertensive LES, and atypical LES relaxation, and in non-spastic esophageal motor disorders like hypotensive LES, IEM, and incomplete LES relaxation^[12,15,37,41]. WT according to disorder was achalasia > DES > nutcracker esophagus^[19,41,43]. The normal basal esophageal WTs at 2 cm and 10 cm above the GEJ were measured as 1.45 ± 0.31 mm and 1.24 ± 0.23 mm, respectively, by Dogan *et al*. The corresponding abnormal values were 2.08 mm and 1.71 mm^[43].

We found no difference in anterior CE WT in patient subgroups divided according to esophageal manometry. In our study, the esophageal measurements were taken at the thyroid gland level, while corresponding values in the literature were measured at the GEJ or 2 or 10 cm above

the GEJ. IEM is characterized by low amplitude esophageal contractions, which could cause ineffective acid clearance and aid the reflux pathogenesis^[37]. In our study, 7 of 14 (50%) patients with abnormal esophageal manometry had acid reflux (3 had DR and 4 had both PR and DR) in 24-h pH monitoring. Two of 5 patients who had IEM disorder, 5 of 7 patients with hypotensive LES and 0 of 2 patients with hypertensive LES had reflux (Table 5). The PR rate was higher than DR rate (70%, 30%) in group A. None of the patients with isolated PR had manometric impairment. Twenty-seven patients (71.1%) had at least one pathology in pH monitoring (acid reflux) and/or manometry. We did not observe any pathology which could cause impairment in esophageal transit in 11 of 38 patients (28.9%). Since we did not investigate non-acid reflux, the probable reflux patterns in these 11 patients are unknown.

We aimed to determine the possible cut-off values for TCEUS findings in patients with refluxate as a predictor of GER or pathologic manometry finding. ELD but not WT and ED showed cut-off values (AUC, $P < 0.05$). ELD (with refluxate) of 4.95 mm had 71% sensitivity and 77% specificity in the estimation of total PR patients (Table 6).

The fact that 24-h pH monitoring and manometry were not performed in subjects without refluxate during TCEUS is a limitation of this study.

Esophageal refluxed material can be recognized in ultrasonographic images. TCEUS can not substitute for 24-h pH monitoring or esophageal manometry, but it can serve as a complementary technique by aiding in the estimation of proximal reflux, GER and motility disorders which could cause impairment in bolus transit.

To our knowledge, there is no study in the available literature showing refluxate presence in the cervical esophageal lumen and measuring the TCEUS parameters at the thyroid gland level transcutaneously while correlating pH monitoring and esophageal manometry findings in adults.

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COMMENTS

Background

Esophageal refluxed material can be recognized in ultrasonographic images. The content of refluxate can be isolated liquid (acid or non-acid nature), isolated gas, or gas/liquid mixture. The content of liquid refluxate, whether acidic or not, did not affect the sonographic appearance. Liquid can be present in the esophageal lumen in gastroesophageal reflux (GER) and esophageal motility disorders. Esophageal ultrasonography is currently being used to evaluate the gastroesophageal junction by transabdominal route, especially in newborns and children, and endosonographic studies have been used especially for motility disorders. The relation between the presence of refluxate in the cervical esophageal lumen and the esophageal pH-metry/manometry findings using transcervical esophageal ultrasonography (TCEUS) has not been studied previously in children and adults.

Research frontiers

We evaluated the possible pathologies in 24-h pH monitoring and esophageal manometry in patients with refluxate in the lumen of the cervical esophagus during TCEUS. In 27/38 (71.1%) patients with refluxate in TCEUS, there was at least one pathologic acid reflux and/or pathologic manometry finding. 24 h pH-metry

and esophageal manometry subgroups were compared statistically according to TCEUS findings [anterior wall thickness (WT) of the esophagus, esophageal diameter (ED), esophageal luminal diameter (ELD)].

Innovations and breakthroughs

Our study is distinct from other studies evaluating the GER and esophageal manometry pathologies with ultrasonographic methods due to our usage of TCEUS. We performed esophageal manometry and 24-h pH monitoring in patients with refluxate in the esophageal lumen. The shortcomings of the study were that 1) we did not perform manometry or pH monitoring in patients without refluxate and 2) we did not evaluate the non-acid reflux.

Applications

Different patient groups and volunteers without refluxate can be evaluated for different study designs.

Terminology

The presence of refluxate in esophageal lumen in TCEUS: The luminal anechoic fluid and/or linear bright stratifying small lines indicating gas in refluxate with the patients not swallowing; The presence of comet-tail artifact: During swallowing, the presence of saliva mixed with air and downward movement of refluxate generated a strong echogenic appearance; TCEUS parameters: Wall thickness (WT) of the esophagus: Distance between adventitia and the mucosa, with 5-7 esophageal wall layers; Esophageal diameter (ED): Distance between the adventitia (outer to outer); Esophageal luminal diameter (ELD) with or without refluxate: Distance between the mucosa (inner to inner).

Peer review

The authors studied transcutaneous cervical esophageal ultrasound (TCEUS) as a possible diagnostic procedure in gastroesophageal reflux. TCEUS has been forgotten in the diagnosis of esophageal diseases, however, it is a non-invasive, available and high sensitive technique. This manuscript is in principle an interesting topic for the readers of World Journal of Gastroenterology.

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Early nasogastric enteral nutrition for severe acute pancreatitis: A systematic review

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Before recommendation to clinical practice, further high qualified, large scale, randomized controlled trials are needed.

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Key words: Enteral nutrition; Nasogastric tube; Severe acute pancreatitis; Systematic review; Meta-analysis

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Abstract

AIM: To evaluate the effectiveness and safety of early nasogastric enteral nutrition (NGEN) for patients with severe acute pancreatitis (SAP).

METHODS: We searched Cochrane Central Register of Controlled Trials (Issue 2, 2006), Pub-Medline (1966-2006), and references from relevant articles. We included randomized controlled trials (RCTs) only, which reported the mortality of SAP patients at least. Two reviewers assessed the quality of each trial and collected data independently. The Cochrane Collaboration's RevMan 4.2.9 software was used for statistical analysis.

RESULTS: Three RCTs were included, involving 131 patients. The baselines of each trial were comparable. Meta-analysis showed no significant differences in mortality rate of SAP patients between nasogastric and conventional routes (RR = 0.76, 95% CI = 0.37 and 1.55, $P = 0.45$), and in other outcomes, including time of hospital stay (weighted mean difference = -5.87, 95% CI = -20.58 and 8.84, $P = 0.43$), complication rate of infection (RR = 1.41, 95% CI = 0.62 and 3.23, $P = 0.41$) or multiple organ deficiency syndrome (RR = 0.97, 95% CI = 0.27 and 3.47, $P = 0.97$), rate of admission to ICU (RR = 1.00, 95% CI = 0.48 and 2.09, $P = 0.99$) or conversion to surgery (RR = 0.66, 95% CI = 0.12 and 3.69, $P = 0.64$), as well as recurrence of re-feeding pain and adverse events associated with nutrition.

CONCLUSION: Early NGEN is a breakthrough in the management of SAP. Based on current studies, early NGEN appears effective and safe. Since the available evidence is poor in quantity, it is hard to make an accurate evaluation of the role of early NGEN in SAP.

INTRODUCTION

Acute pancreatitis (AP) is one of the most common pancreatic diseases, with an incidence rate of 4.9-80/100 000 per year, and there was a trend to increase during the past two decades^[1]. Around 80% of patients with mild acute pancreatitis (MAP) are treatable with a short period of bowel rest, simple intravenous hydration, and analgesia^[1,2]. However, severe acute pancreatitis (SAP) is complicated by systemic inflammatory response syndrome (SIRS), leading to hypermetabolism and high protein catabolism^[3]. Consequently, nutritional stores are rapidly consumed and about 30% of patients with SAP undergo malnutrition^[3,4]. Acute malnutrition is expected to increase morbidity and mortality due to impaired immune function, increased risk of sepsis, poor wound healing, and multiple organ failure^[3]. Thus, current therapy for AP has shifted to intensive medical care, nutrition support, infection control and medicine administration, while early invasive intervention as surgery has been reserved for defined clinical indication^[5,6]. Nutritional management for AP is an important issue and regarded as an indispensable approach.

Oral or enteral feeding may be harmful in AP and is thought to stimulate exocrine pancreatic secretion and consequently autodigestive process^[4]. Up to the mid 1990s, total parenteral nutrition (TPN) and gastrointestinal tract rest have been comprehensively recommended in the acute phase of pancreatitis, which make pancreas at rest to reduce pancreatic exocrine secretion and also meet nutritional need^[6,7]. Intestinal mucosa atrophies during fasting as TPN phase, which would induce bacteria translocation in gastrointestinal tract and cause pancreatic necrotic tissue infection^[8]. Animal experiments and several human studies

have shown that enteral nutrition (EN) is safe and can preserve the integrity of intestinal mucosa to decrease the incidence of infectious complications and other severe complications, such as multiple organ deficiency syndrome (MODS)^[8-10]. Furthermore, EN does not stimulate pancreatic exocrine secretion, if the feeding tube is positioned in the jejunum by nasojejunal or jejunostomy routes^[8,11]. Therefore, TPN or jejunal EN is considered the mainstream of nutritional support for AP.

Recently, some researchers have considered the feasibility of EN through nasogastric (NG) tube to improve the nutrition status of patients with AP in the early phase^[7,12]. However, this breakthrough is potentially opposing to the requirement of pancreatic rest in the acute inflammation phase. The present study was to confirm whether nasogastric EN is safe and effective for patients with AP.

MATERIALS AND METHODS

Search strategy and update

We searched electronic databases of Cochrane Central Register of Controlled Trials (Issue 2, 2006), Pub-Medline (1966-2006), and references from relevant articles. The search strategy used was "Enteral Nutrition" (MeSH) AND ["Pancreatitis" (MeSH) OR "Pancreatitis, Acute Necrotizing" (MeSH)], with limitations to Randomized Controlled Trial, Humans. There was no limitation of publication language. This systematic review will be updated if more randomized controlled trials (RCTs) can be found through the monthly automatic search procedure from National Center for Biotechnology Information (NCBI) at the National Library of Medicine.

Inclusion and exclusion criteria

Only randomized controlled trials were eligible. Eligible patients include those who diagnosed as acute pancreatitis by Atlanta classification, and those with severe diseases assessed by APACHE II criteria, and/or Ranson criteria, and/or Balthazar computer tomography criteria. Any etiology was eligible, and there was no limitation of age, race, and sex distribution. Comparator intervention was considered an early enteral nutritional route through nasogastric tube (NGEN), while control intervention was considered one of the conventional pancreatic-rest nutritional support routes, such as total parenteral nutrition (TPN) or enteral nutrition by nasojejunal tube (NJEN) or jejunostomy tube (JSEN). Additionally, other combined treatments included gastrointestinal decompression, prophylactic antibiotics, fluid management, artificial ventilation or renal replacement therapy for MODS, endoscopic retrograde cholangiopancreatography with endoscopic sphincterotomy for selected biliary patients, and surgery for indicated patients. The primary outcome measure of effectiveness was overall mortality, the secondary outcome measures of effectiveness was hospital stay, complications and their management, while the outcome measures of safety included re-feeding pain recurrence and adverse events related to nasogastric enteral nutrition.

Quality assessment and data collection

To evaluate the methodological quality of included studies,

two reviewers (Jiang K and Chen XZ) assessed the quality of methods used in studies independently. According to the Cochrane Handbook for Systematic Review 4.2.6^[13], we assessed the quality of RCTs using random allocation concealment as adequate (A), unclear (B), inadequate (C), or not used (D); blinding process as double blind (A), single blind (B), unclear (C) or not used (D); intention-to-treat (ITT) as yes (A), unclear (B), or not used (C), or loss, withdrawal, dropout, cross-intervention not reported (D). The criteria proposed by Jadad *et al*^[14] were also used to evaluate the quality of trials.

Data were collected by the two reviewers independently, including study sample (number of each arm), interventions (nutrition management, approach and regimens) and outcomes (overall mortality, time of hospital stay, complications of systematic or local infection, or MODS defined as failure in no less than 2 organs, re-feeding pain defined as pain requiring discontinuation of feeding, elevated serum amylase levels at least two-fold higher than normal^[7], and adverse events related to nutrition), as well as the publication year and country of studies, and the number of withdrawals and dropouts and the reasons.

Any disagreement in quality assessment and data collection was discussed and solved by a third reviewer as the referee.

Statistical analysis

Meta-analysis was performed with the Cochrane Collaboration's RevMan 4.2.9 software. All *P*-values were two-sided and *P* < 0.05 was considered statistically significant. For dichotomous variables, the risk ratio (RR) was calculated with 95% confidence intervals (CI); for continuous variables, weighted mean difference (WMD) was calculated with 95% confidence intervals. Heterogeneity was determined by chi-square test. If any heterogeneity existed, the following techniques were employed to explain it: (1) random effect model, (2) subgroup analysis including different control arms as TPN or EN through nasojejunal or jejunostomy tube, (3) sensitivity analysis performed by excluding the trials which potentially biased the results. Any patients randomly assigned in each trial, but not analyzed in the present meta-analysis, were calculated.

RESULTS

Three RCTs^[15-17] were eligible for the inclusion criteria, and 131 participants were included. Of them, 67 were randomly assigned to NGEN group and 64 to conventional nutritional route group. Conventional routes included TPN and NJEN, but not JSEN. The mean number of samples for each trial was 43.7 (31-50). The baselines of each trial were comparable. The details of included trials are listed in Table 1, and the results of quality assessment in Table 2.

Effectiveness

Overall mortality: Three included RCTs reported the mortality. The overall mortality rate of early NGEN group and conventional route group was 14.9% (10/67) and 18.8% (12/64) respectively, which is consistent with the reported rate^[18]. No heterogeneity was detected (*P* =

Table 1 Details of included RCTs

Reference	Yr	Country	Number of intervention/control	Inclusion/exclusion criteria of participants	Drop-out/withdrawal
[15]	2005	Scotland	27 (NGEN)/23 (NJEN)	Inclusion: both a clinical and biochemical presentation of AP (abdominal pain and serum amylase at least 3 times the upper limit of the reference range), and objective evidence of disease severity (Glasgow prognostic score ≥ 3 , or APACHE II ≥ 6 , or CRP > 150 mg/L). Exclusion: patients under 18 yr of age and pregnant females. Inclusion: severity was defined according to the Atlanta criteria (ie, presence of organ failure and APACHE score of ≥ 8 or CT severity score ≥ 7).	One excluded in NJEN group for misdiagnosed and 2 in NJEN group received NGEN for failure of NJ tube placement.
[16]	2006	India	16 (NGEN)/15 (NJEN)	Exclusion: if there was a delay of more than 4 wk between the onset of symptoms and presentation to the hospital, if they were already taking oral feeding at presentation, if there was acute exacerbation of chronic pancreatitis, or if they were in shock (ie, systolic blood pressure < 90 mmHg at the time of randomization).	One excluded in NJEN group for failure of NJ tube placement.
[17]	2006	Sweden	24 (NGEN)/26 (TPN)	Inclusion: abdominal pain, amylase ≥ 3 times upper limit of normal, onset of abdominal pain within 48 h, APACHE II score ≥ 8 and/or CRP ≥ 150 mg/L and/or peripancreatic liquid shown on CT. Exclusion: AP due to surgery, inflammatory bowel disease, stoma, short bowel, chronic pancreatitis with exacerbation, and patients under 18 yr of age.	One patient from each group was considered as protocol violators due to surgery performed after study inclusion on d 2 in 1 case, and a dislocated tube not accepted to be replaced in the other one

Abbreviations: RCT: Randomized controlled trial; NGEN, nasogastric enteral nutrition; NJEN, nasojejunal enteral nutrition; TPN, total parenteral nutrition; AP, acute pancreatitis; SAP, severe acute pancreatitis; APACHE, acute physiology and chronic health evaluation; CTSL, computer tomography severity index; CRP, C-reactive protein.

Table 2 Quality of included RCTs¹

Study	Randomization method	Allocation concealment	Blind method	ITT method	Jadad score
Eatock <i>et al</i> ^[15] , 2005	Computer-generated random numbers	Adequate	Not used	Yes	3
Kumar <i>et al</i> ^[16] , 2006	Computer-generated random numbers	Unclear	Not used	Unclear	3
Eckerswall <i>et al</i> ^[17] , 2006	Not specified	Adequate	Not used	Yes	2

¹According to the Cochrane Handbook for Systematic Review 4.2.6^[13].

0.61). Meta-analysis showed no significant difference in overall mortality between early NGEN and conventional route groups (RR = 0.76, 95% CI = 0.37 and 1.55, $P = 0.45$) (Figure 1). Sub-group analysis showed no significant difference in overall mortality between NGEN and NJEN groups^[15,16] (RR = 0.67, 95% CI = 0.32 and 1.40, $P = 0.28$), as well as between NGEN and TPN groups^[17] (RR = 3.24, 95% CI = 0.14 and 75.91, $P = 0.47$).

Hospital stay: All the included RCTs reported the mean time of total hospital stay, but 2 trials did not mentioned the standard difference (SD)^[15,17], and were excluded from the meta-analysis. There was no significant difference in the mean time of total hospital stay between NGEN and NJEN groups (WMD = -5.87, 95% CI = -20.58 and 8.84, $P = 0.43$) (Figure 2). The weighted mean time of hospital stay of patients in early NGEN and conventional route groups was 15.4 d and 15.3 d, respectively.

Complications and management: Two RCTs reported

the detailed data on infective complications or MODS^[16,17]. The results of meta-analysis showed no significant difference in infective complications, such as sepsis and infected pancreatic necrosis (RR = 1.41, 95% CI = 0.62 and 3.23, $P = 0.41$) (Figure 3), as well as in heterogeneity ($P = 0.15$). There was no significant difference in MODS (RR = 0.97, 95% CI = 0.27 and 3.47, $P = 0.97$) (Figure 3), as well as in heterogeneity ($P = 0.93$). The rate for admission to intensive care unit was reported in 2 RCTs^[15,17], which was 21.6% (11/51) and 20.4% (10/49) in early NGEN and conventional groups respectively (RR = 1.00, 95% CI = 0.48 and 2.09, $P = 0.99$) (Figure 4). Two trials^[16,17] reported that the rate for surgery was 5.0% (2/40) and 7.3% (3/41) in early NGEN and conventional route groups, respectively with no significant difference between the two groups (RR = 0.66, 95% CI = 0.12 and 3.69, $P = 0.64$) (Figure 4).

Safety

Re-feeding pain recurrence: Two RCTs^[16,17] reported the cases needing to withdraw oral feeding due to recurrent re-feeding pain. Meta-analysis showed no significant difference in the recurrent re-feeding pain (RR = 0.94, 95% CI = 0.06 and 13.68, $P = 0.96$) (Figure 5). The pain recurrence rate was 2.5% (1/40) and 2.4% (1/41) in NGEN and conventional route groups, respectively.

Adverse events associated with nutrition: Meta-analysis showed that the main adverse events associated with nutrition support were diarrhea (RR = 1.59, 95% CI = 0.51 and 4.91, $P = 0.42$), tube displacement (RR = 0.45, 95% CI = 0.09 and 2.30, $P = 0.33$), and withdrawal of feeding due to severe complications (RR = 0.30, 95% CI = 0.03 and 2.76, $P = 0.29$) (Figure 6). Other nutrition-associated complications were found in patients of the NJEN group

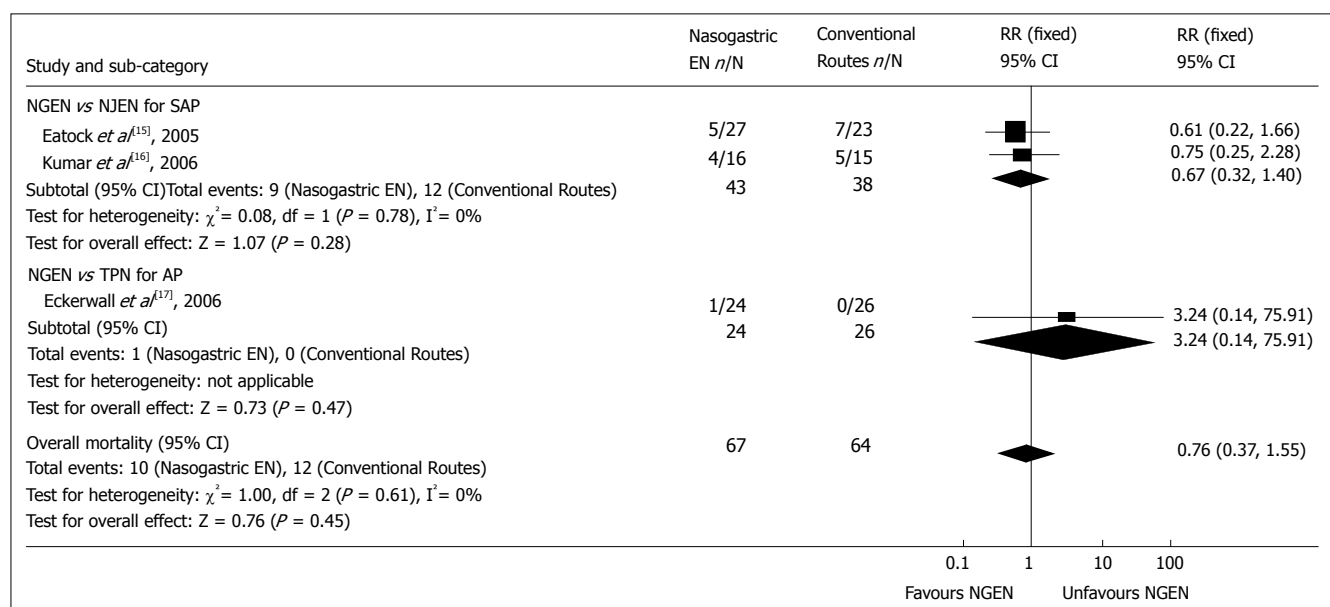


Figure 1 Comparison of overall mortality between nasogastric enteral nutrition and conventional nutritional routes. Abbreviations: AP, acute pancreatitis; SAP, severe acute pancreatitis; EN, enteral nutrition; TPN, total parenteral nutrition; NGEN, nasogastric enteral nutrition; NJEN, nasojejunal enteral nutrition; RR, risk ratio; CI, confidence interval.

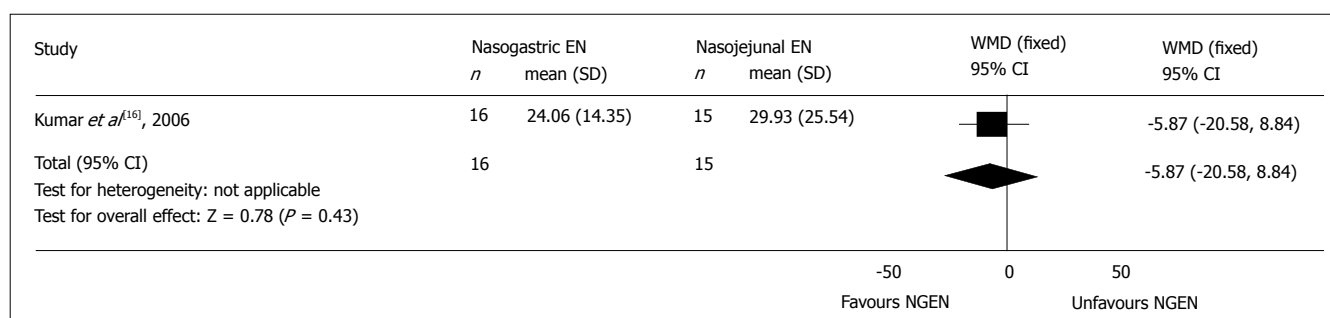


Figure 2 Comparison of total hospital stay (days) between nasogastric and nasojejunal enteral nutrition. WMD, weighted mean difference; SD, standard deviation.

who suffered from cardiorespiratory arrest at the moment of endoscopic tube placement which were successfully resuscitated^[14] and in patients of the NJEN group with sweating and palpitation^[15].

DISCUSSION

The present systematic review was intended to find the feasibility and safety of early nasogastric enteral nutrition in the management of severe acute pancreatitis. We selected 3 randomized controlled trials involving comparison between early NGEN and NJEN or TPN. The total number of samples was limited. All the trials did not perform a blinding process due to the nature of interventions. Two trials reported detailed randomization assignment methods^[15,16], and two were put into practice based on allocation concealment and intention-to-treat method^[15,17]. Sensitivity analysis showed negative results. Potential biases of the present systematic review included selection bias due to the severity criteria of one trial (Eatock 2005)^[15] set at APACHE II > 6, on an international symposium^[19]. Moreover, another concern is the high

mortality (24.5%) in patients with the severity of the illness^[20].

Treatment of SAP has been evolving from routine early aggressive surgical management towards conservative care for patients without evidence of pancreatic infection^[21,22]. However, SAP remains a disease with a poor prognosis^[23]. Artificial nutrition can prevent and provide a long term nutritional support for SAP^[24]. Enteral nutrition is preferred to parenteral nutrition for improving patient outcomes^[25,26], and has largely replaced the parenteral route^[27]. However, early nasogastric enteral nutrition is regarded as a potentially pancreatic-unrest nutritional support route, which is harmful to the early acute phase of AP^[15]. Eatock *et al*^[12] first introduced the early nasogastric feeding into nutritional management of SAP, and then Pandey *et al*^[7] applied oral re-feeding in patients with SAP, suggesting that the nasogastric feeding is feasible in up to 80% cases^[15].

The present meta-analysis showed that early NGEN would be as effective and safe as early NJEN or TPN in SAP patients, without increase in mortality. Pancreatic infection, sepsis, and MODS are the complications

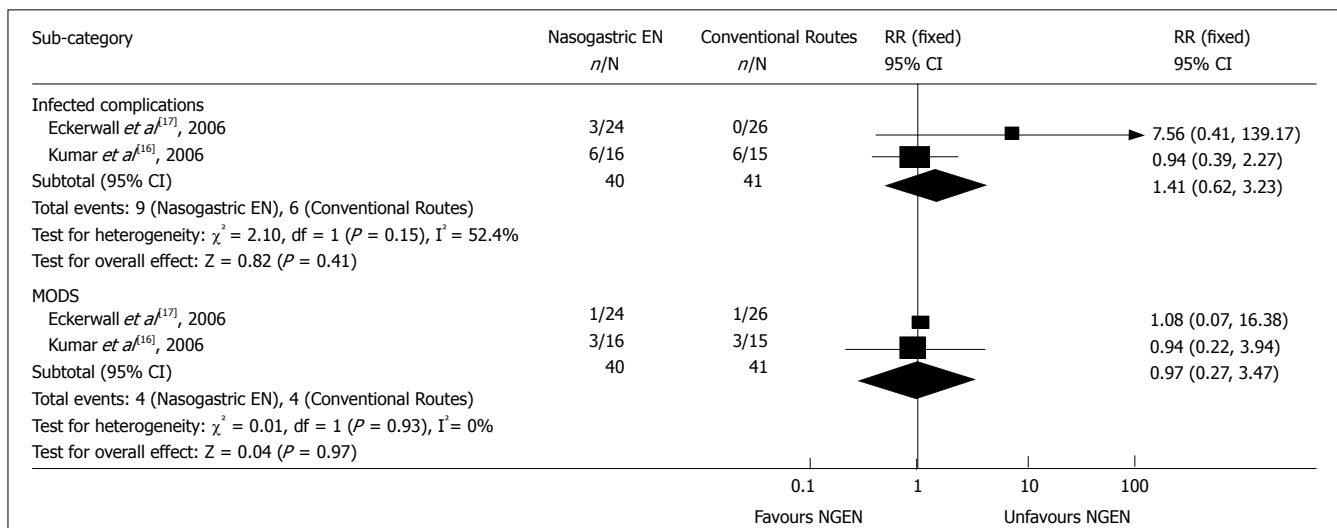


Figure 3 Comparison of complications of acute pancreatitis between nasogastric enteral nutrition and conventional nutritional routes. MODS, multiple organ deficiency syndrome.

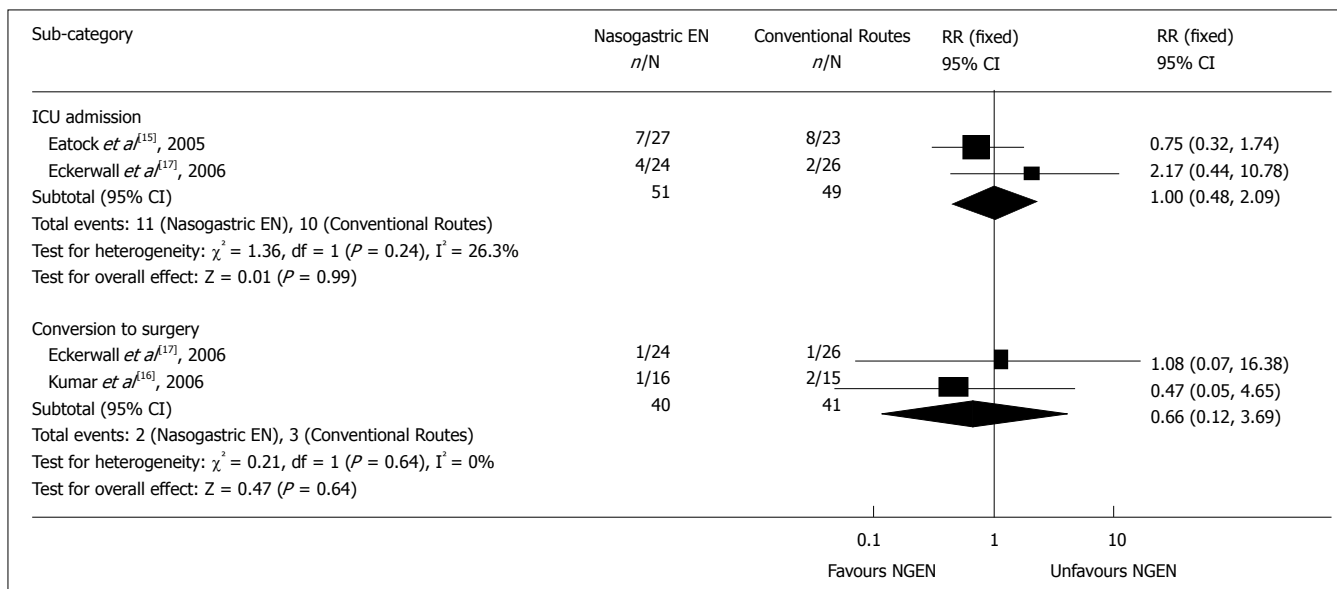


Figure 4 Comparison of management of complications between nasogastric enteral nutrition and conventional nutritional routes. ICU, intensive care unit.

of SAP^[28]. Duration of organ failure during the first week of predicted severe acute pancreatitis is strongly associated with the risk of death or local complications^[29]. Besides, NGEN did not increase severe complications and prolong hospital stay. Bacterial infection is the common complication of acute pancreatitis, and bacterial translocation from the gut is probably the first step in the pathogenesis of these infections^[30]. NGEN could preserve the intestinal permeability including duodenum, proved by the assessment of excretion of polyethylene glycol and antiendotoxin core antibody IgM levels^[17], which would be the best barrier for prevention of certain complications. Interleukin-6 serum levels are elevated very early in patients with necrosis infection^[31], and C-reactive protein (CRP) is considered a valuable independent predictor of mortality^[32]. IL-6 and CRP levels play a similar role in the control of systematic inflammatory response of early

NGEN and TPN groups at each time point^[17]. Moreover, biochemical nutritional parameters, such as serum albumin and prealbumin concentration in early NGEN and NJEN groups, are both well preserved without any significant difference^[16].

After three-month follow-up, about 92% patients in the early NGEN group have no symptoms related to SAP, compared with 82% patients in the TPN group, but all pseudocysts occur in the early NGEN group^[17], suggesting that early nasogastric enteral nutrition is potentially feasible in SAP patients. The clinical outcomes are quite similar to conventional routes, such as early nasojejunal or total parenteral nutrition. However, the incidence of late complications such as pseudocysts is likely higher in early nasogastric enteral nutrition group.

Enteral nutrition has no benefit to the mild acute pancreatitis subset^[24]. However, oral re-feeding is feasible,

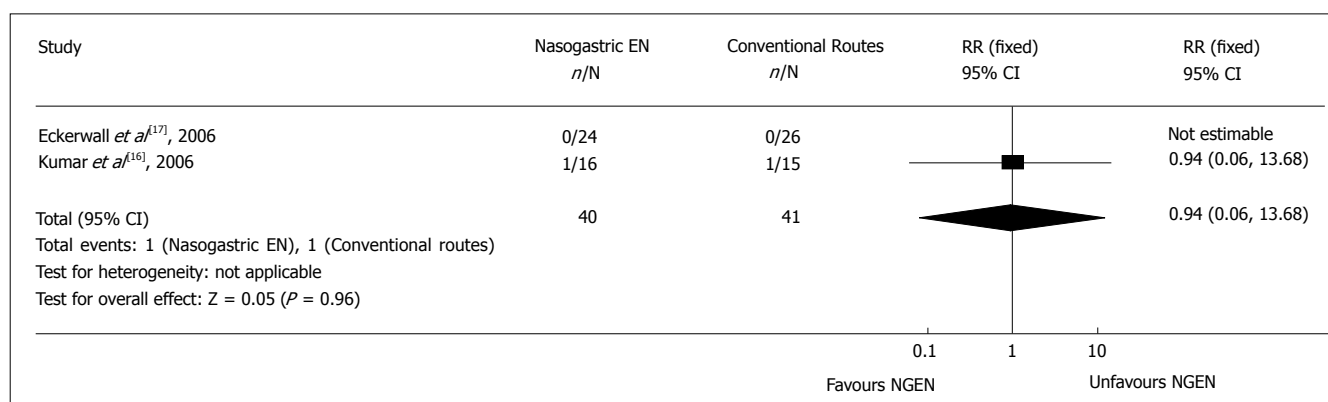


Figure 5 Comparison of refeeding pain recurrence between nasogastric enteral nutrition and conventional nutritional routes.

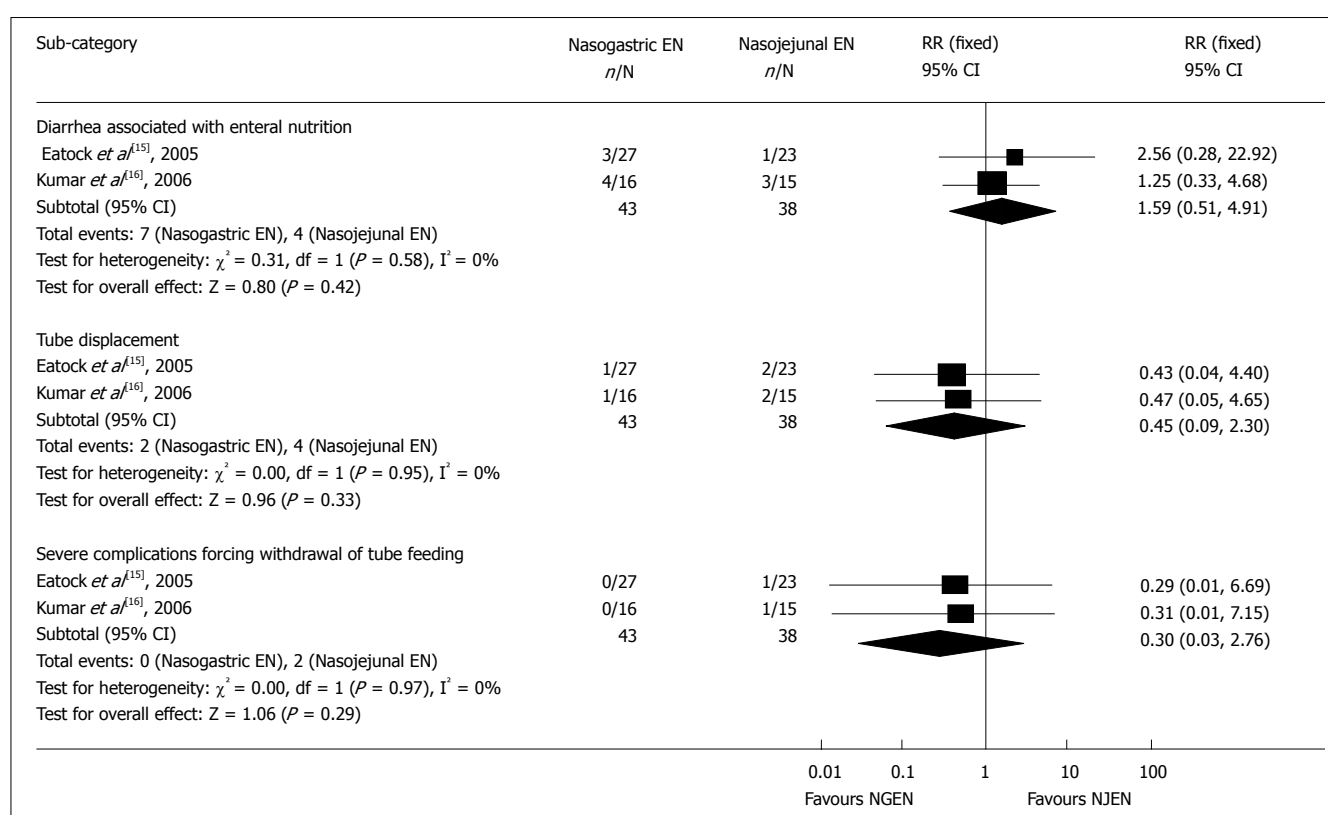


Figure 6 Comparison of nutrition associated adverse events between nasogastric and nasojejunal enteral nutrition (NJEN).

but the proper time of commence needs to be further investigated. Oral re-feeding given 1 wk after onset of the disease is safe in selected MAP patients^[4], while there is no evidence that early oral re-feeding within 1 wk is a feasible management for SAP.

In conclusion, early nasogastric enteral nutritional support route is potentially feasible and safe, which does not increase the rate of mortality, complication and re-feeding pain recurrence in patients with severe acute pancreatitis, and prolong the hospital stay. No major innovations in the treatment of SAP have been introduced in recent years^[23]. It is a breakthrough in enteral nutrition management of severe acute pancreatitis, with a bright future because it is more convenient and cheaper. However, before it is applied in clinical practice, further investigation is necessary to validate its effectiveness, safety

and cost-effectiveness.

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COMMENTS

Background

Nutritional management of severe acute pancreatitis is an important issue

and regarded as an indispensable approach. Total parenteral nutrition and gastrointestinal tract rest have been recommended in the management of severe acute pancreatitis (SAP) since the mid 1990s. Enteral nutrition through jejunal route has been accepted as a safe and effective approach to the management of SAP by preserving the integrity of intestinal mucosa and preventing bacterial translocation. Moreover, some studies have attempted to find the feasibility and safety of early enteral nutrition through nasogastric route.

Research frontiers

Gastrointestinal and pancreatic rest has been regarded as an important factor for management of severe acute pancreatitis. Nevertheless, nasogastric enteral nutrition disobeys this discipline. Whether nasogastric route is able to gain the similar results needs to be further investigated. If possible, serological, radiological or histological appraisal should be considered for the effectiveness and safety of early nasogastric enteral nutrition in the treatment of severe acute pancreatitis.

Innovations and breakthroughs

Early nasogastric enteral nutrition is a breakthrough in the management of severe acute pancreatitis. Previously, it was forbidden for the sake of potentially opposing to the requirement of pancreatic rest in the acute inflammation phase. However, the present systematic review of three randomized controlled trials showed that nasogastric route does not worsen the clinical outcomes compared with the conventional total parenteral or jejunal enteral routes.

Applications

Nasogastric route is much more convenient in clinical practice. Moreover, it is obviously cheaper than nasojejunal tube placement. Based on the present results, nasogastric enteral nutrition can be applied in the early management of severe acute pancreatitis. However, before its application in clinical practice, further investigation is necessary to validate its effectiveness and safety.

Terminology

Severe acute pancreatitis (SAP) is usually accompanied with pancreatic necrosis, systematic inflammatory response syndrome, or organ failure. Total parenteral nutrition (TPN) is the way to give nutrient substances intravenously, bypassing the digestive system. Nasogastric enteral nutrition (NGEN) is the way to provide nutrient substances for patients through a tube placed in the nose up to the stomach. Nasojejunal enteral nutrition (NJEN) is the way similar to NGEN, but the tube is placed up to the jejunum.

Peer review

The authors evaluated the effectiveness and safety of early nasogastric enteral nutrition (NGEN) for severe acute pancreatitis (SAP) compared with conventional nutritional routes. Based on the current studies, early NGEN appears effective and safe, but the available evidence is limited.

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Expression of periostin and its clinicopathological relevance in gastric cancer

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Abstract

AIM: To investigate the expression and localization of periostin in gastric cancer and its clinical relevance.

METHODS: Reverse transcriptase polymerase chain reaction was used to measure periostin mRNA expression. Western blotting was carried out to detect periostin protein expression. Immunohistochemistry was performed to localize and quantify the expression of periostin in benign gastric diseases and gastric cancer, and immunostaining results were correlated with gastric cancer pathological stages.

RESULTS: Periostin expression was low at both mRNA and protein levels in normal gastric tissues, but was overexpressed in gastric cancer tissues. Immunohistochemical staining revealed that periostin was overexpressed in primary gastric cancer, as well as in metastatic lymph nodes, but only faint staining was found in benign gastric ulcers. By quantitative analysis of the immunostaining results, periostin expression was increased 2.5-4-fold in gastric cancer, compared to that in benign gastric disease, and there was a trend toward increasing periostin expression with tumor stage.

CONCLUSION: This observation demonstrated that periostin was overexpressed in gastric cancer and lymph node metastasis, which suggests that periostin plays an important role in the progression and metastasis of gastric cancer.

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Key words: Cell growth; Gastric cancer; Metastasis; Oncogene; Periostin

INTRODUCTION

Gastric cancer remains the fourth most common cancer worldwide. Due to its high incidence and malignant behavior, and the lack of major advances in treatment strategy, it is still the second most frequent cause of death from malignant diseases^[1]. A variety of clinicopathological characteristics may affect the prognosis of gastric cancer, and accumulating evidence has revealed that numerous genetic markers influence the biological behavior of gastric cancer. The abnormal expression of certain genes in cancer cells is closely related to various aspects of tumor progression, including tumor growth, invasion and metastasis, and inactivation of tumor suppressor genes, particularly the p53 or adenomatous polyposis coli gene^[2].

Recently, great efforts have been made to determine the gene expression pattern differences between various types of human cancer and their corresponding normal tissues, although the alterations of a certain number of tumor suppressor genes and oncogenes have been shown to be closely associated with the progression of human cancer. Less is known about the functions of a large number of other genes whose expression patterns are also significantly changed during the tumorigenic process. Particularly interesting is the mesenchyme-specific gene family, normally associated with osteoblasts, which are highly expressed in various types of human cancer^[3,4]. One such candidate gene is that for periostin, which is overexpressed in several types of human cancer, such as breast, colon and ovarian cancer^[5-7]. Furthermore, it is supposed that, at the molecular level, periostin functions as a ligand for alpha (V), beta (3), and alpha (V) beta (5) integrins to support adhesion and migration of ovarian epithelial cells^[5], and periostin activates the Akt/PKB signaling pathway through the alpha (V) beta (3) integrins, to increase cellular survival^[7]. While integrin expression level is supposed to be related to the metastasis and relapse of gastric cancer^[8], the expression pattern of periostin in gastric cancer is still unknown. In this paper we investigated periostin expression profile in gastric cancer and its clinical relevance.

MATERIALS AND METHODS

Patients and tissue collection

Samples from 35 gastric cancers, including five metastatic lymph node samples, were obtained from 25 male and 10 female patients (median age, 56 years; range, 45-79 years) who underwent gastric resection at the University Hospital of the Affiliated Zhong-Da Hospital, Southeast University. Five benign gastric ulcer tissue samples were obtained from three male and two female patients, in whom gastrectomy was performed (median age, 55 years; range, 25-67 years). According to the TNM classification of the Union International Contre le Cancer (UICC), the 35 gastric cancers were classified as follows: five tumors were stage I, eight were stage II, 16 were stage III, and six were stage IV. The vast majority of the tumors were located in the distal part of the stomach (21 cases), and one-third were located in the proximal part of the stomach. Immediately upon surgical removal, tissue samples were either snap-frozen in liquid nitrogen and then maintained at 80°C until use (for RNA extraction), or fixed in 5% formalin and embedded in paraffin after 24 h. All studies were approved by the ethics committees of the Affiliated Zhong-Da Hospital, Southeast University.

RNA extraction and reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA from human gastric tissues was isolated either by the Trizol Reagent (TIANGEN, Beijing, China) according to the manufacturer's instructions. Potentially contaminating DNA was removed by RNase-free DNase I treatment. Primers for the genes of interest were: periostin 193 bp (5'-GCCATCACATCGGACA TA-3' and 5'-CTCCCATAATAGACTCAGAACA-3'), and β -actin 621 bp (5'-ACACTGTGCCCATCTACGAG G-3' and 5'-AGGGGCGCGACTCGTCATACT-3'). The RT-PCR conditions were: for the first strand synthesis of cDNA, 48°C for 45 min and 94°C for 2 min to denature the template; and for second strand synthesis and DNA amplification, 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, for a total of 32 cycles, followed by a single step at 68°C for 7 min.

The products were visualized on 1.5% agarose gels stained with ethidium bromide, and signals were quantified by densitometry using the MetaView analyzing system (version 4.5 Universal Imaging, West Chester, PA, USA). Periostin expression was standardized to β -actin expression assessed from the same cDNA in separate PCR reactions, and run on the same gels. The standardized mean of each triplicate PCR was then expressed relative to the levels in β -actin cDNA. The density value was analyzed by Labimage software (Halle, Germany). $P < 0.05$ was considered as statistically significant.

Protein extraction

Tissues were lysed in 0.5 mL lysis buffer containing 50 mmol/L Tris/HCl (pH 7.5), 150 mmol/L NaCl, 2 mmol/L EDTA (pH 8.0) and 1% SDS with proteinase inhibitors (one tablet/10 mL, Roche Molecular Biochemicals, USA). Protein concentration was determined by the BCA Protein Assay (Pierce, Rockford, IL, USA).

Western blot analysis

Fifty micrograms of protein was separated on SDS-polyacrylamide gels and electroblotted onto nitrocellulose membranes. Membranes were then incubated in blocking solution (5% non-fat milk in 20 mmol/L Tris/HCl, 150 mmol/L NaCl, 0.1% Tween-20; TBS-T), followed by incubation with rabbit anti-periostin antibodies (Abcam, Cambridge, MA, USA; 1:500) at 4°C overnight. The membranes were then washed in TBS-T and incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Life Sciences, Amersham, UK) for 1 h at room temperature. Antibody detection was performed with an enhanced chemiluminescence reaction (Amersham Life Sciences).

Immunohistochemistry

Immunohistochemistry was performed using the streptavidin-peroxidase technique and the DAKO EnVision System (Dako Cytomation, Hamburg, Germany). Consecutive paraffin-embedded tissue sections (4- μ m thick) were deparaffinized and rehydrated. Antigen retrieval was performed by pretreatment of the slides in citrate buffer (pH 6.0) in a microwave oven for 12 min. Thereafter, slides were cooled to room temperature in deionized water for 5 min. Endogenous peroxidase activity was quenched by incubating the slides in methanol containing 0.6% hydrogen peroxide, followed by washing in deionized water for 3 min, after which the sections were incubated for 1 h at room temperature with normal goat serum, and subsequently at room temperature for 1 h with the primary anti-periostin antibody (Abcam; 1:100). Next, the sections were rinsed with washing buffer (Tris-buffered saline with 0.1% BSA) and incubated with biotinylated goat anti-rabbit IgG and streptavidin-peroxidase complex, followed by reaction with diaminobenzidine and counterstaining with Mayer's hematoxylin. In addition, to confirm the specificity of the primary antibody and the technique used, tissue sections were incubated in the absence of the primary antibody and with negative control rabbit IgG. Under these conditions, no specific immunostaining was detected. The quantitative analysis of the immunohistochemistry staining of periostin expression in gastric tissues was performed as previously described^[9]. The staining of each tissue was analyzed to determine the mean optical density (MOD), which represents the concentration of the stain per positive pixels.

RESULTS

Periostin mRNA expression is upregulated in gastric cancer tissues

To clarify the expression of periostin in human gastric tissues, we initially examined the expression of periostin mRNA in five gastric cancer tissues (T) versus normal gastric tissues (N) obtained from the same patients. The expression of β -actin was examined as an internal control. As shown in Figure 1, periostin mRNA was expressed in all five gastric cancer tissues at different levels. In contrast, its staining was detected at a very low level in normal gastric tissues, and quantitative analysis showed that there

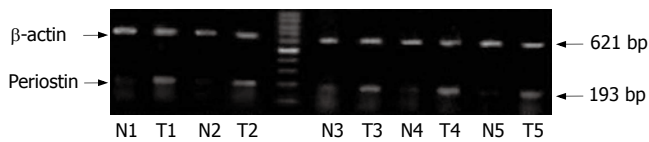


Figure 1 RT-PCR analysis of periostin mRNA expression in human gastric cancer (T) and normal gastric tissues (N). Periostin was differentially expressed in gastric cancer tissues (T) compared with normal gastric tissues (N) from the same patient. The expression of β -actin was used as an internal control.

was a significant difference in periostin expression between gastric cancer and normal tissues ($P < 0.05$, the density ratio between N and T was 0.1912 *vs* 0.8804).

Periostin protein expression in gastric cancer and normal tissues

To determine if the higher level of periostin mRNA expression revealed by RT-PCR analysis was directly linked to increased levels of periostin protein expression, we performed Western blot analysis with protein extracts from matched tissue sections (from which the mRNA samples were extracted). As shown in Figure 2A, periostin was found to be highly expressed in gastric cancer tissues from five different patients (T), whereas only faint periostin expression was found in the normal gastric tissues, with a fivefold overexpression of periostin in cancer tissues compared with normal tissues ($P < 0.05$, the density ratio was 0.8354 *vs* 4.5773) (Figure 2B). Taken together, these data demonstrate that periostin is highly expressed at both mRNA and protein levels in gastric cancer tissues.

Cellular localization of periostin in gastric tissues and its clinical relevance

To investigate the significance of periostin overexpression in gastric cancer further, immunohistochemistry was carried out on five benign gastric ulcers and 35 cases of gastric cancer, which included: five stage I tumors, eight stage II, 16 stage III, and six stage IV tumors, and five metastatic lymph nodes. As shown in Figure 3, the immunostaining indicated that high levels of periostin were present in the areas surrounding cancer cells, as well as in some cancer cells themselves. Periostin was also prominently stained in metastatic lymph nodes. In contrast, periostin expression was low in benign gastric ulcer tissues. Quantitative analysis of the periostin immunohistochemistry results indicated that the average MOD of periostin staining in the stage I-IV tumors was much higher than that in benign gastric ulcers in each group ($P < 0.05$). Furthermore, there was a trend for increasing periostin expression in stage I-IV tumors, although the difference was not statistically significant. In addition, there was no correlation between periostin expression and patient age and gender (data not shown). Taken together, these observations indicated that higher levels of periostin expression were associated with cancer progression and metastasis.

DISCUSSION

Many studies have already demonstrated that interactions

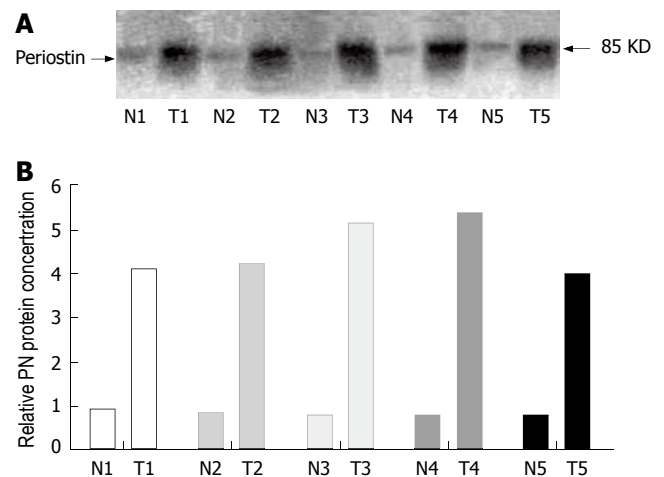


Figure 2 Periostin protein expression in normal and cancer tissue samples. Tissue extracts from normal or gastric cancer tissue samples were subjected to immunoblot analysis with a polyclonal antiperiostin antibody. The results shown in the T and N lanes are for cancer and normal tissues, respectively, from which RT-PCR was performed (A). Over fivefold more periostin was detected in gastric cancer tissues than in normal tissues from the same patient (B).

between integrins of the tumor cell surface and adhesion molecules in the extracellular matrix (ECM) microenvironment may play a critical role in tumor cell migration, survival and growth^[10-12]. The ECM-integrin interactions may also trigger intracellular signaling and activation of certain genes that leads to tumor cell proliferation during metastatic growth^[13], and the formation of blood vessels within the tumor mass^[14]. It is believed that integrins are also important in the attachment and metastasis of gastric cancer^[8]. As a mesenchymal gene, periostin was formerly called osteoblast-specific factor-2, and was originally identified as an 811-amino acid protein secreted by osteoblasts^[15]. It has structural homology with insect fasciclin I and can bind heparin and support adhesion of osteoblasts^[16], which leads to the hypothesis that it functions to recruit and attach osteoblasts to the periosteum.

Periostin is upregulated in colorectal and breast cancer, and their liver metastases, which suggests that it plays a role in promoting growth in these tumors; furthermore, anti-periostin antibodies activate apoptosis and potentiate the effects of 5-fluorouracil chemotherapy in colorectal cancer^[17,18]. Sasaki has reported^[6] that periostin serum level is elevated in breast cancer with bone metastases, and has suggested it as a tumor marker in breast cancer. Since periostin functions as a ligand for integrins, and promotes ovarian cancer cell migration and adhesion^[7], it is reasonable to investigate its role in gastric cancer.

In the present study, we found that periostin was expressed in normal gastric and gastric cancer tissues, while its expression was markedly elevated in cancer tissues. The basal mRNA and protein level of periostin in normal and benign gastric tissues suggest that it plays a role in the normal physiology of the gastrointestinal epithelium, as it was also found that periostin was expressed in normal colon tissue (data not shown). The dramatic increase in periostin in gastric cancer suggests its role in cancer progression. We found that periostin level

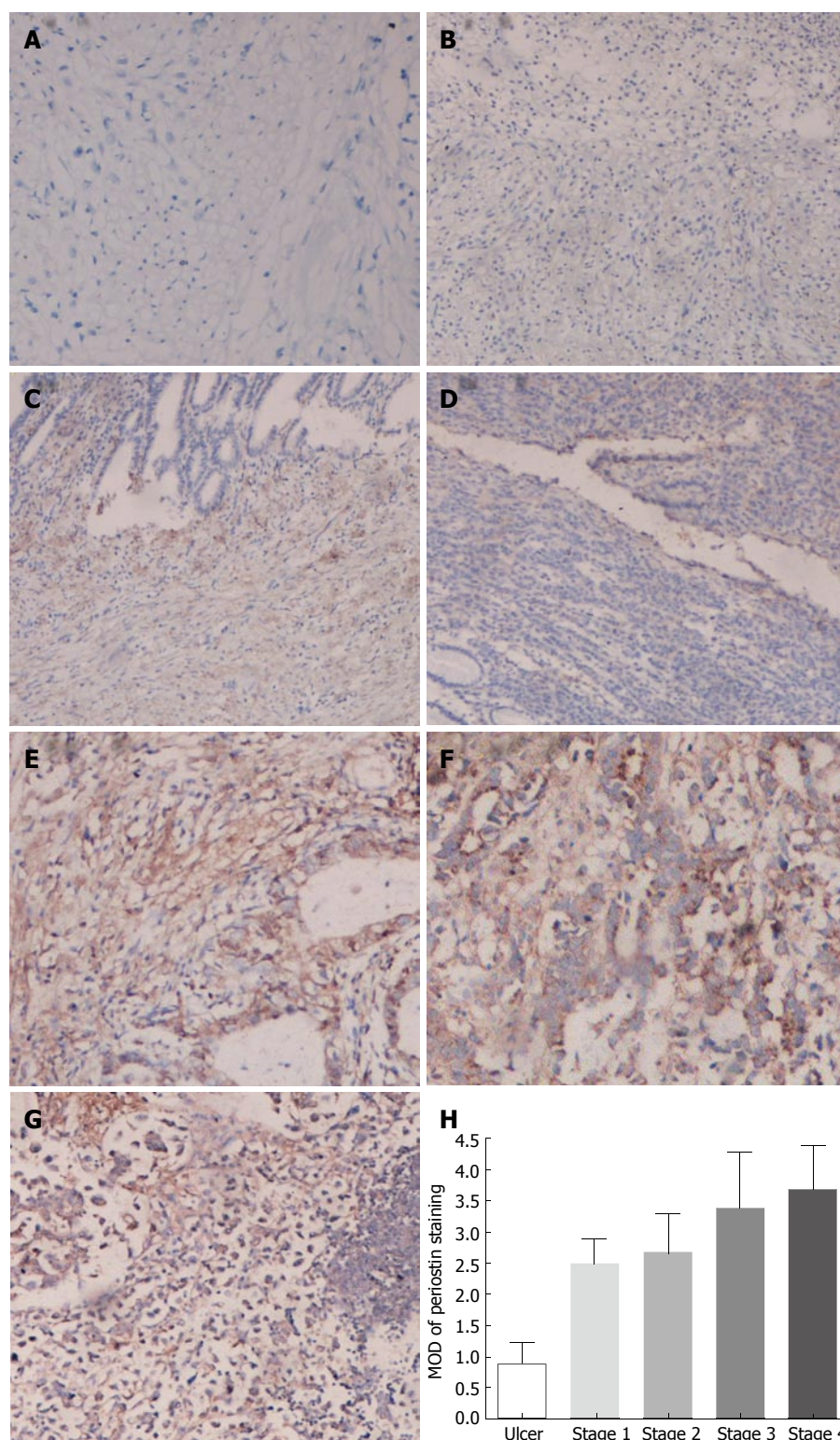


Figure 3 Immunohistochemical analysis of periostin expression in normal gastric tissues, benign gastric ulcers, gastric cancer tissues, as well as lymphoid metastasis from gastric cancer. The tissue sections were immunostained with a polyclonal antibody. The positive staining for periostin protein is shown with a brown color. All sections were counterstained with hematoxylin showing a blue color. (A) Negative control; (B) benign gastric ulcer; (C) stage I gastric cancer; (D) stage II gastric cancer; (E) stage III gastric cancer; (F) stage IV gastric cancer; (G) lymph node metastasis. The average MOD of periostin staining from stage I-IV gastric cancer was significantly higher than that from normal gastric tissues in each group (H) ($P < 0.05$).

was significantly increased in cancer tissues compared with gastric ulcers, and periostin level also increased with tumor-stage progression. As observed in other cancers, increased expression of periostin was associated with advanced stage and cell proliferation, adhesion and migration^[5,19-27], and these results demonstrate that periostin may play a role in the progression of gastric cancer.

However, it has been reported that decreased expression of periostin is associated with progression of bladder cancer in humans, and expression of periostin

mRNA is markedly reduced in a variety of human cancer cell lines^[28,29]. These results differed from our findings that showed that periostin was upregulated in gastric cancer tissues at both the mRNA and protein level. We speculate that there are several explanations for the present findings. First, the expression profile of cancer cell lines may not reflect the *in vivo* expression pattern in certain tumors; second, periostin may have different functions according to different histopathological types of cancer; and third, it is possible that periostin may function differently by

expressing alternative splicing events at the C-terminal region, as five different spliced transcripts of periostin are produced^[15].

In summary, periostin expression was greater in gastric cancer tissues and metastatic lymph nodes compared to that in normal gastric tissues and benign gastric diseases (ulcers), and this increased expression was closely correlated with the TNM stage of gastric cancer. Our results strongly suggest that periostin plays a role in the progression of gastric cancer.

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COMMENTS

Background

The pathogenesis of gastric cancer has been extensively investigated in recent years, and the expression of many genes changes in the progression of carcinogenesis, cell invasion and metastasis. Periostin was originally identified in a mouse osteoblastic library. Its role in tumorigenesis is still unclear.

Research frontiers

Periostin has been suggested to be involved in cell adhesion and tumor formation. The human periostin gene has been shown to be overexpressed in lung cancer. Serum periostin levels are elevated in many kinds of human malignancy, and are correlated with poor prognosis. All of these observations suggest that periostin plays a role in tumorigenesis.

Innovations and breakthroughs

The expression of periostin is variable throughout the gastrointestinal tract. The basic level of this protein expression suggests that it plays a role in the normal physiology of the gastrointestinal epithelium. However, the expression of periostin mRNA differs between primary tumors and their respective cell lines. For example, periostin mRNA expression is low in colorectal cancer and head and neck squamous cell carcinoma cell lines, but higher in the primary tumors. The possibility is that stromal components play a role in stimulating periostin expression. Furthermore, opposite findings have been reported, periostin is down-regulated in bladder cancer, and invasiveness and metastasis are suppressed by periostin.

Applications

This study has implications for the future investigation of mesenchyme-specific genes in the formation of gastrointestinal cancer. Regarding the higher expression of periostin in gastric cancer, at both the mRNA and protein level, it is possible that periostin plays a role in the development of gastric cancer, and further study of this molecule in gastric cancer and its role as a biological marker is warranted.

Peer review

This is a well-written study which investigated the expression of the periostin gene. There has not been much research on this molecule in gastric cancer, and this study revealed that periostin was more highly expressed at the mRNA and protein level in gastric cancer, and the expression level was positively correlated with clinical tumor stage. Further investigation regarding the function and prognostic significance of periostin will be interesting, and this paper is a valuable addition to the literature on gastric cancer.

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Serum transforming growth factor- β 1 level reflects disease status in patients with esophageal carcinoma after radiotherapy

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Abstract

AIM: To evaluate the relationship between changes in serum transforming growth factor β 1 (TGF β 1) level and curative effect of radiotherapy (RT) in patients with esophageal carcinoma.

METHODS: Ninety patients with histologically confirmed esophageal carcinoma were enrolled. Serum samples for TGF β 1 analysis were obtained before and at the end of RT. An enzyme-linked immunosorbent assay was used to measure serum TGF β 1 level. Multivariate analysis was performed to investigate the relationship between disease status and changes in serum TGF β 1 level.

RESULTS: Serum TGF β 1 level in patients with esophageal carcinoma before RT was significantly higher than that in healthy controls ($P < 0.001$). At the end of RT, serum TGF β 1 level was decreased in 67.82% (59/87) of the patients. The overall survival rate at 1, 3 and 5 years was 48.28% (42/87), 19.54% (17/87) and 12.64% (11/87), respectively. Main causes of death were local failure and regional lymph node metastasis. In patients whose serum TGF β 1 level decreased after RT, the survival rate at 1, 3 and 5 years was 61.02% (36/59), 28.81% (17/59) and 18.64% (11/59), respectively. The survival rate at 1 year was 17.86% (5/28) in patients whose serum TGF β 1 level increased after RT, and all died within 18 mo ($P < 0.01$).

CONCLUSION: Serum TGF β 1 level may be a useful marker for monitoring disease status after RT in patients with esophageal carcinoma.

INTRODUCTION

Esophageal carcinoma is one of the most common malignant diseases in China. However, for a large number of patients, treatment is only palliative. The 5-year survival rate has remained about 10% for patients treated with conventionally fractionated radiotherapy (CR) alone. The poor prognosis is the result of both loco-regional treatment failure, seen in up to 80% of cases, and early disease dissemination^[1,2]. Failure of local control remains a significant clinical problem. Therefore, local control to improve survival of patients with esophageal carcinoma patients has been focused on by most investigators. Much emphasis has been placed on the role of physical factors (e.g., total dose and dose per fraction) in improving local control and survival rate. In China, investigators have published their results on esophageal carcinoma, using a schedule named late-course hyperfractionation accelerated radiation therapy (LCHART)^[3-7]. In this way, the survival rate of patients with esophageal carcinoma has been increased from 10% to 30%. However, acute toxicity reactions, mainly esophagitis, have increased. Although physical factors are important, none of these models considers the molecular biological events that may be responsible for the observed heterogeneity in tumor tissue response.

Cytokines play a key role in regulation of cells of the immune system and have also been implicated in the pathogenesis of malignant diseases. Transforming growth factor beta-1 (TGF β 1) is a cytokine with multiple biological functions. It influences the proliferation rate of many cell types, and acts as a growth inhibitor in most but not all cases. In addition, TGF β 1 controls

the process of epithelial cell differentiation. In normal cells, TGF β 1 generally enhances adhesion through increased cell matrix production and decreased proteolysis. Resistance to the negative growth-regulating properties of TGF β 1 has been observed in epithelial and mesenchymal tumors. In addition to acting as a stimulator of angiogenesis, TGF β 1 also influences the growth of tumor cells directly or indirectly. Tumor cells can escape the inhibitory effects of TGF β 1 on normal cells at the post-transcription, receptor or post-receptor level. When tumor cells are insensitive, TGF β 1 can also promote tumor metastasis by enhancing angiogenesis, and adjusting the character of the matrix, or adjusting the body's immune response to tumor growth. Animal experiments and clinical observations have demonstrated the functions of TGF β 1 in radiation-induced injury of normal tissues^[8,9]. It has been implicated in the injury of several organs after irradiation, including the lungs and breasts, especially in radiation-induced pneumonitis (RP)^[10-13]. It also has been proposed that serial measurements of plasma TGF β 1 can be valuable for estimating the risk of RP and deciding whether additional dose-escalation can be safely applied. In recent studies, a relationship between prognosis of many tumors and this cytokine has also been found^[14-17]. These suggest that TGF β 1 may be a promising prognostic marker for some cancer patients. However, Fukai's data have suggested that an elevated systemic TGF β 1 level is not related to tumor progression in esophageal cancer^[18]. For this reason, they think that systemic inflammation or chronic disease, in addition to the tumor itself, may influence plasma TGF β 1 level. However, another study has shown a significant correlation between TGF β 1 level measured in the azygos vein and distant lymph node metastasis in esophageal cancer^[19]. Based on these findings, we hypothesized that serial blood TGF β 1 measurements can be used to identify disease status in patients with esophageal carcinoma treated with conventional doses of radiotherapy (RT), and as a potential predictive marker that may allow us to stratify patients into different treatment groups. Here, we detected serum TGF β 1 level in 90 patients with esophageal carcinoma before and at the end of RT, to investigate the relationship between changes in serum TGF β 1 level and disease status in patients with esophageal carcinoma after RT.

MATERIALS AND METHODS

Patients and pretreatment characteristics

From August 1997 to June 1998, 90 unresectable or medically inoperable patients were enrolled into our clinical trial. Only patients with histologically confirmed esophageal carcinoma were eligible. The additional criteria for eligibility were age \leq 75 years, Karnofsky performance status \geq 70, white blood cell and hemoglobin levels within the normal range, and no prior treatment. The patients' clinical characteristics are listed in Table 1. The pretreatment evaluation generally included medical history and physical examination, complete blood cell count, chest radiography and/or chest computed tomography

Table 1 Patient characteristics

Characteristic	Number of patients
Gender	
Male	64
Female	26
Age (yr)	
Range	42-75
Median	57.80
Pathology	
Squamous cell carcinoma	88
Undifferentiated carcinoma	2
Location	
Upper-thoracic	37
Middle-thoracic	41
Lower-thoracic	12
Length (cm)	
Median	5.91
Range	2-12
Stage (UICC 1997)	
I	8
II a	31
II b	45
III	6

(CT), esophageal barium examination, and ultrasound examination of the abdomen, including the liver, kidneys, spleen and retroperitoneal lymph nodes. Based on the examinations mentioned above, patients were staged according to the TNM staging system of the 1997 American Joint Committee on Cancer staging system.

The study, including the criteria for patient eligibility, diagnostic procedures, fractionation schemes for treatment techniques, collection of blood samples, and tests, was approved by the Ethical Committee of Changhai Hospital. All patients received full information concerning the aim of the study, diagnostic and treatment procedures, medical care, and risks of acute and late sequelae before they entered the trial, and all patients voluntarily gave informed consent.

RT

All patients were given RT alone. A 10 MV X-ray linear accelerator was used for treatment. The design of the radiation fields was based on the diagnosis by CT and barium examination. For all patients, a three-field approach was administered: one anterior and two posterior oblique portals. The width of the fields was adjusted to cover gross tumors with 2-3 cm extended margins, so as to include subclinical lesions. The length of the field covered clinical tumors with a 3-5 cm extended margin at both ends of the lesion. All patients received conventional fractions, 2.0 Gy per fraction, five fractions per week. The total dose given to the tumor was 60-70 Gy/6-7 W. Lung corrections were not performed in this study.

Clinical evaluation of radiation response

At the end of RT, all patients received esophageal barium examination and the clinical radiation response was evaluated according to standard X-ray diagnosis of esophageal carcinoma after RT^[5]. A complete response (CR) was the disappearance of the mass shadow, no narrowing observed in the esophageal lumen, and none or slight

Table 2 Changes in serum TGFβ1 levels before and after RT

Groups	n	Mean value (ng/mL)	t value	P value
Control	15	9.53 ± 6.45	5.287	0.001
All patients				
Before RT	90	41.13 ± 15.41		
After RT	87	36.52 ± 19.26	1.365	0.072
Decreased group				
Before RT	59	42.93 ± 14.37		
After RT	59	25.98 ± 8.39	2.481	0.006
Increased group				
Before RT	28	40.25 ± 16.29		
After RT	28	51.61 ± 19.75	1.827	0.039

rigidity of the esophageal wall without residual ulceration. Partial response (PR) was > 50% reduction in tumor bulk, but < 100% resolution of the disease and a residual shallow ulcer with a diameter < 1.5 cm, despite the disappearance of the mass shadow. Minor response (MR) was definite improvement in the barium esophagogram, but with < 50% regression, with a large residual ulcer crater and/or narrowing of the esophageal lumen, regardless of the residual state of the mass shadow. No change (NC) was no improvement in the X-ray findings, with a deep and large residual ulcer or complete obstruction of the esophageal lumen, regardless of the residual state of the mass shadow.

The main endpoint in this analysis was the relationship between survival rate and change in serum TGFβ1 level. Death from any cause was calculated from the starting date of RT until death or the last follow-up evaluation. After treatment, follow-up included medical oncology visits at 3-mo intervals for 1 year, and then every 6 mo thereafter up to 5 years. The relationship between 1, 3 and 5-year survival rates and change in serum TGFβ1 level was observed.

Preparation of blood samples and tests for TGFβ1

Blood samples (2 mL each) were collected in EDTA tubes and stored for 1-3 h at 4°C, until the samples were centrifuged for plasma removal. Blood samples were centrifuged at 2000 g for 20 min, and only the top 0.5-1.0 mL plasma supernatant (serum) was removed to avoid platelet contamination. The serum samples were kept frozen at -70°C until assayed for TGFβ1. An enzyme-linked immunosorbent assay (ELISA) was used to determine TGFβ1 level. The TGFβ1 ELISA kit was purchased from R&D Systems (Shanghai, China). Serum samples were not subjected to acid/ethanol extraction, and active TGFβ1 was measured using the kit according to the manufacturer's recommended procedures. The control population consisted of 15 samples from normal blood donors.

Statistical analysis

The Statistical Package for Social Sciences, version 10.0. was used for statistical analysis. Serum TGFβ1 levels were expressed as means ± SD. Two sample means were statistically compared using Student's *t* test, assuming an unequal variance. Multiple comparisons between the mean TGFβ1 concentrations were performed using analysis of

Table 3 Immediate response to RT and serum TGFβ1 levels (%)

TGFβ1	n	CR	PR	MR	NC
Decrease	59	32.20 (19/59)	50.85 (30/59)	13.56 (8/59)	3.39 (2/59)
Increase	28	21.43 (6/28)	46.43 (13/28)	25.00 (7/28)	7.14 (2/28)

variance. The Kaplan-Meier model was used to estimate survival, and the differences between them were compared by the log-rank test.

RESULTS

All patients were followed until death or the time of analysis. Three patients were interrupted during RT and were removed from the statistical analysis, and five patients were lost to follow-up, who were counted as being dead at the time they disappeared. Eighty-two patients were followed for 5 years and the follow-up rate was 94.25%.

Changes in serum TGFβ1 level before and after RT

As shown in Table 2, before RT, mean serum TGFβ1 level in patients with esophageal cancer was 41.13 ± 15.41 ng/mL. This concentration was significantly higher than that in controls (9.53 ± 6.45 ng/mL) (*P* < 0.001). At the end of RT, a decreased serum TGFβ1 level was found in 67.82% (59/87) of all patients that completed the schedule. This suggested that TGFβ1 in the blood was produced by the esophageal tumor, and that its decrease may be due to the tumors being controlled by RT.

Clinical response to RT and serum TGFβ1 level

The relationship between clinical response to RT and serum TGFβ1 level is listed in Table 3. In patients with a reduced TGFβ1 level, tumor response rate to RT (CR plus PR) was 83.05% (49/59), and it was 67.86% (19/28) in those showing an increase (*P* < 0.05).

Changes in serum TGFβ1 level and disease status

It was found in stage III patients that serum TGFβ1 level was significantly decreased at the end of RT compared to that before RT. Mean serum TGFβ1 levels before and after therapy in these patients were 42.38 ± 13.65 and 34.76 ± 15.62, respectively (*P* < 0.05). Three months after RT, 11.5% (10/87) of patients had local treatment failure and regional lymph node metastasis. Among these patients, nine out of 28 (32%) patients' serum TGFβ1 levels increased, while only one out of 59 (1.7%) patients had a decreased TGFβ1 level. Comparatively, the difference between change in serum TGFβ1 level and local treatment failure and metastasis was significant (*P* < 0.001). The patterns of failure and metastasis are listed in Table 4.

Changes in serum TGFβ1 level and survival rate

Five patients were lost to follow-up between 1 and 5 years after RT, and were counted as deaths from the day that they died. The overall survival rate at 1, 3 and 5 years was 48.28% (42/87), 19.54% (17/87) and 12.64% (11/87), respectively. Main causes of death were local treatment failure and regional lymph node metastasis. In patients with

Table 4 Characteristics of ten patients

Case	Location	Stage	Response to RT	Failure or metastasis	TGFβ1 (ng/mL)	
					Before	After
1	Upper	III	NC	Lymph nodes M	30.10	42.80
2	Middle	II b	NC	Lymph nodes M	51.95	67.65
3	Upper	II a	PR	Lymph nodes M	18.60	38.20
4	Upper	III	PR	Lymph nodes and bone M	25.93	28.73
5	Middle	III	PR	Trachea M	29.45	29.55
6	Lower	II a	PR	Regional failure	23.05	27.18
7	Middle	II a	PR	Regional failure	40.68	19.88
8	Middle	II b	PR	Regional failure	30.08	62.65
9	Upper	I	CR	Regional failure	40.73	49.03
10	Upper	I	PR	Lymph nodes M	13.00	32.65

decreased serum TGFβ1 level after RT, survival rate at 1, 3 and 5 years was 61.02% (36/59), 28.81% (17/59) and 18.64% (11/59), respectively. The survival rate at 1 year was 17.86% (5/28) in patients with increased serum TGFβ1 levels after RT, and all died within 18 months ($P < 0.01$). The survival curves for patients in the three groups are shown in Figure 1.

DISCUSSION

In the present study, we investigated the relationship between changes in serum TGFβ1 level and curative effect of RT in patients with esophageal carcinoma, and the function of the cytokine TGFβ1 in treatment prognosis or disease status after RT. Data showed that average serum TGFβ1 level in patients with esophageal carcinoma before RT was significantly higher than that of healthy controls, which suggests that TGFβ1 in the blood is produced by esophageal tumors. After RT, serum TGFβ1 level was reduced in 67.82% (59/87) of patients compared to that before RT. This may be because the tumors were controlled by RT. This is believed to be the first report that there is a relationship between serum TGFβ1 level and esophageal carcinoma.

As mentioned above, much emphasis has been placed on the role of physical factors (e.g., total dose and dose per fraction) in improving local control and survival rate of esophageal carcinoma. Although physical factors are important, molecular biological events may be responsible for the observed heterogeneity in tumor tissue response between patients.

Recently, investigators have shown that changes in blood levels of certain cytokines, such as TGFβ1, may predict the risk of radiation-induced lung injury and association with disease progression^[20-22]. In a study of 73 patients receiving high-dose thoracic RT for lung cancer, Anscher *et al*^[23] found that those patients whose plasma TGFβ1 level was normal at the completion of RT were at low risk for subsequent radiation-induced lung injury, whereas the risk of symptomatic lung damage was increased in patients whose TGFβ1 level remained elevated. Subsequent analysis showed that these changes in plasma TGFβ1 correlated with the risk of pulmonary injury, independent of the volume of lung irradiated. TGFβ1 seems to affect tumor angiogenesis and play an

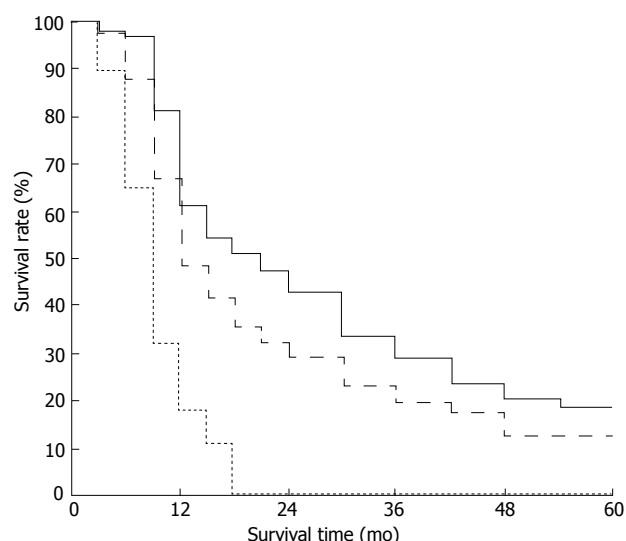


Figure 1 Survival status of all patients, and those with decreased and increased serum TGFβ1 levels. These data show statistically significant differences in the increased group compared with the decreased group and all patients ($P < 0.01$). Solid line indicates the decreased group; broken line indicates all patients; and dashed line indicates the increased group.

important role in tumor progression in non-small cell lung carcinoma. Kong *et al*^[24] measured plasma TGFβ1 concentrations before, during and after RT in 54 patients with lung cancer non-small cell lung cancer (NSCLC), to determine the kinetics of TGFβ1 expression during and after RT, and to correlate plasma TGFβ1 level with disease status after treatment. The results show that in those patients with an elevated plasma TGFβ1 level at diagnosis, monitoring this level may be useful in detecting both disease persistence and recurrence after therapy. Ivanovic *et al*^[25] examined the association between elevated plasma TGFβ1 level and disease progression in advanced breast cancer. Follow-up of six patients indicated a relationship between plasma TGFβ1 and treatment response.

In patients with cervical cancer treated with RT alone, pretreatment plasma TGFβ1 level is a significant prognostic factor for survival and local control, but not for radiation toxicity^[26]. Using concurrent chemoradiotherapy, Yang *et al*^[27] treated 42 patients with biopsy-proven squamous cell carcinoma or adenocarcinoma of the cervix, and assessed serum TGFβ1 level weekly. They have found that sudden elevation of serum TGFβ1 level after the first fraction of brachytherapy is accompanied by greater RT-related morbidity. Lower pretreatment TGFβ1 levels are associated with tumor response to chemoradiation. The conclusion is that serial changes in serum cytokines during chemoradiation may correlate with tumor regression and treatment morbidity.

In breast, gastric, colorectal, prostate, renal and liver cancers, a similar relationship to ours has been found between plasma TGFβ1 level and treatment response^[28-31]. However, in advanced head and neck cancer and NSCLC, no similar relationship between plasma TGFβ1 level and tumor burden was found, and neither to treatment response^[26]. Thus, for these cancers, it can be hypothesized that healthy tissues and the immune system are responsible for the major part of TGFβ1 production and that cancer

cells make only minor contribution to the total plasma TGFβ1 level.

Among patients with local treatment failure and regional lymph node metastasis after RT, there were nine with increased serum TGFβ1 (Table 4). There was a positive relationship between changes in serum TGFβ1 level and local treatment failure and regional lymph node metastasis. All patients with increased serum TGFβ1 died within 18 months after RT, which strongly suggests that serum TGFβ1 level can predict progression in patients with esophageal carcinoma. Our results have been confirmed by other studies on different kinds of carcinoma^[16, 24].

To the best of our knowledge, few of these studies have shown the predictive power of TGFβ1 for disease progression in patients with esophageal carcinoma after RT. The present study demonstrates that a higher serum TGFβ1 level after RT is strongly associated with residual or recurrent tumor or lymph node metastasis. These data suggest that serum TGFβ1 may be useful as a marker for monitoring tumor response to therapy and disease progression.

In conclusion, our findings provide preliminary evidence that significantly elevated serum TGFβ1 level in patients with esophageal carcinoma is associated with poor prognosis. Although the complete mechanism of action and the role of TGFβ1 in esophageal carcinoma remain to be elucidated, our results suggest that this biomarker may be useful for monitoring tumor response to therapy and diseases progression in patients with esophageal carcinoma.

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COMMENTS

Background

TGFβ1 is a cytokine with multiple biological functions. In recent studies, a relationship between prognosis of many tumors and this cytokine has also been found. These suggest that TGFβ1 may be a promising prognostic marker for some cancer patients. Based on these findings, we hypothesized that serial blood TGFβ1 measurements can be used to identify disease status in patients with esophageal carcinoma treated with conventional doses of radiotherapy (RT), and as a potential predictive marker that may allow us to stratify patients into different treatment groups.

Research frontiers

A study has shown a significant correlation between TGFβ1 level measured in the azygos vein and distant lymph node metastasis in esophageal cancer. Few of studies have shown the predictive power of TGFβ1 for disease progression in patients with esophageal carcinoma after RT.

Innovations and breakthroughs

Our findings provide preliminary evidence that significantly elevated serum TGFβ1 level in patients with esophageal carcinoma is associated with poor prognosis.

Applications

TGFβ1 may be useful for monitoring tumor response to therapy and diseases progression in patients with esophageal carcinoma. It may allow us to stratify patients into different treatment groups.

Terminology

Serum TGFβ1 level in esophageal carcinoma reflects disease status. A higher serum TGFβ1 level after RT is strongly associated with residual or recurrent tumor or lymph node metastasis.

Peer review

This paper is a well done and interesting study regarding TGF-beta serum levels and response/survival to radiation therapy in patients with esophageal cancer. The science seems interesting and sound.

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Early steroid withdrawal after liver transplantation for hepatocellular carcinoma

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Abstract

AIM: To evaluate the impact of early steroid withdrawal on the incidence of rejection, tumor recurrence and complications after liver transplantation for advanced-stage hepatocellular carcinoma.

METHODS: Fifty-four patients underwent liver transplantation for advanced-stage hepatocellular carcinoma from April 2003 to June 2005. These cases were divided into a steroid-withdrawal group (group A, $n = 28$) and a steroid-maintenance group (group B, $n = 26$). In group A, steroid was withdrawn 3 mo after transplantation. In group B, steroid was continuously used postoperatively. The incidence of rejection, 6-mo and 1-year recurrence rate of carcinoma, 1-year survival rate, mean serum tacrolimus trough level, and liver and kidney function were compared between the two groups.

RESULTS: In the two groups, no statistical difference was observed in the incidence of rejection (14.3 vs 11.5% , $P > 0.05$), mean serum tacrolimus trough levels (6.9 ± 1.4 vs 7.1 ± 1.1 $\mu\text{g/L}$, $P > 0.05$), liver and kidney function after 6 mo [alanine aminotransferase (ALT): 533 ± 183 vs 617 ± 217 nka/L , $P > 0.05$; creatinine: 66 ± 18 vs 71 ± 19 $\mu\text{mol/L}$, $P > 0.05$], 6-mo recurrence rate of carcinoma (25.0 vs 42.3% , $P > 0.05$), and 1-year survival rate (64.2 vs 46.1% , $P > 0.05$). The 1-year tumor recurrence rate (39.2 vs 69.2% , $P < 0.05$), serum cholesterol level (3.9 ± 1.8 vs 5.9 ± 2.6 mmol/L , $P < 0.01$) and fasting blood sugar (5.1 ± 2.1 vs 8.9 ± 3.6 mmol/L , $P < 0.01$) were significantly different. These were lower in the steroid-withdrawal group than in the steroid-maintenance group.

CONCLUSION: Early steroid withdrawal was safe after liver transplantation in patients with advanced-stage hepatocellular carcinoma. When steroids were withdrawn 3 mo post-operation, the incidence of rejection did

not increase, and there was no demand to maintain tacrolimus at a high level. In contrast, the tumor recurrence rate and the potential of adverse effects decreased significantly. This may have led to an increase in long-term survival rate.

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Key words: Hepatocellular carcinoma; Liver transplantation; Steroids; Tumor recurrence

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INTRODUCTION

Liver transplantation is well recognized as a treatment for prolonging survival in patients with advanced-stage hepatocellular carcinoma^[1]. Obviously, tumor recurrence is the main reason for the poor long-term survival after transplantation in these patients. It has been shown that long-term immunosuppression can facilitate the growth and spread of malignant cells^[2]. There is evidence that steroids play an important role in tumor recurrence after liver transplantation for hepatoma^[3], but whether steroids can be safely withdrawn remains controversial. In this study, we contrasted patients with early steroid withdrawal with those using continuous steroids, in order to establish the validity of the steroid-withdrawal regimen.

MATERIALS AND METHODS

Patients

Fifty-four patients suffering from advanced-stage hepatoma (all exceeding the Milan criterion) underwent liver transplantation between April 2003 and June 2005. There were two immunosuppressive protocols: 28 patients (group A) were given an early steroid-withdrawal protocol and 26 patients (group B) were given a steroid-maintenance protocol. Factors such as age at transplantation, stage of carcinoma, Child-Pugh score, graft cold ischemic time, anhepatic phase, operation time, and mean level of liver function before operation were noted, and these parameters were well matched in both groups (Table 1).

Table 1 Preoperative and intraoperative data for patients in the 2 groups

Parameter	Group A (n = 28)	Group B (n = 26)	P value
Sex (F/M)	1/27	0/26	0.3370
Mean age at OLT	45.7 ± 3.5	47.4 ± 6.3	0.2310
TNM stage of carcinoma			0.9914
II	1 (3.5%)	1 (3.8%)	
III A	8 (28.6%)	7 (26.9%)	
III B	5 (17.9%)	4 (15.4%)	
IV A	14 (50.0%)	14 (53.8%)	
Child-Pugh class			0.5259
A	20 (71.4%)	21 (80.8%)	
B	7 (25.0%)	5 (19.2%)	
C	1 (3.5%)	0 (0.0%)	
Liver and kidney function			
ALT (nka/L)	935.1 ± 383.3	1010.2 ± 536.8	0.5545
T-Bil (μmol/L)	23.1 ± 11.2	20.1 ± 10.8	0.3314
Creatinine (μmol/L)	67.8 ± 22.2	59.8 ± 24.3	0.2218
Graft cold ischemic time (min)	481.6 ± 97.0	462.1 ± 88.0	0.4464
Anhepatic phase (min)	51.5 ± 3.4	50.8 ± 3.1	0.4339
Operation time (min)	375.2 ± 98.1	391.5 ± 116.7	0.5799

OLT: Orthotopic liver transplantation.

Immunosuppressive regime

All the patients took tacrolimus, with a target serum trough level of 6-8 μg/L until 12 mo, and 4-6 μg/L thereafter. Mycophenolate mofetil was prescribed for 1 year at a dose of 0.5-1.0 g/d. Methylprednisolone was given at 500 mg/d intravenously for 3 d, during and after transplantation. Patients in group A received a rapid steroid reduction with the intention of withdrawing steroid by 3 mo. Patients in group B received a slow taper of steroid to prednisone 10 mg/d at 3 mo, and were maintained on this dose thereafter.

Postoperative treatment

ALT, creatinine, total cholesterol and fasting blood sugar were noted regularly after operation. Biopsies were used to establish the diagnosis of rejection on a histological basis when biochemical analysis suggested rejection. The following adjuvant chemotherapy regimen was adopted: E-ADM 40-60 mg/m² on d 1 and C-DDP 20-40 mg/m² on d 2-5, with 28 d as a cycle and 6 cycles in all. Tumor recurrence was confirmed by computed tomography or magnetic resonance imaging.

Statistical analysis

Statistical analysis was performed on preoperative and intraoperative data in the two groups, such as sex, age, stage of carcinoma, Child-Pugh score, liver and kidney function, Graft cold ischemic time, anhepatic phase and operation time. The rejection rate, tumor recurrence rate, patient survival rate and mean levels of biochemical parameters were compared between the two groups. Statistical analyses were conducted using the Statistical Package for the Social Sciences computer program (SPSS for Windows 11.5; SPSS, Chicago, IL, USA). The Student's *t* test and χ^2 test were used to determine statistical

Table 2 Rejection, tumor recurrence, and survival rate

Group <i>n</i>	Rejection rate	Tumor recurrence rate		1-yr survival rate (%)
		6-mo	1 yr	
A 28	14.3	25.0	39.2 ^a	64.2
B 26	11.5	42.3	69.2	46.1

^a*P* < 0.05 vs Group B.

significance between the groups. *P* < 0.05 was considered significant.

RESULTS

There were no significant differences between the two groups for rejection rate and 6-mo tumor recurrence rate. One-year tumor recurrence rate (39.2 vs 69.2%, *P* < 0.05) was significantly higher in the steroid-maintenance group. One-year survival rate was higher in group A than in group B, but the difference was not statistically significant (64.2 vs 46.1%, Table 2).

At 6 mo, the mean serum tacrolimus trough level was (6.9 ± 1.4) μg/L in group A and (7.1 ± 1.1) μg/L in group B, although the difference was not significant. There was no difference in liver and kidney function (ALT and creatinine) between the two groups. However, at 6 mo post operation, the mean levels of total serum cholesterol and fasting blood sugar were significantly lower in group A (Table 3).

DISCUSSION

Corticosteroids, with their multifaceted immunosuppressive properties, have long been considered as a linchpin in the prevention and treatment of transplant rejection. In addition to inhibiting the release and function of cytokines, such as interleukin-2, steroids can also regulate T- and B-lymphocyte apoptosis^[4]. However, there are well-known adverse effects that result in significant morbidity, including hypertension, diabetes, hyperlipidemia, obesity, and infectious complications. The adverse effects of long-term steroid use, even at a low dose, have stimulated interest in the feasibility of steroid-free maintenance immunosuppressive regimens.

This randomized clinical study was focused on a particular group of recipients who suffered from advanced-stage hepatocellular carcinoma before liver transplantation. In this group of patients, the high tumor-recurrence rate may cause the long-term survival rate to decrease sharply. Indisputably, the use of steroids has exacerbated this problem, either *via* a direct negative impact and/or by its adverse effects. One multicenter study has shown that when steroids were withdrawn 3-6 mo after liver transplantation, tumor recurrence was reduced to its lowest level^[2]. A retrospective study of three centers in Italy has found that the risk of hepatoma recurrence in patients with permanent use of steroids was almost fourfold when compared with patients made steroid-free not later than 6 mo after liver transplantation^[3]. Steroids may contribute to tumor recurrence. The potential

Table 3 Biochemical indicator after operation (mean \pm SD)

Group <i>n</i>	FK506 trough levels (μ g/L)	ALT (nka/L)		Creatinine (μ mol/L)		Total cholesterol (mmol/L)	Blood-fasting sugar (mmol/L)
		3-mo	6-mo	3-mo	6-mo	6-mo	6-mo
A (<i>n</i> = 28)	6.9 \pm 1.4	567 \pm 233	533 \pm 183	69 \pm 18	66 \pm 18	3.9 \pm 1.8 ^b	5.1 \pm 2.1 ^b
B (<i>n</i> = 26)	7.1 \pm 1.1	500 \pm 350	617 \pm 217	75 \pm 15	71 \pm 19	5.9 \pm 2.6	8.9 \pm 3.6

^b*P* < 0.01 *vs* Group B.

mechanism of this may be that steroids can inhibit malignant-cell apoptosis and promote migration of these cells. Yazawa *et al*^[5] have reported that glucocorticoids can inhibit human neutrophil-mediated tumor cell cytostasis. Ho *et al*^[6] have found that, in patients with hepatoma, the survival rate is higher when hepatoma cells are negative for corticosteroid receptors, compared with those that are positive. In our study, the 1-year tumor recurrence rate in the steroid-withdrawal group was lower than that in the steroid-maintenance group. This demonstrates that early steroid withdrawal can reduce tumor recurrence. The 1-year survival rate was higher in the steroid-withdrawal group. However, because of the small sample size and short time of follow-up, the difference was not statistically significant.

Early steroid-withdrawal regimens do not increase the rejection rate^[7-11]. Padbury^[12] has reported that, when steroids were withdrawn safely in 140/197 patients (71%), the acute and chronic rejection rate was 4.5 and 3.9%, respectively, and this was similar to the reported rates with steroid-containing regimens. In Jane's study^[13], 499 liver transplant recipients accepted early steroid-withdrawal immunosuppression, and only 9.8% of patients had steroid reintroduction. Stegall's study has shown that early steroid withdrawal after liver transplantation does not increase the fatality rate and the rate of chronic graft dysfunction^[14,15]. In our study, the incidence of acute rejection during the withdrawal phase did not increase, and each episode of rejection had only a modest effect and was steroid-responsive. No graft was lost to immunological causes. The tacrolimus trough levels were similar in the two groups. There was no demand to increase serum tacrolimus levels to prevent extra rejection. Thus, this early steroid-withdrawal protocol was safe in most patients.

The toxicity of steroids includes increased susceptibility to infection (particularly opportunistic organisms), hyperlipidemia, hypertension, diabetes mellitus, osteoporosis and aseptic necrosis, acne, Cushingoid facies, and growth retardation in children. The cumulative toxicity of immunosuppressive agents remains a major source of morbidity and mortality after liver transplantation, therefore, a protocol eliminating the steroid component has been a goal. Stegall's study has shown that steroid withdrawal after adult liver transplantation reduces diabetes, hypertension and hypercholesterolemia, without causing graft loss^[15]. In our study, total serum cholesterol and fasting blood sugar were significant lower in the early steroid-withdrawal group. This effect suggests that corticosteroids are a major causative agent in new-onset diabetes and hypercholesterolemia in liver transplantation recipients. Considering the adverse effects of steroid

treatment, steroid should be withdrawn earlier, except in patients who use prednisone preoperatively, such as primary biliary cirrhosis and sclerosing cholangitis^[13].

This study indicates that steroid withdrawal at 3 mo after liver transplantation is safe and necessary. Early steroid withdrawal does not lead to a high incidence of rejection or a high level of immunosuppressive drugs. In addition, steroid withdrawal may lead to a decreased incidence of tumor recurrence, new-onset diabetes and hypercholesterolemia. The decrease in tumor recurrence and adverse effects may lead to a higher survival rate for liver transplantation recipients with hepatocellular carcinoma. However, the 1-year survival rate in the steroid-withdrawal and steroid-maintenance groups was not significantly different. This result may have been due to the small sample size and short follow-up. Therefore, large long-term follow-up (several years), prospective, randomized and multicenter trials will be necessary to confirm the potential benefit of this regimen for the incidence of tumor recurrence, adverse events, and graft and patient survival.

COMMENTS

Background

Steroids have been the pillars of immunosuppression in organ transplantation for over 50 years. However, the fact that immunological graft loss is rare after liver transplantation, combined with the severe adverse effects of long-term prednisone therapy, supports steroid withdrawal in liver transplantation patients. Especially for patients suffering from advanced-stage hepatoma before liver transplantation, the long-term use of steroids may exacerbated the problem of reduced graft survival and patient survival. However, few clinical studies have focused on this group of recipients.

Research frontiers

Many clinical trials have proven the necessity of steroid withdrawal. The main findings of this study were that early withdraw of steroids was confirmed as a positive posttransplant action with a significant influence in reducing hepatoma recurrence.

Related publications

The present study was a randomized clinical trial of steroid withdrawal after liver transplantation in patients with advanced-stage hepatocellular carcinoma. We have cited several articles from other investigators that report research on steroid withdrawal after liver transplantation.

Innovations and breakthroughs

In prior studies of liver transplantation, little attention has been paid to the immunosuppression of hepatoma transplant recipients. This present clinical trial studied a steroid-withdrawal protocol for this group of recipients. We reached the conclusion that tumor recurrence can be reduced when steroids are withdrawn at 3 mo postoperatively. This protocol can be used as the guide for hepatoma transplant recipients.

Applications

In patients suffering from advanced-stage hepatocellular carcinoma, immuno-

suppression with early steroid withdrawal can reduce tumor recurrence after liver transplantation. In addition, total serum cholesterol and fasting blood sugar decrease sharply in steroid-withdrawal patients. This means that the use of steroids is one of the major causes of new-onset diabetes and hypercholesteremia after liver transplantation. Therefore, this steroid-withdrawal protocol can also be used following liver transplantation for other indications.

Terminology

Advanced-stage hepatocellular carcinoma is the end stage of a primary malignant neoplasm of the liver. Liver transplantation is the treatment for this disease. However, because of the high incidence of tumor recurrence, the outcome is poor. Steroids are a group of hormones that affect carbohydrate, fat and protein metabolism. They also possess pronounced anti-inflammatory activity. They have been used for immunosuppression for over 50 years.

Peer review

This is a brief but well-executed study which underscores what Starzl and others have been writing about for some time: the need to reduce immunosuppression in liver transplant recipients. In the authors' study, steroids were withdrawn successfully 3 mo after operation. This protocol caused a reduction in tumor recurrence and incidence of diabetes and hypercholesteremia after liver transplantation.

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Fever as the only manifestation of hypersensitivity reactions associated with oxaliplatin in a patient with colorectal cancer

Oxaliplatin-induced hypersensitivity reaction

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Abstract

Hypersensitivity reactions (HSR) to oxaliplatin in patients with colorectal cancer include facial flushing, erythema, pruritis, fever, tachycardia, dyspnea, tongue swelling, rash/hives, headache, chills, weakness, vomiting, burning sensations, dizziness, and edema. We report a patient with fever as the sole manifestation of initial HSR, review the literature and discuss the management of HSR. A 57-year-old female with T3N2M0 rectal adenocarcinoma received modified FOLFOX-6. She tolerated the first 8 cycles without any toxicities except grade 1 peripheral neuropathy and nausea. During 9th and 10th infusions, she developed fever to a maximum of 38.3°C with stable hemodynamic status despite medications. During 11th infusion, she developed grade 3 HSR consisting of symptomatic bronchospasm, hypotension, nausea, vomiting, cough, and fever. On examination, she was pale, cyanotic, with a temperature of 38.8°C, BP dropped to 95/43 mm Hg, pulse of 116/min and O₂ saturation of 88%-91%. She was hospitalized for management and recovered in 24 h. Fever alone is not a usual symptom of oxaliplatin HSR. It may be indicative that the patient may develop serious reactions subsequently, as did our patient who developed hypotension with the third challenge. Treatment and prevention consists of slowing the infusion rate, use of steroids and antagonists of Type 1 and 2 histamine receptor antagonists, whereas desensitization could help to provide the small number of patients who experience severe HSR with the ability to further receive an effective therapy for their colorectal cancer.

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Key words: Oxaliplatin; Hypersensitivity reaction; Fever;

INTRODUCTION

Oxaliplatin (C₈H₁₄N₂O₄) (Eloxatin; Sanofi-Aventis), is an organoplatinum in which the platinum atom is complexed with diaminocyclohexane and with an oxalate ligand. It is a third generation platinum which is indicated for the first-line treatment of metastatic colorectal cancer in combination with 5-fluorouracil (5-FU) and leucovorin (LV)^[1] and for the adjuvant treatment of stage III colorectal cancer^[2]. Oxaliplatin is safely administered in the outpatient setting but hypersensitivity reactions (HSR) can occur. Hypersensitivity is defined as an unexpected reaction that can not be explained by the known toxicity profile of the chemotherapeutic agent^[3]. HSR are seen usually with the taxanes (paclitaxel), the platinum compounds, asparaginase, the epipodophyllotoxins and procarbazine^[3]. Doxorubicin and 6-mercaptopurine have also been associated with these reactions. Acute onset and delayed reactions have been described which can cause flushing, pruritis, hypotension, dyspnea, nausea, back pain and rash^[3,4].

We report a patient with fever as the sole manifestation of initial hypersensitivity reaction, who subsequently developed a serious HSR including hypotension with the third challenge.

CASE REPORT

The patient is a 57-year-old female diagnosed with T3N2M0 (Stage III/Dukes C) rectal adenocarcinoma who was treated with oxaliplatin-based regimen in the adjuvant setting. The tumor was 5.7 cm in size and six lymph nodes were positive for cancer. She was started on treatment with adjuvant chemotherapy with modified FOLFOX-6 (mFOLFOX-6; oxaliplatin 85 mg/m² with concurrent LV 400 mg/m² on d 1 followed by IV bolus 5-FU 400 mg/m² followed by a single continuous infusion of 5-FU 2400 mg/m² over 46 h every 2 wk). As per

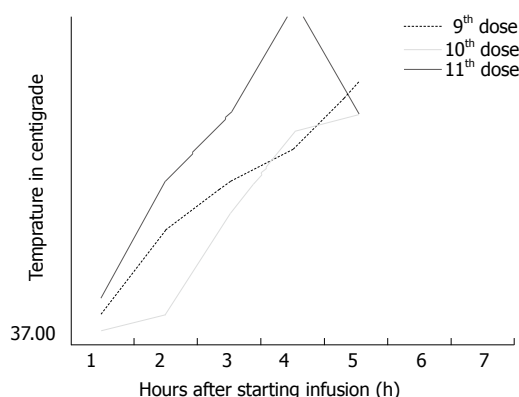


Figure 1 Fever in relation to infusion of oxaliplatin in the present case during 9th-11th cycles of modified FOLFOX-6 regimen in a patient with Stage III rectal cancer.

Table 1 Common terminology criteria for adverse events grading of hypersensitivity reactions (version 3.0)^[26]

Grade	Hypersensitivity reactions
1	Transient flushing or rash; drug fever < 38°C
2	Rash; flushing; urticaria; dyspnea; drug fever ≥ 38°C
3	Symptomatic bronchospasm, with or without urticaria; parenteral medication(s) indicated; allergy-related edema/angioedema; hypotension
4	Anaphylaxis

institutional standard premedication, she received dexamethasone 20 mg and ondansetron 16 mg intravenously. She tolerated 8 infusions without any complications. One and a half hour after starting the 9th infusion, she developed fever of 37.7°C. Other toxicities were grade 1 vomiting and grade 1 diarrhea. She was treated with promethazine (25 mg IV), hydrocortisone (100 mg IV), lorazepam (1 mg IV) and acetaminophen (650 mg PO). During this episode, her BP was 100/60 mm of Hg and pulse of 100/min. Despite the treatment, the fever continued to rise and reached a maximum of 38.8°C (Grade 2). She was discharged with the infusional 5-FU pump after observation and once fever came down to 37.8°C.

Two weeks later when she returned for the 10th infusion, she again developed fever of 37.7°C towards the end of the 2 h infusion. She also had emesis (grade 1) with nausea (grade 1) during oxaliplatin infusion. She was treated with promethazine and acetaminophen. Temperature rose to maximum of 38.4°C (Grade 2). The pulse and BP were preserved. Work-up for infectious etiology including line infection was done and ruled out. Fever defervesced and patient was discharged and returned home.

The patient was pre-medicated with promethazine before cycle 11th. She was planned to receive oxaliplatin over 2-h infusion. One and half hour into the infusion, she complained of itching for which diphenhydramine (25 mg) was given. After this she developed nausea (Grade 1) and vomiting (Grade 1) for which she received promethazine. One hour later she complained of cough and gagging sensation and on examination was found to be pale and cyanosis was visualized on the nail bed. Her temperature rose to 38.8°C (Figure 1) and soon her BP dropped to 95/43

Table 2 Incidence of HSR in pivotal studies of oxaliplatin in metastatic and adjuvant treatment of colorectal cancer

Study	Number of patients	Patients with HSR (%)	Patients with Grade 3/4 HSR (%)
Data from first-line ^[10]	259	12	2
Data from second-line ^[10]	150	10	< 1
MOSAIC (adjuvant) ^[2]	1123	10.3	2.9
Siu <i>et al</i> ^[11] all patients	180	15	2.2
Siu <i>et al</i> ^[11] adjuvant/first-line metastatic	88	10.2	
Siu <i>et al</i> ^[11] second-line metastatic	92	19.6	

HSR: Hypersensitivity reactions.

mm Hg, pulse of 116/min and O₂ saturation of 88%-91% (Grade 3). Two liters of oxygen were administered with nasal canula and the saturations improved to 91%. She was admitted to the hospital. Fever defervesced overnight and blood and urine cultures were negative. Hypotension responded to fluids and patient was discharged the next day. Desensitization was offered but patient refused further therapy with oxaliplatin.

DISCUSSION

HSR have been reported to occur in a minority of patients (12%) who receive oxaliplatin, and as less than 0.55% of the patients develop grade 3 or 4 reactions^[5-8] (Table 1). However, the incidence of HSR to oxaliplatin is rising recently as a result of increasing clinical use both in the adjuvant and metastatic treatment of colorectal cancer. The reactions usually occur after multiple infusions (mean 2-17) cycles of therapy with variable and unpredictable clinical features^[9]. Patients can experience flushing, alterations in heart rate and blood pressure, bronchospasm, back pain, chest discomfort, fever, pruritis, erythema, nausea, and rash^[3,5]. Usually the symptoms are mild but life-threatening anaphylactic reactions can occur. For this reason the FDA and Sanofi-Aventis has included a BOX warning for such reactions^[10]. The incidence of HSR in pivotal phase III trials of oxaliplatin both in metastatic and adjuvant setting is shown in Table 2^[2,10,11].

All platinum compounds are known to cause HSR^[12-15]. Being a platinum derivative, it is not surprising that oxaliplatin can also develop HSR^[5-9]. The differences in the reactions have been summarized in the Table 3.

The pathophysiology of HSR is not well understood. Development of this reaction after multiple infusions in most patients suggests the need for sensitization. Some investigators have described these reactions as Type I (IgE-mediated) allergic reactions^[16]. Santini *et al* reported a case of an idiosyncratic reaction; the serum analysis of the patient showed elevated TNF-α and IL6. The investigator postulated it to be a T-cell mediated reaction^[17]. It was also postulated that oxaliplatin acted as a super-antigen on mononuclear cells and resulted in the release of these cytokines. Other mechanisms that have been suggested include binding of the platinum salts to different peptides of major histocompatibility complex (MHC). In fact, HLA phenotype is a significant determinant of occupational

Table 3 Characteristics of HSR with different Platinum compounds

	Cisplatin ^[12,13]	Carboplatin ^[4,15]	Oxaliplatin ^[5-9]
Incidence	5%-20% (Increased with radiation)	16%	12% (14), < 1% grade 3-4
Initial onset	-	After 6 cycles(range 2-12)	After 7 cycles (range 2-25)
Time of onset	Minutes	Minutes-days	Minutes-hours
Symptoms	Variable(fever, anxiety, pruritus, cough, dyspnea, diaphoresis, angioedema, vomiting, bronchospasm, rash and pruritus, and hypotension	Variable (itching ,rash, chest tightness, Blood pressure changes, facial swelling)	Variable (flushing, alterations in heart rate, dyspnea, back pain, fever, pruritis, erythema, nausea, rash)
Can it be re-introduced?	-	Yes, with slowing the infusion rate (6% discontinued)	Yes, with pre-medications, slowing the infusion rate, and/or desensitization

sensitization to inhaled hapten of complex platinum salts and the strength of this association varies according to the intensity of exposure^[18]. Furthermore, the relationship between hypersensitivity reactions and HLA-haplotype has been described for other drugs^[19]. Additional factors are deemed to be necessary to the immune system for developing the reaction after several infusions.

Fever alone is not a usual symptom of oxaliplatin hypersensitivity. In another study among 39 patients who received a FOLFOX regimen in first line or beyond, the most common manifestations of an allergic reaction included: respiratory (50%), cutaneous (40%), and an anaphylactic shock (7.6%)^[20]. Ulrich-Pur *et al.*^[21] reported a case of 74-year-old man who developed fever hours after receiving the third infusion of oxaliplatin (5-FU + LV d 1 and d 5 every 28 d + mitomycin C d 1). The temperature was recorded to 39°C and lasted for 3 d. Patient had similar episodes of fever with 4th to 6th infusion. A rise in serum levels of IL 6 levels with 5th and 6th infusion corresponded to the rise in temperature. Pre-medication with dexamethasone, clarithromycin and metamizol for 3 d prior to therapy did not prevent the febrile reaction. Because the reactions occurred 1.5 to 2 h after infusion, the authors did not consider it to be hypersensitivity reactions, and thought to be definitely some kind of acquired allergic reaction because the 1st and the 2nd infusions were well-tolerated. It is to be noted that mitomycin C can also cause fever as a reaction in some cases. Thomas *et al.*^[5] reported hypersensitivity reactions in 3 patients; one of them reported fever of 39.6°C 2 h after the infusion of the 9th cycle of oxaliplatin. Fever defervesced overnight and cultures were negative. The patient again developed fever of 39.0°C with rigors and chest tightness several hours after the next cycle. Before the next cycle, the patient was pre-medicated with dexamethasone 20 mg, 6 and 12 h. Thirty minutes before the infusion the patient received solumedrol 125 mg, diphenhydramine 50 mg and cimetidine 30 mg. Despite this, the patient still developed a single spike of 38.3°C without associated symptoms^[5].

When a HSR occurs, the infusion of oxaliplatin should be immediately stopped and replaced by a saline infusion, an intravenous antihistaminic drug and a low-dose corticosteroids administration. In the case of more severe reactions (dyspnoea, sweating, bronchospasm, laryngospasm), immediately administer a high dose of steroid. The steroid dose range between 100 and 1000 mg of hydrocortisone^[9]. After the reaction disappears, the oxaliplatin infusion should not be restarted and the decision to administer the

other scheduled drugs must be taken evaluating the clinical status of the patient after the reaction, the risk of additional toxicity and the clinical utility of the chemotherapy. Mild hypersensitivity reactions to oxaliplatin and other platinum compounds can be ameliorated in some patients through the use of steroids and antihistamines before administration of subsequent cycles. However, premedication cannot prevent all hypersensitivity reactions, and mild reactions may escalate to severe reactions even when steroids and antihistamines are administered prior to oxaliplatin infusion^[22].

Over the years many protocols have been devised to desensitize patients against these life-threatening reactions so that they can benefit from Oxaliplatin. The various approaches have been summarized in the Table 4. One of the approaches is to increase the duration of the infusion. This was described in a retrospective study by Brandi *et al.*^[6] In the review of 124 patients the author reported fewer hypersensitivity reactions as the infusion time was increased from standard 2 to 6 h. In this review, 17 patients out of 124 (13.7%) developed hypersensitivity. Out of the 17 only 2 developed reactions at the end of infusion while in the rest the reaction appeared 10-15 min from start of the infusion. Six of the 17 patients were successfully re-exposed after pre-medication with steroids and antihistaminic drugs. Five patients developed hypersensitivity symptoms again and only one had no further reactions.

Another approach is desensitization to the drug. Lydia *et al.*^[23] reported a 53-year-old female with metastatic colon cancer who was treated with CAPOX (capecitabine 1000 mg/m² per day Monday to Friday with 2 weekly 85 mg/m² oxaliplatin) and bevacizumab (Avastin) 10 mg/kg. With the cycle 5th she developed diaphoresis, hypotension, hypoxia, nausea, and abdominal cramps 12 min from the start of infusion. She was immediately resuscitated with fluid bolus, oxygen, diphenylhydramine (25 mg IV) and dexamethasone (10 mg IV). The patient received cycle 5th through 7th without oxaliplatin. Because she had partial response to combination chemotherapy with oxaliplatin and she refused to take irinotecan (camptosar; Pfizer) due to toxicity profile, it was decided to desensitize her. On d 1 of desensitization, the patient was given prednisone 20 mg every 6 h for 4 doses and was hospitalized the same day. On d 2, 45 min before infusion 8 mg of ondasterone and 20 mg of dexamethasone were administered. Then 30 min before infusion, diphenylhydramine 50 mg and cimetidine 300 mg was administered. Oxaliplatin was administered over 8 h in serial dilutions from 1:10 000 to 1:1 and she

Table 4 Published cases of hypersensitivity associated with oxaliplatin

Reference	Presenting Features	Premedication	Oxaliplatin dose and serial dilution if used	Duration of therapy
Lydia <i>et al</i> ^[23]	Diaphoresis, hypotension, nausea, abdominal cramping rash, coryza	D1-prednisone 20 mg Po Q6 h × 4 D2 45 min pre chemo ondasterone 8 mg + dexamethasone 20 mg iv 30 min prior diphenylhydramine 50 mg iv + cimetidine 300 mg iv	140 mg on d 2 dilutions from 1:10000 to 1:1	8 h
Thomas <i>et al</i> ^[5]	Erythematic, flushing	Dexamethasone 20 mg 6 h and 12 h prior 30 min pre infusion: solumedrol 125 mg iv, diphenylhydramine 50 mg iv, cimetidine 50 mg iv	-	2-4 h
Bhargava <i>et al</i> ^[22]	Palpitation, flushing, hypotension	Dexamethasone starting 24 h prior 30 min before infusion received dexamethasone, diphenylhydramine, hydrocortisone 100 mg iv	1:10000, 1:1000, 1:100, 1:10 each bag infuse over one hour	6-8 h
Meyer <i>et al</i> ^[7]		dexamethasone-famotidine and diphenylhydramine	90% of total dose 1:1000, 1:100 and 1:10 dilution over 90 min	6 h
Lim <i>et al</i> ^[24]	Abdominal distension, heat, pruritis	D 1 diphenylhydramine 50 mg QID 30 min prior to infusion metoclopramide 9 mg, morazepam 2 mg, dexamethasone 5 mg iv	Fixed dose infusion over 24 h with dilute solution (0.15 mg/mL)	24 h
Present case	Fever	-	Refused further oxaliplatin therapy	-

received a total dose of 140 mg. Intravenous epinephrine (1:1000) and diphenylhydramine and methylprednisolone 125 mg were placed at bedside. The patient tolerated the therapy without complications. The next infusion was given as outpatient. Thus desensitization helped this patient to receive an additional three doses. In this report 24-h pre-medication schedule with serial dilution of oxaliplatin over longer period of time was used based on reports used for desensitization of carboplatin. Lim *et al*^[24] also published his case with successful desensitization.

With carboplatin, an intradermal skin test after 6th dose is a good predictor for occurrence of this reaction. However, no intradermal test is recommended before oxaliplatin administration. An intradermal skin test for hypersensitivity to oxaliplatin has been reported in small series to be 75%-80% accurate^[25]. The investigators suggested that desensitization might be considered for patients with a mild to moderate skin reaction in whom oxaliplatin would be beneficial. The investigators also suggested that a challenge should not be attempted for patients with markedly positive skin test reactions. Garufi *et al* reported the skin test to be negative in 15 patients with no previous reactions to oxaliplatin. A positive skin-test reaction is helpful; however, negative results may be seen in some patients who experience hypersensitivity^[25]. Two of 8 patients with prior hypersensitivity reactions to oxaliplatin were reported by Meyer *et al*^[7] to have negative skin tests with oxaliplatin, as did 1 of 3 patients described by Thomas *et al*^[5].

It is expected to see a rising incidence of HSR to oxaliplatin as has been observed as a result of increasing clinical use. Siu *et al*^[11] recently reported epidemiological and clinical features of these reactions in his institution. Among 180 patients, 15% were labeled as allergic to oxaliplatin, the proportion being higher among those receiving oxaliplatin in palliative second-line or above settings (19.6%) than in adjuvant or palliative first-line settings (10.2%). Overall, 2.2% of them developed grade 3-4 reactions. Re-exposure to oxaliplatin in 14 patients resulted in 28.6% HSR with 14.3% reactions of grade 3-4 intensity.

Although the reported incidence of HSR is about 12%

of the patients who receive oxaliplatin (1%-2% grade 3 or 4 in severity), the recent rising incidence of HSR to oxaliplatin observed is the result of increasing clinical use. It is also important to remember that fever can be the sole manifestation of initial HSR in few cases akin to ours; a harbinger that this patient can subsequently develop serious HSR with continued use of oxaliplatin. Therefore, proper recognition and management can prevent a serious HSR. Few patients can be managed with pre-medication with steroids and antihistamines, but the majority of patients require intensive desensitization. Due to the desensitization regimens many patients can successfully continue to receive this agent. Reintroductions have only been reported as single case studies or small cohorts. Large scale validation on desensitization strategies are still missing. Knowledge of this rare but real toxicity of oxaliplatin is paramount since the use of this drug is wide-spread both for metastatic and adjuvant settings in the treatment of colorectal cancer-the second leading cause of cancer mortality in USA.

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CASE REPORT

Retrograde jejunoduodenogastric intussusception due to a replacement percutaneous gastrostomy tube presenting as upper gastrointestinal bleeding

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Abstract

Percutaneous endoscopic gastrostomy (PEG) tube complications can be serious or life threatening. Retrograde intussusception is a very rare complication of PEG tubes with only 9 cases reported in the literature. We describe a case of retrograde intussusception, associated with the use of a Foley catheter as a replacement gastrostomy tube, presenting with upper gastrointestinal bleeding. To our knowledge, this is the first reported case of PEG-related retrograde intussusception successfully managed in a non-surgical manner. Retrograde intussusception likely occurred due to migration of the replacement tube with resultant securing and invagination of the proximal jejunum when the gastrostomy tube was anchored to the abdominal wall.

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Key words: Percutaneous endoscopic gastrostomy; Intussusception; Migration and upper gastrointestinal bleeding

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INTRODUCTION

Since introduction by Gauderer in 1980, percutaneous endoscopic gastrostomy (PEG) has become the preferred procedure for establishing enteral feeding in most clinical

situations^[1]. The complication rate ranges from 3% to 14% depending on the definition used, and mortality approaches 1%^[2]. Retrograde intussusception, defined as the telescoping or invagination of a distal segment of intestine (the intussuseptum) into the receiving proximal end (the intussusipiens), is a very rare complication of gastrostomy tube migration. We report here the first case treated successfully in a non-surgical manner.

CASE REPORT

An 89-year-old female nursing home resident was hospitalized with generalized abdominal pain, coffee ground emesis and change in mental status. Past history revealed hypertension, mild dementia, coronary artery disease, transvaginal hysterectomy and percutaneous endoscopic gastrostomy (PEG) placement six months prior due to poor oral intake. Four weeks prior to admission, the patient's PEG tube was dislodged unintentionally and replaced at the bedside using a 22f Foley catheter, inflating the balloon with 10 CC of sterile water. No testing was performed to confirm intraluminal placement of the Foley at the nursing home.

Physical examination revealed a cachectic elderly woman who was somnolent but arousable. She was afebrile and normotensive. Abdominal examination revealed a distended, diffusely tender abdomen with evidence of erythema and exudate at the ostomy site and normal bowel sounds. The remainder of the physical examination was unremarkable. Pertinent laboratory studies included white blood cell count of 12.9 thousand/ μ L and hemoglobin of 10.7 gm/dL. Abdominal flat and upright radiographs revealed only fecal impaction. An upper endoscopy was performed within 24 h of hospitalization with detection of a large amount of brownish foul smelling fluid within the stomach. A twisted loop of small bowel with mucosal discoloration and sloughing was seen protruding through the pylorus into the gastric body (Figures 1 and 2). A 22 fr Foley catheter was seen tethering the loop of bowel to the body of the stomach. The pyloric channel was not visualized and the intubation of the duodenum was not attempted. A water contrast X-ray series performed immediately after endoscopy showed an antral filling defect with typical coil spring appearance of intussusception. A barium study through the PEG tube showed its tip in the proximal jejunum. The clinical diagnosis of retrograde intussusception was made at this time. A general surgery



Figure 1 Intussuscepted loop of small bowel and size 22 Fr Foley catheter tethering loop to ostomy site in the gastric body.



Figure 2 Close up view of intussuscepted small bowel.

opinion was obtained based on the endoscopic and radiologic findings, but a conservative approach was taken at the request of the patient's family. This included deflation and removal of the Foley catheter, placement of a button replacement PEG at the ostomy site, intravenous hydration, packed red blood cell transfusion and institution of broad-spectrum antibiotics.

There was no overt worsening of her clinical status after deflation/removal of the Foley catheter and button PEG replacement. Repeat upper endoscopy was performed three days after PEG tube replacement. Examination distal to the second portion of the duodenum revealed a segment of continuous mucosal necrosis and discoloration. Biopsies obtained revealed mucopurulent exudates and debris with dense inflammatory infiltrates only. Feeding through the button PEG began the day after the repeat endoscopy and was tolerated well during the remainder of her hospital stay. The patient continued to improve clinically and was discharged, on hospital d 14, initially to a skilled nursing unit for a 2 wk observation period followed by eventual return to her permanent assisted living facility.

DISCUSSION

PEG tube insertion is a simple endoscopic procedure performed worldwide. Multiple complications have been reported (major and minor) including those occurred while placing the PEG and postprocedure^[2]. Common complications include pain at the insertion site, peristomal leak and infection, tube extrusion, transient ileus and cutaneous ulceration^[2,3]. Major complications include gastric or colonic perforation, peritonitis, gastrocolic or colocutaneous fistula, peritonitis and necrotizing fascitis^[4-8]. Bleeding and aspiration are complications that have the potential of turning into serious or life threatening situations^[4]. Retrograde intussusception is very rare and can occur as a complication of gastrostomy tube migration. In the English literature on this subject, only 9 cases have been reported^[9-17]. Nearly all of these cases were secondary to distal migration of a Foley catheter gastrostomy tube lacking an external fixation device or support.

The diagnosis in these cases was made based on radiologic imaging (contrast or barium study, abdominal ultrasound or CT scan) with endoscopy used in 2 cases. All except 1 (postmortem diagnosis) required surgical

resection. The exact mechanism leading to retrograde intussusception is unknown. Oswald *et al.*^[11] suggested that migration of the gastrostomy tube is the initiating event resulting in telescoping of the mobile jejunum over the tip of an impacted gastrostomy tube into the proximal duodenum. Lamont and Rode^[12] have proposed a different mechanism with passage of an inflated Foley gastrostomy catheter migrating past the pylorus into the small bowel, resulting in balloon fixation. Retraction attempts with the balloon inflated result in invagination of the small bowel into the duodenum. Finally, Gasparri and associates^[15] suggest a 3rd potential mechanism with balloon migration resulting in increased intraluminal traction and invagination of the jejunal wall. Attempts at repositioning result in the inflated balloon acting as a lead point with retrograde telescoping of the jejunum back through the duodenum and into the gastric lumen. Following these case reports, new gastrostomy tubes are now fitted with external fixation devices or bolster to prevent distal migration.

Our case illustrates a rare but preventable complication of a gastrostomy tube with uniqueness in terms of therapeutic approach. Unlike previous cases reported in the literature, our patient did not undergo surgical exploration, making a full recovery without evidence of overt sepsis or peritonitis and was eventually discharged to her nursing home. We hypothesize that following replacement of the PEG tube with a Foley catheter, the catheter migrated into and past the C-loop of the duodenum and was pulled back by NH staff without first deflating the balloon, leading to invagination of the mobile portion of the jejunum. The tube was then likely anchored in some manner leading to mucosal ischemia and necrosis but not in a transmural fashion. After deflation of the Foley catheter balloon, blood supply was restored allowing for revascularization of the mucosa.

We recommend that intussusception should be ruled out in a patient with a PEG tube who presents with symptoms of nausea, vomiting, hematemesis or abdominal pain by direct visualization of gastric and duodenal mucosa with EGD and upper GI series with water-based contrast. If intussusception is diagnosed, surgery remains the treatment of choice. However, if identified early or patient's surrogates decline a surgical approach, rapid deflation of the Foley catheter and button PEG replacement can result in a successful conservative approach to retrograde jejuno-duodenal

intussusception with a positive outcome. Prevention of such a complication may be simply achieved by anchoring, with an external bolster, any type of replacement tube to prevent such a migration.

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Acute pancreatitis and cholangitis: A complication caused by a migrated gastrostomy tube

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Abstract

Percutaneous endoscopic gastrostomy (PEG) is generally considered safe with a low rate of serious complications. However, dislocation of the PEG-tube into the duodenum can lead to serious complications. An 86-year old Japanese woman with PEG-tube feeding sometimes vomited after her family doctor replaced the PEG-tube without radiologic confirmation. At her hospitalization, she complained of severe tenderness at the epigastric region and the PEG-tube was drawn into the stomach. Imaging studies showed that the tip of PEG-tube with the inflated balloon was migrated into the second portion of the duodenum, suggesting that it might have obstructed the bile and pancreatic ducts, inducing cholangitis and pancreatitis. After the PEG-tube was replaced at the appropriate position, vomiting and abdominal tenderness improved dramatically and laboratory studies became normal immediately. Our case suggests that it is important to secure PEG-tube at the level of skin, especially after replacement.

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Key words: Percutaneous endoscopic gastrostomy; Complications; Tube migration; Pancreatitis; Cholangitis

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INTRODUCTION

Percutaneous endoscopic gastrostomy (PEG) has gained

broad acceptance as an effective method for achieving enteral access in patients who need chronic nutritional support. The feeding through PEG-tube is convenient, safe, and agreeable. Most of complications of PEG-tube feeding are minor, except for those arising at tube-exchange, which is necessary for a long-term feeding because of degradation of tubes, or incidental tube-removal. It is better to exchange PEG-tubes under fluoroscope in hospital for preventing complications. However, many primary doctors perform at the bed side in patient's home through unavoidable circumstances. This is one of the reasons why serious complications occur when a long-term PEG-tube feeding is needed.

In this report, we describe a case of pancreatitis and cholangitis induced by dislocated PEG-tube, which are the very rare complications of PEG-tube feeding.

CASE REPORT

An 86-year-old Japanese woman with PEG-tube feeding was referred to our hospital for reiterated emesis. She had an attack of cerebral infarction and cerebral embolism at the age of 84 years. Since the disease caused her dysphagia and continuous consciousness disturbance, she received chronic nutritional support by PEG-tube feeding. One month before her hospitalization, a gastrostomy tube (Gastrostomy-tube, Bard Access Systems, Salt Lake City, UT, USA) with a diameter of 18 F, which is fixed by an intragastric balloon (20 mL of water) and an external disc bumper, was replaced by her family doctor in her home without radiologic confirmation, and she sometimes vomited gastric juice and bile without enteric nutrient. Her family noticed that the PEG-tube was sometimes drawn into the stomach. At her hospitalization, she complained of severe tenderness at the epigastric region and the PEG-tube was drawn into the stomach. The distance between the balloon and external disc bumper was 8 cm measured by a scale indicated on the PEG-tube. Laboratory studies revealed $10.4 \times 10^9/L$ white blood cells (normal range: $5.0-8.0 \times 10^9/L$), 97.9 mg/L C-reactive protein (normal range: < 3 mg/L), 1.24 $\mu\text{mol/L}$ total serum bilirubin (normal range: 0.49-2.16 $\mu\text{mol/L}$), 213 U/L aspartate aminotransferase (normal range: 10-34 U/L), 254 U/L alanine aminotransferase (normal range: 5-40 U/L), 553 U/L alkaline phosphatase (normal range: 100-358 U/L), 238 U/L lactate dehydrogenase (normal range: 104-224 U/L), 1191 U/L amylase (normal range: 32-112 U/L), 176 U/L gamma-glutamyltranspeptidase (normal range: 7-29 U/L). A diagnosis of acute pancreatitis and cholangitis was made based on the physical examination and laboratory findings.

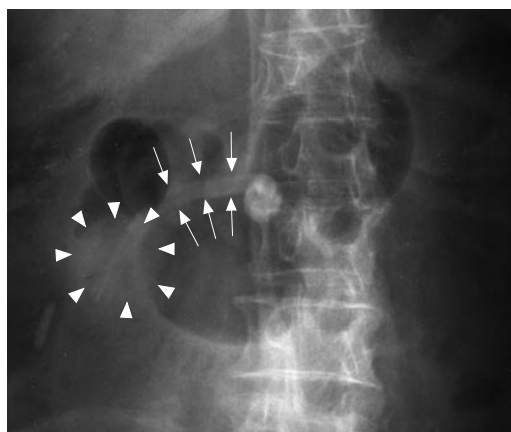


Figure 1 A plain abdominal radiograph showing collected gas in the stomach and duodenum with the tube balloon situated in the second portion of duodenum (tube: arrow, balloon: arrowhead).

Abdominal roentgenography and computed tomography (CT) showed that the tip of PEG-tube with the inflated balloon was migrated into the second portion of the duodenum (Figures 1 and 2), suggesting that it might have disturbed the flow of bile and pancreatic juice at the papilla, and thus was thought to be the cause of cholangitis and pancreatitis. No stone or tumor was found at this region in these studies.

Once the balloon was deflated and fixed at the appropriate position by the balloon re-inflation after the tube was retracted into the stomach. After the PEG-tube was replaced at the appropriate position, vomiting and abdominal tenderness improved on the next day and laboratory studies became normal after two days.

DISCUSSION

PEG has become the preferred method in patients requiring long-term enteral nutrition because of its ease and safety of placement. Previous studies reported that complications are infrequent and a procedure-related mortality is less than 1%^[1,2]. Common procedure-related complications include wound infection, aspiration, hemorrhage, pneumoperitoneum, peritonitis, and common long-term complications include leakage, granulation tissue, unintentional removal, buried bumper syndrome^[3-7]. Obstructive pancreatitis and cholangitis induced by migrated PEG-tube are the very rare complications.

Although PEG-tube feeding is generally considered safe with a low rate of serious complications, dislocation of the PEG-tube into the duodenum can lead to symptoms of obstructive pancreatitis and cholangitis. Because a balloon with PEG-tube is hard to pass through the pyloric ring, a migrated balloon may obstruct the pyloric ring and cause vomiting. In this case, the family doctor might have inserted the PEG-tube too deep into the duodenum, so the tube passed through the pyloric ring and the balloon was inflated in the duodenum. As enteral peristalsis moved the balloon up to papilla of Vater, the flow of bile and pancreatic juice might have been obstructed. These speculations were supported by the frequent PEG-tube traction



Figure 2 A plain CT scan demonstrating the PEG-tube balloon in the second portion of duodenum.

into the stomach noticed by her family, and her repeated vomiting due to small bowel obstruction by the balloon. Roentgenography could easily show the dislocation of PEG-tube which might have been noticed by checking carefully the length of PEG-tube over the skin.

Five cases of acute pancreatitis related to gastrostomy tube migration have been reported^[8-12]. Foley catheter has been used as PEG-tubes in 4 cases^[8-10,12]. This catheter is more likely to migrate because it has no external bumper which prevents dislocation of the tube. In another case using a gastrostomy tube with an external bumper, spontaneous loosening of the external bumper caused the tube migration^[11]. In this case, a technical error might have caused tube dislocation when a new tube was replaced blindly, although the tube has an external bumper.

This case demonstrates that a malpositioned PEG-tube can be an iatrogenic cause of acute pancreatitis and cholangitis. It is important to secure PEG-tube at the level of skin, especially a couple of days after its replacement.

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LETTERS TO THE EDITOR

Portal vein thrombosis: Etiology and clinical outcome of cirrhosis and malignancy-related non-cirrhotic, non-tumoral extrahepatic portal venous obstruction

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Abstract

The etiology and pathogenesis of portal vein thrombosis are unclear. Portal venous thrombosis presentation differs in cirrhotic and tumor-related versus non-cirrhotic and non-tumoral extrahepatic portal venous obstruction (EHPVO). Non-cirrhotic and non-tumoral EHPVO patients are young and present with well tolerated bleeding. Cirrhosis and tumor-related portal vein thrombosis patients are older and have a grim prognosis. Among the 118 patients with portal vein thrombosis, 15.3% had cirrhosis, 42.4% had liver malignancy (primary or metastatic), 6% had pancreatitis (acute or chronic), 5% had hypercoagulable state and 31.3% had idiopathy, 12% had hypercoagulable state in the EHPVO group.

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Key words: Portal vein thrombosis; Cirrhosis; Malignancy; Extrahepatic portal venous obstruction

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TO THE EDITOR

We read with great interest the article "Portal hypertension due to portal venous thrombosis: Etiology, clinical outcomes" by Harmanci *et al*^[1] in the May 14, 2007 issue of World Journal of Gastroenterology. We agree that portal vein thrombosis (PVT) should be considered under two different categories [acute or chronic PVT (noncirrhotic and nontumoral), (b) PVT due to cirrhosis and tumors], as the presentation, clinical course and prognosis are different in the two categories.

Table 1 Clinical and laboratory findings in 118 patients with portal vein thrombosis

Parameters	Extrahepatic portal vein obstruction (n = 50)	Cirrhosis and tumor-related portal vein thrombosis (n = 68)
Male:Female	33:17	51:17
Age (yr) ²	25 (9-40)	52 (23-80)
Hematemesis	36 (72%)	10 (14.7%)
Hypersplenism	30 (60%)	6 (8.8%)
Pain abdomen	18 (36%)	42 (61.8%)
Abdominal distension	8 (16%)	36 (52.9%)
Awareness of splenomegaly	6 (20%)	-
Jaundice	5 (10%)	18 (26.5%)
Cholangitis	1 (2%)	5 (7.4%)
Splenomegaly ² cm ¹	4 (2-14); (n = 35)	2 (2-4); (n = 7)
Hepatomegaly ² cm ¹	2 (1-3); (n = 8)	3 (2-6); (n = 16)
Etiology		
Hepatocellular carcinoma	-	31
Cirrhosis liver	-	18
Metastases liver	-	6
Pancreatic carcinoma	-	7
Cholangio carcinoma	-	3
Carcinoma gallbladder	-	2
Duodenal carcinoid	-	1
Esophageal Varices	35 (70%)	32 (47%)
Gastric Varices	11 (22%)	3 (4.4%)
GoVI	1	2
GoV2	8	1
IGV1	2	
Portal hypertensive gastropathy	8 (16%)	48 (70.4%)
Mild	6	40
Severe	2	8
Hemoglobin ² (gm/dL)	7.2 (1.7-14.7)	8.0 (6.0-11.6)
Total leucocyte count ² (10 ³ /mm ³)	2.9 (1.0-6.8)	6.4 (1.36-29.71)
Platelet count ² (10 ³ /mm ³)	0.49 (0.14-1.54)	1.52 (0.29-2.59)
Bilirubin ² (mg/dL)	1.0 (0.4-18.5)	1.2 (0.5-15.1)
AST ² (U/L)	38 (18-231)	79 (39-961)
ALT ² (U/L)	34 (18-254)	65 (18-1146)
SAP ² (U/L)	264 (110-2849)	348 (140-3140)
Protein/albumin ² (mg/dL)	6.8 (5-7.5)/3.8 (3-4)	6 (5.3-8)/3.3 (2-4.6)
Prothrombin time	2 (1-3)	4 (2-14)
prolongation ² (seconds)		
Portal biliopathy	4 (8%)	
Choledocholithiasis	2	
CBD stricture	2	
EVL sessions ²	3 (2-4)	-
Glue injection (n)	4	-
Superior mesenteric vein thrombosis	11 (22%)	6 (8.8%)
Inferior vena cava thrombosis	4 (8%)	3 (4.4%)
Splenic vein thrombosis	4 (8%)	4 (5.6%)
Deep vein thrombosis	-	1 (1.4%)
HBsAg	0	27 (39.7%)
Anti-HCV	0	3 (4.4%)

¹Centimeter below costal margin; ²Median (range).

Of the 118 cases of PVT admitted to our hospital over the 2-year period (from January 1, 2005 to December 31, 2006), 50 were due to extrahepatic portal vein obstruction (EHPVO) and 68 were due to cirrhosis and tumors. The clinical and laboratory characteristics of 50 patients with EHPVO are given in Table 1. The patients were young and commonly presented with features of hematemesis, hypersplenism, abdominal pain and distension. Ten patients had acute PVT and 2 had presentation as acute Budd-Chiari syndrome, 2 patients had pregnancy and delivered the fetus at term with supportive treatment. Thirty-six patients who presented with hematemesis were managed with endoscopic variceal ligation (2-4 sessions), and four patients were treated with gastric varices glue injection. These patients were maintained on beta-blockers and follow-up endoscopic surveillance. Four patients with symptomatic portal biliopathy were managed with stent placement ($n = 2$) and common bile duct (CBD) stone extraction ($n = 2$). Antithrombin-III deficiency was present in 2 patients, antiphospholipid antibody in 2 patients, factor V Leiden (FVL) mutation in 1 patient and paroxysmal nocturnal hemoglobinuria in 1 patient. All the 6 patients were started on heparin and warfarin with warfarin continued to maintain a 2-3 INR. Four (8%), 2 (4%) and 1 (2%) patients had chronic pancreatitis, acute pancreatitis and liver abscess, respectively. During the mean follow-up period of 9 mo (3-24 mo), none of the patients had symptomatic hypersplenism.

EHPVO affects young individuals who present with well-tolerated bleeding. The etiology and pathogenesis of PVT are unclear. It was initially proposed that umbilical sepsis or catheterization of umbilical veins in the neonatal period is responsible for PVT^[2]. In recent years, the presence of congenital or acquired prothrombotic conditions has been considered an interesting hypothesis for the causation of PVT. FVL mutation is known to be less common among Asians as compared to the population of European descent. Koshy *et al.*^[3,4] reported that FVL mutation has been found in 3% patients with portal vein thrombosis

and prothrombin G20210A gene in patients with portal vein thrombosis in a south Indian study. In our study, FVL mutation was present in 2% of cases.

The clinical and laboratory features of 68 patients in cirrhosis and tumor groups are given in Table 1. The clinical presentation was abdominal pain and distension and jaundice. During a mean follow-up period of 7 mo (range 1-24 mo), 48% of the patients died.

Of 118 cases of PVT admitted to our hospital, 15.3% had cirrhosis, 42.4% had liver malignancy (primary or metastatic), 6% had pancreatitis (acute or chronic), 5% had hypercoagulable state and 31.3% had idiopathy. The higher percentage in idiopathic group might be due to a low prevalence of hypercoagulable factors, abnormality and attribution of umbilical sepsis in childhood. The role of JAK2 mutation in early diagnosis of overt or silent myeloproliferative disease cannot be undermined but requires standardization^[5].

In conclusion, the presentation, etiology and prognosis in non-cirrhotic and non-tumoral EHPVO patients are different from those in cirrhosis and tumor-related portal vein thrombosis patients.

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Colonic gas explosion during therapeutic colonoscopy with electrocautery

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Abstract

Therapeutic colonoscopy with electrocautery is widely used around the world. Adequate colonic cleansing is considered a crucial factor for the safety of this procedure. Colonic gas explosion, although rare, is one of the most frightening iatrogenic complications during colonoscopy with electrocautery. This complication is the result of an accumulation of colonic gases to explosive concentrations, but may be prevented by meticulous bowel preparation. The purpose of this review is to discuss the indications and the types of bowel preparations for therapeutic colonoscopy, and to contribute recommendations for the adequate bowel preparation for colonoscopy with electrocautery.

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Key words: Colonic gas explosion; Electrocautery; Therapeutic polypectomy; Argon plasma coagulation; Polypectomy

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INTRODUCTION

Endoscopic polypectomy and argon plasma coagulation for hemostasis of colonic vascular lesions are considered relatively safe procedures. Among complications that have been reported, gas explosion is rare, but its nature could be dramatic, as perforation could complicate colonic explosion and urgent surgery is needed.

Three factors are necessary to trigger an explosion of colonic gases: presence of combustible gases (hydrogen, methane) produced by the fermentation of non-absorbable carbohydrates in the colon by the colonic bacteria, presence of combustive gas (oxygen), and application of a heat source (electrocautery or argon plasma coagulation)^[1-3]. Five major components of gases have been identified in colon: nitrogen (23%-80%), oxygen (0.1%-2.3%), hydrogen (0.06%-47%), methane (0%-26%), and carbon dioxide (5.1%-29%). Only hydrogen and methane are combustible^[4]. They are produced in the colonic lumen from fermentation of non absorbable (e.g, lactulose, mannitol) or incompletely absorbed (lactose, fructose, sorbitol) carbohydrates by the colonic flora^[4-6]. Concentrations of hydrogen more than 4% and/or methane more than 5% are considered potentially explosive^[7]. Almost half of the patients (42.8%) with unprepared colon have potentially explosive concentrations of hydrogen and methane^[8]. Nevertheless, an explosion with these two gases can occur only if the oxygen concentration is over 5%^[9].

Following bowel preparation with a combination of clear liquids, cathartics, and enemas, mean concentration of hydrogen (0.024% ± 0.007%) and methane (0.0023% ± 0.001%) were below of their minimal explosive concentration^[4]. Thus, safety of therapeutic colonoscopy could be in part related to the quality of preparation before the procedure. An accumulation of colonic gas to potentially explosive concentrations due to poor colon preparation is considered an initiating factor in the complication of colonic gas explosion. Therefore, quality of bowel preparation as well as type of preparation and dietary restrictions are all essential for an uneventful therapeutic colonoscopy.

INDICATIONS FOR COLONOSCOPY WITH ELECTROCAUTERY

The main indication for application of electrosurgical energy is snare colonoscopic polypectomy with blended or pure coagulation current^[10]. Argon plasma has been successfully used for hemostasis of vascular ectasias, for ablation of intestinal polyps or residual adenomatous tissue after colonic polypectomy, and for the endoscopic therapy of postradiation colitis^[11].

Electrosurgical generators

Electrosurgical generators are used to supply electrical energy to endoscopic accessories. When electrical energy is introduced to tissue, it produces excitation of molecules,

which results in generation of heat^[12]. Electrosurgical generators may supply two types of circuits, monopolar and bipolar. Electrosurgical cutting is achieved by a high voltage (> 200 V) continuous current^[10].

Argon plasma coagulation

Argon plasma coagulation is a noncontact electrocoagulation device that uses high-frequency monopolar current conducted to target tissues through ionized argon gas^[10]. Argon plasma delivered through a flexible probe passed through the accessory channel and allows treatment of a large area quickly^[13]. In general, low power and low argon flow rates are used for hemostasis with settings of 40-50 W and 0.8 to 1.2 L/mn, whereas higher settings (70-90 W and over 1.2 L/mn) are used for tissue ablation^[14].

BOWEL PREPARATION

Bowel preparation with purgatives

The selection of purgative used for colon preparation is an important factor that makes the bowel safe for therapeutic procedures. Earlier than 1980's, mannitol was considered as the reference agent for colonic preparation. Explosions during therapeutic colonoscopy have been reported after mannitol preparation and its use is now avoided as cleansing colonic solution^[1-3,5,15-17]. Use of oral mannitol increased hydrogen and methane excretion^[3,5,9,18-19]. Aspiration of colonic gas at the time of colonoscopy showed that mean intracolonic hydrogen concentration was significantly higher after mannitol than after castor oil. Moreover, potentially explosive concentrations were present in 60% of patients given mannitol compared to 0%-20% of patients given castor oil^[3,19]. Fermentation of mannitol by *E.coli* is thought to be responsible for the production of potentially explosive gas mixture after oral mannitol preparation^[20-21]. Significantly higher counts of gas-producing *E.coli* were recovered from patients prepared with mannitol alone compared with mannitol preceded by oral antimicrobials^[20]. Therefore, the use of antibiotics prior to therapeutic colonoscopy could be a measure that would lower the load of intracolonic bacteria. An alternative approach to reduce the risk of explosion of colonic gases if mannitol preparation is used before electrocautery is insufflation of an inert gas such as carbon dioxide instead of air^[3,22].

Since 1990, a major progress occurred with new agents, such as polyethylene glycol electrolyte lavage solution (PEG-ELS) and oral sodium phosphate (NaP) solutions. Several studies agree that these agents provide a climate safe for electrocautery during colonoscopy by decreasing the concentrations of combustible gases^[14,23-26]. The highest hydrogen and methane concentrations after a PEG-ELS preparation are well below the combustible level^[26]. A recent report described a case of colonic gas explosion in a patient that underwent bowel preparation with a polyethylene glycol solution containing sorbitol^[27]. Sorbitol is an important carbohydrate that is daily included in humans diet. Studies have shown that the frequency of sorbitol malabsorption may be as high as 60% in healthy subjects^[3,28]. Thus, fermentation of this malabsorbed carbohydrate by colonic bacteria could result in raised combustible gas concentrations in the colon and explain the explosion that occurred in the above mentioned case report^[27].

Partial bowel preparation by enemas

For lesions that need electrocautery and are located up to the level of sigmoid colon, a flexible sigmoidoscopy with enema preparation is the procedure of choice by the majority of gastroenterologists. However, studies have reported cases of gas explosion in patients prepared by enemas^[29-31]. In these cases, the used enema did not contain any fermentable agents and the extension of colonic preparation was thought to be the initiating factor in this complications. Due to the partial colonic preparation, presence of residual stools above the lesions could enhance gas production and explain gas explosion.

In a prospective study, sixty patients were evaluated to compare the presence of the combustible gases hydrogen and methane during colonoscopy after a PEG-ELS preparation and flexible sigmoidoscopy after phosphosoda enemas alone^[32]. During colonoscopy, the concentrations of hydrogen and methane remained below combustible levels in all patients, whereas 10% of patients had combustible levels of either hydrogen or methane during flexible sigmoidoscopy. Patients had combustible levels even after air insufflation during sigmoidoscopy and the possibility of explosion was clinically significant. Another important observation of this elegant study was that even segments of colon with excess retained stools did not have combustible levels of these two gases. It seems that insufflation of air during colonoscopy equalizes the distribution of combustible gases, overcoming the compartmentation of the colon.

In a more recent study using argon plasma coagulation for hemorrhagic radiation proctitis, incidence of gas explosion was higher after local colon preparation (3/19 sessions) compared with oral preparation (0/53 sessions)^[31]. All three explosions after enema preparation occurred in patients with persistent solid stool above the coagulated lesions. Thus, the presence of stools could constitute the main risk for the colonic explosion.

REVIEW OF PUBLISHED STUDIES

A systematic review of the medical research published in English language from 1952 to October 2006 was performed, by using MEDLINE, SCOPUS, SCIRUS, and EMBASE to obtain studies published on colonic gas explosion. The search terms included were combinations of "colonic explosion" or "gas explosion" with "surgery", "electrocautery", "polypectomy", and "argon plasma coagulation".

A total of 20 cases of colonic gas explosion were identified (Figure 1). Eleven cases of gas explosion during surgery^[2,16,33-39] and 9 cases during colonoscopic procedures have been published. Argon plasma coagulation provided the initiating heat source in five of the nine colonoscopic cases^[29-31], whereas the remaining four cases were associated with endoscopic polypectomy^[1,15,17,27]. Recently, we also experienced a case of colonic explosion during an argon plasma coagulation procedure for postradiation colitis. Although seven successive, uneventful sessions of argon plasma coagulation were performed with enema preparation, gas explosion without colonic perforation occurred upon finishing the last procedure.

Nine of the 20 published cases (45%) of gas explosion

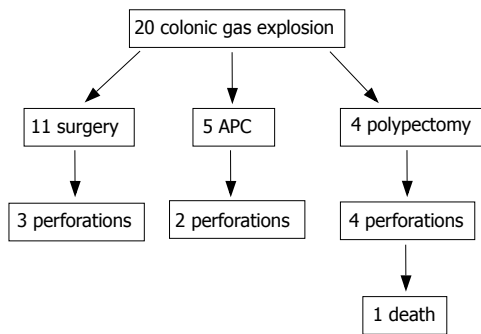


Figure 1 Flow chart of published cases with colonic gas explosion.

were complicated with colon perforation. Perforation was observed in all of the four polypectomy cases^[1,15,17,27], in two cases using argon plasma coagulation^[29,31], and in three cases during electrosurgery^[2,33-34]. One of the four perforations during polypectomy was fatal^[1].

Bowel preparation by ingestion of a mannitol solution was used in 14 cases and of a cleansing solution containing sorbitol in one case^[27]. Preparation by enemas containing no fermentable agent was used in all five cases treated with argon plasma coagulation for post-radiation colitis^[29-31].

CONCLUSION

Colonic gas explosion is a rare, but potentially serious complication during colonoscopy with electrocautery. Accumulation of colonic combustible gases at potentially explosive concentrations due to poor colon preparation is the cause of gas explosion. Cleansing purgatives (PEG, NaP) that make the bowel safe for electrocautery by decreasing the concentrations of the combustible gases are adequate for colon preparation. Argon plasma coagulation carries an increased risk of explosion during sigmoidoscopy following enemas, and it should only be performed after full bowel preparation.

In conclusion, we recommend the following to avoid colonic gas explosion during colonoscopy with electrocautery:

(1) Cleansing solution containing mannitol^[3,8,19] or other malabsorbed carbohydrates (e.g., sorbitol)^[6] should be avoided in the preparation of the colon since intracolonic concentrations of H₂ or/and CH₄ could be at combustible levels.

(2) During flexible sigmoidoscopy after standard enema preparation, concentrations of H₂ or/and CH₄ could be at explosive levels^[32].

(3) Argon plasma coagulation for post-radiation colitis should be performed only after complete bowel preparation with PEG or NaP, to avoid the risk of explosion^[31].

(4) Polypectomy with electrocautery should only be performed after full bowel preparation with PEG or NaP to prevent colonic explosion^[1,15,17,27].

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Effects and mechanisms of silibinin on human hepatoma cell lines

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Abstract

AIM: To investigate *in vitro* effects and mechanisms of silibinin on hepatocellular carcinoma (HCC) cell growth.

METHODS: Human HCC cell lines were treated with different doses of silibinin. The effects of silibinin on HCC cell growth and proliferation, apoptosis, cell cycle progression, histone acetylation, and other related signal transductions were systematically examined.

RESULTS: We demonstrated that silibinin significantly reduced the growth of HuH7, HepG2, Hep3B, and PLC/PRF/5 human hepatoma cells. Silibinin-reduced HuH7 cell growth was associated with significantly up-regulated p21/CDK4 and p27/CDK4 complexes, down-regulated Rb-phosphorylation and E2F1/DP1 complex. Silibinin promoted apoptosis of HuH7 cells that was associated with down-regulated survivin and up-regulated activated caspase-3 and -9. Silibinin's anti-angiogenic effects were indicated by down-regulated metalloproteinase-2 (MMP2) and CD34. We found that silibinin-reduced growth of HuH7 cells was associated with increased activity of phosphatase and tensin homolog deleted on chromosome ten (PTEN) and decreased p-Akt production, indicating the role of PTEN/PI3K/Akt pathway in silibinin-mediated anti-HCC effects. We also demonstrated that silibinin increased acetylation of histone H3 and H4 (AC-H3 and AC-H4), indicating a possible role of altered histone acetylation in silibinin-reduced HCC cell proliferation.

CONCLUSION: Our results defined silibinin's *in vitro* anti-HCC effects and possible mechanisms, and provided a rationale to further test silibinin for HCC chemoprevention.

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies related to a high mortality globally^[1,2]. Recent studies have noted a significant rise in the incidence of HCC in the United States in the past 2 decades^[2]. Less than 1% of HCC patients underwent a radical surgical resection in the US between 1974 and 1996^[3]. HCC's limited treatment remedies and the poor prognosis emphasize the importance in developing an effective chemoprevention for this disease.

Milk thistle (*Silybum marianum*) has been widely utilized as a folk remedy for liver diseases. It is a popular dietary supplement widely used in the United States and Europe^[4]. Silibinin is a polyphenolic flavonoid and the major biologically active compound of milk thistle^[4-6]. It is well known that milk thistle is safe and well-tolerated, and it protects the liver from drug or alcohol-related injury^[7,8]. Studies demonstrated silibinin's inhibitory effects on multiple cancer cell lines, including prostate^[9-12], colon^[13,14], skin^[15-17], bladder^[18,19] and lung cancers^[20]. Recently, we and Varghese *et al*^[21,22] reported silibinin's anti-HCC effects, but further studies are needed to define silibinin's inhibitory effects and mechanisms on human HCC cell growth.

Searching for non-invasive biomarkers is another important field of HCC chemoprevention. Plasma alpha-fetoprotein (AFP) has been used as a clinical marker for diagnosing and monitoring recurrent HCC^[23-25]. However, AFP's value in monitoring effect of HCC chemoprevention has not been tested before.

Phosphatase and tensin homolog deleted on chromosome ten (PTEN), phosphatidylinositol 3'-kinase (PI3K) and Akt (PTEN/PI3K/Akt) pathway has been associated with carcinogenesis^[26]. Activated PI3K-Akt signaling promotes carcinogenesis^[27,28]. PTEN is a negative regulator of PI3K-Akt signaling^[29] and one of the most frequently inactivated genes in malignancies^[30,31]. Akt is a downstream protein kinase of PI3K (PTEN) and is a signal transduction

protein that has been identified as one of the key elements in protecting cells from apoptosis. If unregulated, Akt promotes uncontrolled cell replication^[32,33]. It was reported that silibinin affects Akt expression in prostate cancer cells^[16], but it remains unknown whether silibinin affects HCC growth through a PTEN/PI₃K/Akt pathway in human liver cancer cells.

Histone acetylation modifies nucleosome structure that leads to DNA relaxation, reduces the affinity of histone complexes with DNA, and enhances the access of transcriptional factor to DNA^[34]. Accumulating evidence has indicated that alteration of histone acetylation plays an important role in carcinogenesis^[35,36], but it remains unknown whether it is associated with silibinin's anti-HCC effects.

In the present study, we demonstrated that silibinin significantly inhibited the growth of HuH7, HepG2, Hep3B, and PLC/PRF/5 human HCC cells that was associated with decreased Ki-67 expression, and cell cycle progression by arresting G₁-S transition, and promoted apoptosis. These effects of silibinin were associated with increased PTEN activity and decreased p-Akt production, indicating the role of PTEN/PI₃K/Akt pathway in silibinin-mediated anti-HCC effects. We also demonstrated that silibinin increased AC-H3 and AC-H4 expression, indicating that altered histone acetylation is involved in silibinin-reduced HCC cell proliferation.

MATERIALS AND METHODS

Reagents

The cell culture media were the same, as previously reported^[37,38]. Anti-activated caspase-3 antibody was purchased from Sigma Chemical Co. (St. Louis, MO). The antibodies against human Ki-67, AFP, p-Rb, E2F1, DP1, CD1, CDK4, p21 and p27, activated caspase-9, bcl-2, survivin, CD34, metalloproteinase (MMP)-2, MMP-9, phosphorylated-Akt^{Thr308}, PTEN, AC-histone3 and AC-histone4, and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). PTEN activity assay kit was from Biomol Research Laboratories, Inc (Plymouth Meeting, PA). An EIA kit for cell death detection was from Roche Applies Science (Indianapolis, IN).

Cell culture

Human HCC cell lines, HuH7, HepG2, PLC/PRF/5, and Hep3B cells^[37,38], were used in the present study. All the cells were cultured, as previously reported^[37,38]. The experiments were performed when cells reached about 80% confluence and cultured in FBS-free media for 24 h to synchronize the cell growth^[37,38].

Cell proliferation assay

Cell proliferation was determined using MTT assay, as previously reported^[37,38]. Briefly, the effects of silibinin on HCC cell growth were then determined after 24 h of incubation by optical density absorbance at 490 nm according to the manufacturer's instruction^[37,38].

Apoptosis assays

Apoptosis was determined in duplicate using an EIA kit for cell death detection, as previously reported^[37,38].

Immunoprecipitation (IP) and immunoblot (IB) assays

After 24 h of treatment with silibinin at 25% inhibitory concentration (IC₂₅) or IC₅₀ dose, the cell pellets were lysed and the supernatants were used to detect Ki-67, AFP, p-Rb, E2F1, CD1, CDK4, p21^{waf1/cip1}, p27^{kip1}, bcl-2, survivin, activated caspase-3 and caspase-9, CD34, MMP-2, MMP-9, phosphorylated-Akt^{Thr308}, PTEN, and AC-H3 and AC-H4. The IP assays were same, as previously reported^[37,38]. β -actin was used as an internal control. The relative amount of each protein was quantified by digitally scanning its hybridizing bands, as previously reported^[37,38].

PTEN activity assay

PTEN protein was immunoprecipitated with 10 μ L of rabbit anti-human antibody at 4°C overnight, followed by addition of 25 μ L of anti-rabbit IgG-conjugated agarose beads for 2 h at 4°C, washing and centrifugation. The phosphatase reaction was performed in 50 μ L of assay buffer containing 200 μ mol/L water-soluble diC₈-PIP₃ and the immunoprecipitated PTEN protein. The release of phosphate from the substrate was measured in a colorimetric assay using the Biomol Green Reagent (Plymouth Meeting, PA)^[39]. The OD absorbance at 650 nm was recorded in an ELISA plate reader^[37,38].

Statistical analysis

The descriptive statistics was provided with mean \pm SD. A repeated-measure ANOVA test was used to assess dose dependent effects of silibinin on HuH7, HepG2, Hep3B, and PLC/PRF/5 cells. An independent sample *t*-test was used to assess the effects (i.e., mean differences) of silibinin treatment on apoptosis, and IB results. A *P* value < 0.05 was considered statistically significant.

RESULTS

Potent dose-dependent anti-proliferative effects of silibinin on human HCC cells

Effects of silibinin were initially assessed in HuH7 cells by MTT assay. As shown in Figure 1A, silibinin resulted in a dose-dependent inhibition of HuH7 cell growth. Compared to the control, there was a dose-dependent inhibitory which became significant at the dose greater than 180 μ mol/L (*P* < 0.05). As shown in Figure 1B, silibinin also significantly inhibited the growth of HepG2, Hep3B, and PLC/PRF/5 human HCC cell lines, indicating a wide spectrum of silibinin's inhibitory effects on human HCC cell growth, as previously reported^[21,22]. Because the HuH7 cell line is one of the most commonly used human HCC lines^[37,38], it was then used to further determine silibinin's anti-HCC effects and mechanisms. For further characterization of dose-related mechanistic effect of silibinin, approximate IC₂₅ (i.e., 120 μ mol/L) and IC₅₀ (i.e., 240 μ mol/L) concentration were subsequently used for the remainder of the study.

Ki-67 is a commonly used biomarker for cell proliferation^[40]. Consistent with the data derived from MTT assay, silibinin treatment resulted in a significantly dose-dependent decrease in Ki-67 expression, as shown in Figure 2B (*P* < 0.05). These data further demonstrated

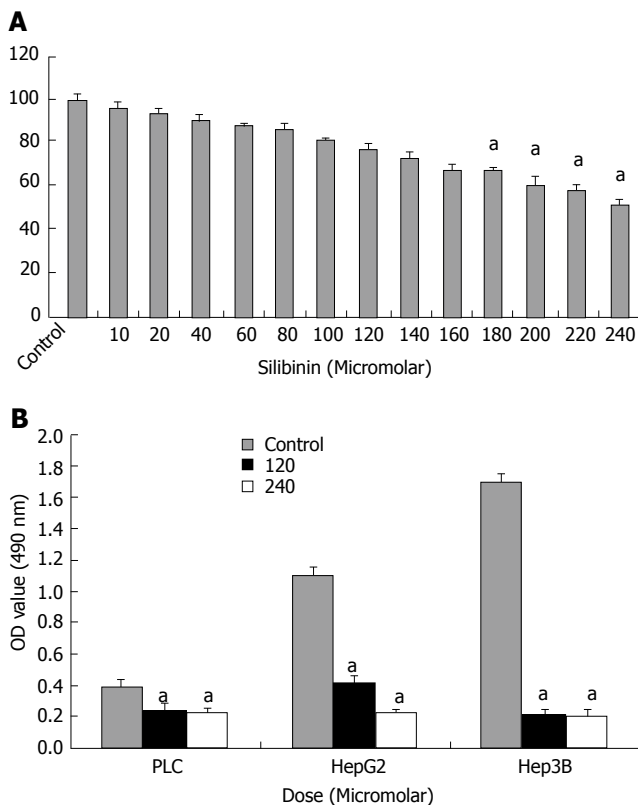


Figure 1 Silibinin's effects on growth of human hepatoma cells. **A:** MTT assay showed silibinin's dose dependent anti-proliferative effects on HuH7 cells. A significant decrease in proliferation compared to control was noted from silibinin $\geq 180 \mu\text{mol/L}$. The IC_{25} is determined to be $120 \mu\text{mol/L}$ and the IC_{50} is determined to be $240 \mu\text{mol/L}$; **B:** Silibinin's effects on other human HCC cell growth. PLC/PRF/5, HepG2, and Hep3B HCC cells were treated with silibinin at IC_{25} and IC_{50} doses for HuH7 cells. Silibinin significantly reduced growth of all three HCC cells in different rates. ^a $P < 0.05$ vs control.

silibinin's significant dose-dependent anti-proliferative effects on human HCC cells.

Effects of silibinin on cell cycle progression

Uncontrolled progression of the cell cycle promotes growth of cancer cells^[41]. A major activity of the CD1/CDK4 complex is to initiate phosphorylation of retinoblastoma (Rb) that then fails to maintain its binding to E2F1, and thus releases the transcription factor to promote cell cycle progression^[42]. Previous studies on other cancer cell lines showed a significant inhibitory effect of silibinin on the cell cycle progression^[11-13]. In the present study, we found that silibinin resulted in a significant dose-dependent inhibition of CD1/CDK4 complex that was associated with reduced Rb phosphorylation and, E2F1/DP1 complex in HuH7 cells ($P < 0.01$), as shown in Figure 2C-E.

By binding to the cyclin/CDK complexes, cyclin dependent kinase inhibitors (CDKIs), such as p21 and p27, halt uncontrolled cell proliferation^[43]. As noted in previous studies on other cancer cell lines^[12-14], we demonstrated that silibinin not only significantly increased p21 and p27 expression ($P < 0.01$), but also increased formation of p21/CDK4 and p27/CDK4 complexes (Figure 2F-I) in a dose-dependent fashion. Thus, our results demonstrate

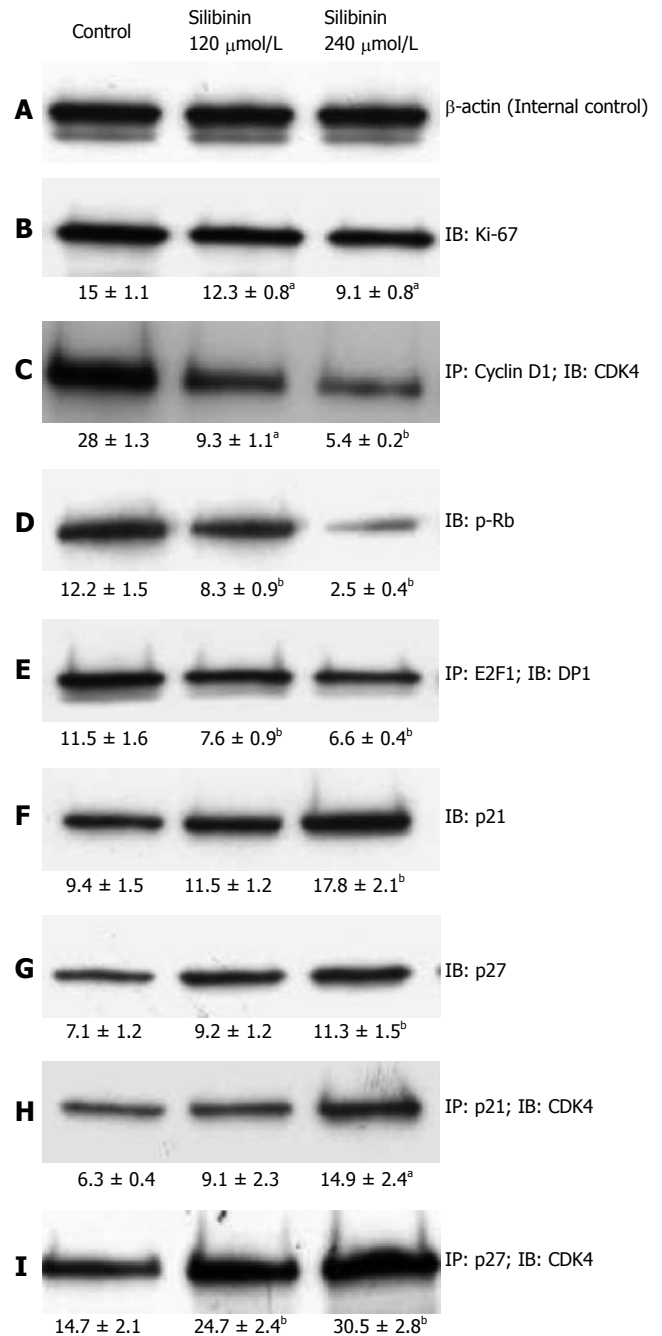


Figure 2 Silibinin's effects on proliferation of HCC cells. After HuH7 cells were treated with silibinin at IC_{25} and IC_{50} doses, immunoprecipitation (IP) and immunoblots (IB) were performed in triplicate for each specimen. A mean densitometer reading was expressed in the respective box and used for statistical analysis. **A:** β -actin for internal control; **B:** Ki-67; **C:** CD1/CDK4 complex; **D:** p-Rb; **E:** E2F1-DP1 complex; **F:** p21^{Waf1/Cip1}; **G:** p27^{Kip1}; **H:** p21^{Waf1/Cip1}/CDK4 complex; **I:** p27^{Kip1}/CDK4 complex. ^a $P < 0.05$, ^b $P < 0.01$, vs control.

silibinin inhibits the growth of human hepatoma cells through inhibiting CDK activity.

Effects of silibinin on AFP production and secretion from HuH7 cells

As shown in Figure 3B, compared to untreated HuH7 cells, silibinin at the dose of $120 \mu\text{mol/L}$ resulted in a significant decrease in AFP production in HuH7 cells ($P < 0.05$) that was associated with a reduced AFP level in the culture medium (Figure 3C, $P < 0.05$).

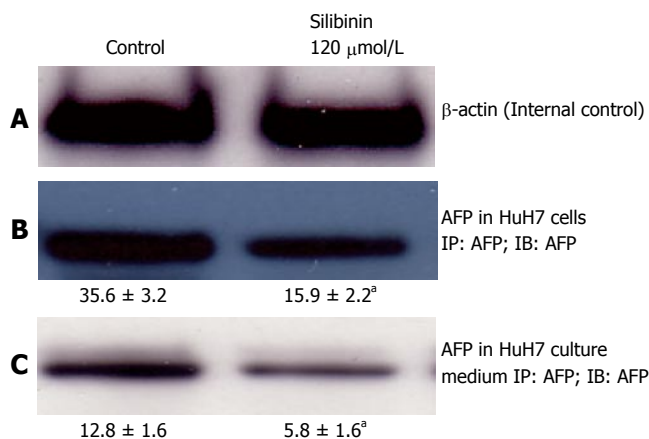


Figure 3 Silibinin's effects on AFP production and secretion from HuH7 cells. **A:** β -actin for internal control; **B:** Silibinin at IC₂₅ dose decreased AFP production in HuH7 cells; **C:** Silibinin at IC₂₅ dose decreased AFP secretion from HuH7 cells. ^a $P < 0.05$ vs control.

Effects of Silibinin on Apoptosis

Apoptosis is another important regulatory step in controlling cancer cell proliferation^[4]. Studies indicated that silibinin induces apoptosis in several malignant cell lines^[13,14,18], but such effects have not been tested in human hepatoma cells. We demonstrated silibinin dose-dependently increases of apoptosis in HuH7 cells, as shown in Figure 4A ($P < 0.01$). To understand the mechanisms of silibinin-induced apoptosis, we examined the expression of Bcl-2, survivin, and activated caspase-3 and 9. Our results showed that silibinin-induced apoptosis did not alter bcl-2 expression (data not shown), but resulted in a dose-dependent inhibition of survivin expression (Figure 4C, $P < 0.01$) that was associated with increased levels of activated caspases-3 and -9 (Figure 4D and E, $P < 0.01$).

Possible effects of silibinin on angiogenesis

In previous studies, silibinin has been reported to inhibit angiogenesis in non-HCC cancer cell lines^[13]. To evaluate whether silibinin affects angiogenesis in human HCC cells, we measured the expression of CD34, a transmembrane glycoprotein on vascular cells associated with angiogenesis^[44], and MMP-2 and MMP-9, which are markers associated with angiogenesis as well as metastatic invasion^[45]. As shown in Figure 5B, silibinin at IC₅₀, but not IC₂₅ dose decreased the expression of CD34 ($P < 0.01$). At the higher dose, Silibinin also resulted in decrease of MMP-2 (Figure 5C, $P < 0.01$), but not MMP-9 (data not shown) in HuH7 cells.

Effects of silibinin on PTEN/PI3K/Akt pathway

It has been reported that the PTEN/PI3K/Akt pathway is involved in cancer growth^[16,46]. As shown in Figure 6B, silibinin-reduced HuH7 cell proliferation was associated with a dose-dependent decrease in p-Akt ($P < 0.01$) in these cells. PTEN is an upstream negative regulator of Akt. It was reported that altered PTEN expression or activity is associated with the pathogenesis of HCC^[47-49]. In the present study, we found that silibinin at IC₅₀ dose did not significantly change PTEN expression (data not shown), but significantly increased PTEN activity (Figure 6E). These results suggested that the PTEN/PI3K/Akt pathway is involved in silibinin-reduced growth of human HCC cells.

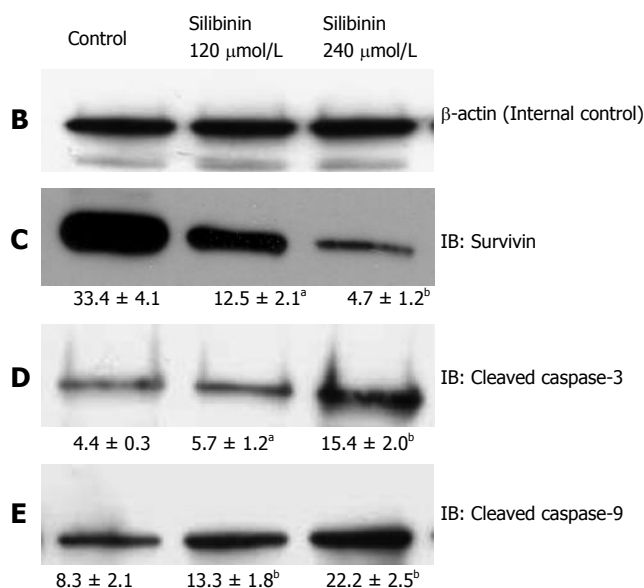
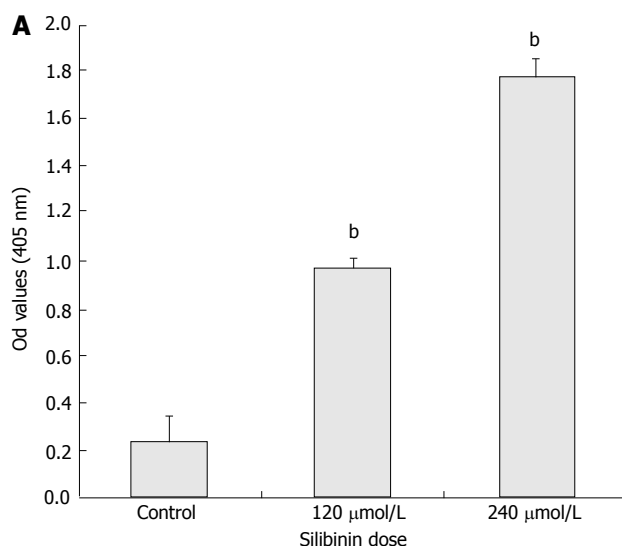


Figure 4 Silibinin's effects on apoptosis in HuH7 cells. **A:** Silibinin at IC₂₅ and IC₅₀ doses significantly promoted apoptosis of HuH7 cells; **B:** β -actin for internal control. Silibinin at IC₂₅ and IC₅₀ doses decreased survivin expression (**C**), but increased activated caspase-3 (**D**), and activated caspase-9 (**E**). ^a $P < 0.05$, ^b $P < 0.01$, vs control.

Effects of silibinin on AC-H3 and AC-H4 expression

We then examined the association of AC-H3 and AC-H4 expression with silibinin-reduced HCC cell growth. Our results demonstrated that silibinin-reduced HuH7 cell growth was associated with increased AC-H3 and AC-H4 expression (Figure 6 C and D, $P < 0.05$). These results suggest that increased AC-H3 and AC-H4 expression may play an important role in silibinin-reduced HCC growth.

DISCUSSION

Searching for an effective chemoprevention of HCC has been an active field of research. Silibinin is a polyphenolic flavonoid and the major biologically active compound of milk thistle. It is well known that milk thistle is safe and well tolerated, and it protects the liver from drug or alcohol-related injury^[7,8]. Recent demonstration of silibinin's anti-HCC

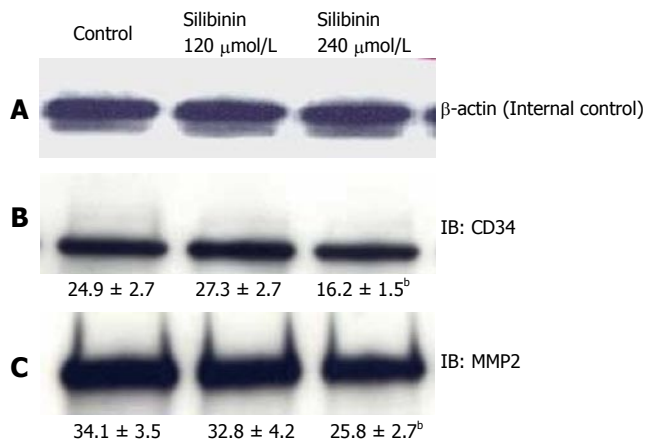


Figure 5 Silibinin's effects on angiogenesis in HuH7 cells. **A:** β-actin for internal control. Silibinin decreased CD34 (**B**), and MMP-2 (**C**). ^b*P* < 0.01 vs control.

effects^[21,22] provided us with a rationale to further define the related effects and mechanisms of HCC chemoprevention. In the present study, we examined the effects and mechanisms of silibinin on growth of human HCC cells.

Using MTT assay^[37,38], we demonstrated that silibinin treatment resulted in a potent inhibition of four different human HCC cell lines, indicating its broad spectrum of anti-HCC effects. We also revealed silibinin's linear dose-dependent inhibition of HuH7 cell growth. Silibinin at IC₂₅ and IC₅₀ doses for HuH7 cells also resulted in reduced growth of HepG2, Hep3B, and PLC/PRF/5 cells, confirming the previous reports^[21,22]. These results promote us to further test silibinin for HCC chemoprevention.

Both PCNA and Ki-67 are biomarkers for cell proliferation^[40]. Singh *et al*^[11] reported silibinin significantly decreases PCNA and Ki-67 expression in nude mice bearing xenografts of human prostate cancer. Consistent with this, we have demonstrated that silibinin significantly reduced Ki-67 expression in HuH7 cells in a dose-dependent fashion. These suggest that silibinin reduces growth of human HCC cells by down regulating their proliferation.

AFP is associated with HCC differentiation and has been widely used for diagnosing HCC and assessing treatment effects or recurrence of HCC in humans^[24,25]. Our results showed that silibinin treatment resulted in significant decrease in AFP production and secretion that was well correlated with growth inhibition of HuH7 cells. These findings suggest silibinin may promote HCC cell differentiation, and AFP may serve as a non-invasive biomarker to determine silibinin's *in vivo* anti-HCC effects.

An uncontrolled G₁-S progression results in continued proliferation with potential malignant transformation and carcinogenesis. Increased CDK4/CD1 complex enhances Rb phosphorylation that results in release of E2F1 from p-Rb/E2F1 complex and promotes E2F1/DP1 complex formation and stimulates cell cycle progression^[42]. Tyagi *et al*^[10] reported that silibinin causes a significant decrease in p-Rb in human prostate cancer cells. Our results indicate that silibinin-inhibited CDK4/CD1 complex formation is one of the important steps that inhibit Rb phosphorylation, followed by reduction of E2F1/DP1 complex formation.

CDKs are important regulators of the activity of the CD1/CDK4 complex. By binding to the cyclin/CDK

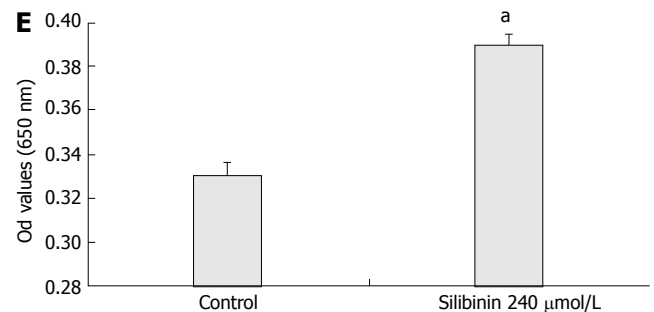
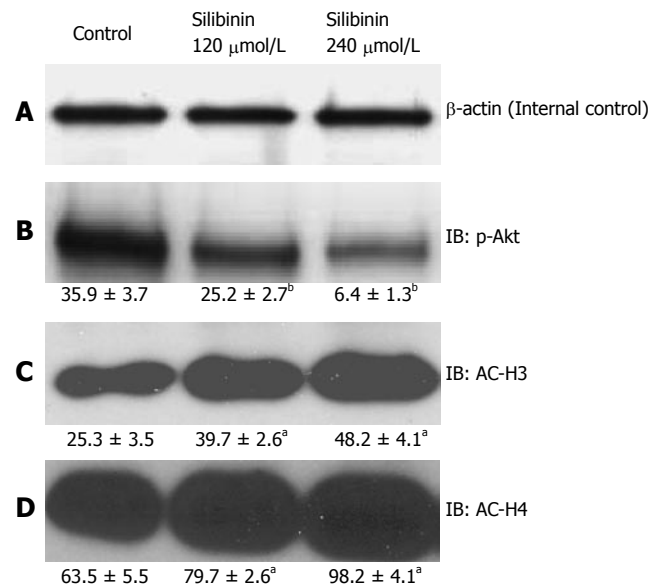


Figure 6 Silibinin's Effects on PTEN, p-Akt, AC-H3 and AC-H4 in HuH7 Cells (**A**). β-actin for internal control. Silibinin significantly decreased p-Akt (**B**); increased AC-H3 (**C**); AC-H4 (**D**). Silibinin at 240 μmol/L significantly increased PTEN activity in HuH7 cells (**E**). ^a*P* < 0.05, ^b*P* < 0.01, vs control.

complexes, two very important CDKs, p21 and p27, inhibit their activities. Varghese, *et al*^[22] reported that silibinin increases levels of p27 in human hepatoma cells. Silibinin was also reported increasing expression of p21 in several non-HCC cancer cells^[14]. In the present study, we demonstrated that silibinin resulted in a significantly dose-dependent increase in both p21 and p27, which was well correlated with their respective binding to CDK4, the bioactive forms of these CDKs. Taken together, our data demonstrated that silibinin reduces cell cycle progression in human hepatoma cells by arresting G₁-S transition that involves a comprehensive signaling of cell cycle modulators.

Previous data on other cancer cell lines have demonstrated that silibinin has effects on the apoptotic control^[10,14,21]. We demonstrated that silibinin causes a significant increase in apoptosis of HuH7 cells which was associated with decreased survivin expression and increased activated caspase-3 and -9. Because survivin can bind with caspases^[19,50,51], our results suggest that silibinin-induced apoptosis of HuH7 cells is mediated by decreased survivin that results in increased caspase-3 and 9 activation.

Angiogenesis is an important aspect of cancer invasion and survival. CD34 is a valuable marker to demonstrate this issue^[44]. A previous study demonstrated that silibinin decreases angiogenesis in colon cancer cells^[15]. In the present study, we revealed that silibinin at IC₅₀ decreased CD34

protein expression. MMP-2 and MMP-9 have been used as markers for angiogenesis and malignant invasion^[45]. It was reported that silibinin resulted in a significant decrease in MMP-2, but not MMP-9 levels in human lung cancer cells^[20]. In the present study, we found that silibinin also resulted in a dose-dependent and significant decrease in MMP-2, but not MMP-9 expression. Although our data suggest that silibinin may reduce angiogenesis in human HCC cells, further *in vivo* studies with quantification of microvessel density^[52] will be needed to validate these findings.

There is growing evidence of PTEN/PI3K/Akt pathway in hepatocarcinogenesis^[27,32,47-49]. PTEN is a tumor suppressor gene and the deletion or inactivation of this gene has been described in a variety of cancer cell lines^[30,33,53]. As a result, the tumor suppressive properties of PTEN relates in part to its ability to down-regulate the Akt pathway and thus inhibit cell proliferation^[32,33]. Paramio *et al*^[54] showed that PTEN decreases p-Rb and resulted in down-regulation of CD1. Furthermore, Weng *et al*^[55] demonstrated through the use of breast cancer cells that PTEN also up-regulates p27 and down-regulates CD1. However, it remains to be determined whether PTEN/PI3K/Akt pathway is involved in silibinin-reduced growth of cancers. In the present study, we found that silibinin significantly increased PTEN activity in association with decreased p-Akt in HuH7 cells. Since silibinin treatment also resulted in significant decrease of p-Rb and proliferation in these cells, it is evident that silibinin alters PTEN activity to assist in cellular growth control through downstream regulation of Akt and also possibly in promoting the up-regulation of p27 and the down-regulation of both p-Rb and CD1 as suggested by previous studies^[54]. It was also reported that overexpression of PTEN reduces survivin expression^[56]. We found that silibinin-mediated increase in PTEN activity and decrease in p-Akt was associated with decreased survivin expression and enhanced apoptosis in HuH7 cells. These data support the notion that PTEN/PI3K/Akt pathway may mediate cancer cell apoptosis by modulating surviving expression, and silibinin may play an important role in this interaction. Additional studies will be needed to further detail the role of PTEN/PI3K/Akt signaling in silibinin-reduced growth of human HCC cells.

Histone acetylation alters chromatin conformation by making promoter regions more accessible to transcription factors and permissive to transcriptional activation^[34]. Studies have reported that histone acetylation is involved in cell proliferation, differentiation, and cell cycle regulation^[34]. Decrease in acetylation status in the cell is associated with carcinogenesis^[35,36]. Our results demonstrated that silibinin-reduced HuH7 cell growth was significantly associated with increased AC-H3 and AC-H4 expression, suggesting that increased histone acetylation may mediate silibinin-reduced HCC growth. Our findings not only indicate silibinin's novel anti-cancer mechanisms, but also provide additional targets for searching new agents for HCC chemoprevention.

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LIVER CANCER

Survivin expression in early hepatocellular carcinoma and post-treatment with anti-cancer drug under hypoxic culture condition

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Abstract

AIM: To investigate the expression of survivin during the early stages of hepatocellular carcinoma (HCC).

METHODS: Immunohistochemical expression of survivin in liver tumor and non-tumor tissue specimens taken from 17 patients was compared. In addition, to determine the survivin expression in response to anti-cancer drugs in early stage HCC, the survivin expression was determined after the treatment of the HCC cells with anti-cancer drugs under hypoxic culture conditions.

RESULTS: Survivin proteins were expressed in 64.7% of cells in early HCC specimens. A correlation between the survivin expression rate in the peritumoral hepatocytes and the rate of expression in the HCC specimens (low-rate group vs high-rate group) was observed. The survivin protein concentration in HCC cells was increased by the combination of hypoxia and anti-cancer drugs.

CONCLUSION: This study suggests that survivin could be used as a therapeutic target in early HCC.

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Key words: Survivin; Hepatocellular carcinoma; Hypoxia

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INTRODUCTION

Hepatocellular carcinoma (HCC) is a major health problem worldwide. There are more than 500 000 new cases diagnosed each year, with an age-adjusted incidence of 5.5-14.9 per 100 000 people^[1]. In some areas of Asia and the Middle East, HCC ranks as the most frequent cancer-related cause of death^[2]. The incidence of HCC is also increasing in Europe and the United States^[3]. A more effective therapy thus needs to be developed from early stages.

Survivin is a member of a family of inhibitors of apoptosis protein (IAP), which has been implicated in both the control of cell division and the inhibition of apoptosis. Specifically, its anti-apoptotic function is associated with the ability to directly or indirectly inhibit caspases. By inhibiting apoptosis and promoting mitosis, survivin facilitates cancer cell survival and growth^[4-8]. Survivin is selectively expressed in the most common human neoplasms and appears to be involved in tumor cell resistance to some anticancer agents and ionizing radiation^[9].

Several preclinical studies have demonstrated that the down-regulation of survivin expression/function by the use of anti-sense oligonucleotide, dominant negative mutants, ribozymes, small interfering RNAs and cyclin-dependent kinase inhibitors increased the rate of apoptosis, reduced tumor growth potential and sensitized tumor cells to various chemotherapeutic drugs and γ -irradiation in *in vitro* and *in vivo* models of various types of human tumors^[9]. Moreover, YM155 is the first agent designed to inhibit survivin. Some early phase clinical studies demonstrated that this novel anticancer agent was well tolerated and shrank tumors in some patients with non-Hodgkin lymphoma and hormone-refractory prostate cancer that recurred after conventional chemotherapy. In addition, interim reports indicate that there are few side effects.

These results suggest the possible efficacy of the survivin inhibitor on HCC. It may be effective for patients with early stages of HCC. Survivin is expressed in HCC^[10]. However, the expression during the early stages of HCC has not been characterized pathologically. In addition, previous results have shown that survivin gene transcription is increased in hypoxic tumor cells^[11]. The well-differentiated HCC has portal blood flow and is not hypervascular^[12]. In order to compare the expression of

survivin and the efficacy of anti-cancer drugs, HCC cells were cultured in a hypoxic environment.

MATERIALS AND METHODS

Patients

The study population included 17 patients (11 men and 6 women; median age 68 years, range 56-81 years) who underwent a tumor biopsy between January 2004 and December 2005 in the Jikei University Daisan Hospital, Tokyo, Japan (Table 1). All patients underwent biopsies to confirm the diagnosis of HCC. These tissue specimens were examined retrospectively. This study was approved by the Jikei University Ethics Committee Institutional Review Board.

Pathologic specimens

Tumor specimens were obtained by tumor biopsies with a 21-G fine-needle aspiration kit. Non-tumorous liver tissue specimens were concurrently obtained by an 18-20-G needle liver biopsy. Formalin-fixed, paraffin-embedded specimens of liver tumor and non-tumor tissues were processed for conventional histological assessment by hematoxylin and eosin (H&E) staining. The tumors were histologically graded as well or moderately differentiated.

Immunohistochemical analysis

For the immunohistochemical analysis, formalin-fixed, paraffin-embedded specimens were used after deparaffinization. A rabbit anti-human survivin polyclonal antibody (Diagnostic BioSystems, USA) was used at dilution of 1:2000 as the primary antibody, which was detected with ENVISION + Rabbit/HRP (Dako, Japan). The specimens were heated in a microwave oven containing antigen retrieval solution (10 mmol/L citrate buffer, pH 6.4) at 121°C for 15 min for the retrieval of the antigens and then cooled to room temperature. 3, 3-Diaminobenzidine and hematoxylin were used for color development and counterstaining, respectively. Cells with brown-colored nuclei were regarded as positive. The mean percentage of survivin-positive HCC cells was determined in three areas at 100 × magnification with the nuclear labeling index (labeled nuclei/500 nuclei). The same method was performed for hepatocytes in non-tumorous biopsy specimens.

HCC cell line cultured in the combination of hypoxia and anti-cancer drugs environment

Human hepatocellular carcinoma cell line FLC-7 was cultured with RPMI-1640 (Invitrogen, Carlsbad, CA) medium supplemented with 100 mL/L heat-inactivated fetal bovine serum (FBS) under conventional conditions at 37°C in a humidified atmosphere containing 50 mL/L CO₂^[13] until the cells were 70%-80% confluent. Cells were then used for culture under hypoxic conditions employing the AnaeroPack for cell (Mitsubishi Gas Chemical Co., Tokyo, Japan) packaging device. The cells were sealed tightly and incubated at 37°C for either 6 or 96 h. In addition, for the anti-cancer drug therapy, the cells were cultured continuously with 0.1 μmol/L farfomycin (EPI)

Table 1 Characteristics of the patients undergoing tumor biopsies (*n* = 17)

Features	Values
Age, yr	68 (56-81)
Sex (Male/Female)	11/6
AFP (ng/mL)	21 (3-444)
HBsAg/HCVAb	3/14
Tumor size (mm)	15 (8-23)
Cirrhosis (positive/negative)	5/12
Differentiation (well/moderate)	11/6

Data are expressed as the medians with ranges in parentheses unless indicated otherwise. HBsAg: Anti-hepatitis B surface antigen; HCVAb: Anti-hepatitis C antibody; Well: Well-differentiated HCC; Moderate: Moderate-differentiated HCC; Normal ranges: AFP (alpha-feto protein) > 20 ng/mL.

Table 2 PCR primer sequences

Name	Forward sequence (5'-3')	Reverse sequence (5'-3')
Survivin	GCCAGTGTTCTCTCT GCTT	GCACITTCITCGCAGT TTCC
β-actin	AGCCATGTACGTAGC CATCC	AAGTGGTGGTGTGCGAC TCTC

containing growth medium for 6 or 96 h. The cytotoxicity (IC₅₀) with EPI of FLC-7 cells determined the medication concentration (Normoxia cultured for 96 h, data not shown).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from the cells using the RNeasy kit (Qiagen, Hilden, Germany). The mRNA was reverse transcribed into cDNA using the Prime script (TAKARA BIO INC, Shiga, Japan). The specific cDNA target sequences for survivin were amplified by a PCR reaction mixture consisting of 1 μL cDNA template, 10 μmol/L each primer (Primer sequences are listed in Table 2), PCR Master Mix (Go taq, Promega, Madison, WI, USA). The PCR conditions were: initial pre-denaturation at 95°C for 5 min; 30 amplification cycles of denaturation at 95°C for 30 s, primer annealing at 55°C for 30 s, and elongation at 72°C for 60 s; and a final extension at 72°C for 5 min. PCR products were analyzed on a 20 g/L agarose/TBE gel electrophoresis and compared to the expression of β-actin as a housekeeping gene.

Western blotting

The harvested cells cultured in either a normal or hypoxic environment for 96 h with or without 0.1 μmol/L of EPI were washed with ice-cold PBS and lysed in ice-cold 5 mL/L Triton X-100 containing 10 mmol/L EDTA. The cell lysate was centrifuged at 15000 *g* for 5 min and the supernatant was used for Western blotting. Thirty micrograms of protein was separated on 150 g/L polyacrylamide gels and transferred onto 0.2-μm nitrocellulose membranes by wet blotting (20 mA for 60 min). Membranes were blocked with blocking buffer (1 × TBS, 1 g/L Tween-20, 1 g/L casein gelatin) for 0.5 h at 37°C and stained with the specific antibody for survivin

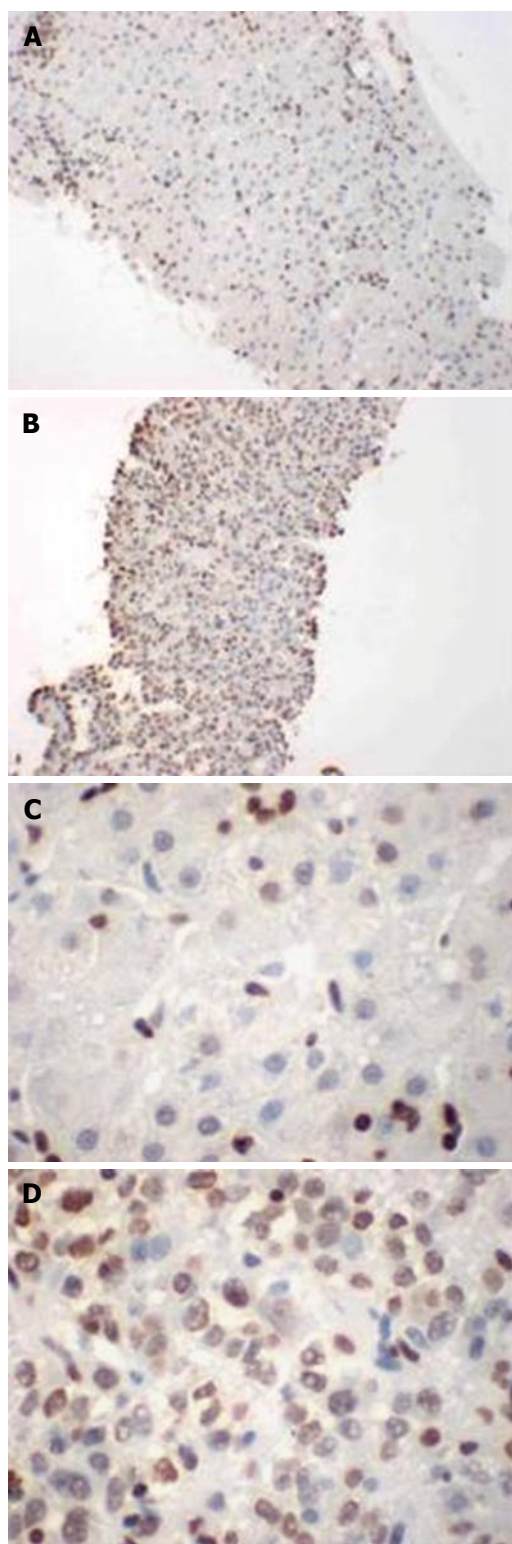


Figure 1 Immunopathological staining of survivin in HCC and peritumoral biopsy tissues. A: Non-tumor biopsy (x 100); B: Tumor biopsy (x 100); C: Non-tumor biopsy (x 400); D: Tumor biopsy (x 400).

(1:1000; Novus Biologicals, Littleton, USA). The complex of antigen with the primary antibody was completely labeled with the secondary antibody, anti-rabbit IgG alkaline phosphatase conjugate (1:2000; Sigma-Aldrich Japan, Tokyo, Japan). The survivin band was visualized with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (Sigma-Aldrich Japan, Tokyo, Japan).

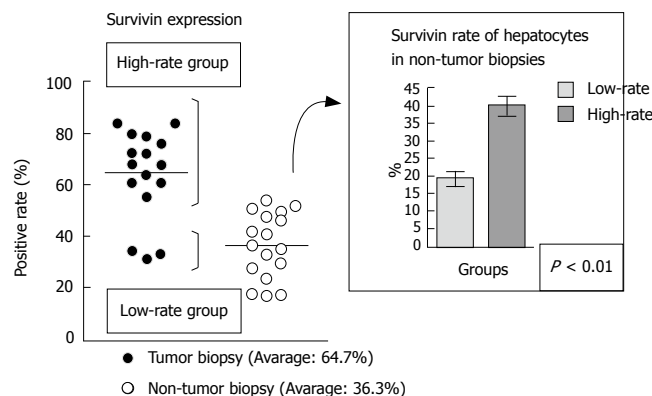


Figure 2 Nuclear survivin expression rates in HCC biopsies and non-tumor biopsies samples. In tumor biopsies, > 500 survivin-expressing HCC cells were counted in three areas at 100 x magnification using the nuclear labeling index. In non-tumor biopsies, > 500 survivin-expressing hepatocyte cell were counted in three areas at 100 x magnification using the nuclear labeling index.

Protein determination

Protein concentration was assayed by a Bio-Rad protein assay kit (Bio-Rad Lab., Tokyo, Japan) using BSA as the standard.

Statistical analysis

Statistical analyses were performed using the Wilcoxon-Mann-Whitney two-sample rank-sum test. A P value less than 0.05 was considered statistically significant.

RESULTS

Rates of survivin expression in early HCC and in non-tumorous liver tissues

In HCC tissues, the rate of survivin expression was determined by counting survivin-positive cancer cells (Figure 1). The average survivin expression rate was 64.7% (median). The samples with a survivin expression rate over 50% were regarded as high-rate group, while the three samples with a survivin expression rate under 50% were regarded as low-rate group (Figure 2). In early-stage HCC with a tumor size > 10 mm ($n = 3$), the expression rate ranged from 67.7% to 83.7%. The expression rate of survivin in HCC had no significant correlation to the level of differentiation of HCC. In non-tumorous liver tissues, survivin expression rates were counted in all hepatocytes. The average of survivin expression was 36.3% (median, Figure 2). A correlation between the survivin expression rate in peritumoral cells and the rate of expression in the HCC specimens (low-rate group vs high-rate group) was observed. A significant difference in survivin expression in the hepatocyte was observed between the high-rate group and low-rate group ($P < 0.01$, Figure 2).

Survivin expression of HCC cells in hypoxic conditions and post-treatment with anti-cancer drugs

The hypoxic environment increased the survivin mRNA expression in both short-term and long-term cultures (Figure 3). Under both normoxia and hypoxia, the survivin mRNA concentrations increased in the presence of anti-cancer drugs in the short-term culture.

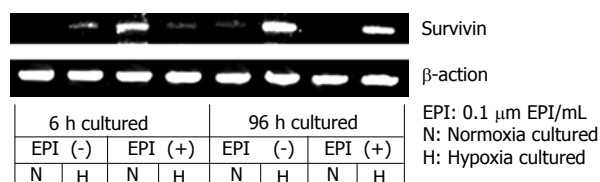


Figure 3 Expression of survivin mRNA in a hypoxic and anti-cancer drug-containing medium. The survivin mRNA expression increased under hypoxia, by anti-cancer drug treatment, and in presence of the both conditions in the 6-h culture. The survivin mRNA expression increased under hypoxia and in the combined conditions of hypoxia and anti-cancer drug in the 96-h culture.

Nevertheless, the survivin mRNA concentrations only increased in the combination of hypoxic culture and anti-cancer drugs in the long-term culture (Figure 3). No survivin protein expression was observed in the hypoxia culture (Figure 4). In contrast, the survivin protein concentration increased with the anti-cancer drug concentrations. Moreover, the survivin protein concentrations increased when cultured in a combination of hypoxia and anti-cancer drugs (Figure 4).

DISCUSSION

The suppression of apoptosis is thought to contribute to carcinogenesis due to several mechanisms, including unusually prolonging the cellular lifespan, facilitating the accumulation of gene mutations and permitting growth factor-independent cell survival^[14]. In addition, since the host's immune system normally eliminates cancer cells by induction of apoptosis, inhibition of this process is critical for cancer cells survival. Several proteins, including the bcl-2 family and the IAP family, are involved in the inhibition of apoptotic signaling^[15-16]. Survivin, a novel member of the IAP family, inhibits the activation of caspase-3 and -7, which are downstream effectors of apoptosis, in cells exposed to apoptotic stimuli^[17-20]. Previous studies have shown that survivin is expressed at a high level in 60%-100% of the most common human tumor types, including colon, pancreas, breast, lung, liver, brain, lymphoma, melanoma and prostate cancers^[21-24]. The elevated expression of survivin is associated with poor patient survival^[25-28]. In the present study, positive nuclear survivin expression was observed in all tumor biopsy samples. It is possible that this result was based on dyeing conditions and the nuclear labeling index. The differential nuclear and cytoplasmic localization of survivin has been shown to be due to differences in the amino-acid sequence of its carboxy-terminal domain^[29]. In HCC, the predominant function of survivin is its cell cycle nuclear distribution, and not the cytoplasmic caspase-3-dependent anti-apoptotic effect^[30]. So in HCC, the prognostic significance of survivin immunostain relates to the cell cycle in nuclei, and not to its cytoplasmic anti-apoptotic effect^[31]. These reports suggest that most cytoplasm in the early HCC samples might be stained moderately under the conditions employed in this study.

In the present study, nuclear survivin is expressed in 64.7% of cells (median) from early-stage HCC specimens.

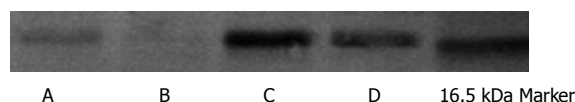


Figure 4 Western blotting showing the expression of survivin protein in the combined conditions of hypoxia and anti-cancer drugs in the 96-h culture. Survivin expression increased under anti-cancer drug-containing medium. Moreover, survivin further increased after the administration of a combination of hypoxia and anti-cancer drug. A: Normoxia; B: Hypoxia; C: Normoxia + 0.1 μ mol/L EPI; D: Hypoxia + 0.1 μ mol/L EPI.

Moreover, in all early HCCs of tumor size > 10 mm ($n = 3$), survivin expression was always above the median average. These data indicate that survivin could be an effective target of gene therapy for HCC, even at an early stage. Moreover, a previous study reported that in surgically removed tissues, the expression of survivin had no correlation with the patient's age, gender, tumor size and differentiation level of HCC^[11]. This is consistent with the rate of nuclear expression in the small biopsy samples from early-stage HCC observed in the present study. However, a recent report showed that alpha-feto protein (AFP) blocked the X-linked inhibitor of apoptosis protein-mediated inhibition of endogenous active caspases in the cytosolic lysates of tumor cells^[32]. Further immunohistological analyses of other proteins of the IAP family must be compared to the clinical parameters in early-stage HCC.

In this study, the survivin expression in HCC samples could be divided into two groups: a high-rate group (rate > 50%) and low-rate group (rate < 50%). Ikeguchi *et al.*^[33,34] detected survivin mRNA over-expression in 21 of 51 (41%) of HCC biopsies, and suggested that this could be useful as a prognostic factor for patients with HCC. From the early stages, the level of nuclear survivin expression may correlate with the prognosis of HCC.

HBV X and HCV core proteins activate NF- κ B and/or STAT-3, which regulate the gene expression for cell survival factors such as the anti-apoptosis proteins, including survivin^[35,36]. A resulting up-regulation of anti-apoptosis factors during HCV or HBV infection may contribute to hepatocarcinogenesis^[35,36]. A previous report demonstrated that HBx promotes the upregulation of survivin expression in hepatoma and normal liver cells, regardless of apoptosis. These findings suggest that survivin and HBx may play important roles in the carcinogenesis of HCC^[37]. Other studies have shown that HCV NS5A protein can stimulate survivin protein expression, and this may result from induced survivin gene transcription^[38]. In the present study, the average nuclear survivin expression was 36.3% in hepatocyte of non-tumor specimens. This result may indicate that the hepatitis virus is associated with survivin expression in peritumoral cells. The expression of survivin has been detected in a variety of pre-neoplastic and/or benign lesions, including polyps of the colon, breast adenomas, Bowen's disease and hypertrophic actinic keratosis^[39], suggesting that expression of survivin may occur during early malignant transformation or following a disturbance in the balance between cell proliferation and death^[9]. The same process may also occur in viral hepatitis.

A previous study reported that inhibition of apoptosis by survivin plays a pivotal role in the metastasis of HCC, and it has some correlation with tumorigenesis. The expression of survivin in the primary lesion can be an indicator of metastasis and the prognosis of HCC^[10]. In this study, a correlation between the survivin expression rate in the non-tumor cells and that in the HCC specimens of the high-rate group (rate > 50%) and low-rate group (rate < 50%) was observed. A significant difference in the survivin expression in the peritumoral hepatocyte was observed between the high-rate group and the low-rate group ($P < 0.01$). The survivin expression of peritumoral cells may, therefore, also be a prognostic factor for patients with HCC. Interestingly, we observed that when the amount of survivin expression was low in the adjacent non-tumor tissues, the corresponding tumor tissues also showed low expression. So in the future, when taking the target therapy of survivin into consideration, a curative effect may be possible if the amount of survivin expression with non-tumorous tissue is evaluated with liver biopsies.

Saitoh *et al*^[40] demonstrated that the portal blood flow is lost before the increase in arterial flow develops in well-differentiated HCC. When the well-differentiated HCC has portal blood flow and is not hypervascular, it shows slow growth^[12]. Yamaguchi *et al*^[41] suggested that this phenomenon is related to hypoxia, because the well-differentiated HCC would be in a transitional stage from the portal blood supply to the arterial blood supply, but the reduction in portal flow appears prior to the increase in arterial flow. Therefore, the current research indicates that the impairment of the normal liver blood system probably causes local hypoxic regions at an early stage of hepatocarcinogenesis and eventually induces angiogenesis^[12]. Recent studies have shown that human solid tumors, even those less than 1 cm in diameter, may have substantial hypoxic fractions^[42-43]. Hence tumor growth is restricted by limited oxygen and nutrients when they are too distant from nearby vessels^[12]. Therefore, to observe the expression of survivin in early-stage HCC, HCC cells were cultured in a hypoxic environment. Previous studies have shown that survivin gene transcription is increased in hypoxic tumor cells^[11]. In the current study, the hypoxic environment increased the survivin mRNA expression in both the short-term and long-term cultures. Moreover, the appearance of survivin protein is thought to control the survivin mRNA levels in the presence of anti-cancer drugs. On the contrary, in the present study, the survivin protein concentrations increased when both hypoxia and anti-cancer drugs were combined. These data suggest that survivin inhibition could therefore potentially be as effective as interventional therapy for the treatment of early HCC.

In conclusion, survivin is expressed at a rate of 64.7% (median) in early HCC. Moreover, survivin protein concentration of HCC cells increases when cultured with anti-cancer drugs under hypoxic conditions. These data suggest that survivin inhibition for early HCC could therefore be potentially useful as an effective interventional radiological treatment modality.

COMMENTS

Background

Several preclinical studies have demonstrated that the down-regulation of survivin expression/function increases the rate of apoptosis, reduces the tumor-growth potential and sensitizes tumor cells to various chemotherapeutic drugs and γ -irradiation in both *in vitro* and *in vivo* models of various types of human tumors.

Research frontiers

Previous reports have shown survivin to be expressed in post-operative HCC tissues. However, it has not yet been fully elucidated regarding whether survivin can be used as a therapeutic target in early HCC.

Innovations and breakthrough

We studied biopsy tissue specimens to confirm the diagnosis of HCC. In all early HCCs of tumor size > 10 mm, survivin expression was always above the median average (64.7%). Moreover, the survivin protein concentrations increased when cultured in a combination of hypoxia and anti-cancer drugs.

Applications

These data suggest that survivin inhibition for early HCC could therefore be potentially useful as an effective interventional radiological treatment modality, such as transcatheter arterial chemoembolization (TACE), etc.

Terminology

In this study, a hypoxia model of cultured HCC cells was employed using an AnaeroPack for cell culture. The Anaeropack is a gas concentration-controlling reagent yielding a hypoxic atmosphere. The principal ingredient of this reagent is sodium ascorbate, which absorbs oxygen and generates carbon dioxide by oxidative degradation. The culture dishes were placed into an airtight jar with the Anaeropack and then the lid was closed. The jar was then incubated at 37°C for 2 h. The concentration of oxygen decreased to less than 1% within 1 h and the carbon dioxide concentration was maintained at about 5% as reported previously.

Peer review

This paper investigated survivin expression in early-stage, small HCC and the results are interesting. The methods and results were clearly written, and the authors gave thoughtful discussions on this topic and their findings. This is an interesting paper that is generally well written.

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COLORECTAL CANCER

RNA interference-mediated gene silencing of vascular endothelial growth factor in colon cancer cells

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Abstract

AIM: To inhibit the expression of vascular endothelial growth factor (VEGF) in colon cancer cell line by RNA interference (RNAi).

METHODS: Followed the service of E-RNAi, we designed and constructed two kinds of shRNA expression vectors aiming at the VEGF gene, then transfected them into colon cancer HT29 cells by lipofectamine™ 2000. The level of VEGF mRNA was investigated by RT-PCR and Northern blotting. The protein expression of VEGF was observed by immunofluorescence staining and Western blotting.

RESULTS: We got two kinds of VEGF specific shRNA expression vectors which could efficiently inhibit the expression of VEGF in HT29 cells. RT-PCR, Northern blotting, immunofluorescence staining and Western blotting showed that inhibition rate for VEGF expression was up to 42%, 89%, 73% and 82%, respectively.

CONCLUSION: The expression of VEGF can be inhibited by RNA interference in HT29 cells.

Key words: RNA interference; Vascular endothelial growth factor; Colon cancer; Northern blotting; Western blotting

Li TJ, Song JN, Kang K, Tong SS, Hu ZL, He TC, Zhang BQ, Zhang CQ. RNA interference mediated gene silencing of vascular endothelial growth factor in colon cancer cells.

INTRODUCTION

Angiogenesis is a common process that is essential for tumor growth beyond 2 mm^[1]. Although numerous growth factors are involved, vascular endothelial growth factor (VEGF), particularly VEGF-A, has been shown to play an important role in tumor angiogenesis^[2]. VEGF, a 45 kDa heparin-binding growth factor, is induced by hypoxia-inducible factor-1a. Binding of VEGF-A to tyrosine kinase receptors, especially VEGFR-2, mediates many key components of angiogenesis, including endothelial cell proliferation, invasion, migration, survival, as well as vessel permeability. VEGF is secreted by most tumors, including tumors of the lung, gastrointestinal tract, kidney, thyroid, bladder, ovary, and cervix, and the level of VEGF is correlated with tumor progression, invasion^[3].

RNAi is the sequence-specific, posttranscriptional gene silencing method initiated by double-stranded RNAs, which are homologous to the suppressed gene. Double-stranded RNAs are processed by Dicer, a cellular RNase III, to generate duplexes of about 21nt with 3'-overhang small interfering RNA (siRNA), which mediate sequence-specific mRNA degradation. RNAi technology is not only an extremely powerful instrument for functional genomic analysis but also a potentially useful method to develop highly specific gene silencing therapeutics^[4-9]. In this study, we constructed vector-based expression systems in which sense and antisense strands of short VEGF sequences were transcribed into the hairpin structure under control of the U6 promoter.

A number of studies are available on VEGF in the treatment of tumors, such as colon cancer and liver cancer^[10-15]. However, due to the "off-target" of RNAi, experiments have to be done to verify the more effective sequence of VEGF before it is used in clinical practice. This study was to find a better VEGF specific RNAi for colon cancer.

MATERIALS AND METHODS

Construction of RNAi vectors

RNAi vectors pShRNA-V1 and pShRNA-V2 were constructed as previously described^[16-19]. In brief,

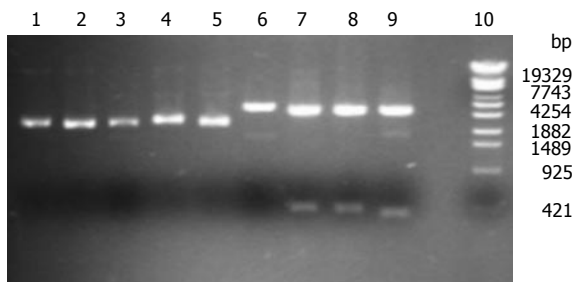


Figure 1 Restriction map of recombinant plasmid pShRNA. Lanes 1-2: pShRNA undigested; Lane 3: undigested null vector pTZU6+1; Lanes 4-5: pShRNA digested by sal I; Lane 6: pTZU6+1 digested by sal I; Lanes 7-8: pShRNA digested by Hind III and EcoR I (2800 + 395 bp); Lane 9: pTZU6+1 digested by Hind III and EcoR I (2800 + 352 bp); Lane 10: λ T14 DNA Marker.

21-nucleotide-long inverted repeats (separated by a 4-nucleotide linker, ttcg) were inserted downstream of the U6 promoter. The transcribed RNA thus comprised a 21-base pair of double-stranded RNAs. Five thymidines were inserted downstream the antisense strand to provide a stop signal for the RNA polymerase. The sense strand of hairpin was homologous to a 21-nucleotide region in the target mRNA. The target sequences were selected following the advice of E-RNAi services (<http://e-rnai.dkfz.de/>). The sequence of V1 and V2 is 5'-TGAAGTT CATGGATGTCTATC-3' and 5'-ACATCACCATGCAG ATTATGC-3', respectively. An irrelevant RNAi control plasmid was constructed for green fluorescent protein (GFP) gene, pShRNA-GFP. The sequence (5'-AGCTG ACCCTGAAGTTCATCT-3') was designed to target the nucleotides 126-144 of the GFP coding region.

Cell culture and transfection

Human colon cancer cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 μ g/mL streptomycin, and 100-units/mL ampicillin. The cells were plated in 24- or 6- well plates at 50%-70% confluence 24 h prior to transfection. Transfection of cells was carried out with LipofectamineTM 2000 reagent (Invitrogen, Carlsbad, CA).

Real-time polymerase chain reaction for VEGF

Total RNA was isolated from cultured cells and real-time polymerase chain reaction (RT-PCR) was performed using the RNeasy and one step RT-PCR kit from Qiagen Corp. RT-PCR of hGAPDH, a housekeeping gene served as a control. The sequences used for primers are 5' ctacctccaccatgccagt-3' (sense) and 5'-aatgtctttctcgcgtctga-3' (antisense) for VEGF (411 bp), 5'-GGCTCTCCAGAACATCAT-3'(sense) and 5'-CACCTGGTGCTCAGTGTA-3' (antisense) for hGAPDH (240 bp). For RT-PCR, two pairs of primers were added into a reaction tube, the program consisted of an initial reverse transcription at 50°C for 30 min, denaturation at 95°C for 10 min, followed by 24 cycles of amplification (denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and extension at 68°C for 1 min) and a final extension at 68°C for 10 min. The products were then separated by electrophoresis on 1.5% agarose gel, the bands were visualized using UV light and analyzed by Genetools software.

Northern blotting analysis

Total RNA was extracted from transfected cells on d 3 post-transfection, and purified using the RNeasy Mini Kit (Qiagen). Twenty micrograms of total RNA was separated on 1.2% agarose-formaldehyde gels and transferred onto a positively charged nylon membrane (Amersham). The presence of VEGF mRNA was probed with 32P-labeled VEGF DNA, which was generated with a random-primed labeling kit (Amersham).

Immunofluorescence staining

Cells were harvested on d 2 post-transfection for analysis, washed once with PBS and fixed with 4% paraformaldehyde in PBS for 20 min at 4°C. After blocked with goat serum, the cells were incubated with monoclonal mouse anti-VEGF for 2 h at 37°C. After three washes, the cells were incubated with Cy3-conjugated rabbit anti-mouse secondary antibodies for 1 h at 37°C and washed three times with PBS. The stained cells were mounted and analyzed under fluorescence microscope.

Western blotting

Cells were harvested on d 3 post-transfection, washed twice with 10 mL of PBS, lysed with SDS buffer, boiled for 5 min, separated by 10% SDS-PAGE gel electrophoresis, transferred onto a nitrocellulose membrane, incubated with VEGF antibodies at a dilution of 1/400 and HRP-conjugated rabbit anti-mouse antibody at a dilution of 1/4000. The HRP substrate was observed on the NC membrane. After three washes, the NC membrane was incubated with actin antibody and HRP-conjugated second antibody. The HRP substrate was observed again.

RESULTS

Identification of recombinant plasmid pShRNA and sequencing

Recombinant plasmid pShRNAs could not be digested by Sal I due to the loss of Sal I site. However, the blank plasmid pTZU6+1 could be lined by sal I. When digested by Hind III and EcoR I, pShRNAs could be separated into two parts (2800 and 395 bp), and pTZU6+1 into 2800 bp and 352 bp. The correct recombinant plasmids were shown in gel electrophoresis and verified by DNA sequencing (Figure 1).

Gel electrophoresis of VEGF

The size of VEGF was 411 bp, and consisted of the Marker in gel electrophoresis. After cloned into T vector, its sequence was verified by DNA sequencing (Figure 2).

VEGF mRNA inhibition in HT29 cells by RT-PCR

The inhibition rate of pShRNA-V1 and pShRNA-V2 was 42% and 40% respectively in HT29 cells compared with the control plasmid pShRNA-GFP (Figure 3A and B).

VEGF mRNA inhibition in HT29 cells by Northern blotting

The inhibition rate of pShRNA-V1 and pShRNA-V2 was 87% and 89% respectively in HT29 cells compared with the control plasmid pShRNA-GFP (Figure 4A and B).

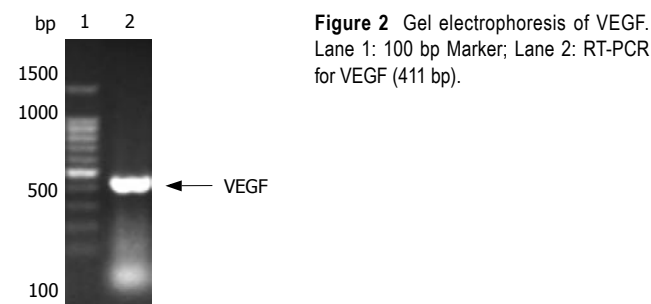


Figure 2 Gel electrophoresis of VEGF. Lane 1: 100 bp Marker; Lane 2: RT-PCR for VEGF (411 bp).

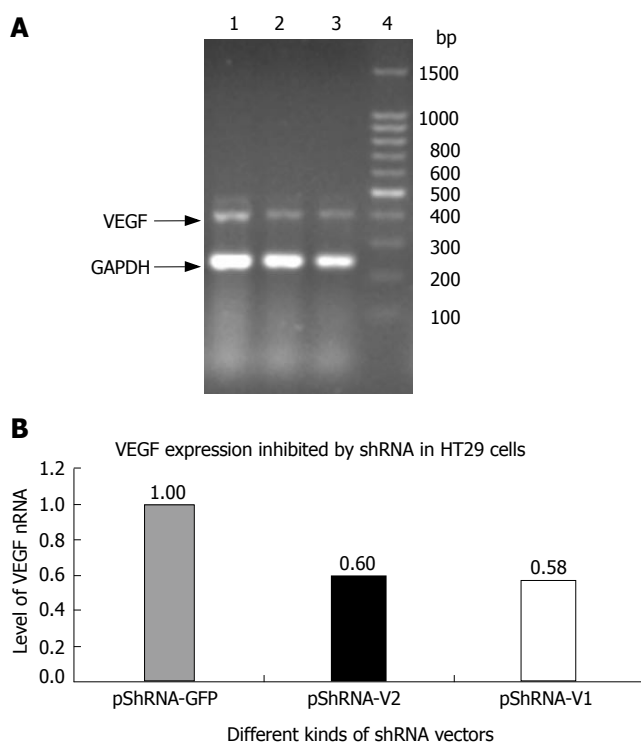


Figure 3 VEGF gene expression inhibited by shRNAs (A) and the inhibition rate of pShRNA-V1 and pShRNA-V2 (B) in HT29 cells. Lane 1: pShRNA-GFP; Lane 2: pShRNA-V2; Lane 3: pShRNA-V1; Lane 4: 100 bp Marker.

VEGF protein inhibition in HT29 cells by immunofluorescence staining

The inhibition rate of pShRNA-V1 and pShRNA-V2 was 63% and 73% respectively in HT29 cells compared with the control plasmid pShRNA-GFP and pTZU6+1 (Figure 5A and B). VEGF was stained red and located in plasma of cells.

VEGF protein inhibition in HT29 cells by Western blotting

The inhibition rate of pShRNA-V1 and pShRNA-V2 was 69% and 82% respectively in HT29 cells compared with the control plasmid pShRNA-GFP and pTZU6+1 (Figure 6A and B).

DISCUSSION

Angiogenesis is a process of generating new capillaries from pre-existing blood vessels, which involves multiple gene products expressed by various cell types. This uncontrolled process of new blood vessel growth from the

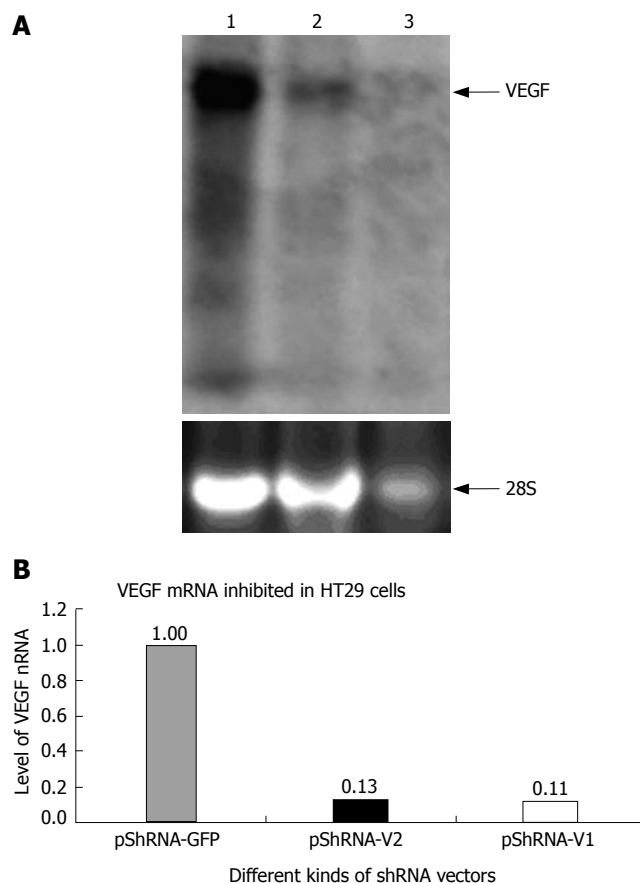


Figure 4 VEGF mRNA inhibited in HT29 cells by Northern blotting analysis (A) and the inhibition rate of pShRNA-V1 and pShRNA-V2 (B). Lane 1: pShRNA-GFP; Lane 2: pShRNA-V1; Lane 3: pShRNA-V2.

preexisting circulation network is an important pathogenic cause of tumor growth^[20-24]. Although several proteins such as hepatocyte growth factor, tumor necrosis factor- α , and fibroblast growth factor 2 (FGF2) have been identified as stimulators of angiogenesis in various settings, the most important angiogenic growth factor is VEGF, which is over-expressed in many human cancers. VEGF expression in tumors can be induced by more than one mechanism. Hypoxia, which is found in most tumors, has long been known to be a potent inducer of VEGF^[25-28].

In this study, shRNAs targeting VEGF efficiently reduced the transcript levels of VEGF mRNAs, and ultimately resulted in the reduction in VEGF protein levels. Furthermore, this inhibition was shown to be highly selective and sequence-specific, since control siRNAs had almost no inhibitory effect on the expression and transcription of VEGF.

There has been a considerable interest in treating a wide range of diseases with RNAi therapeutics^[29,30]. In this study, in addition to the above-reported target sites in the VEGF genome, the specific 21-bp siRNAs targeting VEGF could efficiently and specifically inhibit VEGF expression, suggesting that it is a good method to inhibit the expression of VEGF.

The results of this study demonstrated that the constructed shRNA could efficiently reduce the level of VEGF transcripts and expression, suggest that shRNA-

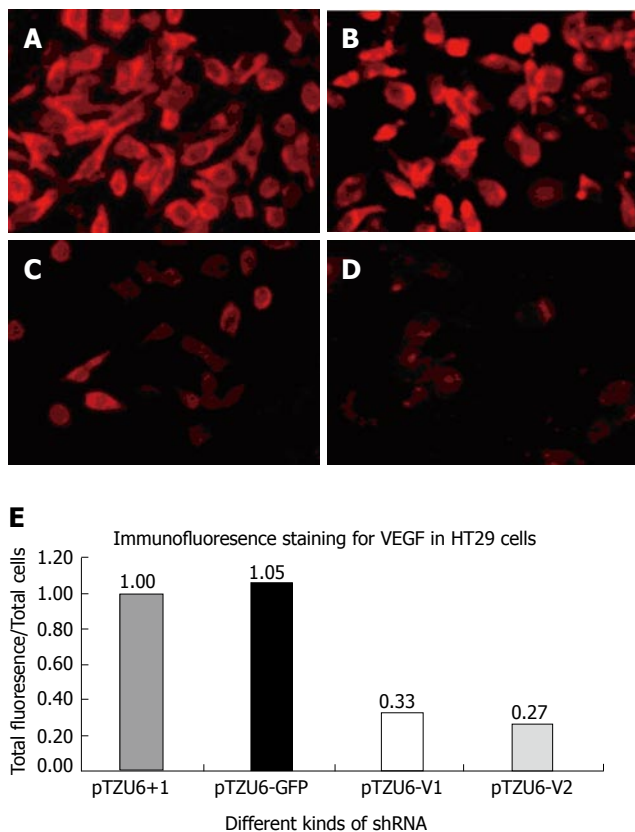


Figure 5 Immunofluorescence staining for pTZU6+1 (A), pTZU6-GFP (B), pTZU6-V1 (C), pTZU6-V2 (D), and the inhibition rate of pShRNA-V1 and pShRNA-V2 (E).

expressing vectors can be used as RNAi-based anti-VEGF therapeutics^[31]. Future studies should be centered on the evaluation of the anti-VEGF efficacy of RNAi vectors in animal models, as well as on the preclinical elucidation using the RNAi technology.

ACKNOWLEDGMENTS

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COMMENTS

Background

Vascular endothelial growth factor (VEGF) has been shown to play an important role in tumor angiogenesis. RNAi is the sequence-specific, posttranscriptional gene silencing method initiated by double-stranded RNAs. A number of studies are available on VEGF used in the treatment of tumors, such as colon cancer, liver cancer. However, due to the "off-target" of RNAi, experiments have to be done to verify the more effective sequence of VEGF before it is used in clinical practice. This study was to find a better VEGF specific RNAi for colon cancer.

Research frontiers

Other researches have applied VEGF in the treatment of tumors, such as colon

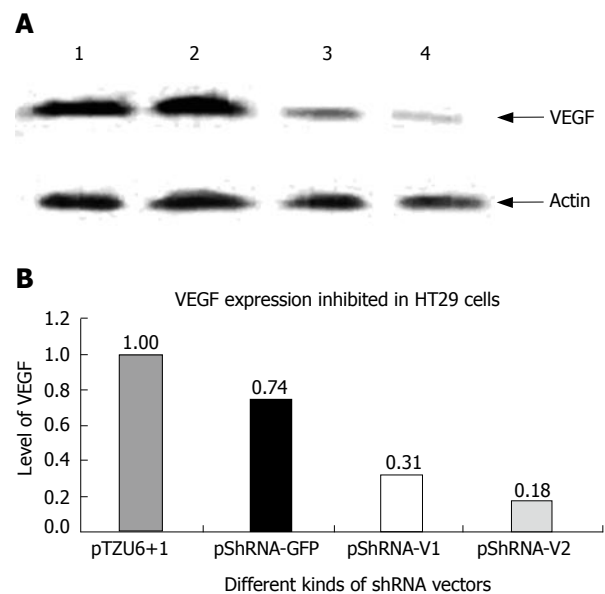


Figure 6 Western blotting for VEGF (A) and the inhibition rate of pShRNA-V1 and pShRNA-V2 (B) in HT29 cells. Lane 1: pTZU6+1; Lane 2: pShRNA-G; Lane 3: pShRNA-V1; Lane 4: pShRNA-V2.

cancer, liver cancer. There has been a considerable interest in treating a wide range of diseases with RNAi.

Innovations and breakthroughs

The results of our study suggest that shRNA-expressing vectors can be used as RNAi-based anti-VEGF therapeutics. Future studies should be centered on the evaluation of the anti-VEGF efficacy of RNAi vectors in animal models, as well as on the preclinical elucidation using the RNAi technology.

Applications

shRNA-expressing vectors can be used as RNAi-based anti-VEGF therapeutics

Terminology

RNAi is the sequence-specific, posttranscriptional gene silencing method initiated by double-stranded RNAs, which are homologous to the suppressed gene. Double-strand RNAs are processed by Dicer, a cellular RNase III, to generate duplexes of about 21nt with 3'-overhang small interfering RNA (siRNA), which mediate sequence-specific mRNA degradation.

Peer review

This manuscript describes the methodology for RNAi of VEGF in HT29 cells by lipofectamine. The process was evaluated quite well. No application of the technique has reported prior to this study.

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Antioxidant therapy for chronic hepatitis C after failure of interferon: Results of phase II randomized, double-blind placebo controlled clinical trial

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Abstract

AIM: To assess the safety and efficacy of antioxidant therapy for patients with chronic hepatitis C virus (HCV) infection.

METHODS: One hundred chronic HCV infection patients failed in interferon treatment were enrolled and randomly assigned to receive combined intravenous and oral antioxidants or placebo, or oral treatment alone. Primary end points were liver enzymes, HCV-RNA levels and histology.

RESULTS: Combined oral and intravenous antioxidant therapy was associated with a significant decline in ALT levels in 52% of patients who received antioxidant therapy vs 20% of patients who received placebo ($P = 0.05$). Histology activity index (HAI) score at the end of treatment was reduced in 48% of patients who received antioxidant therapy vs 26% of patients who received placebo ($P = 0.21$). HCV-RNA levels decreased by 1-log or more in 28% of patients who received antioxidant therapy vs 12% who received placebo ($P = \text{NS}$). In part II of the trial, oral administration of antioxidants was not associated with significant alterations in any of the end points.

CONCLUSION: Antioxidant therapy has a mild beneficial effect on the inflammatory response of chronic HCV infection patients who are non-responders to interferon. Combined antiviral and antioxidant therapy may be beneficial for these patients.

INTRODUCTION

The pathogenesis of hepatitis C virus (HCV) infection involves a complex interaction between viral factors and host immune responses. A major component of the latter involves oxidative stress^[1]. Oxidative stress has been attributed to both host inflammatory processes and induction by viral proteins, with the two mechanisms possibly acting in synergy. HCV non-structural proteins have been shown to induce activation of STAT-3 *via* oxidative stress and Ca^{2+} signaling^[2]. This induction is influenced by the activation of cellular kinases, including p38 mitogen-activated protein kinase, JNK, JAK-2 and Src, and inhibited *in vitro* in the presence of antioxidant 3. It was reported that HCV core protein increases radical oxygen species (ROS) as well as lipid peroxidation products and antioxidant gene expression^[3]. Increased intrahepatic lipid peroxidation products have been observed in HCV transgenic mice, following exposure to carbon tetrachloride^[4]. These processes may contribute to fibrosis and carcinogenesis in chronic HCV^[5]. Analysis of HCV-sub genomic RNA replicon cell lines has shown a drastic reduction in cellular glutathione peroxidase, increasing cellular susceptibility to oxidative stress^[6]. Based on these data, a rationale for antioxidant treatment of chronic hepatitis C was suggested. Currently, the mainstay of treatment of chronic hepatitis C is a combination of pegylated interferons and ribavirin^[7]. However, the overall response rate of its pharmacological efficacy and adverse effects is approximately 55% in genotype I patients^[8].

The use of complimentary and alternative medicine is common in patients with chronic liver disease. In a recent study, 41% of patients reported use of these modalities,

with 20% reporting use of herbal medicine, including silymarin (12%), licorice root (glycyrrhizin) and St. John's wort^[9]. In a review of medicinal herbs for HCV infection, the authors concluded that some of these agents may have an effect on liver enzymes but there is no firm evidence that supports their efficacy on chronic HCV^[10].

In a recently published phase-I, open label clinical trial, 50 patients with chronic HCV infection were treated orally with a combination of seven antioxidative oral preparations (glycyrrhizin, schisandra, silymarin, ascorbic acid, lipoic acid, L-glutathione, and alpha-tocopherol) on a daily basis for 20 wk, along with four different intravenous preparations (glycyrrhizin, ascorbic acid, L-glutathione, B-complex) twice weekly for the first 10 wk^[11]. Normalization of liver enzymes occurred in 44% of patients who had elevated pretreatment ALT levels. A decrease in viral load by one log or more was observed in 25% of the patients. Histological improvement, with two-point reduction in the HAI score, was noted in 36.1% of the patients. The SF-36 quality of life score improved in 26 of 45 patients (58%) throughout the trial. No major adverse reactions were noted. These findings suggest that antioxidant therapy may have a beneficial effect on necro-inflammatory variables in these patients.

The aim of the present trial was to determine the effect of a mixture of antioxidants on the inflammatory response of chronic HCV infection patients who were non-responders to interferon, in a double-blind placebo controlled trial. Two different treatment regimens were studied and compared. The data suggest that combined intravenous and oral antioxidant therapy mildly alleviates the inflammatory response in these patients.

MATERIALS AND METHODS

Subjects

A total of 100 chronic HCV infection patients were enrolled in a double-blind, placebo controlled single-center trial. The study focused on 2 treatment options, with 50 patients in each section. Part I looked at administration of intravenous and oral antioxidant preparations *versus* placebo. Part II tested administration of oral preparations only *versus* placebo. Patients were randomly assigned to treatment or placebo groups, 25 patients in each group. Antioxidants were administered as described below. All experiments were carried out in accordance with the guidelines of the Hebrew University-Hadassah Institutional Committee for Human Clinical Trials. The Israel Ministry of Health Committee for Human Trials approved all experiments.

Inclusion criteria: Eligible participants were male and female chronic HCV infection patients at the age of 18-75 years. All patients were required to display positive HCV RNA on two tests for at least 6 mo prior to enrollment. Diagnosis of chronic HCV infection was based on positive HCV RNA levels with liver biopsy (within 1 year of initiation of study) with or without elevated liver enzymes. Informed consent was obtained from patients prior to participation in the trial. Patients screened for the trial included those in whom treatment with interferon had previously failed, or was contra-indicated. Both partial

responders and non-responders were enrolled. Abstinence from any alternative medications or vitamin supplements for 6 mo prior to initiation of therapy was stipulated.

Exclusion criteria: Patients excluded from the trial included those with decompensated liver disease as evidenced by Child's B or C status; those with creatinine levels above 150 $\mu\text{mol/L}$, hemoglobin levels below 100 mg/L, white blood cell count below $3 \times 10^9/\text{L}$, or platelet count below $1 \times 10^{11}/\text{L}$; those with a history of varices, ascites, or encephalopathy. Also ineligible were patients who exhibited irreversible neurologic deficit or active co-infection with HBV, HAV, HDV, or HIV. Those treated with interferon or pegylated interferon or ribavirin less than 6 mo prior to enrollment or with a history of having received any systemic anti-neoplastic or immune modulatory treatment 6 mo prior to the first dose of study medication were excluded. Evidence of other causes of liver disease or other severe debilitating diseases precluded the patient from participating in this study. Patients who used alcohol or anti-viral medication during the trial, pregnancy or breast-feeding, and had a history of hepatic, renal, or other major organ transplantation were also exclude from the study.

In part I, 50 patients (30 males and 20 females) with a mean age of 56.1 (range, 22-75) years were enrolled in the trial. Sixty-four percent of patients ($n = 32$) had elevated liver enzymes on screening, and 36% ($n = 18$) had normal liver enzymes despite viremia and active inflammation on biopsy. All other markers for additional causes of liver disease were negative. HCV genotype 1, genotype 1a, and genotype 1b were found in 33, 5, and 28 patients, respectively. HCV genotype 2, genotype 3, and genotype 4 were found in 8, 6 and 3 patients, respectively.

In part II, 50 patients (34 males and 16 females) were randomly assigned to treatment ($n = 25$) and placebo ($n = 25$) groups. Their mean age was 57.4 (range 24-75) years. Thirty-five patients had genotype 1 (31 had genotype 1b and 4 had genotype 1a). Two patients had genotype 2a2c, two had genotype 3a, and one had genotype 4c.

Drug administration

Patients in the treatment group received a combination of seven different anti-oxidants orally at the appropriate dose (glycyrrhiza capsules, 500 mg, bid; schizandra capsules, 500 mg, tid; ascorbate capsules, 2000 mg, tid; L-glutathione capsules, 150 mg, bid; silymarin capsules, 250 mg, tid; lipoic Acid capsules, 150 mg, bid; d-alpha tocopherol, 800 IU/d) prepared by Vital Nutrients Middletown (CT, USA), once daily for 24 wk. In addition, all patients in part I of the trial received intravenously a combination of four different preparations (120 mg glycyrrhiza, 10 g ascorbate, 750 mg L-glutathione, 1 mL B-complex) at the appropriate dose, twice a week for the first 10 wk of the study. Medications were prepared by GY&N Nutriment Pharmacology, Unique Pharmaceuticals Company, Palmdale, CA, USA. Dosages were determined based on previous trials in humans. Products for intravenous injection were added to 400 mL of normal saline for infusion. Patients were followed up for an additional 24 wk following completion of the treatment as described below. Patients in the placebo group received identical pills and normal saline intravenously.

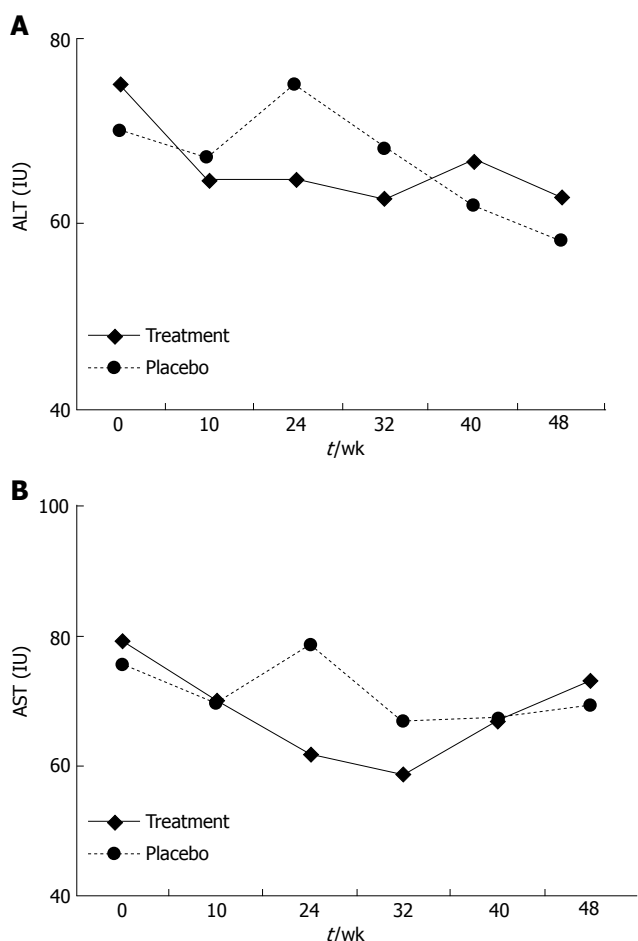


Figure 1 Effect of antioxidant treatment on ALT (A) and AST (B) serum levels in part I with treatment group in solid lines and placebo in dashed lines.

Follow-up parameters

Patients were followed up for clinical, biochemical, virological, and histological parameters. Patients underwent a bi-weekly physical examination throughout the treatment period, and every 4 wk during the follow-up period. Serum bilirubin and liver enzymes, kidney function, prothrombin time, and complete blood counts were checked bi-weekly during the treatment period and every 4 wk during follow-up. HCV RNA was detected every 4 wk throughout the study by standard assays (Roche Diagnostics Systems Inc., Branchburg, NJ). The lower limit of detection for this assay was 100 copies/L. All patients underwent a liver biopsy within 2 years prior to treatment. A repeat biopsy was performed 2 to 6 wk following completion of the antioxidant therapy. Intra-hepatic inflammatory score was evaluated by two blinded pathologists using the standard histological activity index (HAI). Evaluation of the effect of treatment on quality of life was performed using the eighth paradigm (self assessment of general health) of the SF-36 quality of life questionnaire. The questionnaire was completed 2 wk before and within 4 wk after the study.

Primary and secondary variables

The objectives of the study were: (1) evaluation of the safety and tolerability of intravenous and oral administration of glycyrrhizin, antioxidants, and vitamins

in patients with chronic HCV infection; (2) evaluation of the effect of glycyrrhizin, antioxidants, and vitamins on inflammatory indices and HCV viral load. The two primary variables were: (1) safety and tolerability of intravenous and oral administration of glycyrrhizin, antioxidants, and vitamins in patients with chronic HCV infection; (2) amelioration of inflammation and existing liver damage, and reduction in viral load.

Statistical analysis

Data were analyzed using Fisher's exact test. $P < 0.05$ was considered statistically significant.

RESULTS

Safety and adverse effects

In part I, no major adverse effects related to treatment were noted. Side effects possibly related to the mineral corticoid effect of glycyrrhizin treatment included hypokalemia in 6 patients of the treatment group (24%), in 5 patients of the placebo group (20%), and mild exacerbation of hypertension in 1 patient of the treatment group. All cases were mild and treated symptomatically. Two patients in the treatment group developed pedal edema and a sense of bloating. One patient in the treatment group was hospitalized twice due to fever during the trial period which was considered unrelated to treatment.

In part II, One patient in the treatment group had exacerbation of hypertension at wk 6 of treatment, requiring treatment with enalapril and lercanidipine. Subsequent blood pressure during treatment was normal. Six patients in the treatment group had hypokalemia of 2.9 to 3.3 and one patient in the placebo group had hypokalemia of 3.4. One patient in the treatment group had a high systolic blood pressure (up to 190 mmHg) on the day of his second biopsy, and resolved after administration of oral oxazepam. One patient in the treatment group had a weight loss of 5.5 kg during treatment, but regained three kg by wk 32. One patient in the placebo group reported weakness, upper abdominal pain and arthralgia.

Effect of antioxidative treatment on liver enzymes

In part I, at the end of 24 wk of treatment a decline in ALT and AST levels was observed in the treatment group compared to the placebo group. The ALT level at this point was 64.8 IU in the treatment group and 75.04 IU in the placebo group ($P = 0.42$, Figure 1A), the AST level at this point was 61.72 IU in the treatment group and 78 IU in the placebo group ($P = 0.1$, Figure 1B). This effect diminished during the follow-up period. At wk 48, the ALT level was 63 IU in the treatment group and 58.27 IU in the placebo group ($P = 0.68$), the AST level was 73.13 IU in the treatment group and 69.33 IU in the placebo group ($P = 0.75$). At the end of the treatment period, the ALT level improved in 54% of treated patients and in 20% of patients receiving the placebo ($P = 0.05$, Figure 2A), the AST level declined in 50% of treated patients and 20% of patients receiving placebo ($P = 0.02$, Figure 2B). At

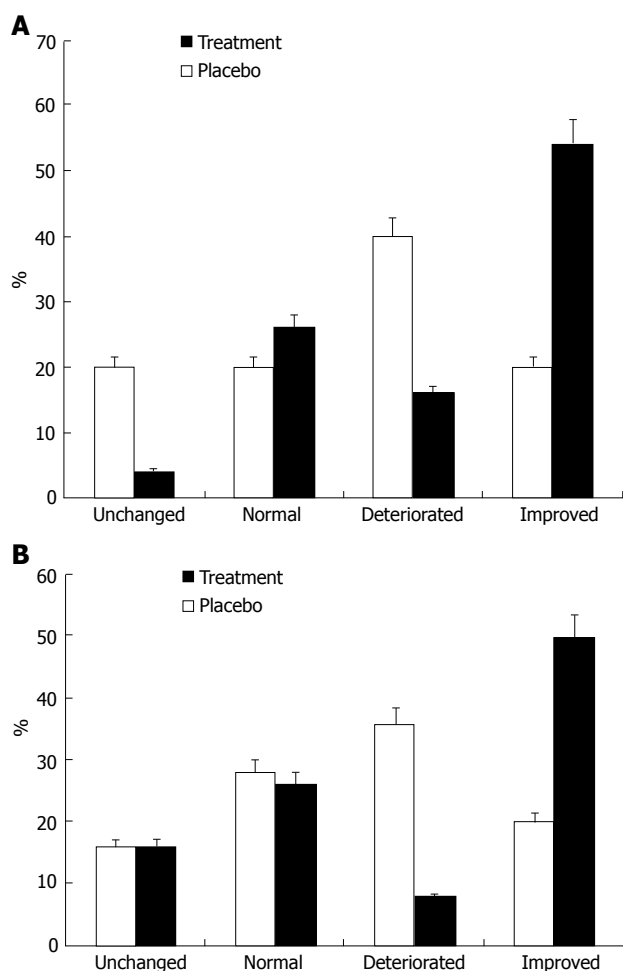


Figure 2 Proportions of patients with improvement in ALT (A) and AST (B).

the end of the follow-up period (at wk 48), the ALT level improved in 46% of patients in the treatment group and in 45% of patients in the placebo group, the AST level improved in 35% of patients in the treatment group and 34% of patients in the placebo group compared to their baseline.

In part II, a minor non-significant decline in liver enzyme levels was noted after 24 wk of treatment. The ALT level at this point was 63.5 IU in the treatment group and 81.81 IU in the placebo group ($P = 0.35$, Figure 3A), the AST level was 65.71 IU in the treatment group and 81.82 IU in the placebo group ($P = 0.24$, Figure 3B). This effect disappeared during the follow-up period. At wk 48, the ALT level was 61.75 IU in the placebo group and 83.94 IU in the treatment group ($P = 0.24$, Figure 3A), the AST level was 69.5 IU in the placebo group and 92.66 IU in the treatment group ($P = 0.39$, Figure 3B).

Effect of antioxidant treatment on biopsy scores

In part I, the HAI reduced from 8.87 before to 8.13 after treatment in the treatment group and from 7.85 to 7.4 in the placebo group ($P = 0.42$). Compared to the pre-treatment score, the total biopsy HAI score at the end of treatment reduced in 48% of patients in the treatment group and 27% of patients in the placebo group, and

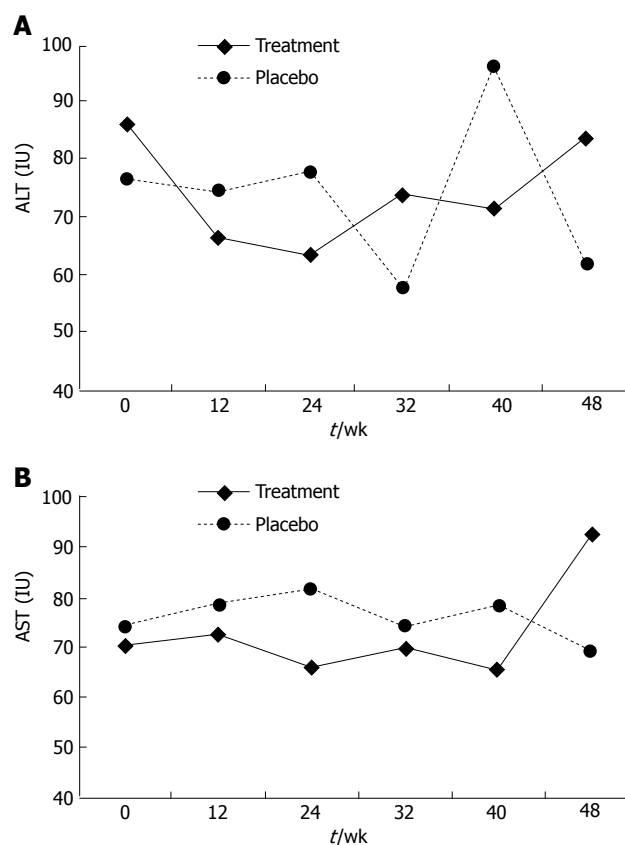


Figure 3 Effect of antioxidant treatment on ALT (A) and AST (B) serum levels in part II with treatment group in solid lines and placebo in dashed lines.

unchanged in 26% of patients in the treatment group and 49% of patients in the placebo group ($P = 0.21$).

In part II, the HAI scores increased from 7.619 to 8.36 in the treatment group, and from 8.53 to 9 in the placebo group ($P = 0.42$).

Effect of antioxidant treatment on HCV RNA serum levels

In part I, antioxidant treatment had no effect on diminishing viral load, and the average log of HCV RNA at wk 24 was 5.08 for the treatment group and 5.04 for the placebo group (Figure 4A).

In part II, similarly, no effect of treatment on viral load was observed. The average log of HCV RNA at wk 24 was 5.36 for the treatment group and 5.28 for the placebo group (Figure 4B).

Effect of antioxidant treatment on quality of life

In part I, data were available from thirty-eight patients. The average baseline quality of life in the treatment group was 52.88 out of a maximum of 100, and the post-treatment value was 52.12 ($P = \text{NS}$). The value in the placebo group was 48.5 before and 47.25 after treatment ($P = \text{NS}$).

In part II, data were available from thirty-seven patients. The average baseline quality of life in the treatment group was 53.1 out of a maximum of 100, and the post-treatment value was 48.8 ($P = 0.50$). The value in the placebo group was 43.44 before and 50.31 after treatment ($P = 0.38$).

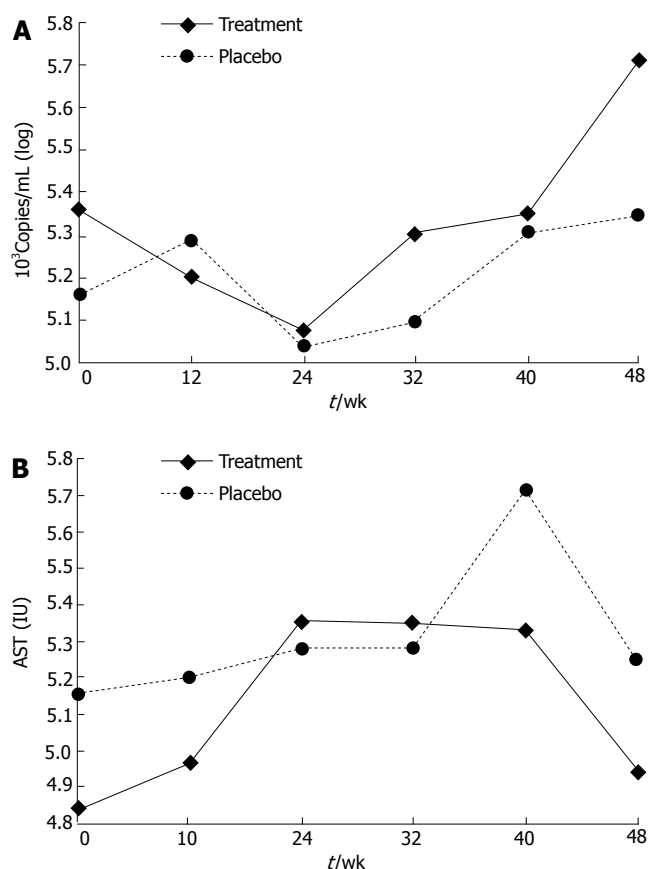


Figure 4 Effect of antioxidant therapy on viral RNA serum levels in part I (A) and part II (B).

DISCUSSION

A combination of intravenous and oral antioxidants could mildly alleviate the intra-hepatic inflammatory response in chronic HCV infection patients who non-responders to interferon therapy. This was manifested as a significant decrease in ALT and AST serum levels, along with improvement in the biopsy inflammatory scores. No effect was noted when oral antioxidants were used alone. The rationale for treatment of chronic HCV infection patients with antioxidants is based on the role of oxidative stress in the pathogenesis of disease progression in animal models and humans. Results of uncontrolled trials suggest that this treatment may have a beneficial effect on inflammatory indices such as liver enzymes and biopsy HAI scores, as well as quality of life^[11]. Intravenous glycyrrhizin was previously tested in patients with chronic HCV infection, and lowered ALT levels (26% *vs* 6% with placebo) within 4 wk were noted. The effect disappears after cessation of therapy^[12]. A retrospective study examining the effects of stronger neo-minophagen C (SNMC), which contains glycyrrhizin as an active component, revealed that treatment with this agent reduces the long term relative risk of developing hepatocellular carcinoma by a factor of 2.49^[13]. When SNMC is used in combination with interferon, a higher rate of improvement in liver enzymes and viral clearance can be observed with no statistical significance^[14]. It was reported that a combination therapy of interferon (IFN) with glycyrrhizin

induces normalization of serum ALT levels in 64.3% of non-responders with serum HVC RNA disappeared in 38.5%^[13]. The efficacy of glycyrrhizin in European studies was less than that reported on Japanese subjects, which can be explained by the genetic polymorphism in drug metabolism^[15].

Silybum marianum (milk thistle) is a herbal medicine commonly used in treatment of liver disease. Its active component, silybinin, has been shown to have anti-fibrotic and antioxidant properties, and hepatoprotective effects in animal models of acetaminophen-induced liver damage^[16]. It was reported that silymarin protects liver cells from other toxins (including ethanol, carbon tetra-chloride, and D-galactosamine) and ischemic injury, radiation, iron toxicity, and viral hepatitis^[17,18]. A combination of three potent anti-oxidants [alpha-lipoic acid (thioctic acid), silymarin, and selenium] induces marked clinical, laboratory, and histological improvements in chronic HCV infection patients^[19,20]. However, a Cochrane systematic review of trials of medicinal herbs in HCV, reported that silybin is significantly reduced in serum AST and GGT levels in only one trial, with no firm evidence for the use of herbal medicines in this condition^[21]. Schizandrae chinensis, a potent anti-oxidant, lowers ALT levels in patients with chronic viral hepatitis^[22]. Administration of glutathione to patients with chronic hepatitis significantly decreases GSH-Px activity of catalase (CAT), and increases superoxide dismutase (SOD) activity^[23,24]. N-acetyl-cysteine, sodium selenite and vitamin E have also been studied as supplementation to interferon therapy for patients with chronic HCV infection^[25]. Vitamin E supplementation increases the chance for a complete response^[25]. A randomized double-blind trial of thioctic acid (alpha-lipoic acid) in chronic hepatitis patients showed that 55% patients have significant improvements in mean ALT levels, and 77% patients have histological improvements on liver biopsy^[26,27]. The results of the present study showed a modest reduction in liver enzymes at the end of 24 wk of treatment in patients receiving the combined intravenous and oral protocol. The proportion of patients on the combination therapy with improvements, was statistically significant, the difference in absolute values did reach significance. In both parts of the trial, the beneficial effect on enzymes did not sustain throughout the follow-up period, which is consistent with some of the previous published data^[13]. The lack of significant improvement on liver biopsy seems to reflect the relatively transient nature of the anti-inflammatory effect of antioxidants in this setting. No significant effect on viral load was noted in either part of the trial. This finding was not surprising given the lack of known direct anti-viral effects of antioxidants. A study reported that induction of gastrointestinal glutathione oxidase (which counteracts oxidative stress) by all trans retinoic acids, down-regulates HCV replicon in cell lines^[6]. Adverse events that are attributable to therapy such as exacerbation of hypertension, pedal edema, and hypokalemia, are mostly related to pseudoaldosteronism associated with the 11-hydroxy-steroid dehydrogenase inhibitory activity of glycyrrhiza and L-glutathione. The excess endogenous cortisol produced by both agents, reacts with the renal

mineral corticoid receptor, promoting an aldosterone-like action. Similar adverse events have also been described^[11]. These findings suggest that it is important to have a close follow-up of patients' blood pressure and serum potassium levels throughout antioxidant therapy.

In the present study, the use of antioxidants had no significant effect on quality of life (QOL) as measured in the general health paradigm of the SF-36 questionnaire which is in contrast with uncontrolled trials, suggesting that antioxidants may exert a beneficial effect on quality of life in chronic HCV infection patients^[11]. The combined intravenous and oral antioxidants had a better effect than oral combination alone, further supporting the potential of the intravenous antioxidants, specifically of glycyrrhiza, as a possible basis for the beneficial anti-inflammatory effect noted. Although not directly tested in the present study, the use of a combination of multiple antioxidants administered both intravenously and orally, may have enhanced the anti-oxidative effect, thus contributing to the observed effect.

The data of the present study support the role of oxidative stress in the pathogenesis of HCV-induced inflammation. The modest effect noted should be viewed in light of the fact that patients enrolled in the trial were non-responders to anti-viral therapy. Therefore, they represent a relatively "hard to treat" subset of chronic HCV infection patients. Treatment in the present study was lasted for a relatively short period of time, less than that of the current treatment with anti-viral drugs. If the main effect of antioxidants is anti-inflammatory, it would be expected that a combination of antioxidants with current anti-viral treatment may be of benefit for these patients. Indeed, previous reports suggest an added value for antioxidants in interferon-treated chronic HCV infection patients. It has been demonstrated that vitamin E-treated patients have a 2.4 times higher chance of obtaining a complete response and a more significant reduction in viral load than patients not treated with vitamin E^[28].

In conclusion, a combination of intravenous and oral antioxidants can reduce intra-hepatic inflammatory response in chronic HCV infection patients who are non-responders to interferon. It is plausible to examine whether such a treatment in combination with anti-viral therapy may be useful.

COMMENTS

Background

The pathogenesis of hepatitis C virus (HCV) infection involves a complex interaction between viral factors and host immune responses. A major component of the latter involves oxidative stress^[1].

Research frontiers

The aim of the present trial was to determine the effect of a mixture of antioxidants on the inflammatory response of chronic HCV infection patients who were non-responders to interferon, in a double blind placebo controlled trial. Two different treatment regimens were studied and compared. The data suggest that the combined use of intravenous and oral antioxidants mildly alleviates the inflammatory response in these patients.

Related publications

Melhem A, Stern M, Shibolet O, Israeli E, Ackerman Z, Pappo O, Hemed N, Rowe

M, Ohana H, Zabrecky G, Cohen R, Ilan Y. Treatment of chronic hepatitis C virus infection via antioxidants: Results of a phase I clinical trial. *J Clin Gastroenterol* 2005; 39: 737-342.

Innovations and breakthroughs

Antioxidant therapy has a mild beneficial effect on the inflammatory response in chronic HCV infection patients who are non-responders to interferon. A combination of antiviral and antioxidant therapy may be beneficial for these patients.

Applications

It is plausible to examine whether such a treatment in combination with anti-viral therapy may be useful.

Peer review

This is the first double blind trial using a combination of several anti oxidants in patients with chronic hepatitis C who failed in interferon therapy. The study was designed well and the results were summarized and discussed appropriately.

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VIRAL HEPATITIS

Establishment and primary application of a mouse model with hepatitis B virus replication

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intermediates were observed in the left, right and middle lobes of the liver. After treatment with polyIC, the level of HBV intermediate DNA in the liver was lower than that in the control mice injected with PBS.

CONCLUSION: A rapid and convenient mouse model with a high level of HBV replication was developed and used to investigate the inhibitory effect of polyIC on HBV replication, which provides a useful tool for future functional studies of the HBV genome.

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Key words: Animal model; Gene expression; Hepatitis B Virus; Hydrodynamic transfection; Polyinosinic-polytidylin acid; Virus replication

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Abstract

AIM: To establish a rapid and convenient animal model with hepatitis B virus (HBV) replication.

METHODS: A naked DNA solution of HBV-replication-competent plasmid was transferred to BALB/C mice *via* the tail vein, using a hydrodynamic *in vivo* transfection procedure. After injection, these mice were sacrificed on d 1, 3, 4, 5, 7 and 10. HBV DNA replication intermediates in the liver were analyzed by Southern blot hybridization. The expression of hepatitis B core antigen (HBcAg) and hepatitis B surface antigen (HBsAg) in the liver was checked by immunohistochemistry. Serum HBsAg and hepatitis B e antigen (HBeAg) was detected by enzyme-linked immunosorbent assay (ELISA). Inhibition of HBV replication was compared in HBV replication model mice treated intraperitoneally with polyinosinic-polytidylin acid (polyIC) or phosphate-buffered saline (PBS).

RESULTS: After hydrodynamic *in vivo* transfection, HBV DNA replication intermediates in the mouse liver were detectable on d 1 and abundant on d 3 and 4, the levels were slightly decreased and remained relatively stable between d 5 and 7, and were almost undetectable on d 10. The expression patterns of HBcAg and HBsAg were similar to that of HBV replication intermediate DNA, except that they reached a peak on d 1 after injection. No obvious differences in HBV DNA replication

INTRODUCTION

Hepatitis B virus (HBV) infection is a major health problem with more than 350 million chronic carriers worldwide, who are at high risk for developing liver cirrhosis and hepatocellular carcinoma^[1,2]. Because the pathogenic mechanisms of HBV remain unclear, molecular-biological studies are required. Until recently, most *in vivo* studies on the mechanism by which HBV causes liver diseases, and the mechanism of anti-HBV drugs, have been performed by using HBV transgenic mice^[3]. However, the acquisition of transgenic mice is very time-consuming and laborious, because expensive microinjection equipment and complicated technology is needed. We have reported previously that high-level expression of HBV surface antigen (HBsAg) in mouse hepatocytes is achieved after injection of an HBsAg expression plasmid *via* the tail vein using a hydrodynamic method^[4]. In this study, we developed a mouse model for HBV replication by using a hydrodynamic procedure that provides a simple, convenient and useful tool for molecular-biological studies on HBV, and for selection of antiviral drugs to treat HBV infection.

MATERIALS AND METHODS

Preparation of plasmid DNA

The HBV replication-competent plasmid used in this study was pHBV4.1, which contains 1.3 copies of the HBV genome (subtype *aym*). This construct has been proven to be able to initiate replication of HBV efficiently after being transfected into human hepatoma HepG2 cell lines^[5,6]. The plasmid DNA was prepared by using a plasmid-purifying Kit (QIAGEN TIP-500) according to the manufacturer's instructions.

Injection of naked plasmid DNA

Male BALB/C mice at specific-pathogen-free (SPF) level, weighing 18–20 g, were provided by the Huaxi Laboratory Animal Center of Sichuan University. Mice were injected *via* the tail vein with plasmid DNA at different doses in 1.8 mL saline within 5–8 s (hydrodynamic *in vivo* transfection)^[7]. The animals were sacrificed at different time points after injection, and the serum and liver were collected. The serum was stored at -20°C. The liver tissue was separated into two parts: one was fixed in 40 g/L neutral-buffered formalin, and the other was snap frozen in liquid nitrogen and stored at -70°C for subsequent analysis of HBV DNA replication intermediates.

Polyinosinic-polytidylin acid (polyIC) treatment

Twenty-four hours after hydrodynamic injection with pHBV4.1, the HBV replication-competent mice were matched by body weight, age and serum levels of hepatitis B e antigen (HBeAg), and were divided into two groups of five mice each. The mice in the experimental group were injected intraperitoneally with 200 µg polyIC (P-0913; Sigma) in 200 µL phosphate-buffered saline (PBS), three times at 24-h intervals, while the control group mice received only 200 µL PBS. The mice were sacrificed 4–6 h after the final injection of polyIC. Liver tissue was frozen in liquid nitrogen and stored at -70°C for DNA extraction and analysis.

Detection of HBV DNA replication intermediates

Frozen liver tissue was mechanically pulverized in liquid nitrogen and HBV DNA replicative intermediates were isolated as described previously, with some modifications^[8]. One hundred and twenty micrograms of liver tissue powder was lysed in 0.6 mL 100 mmol/L Tris hydrochloride (pH 8.0) and 2 mL/L NP40. The lysate was centrifuged for 1 min at 14000 rpm in a microcentrifuge. The supernatant was adjusted to 6.75 mmol/L magnesium acetate plus 200 µg DNaseI/mL, and incubated for 1 h at 37°C. The supernatant was readjusted to 10 mmol/L ethylenediaminetetraacetic acid EDTA, 8 g/L sodium lauryl sulfate, 100 mmol/L NaCl and 1.6 mg pronase/mL, and was incubated for an additional 1 h at 37°C. The supernatant was extracted twice with phenol/chloroform, precipitated with 0.7 volume of isopropanol, and resuspended in 30 µL 10 mmol/L Tris hydrochloride (pH 8.0) and 1 mmol/L EDTA. DNA Southern blot hybridization analysis was performed with 30 µL viral replication intermediates, as previously described^[7]. HBV DNA probe was labeled by

using DIG Luminescent Detection Kit (Roche Applied Science). A digoxigenin-labeled, full-length HBV DNA probe was used for Southern filter hybridization analysis.

Detection of liver HBsAg and HBeAg by immunohistochemistry

The number and location of cells in the liver that expressed HBV core antigen (HBeAg) or HBsAg were assessed by immunohistochemical staining. Liver tissue fixed in formalin was embedded with paraffin and sectioned (3 µm thick). The paraffin-embedded sections in PBS were treated for 15 min at 37°C with 30 g/L hydrogen peroxide, and washed with PBS. After the sections were blocked with normal goat serum for 20 min at 37°C, rabbit anti-HBsAg (Bioscience) or rabbit anti-HBe (NEOMARKERS) primary antibody was applied at 1:100 or 1:150 dilution for 60 min at 37°C, or overnight at 4°C, respectively. After washing with PBS, the secondary antiserum consisting of Polymer-HRP Anti-Rabbit (Zhongshan, Beijing) was applied at 1:350 dilution for 50 min at 37°C, according to the manufacturer's instructions. The antibody-coated sections were washed with PBS, stained with 3', 3'-diaminobenzidine tetrahydrochloride (DAB), and counterstained with hematoxylin, before being mounted.

Serological analysis for HBV antigen

HBeAg and HBsAg analysis was performed with 50 µL mouse serum by using enzyme-linked immunosorbent assay (ELISA). The HBeAg and HBsAg Detection Kits (Shanghai Shiye Kehua Company, China) were used respectively.

RESULTS

Assessment of the appropriate amount of pHBV4.1 plasmid DNA for hydrodynamic injection

To determine an appropriate amount of pHBV4.1 DNA for establishing the mouse model for HBV replication, mice were divided into different groups (two in each group) and injected with various amount of plasmid DNA (5, 10 and 25 µg per mouse). HBV DNA replicative intermediates were examined on d 4 after injection. The results of three independent experiments showed that a significant level of HBV DNA replicative intermediates was detected in mouse liver when the amount of plasmid DNA injected was as low as 5 µg per mouse. The level of HBV replicative intermediates increased when the amount of plasmid DNA was increased to 10 µg per mouse. A further increase in the amount of plasmid DNA to 25 µg per mouse did not result in a significant increase in HBV replicative intermediates in mouse liver. Therefore, we used 10 µg per mouse as the appropriate dose of pHBV4.1 to develop an HBV-replication-competent mouse model (Figure 1).

Establishment of a mouse model with HBV replication

Thirty mice were injected with pHBV4.1 plasmid DNA at 10 µg per mouse using a hydrodynamic procedure. These mice were sacrificed on d 1, 3, 4, 5, 7 and 10 after injection (*n* = 5 at each time point). The level of HBV replication and expression were analyzed.

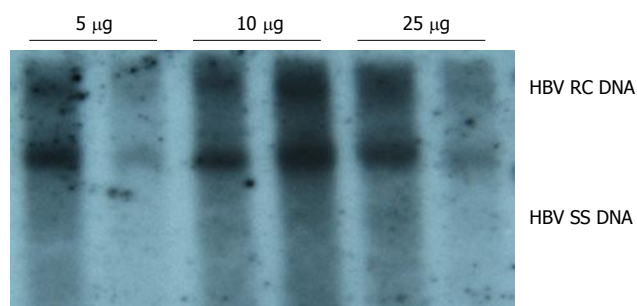


Figure 1 DNA-dose-dependent HBV replication. Various amount of pHBV4.1 in 1.8 mL saline were injected into BALB/c mice within 5-8 s. Mice were sacrificed on d 4 after injection, and HBV DNA intermediates were detected by Southern blotting.

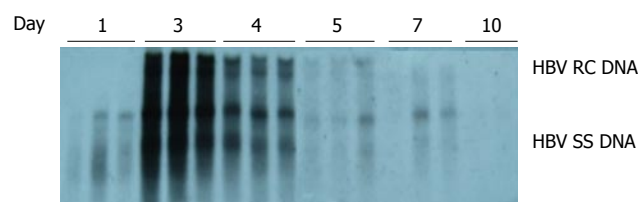


Figure 2 Time-dependent viral replication after hydrodynamic *in vivo* transfection with pHBV4.1. Mice were injected hydrodynamically with 10 µg pHBV4.1 and were sacrificed at different time points after injection. HBV DNA intermediates were detected by Southern blotting.

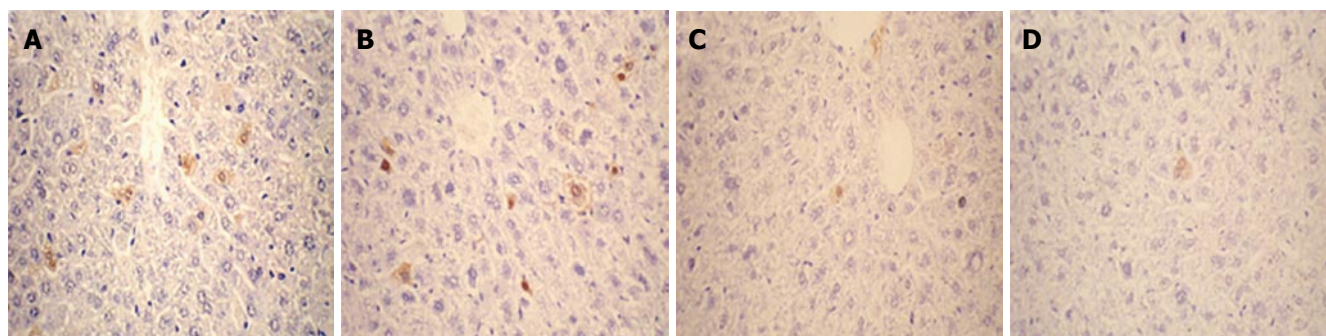


Figure 3 Detection of HBCAg expression in the liver by immunohistochemical staining (DAB, × 40). Mice were injected hydrodynamically with 10 µg pHBV4.1 and were sacrificed at different time points after injection and HBCAg in mouse liver was detected. **A:** 1 d after transfection; **B:** 4 d after transfection; **C:** 7 d after transfection; **D:** 10 d after transfection.

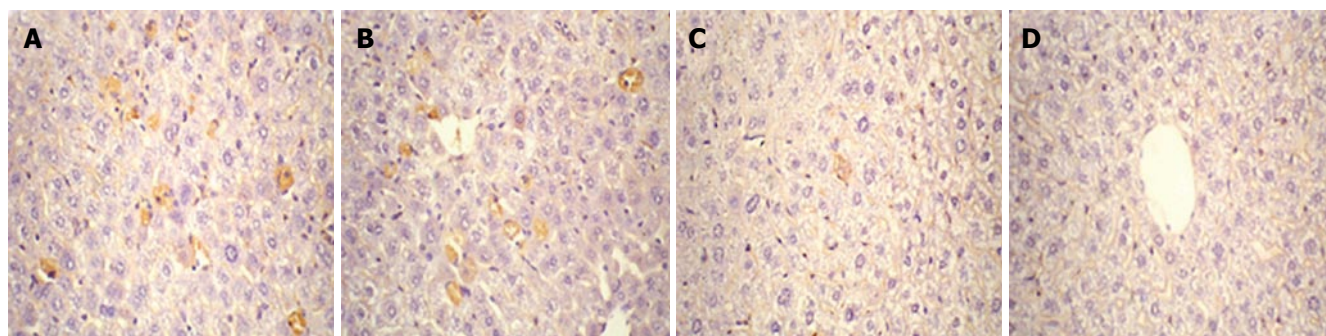


Figure 4 Detection of HBsAg expression in liver by immunohistochemical staining (DAB, × 40). Mice were injected hydrodynamically with 10 µg pHBV4.1 and were sacrificed at different time points after injection and HBsAg in mouse liver was detected. **A:** 1 d after transfection; **B:** 4 d after transfection; **C:** 7 d after transfection; **D:** 10 d after transfection.

HBV replication intermediates

As shown in Figure 2, HBV DNA replication intermediates were detected on d 1 [but without the relaxed circular DNA (RC DNA) form], and were abundant on d 3 and 4. By d 5, the number of HBV replication intermediates decreased, and were eventually undetectable by d 10. These results suggested that HBV could replicate in the liver of the mice, and that the level of HBV replication reached a peak on d 3 and 4 after hydrodynamic transfection.

Expression of HBCAg and HBsAg in mouse liver

Expression of HBCAg and HBsAg in the liver collected on d 1, 4, 7 and 10 after injection was detected by immuno-

histochemistry. As shown in Figures 3 and 4, HBCAg- and HBsAg-positive hepatocytes were randomly distributed throughout the liver lobules, with a tendency for localization in the centrilobular area. HBCAg was present in the nuclei and cytoplasm of the hepatocytes, with most expression in the nuclei. HBsAg staining was only seen in the cytoplasm. The expression of both antigens exhibited a similar time-dependent expression pattern that reached a maximum level on d 1, and remained steady until d 4 after injection, followed by a sharp decline. There were only a few HBCAg- and HBsAg-positive cells in whole liver sections by d 7 and 10.

Serum from mice sacrificed on d 1, 3, 4, 5, 7 and 10

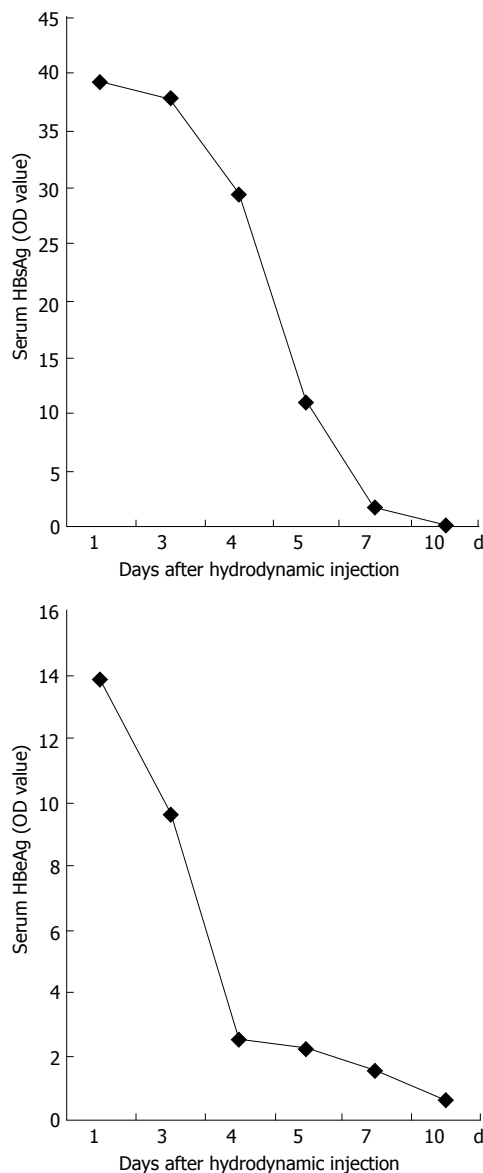


Figure 5 Time-dependent HBV antigen expression in serum after hydrodynamic transfection. Mice were injected with 10 μ g pHBV4.1. At different time points, HBsAg and HBeAg in the serum were measured by ELISA.

after injection was measured by ELISA for the level of HBeAg and HBsAg (OD₄₅₀). Samples were considered positive at OD₄₅₀ \geq 2.1. The mean level of HBeAg or HBsAg expression at each point was derived from five mice. Figure 5 shows that the expression of HBeAg and HBsAg in the serum was detected on d 1, 3, 4 and 5, reached a peak on d 1 after injection, and decreased thereafter. All samples from mice on d 7 and 10 after injection were negative for HBeAg and HBsAg.

HBV replication levels in different parts of mouse liver

To investigate whether the levels of HBV replication in different parts of the liver are similar, mice were injected with 10 μ g pHBV4.1 plasmid DNA and sacrificed 3 d later. The liver of each mouse was divided into three parts: left, right and middle lobules. The level of HBV DNA replication intermediates in each part was analyzed and compared. We found that the level of HBV replication in

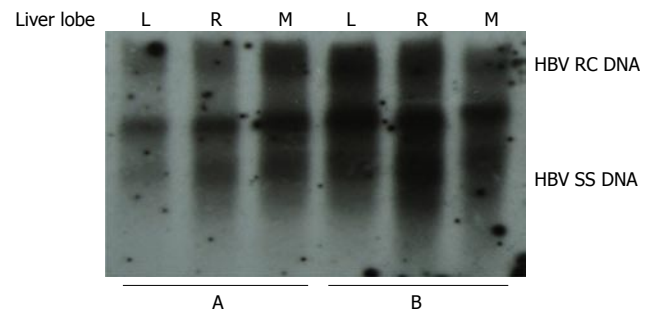


Figure 6 Viral replication in different parts of the mouse liver after hydrodynamic transfection. The results from two mice (A and B) are shown. L: left lobe; R: right lobe; M: middle lobe.

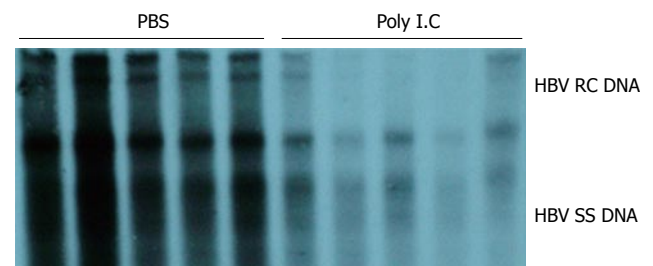


Figure 7 Effect of polyIC on HBV replication in mice. After matched by body weight, age and serum HBeAg, mice were treated with PBS or polyIC. HBV replication levels in the liver of the two groups of mice were compared.

the left, right and middle lobules in each mouse was similar (Figure 6).

Effect of polyIC injection on HBV replication

To investigate whether the mouse model established in this study can be used for the study of HBV, we used it to evaluate the effect of polyIC on HBV replication. As shown in Figure 7, compared with the control animals that were injected with PBS only, HBV DNA replicative intermediates dramatically decreased in the liver from mice injected with polyIC. This result suggested that repetitive polyIC injection decreased the level of viral replication in the liver.

DISCUSSION

HBV is a member of the hepadnavirus family, which contains a group of closely related hepatotropic DNA viruses that infect their respective animal hosts, such as HBV, woodchuck hepatitis virus (WHV)^[9] and duck hepatitis B virus (DHBV)^[10]. HBV naturally infects human and certain non-human primates such as chimpanzees. However, experiments with chimpanzees are limited by cost, availability and ethical considerations. Also, the ability of HBV to infect tupia and the significance of tupia as an HBV animal model remain controversial. Therefore, much current understanding about HBV is derived from studies of WHV and DHBV infection in their natural host^[11]. In addition to their use for the selection of anti-HBV drugs^[12,13], they are also used to study the mechanism of HBV replication by reverse transcription^[14].

and pathogenesis of HBV-related hepatocellular carcinoma^[15-17]. However, these two hepadnaviruses have some differences to HBV in terms of genome structure and transcriptional and replicative regulation; therefore, they cannot serve as a substitute for research into HBV^[18]. Recently, much attention has been paid to HBV transgenic mice, especially HBV-replication-competent transgenic mice with 1.3 copies of the genome of HBV. It is one of the most widely-used animal models in studies on the mechanism of HBV replication^[19-21], the pathogenesis of HBV-related liver diseases^[22-24], and the selection of anti-HBV drugs^[25-27]. However, the production of transgenic mice is very laborious and time-consuming. Therefore, it is necessary to develop a convenient, rapid and useful animal model for HBV-related studies. Hydrodynamic *in vivo* transfection is an efficient, rapid and convenient gene-delivery method, invented in 1999^[7]. We have reported previously that with the hydrodynamic injection of plasmid DNA encoding HBsAg *via* the tail vein into mice, efficient expression of HBsAg was detected mainly in the mouse liver, which persisted for about 10 d. In this study, by taking advantage of this hydrodynamic *in vivo* transfection method, an HBV-replication mouse model was developed by using the HBV-replication-competent plasmid pHBV4.1. The replication of HBV was confirmed by the presence of HBV-replication-intermediate DNA, HBcAg and HBsAg in the mouse liver, and the sera of mice were positive for HBeAg and HBsAg.

For different plasmid DNA, the doses needed for hydrodynamic injection are presumably different; therefore, we first determined the appropriate amount of pHBV4.1 for hydrodynamic transfection. The results of three independent experiments demonstrated that the level of HBV replication was significant and reached a steady-state level when the amount of plasmid DNA injected was as low as 10 µg per mouse. To keep the replication at a high level and also save the plasmid, we used 10 µg per mouse as the appropriate amount of pHBV4.1 to establish an HBV-replication-competent mouse model.

HBV is an enveloped virus containing a 3.2-kb partially double-stranded DNA genome within its nucleocapsids^[28,29]. Upon HBV infection of the hepatocyte, the viral genome is converted to covalently closed circular DNA (cccDNA) in the nucleus. The cccDNA serves as the template for transcription by the host RNA polymerase II, which generates the 3.5-, 2.4-, 2.1- and 0.7-kb viral transcriptions that encode the HBV core (HBcAg and HBeAg) and polymerase polypeptide, the large surface antigen polypeptide, the middle and major surface antigen polypeptides (HBsAg)^[30-32] and the HBx polypeptide^[33]. Besides encoding for the HBV core polypeptide and HBV DNA polymerase that compose the viral capsid, the greater-than-genome-length 3.5-kb pregenomic RNA (pgRNA) is encapsulated and serves as the template for reverse transcription to synthesize HBV replication intermediates^[34-36]. The time-course of the results of our experiments provided the characteristics of HBV replication in the mouse model: within 24 h after transfection, the input DNA apparently reached the nuclei of the hepatocytes, in which it was transcribed and translated to produce HBV-related antigens, and these

antigens were soon secreted into the serum. The time at which HBV antigen expression peaked was on d 1 after transfection. The expression level of viral antigen in mouse liver was stable, but the titer of HBeAg and HBsAg in the serum decreased from d 1 to 4 accordingly. The reason for this difference is unclear at present. Viral replication was not robust on d 1, although the presence of small amounts of single-stranded DNA, the earliest replicative DNA intermediates, indicated that viral replication had begun. By d 3 and 4, viral replication reached its peak level and decreased substantially until d 10, which is the time that HBV replication intermediates could not be detected. All these findings suggest that it probably requires about 2 d for 3.5-kb mRNA to produce HBV replication intermediates by reverse transcription abundantly.

The basic mechanism of the hydrodynamic transfection procedure is that when a large volume of DNA solution is injected rapidly *via* the tail vein, a high hydrostatic pressure develops in the inferior vena cava and causes heart failure, which forces a rapid flow of DNA solution to the liver tissue and DNA molecules into liver cells^[7,37-39]. In this way, digestion of the input DNA by DNAase in the blood is greatly reduced.

To assess whether the levels of gene expression in different liver lobes are different, we compared the level of HBV replication in left, right and middle liver lobes from the same mouse. The results suggested that, for a given mouse, the level of HBV-DNA-replicative intermediates was similar in all parts of the liver. This also suggests that the experimental results were not influenced by the use of different liver tissue parts. As shown in Figures 1 and 2, however, there was some variation in HBV replication between different mice, even under similar experimental conditions. This suggests that if this animal model is to be used for selecting anti-HBV drugs or for other research in which HBV replication levels need to be compared, it is necessary to match the HBV-DNA-replication levels by checking serum HBeAg before treatment, and that repeated experiments are needed to guarantee the reliability and accuracy of the test findings.

As a strong inducer of alpha/beta interferon, polyIC has been widely used in HBV transgenic mice and exerts an inhibitory effect on HBV replication^[40-42]. Therefore, we applied polyIC treatment to the HBV replication mouse model established in this study to investigate whether this model can be used for studies of HBV. We found that polyIC could inhibit HBV replication, which suggests that the mouse model developed in our study can be used for future research on HBV.

To understand the mechanisms of HBV transcription, replication and pathogenesis, it is usually necessary to mutate the HBV genome, and then measure the transcription, replication and expression levels of viral mutants; in this way, the function of various HBV genome sequences can be understood. If HBV transgenic mice are used, it is necessary for every mutated HBV genome to be transferred into mouse fertilized eggs, and then transgenic mice can be cultivated, but that is very time-consuming, costly and laborious. In contrast, by using the HBV-replication-competent mouse model developed in this study, no surgical techniques or special equipment

are needed, which suggests that this model is very simple, convenient, rapid and economical. As a result, this model affords new opportunities for studying HBV; in particular, it is useful for investigating HBV genomic function, especially its role and mechanism in HBV replication through mutation of the HBV genome.

In conclusion, we developed a rapid and convenient mouse model with a high level of HBV replication, and applied it to detecting the inhibitory effect of polyIC on HBV. This provides a very useful tool for functional studies on HBV replication and selection of anti-HBV drugs.

ACKNOWLEDGMENTS

We are grateful to Alan McLachlan (The Scripps Research Institute, La Jolla, CA, USA) for plasmid pHBV4.1.

COMMENTS

Background

Hepatitis B Virus (HBV) infection is a worldwide health problem. Because the pathogenic mechanisms of HBV remain unclear, molecular-biological studies for HBV are required. Until recently, most *in vivo* studies about HBV have been performed by using HBV transgenic mice. However, the acquisition of transgenic mice is very time-consuming and laborious. Therefore, it is necessary to develop a convenient, rapid and useful animal model.

Research frontiers

HBV mouse model developed by using the hydrodynamic *in vivo* transfection method is convenient and useful for HBV-related studies. If the mouse genetic background or plasmid backbone are changed, many different HBV mouse models can be developed for different purposes. By using immunodeficient mice or using AAV as backbone of transferred plasmid, chronic HBV carrier mouse models were established.

Innovations and breakthroughs

In this study, we established a mouse model with HBV replication with naked HBV plasmid through a hydrodynamic procedure and found that for a certain mouse, the levels of HBV DNA replication are similar in left, right, and middle parts of liver tissue. Non-isotope labeled probe and chemiluminescent detection system was used to detect hepatitis B virus replication levels *in vivo* by southern hybridization.

Applications

This mouse model can be used for functional study of HBV genome, for studies about HBV replication mechanism and for selection of anti-HBV drugs.

Peer review

This paper describes the establishment of a mouse model with hepatitis B virus replication, with the aim to provide a tool for selection of antiviral drugs to inhibit HBV replication. The work is well developed, and shows promising results.

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Right liver lobe/albumin ratio: Contribution to non-invasive assessment of portal hypertension

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Abstract

AIM: To study the value of biochemical and ultrasonographic parameters in prediction of presence and size of esophageal varices.

METHODS: The study includes selected cirrhotic patients who underwent a complete biochemical workup, upper digestive endoscopic and ultrasonographic examinations. Albumin/right liver lobe diameter and platelet count/spleen diameter ratios were calculated. The correlation between calculated ratio and the presence and degree of esophageal varices was evaluated.

RESULTS: Ninety-four subjects (62 males, 32 females), with a mean age of 52.32 ± 13.60 years, were studied. Child-Pugh class A accounted for 42.6%, class B 37.2%, whereas class C 20.2%. Esophageal varices (OE) were not demonstrated by upper digestive endoscopy in 24.5%, while OE grade I was found in 22.3% patients, grade II in 33.0%, grade III in 16.0%, and grade IV in 4.3%. The mean value of right liver lobe diameter/albumin ratio was 5.51 ± 1.82 (range from 2.76 to 11.44), while the mean platelet count/spleen diameter ratio was 1017.75 ± 729.36 (range from 117.39 to 3362.50), respectively. Statistically significant correlation was proved by Spearman's test between OE grade and calculated ratios. The *P* values were 0.481 and -0.686, respectively.

CONCLUSION: The right liver lobe diameter/albumin and platelet count/spleen diameter ratios are non-invasive parameters providing accurate information pertinent to determination of presence of esophageal varices, and their grading in patients with liver cirrhosis.

INTRODUCTION

Esophageal varices development is among the major complications of liver cirrhosis, with an estimated prevalence of approximately 50%. The risk of bleeding from varices is 25%-35% with majority of the initial bleeding occurring within 1 year from varices detection^[1]. The mortality from each episode of variceal bleeding is 17%-57%^[2-4]. Bleeding episodes can be predicted by the presence of red signs ("red cherry spots") on the varices, and by the variceal size^[5,6]. The incidence of bleeding can be reduced with nonselective beta-blockers^[7,8]. It is also suggested that prophylactic endoscopic variceal ligation can decrease the incidence of first variceal bleeding and mortality in patients with liver cirrhosis who have large varices^[9,10]. Therefore, annual endoscopic screening is highly recommended for patients with small esophageal varices while the procedure should be conducted once every two years for patients suffering from liver cirrhosis without diagnosed varices^[11,12]. Nevertheless, repeated endoscopic examinations are unpleasant for patients, and have cost impact on health care insurance, while only half of cirrhotic patients have esophageal varices, and up to 30% have large varices. Therefore, the sensitivity and specificity of numerous non-invasive parameters have been investigated for assessment of presence and size of esophageal varices, and risk prediction for bleeding.

We aim to identify non-invasive parameters based on ultrasonographic measurement and laboratory test that have a potential in assessment of presence and size of esophageal varices in patients with liver cirrhosis.

MATERIALS AND METHODS

This study includes 94 patients treated for liver cirrhosis in Clinic for Gastroenterology and Hepatology, Clinical Centre of Serbia in 2006. The diagnosis of cirrhosis

Table 1 Clinical characteristics of patients

Sex (M/F)	62/32
Age (yr; mean \pm SD)	52.32 \pm 13.60
Age range (yr)	17-79
Aetiology of liver disease (alcoholic/infective/autoimmune/other)	43/19/17/15
Child-Pugh class (A/B/C)	40/35/19
Mean size of the right liver lobe (mean \pm SD)	157.71 \pm 23.37
Mean size of the spleen (mean \pm SD)	148.05 \pm 33.73
Mean albumin concentration (g/L; mean \pm SD)	30.79 \pm 7.97
Mean thrombocyte number (mean \pm SD)	134 632.98 \pm 75 937.83
Oesophageal varices grade (0/ I / II / III / IV)	23/21/31/15/4

Table 2 Calculated ratios and varice size

Varices	Right lobe/albumin ratio		Platelet count/spleen ratio	
	mean \pm SD	Range	mean \pm SD	Range
Grade 0	4.19 \pm 1.36	2.76-8.19	1822.53 \pm 804.06	608.70-3362.50
Grade I	5.43 \pm 1.58	3.25-9.44	1072.86 \pm 493.49	366.67-2021.05
Grade II	5.84 \pm 1.25	3.40-8.48	696.46 \pm 439.43	150.00-2283.33
Grade III	6.57 \pm 2.42	4.07-10.00	549.14 \pm 289.90	127.27-1275.00
Grade IV	6.68 \pm 2.52	4.07-11.44	348.19 \pm 231.86	117.39-633.33

was based on clinical features, laboratory test, imaging diagnostics, and liver histology whenever possible.

The following information was collected for each patient: age, gender, etiology of cirrhosis, biochemical parameters (aspartate aminotransferase, alanine aminotransferase, total bilirubin, serum albumin, prothrombin activity, and platelet count), presence and degree of esophageal varices and degree of liver function impairment by Child-Pugh classes. Cirrhosis etiology was determined as viral if hepatitis B surface antigen or hepatitis C serum markers were positive. If viral markers were negative, and the history obtained indicated alcohol consumption of at least 50 g per day in the past five years, liver cirrhosis was considered as alcoholic. Positive immunological markers were characteristic for immunological liver disease. The other studied cases had liver cirrhosis of different etiology (Wilson's disease, α 1 antitrypsin deficiency, hemochromatosis, *etc.*).

All studied subjects underwent ultrasonographic examination of the upper abdomen. The right lobe diameter in the medioclavicular line, as well as the spleen bipolar diameter were measured for three times and the mean value was recorded. One investigator performed all measurements (TA) in order to reduce the inter- and intra-observer errors in assessing the diameters.

Using the laboratory and ultrasonographic values, we calculated two ratios: right lobe diameter/serum albumin concentration and platelet count/spleen diameter.

An experienced endoscopist (SD) who used the grade I-IV classification^[13] did the endoscopies. Varices in the level of mucosa were recognized as grade I, those smaller than 5 mm fulfilling less than 1/3 of the esophageal lumen were recognized as grade II, grade III were varices larger than 5 mm fulfilling more than 1/3 of the esophageal, while grade IV varices occupied more than 2/3 of esophageal lumen. Patients with previous variceal

bleeding, porto-systemic shunts and those taking beta-blockers medications and patients with coexistent illness or infection that could influence the liver and spleen size, were excluded.

Child-Pugh score was calculated using five variables (ascites, encephalopathy, bilirubin, albumin, and prothrombin time). Values 1, 2 or 3 were assigned to each of these variables due to increasing abnormality, and the score calculated as sum of the five variables for each patient. A Child-Pugh score less than 7 was considered as class A, from 7 to 9 as class B, while any score greater than 9 was as class C^[14]. Laboratory test and ultrasonographic and endoscopic examinations were performed within one week.

The Ethic Committee of our institution approved the study and all patients gave an informed consent prior to inclusion into this investigation.

All collected data were analyzed and correlated. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS, version 10.0). Basic descriptive statistics included means, standard deviations, ranges and percentages. For correlation analysis, we used Spearman's test. Differences were considered statistically significant if the two-tailed *P* value was less than 0.05. Sensitivity and specificity, as well as the best cut-off value for the diagnosis of varices were calculated using ROC curves.

RESULTS

Clinical characteristics of the patients are presented in Table 1.

The mean value of the ratio of the platelet count/spleen size was 1017.75 \pm 729.36 (range 117.39-3362.50). The mean value of the ratio of the right liver lobe size/serum albumin concentration was 5.51 \pm 1.82 (range 2.76-11.44).

These ratios were correlated with the presence and size of esophageal varices (Table 2, Figure 1A and B). Spearman's test for nonparametric correlation showed that, the platelet count/spleen size ratio significantly correlated with the presence (*P* = 0.585, *P* < 0.01) and size (*P* = 0.686, *P* < 0.01) of esophageal varices, so does the right liver lobe/serum albumin concentration ratio (*P* = 0.488, *P* < 0.01 and *P* = 0.481, *P* < 0.01 respectively). We also studied the correlation with Child-Pugh score of liver function impairment (Table 3, Figure 2A and B). These ratios had a highly significant correlation (*P* < 0.01) with Child Pugh score. For the platelet count/spleen size ratio, *P* = -0.585, while for the right liver lobe/serum albumin concentration ratio, *P* = 0.491.

We counted the best cut-off value of the right liver lobe/serum albumin concentration ratio for diagnosis of varices. For the value of 4.425, the sensitivity was 83.1% and the specificity was 73.9% (Figure 3).

DISCUSSION

Incidence of esophageal varices in patients with liver cirrhosis is approximately 90%, but only 9%-35% have been described^[15-17]. Variceal bleeding in cirrhotic

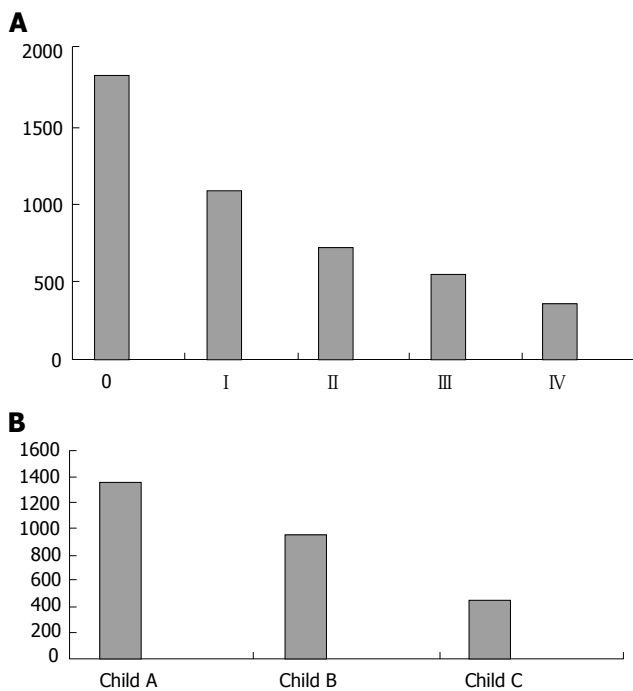


Figure 1 A: Platelet count/spleen ratio in correlation to varice size; B: Platelet count/spleen ratio in correlation to Child-Pugh score.

Table 3 Calculated ratios and Child score for hepatic insufficiency

Child-Pugh score	Right lobe/albumin ratio		Platelet count/spleen ratio	
	mean \pm SD	Range	mean \pm SD	Range
A	4.59 \pm 1.38	2.76-8.19	1355.34 \pm 796.62	365.52-3362.50
B	5.92 \pm 1.60	3.25-9.44	946.67 \pm 612.48	127.27-2975.00
C	6.68 \pm 2.12	4.07-11.44	437.97 \pm 215.99	117.39-900.00

patients is associated mainly with fatal outcome. Portal hypertension is an essential element of survival in cirrhotic patients^[5]. Therefore, regular control and evaluation of esophageal varices with timely introduction of nonselective beta-blockers and variceal ligation play an important role in prevention of bleeding. Endoscopy is an invasive and costly diagnostic procedure. Therefore, introduction of non-invasive parameters for assessment of presence and size of esophageal varices is a major goal of numerous studies.

Platelet count and spleen size were the most frequently explored non-invasive parameters in recent studies. Zaman^[15] reported that patients with platelet counts of less than 88 000/mm³ have five times greater likelihood of having large esophageal or gastric varices as compared with the patients with higher platelet counts. Ng^[16] identified correlation between presence of ascites, thrombocytopenia, hyperbilirubinemia and larger varices in the Chinese population. Similarly, Chalasani^[11] concluded that large esophageal varices are predictable in thrombocytopenic patients who have enlarged spleen, while platelet count of less than 88 000/mm³ indicates a higher risk for esophageal bleeding. The same study also demonstrated that endoscopy is incrementally cost-effective. Madhura reported that platelet count less than 68 000/mm³ have a

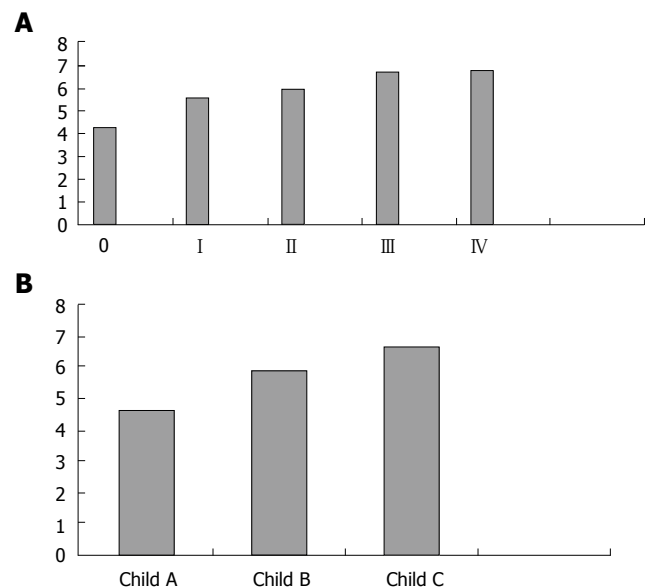


Figure 2 A: Right lobe/albumin ratio in correlation to varice size; B: Right lobe/albumin ratio in correlation to Child-Pugh score.

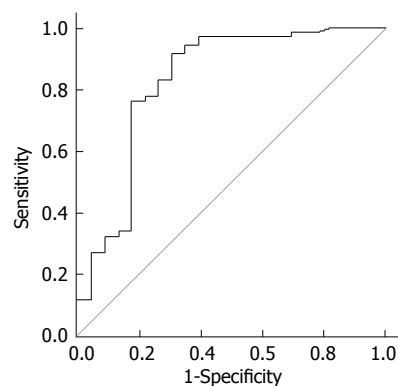


Figure 3 ROC curve for diagnosis of esophageal varices using right lobe/albumin ratio.

larger discriminatory value^[12].

Splenomegaly and hypersplenism are prevalent in patients with liver cirrhosis and portal hypertension^[11]. Thrombocytopenia is a common and highly specific manifestation of hypersplenism, but with low sensitivity for presence of portal hypertension^[18]. It is suggested that the main mechanism of thrombocytopenia is splenic sequestration and pooling^[19], and other mechanisms are also involved. Madhura and co-authors reported that 32% of patients had platelet count less than 68 000/mm³ without detectable splenomegaly, which might be explained by insufficient synthesis of thrombopoietin. It is also indicated that platelet count and thrombopoietin level are returning to referent values following liver transplantation^[20]. Other potential explanations for this phenomenon are presence of antithrombotic antibodies and thrombocyte associated immunoglobulin, which can be found in the sera of patients with liver diseases^[21].

Some investigators reported that the use of non-invasive indicators is invalid for avoiding the endoscopic screening in patients with primary biliary cirrhosis and

sclerosing cholangitis. The probability of esophageal varices presence is lower in these patients if they have platelet count less than $200\,000/\text{mm}^3$, possibly due to the fact that thrombopoietin production is preserved in primary biliary cirrhosis and sclerosing cholangitis^[22].

It is well documented that the spleen size is correlated with severity of esophageal varices^[23-25]. Watanabe^[25] calculated the splenic ratio (length \times width \times height of the spleen size on computed tomography) and showed that patients having a ratio greater than 963 cm^3 have esophageal varices, and that a high ratio may predict esophageal bleeding in patients with liver cirrhosis. It is concluded that ultrasonographic measurement of splenic cranio-caudal diameter results in lower inter- and intra-observer variability unlike Doppler sonographic assessment of hepatic flow^[26,27]. The value of Doppler sonographic assessment of portal hypertension is controversial. It is indicated that congestive ratio (counted from the cross section area and mean velocity of portal flow)^[26,28,29], as well as resistance ratio of the hepatic artery^[30] and splenic artery^[24] are correlated with portal hypertension. Portal hemodynamic parameters (mean and maximum portal vein velocity) seem to be weak in assessment of portal hypertension^[31]. In addition, it is important to keep in mind that performing Doppler sonographic examination requires sophisticated skills and equipment.

The pathophysiologic mechanisms are combined based on the integration of two non-invasive parameters, i.e., platelet count and spleen size into one ratio. Calculation of this ratio is very easy for routine clinical practice. Gianinni and co-authors^[32,33] reported the results of a retrospective and prospective study and concluded that this ratio is sensitive for prediction of presence and size of esophageal varices. The same study group suggests as especially important, the use of non-invasive parameters in the diagnostic algorithm for identifying patients without esophageal varices. A cut-off value of 909 has been proposed for platelet count/spleen size ratio^[34]. Patient having the ratio greater than cut-off value should not receive nonselective beta-blockers prophylactic therapy because they are less likely to develop esophageal varices. These patients should less frequently undergo endoscopy.

Taking into account the results of previous studies in the field, we also combined laboratory and ultrasonographic parameters and counted an original ratio. For the first time we reported the value of the right liver lobe/serum albumin concentration in assessment of portal hypertension^[35]. We used serum albumin concentration as a parameter of liver function in combination with right liver lobe size.

Previously published studies reported that platelet count/spleen size ratios are good predictors of esophageal varices. Despite a good correlation between these ratios and esophageal varices grade, it is unlikely that these ratios could be used to exclude patients from initial endoscopic screening. Nevertheless, these ratios may serve for selection of patients who need more frequent endoscopies. These ratios will help identify patients at higher risks for development of esophageal varices. It will provide insight into the relationship between clinical, biochemical,

hematological and imaging abnormalities and development of clinically significant esophageal varices. The right liver lobe diameter/albumin, as well as platelet count/spleen diameter ratios are non-invasive parameters that can provide accurate information pertinent to determination of the presence and grade of esophageal varices in patients with liver cirrhosis. Further validation of the results will be achieved through long-term follow-up of the patients and a larger number of studied subjects.

COMMENTS

Background

Esophageal varices are frequent in patients with liver cirrhosis. There is an increasing mortality rate of cirrhotic patients due to high risk of bleeding. The correlation of varices size and risk of bleeding is well-known, yet non-invasive techniques for variceal size estimation have not been intensively studied.

Research frontiers

Valuable laboratory tests and clinical examination may help predict the risk of esophageal varices bleeding. This study shows that excellent correlation exists between platelet count/spleen size and size of esophageal varices, so does the right liver lobe/serum albumin concentration ratio. In addition, the authors suggest that the use of non-invasive and less expensive methods may be of benefit for patients and consumers.

Innovations and breakthroughs

Accumulated literature shows that it is necessary to investigate non-invasive parameters in liver cirrhosis for prediction of the size of esophageal varices. Development of these methods may decrease the exposure of cirrhotic patients to repeated endoscopic procedures.

Applications

This can be used for non-invasive estimation of esophageal varice size in cirrhotic patients.

Peer review

The manuscript reports the results from an investigation of non-invasive parameters in estimation of variceal size and risk for bleeding. The authors conclude that combination of laboratory and ultrasonographic parameters such as right liver lobe/serum albumin concentration may be useful in assessment of portal hypertension. The appropriate figures and tables are supporting the results.

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RAPID COMMUNICATION

Liver biopsy in a district general hospital: Changes over two decades

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Abstract

AIM: To study liver biopsy practice over two decades in a district general hospital in the United Kingdom.

METHODS: We identified all patients who had at least one liver biopsy between 1986 and 2006 from the databases of the radiology and gastroenterology departments. Subjects with incomplete clinical data were excluded from the study.

RESULTS: A total of 103 liver biopsies were performed. Clinical data was available for 88 patients, with 95 biopsies. Between 1986 and 1996, 18 (95%) out of the 19 liver biopsies performed were blind and 6 (33%) were for primary biliary cirrhosis. Between 1996 and 2006, 14 (18%) out of 76 biopsies were blind; and the indications were abnormal liver tests (33%), hepatitis C (12%) and targeted-biopsies (11%). Liver biopsies were unhelpful in 5 (5%) subjects. Pain was the most common complication of liver biopsy (5%). No biopsy-related mortality was reported. There was a trend towards more technical failures and complications with the blind biopsy technique.

CONCLUSION: Liver biopsies performed in small district hospitals are safe and useful for diagnostic and staging purposes. Abnormal liver tests, non-alcoholic fatty liver disease and targeted biopsies are increasingly common indications. Ultrasound-guided liver biopsies are now the preferred method and are associated with fewer complications.

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Key words: Complication; District general; Indication;

INTRODUCTION

Since the first reported percutaneous liver biopsy in 1923, the liver biopsy has become widely used in the investigation of liver disease and is currently the gold standard for confirming the diagnosis and for staging of liver disease^[1]. However, it remains an invasive procedure, with a mortality risk ranging between 0.01% and 0.17%^[2]. Studies have shown that less than one-third of the biopsies altered the treatment^[3]; hence biopsies should only be performed in patients who would benefit from this procedure.

Over the last few decades, there has been a significant improvement in diagnostic and imaging techniques, as well as in the drug therapy of liver disease^[1]. These have led to changes in our management of liver diseases. At the same time, changes in socio-economic status and life-style, have resulted in increased prevalence of obesity^[4,5] and rising incidence of non-insulin diabetes mellitus^[6,7]. Non-alcoholic steatohepatitis (NASH), the hepatic manifestation of the metabolic syndrome or insulin-resistant state^[8], is now one of the most common indications for liver transplantation in the USA^[9]. Consequently, the indications for liver biopsies in the UK will be expected to evolve over time.

We reviewed all liver biopsies performed in a single district general hospital (DGH) in the UK over two decades. We examined the indications, findings and complications of liver biopsy and explored the changes in our practice over the last two decades.

MATERIALS AND METHODS

Patients

All patients who had a liver biopsy were identified from the databases of the radiology and gastroenterology departments. Relevant clinical and laboratory information was collected retrospectively. Patients whose clinical information was incomplete were excluded from the analysis.

Table 1 Patient demographics and clinical features

	Median values (range)	Normal values
Age (yr)	57 (25-86)	
Sex (F/M)	51:37	
Liver biochemistry		
ALT (U/L)	66.5 (11-1103)	10-31
Bilirubin ($\mu\text{mol/L}$)	13 (5-430)	1-17
GGT (U/L)	221 (20-1394)	0-50
ALP (U/L)	145 (44-994)	45-145
Platelets ($\times 10^9/\text{L}$)	239 (93-813)	150-500
Indications for biopsy:		
Acute hepatitis	5	
Chronic hepatitis/Chronic elevated liver tests/Staging of disease	75	
Abnormal imaging	8	
Disease follow-up	3	
Routes of biopsy:		
Blind	32 (3 ¹)	
USS guided	60 (1 ¹)	
TJB	2	
Laparoscopic	1	

ALT: Alanine aminotransferase; GGT: Gamma glutamyltransferase; ALP: Alkaline phosphatase; USS: Ultrasonography; TJB: Transjugular liver biopsy.
¹Failed.

Liver biopsy protocol

In the absence of any contraindications, liver biopsies were performed when clinically indicated, as determined by the supervising clinician. The clinician performing the biopsy obtained an informed consent.

A full blood count (FBC) and clotting profile were obtained one day prior to the biopsy to ensure that the platelet counts were $> 80\,000/\text{mm}^3$ and the international normalized ratio (INR) was < 1.3 ^[1,2,10]. Appropriate platelet and clotting factors were given as necessary. An abdominal ultrasound of the liver was performed within six months of biopsy to ensure that there were no anatomical variants, biliary dilatation or focal or cystic lesions that may require a targeted biopsy^[1,11]. The presence of moderate to severe ascites was considered a contraindication to percutaneous biopsy^[1]. If considered safe and feasible, patients with massive ascites underwent paracentesis until completely dry, prior to the liver biopsy.

All liver biopsies were performed either as a 'blind' procedure or under ultrasound (USS)-guidance, depending on the personal preference of the clinician or the availability of a radiologist. Patients were discharged after six hours if the procedure was uncomplicated, and if the patient was clinically stable and pain free. A transjugular liver biopsy (TJB) was performed in patients with contraindications to a percutaneous biopsy.

Liver biochemistry, serology and immunology

All patients had liver biochemistry (serum bilirubin, alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyltransferase (GGT)) taken at the time of the initial clinical assessment. The normal ranges of these tests were: INR, 1; platelets $150\text{--}500 \times 10^9/\text{L}$; bilirubin, $1\text{--}17 \mu\text{mol/L}$; ALT, $10\text{--}31 \text{ U/L}$; ALP, $45\text{--}145 \text{ U/L}$; GGT

$0\text{--}50 \text{ U/L}$. Serum aspartate transaminase (AST) is not part of the routine liver panel at our centre. A liver screen comprising of immunological tests (anti-nuclear antibodies, anti-mitochondrial antibodies, anti-smooth muscle antibodies) for autoimmune diseases, serological tests for hepatitis B and C viruses (hepatitis B surface antigen, anti-hepatitis C antibodies) and serum levels for metabolic disease (ceruloplasmin, alpha-1 antitrypsin, ferritin) was performed in all patients with abnormal liver biochemistry or abnormal USS. Patients with elevated ferritin levels had additional blood samples taken for haemochromatosis gene (C282Y/H63D) mutation analysis.

Usefulness of liver biopsy

In the present study, the usefulness of a liver biopsy was determined qualitatively by a) the success in establishing the diagnosis and/or b) any change (s) in the management based on the results of the liver biopsy

RESULTS

Patient population

A total of 103 liver biopsies were obtained between 1986 and 2006. Clinical data was available on 88 patients who had 95 biopsies. The number of successful liver biopsies was 91; 88 had one biopsy, 7 (4 failed 1st attempt; 3 follow-up biopsies) had two biopsies, while none of the subjects had > 2 biopsies. The median age of the patients was 57 (range, 25-86) years; 51 patients were female. 5 patients had acute hepatitis and 8 patients had focal lesions in the liver on abdominal imaging. The remaining patients underwent staging biopsies or diagnostic biopsies for chronic hepatitis or chronic elevations in liver function tests.

Liver function tests, platelets and clotting profile

The median INR was 1 (range 0.9-1.6). All patients had platelet levels $> 80\,000/\text{mm}^3$ (median 239; range 93-813). The median values of the liver tests were as follows: ALT 66.5 (range 11-1103) U/L, ALP 145 (range 44-994) U/L, GGT 221 (range 20-1394) U/L and bilirubin 13 (range 5-430) $\mu\text{mol/L}$ (Table 1).

Liver biopsy

Between 1986 and 1996: Number of patients and approach used: Eighteen patients underwent 19 biopsies. One biopsy was USS-guided and 18 were 'blind' biopsies (94.7%). One patient needed a repeat biopsy because of inadequate sample (blind) at the first attempt. The median size of liver biopsies was 1.5 cm (range: 0.5-2.5) (Table 2).

Liver biopsy findings: One-third of biopsies were performed for primary biliary cirrhosis (PBC). Two patients had elevated serum ferritin levels but only one was homozygous for the C282Y haemochromatosis mutation. Liver biopsy of the second patient showed features of porphyria cutanea tarda that was confirmed on faecal and plasma porphyrin tests.

Between 1996 and 2006: Number of patients and approach used: A total of 70 patients underwent 76 liver

Table 2 Liver biopsies between 1986 and 1995

Number of successful liver biopsies	Diagnosis pre-biopsy	History	Abnormal liver tests	Viral serology	Autoimmune / Immunoglobulins	Imaging	Others	Final Diagnosis
6	PBC	Y	Y	N	Y	Normal	n/a	PBC
3	AIH	N	Y	N	Y	Normal	n/a	AIH: 3 (staging)
1	LKM	N	Y	N	Y	Spleen +	n/a	Possible overlap AIH/PBC
3	ALD	Y	Y	N	N	Normal/Spleen +	n/a	ALD: 3 (cirrhosis in 1)
3	Abnormal Liver tests	N	Y	N	N	Echogenic liver	Elevated ferritin	NAFLD (simple steatosis): 3
1	ALD/Iron overload	Y	Y	N	N	Normal	Elevated ferritin	Porphyria cutanea tarda (plasma porphyrin 618, faecal porphyrin +)
1	Iron overload	N	Y	N	N	Normal	Elevated ferritin and homozygous C282Y	Primary haemochromatosis

Total number of biopsies performed: 19; Failed biopsies: 1; Successful biopsies: 18. Y: Present; N: Negative or not present; n/a: Not applicable; PBC: Primary biliary cirrhosis; AIH: Autoimmune hepatitis; ALD: Alcoholic liver disease; NAFLD: Non-alcoholic fatty liver disease; LKM: Liver, kidney, microsomal antibody; AMA: Anti-mitochondrial antibody.

biopsies; 59 biopsies were USS-guided and 14 (18.4%) were 'blind' procedures. Two patients had contraindications to a percutaneous biopsy (one had large volume ascites with raised INR and one had elevated INR), and underwent TJB. Laparoscopic liver biopsy was performed in one patient during laparoscopy performed for abdominal pain. Three patients needed a second biopsy after a failed initial attempt (2 blind, 1 USS-guided) while a further 3 had follow-up biopsies. The median size of liver biopsies was 1.7 (range, 0.1-3) cm (Table 3).

Liver biopsy findings: One-third of the biopsies were performed for undiagnosed elevation in the liver function tests and 12% were for staging of hepatitis C infection. Out of 59 USS-guided biopsies, 8 were performed in patients with focal lesions seen on abdominal imaging. Seven out of eight patients were found to have metastatic lesions while one patient had macrovesicular steatosis.

Changes in indications and/or findings over two decades

Between 1996 and 2006, 26% of patients were diagnosed with non-alcoholic fatty liver disease (NAFLD) (including non-alcoholic steatohepatitis), compared with 16.7% between 1986 and 1995. In the last ten years, targeted biopsies were also more common (11%; Table 3) but only one patient underwent biopsy for PBC.

Usefulness of liver biopsy

Diagnosis: In patients with elevated liver function tests but non-specific liver screen, liver biopsy confirmed the diagnoses in 25 out of 27 patients. It provided histological confirmation of metastases in 7 patients and was useful in confirming the overlap syndrome (autoimmune hepatitis/PBC) in 3 patients. Non-specific features were seen in 5 patients (5%).

Staging disease and treatment: Biopsy findings were used to stage autoimmune hepatitis, haemochromatosis, hepatitis B, hepatitis C, alcoholic liver disease and NAFLD.

Those with moderate to severe fibrosis and cirrhosis at liver histology were placed on six-monthly hepatocellular carcinoma USS screening programme and had an upper gastrointestinal endoscopy to assess the presence and size of varices. Six patients with NASH were followed on a regimen of exercise and dietary changes, with tight glycaemic and blood pressure control. Nine patients with hepatitis C infections were treated with pegylated interferon and ribavirin.

Complications: Five patients experienced severe pain, requiring hospital admission and treatment with opiates. One patient developed a sub-capsular haematoma (blind biopsy); one patient developed pneumothorax (blind biopsy) that was managed conservatively; and another patient had an intra-abdominal bleed during TJB, requiring admission to the intensive care unit. There was no biopsy-related mortality.

DISCUSSION

In the present study, we observed an increasing number of liver biopsies being performed for elevated liver function tests. NAFLD was the most common diagnosis (70%) in these patients. When all the biopsies were taken in account, NAFLD accounted for nearly 30% of biopsies in our study population. These findings are consistent with the results of previous studies^[12-14], and likely reflect that an increasing proportion of our population suffers from obesity^[15-18] and non-insulin dependent diabetes mellitus^[19,20]. Although the diagnosis of NAFLD can often be made clinically in patients with a combination of elevated liver tests, negative liver screen, 'bright' liver on abdominal USS and the presence of metabolic risk factors such as hypertension, non-insulin dependent diabetes mellitus, hyperlipidaemia and obesity^[16,21,22], the distinction between simple steatosis and steatohepatitis can only be made by liver biopsy^[23,24]. This is important because simple steatosis is considered a benign condition, while steatohepatitis can progress to

Table 3 Liver biopsies between 1996 and 2006

Number of successful liver biopsies	Diagnosis pre-biopsy	History	Abnormal liver tests	Viral serology	Autoimmune/Immunoglobulins	Imaging	Others	Final Diagnosis
24	Abnormal liver tests	Non-specific Abdominal pains in 2	Y	N	SMA in 1 IgG in 1	Normal/ Echogenic	n/a	Simple steatosis: 12 NASH: 4 Autoimmune (including overlap): 2 Drug-related: 2 Microhamartomas: 1 (laparoscopy) Non-specific: 2 Venous congestion: 1
6	NAFLD	Metabolic features present	Y	N	IgA elevated in 2	Echogenic 3	n/a	NASH: 1 Simple steatosis: 4 Normal: 1
3	Alcohol	Strong alcohol history	Y	N	Elevated IgG in 1 Autoimmune normal	Normal 2 Ascites 1	n/a	ALD: 1 (staging) ALD: 1 (diagnosis; transjugular) AIH: 1
6	Iron overload	N	Y in 2	N	N	Echogenic 3	Elevated Ferritin in all	C282Y/C282Y: 4 C282Y/H63D: 1 NAFLD (simple steatosis): 1
2	Alcohol/Iron overload?	Strong alcohol history	Y	N	Elevated IgA	Echogenic 2	Elevated Ferritin in 2	C282Y/H63D + alcohol features: 2
8	Liver lesions on imaging	N	Y in 2	N	N	Multiple lesions noted on US/CT	n/a	Lung primary: 3 GIST: 1 GI tract: 2 Pancreas: 1 NASH: 1
9	Hepatitis C	Y	Y	PCR positive	N	Normal	n/a	Hepatitis C (staging) Genotype 1:6 Genotype 3:3
1	Hepatitis B	Y	Normal	Y	N	Normal	n/a	Hepatitis B (staging)
1	PBC	N	Y	N	Elevated IgM AMA: 1: 640	Normal	n/a	PBC
8	AIH	N	Y	N	AIH: ANA + SMA in 3 Isolated SMA in 1 LKM + AMA in 1 Non-specific: Isolated SMA in 2 ANA only in 1	Normal (7); Splenomegaly in 1	n/a	AIH: 3 (staging) (transjugular in 1) AIH: 1 (diagnosis) Possible AIH/PBC overlap: 1 Non-specific: 3
2	Drug induced	Y	Y	N	Polyclonal increase in Ig	Normal	n/a	Drug-cholestasis: 1 NAFLD(simple steatosis): 1
3	Miscellaneous	Y	Y in PUO	N	N	Normal	n/a	PUO: 1 Methotrexate: 2 (staging)

Total number of biopsies: 76; Failed biopsies: 3; Number of successful biopsies: 73. Y: Present; N: negative or not present; n/a: Not applicable. PBC: Primary biliary cirrhosis; PUO: Pyrexia of unknown origin; AIH: Autoimmune hepatitis; ALD: Alcoholic liver disease; NAFLD: Non-alcoholic fatty liver disease. NASH: Non-alcoholic steatohepatitis; LKM: Liver, kidney, microsomal antibody; SMA: Smooth muscle antibody. ANA: Anti-nuclear antibody; AMA: Anti-mitochondrial antibody; IgG: Immunoglobulin G; IgA: Immunoglobulin A; IgM: Immunoglobulin M; US: Ultrasound; CT: Computed tomography.

cirrhosis^[25]. Aggressive control of metabolic risk factors has been shown to improve the liver function tests and liver histology^[26-31]. Although it is not feasible to biopsy all patients with possible NAFLD, this should be considered

in patients who are older (over 40 years), obese or have non-insulin dependent diabetes^[18,32].

With improvements in chemo-radiotherapy regimens, many patients with metastatic diseases are offered neo-

Table 4 Liver biopsies for acute hepatitis

Acute hepatitis	Pre-biopsy diagnosis	Final diagnosis
1	Cause unknown	Likely drug-induced; prednisolone started
2	Cause unknown	Mild steatohepatitis
3	Pyrexia of unknown origin	Tuberculosis excluded
4	Cause unknown	No specific features
5	Cause unknown	Microhamartomas with cholestatic hepatitis

adjuvant and adjuvant therapy. Hepatic resection for colorectal metastases limited to the liver, has become the standard of care^[33,34]. Imaging guided biopsy of liver lesions is being performed increasingly to confirm metastases and to determine the primary tumor. By contrast, a liver biopsy is no longer necessary to make a diagnosis of PBC. A persistently elevated E2AMA strongly suggests PBC, even in asymptomatic patients^[35]. Moreover, Cox-regression analyses have shown that the main prognostic marker in PBC is serum bilirubin^[36]; thus indicating that the presence of fibrosis or cirrhosis in PBC is of limited prognostic use.

Liver biopsies are currently recommended in patients with chronic hepatitis C before treatment to stage and grade the disease^[37,38]. Six patients with genotype 1 disease and three with genotype 3 disease received combination treatment with pegylated interferon and ribavirin. Since over 75% of patients infected with genotype 3 hepatitis C have a sustained virologic response after six months of combination treatment with pegylated interferon and ribavirin^[39], a liver biopsy may be unnecessary in some of these patients. In the future, non-invasive fibrosis markers are likely to play a bigger role in staging liver disease. We found that in patients with isolated autoantibodies or complex clinical and laboratory features, a liver biopsy was able to confirm the disease processes. For example, in a patient with excessive alcohol consumption who also had elevated immunoglobulin levels, liver biopsy indicated a diagnosis of autoimmune hepatitis instead of alcoholic hepatitis.

Liver biopsies from five patients showed non-specific features (5%). It has been suggested that for evaluation of diffuse liver disease, a core of at least 1.5cm is required^[40] as it provides at least 6 to 8 portal tracts for adequate histological assessment. Although the median biopsy size in the study was 1.5 cm, many samples were inadequate, with the smallest recorded size being 0.1 cm. Three of these patients had biopsy fragments measuring less than 1cm in size, which may account for the non-specific findings. Liver biopsies are generally not required in patients with acute hepatitis^[1]. We however, found it helpful in three patients (Table 4).

The most common complication of liver biopsy is abdominal discomfort. Hospitalization is needed in up to 3% of patients for pain and hypotension^[41,42], but major complications are rare^[43]. At our centre, five patients (5.6% of patients, 5.2% of biopsies) were admitted for severe pain while another patient developed clinically significant intraperitoneal bleed after a TJB and required intensive care treatment. Consistent with published reports^[44,45],

we found a trend towards greater technical failures and complications with the 'blind' biopsy technique. Three out of four patients who needed a second liver biopsy had their initial biopsies performed 'blind'; two other patients, one with a pneumothorax and the second with sub-capsular haematoma also had 'blind' biopsies. In the last decade, nearly 78% of all biopsies in our unit were USS-guided. This increase is likely to be related to the increasing availability and safety of USS-guided liver biopsy^[46,47].

Although our study population was small, it is reflective of the current DGH practice in the UK. Post-liver transplant, protocol liver biopsies are performed at the tertiary transplant units, hence the absence such data in the present study.

In conclusion, our findings confirm that liver biopsies in the DGH are safe and useful in the evaluation and staging of liver diseases. USS-guided rather than 'blind' liver biopsies are likely to be the preferred technique by patients and clinicians. NAFLD rather than PBC or viral hepatitis, will increasingly constitute the majority share of the liver biopsy workload.

COMMENTS

Background

Liver biopsy is a widely used tool in the investigation of liver diseases. However, it is an invasive procedure and studies have shown that less than one-third of the biopsies actually alter the management of patients. A liver biopsy should therefore only be performed in those patients who would benefit from such a procedure. Over the last two decades, changes in the socio-economic status and life-style have led to alterations in disease profiles. There is an increasing burden of obesity, type II diabetes mellitus and non-alcoholic steatohepatitis. Non-alcoholic steatohepatitis is now one of the most common causes of chronic liver disease and indications for liver transplantation in the USA. Concurrently, there have been rapid improvements in diagnostic and treatment options for patients with liver disease. With such changes, we expect that indications for liver biopsy will also evolve over time.

Research frontiers

Between 1986 and 1996, liver biopsies were performed mainly in patients with primary biliary cirrhosis as part of the staging process. With the development of reliable immunological markers designed to confirm the diagnosis, and prognostic markers to predict outcome of primary biliary cirrhosis, liver biopsy is no longer necessary. Between 1996 and 2006, the majority of biopsies (> 40%) were performed for raised liver function tests and hepatitis C staging. Non-alcoholic fatty liver disease was also found to be more common. In the present study, only 5% of biopsies showed non-specific features and were therefore unhelpful in patient management. The rest of the biopsies confirmed the diagnoses of the primary liver disease or the presence of metastasis. Patients with end-stage liver disease were placed on regular surveillance and those with treatable diseases were managed according to the treatment protocols. The complication rates after a liver biopsy in a district general hospital were found to be similar to the published rates. Between 5% and 6% of patients were admitted for severe pain. There was a trend towards increasing technical failures and complications with blind liver biopsy. In the last decade, ultrasound-guided biopsies were obtained more frequently than blind biopsies.

Innovations and breakthroughs

This study confirms that a liver biopsy is safe and useful in evaluating and staging of liver disease, even in district general hospitals. The risks from a biopsy remains small, but ultrasound-guided biopsies should be the preferred technique. In the future, non-alcoholic fatty liver disease will account for the vast share of the biopsy workload.

Applications

Useful for practicing clinicians in smaller hospitals as it confirms the safety and

utility of a liver biopsy. Ultrasound-guidance may be safer. Non-alcoholic fatty liver disease is increasingly common.

Peer review

This is a very nice review that describes the historical changes in liver biopsy methodology over the past 20 years. The data presented is clear and concise and has been described clearly in the text.

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Effects of a 24-week course of interferon- α therapy after curative treatment of hepatitis C virus-associated hepatocellular carcinoma

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related HCC occurred during persistent viral infection. Eradication of HCV is essential for the prevention of HCC recurrence and improvement of survival.

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Key words: Hepatitis C virus; Hepatocellular carcinoma; Recurrence; Survival; Sustained virological response

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Abstract

AIM: To assess whether a 24-wk course of interferon (IFN) could prevent hepatocellular carcinoma (HCC) recurrence and worsening of liver function in patients with hepatitis C virus (HCV)-infected patients after receiving curative treatment for primary HCC.

METHODS: Outcomes in 42 patients with HCV infection treated with IFN- α , after curative treatment for primary HCC (IFN group), were compared with 42 matched curatively treated historical controls not given IFN (non-IFN group).

RESULTS: Although the rate of initial recurrence did not differ significantly between IFN group and non-IFN group (0%, 44%, 61%, and 67% vs 4.8%, 53%, 81%, and 87% at 1, 3, 5, and 7 years, $P = 0.153$, respectively), IFN group showed a lower rate than the non-IFN group for second recurrence (0%, 10.4%, 28%, and 35% vs 0%, 30%, 59%, and 66% at 1, 3, 5 and 7 years, $P = 0.022$, respectively). Among the IFN group, patients with sustained virologic response (SVR) were less likely to have a second HCC recurrence than IFN patients without an SVR, or non-IFN patients. Multivariate analysis identified the lack of SVR as the only independent risk factor for a second recurrence, while SVR and Child-Pugh class A independently favored overall survival.

CONCLUSION: Most intrahepatic recurrences of HCV-

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignant neoplasms worldwide. Chronic infection with hepatitis C virus (HCV) has been causally associated with HCC^[1-3]. Recent advances in imaging and treatment have brought about some improvement in prognosis of patients with HCV-related HCC, but outcomes are still unsatisfactory. The 5-year survival rate is only 50%-70%, even after curative treatment such as hepatic resection or local ablation^[4]. Reasons for this unfavorable prognosis are considered to include high intrahepatic tumor recurrence rates and sustained hepatic damage, both resulting from HCV infection^[5].

Even after curative hepatic resection for HCV-related HCC, the rate of intrahepatic tumor recurrence within 1 year is 20%-40%, rising to about 80% by 5 years^[4,6-8]. Intrahepatic recurrence of HCC may result from intrahepatic metastasis originating from the primary HCC, or from ongoing multicentric carcinogenesis related to chronic HCV infection. Underlying HCV-related hepatic damage may also compromise hepatic functional reserve, and worsen clinical outcome. Thus prevention of HCC recurrence and preservation of liver function are both high priorities for improving prognosis of patients with HCV-

related HCC.

Interferon (IFN) therapy for patients with HCV infection is effective in reducing serum alanine transaminase (ALT) activity and in eradicating HCV^[9,10], and thus IFN could have value in minimizing hepatic necrosis, inflammation and fibrosis, as well as reducing the incidence of HCC. Several recent studies have reported that IFN therapy, even after curative treatment for HCV-related HCC, could prevent HCC recurrence and improve survival^[11-17]. Unfortunately, since these studies are characterized by differing IFN regimens, definitions of IFN responses, and background characteristics of patients, results have varied and no standard IFN regimen has been established for after curative treatment of HCV-related HCC. As well, the mechanisms by which IFN suppresses HCC recurrence, including possible direct anti-tumor and anti-inflammatory effects, remain uncertain.

In the present study, recurrence and survival outcomes in matched historical controls were compared with those in patients receiving a 24-wk course of IFN- α therapy after receiving curative treatment for HCC.

MATERIALS AND METHODS

Patients

We retrospectively reviewed 495 consecutive patients treated for primary HCC associated with HCV infection at Hiroshima University Hospital from March 1992 to March 2004. Of these, 384 with HCC initially underwent therapeutic intervention with curative intent. Curative treatment was defined as complete tumor eradication, with no residual tumor visible by computed tomography, or resection of all evident tumor tissue. Medical treatment included percutaneous radiofrequency ablation (RFA), ethanol injection, and microwave coagulation therapy (MCT). Surgical treatment included hepatic resection and ablation during laparotomy.

Among these 384 patients, we administered IFN therapy to 42 who met the following eligibility criteria: age under 70 years; up to three tumors with none exceeding 30 mm in diameter, or a solitary tumor less than 50 mm in diameter; tumor-node-metastasis (TNM) stage I, II, or III; detectable serum HCV RNA; seronegativity for hepatitis B surface antigen; chronic hepatitis or compensated cirrhosis with a Child-Pugh class of A or B; platelet count above 70 000/ μ L; absence of local recurrence during the follow-up period; and absence of ectopic intrahepatic recurrence within 24 wk after treatment for primary HCC. We used the TNM classification system of the Liver Cancer Study Group of Japan as the staging system for HCC^[18]. Underlying liver conditions such as hepatitis or cirrhosis were confirmed by laboratory, pathologic and radiologic examinations. We classified liver function in chronic hepatitis as Child class A because chronic hepatitis is a known pre-cirrhotic condition. There were only a few chronic hepatitis cases: three in the IFN group and four in the non-IFN group.

As historical control subjects, we selected 42 patients with no IFN therapy after treatment for primary HCC (non-IFN group). These 42 patients, who met the eligibility

Table 1 Patient characteristics

	IFN group (n = 42)	Non-IFN group (n = 42)	P
Median age in years (range)	62 ¹ (45-69)	63 ¹ (40-69)	NS
Gender (male/female)	36/6	29/13	NS
Alb (g/dL)	3.9 ¹	3.9 ¹	NS
PLT ($\times 10000/\mu$ L)	12 ¹	11.5 ¹	NS
ICG R-15 (%)	17 ¹	18 ¹	NS
CH or Child A/B	35/7	35/7	NS
Size of main tumor (mm)	20 ¹ (10-50)	15 ¹ (10-50)	NS
AFP (ng/mL)	26 ¹	31.4 ¹	NS
No. of HCC (single/two or three)	30/12	36/6	NS
Stage (I / II or III)	14/28	23/19	NS
Treatment of HCC (medical/surgical)	18/24	20/22	NS

IFN: interferon; Alb: albumin; PLT: platelet; ICG-R15: indocyanine green retention at 15 min; CH: chronic hepatitis; AFP: alpha-fetoprotein; HCC: hepatocellular carcinoma. ¹Median.

criteria noted above, were matched by age, gender, tumor size, TNM stage of HCC, serum albumin, platelet counts, and Child-Pugh class with patients who received IFN therapy (IFN group).

Thus, a total of 84 patients (42 in the IFN group and 42 in the non-IFN group) were enrolled. All agreed to participate in the research protocol, which was approved by the hospital research ethics board. Table 1 shows the baseline characteristics of the two groups, indicating no significant differences for age, gender, liver function, tumor characteristics, or therapeutic methods used against HCC.

IFN therapy

In the IFN group, patients received 6 MIU of natural IFN- α (human lymphoblastoid IFN, Sumiferon; Dainippon Sumitomo Pharmaceuticals, Osaka, Japan) intramuscularly every day for 2 wk, followed by three times weekly for 22 wk. IFN therapy began within 24 wk after the initial treatment for HCC. All patients were evaluated every week in an outpatient setting during IFN treatment. Qualitative detection of HCV-RNA was performed by a standardized qualitative reverse transcription-polymerase chain reaction (RT-PCR) assay at every 4 wk during and after IFN treatment.

Among the patients who received IFN therapy, 28 were of HCV genotype 1 and 14 were of HCV genotype 2. These 42 patients had various pretreatment viral loads. Twenty patients (genotype 1, $n = 11$; genotype 2, $n = 9$) had high viral loads (≥ 100 kIU/mL by PCR), and 22 (genotype 1, $n = 17$; genotype 2, $n = 5$) had low viral loads (≤ 100 kIU/mL by PCR). The 42 patients were divided into two subgroups according to virologic response, i.e., patients with vs without a sustained virologic response (SVR). SVR was defined as the sustained absence of serum HCV RNA for more than 24 wk after completion of IFN treatment. Absence of SVR included both persistent viremia (no response) and transient viral disappearance (transient response) during or after IFN therapy. Biochemical response was defined as ALT activity declining to a value within the normal reference range in the presence of viremia.

Follow-up

After curative treatment for primary HCC, all patients studied underwent liver function tests, serum tumor marker assays, such as those for α -fetoprotein (AFP) and protein induced by vitamin K absence or antagonist II (PIVKA-II) every month, abdominal ultrasonography every 3 mo, and dynamic computed tomography (CT) every 6 mo. If recurrence of HCC was suspected, additional examinations including CT during arteriography or tumor biopsy were performed. Recurrence of HCC was defined as any new nodules indicated by CT as hyperattenuation during hepatic arteriography or by hypoattenuation in CT performed during arteriportography. Hypovascular HCC was confirmed histopathologically after fine-needle aspiration biopsy. Patients with recurrent HCC were treated medically or surgically, with curative intent if possible.

In IFN patients, including those with or without SVR, and in the non-IFN group, we compared both the rate of HCC recurrence and the survival rate. We also sought to identify significant prognostic indicators for survival and recurrence after curative treatment of primary HCC.

Statistical analysis

Chi-squared and Fisher exact tests were used for categorical variables, while Student's *t* test and the Mann-Whitney *U* test were used for continuous and ordinal variables, as appropriate. The Kaplan-Meier method was used to assess cumulative survival and recurrence rates, calculated from the date of diagnosis to the date of disease recurrence or death. Surviving patients and those who died of causes unrelated to the liver were defined as censored cases, while patients who died of causes related to the liver were defined as non-censored cases. The log-rank test was used to compare survival and recurrence curves. Univariate and multivariate predictors of survival or recurrence time were determined using the Cox proportional hazard model. Hazard ratios and their 95% confidence intervals (95% CI) were computed. $P < 0.05$ was considered to indicate statistical significance. The JMP version 5.1 statistical software package (SAS Institute, Cary, NC, USA) was used for analysis of data.

RESULTS

Virologic and biochemical responses to IFN therapy and side effects

The 42 patients receiving IFN therapy included 29 in the SVR group and 13 in the group without SVR (10 transient virological responders, 3 with no virological response). In the group without SVR, 7 biochemical responders who had a normalized ALT included 5 with transient virological responses and 2 with no virological response. Although there was no significant difference in the population of patients with HCV genotype 1 between the SVR and non-SVR group, patients in the former had significantly lower pre-IFN viral loads than patients in the latter group. In the SVR group, 24 patients received full-dose IFN therapy without dose reduction, while five patients received a reduced dose of IFN until completion

of treatment. In the group without SVR, one patient with no response discontinued IFN treatment at 16 wk because of a recurrence of HCC, while three patients with a transient response discontinued treatment because of generalized fatigue. The remainder of the group without SVR received the full course of IFN therapy. Thus, most patients were able to complete the 24-wk course.

Recurrence of HCC

In the IFN group, first recurrences of HCC developed in 20 patients after the initial treatment for HCC during a median follow-up period of 32 mo. Of these recurrences, 10 were in patients with SVR (10/29) and 10 in patients without SVR (10/13), including 7 transient virological responders and 3 with no virological response. For the 7 biochemical responders without SVR, HCC recurred in 6 patients, including 5 transient virological responders and 1 with no virological response. Of these 20 patients with recurrence, 18 were treated with local ablation therapy or surgical resection without leaving any residual tumor. The remaining 2 patients developed uncontrolled multiple HCC and were excluded from the subsequent study concerning the next recurrence. One died of HCC, while the other was treated repeatedly with hepatic arterial infusion, and has survived. Three patients in the SVR group and 7 in the group without SVR (5 transient virological responders and 2 with no virological response) had a second recurrence of HCC. Of these 10 patients with a second recurrence, 3 (2 transient virological responders and one with no virological response) developed uncontrolled HCC, while others were treated curatively with hepatic resection or local ablation therapy. In the non-IFN group, a first recurrence of HCC occurred in 30 patients during a median follow-up period of 31 mo. HCC recurred in 11 of the 17 who had a normal ALT level. Among the 30 patients with recurrent HCC, 25 were treated with local ablation therapy or surgical treatment, with no residual tumor. The remaining 5 patients who did not undergo curative therapy were treated repeatedly with transarterial chemoembolization. A second recurrence developed in 15 of the 25 patients who had curative treatment for a first recurrence. Among these 15 patients, 10 were treated curatively (9 with local ablation and 1 with hepatic resection). The remaining 5 patients had uncontrolled multiple HCC as their second recurrence.

Overall cumulative rates for first and second recurrence of HCC were compared between the groups. The 1-, 3-, 5- and 7-year rates for first recurrence in the IFN and non-IFN group were 0% *vs* 4.8%, 44% *vs* 53%, 61% *vs* 81%, and 67% *vs* 87%, respectively (Figure 1A, $P = 0.153$; no significant difference between groups). However, the 1-, 3-, 5-, and 7-year rates for second recurrence in the IFN and non-IFN group were 0% *vs* 0%, 10.4% *vs* 30%, 28% *vs* 59%, and 35% *vs* 66%, respectively (Figure 1B, $P = 0.022$). Thus, the second-recurrence rate was significantly lower in the IFN group than in the non-IFN group.

Next, the recurrence rates of HCC were compared between the SVR group, the non-SVR group and the non-IFN group. The rate of first recurrence was significantly lower in the SVR group than in the non-SVR and non-IFN group (Figure 2A). The rate of second recurrence in the

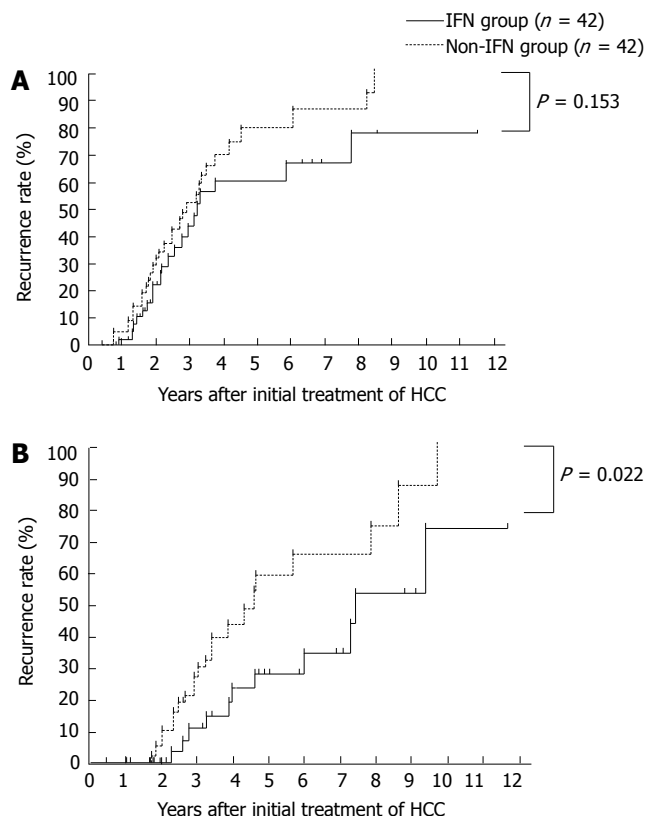


Figure 1 Cumulative recurrence rates after curative treatment of HCC. **A:** Rates of first recurrence compared between IFN and non-IFN groups, showed no significant difference ($P = 0.153$); **B:** Rates of second recurrence compared between IFN and non-IFN groups. The second recurrence rate for the IFN group was lower than that for the non-IFN group ($P = 0.022$).

SVR group was also lower than that in the non-SVR and non-IFN groups; this decrease was significantly greater than that for the rate of first recurrence (Figure 2B). No significant difference was seen in cumulative rates for first or second recurrence between the non-SVR and non-IFN groups. We also confirmed that biochemical responders in the non-SVR and non-IFN groups showed similar Kaplan-Meier curves for cumulative recurrence (data not shown). Recurrence curves were similar between the non-SVR group, including biochemical responders, and the non-IFN group, therefore, we defined these two groups as "non-SVR status" for statistical analysis. Factors found to be significantly associated with first recurrence by univariate analysis were tumor size (≥ 20 mm) and non-SVR status ($P = 0.019$, $P = 0.0067$, respectively). Multivariate analysis showed that no independent risk factor was associated with the first recurrence of HCC (data not shown), although non-SVR status tended to be associated with first recurrence ($P = 0.0657$). As shown in Table 2, univariate analysis indicated that non-SVR status, low platelet count ($< 100\,000$) and high indocyanine green retention ($\geq 20\%$) were significantly associated with second recurrence. Multivariate analysis identified only SVR status as a significant independent inhibiting factor for second recurrence of HCC.

Survival of patients

During the observation period, 13 of the total patients

studied died of liver disease. Nine died of HCC and 4 of liver failure. When we compared cumulative survival rates between the IFN and the non-IFN groups (Figure 3A), the respective rates were 100% *vs* 95% at 3 years, 100% *vs* 72% at 5 years, and 86% *vs* 63% at 7 years. The cumulative survival rate was significantly higher in the IFN group than in the non-IFN group ($P = 0.039$). Median survival time following the first treatment of HCC was 52.3 mo (range, 12-158) in the IFN group and 51.8 mo (range, 11-126) in the non-IFN group. In the IFN group, 2 patients died of advanced HCC, 1 with an SVR and the other without. No patients in the IFN group died of hepatic failure. In the non-IFN group, 7 patients died of HCC and four of hepatic failure.

Figure 3B shows cumulative survival curves for the SVR, non-SVR and non-IFN groups. The rate of survival in the SVR group was significantly better than that in the non-IFN group ($P = 0.029$), while no significant difference was evident between the non-SVR and non-IFN group ($P = 0.248$).

Pretreatment factors found to be significantly associated with survival by univariate analysis subsequently were evaluated by Cox regression analysis to determine independent factors. Multivariate analysis showed that SVR status and Child-Pugh class A were independent factors favorably associated with long survival (Table 3).

Liver function

Compared with the non-IFN group, patients who received IFN therapy were less likely to have worsening of hepatic dysfunction. For the SVR, non-SVR and non-IFN groups, we compared the average score for Child-Pugh classification at initial treatment of HCC with that at the time of data analysis. Median observation time was 59.8 mo in the SVR group, 45 mo in the non-SVR group, and 51.8 mo in the non-IFN group. There were no significant differences in the Child-Pugh classification score among these three groups at the time of initial treatment of HCC; however, at the time of data analysis, scores in the non-IFN group were significantly worse than in the SVR group ($P = 0.003$). No significant difference was seen between the non-SVR and non-IFN groups (Figure 4).

DISCUSSION

The present study compared historical control subjects with no IFN treatment with other subjects who were treated with IFN. Background characteristics showed no significant difference between the groups. IFN and non-IFN group did not differ significantly in their rate of first recurrence, but did differ significantly in their rate of second recurrence. According to IFN response, the recurrence rate in the SVR group was significantly lower than that in the non-SVR and non-IFN group, while recurrence rates in the non-SVR and non-IFN group did not differ significantly. Thus, SVR (i.e., HCV eradication) was the most important, and only, inhibiting factor for decreasing risk of HCC recurrence, associated with a 24-wk course of IFN- α therapy following HCC treatment.

Although several recent studies have reported the

Table 2 Factors associated with second recurrence

Variables	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	P	Hazard ratio	95% CI	P
SVR	0.454	0.246-0.728	0.0005	0.457	0.243-0.757	0.0015
PLT > 100 000/ μ	0.553	0.373-0.814	0.003	0.694	0.445-1.069	0.0973
ICG R-15 (< 20%)	0.667	0.450-0.965	0.032	0.685	0.447-1.035	0.0721

Cox's proportional hazards model was used.

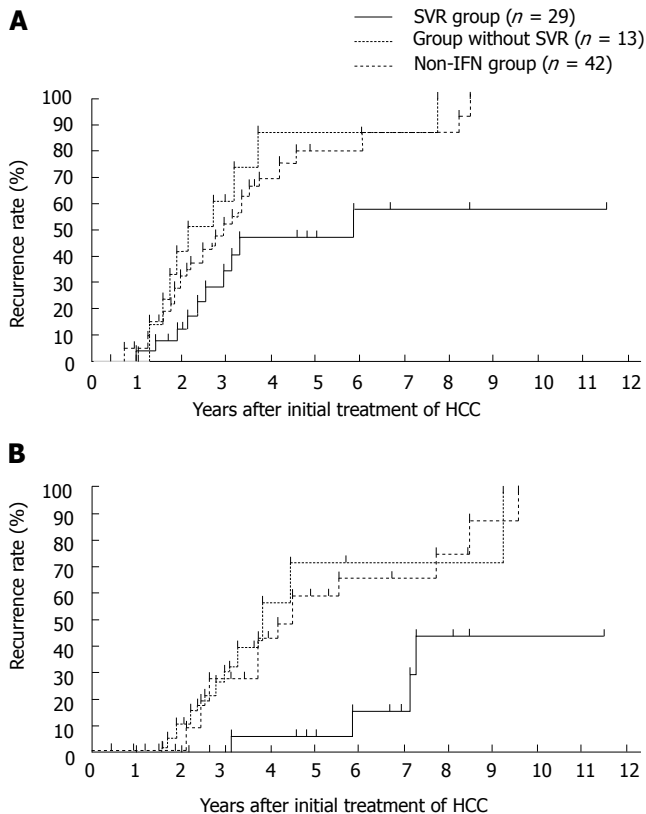


Figure 2 Cumulative recurrence rates according to SVR to IFN therapy after curative treatment of HCC. **A:** Rates of first recurrence compared among SVR, non-SVR and non-IFN groups. The rate of first recurrence of HCC in the SVR group was significantly lower than in the non-SVR and non-IFN groups ($P = 0.002$, $P = 0.016$, respectively). No significant difference in first recurrence rate was seen between the non-SVR and non-IFN groups ($P = 0.381$); **B:** Rates of second recurrence compared among the three groups. Second recurrence of HCC was suppressed in the SVR group compared with the non-SVR and non-IFN groups ($P = 0.0037$, $P = 0.0019$, respectively), and to a more pronounced degree than for the first recurrence rate. No significant difference in second recurrence rate was seen between the non-SVR and non-IFN groups ($P = 0.90$).

efficacy of chemoprevention with IFN after treatment of HCV-related HCC, the basis of this benefit has not been determined, since IFN has a variety of biologic effects, including antiviral, antiproliferative, immunomodulatory^[19-22] and anti-fibrogenic^[23,24] activities; growth inhibition through changes in signal transduction^[19,25,26]; and activation of natural killer cells^[27] and T cells^[28,29]. Through these various effects, IFN therapy is thought to suppress tumor recurrence directly and/or indirectly.

Sakaguchi *et al.*^[15] have reported that low-dose, long-term, intermittent IFN- α therapy can, by a direct anti-cancer effect, inhibit intrahepatic metastasis but not

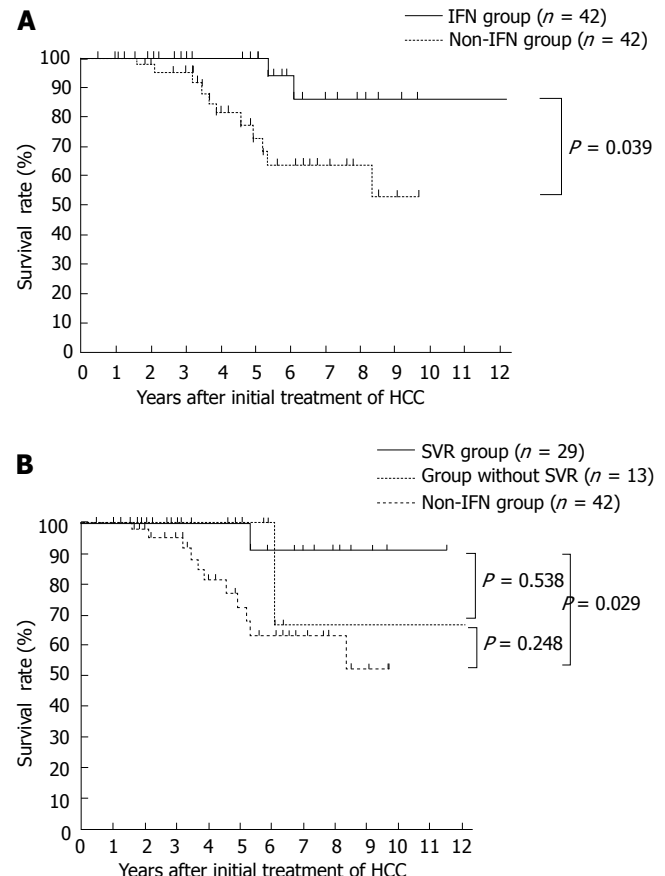


Figure 3 Cumulative survival rates after curative treatment of HCC. **A:** Comparison of cumulative survival rates in the IFN and non-IFN groups. The cumulative survival rate was significantly higher in the IFN group than in the non-IFN group ($P = 0.039$); **B:** Comparison of cumulative survival rates in the SVR, non-SVR and non-IFN groups. Although no significant overall difference was found between the SVR and non-SVR groups ($P = 0.538$), the SVR group had a particularly high survival rate compared with the non-IFN group ($P = 0.029$).

multicentric occurrences. Lai *et al.*^[29] have reported that IFN- α therapy is effective in advanced HCC. Several experimental studies have shown that IFN inhibits the growth of a human hepatoma cell line^[11,15]. In partial disagreement, however, Nishiguchi *et al.*^[12,14], Suou *et al.*^[16] and Shiratori *et al.*^[17] have reported that the rate of HCC recurrence was not different between IFN and non-IFN group during the first few years, but later became significantly lower in the IFN group. They suggested that IFN reduced HCC recurrence in the later period of observation by suppressing multicentric occurrence, as an indirect anti-tumor effect that was related to sustained hepatic inflammation. Although the present study did not have a randomized controlled design, and details of the

Table 3 Factors associated with survival

Variables	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	P	Hazard ratio	95% CI	P
SVR	0.409	0.096-0.922	0.028	0.329	0.076-0.761	0.006
Child-Pugh class A	0.521	0.299-0.922	0.027	0.463	0.238-0.875	0.019
ICG R-15 (< 20%)	0.551	0.286-0.968	0.038	0.724	0.351-1.429	0.350

Cox's proportional hazards model was used.

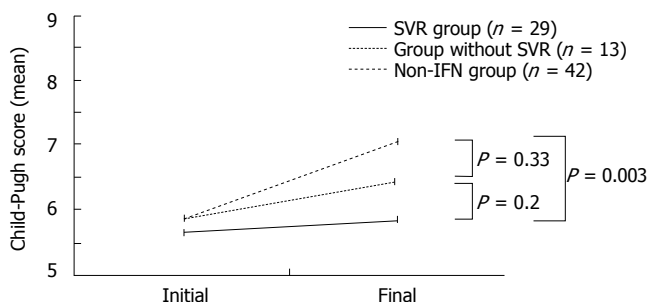


Figure 4 Influence of IFN therapy after curative treatment of HCC on Child-Pugh scores. IFN-treated patients were less likely to show deterioration of hepatic function. In particular, liver function scores in the SVR group were significantly better preserved than in the non-IFN group ($P = 0.003$). Median observation time was 59.8 mo in the SVR group, 45 mo in the non-SVR group, and 51.8 mo in the non-IFN group.

IFN protocol differed from those of others, the long-term results appear to be similar among studies. Recurrence during the first few years might involve undetectable intrahepatic metastasis, or a potential malignant tumor already existing at the time of treatment of the primary HCC; afterward, HCC might recur as multicentric new liver tumor, accompanied by sustained hepatic necrosis and inflammation. Although a direct anti-cancer effect of IFN might to some extent have directly inhibited HCC recurrence, our IFN doses were insufficient to suppress intrahepatic metastatic tumors because there was only a 24-wk treatment. Therefore, in our study, we believe that IFN therapy suppressed HCC recurrence less by a direct anti-tumor effect than by an indirect effect through inhibition of the chronic inflammation associated with HCV infection in the later period of observation.

Several studies have reported that recurrence was suppressed not only in virologic responders to IFN, but also in biochemical responders, even though HCV was not eradicated^[12-14]. However, the recurrence rates in our study did not differ significantly between biochemical responders and the non-IFN group. HCV eradication appeared to stand alone as an IFN effect capable of inhibiting recurrence, with eradication having a stronger influence against second recurrence than the first. The differences between the results of the various studies might be due to several reasons. In most previous studies, IFN therapy was given for more than 48 wk, compared with our 24 wk. Differences may also have been present in underlying hepatic inflammatory conditions such as chronic hepatitis and cirrhosis. Although such differences introduce some uncertainty to the conclusions, several recent studies suggest that HCV core protein might directly participate in hepatocarcinogenesis^[28,29], which supports the importance

of virus eradication.

Although some other recent studies have reported that IFN therapy following HCC treatment also improves liver function and survival of patients with HCV-related HCC, which of the specific IFN actions is important for these benefits remains unknown. We found that overall survival rate and preservation of liver function were significantly better in the SVR group than in the other groups, even including biochemical responders, with all subgroups without SVR resembling non-IFN patients. Favorable independent factors associated with survival by multivariate analysis were SVR and Child-Pugh class A. Thus, with a 24-wk course of IFN- α therapy, HCV eradication appears necessary for prolonging survival, suppressing HCC recurrence, and preserving liver function.

As stated above, effective management of HCV infection is needed, as well as direct treatment of the primary HCC. Although our study had limitations, such as the use of historical controls and a small number of patients, we could demonstrate a clear requirement for HCV eradication to improve survival after a short-course IFN- α therapy. Ribavirin combination or pegylated IFN therapy are considered more effective in HCV eradication than conventional IFN monotherapy^[32-34]. Several studies have indicated that pegylated IFN therapy is superior to conventional IFN when administered for 48 wk^[34-41]. Pegylated IFN therapy, with or without ribavirin, may improve prognosis in selected patients with no sustained initial response to conventional IFN. For patients who cannot undergo standard-dose IFN therapy because of limited hepatic reserve or thrombocytopenia, low-dose IFN therapy for a longer course might be effective. Nonetheless, further studies with larger controlled groups and long-term follow-up need to be performed to establish what constitutes optimal management of HCV infection after HCC treatment.

COMMENTS

Background

Risk of multicentric recurrence of hepatocellular carcinoma (HCC) and liver function deterioration remains high in hepatitis C virus (HCV)-infected patients even after receiving curative treatment for primary HCC. Most intrahepatic recurrences occurred during persistent viral infection. Although several recent studies have reported the efficacy of chemoprevention with interferon (IFN) therapy after treatment of HCV-related HCC, there was no standard IFN regimen. We investigated whether 24-week course of IFN- α therapy following curative treatment for primary HCC associated with HCV infection could suppress HCC recurrence and improve prognosis.

Research frontiers

To obtain sustained virological response (SVR) was important for suppression of HCC recurrence and for long-term survival in a 24-week course of IFN- α therapy.

Innovations and breakthroughs

Our study demonstrated that only SVR status by a 24-wk IFN- α therapy was the most important factor for decreasing risk of HCC recurrence in the later period of observation including second recurrence.

Applications

This study demonstrated that compared with non-IFN and non-SVR group, SVR group decreased the rate of recurrence, preserved liver function, and prolonged survival time in a 24-wk course of IFN- α therapy.

Peer review

This is a matched historical case controlled study concerning about the effect of 24-week short course IFN- α therapy after receiving curative treatment for primary HCC. The paper is well written and the results show that the most important factor associated with the improvement of prognosis is the SVR status.

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Pancreatic fistula after pancreaticoduodenectomy: A comparison between the two pancreaticojejunostomy methods for approximating the pancreatic parenchyma to the jejunal seromuscular layer: Interrupted vs continuous stitches

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Abstract

AIM: The purpose of this study is to find a better operative technique by comparing interrupted stitches with continuous stitches for the outer layer of the pancreaticojejunostomy, i.e., the stitches between the stump parenchyma of the pancreas and the jejunal seromuscular layer, and other risk factors for the incidence of pancreatic leakage.

METHODS: During the period January 1997 to October 2004, 133 patients have undergone the end-to-side and duct-to-mucosa pancreaticojejunostomy reconstruction after pancreaticoduodenectomy with interrupted suture for outer layer of the pancreaticojejunostomy and 170 patients with a continuous suture at our institution by one surgeon.

RESULTS: There were no significant differences between the two groups in the diagnosis, texture of the pancreas, use of octreotide and pathologic stage. Pancreatic fistula occurred in 14 patients (11%) among the interrupted suture cases and in 10 (6%) among the continuous suture cases ($P = 0.102$). Major pancreatic leakage developed in three interrupted suture patients (2%) and zero continuous suture patients ($P = 0.026$). In multivariate analysis, soft pancreatic consistency (odds ratio, 5.5; 95% confidence interval 2.3-13.1) and common bile duct cancer (odds ratio, 3.7; 95% CI 1.6-8.5) were predictive of pancreatic leakage.

CONCLUSION: Pancreatic texture and pathology are the most important factors in determining the fate of pancreaticojejunal anastomosis and our continuous suture method was performed with significantly decreased occurrence of major pancreatic fistula. In conclusion, the continuous suture method is more feasible and safer in performing duct-to-mucosa pancreaticojejunostomy.

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Key words: Pancreaticoduodenectomy; Pancreaticojejunostomy; Pancreatic fistula

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INTRODUCTION

Despite improvement of the operative technique, materials and instruments, pancreatic fistula after pancreaticoduodenectomy is the most common and serious complication. Recent large series have reported that the failure rate of the pancreaticoenteric anastomosis is 9%-18%^[1-6], a complication rate not far improved from Dr. Whipple's report of a 19.5% fistula rate more than 50 years ago^[7]. A number of methods for reducing the incidence of pancreatic fistula have been proposed and tested. Many of these involve technical aspects of the anastomosis, including the site of reconstruction (pancreaticogastrostomy versus pancreaticojejunostomy)^[8,9], the anastomotic technique (duct-to-mucosa anastomosis versus stump invagination)^[10,11], the use of biologic adhesive^[12,13] and the use of intraoperative transanastomotic stents^[14]. In addition, in order to determine how to prevent pancreatic fistula, the risk

factors for pancreatic fistula have been extensively studied. They include the patient's comorbid illness^[8], age^[15], texture of the pancreas^[4], pancreatic duct size^[16], intraoperative blood loss^[15] and the surgeon's experience^[15].

The present study tested the hypothesis that using continuous stitches for the outer layer of the pancreaticojejunostomy (i.e., the stitches between the stump parenchyma of the pancreas and the jejunal seromuscular layer) is a better operative technique than using interrupted stitches in terms of safety and efficiency.

MATERIALS AND METHODS

During the period from January 1997 to October 2004, 133 patients underwent duct-to-mucosa pancreaticojejunostomy reconstruction after pancreaticoduodenectomy with the interrupted suture method for the outer layer of the pancreaticojejunostomy and 170 patients underwent the procedure with the continuous suture method at our institution. From 1997 to 2000, the interrupted suture method was performed, and from 2001 to 2004, the continuous suture method was performed. The operations were performed by one surgeon who had experienced more than 500 cases of pancreaticoduodenectomy before this study.

We retrospectively reviewed the medical records of the patients who underwent duct-to-mucosa pancreaticojejunostomy after pancreaticoduodenectomy noting parameters such as the existence of pancreatic fistula, age, sex, preoperative symptoms, preoperative laboratory tests results, amount of intraoperative bleeding, and postoperative octreotide usage. Postoperative octreotide was given subcutaneously (dose 100 mg every 8 hours) for the patients considered high risk for pancreatic fistula based on gland texture and duct size.

Pancreaticoduodenectomy was performed with conventional pancreaticoduodenectomy or pylorus-preserving pancreaticoduodenectomy (PPPD). Anastomosis for the remnant pancreas was performed between the pancreas and jejunum by a two layer pancreaticojejunostomy. The outer layer consisted of the remnant pancreatic parenchyma and the seromuscular layer of jejunum and interrupted suture or continuous suture between these two was performed with 5-0 polypropylene (Prolene[®], Ethicon, Somerville, NJ). The inner layer consisted of the pancreatic duct and mucosa of the jejunum, and interrupted suture for duct-to-mucosa was performed with 5-0 polydioxanone (PDS[™] II, Ethicon, Somerville, NJ). A silastic polyethylene tube was inserted into the pancreatic duct as a stent for all patients, and external drainage was done (Figure 1).

Two or three drains were routinely placed anterior and posterior to the pancreatico-jejunal anastomosis and exteriorized through the lateral abdominal wall.

A pancreatic fistula was defined as the drainage of more than 30 mL of fluid with an amylase level higher than 600 U/dL on or after postoperative week 1^[17]. Also, three grades of fistula severity (A, B, C) were classified according to the International Study Group for Pancreatic Fistulas (ISGPF) clinical criteria^[18] as follows: Grade A

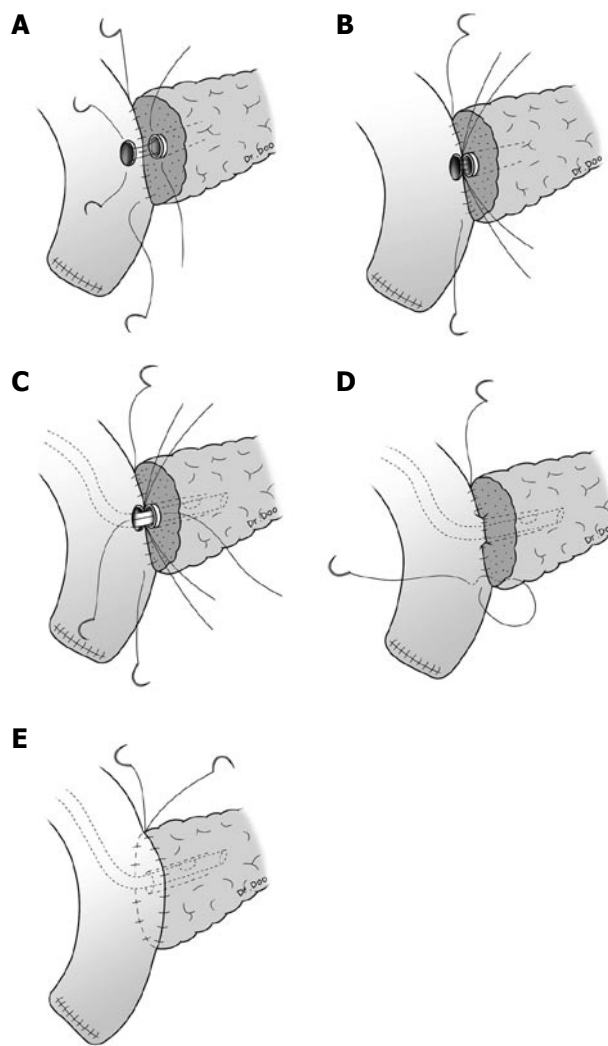


Figure 1 Continuous suture method for the outer layer of pancreaticojejunostomy. **A:** The posterior outer layer consisted of the remnant pancreatic parenchyma and the seromuscular layer of jejunum and continuous suture between these two was performed with 5-0 polypropylene (Prolene[®], Ethicon, Somerville, NJ); **B:** The posterior inner layer consisted of the pancreatic duct and mucosa of the jejunum, and interrupted suture for duct-to-mucosa was performed with 5-0 polydioxanone (PDS[™] II, Ethicon, Somerville, NJ); **C:** A silastic polyethylene tube was inserted into the pancreatic duct and external drainage was done; **D:** For anterior inner layer consisted of the pancreatic duct and mucosa of the jejunum, interrupted suture was performed; **E:** Continuous suture for anterior outer layer was performed.

fistulas are transient, asymptomatic fistulas, with only elevated drain amylase levels and treatments or deviation in clinical management are not required. Grade B fistulas are symptomatic, clinically apparent fistulas that require diagnostic evaluation and therapeutic management. Grade C fistulas are severe, clinically significant fistulas that require major deviations in clinical management and aggressive therapeutic interventions are unquestionably warranted. Major pancreatic leakage was defined as the drainage of more than 200 mL of fluid or the development of an intra-abdominal abscess.

Pancreatic fistulas, according to the operative methods and clinicopathologic factors causing pancreatic fistula, were analyzed.

Table 1 Comparison of clinical characteristics between the interrupted suture group and the continuous suture group *n* (%)

	Interrupted suture group (<i>n</i> = 133)	Continuous suture group (<i>n</i> = 170)	<i>P</i> value
Age (yr)	58.2 ± 12.4	60.4 ± 10.9	0.072
Male:Female	1.8:1	1.5:1	0.484
Pancreas texture			0.893
Hard	35 (26)	52 (31)	
Firm	86 (65)	76 (45)	
Soft	12 (9)	42 (24)	
Use of prophylactic octreotide	83 (62)	122 (72)	0.072
Jaundice	59 (44)	69 (41)	0.643
Diabetes mellitus	21 (16)	33 (19)	0.544
Diagnosis			0.433
Ampulla of Vater cancer	35 (26)	45 (26)	
Common bile duct cancer	38 (29)	46 (27)	
Pancreatic cancer	34 (25)	57 (34)	
Duodenal cancer	6 (5)	6 (4)	
Etc.	20 (15)	16 (9)	

Etc.: Ampulla of Vater adenoma, choledochal cyst, chronic pancreatitis, duodenal GIST, gallbladder cancer, intraductal papillary mucinous tumor, islet cell tumor of pancreas, peripancreatic neurilemmoma, pseudocyst, serous cystic adenoma.

Data comparisons between the two groups were made using the χ^2 test for qualitative parameters, a Student's *t*-test for quantitative parameters and logistic regression for determining the effect of multiple risk factors on pancreatic leakage. A value of *P* < 0.05 was accepted as significant.

RESULTS

There were no significant differences in mean age, sex, prophylactic use of octreotide, pancreas texture, stage, and indication for pancreaticoduodenectomy between the two groups (Table 1).

Although there was no significant difference in the mean total operation time between the interrupted suture and continuous suture groups, there was a statistically significant difference in the mean operation time of the outer layer anastomosis of pancreaticojejunostomy. The mean operation time of the interrupted suture method for the outer layer anastomosis of pancreaticojejunostomy was 35.4 ± 4.8 minutes, and the mean operation time of the continuous suture method was 29.1 ± 3.9 min (*P* < 0.001).

Pancreatic fistula occurred in 14 patients (11%) of the 133 patients who underwent interrupted suture for the outer layer and in 10 patients (6%) of the 170 patients who underwent continuous suture (*P* = 0.102). There were 5 grade A fistulas, 6 grade B fistulas, and 3 grade C fistulas in the interrupted suture group. There were 4 grade A fistulas, 5 grade B fistulas, and no grade C fistula in the continuous suture group. There was no significant difference between the two groups (*P* = 0.085). Major pancreatic leakage occurred in three patients (2%) in the interrupted suture group and zero patients in the continuous suture group (*P* = 0.026). For one patient of the interrupted suture group

Table 2 Comparison of postoperative complications and mortality between the interrupted suture group and the continuous suture group *n* (%)

	Number of patients		<i>P</i> value
	Interrupted suture group (<i>n</i> = 133)	Continuous suture group (<i>n</i> = 170)	
Pancreatic fistula	14 (10.5)	10 (5.9)	0.102
ISGPF grade			0.085
Grade A	5 (3.8)	4 (2.4)	
Grade B	6 (4.5)	6 (3.5)	
Grade C	3 (2.3)	0	
Major pancreatic fistula	3 (2.3)	0	0.026
Disruption	1 (0.8)	0	
Daily drainage > 200 cc	0	0	
Intra-abdominal abscess	2 (1.5)	0	
Pseudoaneurysm	0	2	0.128
Reoperation for pancreatic fistula	1 (0.8)	0	0.199
Hospital mortality	0	0	

who experienced pancreaticojejunal anastomotic rupture, externalization of the pancreatic duct was performed at the 10th postoperative day. For the other two patients of the interrupted suture group with major pancreatic leakage, percutaneous drainage was added.

Two patients of the continuous suture group with distal common bile duct (CBD) cancer developed pseudoaneurysm with preceding pancreatic fistula. They were successfully managed by radiologic intervention. There was no postoperative hospital mortality in the two groups (Table 2).

Of the total of 303 patients, 24 patients (8%) developed postoperative pancreatic fistula. There were no significant differences in age, sex, preoperative bilirubin and albumin levels, operation methods, amount of intraoperative bleeding, total operation time, postoperative prophylactic octreotide usage, and stage between the pancreatic fistula group and the non-pancreatic fistula group. There was a significant difference in pathologic features between the pancreatic fistula group and the non-pancreatic fistula group (*P* = 0.039). When the pathologic features were divided into CBD cancer and non-CBD cancer, there was a significant difference (*P* = 0.004). The consistency of the remnant pancreas correlates strongly with subsequent postoperative fistula rates. In the non-pancreatic fistula group, 43 (15%) were classified as soft and 85 as hard. In the pancreatic fistula group, 11 (46%) were classified as soft and 2 as hard (*P* < 0.001) (Table 3). In multivariate analysis, soft pancreatic consistency (odds ratio, 5.5; 95% confidence interval 2.3-13.1) and CBD cancer (odds ratio, 3.7; 95% confidence interval 1.6-8.5) were predictive of pancreatic leakage.

DISCUSSION

Recently, pancreaticoduodenectomy has become popularized as the standard treatment for various benign and malignant diseases of the periampullary region, including the pancreas head. Although the mortality rate has markedly decreased, the incidence of pancreatic fistula, the most catastrophic complication, remains high

Table 3 Perioperative risk factors for pancreatic fistula

	Pancreatic fistula (+) (n = 24)	P value
Age (yr)	62.3 ± 10.2	0.565
Male:Female	2:1	0.582
Preoperative disease		
Diabetes mellitus	4 (7%, 4/54)	0.959
Laboratory findings		
Hypoalbuminemia (3 < g/dL)	3 (9%, 3/33)	0.729
Hyperbilirubinemia (> 2 mg/dL)	14 (7%, 14/195)	0.665
Pathologic feature		0.039
Ampulla of Vater cancer	4 (5%, 4/80)	
Common bile duct cancer	13 (16%, 13/84)	
Pancreatic cancer	4 (4%, 4/91)	
Duodenal cancer	0 (0%, 0/12)	
Others	3 (8%, 3/36)	
Type of resection		0.097
PPPD	22 (10%, 22/218)	
Whipple's op.	2 (2%, 2/85)	
Outer layer suture method		0.102
Interrupted	14 (11%, 14/133)	
Continuous	10 (6%, 10/170)	
Pancreas consistency		< 0.001
Hard	2 (2%, 2/87)	
Firm	11 (7%, 11/162)	
Soft	11 (20%, 11/54)	
Total operative time (min)	383 ± 52	0.515
Estimated blood loss (mL)	564 ± 220	0.831
Use of prophylactic Octreotide	14 (7%, 14/205)	0.317
Lymph node metastasis		0.351
Yes	17 (9%, 17/197)	
No	7 (7%, 7/106)	
Positive resection margin		0.105
Yes	1 (4%, 1/26)	
No	23 (8%, 23/277)	

after pancreaticoduodenectomy. After the first successful pancreaticoduodenectomy was performed by the German surgeon Kausch in 1912^[19], the risk factors for pancreatic anastomotic leakage and methods aimed at prevention of pancreatic anastomotic leakage have been extensively studied.

Several factors related to pancreatic anastomotic leakage have been described in the literature. They can be conveniently divided into disease factors (pancreatic texture^[4], pancreatic pathology^[8], pancreatic duct size^[16], pancreatic juice output^[8]), procedure-related factors (intraoperative blood loss^[15], operative techniques^[15]) and patient factors (age^[15], sex^[8], comorbid illness^[8], jaundice^[8]). Still, it is very difficult to predict the relationship of the risk factors and pancreatic fistula, and many studies have revealed heterogeneous results. In our study, two risk factors, common bile duct cancer and soft pancreatic texture were correlated with increased incidence of pancreatic fistula. The relative proportion of bile duct and ampulla of Vater cancers is much larger in Korea than in Western countries. The reason is not clear. The high incidence of hepatolithiasis, choledocholithiasis, choledochal cyst, and clonorchiasis in Korea is a possible explanation, at least in a proportion of cases. Unlike in the Johns Hopkins series^[8], the leakage rate was lower in cases of ampulla of Vater cancer (5%, 4/80) than in others (9%, 20/223). Patients with ampulla of Vater cancer are diagnosed early, and their general condition is

relatively good. More importantly, most of the patients have a dilated pancreatic duct and a firm pancreas, which facilitates the anastomosis and its healing. On the other hand, most of the patients with common bile duct cancer have a non-dilated pancreatic duct and a soft pancreas. The increased fistula rates with soft pancreatic texture may be interpreted by three explanations. First, most soft pancreases have no pancreatic duct dilatation, which makes secure duct-to-mucosa anastomosis difficult^[20,21]. Second, a soft pancreas is more easily injured directly or *via* ischemia by stitches placed between the pancreas parenchyma and the seromuscular layer of the jejunum^[20]. Third, a soft pancreas has a good exocrine function, secreting more pancreatic juices rich in proteolytic enzymes^[20-22].

Prophylactic use of octreotide is expected theoretically to reduce the incidence rate of the pancreatic fistula through decreasing pancreatic juice secretion. However, randomized trials from Europe and United states showed opposite results^[23-26]. In our study, there was no significant difference in the incidence rate of pancreatic fistula between postoperative prophylactic octreotide usage group and no usage group ($P = 0.347$). To prove the effect of octreotide in reducing the amount of postoperative pancreatic fistula, more organized randomized controlled studies are needed.

Various techniques for managing the pancreatic remnant have been studied with the aim of reducing the anastomotic leakage rate, including varying the site of the jejunum for pancreaticojejunostomy (end *vs* side), varying the type of anastomosis (duct-to-mucosa *vs* invagination)^[10,11], comparing the difference between pancreaticogastrostomy and pancreaticojejunostomy^[8,9], use of fibrin glue^[12,13] and pancreatic duct stenting^[14]. Unfortunately, randomized trials on these technical measures are scarce^[11]. As a result, there is no universal agreement on which operative technique is safer and less prone to pancreatic leakage^[11].

Because our study is not a prospective randomized controlled study, there is potential for a beta error. However, it could be thought as a periodic randomized study. The operations were performed by one surgeon who had experienced more than 500 cases of pancreaticoduodenectomy before this study, and the improved results are unlikely associated with a learning curve by doing more procedures.

Although there was no significant difference between the interrupted suture and continuous suture methods for preventing pancreatic fistula in our study, the incidence of major pancreatic fistula decreased significantly in the continuous suture group ($P = 0.026$). Theoretically, the continuous suture method has many advantages over the interrupted suture method^[27-32]. First of all, a more even distribution of tension is possible in the continuous suture between the pancreatic parenchyma and jejunum^[27]. Due to the coiled spring effect, the continuous suture method provides a reduction in the likelihood of focal tissue ischemia^[27], an increase in tensile strength^[27], and a reduction of the risk of pancreaticojejunal rupture. A continuous suture provides an enhanced air and watertight seal^[28,29]. As our study shows, a continuous suture reduces anastomosis time^[30,31]. A continuous suture is technically

easier^[30,31] and costs less^[31,32]. For continuous sutures, we use one polypropylene (Prolene*, Ethicon, Somerville, NJ) thread, and the thread costs 5.7 dollars. However, for interrupted sutures, we use 16 polypropylene sutures and it costs 86 dollars more than continuous suture.

The study of two methods for approximating the pancreatic parenchyma to the jejunal seromuscular layer in duct-to-mucosa pancreaticojejunostomy revealed that the incidence of major pancreatic fistula decreased significantly and operation time is reduced significantly in the continuous suture group. In conclusion, the continuous suture method is more feasible and safe to use in performing pancreaticojejunostomy.

COMMENTS

Background

Leakage of the pancreaticojejunal anastomosis has been a major complication after pancreaticoduodenectomy. Over the past decades, various measures directed towards prevention of pancreatic leakage have been studied.

Research frontiers

The purpose of this study is to find better operative technique as comparing the interrupted stitches with the continuous stitches for the outer layer of the pancreaticojejunostomy, ie. the stitches between the stump parenchyma of the pancreas and the jejunal seromuscular layer, and other risk factors for the incidence of pancreatic leakage.

Innovations and breakthroughs

Soft pancreatic consistency (odds ratio, 5.5; 95% confidence interval 2.3-13.1) and CBD cancer (odds ratio, 3.7; 95% confidence interval 1.6-8.5) were predictive of pancreatic leakage and our continuous suture method performed with significantly decreasing major pancreatic fistula.

Applications

The study of two methods for approximating the pancreatic parenchyma to the jejunal seromuscular layer in duct-to-mucosa pancreaticojejunostomy revealed that the incidence of major pancreatic fistula decreased significantly and operation time is reduced significantly in the continuous suture group. In conclusion, the continuous suture method is more feasible and safe to use in performing pancreaticojejunostomy.

Terminology

A pancreatic fistula was defined as the drainage of more than 30 mL of fluid with an amylase level higher than 600 U/dL on or after postoperative week 1. Also, three grades of fistula severity (A, B, C) were classified according to ISGPF clinical criteria as follows: Grade A fistulas are transient, asymptomatic fistulas, with only elevated drain amylase levels and treatments or deviations in clinical management are not required. Grade B fistulas are symptomatic, clinically apparent fistulas that require diagnostic evaluation and therapeutic management. Grade C fistulas are severe, clinically significant fistulas that require major deviations in clinical management and aggressive therapeutic interventions are unquestionably warranted. Major pancreatic leakage was defined as the drainage of more than 200 mL of fluid or the development of an intra-abdominal abscess.

Peer review

As this was a retrospective, non-randomized study in which the continuous suture was used after experience with 133 patients in whom an interrupted suture technique was used, the "better" results with the continuous suturing may simply be a learning curve.

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Type 2 diabetes mellitus and CA 19-9 levels

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be caused by diabetes. We propose that a higher cut-off value of CA 19-9 should be used in diabetics to differentiate benign and malignant pancreatic disease, and subtle elevations of CA 19-9 in diabetics should be considered as the indication of exocrine pancreatic dysfunction.

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Key words: CA 19-9; Diabetes mellitus; Chronic pancreatitis

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Abstract

AIM: To prospectively investigate serum CA 19-9 levels in type 2 diabetic patients in comparison with age- and gender-matched control subjects.

METHODS: We recorded duration of diabetes and examined fasting glucose levels, HbA1c levels and serum CA 19-9 levels in 76 type 2 diabetic patients and 76 controls. Abdominal CT was performed in order to eliminate abdominal malignancy in the diabetic and control groups.

RESULTS: The average CA 19-9 level was 46.0 ± 22.4 U/mL for diabetic patients whereas it was 9.97 ± 7.1 U/mL for the control group ($P < 0.001$). Regression analysis showed a positive correlation between diabetes and CA 19-9 independent from age, gender, glucose level and HbA1c level ($t = 8.8$, $P < 0.001$). Two of the diabetic patients were excluded from the study because of abdominal malignancy shown by CT at the initial evaluation. For all patients, abdominal CT showed no pancreatic abnormalities.

CONCLUSION: CA 19-9 is a tumor-associated antigen, which is elevated in pancreatic, upper gastrointestinal tract, ovarian hepatocellular, and colorectal cancers, as well as in inflammatory conditions of the hepatobiliary system, biliary obstruction and in thyroid diseases. Diabetes has been claimed to be a risk factor for pancreatic cancer, which is increasing its incidence and has one of the lowest survival rates of all cancers. CA 19-9 is used in the diagnosis of pancreatic cancer but is also a marker of pancreatic tissue damage that might

INTRODUCTION

CA 19-9 is a tumor-associated antigen that was originally defined by a monoclonal antibody produced by a hybridoma prepared from murine spleen cells immunized with a human colorectal cancer cell line. CA 19-9 is elevated in pancreatic cancers, in cancers of the upper gastrointestinal tract, in ovarian cancer, hepatocellular cancer, colorectal cancer, as well as in inflammatory conditions of the hepatobiliary system and in thyroid diseases^[1]. It may also be elevated in malignant and benign cases of biliary obstruction^[2].

Diabetes has been claimed to be a risk factor for pancreatic cancer, which is increasing its incidence and has one of the lowest survival rates of all cancers^[3].

The association between diabetes and pancreatic cancer remains controversial. A meta-analysis of 20 studies found a two-fold increased risk of pancreatic cancer among diabetic patients of 5 years duration, suggesting that diabetes is a risk factor for the tumor^[4]. Other studies have concluded that cancer preceded and caused diabetes^[5]. There are even some studies suggesting that diabetes protects against pancreatic cancer^[6].

CA 19-9 is used in the diagnosis of pancreatic cancer but is also a marker of pancreatic tissue damage that might be caused by diabetes. Therefore it is necessary to define the normal range of CA 19-9 in type 2 diabetic patients in order to eliminate additional interventional approaches. In this study, we prospectively evaluated serum CA 19-9 levels in type 2 diabetic patients in comparison with age- and gender-matched control subjects.

MATERIALS AND METHODS

76 consecutive, type 2 diabetic patients and 76 age- and gender-matched healthy controls comprised the study groups. We recorded duration of diabetes and examined fasting glucose levels, HbA1c and serum CA 19-9 levels. CA 19-9 was measured with a chemiluminescence immunoassay (Roche Diagnostics E 170 analyser). The normal range of CA 19-9 was defined as less than 27 U/mL. Abdominal CT was performed in order to eliminate abdominal malignities for the diabetic and control groups.

RESULTS

Characteristic features of the control and study groups are shown in Table 1. Two of the diabetic patients were excluded from the study because of abdominal malignancy found with CT at the initial evaluation (one had an adrenal mass and the other prostatic carcinoma).

Average CA 19-9 levels were 46.0 ± 22.4 U/mL in diabetic patients whereas it was found to be 9.97 ± 7.1 U/mL in the control group ($P < 0.001$). Regression analysis showed a positive correlation between diabetes and CA 19-9, independent from age, gender, glucose level and HbA1c level ($t = 8.8$, $P < 0.001$) (Figure 1). For all patients, abdominal CT showed no pancreatic abnormalities.

DISCUSSION

Diabetes mellitus is a chronic inflammatory disease of the pancreas. The mechanisms of glucose intolerance include insulin resistance and destruction of islet beta cells. Most of these conditions damage exocrine tissue and islet cells and both of these systems are anatomically and functionally related. There are no capsules or basement membranes around islets and there are cell-to-cell contacts between exocrine and endocrine cells. There are direct connections between the capillaries of islets and acini that underlie the regulatory connections between islet hormones and exocrine pancreatic secretion, or in other words the relation between parenchymal disease and beta-cell dysfunction^[3].

Insulinitis is a progressive phenomenon in diabetes mellitus. The United Kingdom Prospective Diabetes Study evaluated beta cell function in diabetes using the homeostasis model assessment and found that beta cell function was already reduced by 50% at diagnosis and that there was subsequent deterioration regardless of therapy^[7]. Diabetes can even be described as the last step of chronic pancreatitis with the new developing concepts of pathogenesis^[8].

On the other hand, patients with chronic pancreatitis often suffer from endocrine pancreatic dysfunction, which leads to a secondary form of diabetes. This form of diabetes accounts for $< 1\%$ of all diabetic cases^[8]. Eighty percent of patients with chronic pancreatitis develop an overt diabetes mellitus in the long run and diabetes mellitus is an independent risk factor for mortality in patients with chronic pancreatitis^[9,10]. Chronic pancreatitis is a risk factor for pancreatic cancer^[11] and the same is also true for diabetes^[3]. Therefore diabetic patients have to be followed up for pancreatic cancer.

CA 19-9 is a tumor marker used for the diagnosis of

Table 1 Sex distribution and mean age of patients

	Sex		Mean age (yr)
	Male	Female	
Type II DM	19	55	53.3
Control	17	59	52.5

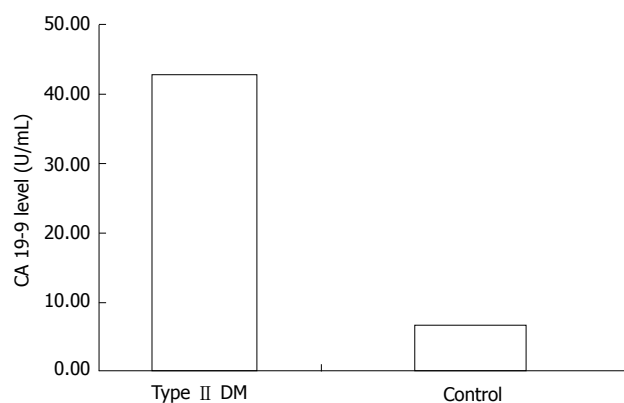


Figure 1 Mean CA 19-9 levels in Type II DM and Control groups.

pancreatic cancer with a sensitivity of 70%-90% and a specificity of 68%-91%^[12]. The main limitation of CA 19-9 is that it can also be elevated in patients with nonmalignant obstructive jaundice, thyroid disease and ovarian diseases resulting in impaired specificity of the marker. The solution to this problem was found with the use of higher cut-off values and a combination of tumor markers in differential diagnosis of gastrointestinal cancers. Lewis blood group status is also important for the interpretation of CA 19-9 because 10% of the Caucasian population who are Lewis genotype negative are unable to express CA 19-9^[13]. Diabetes is also associated with an increased risk of hepatocellular carcinoma^[14].

Taking the close relation between exocrine and endocrine pancreatic function into consideration, the use of CA 19-9 in diabetics is controversial. The relationship between diabetes mellitus and CA 19-9 has been studied by Benhamou *et al*^[15] and they investigated the relationship between CA 19-9 and metabolic control of diabetes in 51 adult patients. They concluded that CA 19-9 in diabetic patients is raised in acute metabolic situations, which correlated very well with blood glucose concentration.

In contrast to Benhamou *et al*, 64 diabetic patients examined by Banfi *et al*^[16] showed no correlation between CA 19-9 and biochemical markers of metabolic compensation in diabetes. In our study we also found that CA 19-9 levels were higher in diabetic patients in comparison to controls ($P < 0.001$). The elevation of CA 19-9 in diabetics was less than twice of the normal range. Regression analysis showed a positive correlation between diabetes and CA 19-9 independent from age, gender, glucose level and HbA1c level ($t = 8.8$, $P < 0.001$). We could not identify any pancreatic or gastrointestinal cancer in the diabetic patients with a high level of CA 19-9, which is consistent with the concept of exocrine pancreatic dysfunction in diabetes.

We propose that a higher cut-off value for CA 19-9

should be used for diabetics to differentiate benign and malignant pancreatic disease, and subtle elevations of CA 19-9 in diabetics should be considered as an indication of exocrine pancreatic dysfunction.

COMMENTS

Background

Ca 19-9 is a widely used antigen in the diagnosis of pancreatic and gastrointestinal cancers but its elevation might also be due to some nonspecific causes. Chronic inflammation such as diabetes mellitus can lead to pancreatic tissue damage and to a nonmalignant cause of high Ca 19-9 levels. Therefore the "normal" interval of Ca19-9 should be defined in type 2 diabetic patients.

Research frontiers

Insulinitis is a progressive phenomenon in type 2 diabetes and United Kingdom Prospective Diabetes Study confirmed this entity. Chronic pancreatitis is a risk factor for diabetes and also for pancreatic cancer. The studies by Benhamou and Banfi found controversial data regarding the correlation between Ca 19-9 and diabetes.

Innovations and breakthroughs

Elevation of Ca 19-9 in type 2 diabetics has been associated with bad metabolic control in previous studies and no morphological studies was done. We performed abdominal CT in all the patients in order to exclude any pancreatic malignancy and found out that Ca 19-9 was elevated in all the diabetic patients.

Applications

We have to define a new cut-off value for Ca 19-9 level in type 2 diabetics in order to eliminate unnecessary investigations for pancreatic cancer in this subgroup. This article can lead to further study the effect of insulinitis and chronic pancreatitis on Ca 19-9 expression in experimental and morphological studies.

Terminology

Ca 19-9 is a tumor-associated antigen which is originally defined by a monoclonal antibody that has been produced by a hybridoma prepared from murine spleen cells immunized with a human colorectal cancer cell line.

Peer review

This study has interesting scientific and innovative contents, and good readability.

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RAPID COMMUNICATION

Holistic Acupuncture approach to idiopathic refractory nausea, abdominal pain and bloating

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Abstract

AIM: To evaluate the effectiveness of a holistic acupuncture approach on nausea, pain, bloating and electrogastrogram (EGG) parameters in patients with intractable symptoms.

METHODS: Twelve patients with no or mild nausea (those without nausea had bloating or pain) and 10 with a history of moderate to severe nausea were referred for acupuncture. All underwent an EGG and were treated at acupuncture points PC6, SP4 and DU20. Visual analog scales (VAS) assessing severity of nausea, pain and bloating were obtained before and after acupuncture treatment. Nineteen patients received three and three patients received two treatments.

RESULTS: VAS scores for nausea reflected the clinical assessment and differed significantly between mild and moderate/severe nausea groups. Acupuncture significantly improved severity of nausea in both groups with improved pre-treatment nausea between the first and third treatments in the moderate/severe nausea group. Pain scores improved with acupuncture in the mild nausea group only and bloating improved only with the first treatment in this group. Patients with bloating with VAS scores greater than 35 pre-treatment improved with acupuncture and over all VAS scores for pain improved with treatment. Acupuncture increased the power in the 2.7 to 3.5 cpm range in the EGG.

CONCLUSION: In this uncontrolled clinical study, a holistic acupuncture approach significantly improved nausea in patients with refractory symptoms and increased the power in the 2.7-3.5 cpm component of the electrogastrogram. Bloating and pain VAS scores improved acutely with treatment. This study suggests that acupuncture may be effective in this refractory group of patients and further study using appropriate controls is warranted.

INTRODUCTION

Nausea is a debilitating symptom that severely impacts on quality of life. Surveys of patients with functional dyspepsia (chronic or recurrent abdominal pain without an anatomic or inflammatory cause) and patients undergoing chemotherapy, identify nausea as one of the symptoms with the greatest negative impact on quality of life^[1,2]. It is a complicated symptom to study and treat because it is often a co-morbid symptom and multiple factors impact on its etiology and severity.

Nausea is a prominent symptom in approximately 20% of patients recruited to dyspepsia studies in whom pain is the major complaint^[1]. Patients who present with chronic or recurrent nausea in whom no biochemical, anatomic or inflammatory abnormalities can be found, are diagnosed as having functional nausea^[3]. Efforts to determine a single pathophysiologic process in patients with functional nausea have been unsuccessful. Some patients have gastroparesis, or delayed emptying of solid foods from the stomach, either related to other medical conditions such as diabetes mellitus or without any obvious underlying systemic disorder. Others have normal gastric emptying despite severe nausea. Even when a condition such as gastroparesis is identified, it is unclear whether the symptom of nausea can be directly ascribed to that condition^[4]. Symptoms of nausea can vary in severity over time despite persistent delayed gastric emptying, suggesting that gastroparesis alone does not explain the severity of nausea. Medications for treatment of nausea are limited to those that increase gastric or duodenal contractile activity, the prokinetic agents, and those affecting the chemoreceptor trigger zone^[3]. Many prokinetic and centrally acting medications have significant side effects. Psychological factors are often important. Even following chemotherapy, a condition where nausea is known to be a direct effect of medications, the severity of nausea is impacted by psychological stress from family

relations^[5]. Psychotropic agents may be helpful in the treatment of nausea, but many have side effects that may be counterproductive to gastric function, such as the anticholinergic action of tricyclic antidepressants.

The complexity of the processes contributing to nausea does not lend itself to the usual "modern" medical approach to disease, which includes treating each abnormal condition with separate medications. In contrast, the ancient Chinese paradigm of medicine involves a holistic approach that uses a paradigm of deviation from balance in energy or "Qi" and accepts the concept that many conditions that would appear to involve disparate organ systems may be intertwined resulting in "imbalance". Improvement in symptoms requires the treatment of multiple conditions to restore balance^[6].

Acupuncture has been used since antiquity to treat patients with nausea⁷ and has been subjected to clinical study. It has been used with moderate success in patients with chemotherapy induced nausea and post-operative nausea^[7,8,9]. A search of the literature revealed no reports of its use in treating functional nausea.

The aims of this study were to examine the effect of acupuncture, using a holistic approach, in patients referred for nausea and/or abdominal pain, refractory to routine medical treatment. We examined the effect on the symptoms of nausea, bloating, and pain and on gastric motor activity, as assessed by electrogastrography. The holistic approach included acupuncture therapy at three points, PC6, SP4 and DU20. These are described in detail in the methods section.

MATERIALS AND METHODS

Subjects and treatment

Charts were reviewed of 22 patients (3 male, 19 female. Mean 44.1 ± 2.7 years, range 17-66) who were treated with acupuncture between June 2003 and December 2005. Patients were referred to Hershey Medical Center for care and treatment with prokinetic agents and usual therapy had failed. All patients were interested in pursuing alternative medical therapy. All patients had undergone the same treatment plan. Charts were reviewed for the history of the severity and duration of nausea, abdominal pain and bloating and prior gastric emptying studies. Fourteen of the patients had a documented diagnosis of depression on their chart. The other patients had no documentation of psychiatric disorders but no documentation stated specifically that there were no psychological issues. From the chart narrative, patients were determined to have either no nausea or mild nausea (those with no nausea complained primarily of abdominal bloating and pain) or moderate to severe nausea. The severity of each symptom was also assessed before and after each acupuncture treatment using a visual analog scale (VAS) with 0 for no symptoms and 100 for the worst symptom during the first three treatments. The assessment by the physician in clinic was not known to the subject who completed the VAS score and was based on patients' history of the complaint. EGGs were also performed on all patients^[10]. During the treatment sessions, the acupuncture points P6, SP4 bilaterally and DU20 were needled for 30 min, while the EGG was recorded. A

VAS was completed for each of the symptoms of nausea, pain and bloating before and after each acupuncture treatment. Patients returned for at least one or two additional treatments. Nineteen patients had three treatment and three patients underwent two treatments. The average duration between treatments was 15 ± 1.9 d (mean \pm SE), range 7-33 d. The study was approved by the Institutional Review Board of the Hershey Medical Center (November 8, 2006).

Electrogastrogram (EGG) recording and analysis

All patients underwent a clinical EGG following our standard protocol^[10]. Patients fasted overnight. On the day of treatment, each patient consumed a standard 200 Kcal snack of 8oz of apple juice and two slices of toast. Each subject then fasted for an additional two hours after which time EGG recordings were obtained. On the first treatment day, a water load test was performed after 15 min of baseline EGG. EGG recording was then continued during the 30 min of acupuncture treatment. Signals were passed through 0.016 Hz high-pass filter and a 0.25 Hz low-pass filter. The EGG signal was reviewed to remove areas of artifact and the resulting EGG signal was digitized for computer analysis and subjected to fast Fourier transform to extract the frequency information present in the EGG signal. A running spectral analysis of the component wave form was followed over time. The percent power in the frequency ranges of 1.0-2.5 cpm, 2.5-3.7 cpm and 3.7 to 10 cpm were recorded.

Water load test

A water load test was performed at the first treatment session. After a 15 min baseline EGG was recorded, the patients ingested water "until full" within five minutes. The water was non-carbonated spring water at room temperature (23°C). The volume (mL) of water drunk was recorded^[10].

Visual analog scale (VAS) for symptoms

A visual analog scale (VAS) with a score from 0-100 points was utilized to assess the intensity of each of the following visceral sensations: nausea, pain, bloating, with 0 being none and 100 being unbearable. Each subject completed a VAS score prior to the study and before and after each acupuncture treatment.

Acupuncture points

Acupuncture points P6, SP4 and DU20 were selected for this treatment regimen^[7]. The point PC6 (Neiguan) is on the pericardium meridian of hand-jueyin. It is located 2 cm above the transverse crease of the wrist, between the tendons of *muscularis palmaris longus* and the *flexor radialis*. The traditional indications for needling of the PC6 point are stomach ache, nausea, vomiting, hiccough, mental disorders, epilepsy, insomnia, febrile disease, irritability, and malaria. The point SP4 (Gong sun) lies on the Spleen meridian of the foot-taiyin. It is located in the depression distal and inferior to the base of the first metatarsal bone. The traditional indications for treatment of the SP4 point relate to the gastrointestinal (GI) or central nervous system and are abdominal pain and distension, diarrhea, dysentery,

Table 1 Demographics and clinical characteristics of patients in the mild or severe nausea groups

	Mild nausea (<i>n</i> = 12) symptom score 0 or 1	Severe nausea (<i>n</i> = 10) symptom score 2 or 3	<i>P</i>
Gender	10F:1M	9F:1M	NS
Age (yr ± SE)	47.5 ± 2.9	40 ± 4.6	NS
Weight (lbs ± SE)	143 ± 7	150.8 ± 10.4	NS
Diabetes	1/12	1/10	
Severity of nausea ¹	0.25 ± 0.04	2.6 ± 0.15	<i>P</i> < 0.0001
Severity of pain ¹	2 ± 0.3	1.1 ± 0.37	NS
Severity of bloating ¹	2.3 ± 0.37	1.2 ± 0.5	NS
Duration of symptoms in months (mean ± SE)	24.9 ± 5 Range 7-60	15.3 ± 3.4 Range 4-36	NS ²

¹Symptom key: 0-none, 1-mild, 2-moderate, 3-severe (based on narrative and complaint in patient chart). ²Using Mann-Whitney test for non-parametric data.

borborygmus, nausea, vomiting, hiccough, and insomnia. The point DU20 (Baihui or GV 20) lies on the Governor vessel. It is located in the midline of the head, at the intersection of the line connecting the apices of both ears and a line that lies in the midline in the sagittal orientation on the head. The traditional indications for using the DU20 point are vertigo, headache, tinnitus, nasal obstruction, aphasia by apoplexy, coma, mental disorders, prolapse of the rectum and the uterus. It was felt that this combination of points would treat specific nausea symptoms and gastrointestinal complaints as well as depression, which was felt to be a common problem for many patients with refractory nausea.

Statistical analysis

The charts were reviewed for age, weight, gender, and assessment of the severity of the symptoms of nausea, vomiting, bloating and pain at the clinic visit. As the degree of nausea was variable patients were analyzed in two groups based on the severity of nausea determined by history at the initial clinic assessment-either in a "none to mild" nausea group or a "moderate to severe" nausea group. Separation of patients into the two groups was based on clinical assessment of the chart and was determined prior to any knowledge of the outcome of treatment.

The effect of acupuncture before and after treatment in the different groups and its effect on the parameters of VAS scores for nausea, bloating and pain and power of EGG in the different frequencies was analyzed using ANOVA. If a significance level of *P* < 0.05 was determined, direct comparisons between specific parameters was performed using a student *t* test (two-tailed) for parametric data or Mann-Whitney test for non-parametric data. Data are presented as mean ± SE.

RESULTS

Patients were primarily analyzed as two groups, based on

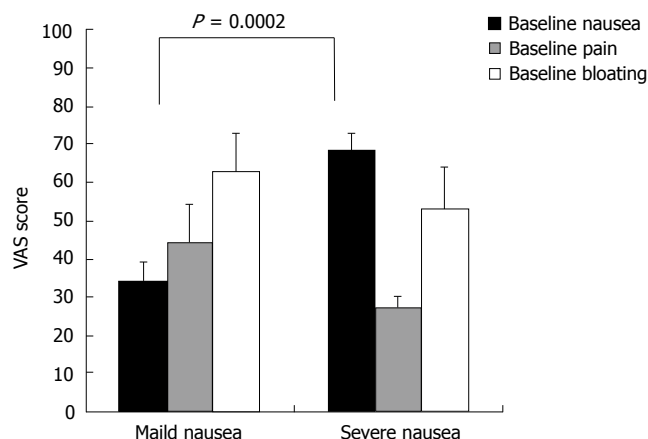


Figure 1 VAS score for symptoms of nausea, abdominal pain and bloating at baseline for patients in either the mild or severe nausea groups. A significant difference was seen in the VAS score for nausea between the groups (*P* = 0.0002).

an assessment of the severity of nausea as determined by obtaining a history from patients at their initial clinic visit. Those with no or mild nausea were grouped in the mild nausea group (*n* = 12), those with moderate or severe nausea were grouped in the severe nausea group (*n* = 10). The demographics and clinical features are shown in Table 1. In the group who complained of mild to no nausea, nine patients did not complain of nausea, but complained more of pain and/or bloating. The frequency of nausea (25% vs 100%) and the severity of nausea was significantly different between the groups (*P* < 0.05). There was no difference in either the prevalence of abdominal pain or bloating or severity of these symptoms between the two groups. The duration of nausea when present was between seven months and five years in the mild nausea group and between four months and three years in the severe nausea group (not significant [NS]). Only one out of each group was diabetic.

At the first visit for acupuncture treatment, the VAS scores for nausea were significantly greater in the severe nausea group than the mild nausea group (68.2 ± 4.4 vs 33.3 ± 5.8 , *P* < 0.0002, Figure 1) and these subjects tolerated the water load test more poorly than did the mild nausea group (429 ± 33.9 vs 642 ± 22 mL, *P* < 0.0001). There was no significant difference in VAS scores for bloating or pain at baseline between the two groups. Bloating was the predominant symptom in the mild nausea group while both nausea and bloating were equally prominent in the severe nausea group.

Effect of acupuncture on symptoms

Acupuncture significantly improved the VAS score for nausea in both the mild nausea and severe nausea groups (Figure 2A). There was improvement in the pre-acupuncture nausea score between the first and third treatment in the severe nausea group (*P* < 0.03) suggesting a persistent effect between treatments. In the mild nausea group, there was no significant difference in the baseline score between the first and third treatment.

Acupuncture was less effective for the symptoms of pain or bloating (Figure 2B and C). Pain scores were significantly improved during acupuncture in the mild nausea

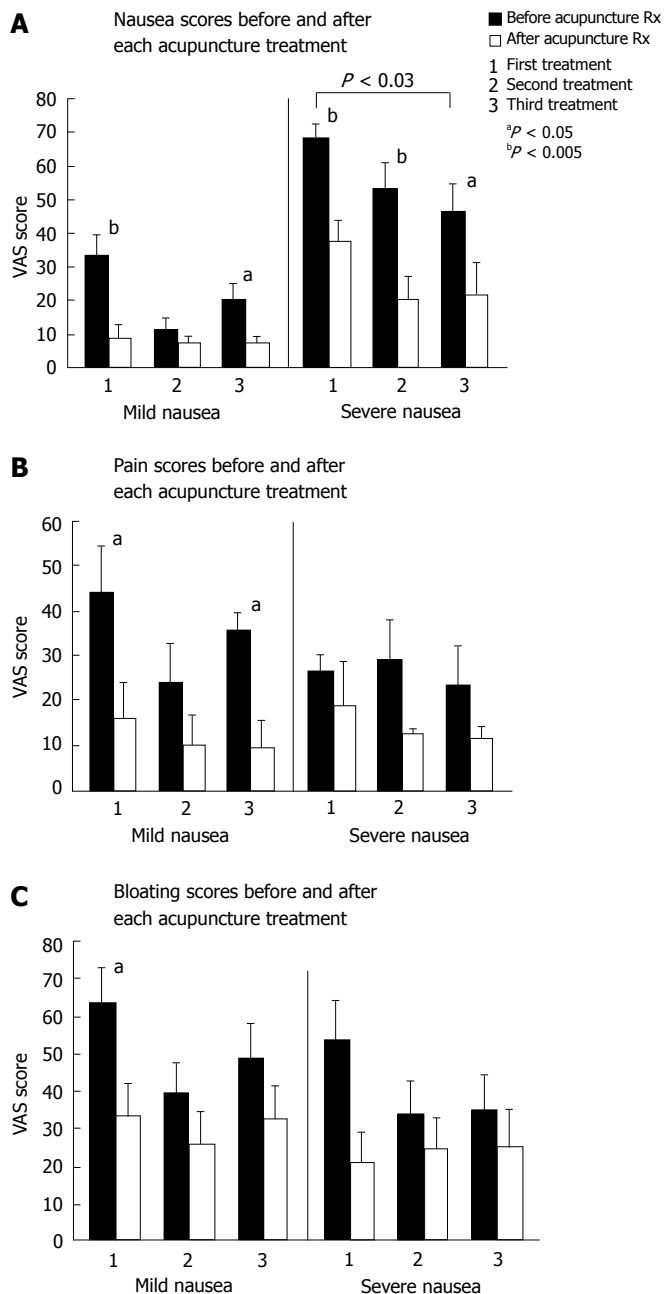


Figure 2 Effect of acupuncture on the symptoms of nausea (A), abdominal pain (B) and bloating (C) in the patients with mild or severe nausea. The response to each acupuncture treatment is shown.

group. No significant improvement was seen in the severe nausea group although the trend was towards improvement. Although bloating improved during the first acupuncture treatment in the mild nausea group, no significant improvement was seen in the severe nausea group.

Additional categorization of patients examined the response of bloating and pain to acupuncture by the pre-treatment VAS score, as the examining physician did not score the symptoms of pain and bloating clinically. The VAS for bloating improved with the first acupuncture treatment ($n = 22$) from 56.2 ± 7.7 to 30.4 ± 5.4 ($P < 0.01$). The overall pain score for all patients decreased from 35.9 ± 7.8 to 17.8 ± 0.8 ($P = 0.08$) with the first treatment. We also compared responses to acupuncture in patients with mild vs moderate/severe symptoms of bloating or pain.

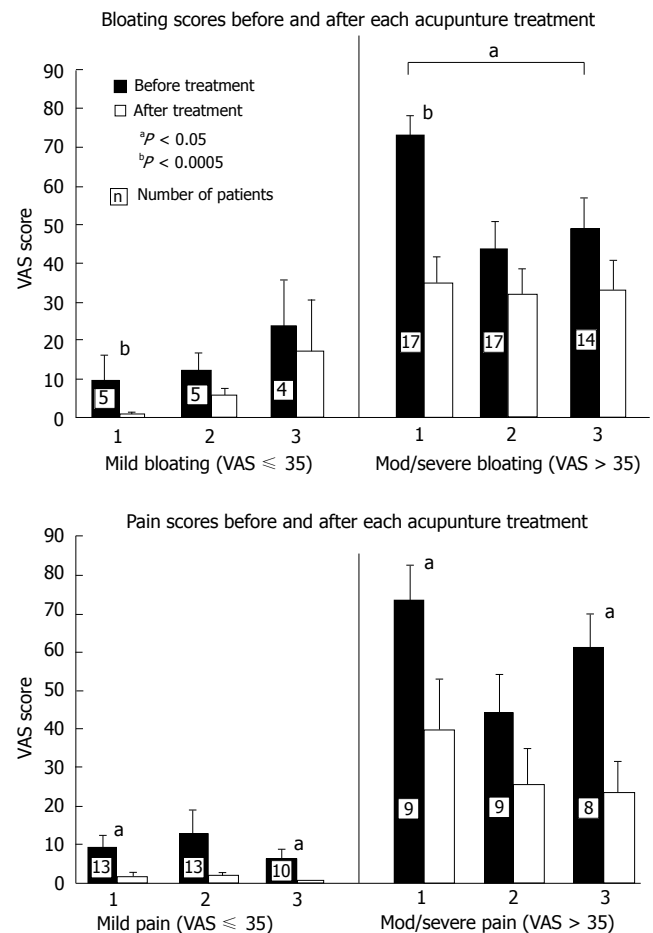


Figure 3 Effect of acupuncture on the symptoms of bloating and pain. Response of patients with a baseline pre-treatment VAS of > 35 (moderate/severe) or ≤ 35 (mild) are shown. The numbers of patients in each group are shown for each treatment session. A significant improvement was seen with acupuncture at the first treatment only for bloating although there was a significant decrease in pre-treatment bloating between the first and third treatment. Acupuncture improved pain with most treatments but the improvement was not sustained.

We chose an arbitrary cut off of an initial VAS score of 35 out of 100 to differentiate patients with mild bloating or pain from those with moderate or severe bloating or pain (Figure 3). The VAS for bloating improved with initial treatment in those with a baseline VAS score of greater than 35. While there was no acute improvement with subsequent treatments, there was a significant improvement in the pre-treatment VAS for bloating over the three treatments (Figure 3). Acupuncture treatment resulted in improvement of the VAS score for pain in both those with severe/moderate pain and mild pain after the acute acupuncture treatment (Figure 3). However, there was no significant improvement in pre-treatment pain between the first and third treatments.

Effect of acupuncture on EGG

Acupuncture resulted in a significant improvement in the percent of power in the normal frequency. At baseline, there was no difference in overall EGG diagnosis between the severe and mild nausea groups. When comparing the power in the different frequency components before and after acupuncture, there was a significant improvement in the power in the 2.5-3.7 cpm between the pre-acupuncture

and post-acupuncture EGG following the first and second treatments in the severe nausea patients. The percent power in this range improved from 23.4 ± 7.5 to 30.8 ± 10 following the first treatment ($P < 0.05$) and from 19.35 ± 10 to 31.2 ± 10.8 with the second treatment ($P < 0.03$). The third treatment increased the percent power in this frequency range from 28.1 ± 11.3 to 37.3 ± 15 (NS). In the mild nausea group, the percent power in this normal frequency range also improved comparing pre- and post-acupuncture treatment but did not reach statistical significance. There was no correlation between the percent power in the 2.5-3.7 cpm range and the symptoms of bloating or pain.

DISCUSSION

We report the results of our experience with the use of acupuncture in patients with functional dyspepsia and nausea. A significant improvement in the level of nausea was seen following acupuncture in patients with none to mild nausea (mild nausea group) and those who complained of moderate to severe nausea (severe nausea group). A significant response was seen in both, suggesting that acupuncture given in the holistic method was effective for both mild to severe nausea. Only one patient in each group was diabetic and most had failed other treatment modalities. A review of Pubmed using the search term "acupuncture and nausea" revealed no prior reports of the use of acupuncture for functional dyspepsia or nausea, other than one study of 15 patients with diabetes and symptoms suggestive of gastric dysmotility^[11]. There was a significant improvement in the VAS score for pain in the mild nausea group and the first treatment only improved bloating in the mild nausea group. This difference in response between complaints of nausea and the other complaints of pain and bloating suggest that the response seen was not purely placebo. This is also the first reported study in which acupuncture was given in a holistic approach for nausea.

Multiple studies report the use of acupuncture in the treatment of chemotherapy induced nausea, post-operative nausea and nausea in pregnancy. Acupuncture is effective in most studies without controls, but its effectiveness is less noticeable if compared to a placebo or sham treatment^[7-9]. Controlled studies of acupuncture in the treatment of chemotherapy induced nausea and vomiting suggest a moderate effect with less acute vomiting episodes and an improvement in the mean worst nausea severity in patients treated by acupressure and concurrent anti-emetics^[8]. Following elective gynecological surgery, a greater percentage of patients treated with acupressure of PC6 point (also known as P6 point) reported no nausea or vomiting compared to sham treated patients^[9]. Several studies suggest effectiveness of acupuncture/acupressure in treating nausea of pregnancy. A randomized controlled trial of acupressure at P6 *vs* placebo showed improvement of symptoms of nausea over time in both groups with nausea symptoms being less severe in the acupressure treated group^[12]. Others report an improvement in duration but not intensity of nausea in pregnancy compared to placebo treatment^[13]. Although well-conducted placebo controlled studies failed to show a difference compared to

sham treatment, acupuncture may prove to be as effective as medications^[13,14].

Comparison between studies is difficult because the criteria for recruitment of subjects and presence of potentially confounding factors, such as depression, are often not discussed. Most of the trials involve acupuncture at one or two acupuncture points only, mainly P6, and are therefore different from the usual clinical holistic approach when using acupuncture to treat a patient. A review of several studies suggests that these studies may be underpowered and a true difference between placebo and sham may be missed^[15].

Our treatment included acupuncture at three points, two related to the GI tract and one at a point recommended for psychological complaints. These points were chosen based on clinical experience and training and our observation that many of the subjects with nausea have psychological distress. We did not formally test for any psychiatric diagnosis, as this was a clinical treatment approach. The results suggest that acupuncture at the points chosen for this study may be effective in treating nausea and less for pain or bloating when patients are grouped by degree of nausea. When the subjects are grouped into moderate/severe or mild bloating or pain by their first pre-treatment VAS score, an improvement in VAS score was seen in bloating and pain. It is unclear if this can be extrapolated to treatment of functional bloating or pain as the degree of symptom was assessed by the pre-treatment VAS score and it is unknown if this correlates with the degree of chronic symptom complaint. There was an increase in the power of gastric myoelectric activity in the normal range, 2.5-3.7 cpm, as assessed by EGG. Whether this is a primary effect of acupuncture or secondary to a central nervous system response cannot be determined in this study. These data support further study using a placebo-controlled approach.

The mechanism of the action of acupuncture is unknown. There are few studies in patients examining the effect in nausea in humans. Hu *et al*^[16] demonstrated that acupressure at the PC6 point decreased symptoms of nausea and decreased the gastric dysrhythmia induced byvection in an optokinetic drum. An increase in the percent of normal frequency in the EGG has been reported in diabetic patients with symptoms suggesting an action of acupuncture on gastric motor function^[17]. In these subjects, the ST36 points and electrical stimulation of needles was used. In humans, acupuncture and manipulation at the PC6 point in healthy volunteers selectively activated regions of the superior frontal gyrus, anterior cingulate gyrus and dorsomedial nucleus of the thalamus as well as areas of the cerebellum^[17]. The subjects did experience more unpleasantness with active acupuncture than with sham treatment which may explain the cerebral cortical findings. The cerebellar findings may be more specific to the action of acupuncture at PC6, as activation of this brain area is probably unrelated to the pain of needle placement.

In animals, acupuncture of the PC6 and ST36 points results in an increased rate of gastric emptying and an increase in the percent of time that the dominant frequency is within the normal range^[18]. Based on the effect of acupuncture on the heart rate variability, it was concluded that this effect was associated with increased vagal activity. A

central effect of electroacupuncture has also been suggested by Tatewaki *et al*^[19] who demonstrated that electroacupuncture at PC6 reduced the number of retching and vomiting episodes following vasopressin injection in dogs, an anti-emetic effect which was abolished by pretreatment with naloxone. Whether this proves to be similar for humans is unknown. The effect of acupuncture is complex in animals, with studies showing that in the rat, electrical stimulation of the ST36 point can induce either excitatory or inhibitory effects on gastric contraction^[20]. Multiple variables are possible to explain differences in animals: the site of stimulation and the intensity and type of stimulation. These effects in animals support a true physiologic response to stimulation of acupuncture points.

In humans, additional variables are present. These include co-morbid conditions, particularly other pain or psychological conditions. The effect may be influenced by belief in the treatment that may contribute to a placebo effect, or may be associated with central nervous system biological responses that may impact on response to acupuncture *via* a non-placebo mechanism. For example, a study by Pariente *et al*^[21] demonstrated that real acupuncture stimulated activity in the brain, as assessed by fMRI, to a greater extent than placebo and that belief in acupuncture affected the response at other areas of the brain. Our clinical study was not designed to test the effect of placebo or belief in the response. However, the differential effect of acupuncture on the symptoms of nausea compared to pain and bloating in the same subjects suggests that the effect on nausea may not be entirely placebo. These results would support the value of a rigorously designed study to examine the effects of acupuncture on the symptoms of patients with functional nausea.

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COMMENTS

Nausea is a common and debilitating symptom complaint. In many patients the underlying pathophysiology is unknown and likely represents a combination of gastric pathophysiology and psychological issues. There are few medications available, many of which have significant side effects. The traditional approach of acupuncture allows several likely underlying conditions to be treated at the same time.

Research frontiers

While acupuncture has been studied in treatment of nausea of chemotherapy, postoperatively and during pregnancy, no studies have been published on its use in functional nausea. Most published studies also use acupuncture and one site, which is a different approach to the general clinical acupuncture approach. Based on experience and the finding that 14/22 subjects had documented history of depression, a holistic approach which treated multiple clinical conditions was used. The same acupuncture points were used in all patients.

Innovations and breakthroughs

The study showed a significant improvement in degree of nausea in subjects who presented with moderate/severe nausea. The degree of nausea improved over the three treatment visits suggesting a lasting effect. Both bloating and pain were improved by acute treatment with acupuncture, but it is unclear whether the pre-treatment VAS score accurately reflects the severity of chronic complaints by the patients. The main innovation in this study was the holistic approach to treat both gastrointestinal and psychological conditions concurrently.

Applications

Data from this study would support the need for a controlled study to examine the effect of treatment and to control for factors such as concurrent psychological conditions and belief in acupuncture as a treatment.

Terminology

Functional nausea-symptom of nausea in the absence of anatomic or biochemical abnormalities, such as pregnancy, ulcer disease, obstruction *etc.* Dyspepsia-chronic upper abdominal pain. The symptom may be associated with ulcer, but functional dyspepsia is a condition with similar symptoms but in the absence of identifiable pathology in the stomach.

Peer review

It is an interesting paper integrating Traditional Chinese Medicine (acupuncture) with Western Medicine. The author has included a lot of thought provoking factors in this paper, such as the causes of nausea, the Chinese medical theory of imbalance, the conducted placebo controlled studies failed to show a difference compared to sham treatment, activation of brain region related to needle stimulation, and that acupuncture affected the response, and so on.

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Epidemiology of upper gastrointestinal cancers in Iran: A sub site analysis of 761 cases

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Abstract

AIM: To define the sub site distribution of upper gastrointestinal cancers in three provinces of Iran.

METHODS: The study was carried out in three provinces in Iran: Ardabil, Golestan, and Tehran. In Ardabil and Golestan, the data was collected from the sole referral center for gastrointestinal cancers and the local cancer registry. For Tehran province, data from two major private hospitals were used. All gastric and esophageal cancer patients diagnosed during the period from September 2000 and April 2002 were included in the study.

RESULTS: A total of 761 patients with upper gastrointestinal cancers were identified, 314 from Ardabil, 261 from Golestan, and 186 from Tehran. In Tehran, the relative rate of cancer increased from the upper esophagus to the distal stomach. In Golestan, the reverse pattern was observed. In Ardabil, the mid portion (distal esophagus and proximal stomach) was involved most frequently.

CONCLUSION: There were considerable variations in the sub site of upper gastrointestinal cancers in the three provinces studied. We cannot provide any explanation for this variation. Further research aimed at explaining the discrepancies in sub site distribution of upper gastrointestinal cancers may help identify important risk factors.

INTRODUCTION

Gastric cancer is the third most common malignancy worldwide^[1]. According to a recent report by Iran's ministry of health, cancer is the third most common cause of mortality, constituting 14% of all deaths in Iran. Upper gastrointestinal (GI) cancers cause 55% of all cancer-related deaths in Iran, with gastric cancer being the most common. Gastric cancer accounts for nearly 50% of all GI cancers^[2]. The incidence of squamous cell carcinoma of the esophagus and cancer of the stomach are very high as compared to their incidence in western countries^[3,4].

A major problem in classifying upper GI cancers is the lack of a universally accepted and clearly reproducible anatomic landmark separating the gastric cardia from the distal esophagus. Even when the landmarks are defined, cancer frequently destroys the anatomy to the extent that the landmarks become unrecognizable. Therefore, misclassification of gastric cancer occurs frequently^[5,6].

Recently, several studies have indicated an increase in the incidence of adenocarcinoma of the distal esophagus compared to cancer of the gastric cardia in western countries. These studies postulate that a significant proportion of adenocarcinomas of the distal esophagus may have been misclassified as cancer of gastric cardia, leading to the apparent increase in cardia cancer^[6,7].

In view of recent recognition of the importance of the sub site involvement in upper GI cancers, we aimed to examine the sub sites of upper GI cancers in Tehran and two provinces in north Iran.

MATERIALS AND METHODS

Subjects

The study was carried out in three provinces: Golestan, Ardabil, and Tehran. Golestan, located in the south-east of the Caspian Sea, is distinguished from other Caspian littoral provinces by an ethnic composition that has 40% Turkmen ethnicity. This province is located on longitude 51°-56° E, and latitude 36°-38° N, with an altitude of 50-500 meters above sea level.

Ardabil is situated near the south-western border of the Caspian Sea on 48°-49° E, 38°-39° N, and altitude of 3010 m. In this mountainous land, 98% of the population is of Azeri ethnicity.

Tehran is the most densely populated province of Iran and is located between the other two provinces (51°-52° E, 35°-36° N, altitude 1100-1700 m). Tehran has a diverse ethnicity which is representative of the entire Iranian population. Western lifestyle is prevalent in Tehran. By contrast; traditional lifestyle is still dominant in Golestan and Ardabil.

Methods

The data was collected from three different sources. The first source was Atrak clinic located in Gonbad, the main Turkmen-resident city of Golestan. Atrak clinic is the sole referral center for gastroesophageal cancers in the region and it also hosts an active cancer surveillance program. We estimate that over 95% of cancers in the region are registered in this center.

The second source was Aras clinic established in Ardabil for the management of gastroesophageal cancers. This clinic is also the sole referral center for such cancers in the Ardabil province and is a host to another active cancer surveillance program registering over 95% of cancers in the region.

The third source was two private general hospitals in Tehran. These two hospitals together are the primary referral centers for nearly 2 million upper socioeconomic class residents of Tehran.

All patients with gastric and esophageal cancer diagnosed during the period from September 2000 to April 2002, were included in the study. An essential inclusion criterion was that the subjects were born in the specified provinces and had been residing there for at least the last 10 years.

Endoscopic and pathologic tests were performed by the same team, according to the study protocol in the regions^[8]. Uniform endoscopic, surgical, and histologic criteria were used to define the tumor sub site.

The sub-site of esophageal tumors was recorded as upper, middle, or lower based on the endoscopy or surgery report. If the location of the main tumor bulk was not obvious, the cancer was recorded as "esophageal".

Non-cardia gastric cancers were defined as those where the center of the tumor was located over 2 cm distal to the gastro-esophageal junction. The gastro-esophageal junction was defined as the most proximal site of the gastric folds. Non-cardia gastric cancers were further divided into antrum, body, and fundus, according to the location of the main bulk of tumor. If the main bulk was

Table 1 The demographic characteristics of patients in the study

	Ardabil	Golestan	Tehran	Total
Patients No.	314	261	186	761
Age, yr (SD)	63 (10.8)	64.4 (11.8)	60.1 (12.7)	62.8 (11.7)
Male/Female	224/90	161/100	123/63	508/253
Urban/Rural residents	97/217	85/176	186/0	368/393

not obvious, the cancer was recorded as "gastric".

Adenocarcinomas of the gastro-esophageal junction area were classified according to the WHO/IARC guidelines as a tumor which crosses the gastro-esophageal junction regardless of the main site of the tumor bulk. Adenocarcinoma of esophagus was located entirely above the gastro-esophageal junction, and adenocarcinoma of gastric cardia was located below the gastro-esophageal junction and was centered within 2 cm from the junction.

Statistical analysis

Statistical analysis was performed using SPSS for windows, version 11.5. Analysis of variance was applied for comparison between normal numeric variables and chi-square for categorical variables.

RESULTS

A total of 761 patients with a confirmed diagnosis of gastric and esophageal cancer were identified. Of the patients included, 41.3% were from Ardabil, 34.3% from Golestan, and 24.4% from Tehran. The demographic characteristics are shown in Table 1.

The mean age of the subjects was 63 years (SD 12, range 18-96). There was no difference with respect to the age of the subjects in the three provinces (ANOVA, $F = 2.7$, $P = 0.64$). The male to female ratio was 2.0; there was no difference in the sex ratios between the three provinces ($\chi^2 = 9.16$, $P = 0.10$).

None of the subjects from Tehran resided in rural regions. This observation is in agreement with the fact that 87% of Tehran's population resides in urban areas. In Ardabil and Golestan provinces this ratio was 43.1%, and 54.5% respectively.

In 54 (7%) cases, the location of the tumor sub site was determined by review of the surgical report. Endoscopic data was used in the remaining subjects. Localization to a specific sub site was not possible in 5.2% of gastric cancers and 2% of esophageal cancers. The details of the anatomic distribution of cancers are shown in Table 2.

DISCUSSION

We observed distinct patterns of sub site involvement of upper GI cancers in the three geographic regions of Ardabil, Gonbad and Tehran. Tehran province had a higher rate of antral cancer compared to esophageal or junctional cancers. By contrast, in Ardabil and Golestan provinces, junctional and esophageal cancers were more common respectively, compared to antral cancers.

Table 2 The distribution of upper gastrointestinal cancers in three provinces of Iran *n* (%)

	Ardabil	Golestan	Tehran	Total
Stomach	178 (56.7)	93 (35.6)	137 (73.7)	408 (53.6)
Antrum	49 (15.6)	18 (6.9)	64 (34.4)	131 (17.2)
Body	47 (15.0)	32 (12.3)	29 (15.6)	108 (14.2)
Fundus	0 (0)	1 (0.4)	3 (1.6)	4 (0.5)
Cardia	82 (26.1)	41 (15.7)	21 (11.3)	144 (18.9)
Unspecified	0 (0)	1 (0.4)	20 (10.8)	21 (2.8)
Junction	24 (7.6)	13 (5.0)	13 (7.0)	50 (6.6)
Esophagus	112 (35.7)	155 (59.4)	36 (19.4)	303 (39.8)
Lower	41 (13.1)	42 (16.1)	21 (11.3)	104 (13.7)
SCC	26	41	18	85
ADC	15	1	3	19
Mid	57 (18.2)	91 (34.9)	9 (4.8)	157 (20.6)
Upper	14 (4.5)	19 (7.3)	3 (1.6)	36 (4.7)
Unspecified	0 (0)	3 (1.1)	3 (1.6)	6 (0.8)
Total	314	261	186	761

SCC: Squamous cell carcinoma; ADC: Adenocarcinoma.

The disparity in the relative frequencies of upper GI cancers in different regions could be explained by variations in the environmental and genetic factors. There are several reports that dietary, behavioral, life style and environmental factors may play a causative role in high-risk populations^[9].

HP infection is a well recognized factor involved in the pathogenesis of gastric cancer^[10,11]. All the three provinces studied have *HP* prevalence rates of nearly 90%^[12,13]. Therefore, in the present study, *HP* infection is unlikely to have accounted for the observed disparity in the sub-site prevalence of cancer.

Gastro-esophageal reflux (GER) is another risk factor associated with lower esophageal and cardia cancers^[14]. The prevalence of GER, defined as having reflux symptoms at least once a week, has been found to be 21.2% in Tehran^[15] and 30% in Golestan^[16,17]. The endoscopic prevalence of erosive GER in Ardabil was 34.7%^[12]. The progressive increase in the prevalence rate of GER from Tehran to Golestan to Ardabil correlates with the increase in the rate of cancer of gastric cardia in these provinces, from 11.3% in Tehran to 15.7% in Golestan and 26.1% in Ardabil (Table 2). It is possible that the prevalence of GER may explain the differences in cancer rate, especially that of cardia cancer. However, other explanations are required to determine the basis of the other cancer variations in the present study.

In the west, adenocarcinoma of the distal stomach (non-cardia cancer) has shown a decreasing incidence over the last 50 years^[9]. By contrast, adenocarcinoma of the cardia of the stomach and the adjacent gastro-esophageal junction has increased over the past 25 years. This increase is occurring at rate that exceeds that of any other cancer^[18-21]. The opposite trends in the incidence may indicate distinct etiologies for the cancer at these two sub sites of the stomach. Moreover, cancers at these sub sites differ with respect to the underlying gastric phenotype. Non-cardia gastric cancers occur in patients with *HP*-induced atrophic gastritis and accompanying hypochlorhydria. These patients usually have elevated

serum gastrin and low serum pepsinogen levels^[10,22]. On the other hand, cancer of the cardia and gastro-esophageal junction occur in subjects with normal acid secretion and is generally not associated with *HP* infection^[10,23].

Although the distinctive patterns observed in gastric cardia and non-cardia cancers have received much attention in explaining the underlying etiology of cancer, junctional cancers have not excited much interest.

One type of cancer pattern is observed in western countries where the incidence of junctional cancer is increasing while that of non-junctional esophageal cancer (SCC) is decreasing. The other pattern is observed in high incidence regions such as Linxian in China and North Iran. In these areas, an increase in junctional cancer is accompanied by high rates of esophageal SCC (not only of the lower esophagus), which is in sharp contrast to the pattern in the west where esophageal SCC is uncommon^[8,12]. As with the case of cardia *vs* non-cardia gastric cancers, it is possible that distinct etiologies are involved in the pathogenesis of junctional cancers such that junctional cancers associated with high rates of esophageal SCC may have different etiologies than those not associated with high rates of high esophageal SCC.

In summary, the present study represents the largest report from Iran identifying sub site involvement of upper GI cancers. We observed distinct pattern of cancers in the three provinces of Tehran, Ardabil, and Golestan. There is no clear explanation for this disparity, although we postulate that different etiologic factors are likely to be involved.

COMMENTS

Background

Gastric cancer is the world's third most common malignancy. Defining the exact origin of gastric cancers is sometimes difficult especially when the gastro-esophageal junction is involved. This difficulty often leads to misclassification of cancers, especially those arising from the gastric cardia and the lower esophagus.

Research frontiers

The incidence of cancer of the gastric cardia and the lower esophagus has shown a rapid change in several areas of the world. This rapid change offers a unique opportunity to study the etiology of these cancers. Many scientists are working on the epidemiologic characteristics of these changes in order to identify the etiologic factors. Thus, the exact determination of the sub site of origin of cancers and their epidemiology is very important.

Innovations and breakthroughs

The decreased prevalence of *H. pylori* infection in some areas is believed to have resulted in a decrease in the incidence of gastric cancers. On the other hand, the increase in prevalence of gastro-esophageal reflux disease is believed to have lead to an increase in lower esophageal cancer.

Applications

We observed different patterns of sub site involvement in different parts of Iran. This difference could indicate different etiologies. Further epidemiologic studies aimed at identifying the probable etiologic factors are required.

Terminology

Gastric cardia is the most proximal 2 cm of stomach located distal to the gastro-esophageal junction.

Peer review

In this prospective study, the authors studied the sub site distribution of upper

gastrointestinal cancers in three provinces of Iran and they found that there are considerable variations in the sub site of upper gastrointestinal cancers in the three provinces studied.

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Colorectal neoplasm: Magnetic resonance colonography with fat enema-initial clinical experience

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echo with inversion recovery sequence is feasible in detecting colorectal neoplasm larger than 10 mm.

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Key words: Magnetic resonance colonography; Contrast-to-noise ratio; Virtual endoscopy; Colorectal neoplasm; Fat contrast medium

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Abstract

AIM: To assess Magnetic resonance colonography with fat enema as a method for detection of colorectal neoplasm.

METHODS: Consecutive twenty-two patients underwent MR colonography with fat enema before colonoscopy. T1-weighted three-dimensional fast spoiled gradient-echo with inversion recovery sequence was acquired with the patient in the supine position before and 75 s after Gadopentetate Dimelumine administration. Where by, pre and post MR coronal images were obtained with a single breath hold for about 20 s to cover the entire colon. The quality of MR colonographs and patients' tolerance to fat contrast medium was investigated. Colorectal neoplasms identified by MR colonography were compared with those identified on colonoscopy and sensitivity of detecting the lesions was calculated accordingly.

RESULTS: MR colonography with fat enema was well tolerated without sedation and analgesia. 120 out of 132 (90.9%) colonic segments were well distended and only 1 (0.8%) colonic segment was poor distension. After contrast enhancement scan, mean contrast-to-noise ratio (CNR) value between the normal colonic wall and lumen was 18.5 ± 2.9 while mean CNR value between colorectal neoplasm and lumen was 20.2 ± 3.1 . By Magnetic resonance colonography, 26 of 35 neoplasms (sensitivity 74.3%) were detected. However, sensitivity of MRC was 95.5% (21 of 22) for neoplasm larger than 10 mm and 55.6% (5 of 9) for 5-10 mm neoplasm.

CONCLUSION: MR colonography with fat enema and T1-weighted three-dimensional fast spoiled gradient-

INTRODUCTION

Conventional colonoscopy, with its ability to visualize the mucosa directly, is the standard procedure for evaluating the colon^[1]. But poor patients' acceptance and high cost limit the utility of the technique for colorectal screening^[2]. This catalyzes the investigation for non-invasive methods to visualize the colon. A great number of studies about computed tomography (CT) colonography have been reported in the United States and Europe^[3-6]. But CT colonography has its intrinsic limitation of exposing patients to ionizing radiation and magnetic resonance colonography (MRC) has theoretic capacity for originating soft-tissue contrast 10-1000 times greater than that on CT^[7,8]. So the future of colorectal screening has shifted to MRC.

To date, most of the MRC research needs administration of a rectal enema containing paramagnetic contrast medium and the acquisition of three-dimensional (3D) gradient-echo pulse sequence^[9-12]. This approach directly demonstrates the lumen but not the bowel wall and requires an effective breath hold of up to 30 s. In addition, enema containing paramagnetic contrast medium needs to be compounded by staff themselves and has high cost. These factors may limit its widespread application in the symptomatic and screening population.

Recently, several MRC studies used air, carbon dioxide and water as intraluminal contrast medium which provides negative contrast within bowel lumen and have a high accuracy in diagnosing colorectal neoplasm^[13-16]. But there are few patients in the MRC studies with air enema and the data required from MRC with water enema could not provide the magnetic resonance virtual endoscopy (MRVE) mode.

In this paper, we evaluated the feasibility of detection of colorectal neoplasm with fat enema in MRC using T1-weighted 3D fast spoiled gradient-echo with inversion recovery sequence.

MATERIALS AND METHODS

Materials

The study was approved by the Ethics Committee of the Fudan University Cancer Hospital. All patients who participated in the study were requested to review and sign a written informed consent. No patient has a history of severe arrhythmia and glaucoma. MRC was performed in consecutive 22 patients (9 men, 13 women) aged 46-86 (mean age 58.6) years. Owing to rectal bleeding, positive fecal occult blood test or altered bowel habits, these patients had been referred for conventional colonoscopy.

Exclusion criteria included contraindications to magnetic resonance imaging, such as the presence of a pacemaker, metallic implants in the central nervous system, as well as claustrophobia.

Methods

Following a standard bowel preparation (oral ingestion of 0.5 L of 20% mannite and 1 L of 5% glucose sodium chloride solution), MRC was performed with a 1.5T MR system (Signa twinspeed with excite; GE medical system, Milwaukee, Wis). No sedative or analgesic agents were administered. An eight-channel body coil was used for signal transmission and reception to permit coverage of the entire colon. To minimize bowel peristalsis, 20 mg of raceanisodamine hydrochloride (No.1 Biochem & Pharm, Shanghai, China) were injected intramuscularly.

Following placement of a rectal enema tube (202-26, Shuangling Medical Device, China), fat contrast medium (Kangque; Atai medical system, Inner Mongolia, China) were gently insufflated into the colon at up to 1 m of hydrostatic pressure with the patient lying in the prone position. Fat insufflation was stopped when the patient began to feel uncomfortable. Reinsufflation was not a routine procedure. The administered volume was 1200-1500 mL.

MRC was performed using T1-weighted three-dimensional fast spoiled gradient-echo with inversion recovery sequence. Coronal images were obtained in the supine position with a single breath hold that lasted about 20 s to cover the entire colon. Imaging parameters included: repetition time 4 ms, echo time 2 ms, flip angle 14°, field of view 38-42 cm, matrix 256 × 256 and section thickness 5 mm. Zero interpolation was applied in coronal plane, rendering an effective section thickness of 2.5 mm and a matrix of 512 × 512. About 68 sections were required. Subsequently, using an automatic injector (Optistar, Mallinckrodt; Tyco, Missouri, USA), gadopentetate dimelumine (Magnevist; Schering, Berlin, Germany) was intravenously administered by the basilic vein. Injection parameters included a dosage of 0.2 mmol/kg and a flow rate of 2.0 mL/s, followed by rapid injection of 10 mL of normal saline at the same rate. After a delay of 75 s, 3D acquisition was repeated with identical imaging parameters with the patient in the supine position.

The 3D data sets were transferred to a post processing

workstation (Advantage 4.0; GE Medical System) and evaluated by two radiologists who were blinded to the colonoscopic data. All the data sets were assessed in the multiplanar reformation and MRVE mode. For the purpose of analysis, the entire colon was divided into six segments: cecum, ascending colon, transverse colon, descending colon, sigmoid colon, and rectum. The colonic distension was classified by scores as follows: score 1 = well distension; score 2 = moderate distension, if it was distended enough to visualize the mucosa and fat interface; and score 3 = poor distension, if it was collapsed. For quantitative assessment, region of interest (ROI) was placed in the lumen, all the colonic neoplasm and the adjacent healthy colonic walls. Image noise, defined as the standard deviation of signal intensities (SI) measured in a ROI placed outside the body, were determined^[14]. On the basis of these measurements, contrast-to-noise ratio (CNR) values were calculated: $CNR = [SI(\text{colonic wall}/\text{colonic lesion}) - SI(\text{lumen})]/\text{noise}$.

Localization and size of all detected colonic neoplasm was recorded. Colorectal neoplasm was classified based on size: (a) < 5 mm; (b) 5-10 mm; (c) > 10 mm in diameter. Extracolonic lesions, including hepatic metastasis, were also recorded as additional findings. Colonoscopic findings were considered as the standard of reference.

RESULTS

All patients completed the MRC without any complication, but 4 patients did not complete colonoscopy for obvious stenosis of colon. Of the 132 colonic segments examined, distensions were documented as follows: 120 (90.9%) had a score of 1, 11 (8.3%) had a score of 2, and 1 (0.8%) had a score of 3. Distinct motion artefacts affecting diagnosis were not found.

Mean CNR values were documented as follows: between the normal colonic wall and lumen, pre contrast enhancement scan 8.4 ± 2.1 , post contrast enhancement scan 18.5 ± 2.9 ; between colorectal neoplasm and lumen, post contrast enhancement scan 20.2 ± 3.1 .

MRC revealed 26 neoplasms including an inflammatory neoplasm (Figure 1), 16 carcinomas (Figure 2) and 9 polyps in 22 patients. The size of five polyps (Figure 3) was between 5 mm and 10 mm and the size of the other 21 neoplasm (Figure 4) was larger than 10 mm in diameter. In patients, there were no false positive MR findings. However, four polyps less than 5 mm, four 5-10 mm polyps and one 20 mm polyp were not found by MRC but by colonoscopy. The 20 mm polyp was a flat polyp. On MRC, neoplasm larger than 10 mm had a higher sensitivity and neoplasm less than 10 mm had a lower sensitivity. The sensitivity for neoplasm of a diameter of < 5 mm, 5-10 mm, and > 10 mm was 0% (0/4), 55.5% (5/9), and 95.5% (21/22). The overall sensitivity was 74.3% (26/35).

MRC permitted assessment of extracolonic organs. Hepatic metastases were discovered in two patients. Hepatic and/or renal cysts were seen in three patients.

DISCUSSION

MRC is a promising, less intrusive imaging technique that can image the entire colon. The potential advantages of

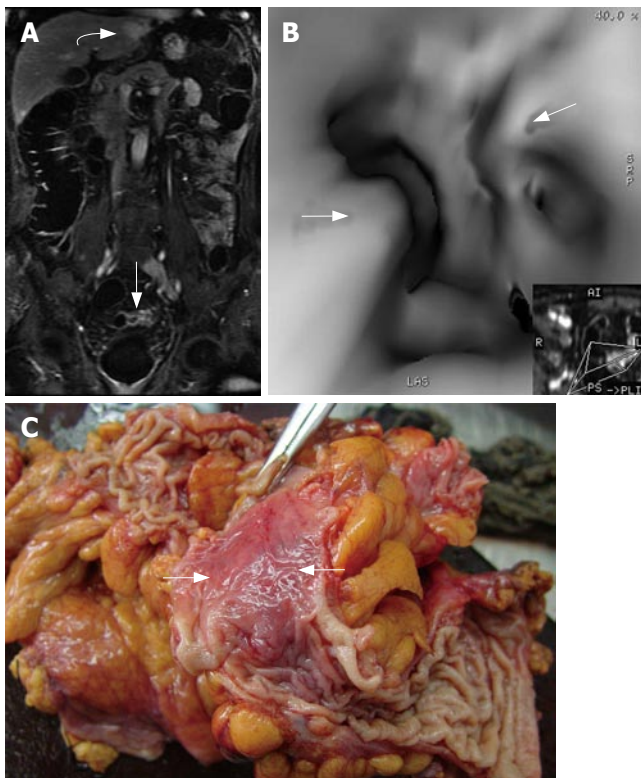


Figure 1 74-year-old man with history of altered bowel habits. **A:** The thickened and increased-contrast-uptake bowel wall of sigmoid colon is seen (white arrow) in the coronal gadolinium enhanced T1-weighted image. In liver, an obvious high-intensity neoplasm is discovered (white curved arrow). Subsequent MR scan confirms the diagnosis of a hepatic angioma; **B:** MR virtual endoscopic rendering confirms stenosis of sigmoid colon (white arrows); **C:** Post-operation pathology confirms the condition of an inflammatory neoplasm. Specimen shows inflammatory mucosa change (white arrows).

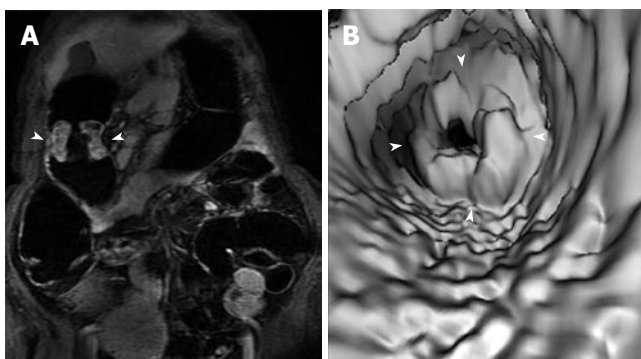


Figure 2 79-year-old woman with history of right upper quadrant pain. **A:** Coronal gadolinium enhanced T1-weighted image shows an obviously enhanced 6 cm carcinoma (white arrowheads) in the transverse colon; **B:** Virtual endoscopic rendering confirms the carcinoma (white arrowheads).

MRC over CT colonography include powerful multi-planar data acquisition, superior contrast resolution and absence of ionizing radiation^[8].

To date, most MRC techniques require a water enema containing paramagnetic contrast material gadolinium at 10 mmol/L concentration^[9,11,12]. Despite Luboldt *et al*^[9] reporting promising results, the gadolinium enema may limit extensive use of MRC. Given 10 mmol/L as optimal concentration of gadolinium, 1.5 L enema would need 30 mL of 0.5 mol/L gadopentetate dimeglumine. The

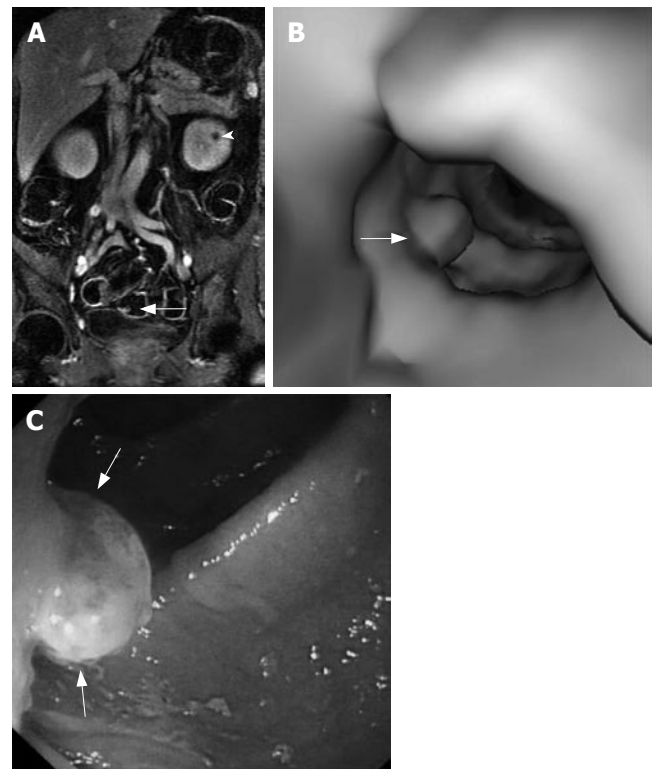


Figure 3 54-year-old man with positive results of FOBT. **A:** Coronal gadolinium enhanced T1-weighted image shows a 7 mm polyp (white arrow) in the sigmoid colon and a cyst (white arrowhead) in the left kidney; **B:** Virtual endoscopic rendering shows a well-defined filling defect (white arrow) in the sigmoid colon; **C:** Endoscopy confirms the polyp (white arrows).

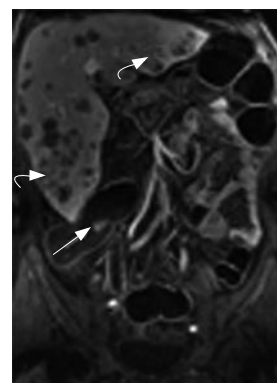


Figure 4 66-year-old man with history of right lower quadrant pain. Coronal gadolinium enhanced T1-weighted image shows a 1.1 cm polyp (white arrow) covered by residual water in the ascending colon and multiple hepatic metastases (white curved arrows).

cost of dilute gadolinium enema would be considered as big setback to MRC. In addition, a 3D gradient echo sequence method that is designed to generate maximal signal from interior of the lumen and depress all the other tissue including the luminal wall was performed. Colorectal neoplasms were identified solely on filling defects in the bright colonic lumen. Hence the differentiation between polyps or carcinomas and residual fecal materials or air bubbles proved to be difficult and sometimes even impossible. So the MRC technique needs to be further amplified by the acquisition of 2D gradient echo sequence datasets. And to compensate for the residual air in the lumen, the MRC requires data acquisition both in the supine and the prone patient positions, which results in the complicated examination and longer examination time.

Recently, MRC has been used with air or carbon

dioxide insufflation and Susceptibility artefacts which thought to be a big obstacle had been minimized by using a half-Fourier single-shot fast spin-echo (SE) sequence or a short echo time (TE) multi-slice half-Fourier acquisition single-shot turbo spin-echo (HASTE) sequence^[14,17,18]. Although air is cheap and abundant, HASTE sequence acquires two or more breath hold acquisitions to cover the entire colon, and can not afford virtual endoscopic observation. In addition, the MRC with air or carbon dioxide insufflation analysis had shortage of clinical cases.

The fat contrast medium that we used in MRC mainly contains salad oil, acacia, menthol and distilled water. It is not absorbed by gastrointestinal tract and has no obvious side-effects. The estimated price of 500 mL of fat contrast medium is \$1.25. It is cheaper than paramagnetic contrasts such as gadolinium and less sensitive to susceptibility artefacts than air. It could provide negative contrast within the bowel lumen with a fat-depression sequence. On T1WI, the intensity of fat contrast medium was slightly lower compared to pericolic fat; however it was too low to visualize air-fat interface when the fat-depression sequence was used. But the intensity of residual water in colon on T1-weighted three-dimensional fast spoiled gradient-echo with inversion recovery sequence was higher than that of fat, which led to distinction of fat-water interface. At the same time, residual water blurred colonic wall and neoplasm, even resulting in neoplasm being missed in diagnosis. But after gadolinium administration, the intensity of colonic wall and neoplasm increased considerably, and the intensity difference between colonic wall, neoplasm and water became distinct. Thus the diagnosis of neoplasm obscured by residual water could be done without a need to change patients' position.

Residual fecal materials can not take up paramagnetic contrast, so it is easy to differentiate between residual fecal materials and neoplasm. This form of direct visualization of the colonic wall and colorectal pathologies stemming from it can reduce the possibility of false positive findings that might mimic neoplasm in MRC with gadolinium solution.

T1-weighted three-dimensional fast spoiled gradient-echo with inversion recovery sequence is a 3D SPGR acquisition that automatically uses a Partial K spatial filling technique and a segmented special technique. Thus T1-weighted three-dimensional fast spoiled gradient-echo with inversion recovery sequence allows shortening of acquisition time, enlargement of spectrum of scan, optimization of fat saturation, and boosting spatial resolution. However, it is essential that the patients maintain the breath-hold position throughout the data acquisition. Minor movements can lead to significant motion artefacts. After being trained, all patients can hold their breath for approximately 25 s. The mean acquisition time of 20 s for covering entire colon can be tolerated by the patients. And thus T1-weighted three-dimensional fast spoiled gradient-echo with inversion recovery sequence is able to accomplish MRC.

In terms of lesion detection, the sensitivity of barium enema in detecting polyps of 10 mm or larger has been reported to be varied between 39% and 56%^[19]. For CT colonography, the sensitivities of detecting > 9 mm, 6-9

mm and < 6 mm polyps were 85%, 70% and 48% and specificities of detecting > 9 mm, 6-9 mm and < 6 mm polyps were 97%, 93% and 92%, respectively^[20]. Using a water enema containing paramagnetic contrast material as a contrast medium, MRC of colorectal neoplasm larger than 10 mm has been reported to have a sensitivity of 93%, specificity of 99%, positive predictive value of 92%, and negative predictive value of 98%^[9]. While using water as a contrast medium, sensitivity was 93% and specificity was 100% for colonic neoplasm exceeding 5 mm^[15]. However, colorectal neoplasm less than 5 mm are often missed in diagnosis^[9,15,16,21]. In contrast, MRC using air has shortcomings of low sensitivity, limited coverage and signal drop-off at the borders of the field of view as well as needs further large-scale studies^[14,22]. Although the number of patients in our study was small, our results suggest that MRC with fat enema could correctly detect neoplasm larger than 10 mm and neoplasm less than 5 mm would most likely were missed.

This technique can be helpful in patients with colonic carcinoma. It can demonstrate the entire colon and exclude synchronous colorectal neoplasm, as well as detect extra-colonic involvement, such as hepatic metastases.

But there are three limitations of this study. The first is the relatively large slice thickness, 5 mm slice thickness. It may be a reason for missing colorectal neoplasm less than 5 mm in diameter. With technical advancements, it is more likely that colorectal neoplasm less than 5 mm would be identified on MRC with fat enema. The second is that patients required a standard bowel preparation, which results in the limitation of patient acceptance for the technique. Recent studies have shown that fecal tagging technique which does not need a cleansed colon can improve patient acceptance, evaluate the majority of colonic segments inaccessible with conventional colonoscopy and depict polyps exceeding 5 mm in size^[23-25]. The third is the small number of the patients and almost all patients with colorectal carcinoma that have large neoplasm. It could be a reason for high sensitivity.

In summary, the present data indicates that MRC with fat enema is a promising alternative to colonoscopy for the detection of colorectal neoplasm larger than 10 mm. Fat contrast medium distention seems to be well tolerated by patients. In the future, advancements in technique together with large-scale clinical cases analysis might improve the sensitivity of the method and hence reliability of its results.

COMMENTS

Background

MR colonography (MRC) is an effective diagnostic tool for colorectal lesions. Currently two techniques were introduced for MRC based on the signal in the colorectal lumen: "bright lumen" and "dark lumen". Usually, bowel cleansing is essential for two techniques. Bright lumen MRC can be performed by fast T1 weighted 3D gradient-echo acquisitions within the conforms of a single breath hold and requires a rectal enema containing paramagnetic contrast. Dark lumen MRC is based on contrast generated between an enhancing colorectal wall and a homogeneously dark colonic lumen. The technique requires intravenous administration of paramagnetic contrast and administration of a water or air enema.

Research frontiers

Recently, several studies laid emphasis on dark lumen MRC. Compared with bright lumen MRC, dark lumen MRC has considerable advantages including reduced examination and post-processing time, good differentiation between polyps and stool, direct analysis of the bowel wall and comprehensive assessment of parenchymal abdominal organs within the field of view. However, bowel cleansing is not well accepted by more than half of patients. To assure high patient acceptance of MRC, fecal tagging has been introduced. It is based on the principle of altering the signal intensity of fecal material by adding contrast compounds to regular meals. For fecal tagging, a paramagnetic MR contrast medium and a highly concentrated barium sulphate containing contrast are administered for bright lumen MRC and dark lumen MRC, respectively.

Innovations and breakthroughs

MRC with fat enema is a technique that could generate a dark colonic lumen with a fat-depression sequence. So it has almost all of the advantages of dark lumen MRC. The data sets required could render an endoluminal virtual view through post-processing. In addition, fat contrast medium is cheaper than paramagnetic contrasts such as gadolinium and less sensitive to susceptibility artefacts than air.

Applications

MRC with fat enema is a promising alternative to colonoscopy for the detection of colorectal neoplasms larger than 10 mm. Moreover, it could depict the infiltration of extra-colonic fat and evaluate other abdominal organs.

Terminology

MRC is based on 3D datasets required from cross sectional images. Compared with a conventional colonography, MRC is not limited to endoscopic viewing. Multiplanar reformation analysis can not only depict the colonic lumen and colonic wall, but also assess parenchymal abdominal organs.

Peer review

The authors assessed Magnetic resonance colonography with fat enema as a method for detection of colorectal neoplasms. Colorectal neoplasms identified by MR colonography were compared with those identified on colonoscopy and sensitivity of detecting the lesions was calculated accordingly. They concluded that MR colonography with fat enema and T1-weighted three-dimensional fast spoiled gradient-echo with inversion recovery sequence is feasible in detecting colorectal neoplasms larger than 10 mm.

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RAPID COMMUNICATION

Treatment of gastric outlet and duodenal obstructions with uncovered expandable metal stents

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Key words: Gastric outlet obstruction; Duodenal obstruction; Expandable metal stent

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Abstract

AIM: To investigate and evaluate the technical feasibility and clinical effectiveness of fluoroscopically guided peroral uncovered expandable metal stent placement to treat gastric outlet and duodenal obstructions.

METHODS: Fifteen consecutive patients underwent peroral placement of Wallstent™ Enteral Endoprosthesis to treat gastric outlet and duodenal obstructions (14 malignant, 1 benign). All procedures were completed under fluoroscopic guidance without endoscopic assistance. Follow-up was completed until the patients died or were lost, and the clinical outcomes were analyzed.

RESULTS: The technique success rate was 100%, and the oral intake was maintained in 12 of 14 patients varying from 7 d to 270 d. Two patients remained unable to resume oral intake, although their stents were proven to be patent with the barium study. One patient with acute necrotizing pancreatitis underwent enteral stenting to treat intestinal obstruction, and nausea and vomiting disappeared. Ten patients died during the follow-up period, and their mean oral intake time was 50 d. No procedure-related complications occurred. Stent migration to the gastric antrum occurred in one patient 1 year after the procedure, a tumor grew at the proximal end of the stent in another patient 38 d post-stent insertion.

CONCLUSION: Fluoroscopically guided peroral metal stent implantation is a safe and effective method to treat malignant gastrointestinal obstructions, and complications can be ignored based on our short-term study. Indications for this procedure should be discreetly considered because a few patients may not benefit from gastrointestinal insertion, but some benign gastrointestinal obstructions can be treated using this procedure.

INTRODUCTION

Gastroduodenal obstruction is usually a preterminal event in patients with advanced malignancies of the stomach, pancreas, and duodenum. Surgery is associated with a high complication rate, and relatively high morbidity and mortality rates, due to poor nutrition and general status or progressing tumor infiltration in these patients^[1-8]. Fluoroscopically guided peroral placement of self-expandable metal stents to treat gastric and duodenal obstructions have been demonstrated to be an effective alternative to surgical bypass with lower morbidity and mortality rates, shorter hospitalization, and a lower cost of the overall treatment^[1,3,5,6,8-10]. Various stents including vascular stents have been used to treat gastric and duodenal obstructions before the design of special duodenal stents^[1]. Here, we report our experience in the treatment of gastric and duodenal obstructions using uncovered Wallstent Enteral Endoprosthesis in recent years. There have been few reports on enteral stenting for benign gastrointestinal obstructions^[2,11,12]. Here, we discuss our methods used for intestinal stenting for one patient with acute necrotizing pancreatitis. We also discuss our opinions on the placement technique, indications, complications, and follow-up results.

MATERIALS AND METHODS

Subjects

A total of 15 consecutive patients underwent fluoroscopically guided peroral placement of Wallstent Enteral Endoprosthesis to treat gastroduodenal obstructions from June 2004 to December 2006, in our department. There were 11 men and 4 women with an average age of 62.4 years (age range, 32-87 years). The underlying causes of

Table 1 Patient information

Case	Sex/age (yr)	Underlying disease	Stent size (diameter × length, mm)	Duration of oral intake after stenting	Follow-up time	Outcome
1	F/68	Ampullary cancer	22 × 90	At least 10 d	10 d	
2	M/72	Gastric cancer	22 × 60	15 d	24 d	Dead
3	F/39	Pancreatic cancer	20 × 90	At least 4 mo	4 mo	
4	F/65	Pancreatic cancer	22 × 90	38 d	74 d	Dead
5	M/86	Pancreatic cancer	20 × 90	80 d	85 d	Dead
6	M/87	Pancreatic cancer	20 × 60	At least 20 d	20 d	
			22 × 60			
7	M/80	Ampullary cancer	20 × 60	9 mo	10 mo	Dead
8	F/72	Colon cancer	22 × 90			
			22 × 60	22 d	26 d	Dead
9	M/66	Gallbladder cancer	22 × 90	7 d	50 d	Dead
10	M/33	Acute necrotizing pancreatitis	20 × 60			
			22 × 60		1 y	
			22 × 90			
11	M/67	Duodenal cancer	20 × 90	60 d	70 d	Dead
12	M/68	Pancreatic cancer	20 × 90	7 d	10 d	Dead
13	M/48	Pancreatic cancer	20 × 90	0 d	6 d	Dead
14	M/32	Colon cancer	20 × 60	At least 7 d	7 d	
15	M/53	Esophageal cancer	20 × 60	0 d	40 d	Dead

obstructions were pancreatic carcinoma ($n = 6$), ampullary carcinoma ($n = 2$), colon carcinoma ($n = 2$), gastric cancer ($n = 1$), duodenal cancer ($n = 1$), gallbladder cancer ($n = 1$), esophageal carcinoma ($n = 1$), and acute necrotizing pancreatitis ($n = 1$) (Table 1). The diagnosis was established by means of CT ($n = 7$), endoscopy ($n = 2$) or after surgery ($n = 6$). Gastrointestinal obstructions were confirmed by a barium study ($n = 11$), endoscopy ($n = 2$), and CT ($n = 2$). The sites of obstructions were the gastric antrum ($n = 1$), pylorus ($n = 1$), gastrointestinal anastomotic stoma ($n = 1$), duodenum ($n = 12$, including duodenal metastasis from colon carcinoma in two patients). Fourteen patients with malignant obstructions were considered inoperative because of the risk of recurrence after surgery, extensive tumor growth, metastatic cancer or the presence of peritoneal seeding or distant metastasis. They all presented with severe nausea and recurrent vomiting. The patient with acute necrotizing pancreatitis developed severe intestinal obstruction following surgery. Although jejunostomy had been performed, nausea and vomiting persisted in this patient, and duodenal obstruction was finally detected. There was no amelioration in conditions after 4 months, so we decided to insert a metal stent in order to relieve the obstructive syndrome.

Methods

The Wallstent Enteral Endoprosthesis (Boston Scientific Medi-Tech, USA) was used in all patients. The sizes of the stents were as follows: 20 mm (diameter) × 60 mm (length), 22 mm × 60 mm, 20 mm × 90 mm, and 22 mm × 90 mm. Informed consent was obtained from all patients before all procedures. Stent placement procedures were performed according to a standard technique as described in the literature^[1,13,14]. More than 2 d before the stent placement, a barium study was performed to evaluate the site, length, and severity of the obstruction. A nasogastric tube was placed overnight to empty the stomach, and was removed just before the stent insertion. All procedures were performed using a digital angiographic unit with the patient in the supine position. Pharyngeal

anesthesia was usually performed using lidocaine aerosol spray ($n = 13$), but basal anesthesia ($n = 1$) or intravenous general anesthesia ($n = 1$) was also offered depending on the patients' or their relatives' demands. A catheter and guiding wire were inserted perorally into the esophagus under fluoroscopic guidance, and were advanced into the stomach. A small amount of contrast medium was injected through the catheter to identify the gastric outlet or the site of obstruction. The guiding wire and catheter were carefully manipulated to cross the obstruction using a standard catheter technique. After the catheter and guiding wire were located distal to the obstruction and the length of the obstruction was measured, the wire was exchanged for a 260-cm super-stiff guide wire to provide better support for the stent insertion. The Wallstent Enteral Endoprosthesis was then performed under fluoroscopic monitoring to ensure the perfect position of the stent. A stent 4 cm longer than the obstruction length was routinely chosen to completely overlap the affected region. Balloon dilation was performed when the stent assembly could not pass the stenosis. We also performed balloon dilation after stent deployment in 2 of our patients, which was a different procedure from the literature^[1].

The patients were monitored hourly for at least 8 hours after the procedure. If they did not complain of abdominal pain and the hemoglobin level was normal, they were allowed to resume oral intake of liquids. Their diet progressed to soft or solid foods when they could tolerate liquids well. A barium study or endoscopy was performed to verify the position and patency of the stent in cases of bad tolerance of foods or recurrence of nausea and vomiting.

Follow-up was continued by phone calls after patient discharge, and a further barium study or endoscopy was suggested only when symptoms recurred.

RESULTS

Peroral stent deployment was successfully completed under fluoroscopic guidance in our 15 patients, indicating

a technique success rate of 100%. No procedure-related complication occurred. Two stents were needed in 2 patients, and three stents were needed in one due to the lengths of the obstructions. Therefore, a total of 19 stents were used in our 15 patients. The follow-up period was 6 d to 365 d (mean = 80.5 d). Stent migration to the gastric antrum occurred in one patient 1 year after the procedure, and tumor ingrowth at the proximal end of the stent was detected in another patient 38 d after stent insertion. Oral intake was maintained in 12 of 14 patients (85.7%) varying from 7 d to 270 d. Two patients were still unable to resume oral intake, although their stents were proven to be patent with the barium study. The patient with acute necrotizing pancreatitis (benign gastrointestinal obstruction) underwent enteral stenting in order to treat the intestinal obstruction, and nausea and vomiting disappeared after the procedure. Ten patients died during the follow-up period, and their mean oral intake time was 50 d (0 d to 270 d). The time between their death and the stent insertion varied from 6 d to 300 d (mean = 68.5 d).

Percutaneous transhepatic biliary drainage (PTBD) and/or bile duct stent embedding were performed before enteral stenting in 13 patients, 4 of whom further underwent biliary interventional procedures as jaundice recurred after the procedure. The times between the relapse of jaundice and the enteral stent procedure were 7 d, 14 d, 15 d, and 130 d for these 4 patients, respectively. No evidence showed that jaundice recurred because of enteral stenting.

Three of the 14 patients (malignant gastrointestinal obstructions) underwent transcatheter arterial chemotherapy (TAC), and their nutritional status improved after the procedure. Their survival time seemed to be longer than that of the others. However, we did not analyze the influence of TAC considering the small number of patients.

DISCUSSION

A malignant gastrointestinal obstruction is usually a preterminal event in patients with pancreatobiliary, stomach or duodenum malignancies. Such patients often cannot undergo curative resections because of the inoperability or unresectability of their tumors. Palliative surgery is associated with a high complication rate, and relatively high morbidity and mortality rates, due to poor nutritional and general status or progressing tumor infiltration in such patients^[1-8]. Fluoroscopically guided peroral placement of self-expandable metal stents to treat gastric and duodenal obstructions have been demonstrated to be an effective alternative to surgical bypass with lower morbidity and mortality rates, shorter hospitalization, and a lower cost of the overall treatment^[1,3,5,6,8-10]. Various stents including vascular stents have been used for the treatment of gastric and duodenal obstructions before the availability of specially designed duodenal stents^[13]. However, only expandable metal stents have been approved by the Food and Drug Administration for the treatment of gastrointestinal obstructions due to cancer risks^[5,15]. We chose the Wallstent Enteral Endoprosthesis (Boston Scientific Medi-Tech, USA) because of its flexibility and safety. Covered stents have been used to prevent tumor

ingrowth or overgrowth^[4,6,10,12,16-19]. Because we thought the expected survival time of these patients was limited, and tumor ingrowth or overgrowth did not need to be considered^[19], we chose to use uncovered metal stents to obviate migration. In fact, only one case of tumor ingrowth was identified in our 15 patients 38 d after stent insertion, with an ingrowth rate of 6.67%. Additionally, the implantation of peroral uncovered stents is technically easier than covered stents^[1,6,8,16-18].

According to the literature, fluoroscopic stent placement for duodenal obstructions is a technically more difficult procedure than for gastric outlet obstructions, not only because of a loop formation of the stent delivery system in the distended stomach, but also because of the curved configuration of the duodenal C loop^[1,16,17,20]. Endoscopic assistance and gastrostomy have been used after guide wire navigation to advance past the duodenal obstructions^[1,3,16]. In our series, the site of obstruction was located in the duodenum in 12 patients, but no endoscopic assistance or gastrostomy was required in any of them because of the flexibility of the endoprosthesis system and the patience of the interventional radiologists. PTBD before enteral stenting is considered to be necessary by some authors, but there are different opinions^[1,18]. Thirteen patients in our series developed jaundice and underwent PTBD or biliary stenting before gastrointestinal obstructions occurred, and enteral stenting was scheduled. Three of them underwent a second biliary intervention after the enteral stenting, but there was no evidence showing that enteral stenting caused recurrence of jaundice.

TAC was performed in three patients who showed a relatively longer survival time than those who could not tolerate TAC. Considering the small number of patients, we did not analyze the influence of TAC on survival time. Further studies are required to elucidate this relationship.

Nausea and vomiting disappeared or mitigated, and oral intake was maintained in 12 of 14 (85.7%) patients with malignant gastrointestinal obstructions ranging from 7 d to 270 d (mean = 54.7 d). Two patients were still unable to resume oral intake while nausea and vomiting continued after the procedure, although their stents were proven to be patent according to the barium study. A clinical success rate of 85.7% is acceptable because many factors may contribute to the symptoms in malignant gastrointestinal obstructions^[21]. Further studies on the indications of enteral stenting for malignant gastric and duodenal obstructions are warranted.

The patient with acute necrotizing pancreatitis (benign gastrointestinal obstruction) underwent enteral stenting to treat the intestinal obstruction, and nausea and vomiting disappeared after the procedure. His symptoms of nausea and vomiting continued for over 4 mo even after jejunostomy had been performed. Therefore, we decided to perform enteral stenting after discussion with his surgeons. The convention has been that stent placement is rarely indicated for benign gastrointestinal strictures^[12,14], and further studies are needed to elucidate such issues. No procedure-related complication occurred, and only two complications were observed during the follow-up period. Stent migration to the gastric antrum happened in one

patient 1 year after the procedure, and a gastric ulcer with hemorrhage was found around the proximal end of the stent by endoscopy. The patient refused further treatment, and left. Tumor ingrowth at the proximal end of the stent was detected by the barium study in another patient 38 d after stent insertion. Due to progressing tumor infiltration and a hepatic abscess leading to deterioration, a second stent implantation could not be performed, and the patient died 36 d later.

In conclusion, fluoroscopic peroral placement of self-expandable metal stents is a safe and effective treatment for malignant gastric outlet and duodenal obstructions. The technique success rate is high, and complications can be ignored due to the short survival time of the patients in our study. However, indications should be carefully considered before the procedure because a minority of patients may not benefit from gastrointestinal insertion, while some benign gastrointestinal obstructions can also be treated with this procedure.

COMMENTS

Background

Gastroduodenal obstruction is usually a preterminal event in patients with advanced malignant diseases. It can lead to severe malnutrition and even death in the patients. Many studies have reported that enteral stents can be used to achieve gastroduodenal patency, and the effects have been satisfactory.

Research frontiers

Stents have been widely used in interventional procedures. A research hotspot has been to modify the craft of stents to obtain more flexible and durable materials. Various covered stents have been studied to improve effectiveness and reduce adverse reactions.

Innovations and breakthroughs

In previous applications of stents to treat gastroduodenal obstructions, it was found that tumor ingrowth or overgrowth could cause restenosis in uncovered stents, while migration might happen in covered stents. Endoscopic assistance or gastrostomy has been used after guide wire navigation to advance past the duodenal obstructions. Here, we proved that fluoroscopic peroral placement of uncovered metal stents can be safe and effective for most cases.

Applications

The study results suggest that fluoroscopic peroral placement of uncovered metal stents is safe and effective to treat gastroduodenal obstructions. However, indications should be discreetly considered before the procedure.

Peer review

This is a clinical study in which authors investigate and evaluate the technique feasibility and clinical effectiveness of fluoroscopically guided peroral uncovered expandable metal stent placement to treat gastric outlet and duodenal obstructions.

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RAPID COMMUNICATION

Effects of *H pylori* therapy on erythrocytic and iron parameters in iron deficiency anemia patients with *H pylori*-positive chronic gastritis

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efficacy of ferrous succinate therapy in IDA patients with *H pylori*-positive chronic gastritis.

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Key words: *H pylori*; Iron deficiency anemia; Oral iron treatment; Chronic gastritis

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Abstract

AIM: To elucidate the influences of *H pylori* infection on oral iron treatment for iron deficiency anemia (IDA).

METHODS: A total of 86 patients were divided into two groups: group A, receiving ferrous succinate combined with triple therapy for *H pylori* eradication, and group B (control), treated with ferrous succinate only. During treatment of IDA, dynamic changes in hemoglobin (Hb) level, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), serum iron (SI), and serum ferritin (SF) were compared between the groups.

RESULTS: Hb was slightly higher in group A at d 14 after the start of triple therapy for *H pylori* eradication ($P > 0.05$). After the therapy, the increase of Hb in group A became significantly faster than that in group B ($P < 0.05$). At d 56, the mean Hb in group A returned to the normal level, however, in group B, it was lower than that in group A ($P < 0.05$) although it had also increased compared with that before oral iron treatment. The MCV and MCH in group A recovered to the normal level, and were much higher than those in group B ($P < 0.05$) at d 21. In Group B, the MCV and MCH remained at lower than normal levels until d 42 after the start of therapy. And then, they reached a plateau in both groups and the differences disappeared ($P > 0.05$). The SF in group A was higher than that in group B ($P < 0.05$) 28 d after the treatment and its improvement was quicker in group A ($P < 0.05$), and the difference between the two groups was even more significant ($P < 0.01$) at d 56. The SI in group A was higher than that in group B ($P < 0.05$) at d 14 and this persisted until d 56 when the follow-up of this research was finished.

CONCLUSION: Treatment of *H pylori* can enhance the

INTRODUCTION

Spontaneous iron excretion in adults is minimal and iron deficiency anemia (IDA) is generally attributed to abnormal blood loss^[1]. IDA is often caused by gastrointestinal bleeding due to peptic ulcer and chronic gastritis, or in women of reproductive age by increased menstrual flow and the greater requirements of pregnancy. Recently, several seroepidemiological studies have suggested a link between IDA and *H pylori* infection^[2,3]. A high percentage of *H pylori*-positive IDA patients exhibit atrophic changes in the gastric body, and the remainders have chronic superficial gastritis extending to the fundic mucosa.

H pylori infection has even been implicated as a cause of IDA refractory to oral iron treatment^[4,5]. Some patients with refractory IDA have no gastrointestinal symptoms but *H pylori* gastritis, as the only cause of their anemia^[6]. Most dietary iron is in the non-hemic ferric form, and an acidic intragastric pH is needed to reduce it to the ferrous form for absorption. This reaction is promoted by gastric acidity and ascorbic acid (AA), which is thus considered the most potent regulator of iron absorption^[7]. Patients with *H pylori* gastritis showed an increase in intragastric pH with a median of > 3 , a pH that is known to be critical in the process of iron absorption. Moreover, AA is actively secreted from plasma to the gastric juice^[8], but the concentration of AA in the gastric juice of patients with *H pylori* gastritis and IDA is clearly reduced in comparison with both healthy and non-anemic *H pylori*-positive controls^[9-11].

The availability of convenient, non-invasive methods for identifying *H pylori* gastritis has greatly facilitated the recognition of infected patients, resulting in progressive

awareness of its influences and possible role in the causation of IDA^[12]. Therefore, we first examined the prevalence of *H. pylori* infection and serum markers of iron deficiency. We then evaluated the effects of subsequent *H. pylori* eradication on the response to oral iron therapy, which could provide valuable information for further clinical applications.

MATERIALS AND METHODS

Patients

From January 2002 to December 2005, 86 IDA adult patients with *H. pylori*-positive chronic gastritis were enrolled. The participants comprised 36 women and 50 men; median age 53 years, range 18-76. According to Sydney System, the patients were diagnosed as having atrophy, intestinal metaplasia, or antral versus body gastritis, in 34, 27, and 25 cases, respectively. According to the criteria^[13], IDA was defined as hemoglobin (Hb) concentration < 120 g/L in the men and < 110 g/L in the women, serum ferritin (SF) < 12 µg/L, mean corpuscular hemoglobin (MCH) < 27 pg, and mean corpuscular volume (MCV) < 80 fL. Obvious causes of blood loss, such as active gastrointestinal hemorrhage, menometorrhagia or heavy menstrual loss, and any other non-gastrointestinal disease likely to cause IDA, were exclusion criteria for this study. Also excluded from the study protocol were patients with previous gastric surgery, and those who received anti-*H. pylori* treatment or anti-secretory drugs before. Patients who were lactating or pregnant and those with malnutrition or cancer were also excluded.

Methods

The ¹⁴C-urease breath test was performed as described previously^[14]. Briefly, the patients fasted overnight and were then given urea labeled with 37 kBq of ¹⁴C dissolved in water. Breath samples were collected before and 15 min after ingestion, when enzymolysis of labeled urea occurred and ¹⁴CO₂ had been released into the peripheral circulation. The ¹⁴CO₂ in these samples was trapped in 1 mmol/L of hyamine hydroxide in ethanol and transferred into scintillation liquid. The ¹⁴C content was measured in Bq mode using a liquid β-scintillation counter (Headway, China). Samples with < 3.33 Bq were considered negative for *H. pylori*, while samples with > 3.33 Bq were considered positive. The peripheral hemogram including MCH and MCV was measured with a Sysmex automated analyzer (Kobe, Japan). Biochemical assays for serum iron (SI) and Hb were performed as routine. SF was determined with a chemiluminescent immunometric analyzer and kit from Bayer Co. (Germany).

The 86 patients were randomly divided into two groups of equal size. Group A comprised 43 cases and received oral iron treatment combined with triple therapy for *H. pylori* eradication, while the 43 cases in group B received oral iron treatment only as controls. Oral iron treatment comprised ferrous succinate (with an Fe²⁺ content of 34%-36%) 200 mg supplemented with ascorbic acid 100 mg three times daily. Treatment was given until the SF recovered to normal. Triple therapy for *H. pylori* eradication included deuterio-bismuth citrate 240 mg × 2/d, amoxicillin 500 mg × 2/d, and metronidazole 400 mg × 2/d for 2 wk; in all cases, *H. pylori* was eradicated.

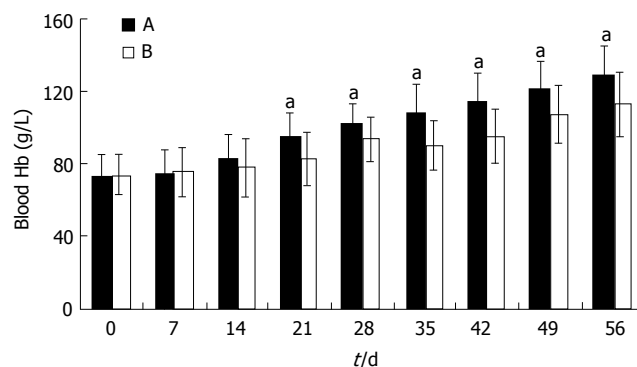


Figure 1 Blood Hb during iron treatment (mean ± SD, *n* = 43; ^a*P* < 0.05 vs group B).

Follow-up

The mean Hb, MCH, and MCV in the peripheral blood of the two groups were recorded before and at d 0, 7, 14, 21, 28, 35, 42, 49, and 56 after iron treatment. SI and SF were compared before and at d 0, 14, 28, 42, and 56.

Statistical analysis

Each assay was made at least three times and data were expressed as the mean ± SD. The differences between groups were determined using *t* test for paired analysis. *P* < 0.05 was considered statistically significant.

RESULTS

Influence of *H. pylori* on Hb

Hb was slightly higher in group A at d 14 after the start of triple therapy for *H. pylori* eradication, but the difference was not significant (*P* > 0.05). After the complete course of triple therapy, the increase of Hb in group A became significantly faster than that in group B (*P* < 0.05). At d 56, the mean Hb in group A returned to the normal level referred to the criteria. In group B, the mean Hb had also increased compared with that before oral iron treatment, but it was still lower than that in group A (*P* < 0.05, Figure 1).

Influence of *H. pylori* on MCV and MCH

Similar to the trend seen for Hb, the increase of MCV and MCH in group A was not significantly greater than that in group B (*P* > 0.05). At d 21, the MCV and MCH in group A became normal, and were much higher than those in group B (*P* < 0.05). In group B, the MCV and MCH remained at lower than normal levels until 42 d after the start of therapy. At this time, the MCV and MCH reached a plateau in both groups and the differences disappeared (*P* > 0.05, Figures 2 and 3).

Influence of *H. pylori* on iron status

There were no significant differences in parameters of iron status between the two groups at the start of treatment (*P* > 0.05), but after 28 d the SF in group A was higher than that in group B (*P* < 0.05). Thereafter, the improvement of SF was much quicker in group A, and at d 56 the difference between the two groups was even more significant (*P* < 0.01). At d 14, the SI in group A was higher than in group B (*P* < 0.05) and this persisted until 56 d after the start of oral iron treatment, when the follow-up of this research was finished (Table 1).

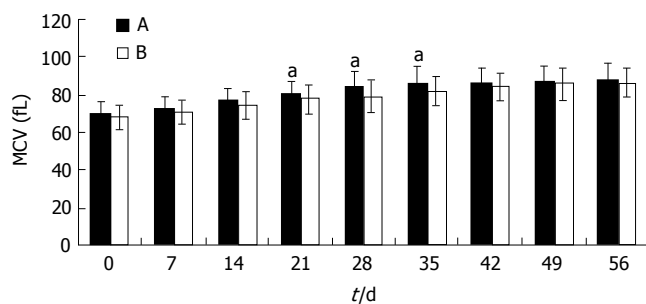


Figure 2 MCV during iron treatment (mean \pm SD, $n = 43$; ^a $P < 0.05$ vs group B).

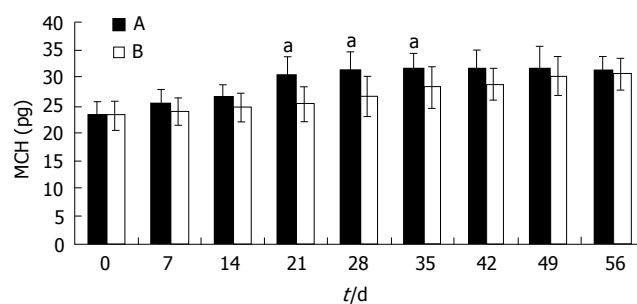


Figure 3 MCH during iron treatment (mean \pm SD, $n = 43$; ^a $P < 0.05$ vs group B).

Table 1 Alterations of SI ($\mu\text{mol/L}$) and SF ($\mu\text{g/L}$) during iron treatment (mean \pm SD, $n = 43$)

Groups	d 0		d 14		d 28		d 42		d 56	
	SI	SF	SI	SF	SI	SF	SI	SF	SI	SF
A	3.2 \pm 1.1	7.2 \pm 1.6	11.3 \pm 3.2 ^a	9.4 \pm 2.6	13.2 \pm 3.4 ^a	11.3 \pm 2.6 ^a	18.2 \pm 3.6 ^a	18.2 \pm 5.3 ^a	23.2 \pm 4.7 ^a	38.5 \pm 9.2 ^a
B	3.3 \pm 1.2	7.5 \pm 1.8	8.9 \pm 2.9	8.8 \pm 2.4	9.2 \pm 2.2	9.6 \pm 2.7	13.2 \pm 2.9	13.6 \pm 3.2	18.2 \pm 3.7	21.6 \pm 5.6

^a $P < 0.05$ vs group B.

DISCUSSION

H. pylori infection has been reported to have various manifestations in adolescents and adults, including IDA. A double-blind, placebo-controlled trial performed in adolescents with IDA and *H. pylori* infection suggested that SF was significantly lower in the *H. pylori*-infected group and that *H. pylori* eradication led to resolution of iron deficiency^[15,16]. A large serosurvey of *H. pylori* in adults also found that SF was significantly lower in adults positive for *H. pylori* IgG than that in non-infected controls^[17,18]. Based on our medical records of adult patients with chronic gastritis, we found that IDA occurred more frequently in *H. pylori*-positive cases. The coexistence of *H. pylori* gastritis in the 86 IDA adult patients enrolled in this study was determined by endoscopy and the urease breath test. To avoid influences from other clinical factors, the subjects were selected according to strict criteria.

It has recently been shown that extension of gastritis to the corporal mucosa occurs in a higher percentage of patients with *H. pylori* infection and IDA compared with non-anemic *H. pylori*-infected controls^[19]. The blood loss in chronic gastritis, and bleeding from duodenal or gastric ulcers related to *H. pylori* infection, plays an important role in the development of iron deficiency in adults. In response to *H. pylori* chronic gastric inflammation, the epithelial cells in the mucosa are damaged, leading to detachment and apoptosis. In the absence of bleeding lesions, the possible mechanisms by which *H. pylori* is involved in the development of IDA remain unclear. Preliminary studies suggest that the growth and proliferation of *H. pylori* requires iron from the host and that some *H. pylori* strains have a specific ability to interfere with iron metabolism by binding iron to their outer membrane proteins^[20]. However, other studies have demonstrated that neither virulence factors such as Cag-A4 nor mutations in the bacterial genes involved in iron uptake are associated with IDA. Given the high prevalence of *H. pylori* infection, this hypothesis cannot explain why not all patients with *H. pylori* gastritis develop IDA^[21].

Besides the occult gastrointestinal bleeding and competition for dietary iron, *H. pylori* infection can affect the gastric body and initiate the development of atrophic body gastritis that can in turn cause decreased gastric acid secretion and increased intragastric pH^[5,22]. *H. pylori* infection adversely influences the composition of the gastric juice; for example, in terms of its acidity and ascorbate content, both of which are critical for normal iron absorption^[23]. These findings suggest that the physiological mechanisms that are necessary for the absorption of alimentary iron in the duodenal mucosa are impaired in patients with *H. pylori* gastritis and IDA. Thus, we planned to determine the relationship between *H. pylori* infection status and indices of IDA such as the peripheral hemogram and SI. These indices were compared between group A, which received triple therapy combined with oral iron treatment, and group B, which received oral iron treatment only.

Before the completion of *H. pylori* eradication, there were no significant differences in parameters reflecting iron status between the two groups, though the indices were slightly higher in group A. After the 2 wk triple therapy was finished, our observations indicate that the response to oral iron treatment was significantly greater in group A than in group B. Since supplementation with ascorbic acid has been commonly shown to reduce the pH of gastric juice thereby increasing iron absorption, its influence was not evaluated in this research. It has been clearly demonstrated in previous studies that *H. pylori* eradication can reverse the negative effect of *H. pylori* infection on iron absorption and lead to improvement of IDA in case series and in clinical trials in both children and adults^[4,15,16]. Successful *H. pylori* eradication resulted first in a significant post-treatment decrease of serum gastrin and *H. pylori* IgG antibody titers, and then an increase in the peripheral hemogram and SI. This normalization of iron metabolism in *H. pylori*-positive patients increased the MCV and MCH to a plateau similar to the normal level.

The *H. pylori* gastritis is increasingly considered a pos-

sible cause of IDA refractory to oral iron treatment, and eradication of *H pylori* may be followed by an improved response to oral iron in previously refractory IDA patients^[4,5]. In this study, *H pylori* infection slowed the trend of recovery from IDA recovery, but the indices in group B still reached normal levels. In our opinion, *H pylori* eradication is necessary for the resolution of IDA with a lower risk of recurrence. Patients with *H pylori* infection and IDA should receive oral iron and triple therapy simultaneously.

COMMENTS

Background

Iron deficiency anemia (IDA) is often caused by gastrointestinal bleeding due to peptic ulcer and chronic gastritis. *H pylori* infection, which has been proved to play the main role in peptic ulcer and chronic gastritis, has been implicated as a cause of IDA refractory to oral iron treatment. The role of *H pylori* and its eradication in IDA in patients with *H pylori*-positive gastritis has been unclear.

Research frontiers

In some cases, refractory IDA is not sensitive to oral iron treatment, especially in patients with *H pylori*-positive gastritis. It is important to elucidate the relation of IDA to *H pylori* infection and the effect of *H pylori* eradication on the treatment of IDA.

Related publications

Related publications are rare.

Innovations and breakthroughs

This study showed that the *H pylori* infection could slow the trend of recovery from IDA and that *H pylori* eradication is necessary for the resolution of IDA with a lower risk of recurrence. The efficacy of simultaneous oral iron and triple therapy was evaluated.

Applications

For the treatment of IDA in patients with *H pylori*-positive chronic gastritis, it is effective and necessary to eradicate *H pylori* infection, which can lead to satisfactory recovery of this category of IDA.

Terminology

H pylori infection: *H pylori* infection is the main cause of peptic ulcer and chronic gastritis. IDA: iron deficiency anemia is generally attributed to abnormal blood loss, which is often caused by gastrointestinal bleeding due to peptic ulcer and chronic gastritis, or in women of reproductive age by increased menstrual flow and the greater requirements of pregnancy.

Peer review

This is a good review on an interesting topic with an appropriate number of patients. The major point greatly enhances the conclusions.

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RAPID COMMUNICATION

Heme oxygenase-1 induction by hemin protects liver cells from ischemia/reperfusion injury in cirrhotic rats

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Abstract

AIM: To investigate the potential protective effect of HO-1 on cirrhotic liver cells in rats.

METHODS: Male Wistar rats included in the current study were randomly divided into 5 groups as follows: normal (N) group; liver cirrhotic (LC) group; sham (S) group; I/R group and I/R + hemin group. The model for inducing liver cirrhosis in rats was established according to a previously published protocol. Following this the segmental hepatic ischemia reperfusion operation was carried out. The rats were treated with 30 μ mol/kg hemin (HO-1 inducer, ferric protoporphyrin IX chloride) i.p. or 0.9% NaCl (control) 24 h and 12 h before hepatic ischemia for 30 min or sham laparotomy. Blood was collected for serum enzymatic measurement 6 and 12 h after reperfusion or sham laparotomy. HO-1, NF- κ B and caspase-3 expressions were assessed by immunohistochemical analysis.

RESULTS: The expressions of proteins are inversely correlated to the gray values. HO-1 expression in the I/R + hemin group was increased significantly than I/R group at 6 h and 12 h after hepatic I/R (6 h: 112.0 ± 8.3 vs 125.1 ± 5.7 , $P < 0.01$; 12 h: 120.8 ± 11.0 vs 132.4 ± 6.2 , $P < 0.01$). Hemin improved serum manganese superoxide dismutase (MnSOD) (6 h: 131.3 ± 17.6 vs 107.0 ± 13.9 , $P < 0.01$; 12 h: 141.4 ± 12.5 vs 118.3 ± 10.2 , $P < 0.01$), lessened liver cell injury, decreased caspase-3 (6 h: 166.7 ± 8.1 vs 145.5 ± 14.6 , $P < 0.01$; 12 h: 172.8 ± 3.8 vs 148.0 ± 6.5 , $P < 0.01$) and NF- κ B expression (6 h: 150.2 ± 8.6 vs 139.7 ± 6.0 , $P < 0.01$; 12 h: 151.1 ± 5.9 vs 148.1 ± 5.3 , $P > 0.05$) and serum alanine aminotransferase (ALT) (6 h: 413.3 ± 104.1 vs 626.8 ± 208.2 , $P < 0.01$; 12 h: 322.2 ± 98.8 vs 425.8 ± 115.4 , $P < 0.05$), aspartate aminotransferase (AST) (6 h: 665.2 ± 70.1 vs 864.3 ± 70.4 , $P < 0.01$; 12 h: 531.1 ± 98.6 vs 664.4 ± 115.6 , $P < 0.01$), malondialdehyde

(MDA) levels (6 h: 11.1 ± 2.17 vs 13.5 ± 2.01 , $P < 0.01$; 12 h: 9.36 ± 1.10 vs 10.8 ± 1.62 , $P < 0.05$) in the I/R + hemin group when compared with the I/R group.

CONCLUSION: These results suggest that HO-1 plays an important role in protecting liver cells from hepatic I/R injury in cirrhotic rats by decreasing oxidative stress, apoptosis and inflammation.

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Key words: Heme oxygenase-1; Ischemia reperfusion; Caspase-3; Nuclear factor- κ B; Liver cirrhosis

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INTRODUCTION

Gastrointestinal bleeding, liver surgery and liver transplantation may lead to hepatic ischemia/reperfusion (I/R) injury. Previous studies have shown that pro-inflammatory, apoptosis, oxidative stress and microcirculation dysfunction are strongly correlated with hepatic I/R^[1,2]. Many patients presenting with hepatic ischemia have liver cirrhosis disease. Some studies have shown that cells from a cirrhotic liver are more fragile than the cells from a normal liver. Similarly, other studies have found that the anti-oxidative capacity during stress decreases in patients with hepatic decompensated cirrhosis^[3,4], and further, the tolerance time of ischemia is shorter in cirrhotic rats than in normal rats^[5].

Heme oxygenases (HOs), the rate-limiting enzymes in heme catabolism, catalyzes the oxidative degradation of heme into carbon monoxide (CO), free iron, and biliverdin. Three isoforms of the enzyme have been identified: The inducible HO-1, also known as heat shock protein 32, the constitutive HO-2, and a not fully defined HO-3. It has been found that HO-1 serves as a protective gene by virtue of anti-inflammatory, anti-apoptotic and anti-oxidative actions and improves microcirculation in many cell types^[6,7].

Amersi *et al*^[8] reported that up-regulation of HO-1 protects genetically fat Zucker rat livers from I/R injury.

Some researchers have demonstrated that HO-1 has potent protecting effects against I/R injury in various organ systems^[9,10]. Kaizu *et al.*^[11] reported that prior induction of the HO-1 protein may lead to anti-inflammatory and anti-apoptotic effects on warm renal I/R injury.

HO-1 has been shown to protect liver cells from I/R injury^[12,13]. However, there are few reports regarding the protective effect of HO-1 on liver cells affected by the pathological condition of cirrhosis. Investigations into the role of HO-1 in protecting liver cells from hepatic I/R injury in cirrhotic rats and the mechanism by which it achieves this protective effect have great significance. Thus, the objective of the present study was to investigate whether upregulating HO-1 would result in reduced damage to liver cells as a result of hepatic I/R in a liver cirrhotic rat model. Secondly, we investigated the possible mechanism/s by which HO-1 exerts its protective effect.

MATERIALS AND METHODS

Rat cirrhosis and grouping

Adult male Wistar rats (weighting 200–250 g) were obtained from the experimental Animal Center of Xi'an Jiaotong University. Ethical approval for this study was obtained from the Ethical Committee on Animal Experiments at the Medical College of Xi'an Jiaotong University. The rats had free access to food and water, were kept in an air-conditioned room at 23°C, with a 12 h/12 h light/dark cycle, and were handled humanely. Liver cirrhosis was induced by subcutaneous injection of 400 mL/L CCl₄-olive oil solution twice a week at an initial dose of 5 mL/kg. Subsequent doses were adjusted to body mass changes at a dose of 3 mL/kg for 11 wk as described previously^[14,15]. The rats in the normal group received the same dose of pure olive oil only. The I/R operation was carried out in wk 12.

All 91 animals were randomly divided into 5 groups as follows: Normal (N) group; liver cirrhotic (LC) group; sham (S) group (cirrhotic group operated); I/R group and I/R + hemin group. The last three groups were divided into two subgroups for sampling at different time-points. The I/R + hemin group was administered 30 µmol/kg hemin^[16] (HO-1 inducer, Sigma chemical Co., USA) 24 h and 12 h before hepatic I/R. Hemin solution was prepared under subdued lighting by dissolving the compound in 1 mL of 0.2 mol/L NaOH, adjusting the pH to 7.4 with 1 mol/L HCl, and diluting the solution with 9 g/L NaCl^[11]. The stock concentration was 5 g/L. The solution was kept in the dark and used within 1 h. The concentration dose of hemin used in the present study was selected according to previous reports.

Hepatic I/R model

Rats were anaesthetized using ketamine hydrochloride (800 mg/kg) intraperitoneally. The laparotomy was performed through a midline abdominal incision. Partial (70%) hepatic ischemia of the median and the left lobes was induced by placing non-crushing microvascular clamps around the appropriate branches of the portal vein and hepatic artery for 30 min. The portal branches to the right

and caudate lobes were left open to prevent mesenteric venous congestion^[17]. Reperfusion was initiated by removal of the clamps. Hepatic I/R injury was induced by 30 min of ischemia followed by 6 h and 12 h of reperfusion. After 30 min of ischemia, the clamps were removed allowing the liver to reperfuse, and the wound was closed with 3-0 silk. Sham operated rats underwent isolation of the portal vein and hepatic artery without occlusions. At the indicated time after the start of reperfusion, rats were anesthetized and sacrificed. Blood was collected from the portal vein and liver samples were taken from the left lobe. The blood samples were centrifuged to obtain the serum for the biochemical analyses, and the left lobe liver tissue samples (0.8 cm × 0.4 cm × 0.4 cm) were fixed in 40 g/L paraformaldehyde for immunohistochemical analysis.

Immunohistochemical analysis of HO-1, NF-κB and caspase-3 expression

Serial 5 µmol/L sections were prepared after the samples had been dehydrated in graded ethanol solutions, cleared in chloroform and embedded in paraffin. Immunohistochemical staining was performed to detect expression of NF-κB (diluted 1:300; Boster, China), caspase-3 (diluted 1:100; Boster, China), and HO-1 (diluted 1:100; Boster, China) using the MaxVision™ kit-5004 (Maxin Biotechnology Co., Fujian, China). Sections were boiled in 0.01 mol/L citrate buffer (pH 6.0) to retrieve the antigens. Endogenous peroxidases were inactivated by immersing the sections in 30 mL/L hydrogen peroxide for 20 min. The sections were then incubated overnight at 4°C with the relevant primary rabbit anti-rat polyclonal antibodies. After washing, the sections were overlaid with peroxidase-conjugated goat anti-rabbit secondary antibodies (MaxVision™ kit-5004, Maxin Biotechnology Co., Fujian, China) for 15 min. The chromogenic reaction was developed with diaminobenzidine. Some sections were counterstained with hematoxylin. The immunohistochemical signal was analyzed using an image acquiring and analysis system (QWin500CW, Leica, Germany). At least four random fields of each section were examined at a magnification of × 400, and analyzed using a computer image analysis system (Image-Pro Plus, Media Cybernetics, Silver Spring, MD). The expressions of proteins are inversely correlated to the gray values.

Serum enzyme analysis

For the assessment of hepatic injury, serum levels of aspartate transaminase (AST) and alanine transaminase (ALT) activities were measured using an automatic biochemical analyzer (Vitros 250, Johnson & Johnson Co., USA). Serum Malondialdehyde (MDA) and superoxide dismutase (SOD) activity were measured with a commercially available kit (Nanjing Jiancheng Bioengineering Institute, China, NJBI). Total SOD and Cu/Zn-SOD activities were measured. Mn-SOD activity was calculated as the difference between total SOD and Cu/Zn-SOD activity.

Statistical analysis

All results were expressed as mean ± SD. The statistical analysis was performed using a one-way analysis of variance. The results were considered statistically significant if $P < 0.05$.

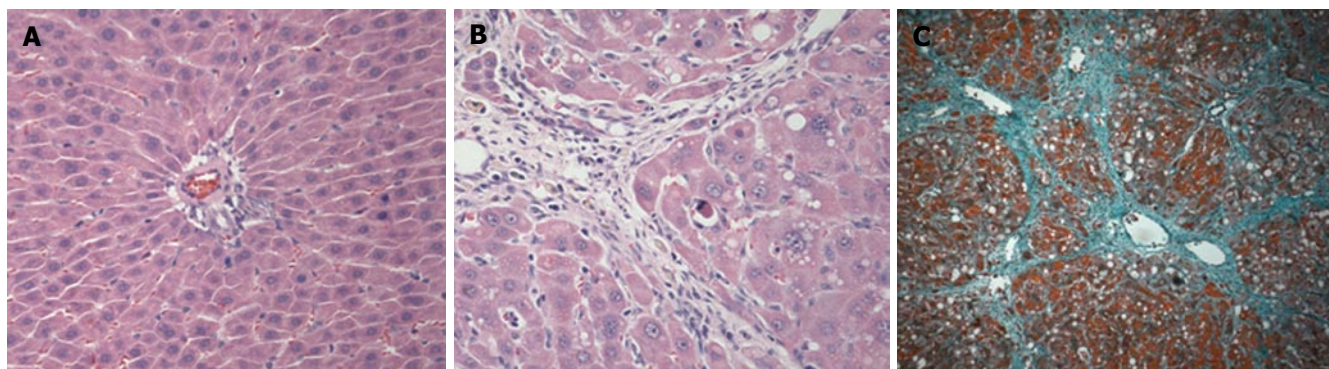


Figure 1 A: No evidence of collagen deposition was observed in the liver of the normal group (HE, × 400); B: The collagens deposited in portal areas of liver cirrhosis group (HE, × 400); C: Severe fibrosis with regenerating nodules was observed in liver cirrhosis group (Masson, × 100).

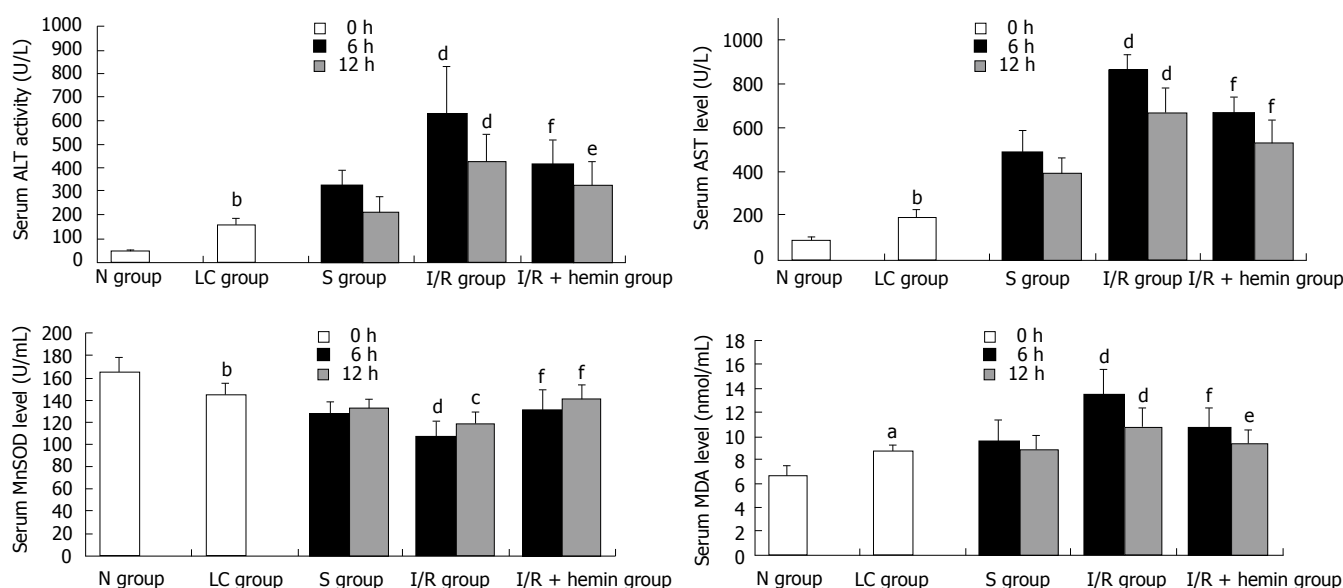


Figure 2 Serum ALT, AST activity, MnSOD and MDA levels of each group. N group: normal group; LC group: liver cirrhosis group; S group: sham group; I/R group: the rats underwent hepatic ischemia/reperfusion operation; I/R + hemin group: the rats were administered with 30 μ mol/kg hemin before hepatic ischemia/reperfusion operation. 6 h: 6 h after reperfusion. 12 h: 12 h after reperfusion. After hepatic ischemia/reperfusion, the ALT, AST and MDA levels were increased and the levels were all decreased after hemin administration. The level of MnSOD was low in I/R group, and after giving hemin, its level in I/R + hemin group was higher than I/R group. Values are mean \pm SD. ^b $P < 0.01$ vs N group, ^c $P < 0.05$ vs N group, ^d $P < 0.01$ vs S group, ^e $P < 0.05$ vs S group, ^f $P < 0.01$ vs I/R group, ^g $P < 0.05$ vs I/R group.

Table 1 Gray values of caspase-3, NF- κ B and HO-1 of rats (mean \pm SD)

Group	n	NF- κ B	Caspase-3	HO-1
Normal	6	172.0 \pm 1.7	188.3 \pm 3.5	158.9 \pm 2.6
Liver cirrhosis	10	164.1 \pm 3.5 ^b	176.8 \pm 4.9 ^b	148.1 \pm 3.7 ^b
Sham	6 h	159.4 \pm 4.7 ^d	177.2 \pm 2.7 ^d	145.4 \pm 2.9 ^d
	12 h	159.6 \pm 4.2 ^d	178.5 \pm 3.9 ^d	146.8 \pm 3.8 ^d
I/R	6 h	139.7 \pm 6.0	145.5 \pm 14.6	125.1 \pm 5.7
	12 h	148.1 \pm 5.3	148.0 \pm 6.5	132.4 \pm 6.2
I/R+hemin	6 h	150.2 \pm 8.6 ^d	166.7 \pm 8.1 ^d	112.0 \pm 8.3 ^d
	12 h	151.1 \pm 5.9 ^c	172.8 \pm 3.8 ^d	120.8 \pm 11.0 ^d

^b $P < 0.01$ vs N group; ^c $P > 0.05$ vs I/R group; ^d $P < 0.01$ vs I/R group.

RESULTS

Changes in the CCl₄ induced liver cirrhotic rats

To confirm the occurrence of liver cirrhosis, the liver tissues were examined following hematoxylin and eosin

staining (HE staining) and Masson's trichrome staining (Figure 1). Biochemical analyses of the serum and immunohistochemical analysis of the liver samples were also performed. The levels of serum ALT and AST activities are markers for hepatic injury, MDA and MnSOD reflect the anti-oxidative ability. Following the administration of CCl₄ for 11 wk, the activities of ALT, AST and MDA in the LC group were increased when compared with those observed in the N group. The MnSOD in the LC group was decreased when compared with the N group (Figure 2).

Hemin induced HO-1 high expression in liver tissue

Animals in the I/R + Hemin group were given hemin prior to the induction of I/R. We examined the changes in HO-1 expression in liver tissues after I/R using the gray value method. HO-1 expression in the I/R + hemin group was increased significantly, especially 6 h after reperfusion (Table 1 and Figure 3). Hepatic I/R resulted in an increase in serum ALT, AST and MDA levels in the I/R group

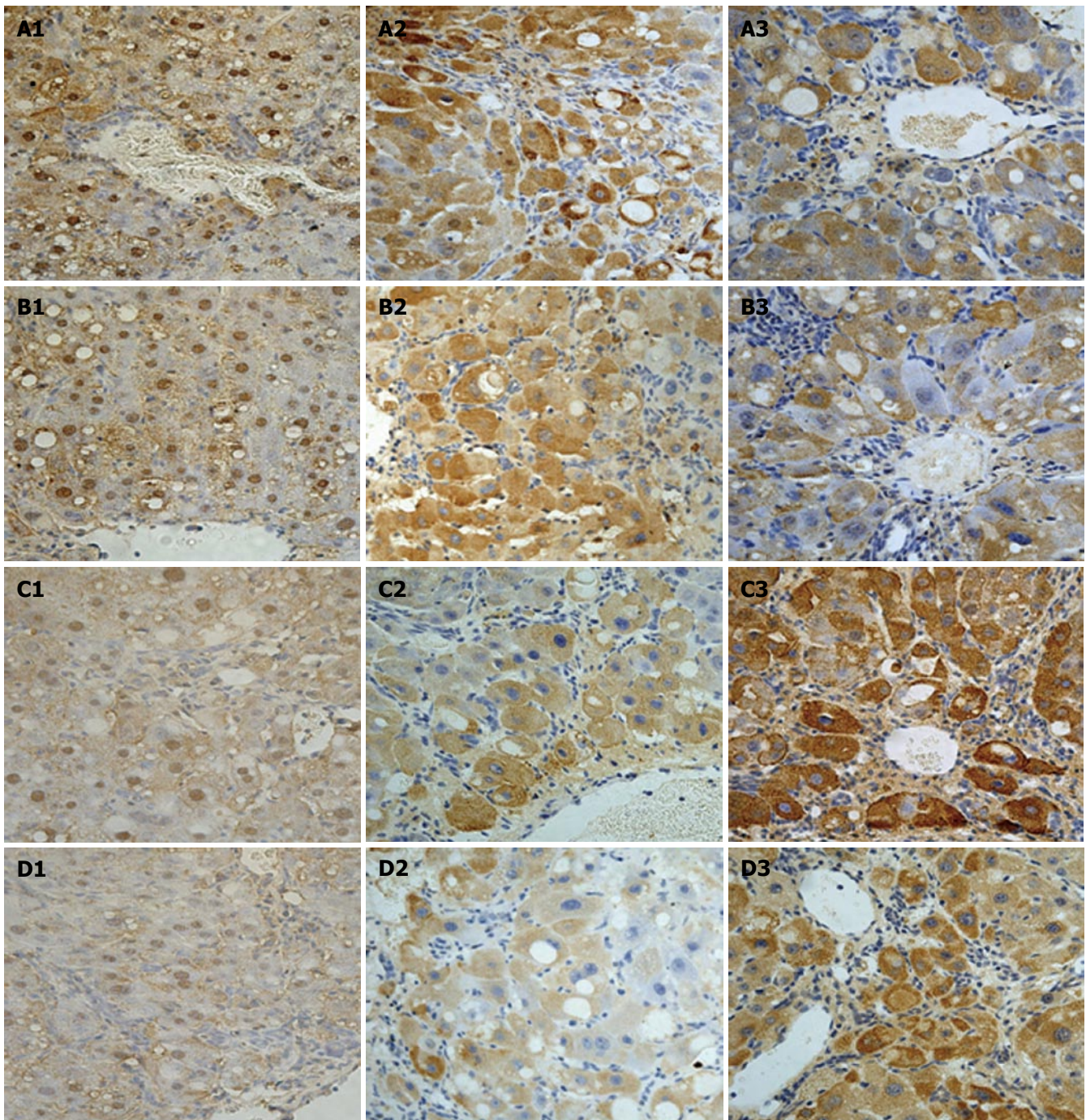


Figure 3 Immunohistochemistry of NF- κ B, caspase-3 and HO-1 at 6 h and 12 h after hepatic ischemia/reperfusion in cirrhotic rats. **A:** I/R group, 6 h after reperfusion; **B:** I/R group, 12 h after reperfusion; **C:** I/R + hemin group, 6 h after reperfusion; **D:** I/R + hemin group, 12 h after reperfusion; 1: NF- κ B: Most positive NF- κ B expression was in the nuclear of liver cells. After giving hemin to induce HO-1, the positive expression of NF- κ B in I/R + hemin group was decreased compared with I/R group at 6 h after reperfusion ($P < 0.01$). There is no difference between I/R group and I/R + hemin group at 12 h after reperfusion ($P > 0.05$) (Figure A1-D1); 2: Caspase-3: The positive caspase-3 expression was in the cytoplasm of hepatic cells. After hemin administration, the caspase-3 expression was decreased at 6 h and 12 h after reperfusion when compared with I/R group ($P < 0.01$) (Figure A2-D2); 3: HO-1: The positive HO-1 expression was in the plasma of hepatic cells. After hepatic ischemia/reperfusion, the expression of HO-1 was increased in I/R group, and through giving hemin, the expression of HO-1 was higher than I/R group at 6 h and 12 h after reperfusion ($P < 0.01$) (Figure A3-D3).

compared with the S group, especially at 6 h after reperfusion. In contrast, the I/R + hemin group had significantly lower serum ALT, AST and MDA levels at both time-points relative to the I/R group (Figure 2). MnSOD levels were markedly decreased in the I/R group at 6 h and 12 h after hepatic I/R compared with that of the S group. However, hemin administration increased MnSOD levels in the I/R+hemin group at both time-points (Figure 2).

Caspase-3 and NF- κ B immunohistochemical staining

To examine the protective effects of elevated HO-1 expression on apoptosis and inflammation in the liver following hepatic I/R, caspase-3 and NF- κ B expression in the liver were assessed by immunohistochemical staining. Most positive NF- κ B expression was observed in the nucleus of liver cells, while caspase-3 expression was localized in the cytoplasm of hepatocytes. After hepatic I/R, the expres-

sion of NF- κ B and caspase-3 was significantly stronger with a lower gray value in the I/R group compared with the S group ($P < 0.01$). After giving hemin to upregulate HO-1, caspase-3 expression in the I/R + hemin group was decreased 6 h and 12 h after reperfusion and the gray values were increased ($P < 0.01$). Following upregulation of HO-1, the expression of NF- κ B in the I/R + hemin group was lower 6 h after reperfusion than in the I/R group ($P < 0.01$), but there was no difference 12 h after reperfusion between the two groups ($P > 0.05$, Figure 3).

DISCUSSION

In this study, we have shown that HO-1 upregulation following hemin administration protects cirrhotic rats from hepatic I/R injury. Hepatic injury induced by I/R has been proposed as a key clinical problem associated with liver upper gastrointestinal bleeding, liver surgery or liver transplantation. There are two distinct phases of liver injury after I/R. The initial phase (< 2 h after reperfusion) is characterized by oxidant stress, where production and release of reactive oxygen species (ROS) appears to directly result in hepatocellular injury. The late phase of liver injury, from 6 h to 48 h after hepatic reperfusion, is an inflammatory disorder mediated by recruited neutrophils^[18-20]. Our results regarding the mechanism leading to I/R injury will undoubtedly provide important clues for developing an approach to decreasing liver cell damage caused by I/R. Protection against hepatic I/R injury is, clinically, one of the most critical problems in liver cirrhotic patients.

Recently, it was demonstrated that HO-1 upregulation ameliorates hepatic I/R injury^[12,13]. In addition, Coito *et al*^[21] reported that HO-1 gene transfer inhibits inducible nitric oxide synthase expression and protects genetically fat Zucker rat livers from I/R injury. In the present study, our data support the hypothesis that HO-1 provides a protective effect against I/R injury in cirrhotic rats via anti-oxidative, anti-apoptotic and anti-inflammatory actions. Our results also highlight that administration of hemin may be an important new therapy for the treatment of hepatic I/R. Although these mechanisms are complicated and largely unknown, our findings suggest the possibility that upregulation of HO-1 may reduce the potential risk in hepatocellular I/R complicated with liver cirrhosis.

HO-1 is one of the most critical of the cytoprotective mechanisms activated during cellular stress, exerting anti-oxidative and anti-inflammatory functions, modulating the cell cycle, and maintaining the microcirculation. The up-regulation of HO-1 is thought to be a protective response from cellular stress following ischemia, inflammation and radiation. Given the multi-factorial cytoprotective properties of HO-1, it is used as a novel strategy to prevent I/R injury^[22]. In line with this, we have found that hemin administration significantly protects the cells in the hepatic I/R as compared with control treatment.

The role of SOD in hepatic I/R has received more attention. There are three isoforms of SOD. The Cu/Zn-SOD is located in the cytosol, the MnSOD is primarily a mitochondrial enzyme, and extracellular SOD is usually found on the outside of the plasma membrane interacting with matrix component. Marczin *et al*^[23] reported that, by

utilizing adenoviral gene transfer, overexpression of Mn-SOD may reduce the extent of *in vivo* regional I/R injury in the rat heart. Similarly, in the current study we found that MnSOD levels were increased, and the injury due to I/R was lessened, after giving hemin to induce HO-1 expression. Based on this, we suggest that HO-1 has an anti-oxidant function during hepatic I/R.

MDA is the main product of lipid peroxidation, and is reactive with thiobarbituric acid (TBA). MDA concentration is therefore generally presented as the total level of lipid peroxidation products^[24]. As the end product of lipid peroxidation MDA can produce ozone, which reacts rapidly with cellular structures, generates hydrogen peroxide and other active oxygen species and causes peroxidation and denaturation of membranes^[25]. Our findings suggest that HO-1, induced by hemin administration, decreases the serum MDA level after hepatic I/R. Therefore, we speculate that high HO-1 expression induced by hemin may decrease the lipid peroxidation and play an anti-oxidative role in hepatic I/R.

Apoptosis plays a vital role in lethal hepatic I/R injury as indicated by many studies in various animal models^[2,26]. There are two major pathways of apoptosis, and caspase-3 is the final executor of apoptosis^[27]. Although the TUNEL assay is a very sensitive and widely used method^[28,29], data is also available suggesting that caspase-3 immunohistochemistry is an easy, sensitive, and reliable method for detecting and qualifying apoptosis in tissue sections^[30]. In the present study, to determine the effect of HO-1 on apoptosis, we carried out an immunohistochemical analysis of caspase-3. The expression of caspase-3 was elevated following hepatic I/R. However, caspase-3 expression was lower in the I/R + hemin group than in the I/R group. These data suggest that the increase in HO-1 following treatment with hemin results in a decrease in apoptosis during hepatic I/R in liver cirrhotic rats. Our findings in this regard are consistent with other studies^[31].

NF- κ B is activated during I/R of the liver, and plays an important and complex role in the gene expression of proinflammatory cytokines (TNF- α and IL-1), chemokines and adhesion molecules (ICAM-1 and VCAM-1), which will lead to the tissue injury^[32]. In the present study, the level of NF- κ B at 6 h after reperfusion was decreased in the I/R + hemin group compared with the I/R group. However, there was no statistical difference 12 h after reperfusion between the I/R group and the I/R + hemin group. We believe that high levels of HO-1 expression may decrease NF- κ B expression to lessen the inflammatory reaction at 6 h after reperfusion.

Hepatic I/R injury may cause more severe tissue damage in cirrhotic liver than in normal livers. Our data show that HO-1 overexpression, induced by hemin, plays a crucial role in protecting liver cells from hepatic I/R injury in cirrhotic rats. Our results also raise the possibility of a new treatment for reducing hepatic I/R injury in the pathological condition of cirrhosis.

COMMENTS

Background

Gastrointestinal bleeding, liver surgery and liver transplantation may lead to

hepatic ischemia/reperfusion (I/R) injury. Previous studies have shown that pro-inflammatory, apoptosis, oxidative stress and microcirculation dysfunction are strongly correlated with hepatic I/R. Many patients presenting with hepatic ischemia have liver cirrhosis disease. Some studies have shown that cells from a cirrhotic liver are more fragile than the cells from a normal liver. Similarly, other studies have found that the anti-oxidative capacity during stress decreases in patients with hepatic decompensated cirrhosis, and further, the tolerance time of ischemia is shorter in cirrhotic rats than in normal rats.

Research frontiers

Three isoforms of the Heme oxygenases (HOs) have been identified: The inducible HO-1, also known as heat shock protein 32, the constitutive HO-2, and a not fully defined HO-3. It has been found that HO-1 serves as a protective gene by virtue of anti-inflammatory, anti-apoptotic and anti-oxidative actions and improves microcirculation in many cell types.

Related publications

Researchers have demonstrated that HO-1 has potent protecting effects against I/R injury in various organ systems. It has also been reported that upregulation of HO-1 protects genetically fat Zucker rat livers from I/R injury and that prior induction of the HO-1 protein may lead to anti-inflammatory and anti-apoptotic effects on warm renal I/R injury. HO-1 has been shown to protect liver cells from I/R injury. Investigations into the role of HO-1 in protecting liver cells from hepatic I/R injury in cirrhotic rats and the mechanism by which it achieves this protective effect have great significance.

Innovations and breakthroughs

There are few reports regarding the protective effect of HO-1 on liver cells affected by the pathological condition of cirrhosis. The objective of the present study was to investigate whether upregulation of HO-1 would lessen the liver cell damage caused by hepatic I/R in a liver cirrhotic rat model and, secondly, to study the possible mechanisms for its protective effect. Our data support the hypothesis that HO-1 has a protective effect from I/R injury in cirrhotic rats. Our data further suggest that this effect is mediated by the anti-oxidative, anti-apoptotic and anti-inflammatory actions of HO-1, and highlight that administration of hemin may be an important new therapy method in hepatic I/R. Although many of these mechanisms are still unknown, our findings suggest the possibility that upregulation of HO-1 may reduce the potential risk in hepatocellular I/R complicated with liver cirrhosis.

Applications

Hepatic I/R injury may cause more severe tissue damage in a cirrhotic liver than in a normal liver. Our data have shown that HO-1 over-expression, induced by hemin, plays a crucial role in protecting liver cells from hepatic I/R injury in cirrhotic rats, and raises the possibility of a new treatment in hepatic I/R injury in the pathological condition of cirrhosis.

Terminology

Heme oxygenases (HOs): Heme oxygenases (HOs) are the rate-limiting enzymes in heme catabolism. HOs catalyse the oxidative degradation of heme into carbon monoxide (CO), free iron, and biliverdin. Three isoforms of the enzyme have been identified: HO-1, HO-2 and HO-3. Superoxide dismutase (SOD): Superoxide dismutase (SOD) is a ubiquitous enzyme with an essential function in protecting cells against oxidative stress by catalytic removal of superoxide radicals and conversion to H₂O₂ by the dismutation reaction.

Peer review

In this manuscript, Hui Xu *et al* have analyzed the beneficial effects of hemin-dependent induction of hepatic heme-oxygenase-1 expression in cirrhotic livers after ischemia/reperfusion which is an interesting paper.

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Sequential stenotic strictures of the small bowel leading to obstruction

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Abstract

Small bowel obstructions (SBOs) are primarily caused by adhesions, hernias, neoplasms, or inflammatory strictures. Intraluminal strictures are an uncommon cause of SBO. This report describes our findings in a unique case of sequential, stenotic intraluminal strictures of the small intestine, discusses the differential diagnosis of intraluminal intestinal strictures, and reviews the literature regarding intraluminal pathology.

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Key words: Small bowel obstruction; Stenosis; Stricture

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INTRODUCTION

Small bowel obstructions (SBOs) are most commonly caused by adhesions, hernias, neoplasms, or inflammatory strictures^[1], with most caused by extraluminal adhesions due to postoperative inflammatory changes^[2]. Intraluminal strictures are less common and are primarily caused by congenital defects, inflammation, ischemia, neoplasms, or radiation. We present an unusual case of sequential, stenotic strictures of the small intestine with no clearly defined etiology.

CASE REPORT

A 43-year-old Hispanic female presented to a surgical service for evaluation of SBO. Four days prior to presentation she developed nausea and mid-epigastric, cramping abdominal pain. She had not been able to tolerate liquids or solids for 2 d.

She had a 22-year history of chronic abdominal pain. An outpatient work-up involving an abdominal computed tomography (CT) scan the previous year revealed dilated loops of small intestine, with areas of diffuse thickening of the intestinal wall, but no evidence of obstruction. An esophagogastroduodenoscopy revealed gastritis but no major pathology. Stool cultures sent for *Clostridium difficile* toxin, ova, and parasites (Iodine and trichrome staining for worms (i.e., *Ascaris*, *Necator*, *Enterobius*, *Taenia*), amoeba (i.e., *Entamoeba*), flagellates (i.e., *Giardia*), cryptosporidium, and others) were negative. Her surgical history included bilateral tubal ligation, open appendectomy, and laparoscopic cholecystectomy. She had no personal or family history of trauma, cancer, or inflammatory bowel disease.

The patient was awake and alert with stable vital signs. Her abdomen was soft and mildly distended, with mid-epigastric tenderness upon palpation. She had no peritoneal signs. Her laboratory test values were all within normal limits.

A CT scan of the abdomen revealed a proximal SBO with dilated loops of small intestine and a transition point in the mid jejunum. Distal to the transition point, the intestine was thickened for several centimeters (Figure 1). Distal to the thickened portion, the intestine was normal in appearance. The terminal ileum and colon were normal, and no retroperitoneal lymphadenopathy was present.

On exploratory laparotomy, we found a thickened, dilated proximal small intestine with a clear transition point to the decompressed distal jejunum. The intestine was pink and viable. No extraluminal bands, adhesions or hernias were present. The duodenum and ligament of trietz appeared normal. Distal to the duodenum, the jejunum became thickened and dilated. We found deposits of mesenteric fat on the serosal edge of the intestine every 5-10 cm. Associated with these areas of mesenteric fat were palpable intraluminal stenoses. The rest of the intestine was normal in appearance. There were no intestinal or mesenteric masses. Strong pulses were palpated in all of the mesenteric vessels. We resected the thickened portion of jejunum and mesentery, and performed a jejunojejunum anastomosis.



Figure 1 CT scan of the abdomen. A CT scan of the abdomen revealed an SBO with dilation of small-intestinal loops, and a transition point in the mid-jejunum. A distended small was observed anteriorly of the air fluid level, and distal to this, a dilated loop and cross section was filled with feces, the so-called small bowel feces sign, which is indicative of SBO. Distal to the obstruction, there was a clear transition point in the mid-jejunum.

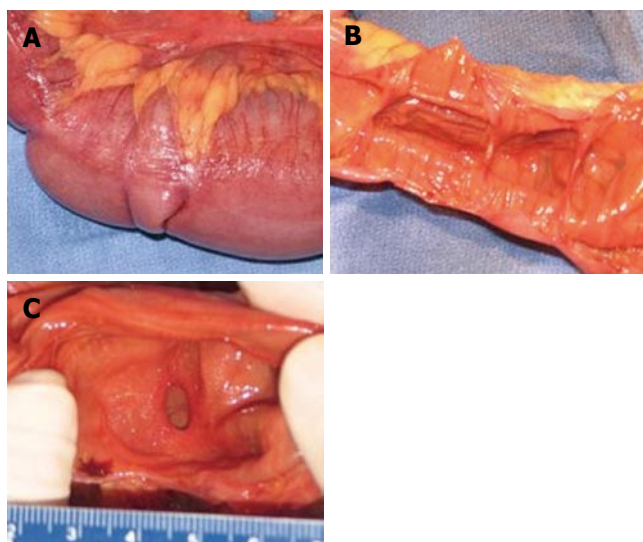


Figure 2 Gross specimens. Seventy-six centimeter segment of small intestine. On gross examination, fat surrounded the serosal surface of the intestine at irregular intervals, at the mesenteric border (A). Associated with these deposits of fat were 14 areas of mucosal stricture. In the normal intestine, the luminal diameter ranged from 4.7 to 9.5 cm. In the areas of luminal constriction, the diameter was 0.7 to 1.0 cm (B, C).

A 76-cm segment of small intestine was resected. On gross examination, fat was seen surrounding the serosal surface of the intestine at irregular intervals at the mesenteric border (Figure 2A). Associated with these deposits of fat were 14 areas of mucosal stricture causing luminal constriction (Figure 2B and C). The lumen between the constrictions was dilated and showed mucosal thinning. In the mesentery, four benign reactive perienteric lymph nodes were present.

On microscopic examination, the intestinal strictures contained mucosa and submucosa. Muscularis propria was not present within the webs (Figure 3). There was no evidence of inflammatory bowel disease, malignancy, or pathologic inflammation of the bowel or mesentery.

Postoperatively, bowel function was slow to return to

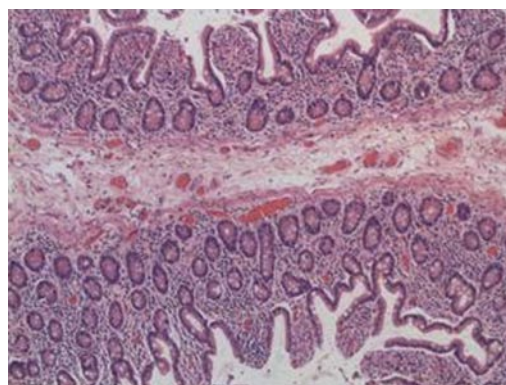


Figure 3 Microscopic image. On microscopic examination, the intestinal webs contained mucosa and submucosa. Muscularis propria was not present within the webs. There was no evidence of inflammatory bowel disease, malignancy, or pathologic inflammation of the bowel or mesentery.

normal. The patient had minimal bowel sounds and did not pass flatus until postoperative day (POD) 8. After POD 8, the patient progressed quickly; first, she tolerated a clear liquid diet and then a regular diet. On POD 10, the patient had an upper gastrointestinal barium study with a small intestine follow through, which showed normal motility, prompt emptying through the anastomosis, and barium reached the cecum within an hour. She was discharged on POD 10 and had no postoperative complications.

DISCUSSION

SBOs are most commonly caused by adhesions, hernias, neoplasms, or inflammatory strictures^[1], with most caused by extraluminal adhesions due to postoperative inflammatory changes^[2]. Although our patient had a history of abdominal surgery, she had no evidence of adhesions or extraluminal pathology. Her SBO was secondary to intraluminal strictures.

Intraluminal strictures are primarily caused by congenital defects, inflammation, ischemia, neoplasms or radiation. Congenital atresia is a common cause of neonatal intestinal obstruction. Jejunoileal atresia primarily results from intrauterine vascular events and tends to present early in life^[3]. Late presentation of jejunal atresia has been reported; however, this is associated with other abnormalities^[4]. It is unlikely that our patient had such a condition that had remained undiagnosed since birth.

Inflammatory bowel diseases, such as Crohn's disease, may also cause intestinal strictures. Crohn's disease presents with full thickness inflammation of the intestinal wall. Obstruction occurs through either acute inflammation leading to occlusion, or through chronic inflammation leading to strictures. Our patient had no clinical or pathologic evidence of acute or chronic inflammatory bowel disease. Chronic inflammatory bowel disease strictures also tend to be longitudinal and localized to the ileum^[5,6]; whereas our patient's strictures were distinct, concentric jejunal bands. Intestinal ischemia may also cause intestinal strictures and usually occurs in the presence of trauma, drug use, or vascular disease. The patient had no history of vascular disease or abdominal

trauma, and reported no history of postprandial abdominal pain or fecal blood, which might indicate chronic intestinal ischemia. Cases of intestinal ischemia usually reveal some degree of mucosal ulceration and necrosis, with associated intestinal fibrosis and vascular disease^[7]. A Medline search revealed no previous cases of sequential, stenotic strictures of the small intestine.

Intraoperatively, the intestine was viable and the mesenteric vessels had strong pulses. On pathologic examination, the patient had no evidence of mucosal irritation, intestinal fibrosis, or vascular pathology.

Malignancies may be another cause of intraluminal strictures. Benign tumors are the most common (i.e., leiomyomas); however, malignant lesions are more likely to have a symptomatic presentation. Melanoma is the most common metastatic malignancy of the small intestine and adenocarcinoma is the most common primary malignancy^[8]. Additionally, radiation therapy used to treat intraabdominal masses may cause intestinal webs or scarring^[9]. However, the patient had no history of malignancy or radiation therapy, and the pathology revealed no evidence of malignancy.

Chronic nonsteroidal anti-inflammatory (NSAID) usage may also cause intraluminal pathology^[10]. With newer imaging modalities such as capsule endoscopy, the small intestine is increasingly being recognized as a site of NSAID-induced toxicity. Mucosal damage is primarily mediated by a cyclooxygenase-independent mechanism, but may also be caused by cyclooxygenase-dependent mechanisms^[10]. NSAID usage may cause either longitudinal or concentric strictures; however, our patient had no history of chronic NSAID usage, and there was no pathologic evidence of mucosal ulceration or inflammation.

Other possible sources of intestinal stricture are parasites (e.g., *Ascaris*) or bacteria (e.g., *Mycobacterium tuberculosis*); however, the patient had no evidence of bacterial or parasitic infection.

In conclusion, in this report, we describe our finding

of sequential, stenotic intraluminal strictures of the small intestine, which led to SBO. The unusual sequential, concentric nature of the strictures, along with the lack of evidence of inflammatory bowel disease, vascular disease, or malignancy is believed to be unique. This lack of a clearly defined etiology of the patient's strictures made this an intriguing case.

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CASE REPORT

Occasional finding of mesenteric lipodystrophy during laparoscopy: A difficult diagnosis

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Abstract

Mesenteric lipodystrophy is a rare pathological condition affecting the mesentery. Its initial presentation is typically asymptomatic. Pathological characteristics are unspecific, and generally attributed to inflammation, unless the diagnosis is suspected. Laparoscopy done for other reasons has been, as in this case, unsuccessful in providing evidence for the correct diagnosis, thus requiring laparotomy due to lack of diagnostic tissue. After 6 mo no further medical therapy is required, as the patient remains asymptomatic. Discussion of this case and a brief review of the literature are presented in the following paragraphs.

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Key words: Mesenteritis; Lipodystrophy; Laparoscopy; Panniculitis; Sclerosing mesenteritis

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INTRODUCTION

Mesenteric lipodystrophy is an uncommon diagnosis with few, if any, symptoms at presentation. Only 200 cases have been reported in the world literature^[1]. These cases mirror the presentation and symptoms of this case. Therefore, the diagnosis requires the astute clinical

suspicion of the surgeon, radiologist, and pathologist, as it may resemble other diseases which require different therapeutic approaches. Diagnosis is usually achieved through laparotomy performed for a mesenteric mass with lipomatous characteristics. The first report is attributed to Jura^[2], an Italian surgeon, who described a condition of "retractile sclerosing mesenteritis" in 1924. The term was reprinted by Durst^[3], who reviews 68 cases previously described in the literature and defines it as a benign disease. Today most articles describe the pathologic condition as "sclerosing mesenteritis", and divide it into three major phases, each regarding a progressively worsening state. The pathologic findings begin with fat necrosis and end with regenerative fibrosis. Within this progress of disease state, fat necrosis predominates in the first step, therefore called mesenteric lipodystrophy. This is then followed by mesenteric panniculitis, characterized by intense inflammatory reaction, and lastly by retractile mesenteritis, when fibrosis becomes the main feature.

CASE REPORT

An obese (body mass index = 41), 56-year old Caucasian man underwent laparoscopy for the repair of an umbilical hernia (> 3 cm). On exploration chylous ascites was noted in the pouch of Douglas and taken for examination. Because of the patient's obesity no mass or other abnormalities were noted, with the exception of cerulean stains on the surface of the mesentery, at first attributed to an unknown previous pancreatitis. A laparoscopic repair was performed, using a polytetrafluoroethylene (PTFE) mesh. The recovery was uneventful with dismissal on post operative day five. The patient did have chylous drainage in the first two days which then ceased by dismissal. Cytological and chemical examination of the fluid was negative for malignant cells, even though mononuclear inflammatory cells with enlarged cytoplasm were noted. Abdominal ultrasound and blood chemistry were normal, comprising amylase, lipase, lipidic asset, carcino-embryonal antigen (CEA) and Ca 19-9. After a month, a follow-up computed tomography (CT) scan (Figure 1) revealed hyperdensity of mesenteric fat, with regular margins, and radiological characteristics of a mesenteric lipoma (8 cm wide). Exploratory laparotomy was then performed to exclude definitely any malignancy. The mass was biopsied, however a complete removal was impossible due to its encasement of the superior mesenteric vessels

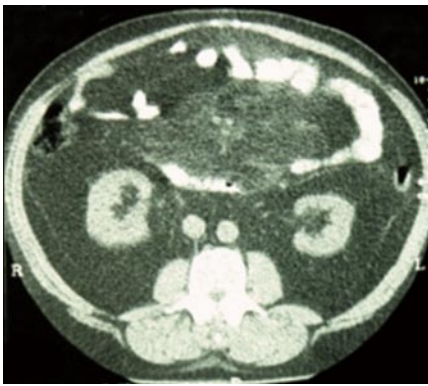


Figure 1 CT scan visualizing a mesenteric lipoma-like mass.

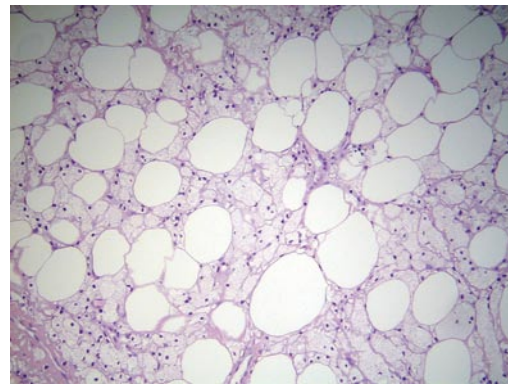


Figure 3 Mesenteric adipose tissue showing lipid necrosis and foamy macrophages (HE, x 10).



Figure 2 Intraoperative view of pseudonodular thickening of the mesentery.

(Figure 2). The ileum showed chronic ischemic condition with scars on the serosal surface. Macro-biopsies were performed which excluded neoplastic origin and diagnosed fatty necrosis, with scarce fibrotic component, adipose cells with foamy cytoplasm, infiltration of lipid-laden macrophages and foreign body giant cells (Figure 3). The patient was asymptomatic in the next six months, without any medical therapy, and did not complain of any digestive discomfort. The follow-up CT after three months was unchanged, except for a minimal reduction in the diameter of the mesenteric enlargement.

DISCUSSION

A nosological classification has been recently proposed^[4] for this rare pathology with unknown origin, defining under the word of "sclerosing mesenteritis", a disease previously described as mesenteric panniculitis, retractile mesenteritis, isolated lipodystrophy, mesenteric lipogranuloma, mesenteric manifestation of Weber-Christian disease, *etc.* Only three large series have been collected in Literature, one by Durst^[3], one by Kipfer^[5] and one more recently by Emory^[4] of 68, 53 and 42 cases, respectively. Still this disease is largely unknown to many surgeons and pathologists. It usually affects males (2-3:1), in the fifth or sixth decade of life (age range 20-80 years), and might take on different clinical aspects. In almost half of the cases (as in our patient) the mass is incidentally

discovered during abdominal surgery (i.e., during cholecystectomy). The remaining patients might complain of various degrees of intestinal obstruction, from post-prandial discomfort to acute occlusion. Other uncommon clinical findings may be fever, protein, losing enteropathy or abdominal mass. The biochemistry is mostly unhelpful in diagnosis. CT scan appearance varies from increased attenuation ("misty mesentery") to solid soft-tissue mass, which might envelope the mesenteric vessels preserving a fatty area around ("fat ring sign")^[6,7]. Other tumors of the mesentery may show a similar radiologic appearance, like lipoma, lymphoma, carcinoid tumor, carcinomatosis or tuberculosis, mesothelioma, edema or hematoma (from cirrhosis, trauma, hypoalbuminemia, heart failure or vasculitis)^[8]. Percutaneous needle aspiration or biopsy can give a hint to diagnosis, but generally laparotomy and surgical biopsy is required. More recently laparoscopic biopsy has been described^[9,10], but a pre-operation suspicion is mandatory, given, for example, by a CT scan. In our case, obesity (hiding the diffuse thickness of the mesentery) and the peritoneal aspect (mimicking that of a pancreatitis) misled laparoscopic diagnosis. A CT scan, performed after dismissal, led to laparotomy, in order to obtain a radical excision of the described mass. Intraoperative findings might be a diffuse thickening of the mesentery, a single tumor, multiple tumors or also a various mix of the nodular and hypertrophic components. Also the mesocolon may be involved, as well as, rarely, mesoappendix, peripancreatic area, omentum and pelvis. Its gross appearance explains how easy it is to mimic many other intra-abdominal neoplastic diseases. Histology displays different grades of involvement. Lipodystrophy is characterized by mesenteric infiltration by lipid-filled macrophages in the narrow septa inside the adipose tissue, as inflammation increases (panniculitis) lymphocytic infiltrate and lipid cystic necrosis can be noted. The last stage is distinguished by a diffuse presence of necrosis and fibrosis that contribute to tissue retraction as seen in retractile mesenteritis. Also calcifications and giant multinucleated cells have been seen in some cases. In the case presented, macrophage infiltration was the main feature, even if a component of fatty necrosis and fibrosis was evidenced. Haematoxylin and eosin stain

is generally enough to provide a correct diagnosis, but also immunochemistry might prove useful in some cases (between antibodies' panel nuclear beta-catenin has proved interesting)^[11]. The differential diagnosis comprises foreign body reaction (more fibrosis and fewer lipid laden macrophages) and Weber-Christian disease (predominantly localized in the subcutaneous tissue and with enhanced lymphocytic infiltrate)^[12]. Lipomatosis, lymphoma and retroperitoneal fibrosis have quite different microscopic features, and no fatty necrosis^[13]. Although the aetiology remains obscure, association with other diseases has been described. AIDS^[14], non-Hodgkin lymphoma^[15], tuberculosis^[1], cholesterol ester storage disease^[16] have been found together with sclerosing mesenteritis. Drug therapy is not standardized, and should be based on the stage of the disease. In the first stage, when fat necrosis is predominant, authors agree not to treat the disease as it can regress spontaneously. Chronic inflammation requires therapy based on corticosteroids and various types of immunosuppressants. Good results are reported with cyclophosphamide, colchicine, azathioprine and also with oral progesterone^[17]. As intense fibrosis appears, bowel obstruction may occur. Intestinal resections, bypasses or neostomy might be required^[18,19].

In summary, diagnosis of this unspecific, benign inflammatory disease is a challenge, both for the radiologist, surgeon and pathologist. Some authors^[20] suggest that this condition might be more common than reported. In fact, as Poniachik *et al*^[21] has supposed, the suspect of sclerosing mesenteritis has to be arisen in order to prevent such conditions to be catalogued as "unspecific inflammatory process". Since we also met the difficulty in diagnosis by laparoscopic means, especially for large masses in obese patients, we acknowledge that laparotomy is required to achieve complete removal or at least a macro-biopsy of the mass, as pathologic examination on frozen section might be incomplete, and radiology is unhelpful.

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Complications and treatment of migrated biliary endoprosthesis: A review of the literature

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Abstract

Endoscopic biliary stent insertion is a well-established procedure. It is especially successful in treating postoperative biliary leaks, and may prevent surgical intervention. A routine change of endoprosthesis after 3 mo is a common practice but this can be prolonged to 6 mo. We reported a colonic perforation due to biliary stent dislocation and migration to the rectosigmoid colon, and reviewed the literature.

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Key words: Biliary endoprosthesis; Migrated biliary stent; Colonic perforation; Biliary stent complications

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INTRODUCTION

Endoscopic biliary stent insertion is a well-established treatment for hepatic, biliary or pancreatic disorders (e.g., chronic pancreatitis or pancreatic carcinoma). It is especially successful in treating postoperative biliary leaks, and may prevent surgical intervention^[1,2]. Stenting of the biliary duct can be performed with a variety of prosthesis that can

differ by size, design and material^[3,4].

The available biliary endoprosthesis can be classified by material into two categories: plastic (i.e., polyethylene, polyurethane and "Teflon") and metallic stents. Plastic endoprosthesis are less expensive but have a higher risk of clogging and dislocation^[5]. On the other hand, they are easier to remove or to change.

A routine change of an endoprosthesis after 3 mo is common practice, but this can be prolonged to 6 mo^[6]. To avoid stent migration, the biliary stent should be placed across the sphincter of Oddi^[7]. Distal stent migration is an infrequent late complication, but occurs in up to 6% of cases^[3,8]. The majority of biliary endoprosthesis pass through the intestine without any problems. Infrequently, however, stents get stuck in the bowel, leading to complications.

Endoscopic retrieval is often possible and surgical intervention is rarely necessary^[9,10]. The most common site of a migrated biliary stent is the duodenum^[11-17], whereas complications in the rest of the small intestine^[18,19] or colon^[20-30] are rare.

CASE REPORT

A 65-year-old female patient presented with 4 d of persistent diffuse colicky abdominal pain and localized peritonitis in the supra-symphysial area. Normal leukocytes, elevated C-reactive protein (14.5 mg/dL; normal value < 0.5) and simultaneous cystitis (6500 bacteria/ μ L; normal value < 600) were found. Three months previously, the patient had undergone a laparoscopic cholecystectomy 4 wk after acute cholangitis with choledocholithiasis. The intraoperatively placed easy-flow drainage showed early postoperative biliary secretion. Endoscopic retrograde cholangiography was performed and showed a small peripheral leakage from the liver. A residual bile duct stone that was causing partial obstruction was removed. A 12 French 10 cm plastic stent was placed in the common bile duct without sphincterotomy. Biliary drainage stopped concurrently. The patient was discharged on the ninth postoperative day. A biliary stent extraction was advised at 4-6 wk later, but this was not carried out because of the patient's non-compliance.

A computed tomography (CT) scan was performed because of extensive pain. A biliary stent dislocation and migration to the rectosigmoid colon was detected. The CT scan showed rectal perforation, and the stent was found in

Table 1 Colonic perforation due to biliary stent migration. Review of the literature to January 2007

Reference	Year	Stent	Material	Type of bile duct lesion	Time to migration	Complication	Therapy
Anderson <i>et al</i> ^[28]	2006	Uk	Uk	Benign	5 mo	Sigmoid diverticula perforation	Endoscopy
Wilhelm <i>et al</i> ^[27]	2003	Straight	Uk	Benign	18 mo	Colovesicular fistula	Surgery
Diller <i>et al</i> ^[19]	2003	7 French, 10 cm	Teflon	Benign	1 mo	Stuck in sigmoid diverticula	Endoscopy
Elliott <i>et al</i> ^[21]	2003	10 French, 10 cm	Uk	Benign	4 mo	Sigmoid perforation	Surgery
		10 French, 10 cm					
Figueroa <i>et al</i> ^[29]	2001	Straight	Polyethylene	Benign	3 mo	Colocutaneous fistula	Surgery
Klein <i>et al</i> ^[26]	2001	Straight	Teflon	Benign	3 yr	Sigmoid diverticular perforation	Surgery
		7 French, 5 cm,					
Storkson <i>et al</i> ^[30]	2000	Straight	Plastic	Benign	2 wk	Sigmoid perforation	Surgery
Lenzo <i>et al</i> ^[22]	1998	7 French, 7, 5 cm	Polyethylene	Benign	1 mo	Sigmoid diverticula perforation	Surgery
Baty <i>et al</i> ^[20]	1996	Uk	Polyethylene	Malign	1 mo	Sigmoid diverticula perforation	Surgery
Schaafsma <i>et al</i> ^[23]	1996	Straight	Uk	Benign	6 mo	Sigmoid perforation	Surgery
D'Costa <i>et al</i> ^[24]	1994	Uk	Uk	Malign	Uk	Sigmoid perforation	Surgery

Uk: unknown.

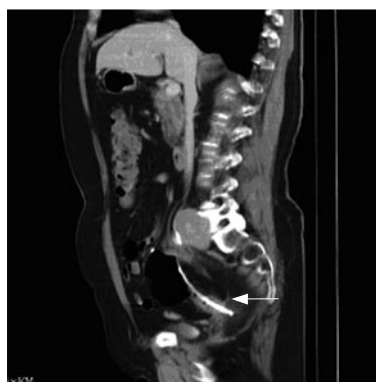


Figure 1 CT-scan of colonic perforation caused by migrated biliary endoprosthesis. Arrow marks dislocated stent.

the rectal mesentery (Figure 1). A rectal resection was performed. After an uncomplicated postoperative recuperation, the patient was discharged on the seventh postoperative day.

DISCUSSION

The incidence of postoperative biliary fistula is elevated in settings of acute or chronic inflammation^[32-34]. Treatment options are surgical biliary leak repair, percutaneous biliary drainage, and endoscopic biliary drainage.

Endoscopic placement of a biliary endoprosthesis is a well-established procedure for treatment of biliary outflow obstruction. The most frequent complication associated with bile duct stents is early occlusion caused by clogging, with resultant cholangitis, or by tumor over-growth. Stent dislocation and proximal or distal migration are uncommon, with an overall incidence of up to 6%^[3]. Several complications of stent migration have been reported and can be classified into categories of penetration, intestinal perforation, and obstruction. Other organs are usually unaffected^[9,10].

Most ingested foreign bodies pass through the intestine without major problems. Neither size nor shape of the stent predicts the likelihood of successful conservative management. In all patients, a close follow-up after biliary stent placement is mandatory. Patients with diverticular disease, hernia or intra-abdominal adhesions, are

especially at risk. Intestinal-wall weakness and increased resistance during bowel movement can produce localized complications^[26,39].

Lesions of the intestinal wall due to biliary-stent migration have been reported in the duodenum, and are related to retroperitoneal fixation of this intestinal segment. Penetration requires adherence between the perforated and the penetrated organ, and does not induce intra-abdominal contamination, but eventually causes fistulae (i.e., interenteric or biliocolic^[36], colovaginal^[25] or colovesicular^[27]).

Downstream migration is more frequent in benign than in malignant biliary duct strictures^[9,35]. Benign stenoses are not as tight because of regression of inflammatory reactions after placement of the stent. By contrast, tumor growth can anchor a stent in malignant stenoses.

In the case of intestinal perforation, surgical stent removal and drainage of consecutive intra-abdominal or retroperitoneal abscesses is mandatory. A pelvic abscess^[28], and colovesicular^[27] or colocutaneous^[29] fistula are typical consequences (Table 1). A case of necrotizing fasciitis due to colonic perforation has also been reported^[31].

A review of the literature published to January 2007 has revealed 11 cases of colonic perforation due to biliary stent migration, with the majority being straight plastic endoprostheses^[10,17,26,27,29,38,40].

In conclusion, endoscopic insertion of biliary stents is a useful and powerful procedure for short-term decompression of the biliary system. In the case of long-term therapy, stent-migration-associated complications have to be considered as a differential diagnosis that may lead to life-threatening situations. A correct diagnosis is sometimes difficult to make because of the absence of typical symptoms.

A migrated biliary stent, symptomatic or not, should be removed immediately^[19]. In cases of intestinal perforation, immediate surgical therapy is required. In cases of benign lesions of the bile duct, the stent should be either removed early to decrease the risk of secondary complications, or replaced regularly to prevent stent obstruction, infection or migration.

Caution should be exercised when straight plastic stents are inserted, and these should be closely followed-up.

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CASE REPORT

Sigmoid colon endometriosis treated with laparoscopy-assisted sigmoidectomy: Significance of preoperative diagnosis

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Abstract

We present a female patient with sigmoid colon endometriosis who was diagnosed correctly preoperatively and underwent minimally invasive surgery. She was admitted to our hospital with rectal bleeding and constipation. We performed several workups. Colonoscopy and endoscopic ultrasonography showed sigmoid colon stenosis caused by submucosal tumor, and magnetic resonance imaging revealed a sigmoid colon tumor displaying signal hypointensity on both T1- and T2-weighted imaging. However, colonoscopic ultrasonography-assisted needle aspiration biopsy could not specify tumor characteristics. From these examinations, the lesion was diagnosed as sigmoid colon endometriosis and laparoscopy-assisted sigmoidectomy was performed. Pathological diagnosis from the resected specimen was identical to preoperative diagnosis, i.e., colonic endometriosis. Since differential diagnosis of intestinal endometriosis seems difficult, a cautious preoperative diagnosis is required to select treatments including minimally invasive surgery.

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Key words: Intestinal endometriosis; Preoperative diagnosis; Laparoscopy-assisted surgery

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INTRODUCTION

Endometriosis is a common benign disease among women of reproductive age, and affects the intestinal tract in 15%-37% of all patients with pelvic endometriosis^[1]. The disease is thus comparatively common, but preoperative diagnosis is reportedly difficult^[2-4]. Herein, we report a case of sigmoid colon endometriosis that was diagnosed preoperatively using magnetic resonance imaging (MRI) and then treated by minimally invasive surgery.

CASE REPORT

A 43-year-old woman was admitted to our hospital with chronic constipation and decreased stool caliber for 3 mo before admission. Rectal bleeding was also reported, but was not associated with her menstrual cycle. She did not have any other symptoms, including abdominal pain. She was 14-years-old at first menstruation and her cycle was irregular at every 27-50 d, lasting 7-12 d. The patient was nulliparous and had not had an abortion.

Physical examination revealed no specific findings, including abdominal pain or palpable masses in the abdomen.

Laboratory examination showed an elevation of cancer antigen (CA) 125 level to 90.5 IU/mL, but other values were within normal limits.

Gynecological examination revealed a normal vagina, uterus and uterine cervix. Transvaginal ultrasonography revealed multiple myomas with diameters of 1-2 cm in the uterine wall, but no abnormality in either ovary.

Barium enema showed stenosis of the sigmoid colon caused by tumor, but the mucous membrane was smooth (Figure 1).

Colonoscopy also revealed sigmoidal stenosis with a smooth mucosal surface. Endoscopic ultrasonography was performed at the same time and revealed an iso- to high-echoic lesion in the 3rd echoic layer (submucosal layer) (Figure 2). In addition, endoscopic ultrasonography-guided fine needle aspiration biopsy (EUS-FNAB) was attempted, but sufficient specimens could not be obtained due to severe stenosis of the sigmoid colon.

MRI was performed for evaluation and differential

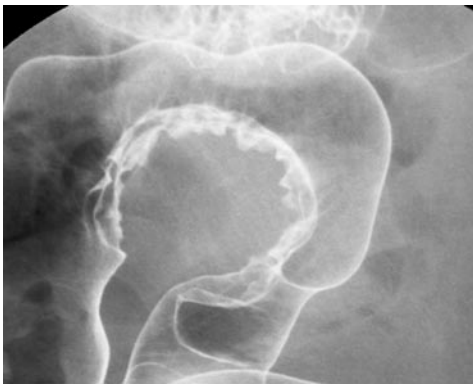


Figure 1 Barium enema shows sigmoidal stenosis with smooth inner lumen.

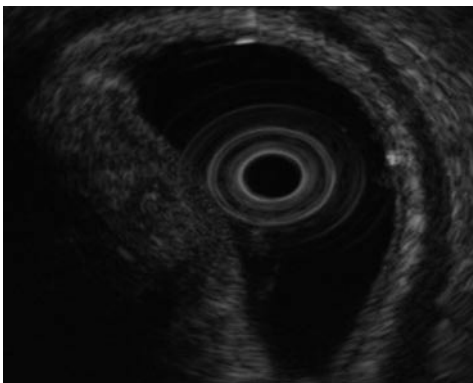


Figure 2 EUS shows iso to high echoic submucosal tumor in the 3rd echoic layer.

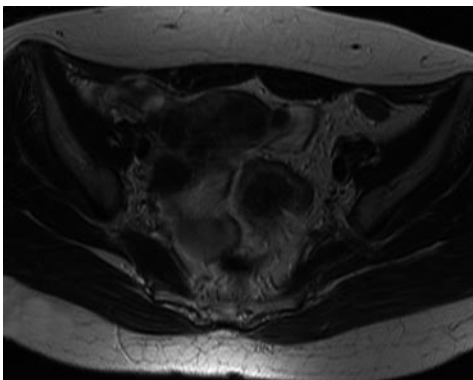


Figure 3 MRI (T2 weighted image) shows a sigmoidal tumor lesion with low signal intensity.

diagnosis of the submucosal tumor, revealing a tumor 3 cm in diameter in the sigmoid colon with signal hypointensity on both T1- and T2-weighted imaging (Figure 3; T2-weighted image only). Several small myomas were also seen in the uterus.

The sigmoid tumor was diagnosed as intestinal endometriosis from these examinations, for the following reasons: (1) the patient was a woman of reproductive age; (2) tumor was located in the sigmoid colon; (3) a submucosal tumor containing muscle and/or fibrous components was detected on MRI. As stenosis of the colon was severe and the patient wanted to have children

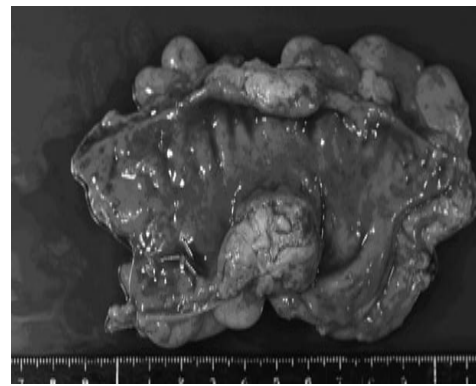


Figure 4 Gross examination of the resected specimen.

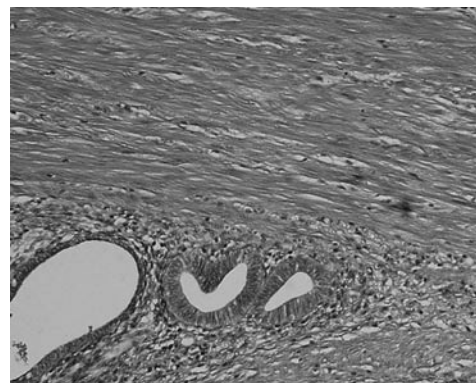


Figure 5 Tumor comprising endometrial-like glands and smooth muscle cells (× 200).

in the near future, we selected sigmoid resection by laparoscopy-assisted sigmoidectomy with a minimal skin incision to prevent postoperative abdominal adhesions. Surgery was performed after informed consent was obtained from the patient.

Three trocars, including a 5-mm laparoscope, were introduced and a 4-cm minilaparotomy was performed in the left lower abdomen to retrieve the resected specimen and anastomose the colon. The operation was successful and she was discharged uneventfully 7 d after surgery.

Gross examination of the resected specimen revealed a 30 mm × 20 mm × 15 mm hard submucosal mass, and the serous membrane of this site was indented to the tumor (Figure 4). No abnormality in the mucosal surface was identified.

Histological examination revealed a mixture of smooth muscle cells and endometrial glands, without atypical cells and goblet cells, so the diagnosis of sigmoid endometriosis was confirmed (Figure 5).

DISCUSSION

Endometriosis is histologically defined as the presence of endometrial-like tissue outside the uterus^[5]. This condition is thought to affect the intestinal tract in 15%-37% of patients with pelvic endometriosis^[1]. The disease is not particularly rare among women of childbearing age, but many authors have reported that correct preoperative

diagnosis is difficult^[2-4]. This is because the symptoms such as abdominal pain, hemostool and decreased stool caliber are not specific for this disease, and hemostool or rectal bleeding are thought to result from mucosal injury during passage of stools through the narrowed intestinal lumen^[2]. Some patients reportedly display symptoms associated with the menstrual cycle^[2,4], but these patients represent only about 40% of all patients with endometriosis^[6]. In addition, some reports have described aggravation of inflammatory bowel disease and irritable bowel syndrome during menstruation^[7]. Symptoms alone are thus not helpful for differential diagnosis, and a similar situation is seen for laboratory examination of the blood. Mol *et al*^[8] reported a systematic review of the diagnosis of endometriosis, and concluded that serum CA125 level may be elevated in endometriosis, but this measurement had no value as a diagnostic tool compared to laparoscopy.

Imaging examination is thus essential for the preoperative diagnosis of intestinal endometriosis, but some reports have described preoperative confusion between this disease and cancer according to colonoscopy and CT with barium enema, particularly in patients with lesions involving the mucosal surface^[3,4]. In such patients, MRI is helpful for differential diagnosis. In a typical endometrial lesion, MRI showed signal hyperintensity on T1-weighted imaging and signal hypointensity on T2-weighted imaging^[9]. However, smooth muscle components are reportedly recognized frequently in endometrial lesions^[10]. In such lesions, as seen in the present case, MRI indicates signal hypointensity on both T1- and T2-weighted imaging, and differential diagnosis from other diseases such as cancer and gastrointestinal stromal tumor is thus difficult. In fact, Chapron *et al*^[11] reported that MRI specificity for deeply infiltrating endometriosis was 97.9%, but sensitivity was only 76.5%.

Endoscopic ultrasonography is also a useful and noninvasive examination for the diagnosis of intestinal endometriosis. Sensitivity and specificity are reportedly about 97% for the diagnosis of rectal involvement in patients with known pelvic endometriosis^[12]. In addition, EUS-FNAB provides accurate tissue and may be the only procedure for correct preoperative diagnosis of intestinal endometriosis, but the overall specificity, sensitivity and accuracy of EUS-FNA for neoplasms of the gastrointestinal tract are reportedly 88%, 89% and 89%, respectively^[13].

Biscaldi *et al*^[14] reported the usefulness of multislice CT combined with distention of the colon by rectal enteroclysis for intestinal endometriosis. The sensitivity was 98.7% and specificity was 100% in identifying women with intestinal endometriosis. This method is thought to be very helpful for diagnosing intestinal endometriosis, but requires bowel preparation, such as the need for a low-residue diet for 3 d, drinking of 4-6 doses of a granular powder dissolved in 500 mL of water per dose and intravenous administration of iodinated contrast medium. This technique is thus inappropriate for patients with obstructive symptoms or

allergy to iodinated contrast medium.

Among these examinations, we consider MRI and EUS (and/or EUS-FNAB) are the most useful examinations for intestinal endometriosis. However, it is important to perform valuable examinations for diagnosis of intestinal endometriosis, including radiological, histological and etiological examinations, as the condition basically involves a benign lesion requiring minimally invasive treatment. This disease should also be considered in the differential diagnosis for women of childbearing age presenting with gastrointestinal tract symptoms.

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Reactive lymphoid hyperplasia of the liver: A case report and review of literature

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lymphoma; Hepatocellular carcinoma; Autoimmune thyroiditis; Immunohistochemistry

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Abstract

A case of a 53-year-old female patient with reactive lymphoid hyperplasia (RLH), clinically designated as pseudolymphoma of the liver is described in this article. The patient was admitted to our hospital for further evaluation of hepatic tumors incidentally discovered at another hospital. Various diagnostic methods, including ultrasonography (US), computerized tomography (CT), magnetic resonance imaging (MRI) and hepatic angiography displayed three small lesions in the liver with outstanding findings consistent with hepatocellular carcinoma (HCC). Surgical resection was performed and the three lesions were microscopically diagnosed as RLH of the liver. The lesions comprised a massive infiltration of lymphoid cells with follicles and hyalinized inter-follicular spaces. Immunohistochemical examination revealed that infiltrating lymphocytes had no prominent nuclear atypia and polyclonality. RLH of the liver is a very rare condition and only twelve cases have been reported in the English literature. Majority of the reported cases were middle-aged women and about half of them had some immunologic abnormalities such as autoimmune thyroiditis, Sjogren's syndrome, primary immunodeficiency, primary biliary cirrhosis. Since they are often clinically misdiagnosed as HCC, surgery is the choice of treatment for these patients. Although their pathology resembles malignant lymphoma, the clinical course is completely benign. The authors propose that RLH of the liver can be discriminated from HCC by its clinical features.

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Key words: Reactive lymphoid hyperplasia; Pseudo-

INTRODUCTION

Reactive lymphoid hyperplasia (RLH) is a benign nodular lesion, histopathologically characterized by marked proliferation of non-neoplastic, polyclonal lymphocytes forming follicles with an active germinal center. The lesion is encountered in various organs such as the orbit^[1], lung^[2], skin^[3,4] and gastrointestinal tract^[5-7]. However, disease of the liver is quite rare, with only 12 cases reported in the English literature. We have recently encountered a patient with 3 RLH lesions occurred in the liver. In this paper, the clinicopathological and radiographic characteristics of this unique disorder are discussed. Problems in differential diagnosis from malignant diseases, including malignant lymphoma and hepatocellular carcinoma, are also discussed.

CASE REPORT

A 53-year-old Japanese woman with a history of autoimmune thyroiditis was admitted to our hospital for further evaluation of hepatic lesions incidentally discovered on abdominal ultrasonography. The patient was asymptomatic on admission and her condition was generally good. Physical examination revealed no abnormalities. Relevant laboratory tests disclosed slightly elevated lactate dehydrogenase activity (547 IU/L; normal range: 120-245 IU/L), thymol turbidity (7.2 U/L; normal range: 0.0-4.0 U/L) and zinc sulfate turbidity (13.0 U/L; normal range: 4.0-12.0 U/L), although other hepatic function tests including serum aspartate aminotransferase, serum alanine aminotransferase and total bilirubin were within normal range. Levels of serum thyroid-stimulating hormone (10.22 μ U/mL; normal range: 0.35-3.73 μ U/mL) and antinuclear antibody (80 \times ; normal range: $< 40 \times$) were elevated due to autoimmune thyroiditis. Both hepatitis B surface antigen and anti-hepatitis C virus antibody were negative. Tumor markers including alpha-fetoprotein and protein induced by vitamin K antagonist-II were negative.

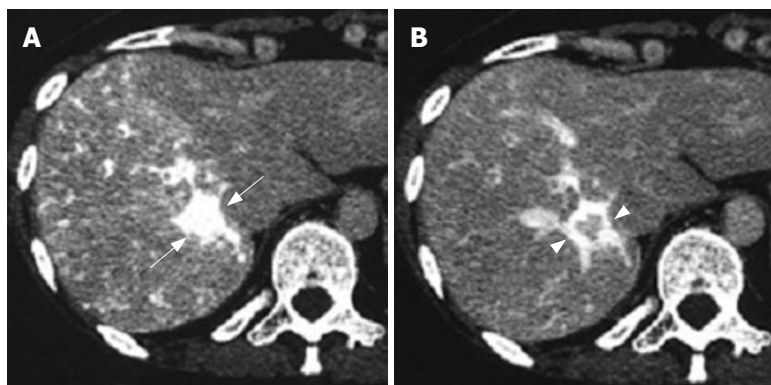


Figure 1 CT angiography showing the conspicuously enhanced lesion in segment 7 through the early phase (arrows) (A) and intensified tumor rim enhancement through the delayed phase along with radial enhancement (arrowheads) (B).

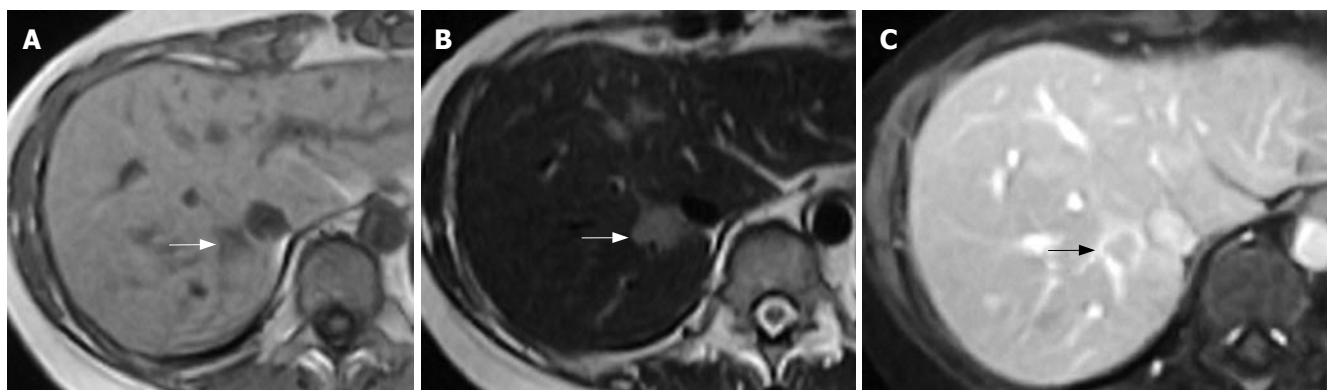


Figure 2 MRI displaying a hypointense signal on T1-weighted imaging (arrow) (A), a hyperintense signal on T2-weighted imaging of the lesion (arrow) (B), and enhanced tumor rim in the delayed phase of MRI (arrow) (C).



Figure 3 Macroscopy of tumor in segment 7 demonstrating the lesion as a well-circumscribed, white-yellowish nodule on the cut surface.

Precise US examination disclosed 2 hypoechoic nodules in the posterior segment and 1 nodule in the medial segment of the liver (13, 11 and 8 mm in diameter) respectively. Dynamic CT performed in the planes of lesions demonstrated a slight hyperdensity in the arterial phase followed by hypodense areas with pronounced enhancement along the tumor rims in the portal phase. The lesion in segment 7 was conspicuously enhanced through the early phase on CT angiography (Figure 1A). The tumor rim was continually intensified through the delayed phase, and radial enhancement toward surrounding liver parenchyma was also recognized (Figure 1B). The lesion was identified as a portal flow defect on CT with arterial portography. On MRI, tumor gave a hypointense signal on T1-weighted imaging (Figure 2A) and a hyperintense signal on T2-weighted imaging (Figure 2B). The tumor rim

was underscored as an annular enhancement in the delayed phase, similar to dynamic CT (Figure 2C). Other lesions within segments 4 and 6 showed equivalent hemodynamics to the tumor in segment 7. Hepatic angiography of the 3 lesions also demonstrated distinct tumor staining in the arterial phase, following dense staining along tumor rims in the parenchymal-venous phase. As all images suggested hepatocellular carcinoma, posterior segmentectomy and partial resection of the medial segment of the liver were performed.

The resected liver displayed smooth surfaces and normal appearance. On the cut section, lesions were well-circumscribed, but not encapsulated white-yellowish nodules with irregular shapes (Figure 3). The 3 nodules exhibited similar microscopic findings, comprising a massive infiltration of lymphoid cells with follicles and hyalinized interfollicular spaces at low magnification (Figure 4A). Lymph follicles varied in size and shape with germinal centers composed of small or large lymphoid cells and tingible body macrophages. Interfollicular areas mostly comprised mature lymphocytes with no prominent nuclear atypia and a small amount of infiltrated plasma cells and histiocytes (Figure 4B). Strands of amorphous, somewhat-dense hyalinized material accompanied with capillaries were observed in interfollicular areas (Figure 4C). In some parts, fibrous materials aggregated as nodular structures of various sizes. Bile ductule proliferation was seen along the lesion edges. Portal areas apart from nodules showed irregular expansion with infiltration of small mature lymphocytes (Figure 4D). Immunohistochemical studies on

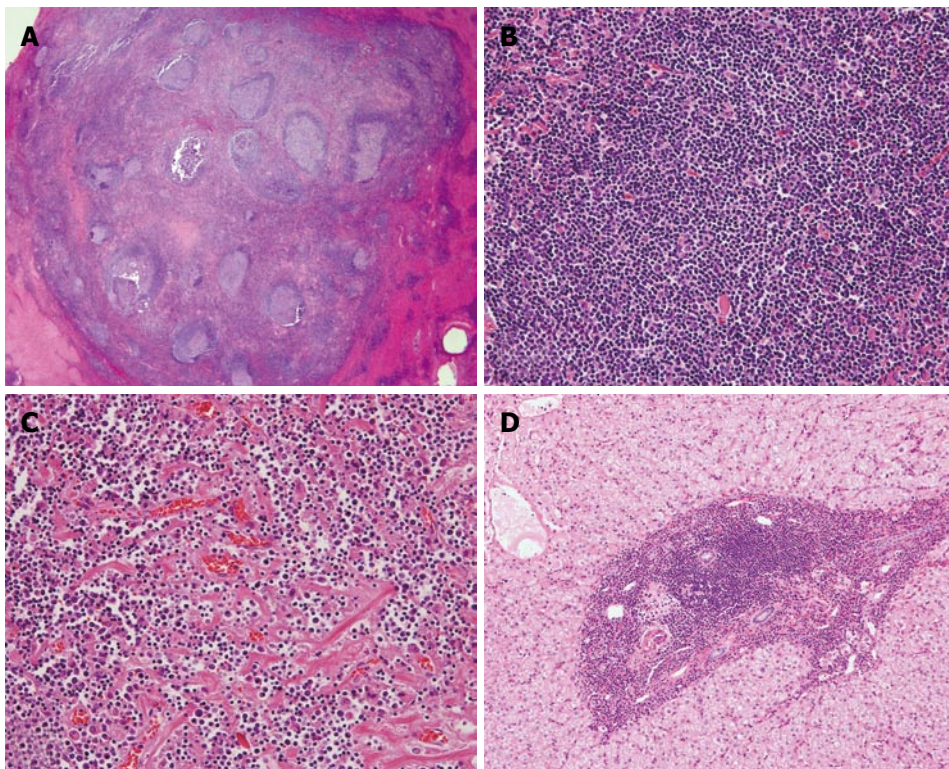


Figure 4 Microscopy revealing lesions comprising massive lymphoid cell infiltration with prominent follicles and hyalinized interfollicular spaces at low magnification (HE, X 2.5) (A), interfollicular areas mainly comprising mature lymphocytes with no nuclear atypia (HE, X 50) (B), strands of amorphous hyalinized material accompanying capillaries in interfollicular areas (HE, X 50) (C), and portal areas apart from the nodules with irregular expansion of infiltration of small mature lymphocytes (HE, X 25) (D).

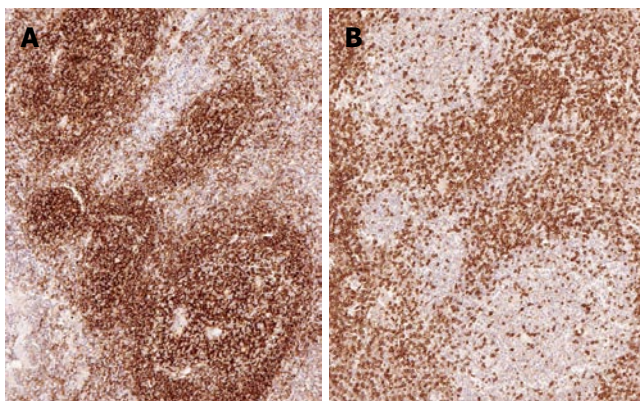


Figure 5 Immunohistochemistry discovering follicles mainly comprising CD20-positive lymphocytes (X 50) (A), and interfollicularly and circumferentially distributed CD3-positive cells compared to follicles (X 50) (B).

paraffin-embedded tissue sections for CD3, CD5, CD10, CD20, bcl-2 and both κ and λ immunoglobulin light chains were undertaken using the avidin-biotin-peroxidase complex technique. Follicles mainly comprised CD20-positive lymphocytes, while CD3-positive cells were distributed interfollicularly and circumferentially around follicles (Figure 5). Distributions were similar to that seen in reactive hyperplasia of lymph nodes. Lymphoid cells in follicles showed ordinary immunoreactivity, while cells in the mantle zone were positive for CD5 and cells in the germinal center were positive for CD10. Bcl-2 reacted only to lymphocytes in marginal or circumferential areas of follicles, but not in central regions. Scattered plasma cells were positive for both κ and λ light chains at an equal frequency. These immunohistochemical results indicate that lymph follicles were of reactive origin, but not of neoplastic, and

that the lesion was composed of polyclonal lymphocytes and plasma cells. A genetic investigation of clonality in the immunoglobulin heavy chains (IgH) using a previously reported polymerase chain reaction method^[6] with DNA from paraffin-embedded tissue was also performed. No clonal IgH gene rearrangement was detected with satisfactory negative and positive controls. The 3 lesions were thus pathologically diagnosed as RLH of the liver.

Postoperative course was relatively good and the patient experienced no signs of recurrence for 22 mo.

DISCUSSION

Although RLH can be found in various organs, including the gastrointestinal tract^[5-7], orbit^[1], lung^[2] and skin^[3,4], its occurrence in the liver is rare. To the best of our knowledge, only 14 lesions in 12 other cases of hepatic RLH of the liver have been reported in the English literature since the first case report by Snover *et al*^[9] in 1981, with this case representing the thirteenth (Table 1). RLH occurs predominantly in middle-aged women, with a mean age of 54.1 years (range 15-72 years, Table 1). Although the pathogenesis remains unknown, an association between development of hepatic RLH and systemic or local immunological abnormalities has been suggested in earlier reports^[10,11]. In fact, 6 of 13 patients, including the present, have displayed some immunological complications, such as autoimmune thyroiditis^[10], Sjogren's syndrome^[11], primary immunodeficiency (PI)^[9], and primary biliary cirrhosis (PBC)^[12] (Table 1). Two cases have been reported in association with hepatitis B (HB) virus or hepatitis C (HC) virus infection^[13,14] (Table 1). The patient with hepatitis B was treated with interferon (IFN)- α ^[13]. Whether hepatitis viral infection is involved in hepatic RLH remains unclear. However,

Table 1 Reported cases of RLH of the liver

Case	Reference #	Age (yr)	Sex	Complications	Needle biopsy	Preoperative diagnosis	Tumor number	Tumor size (mm)	Treatment
1	9	15	F	PI	Done	Cirrhosis			
2	13	42	F	HB	None	HCC	3	15, 14, and smaller	Resection
3	10	47	F	Autoimmune thyroiditis	None	HCC	1	17	Resection
4	11	49	F	Sjogren's syndrome	Done	Suspicious HCC	1	20	Resection
5	12	52	F	PBC	None	PBC	1	4	Transplantation
6	12	56	F	PBC	None	PBC	1	15	Transplantation
7	12	56	M	Diverticulitis	None	Not reported	1	7	Resection
8	19	59	F	Diabetes	None	Malignant tumor	1	9	Resection
9	16	66	F	Diabetes	Done	Suspicious lymphoma	1	15	Resection
10	17	67	F	None	None	HCC	1	20	Resection
11	18	69	F	Renal cell carcinoma	Done	Metastatic tumor	1	17	Resection
12	14	72	M	Gastric carcinoma HC	None	HCC	1	17	Resection
13	Our case	53	F	Autoimmune thyroiditis	None	HCC	3	15, 12, and 10	Resection

the findings in these patients indicate that continuous stimulation from cytokines such as IFN- α or hepatitis viruses may cause RLH of the liver.

Saltzstein *et al*^[15] has defined RLH as propagation of lymph-follicles constructed with lymphoid cells without cytological atypia, accompanied with a conspicuous, reactive germinal center. Katayanagi *et al*^[16] and Tanizawa *et al*^[17] have also proposed RLH as a localized lesion well-demarcated from the surrounding tissues and characterized by the presence of hyperplastic lymphoid follicles with polymorphic and polyclonal cell populations composed of small mature lymphocytes, mature plasma cells, macrophages and stromal fibrosis. The nodular lesion described herein seems to satisfy these histological criteria.

When RLH is diagnosed, malignant lymphoma must be excluded from the differential diagnosis. In the present case, immunohistochemical examination was performed to rule out malignant lymphoma. Germinal centers were considered non-neoplastic, based on negative results for bcl-2. In addition, lymphoid cells, including plasma cells, proliferating in the interfollicular area displayed polyclonal immunophenotypes. The results of a genetic investigation pertaining to IgH clonality also supported the histological findings. A diagnosis of RLH of the liver thus seems justified.

The lesion has been often misdiagnosed as a malignant tumor, since features are shared with hepatocellular carcinoma on various imaging modalities, namely detection as a hypoechoic mass on US, low density on CT with or without enhancement, low intensity on T1-weighted imaging and high intensity on T2-weighted imaging with magnetic resonance imaging, and tumor staining on hepatic angiography. Distinguishing RLH from hepatocellular carcinoma (HCC) is thus extremely difficult. Hepatectomy has therefore been performed for many patients^[10-14,16-19] (Table 1). In the present case, the tumor rim displayed a sustained intensity accompanied with radial enhancement in addition to intratumoral hyperintensity on various imaging modalities. This prominent finding may represent increased capillaries within the nodule and massive infiltration of lymphocytes into portal areas around the lesion. Needle biopsy was indicated for only 4 patients in past reports^[9,11,16,18] (Table 1), but it is not helpful for a confident diagnosis of this disease.

In conclusion, accumulation of reported cases with this

benign lesion, including our patient, reveals some knowledge concerning the characteristics of this rare condition, such as female predominance, small tumor size, coexistent immunological disorder, and imaging features resembling HCC. The authors would like to emphasize that RLH should be included in the differential diagnosis of small hepatic lesions, particularly in middle-aged women with suspected HCC.

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CASE REPORT

Clindamycin-induced acute cholestatic hepatitis

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Abstract

We report a case of acute hepatotoxicity in a 42-year-old woman after administration of clindamycin for a dental infection. After 6 d of treatment, she had fatigue, nausea, vomiting, anorexia, pruritus and jaundice. Her laboratory analysis showed alanine aminotransferase (ALT), 1795 IU/L (normal range 0-40); aspartate aminotransferase (AST), 1337 IU/L (normal range 5-34); alkaline phosphatase (ALP), 339 IU/L (normal range 40-150); γ -glutamyl transpeptidase (GGT), 148 IU/L (normal range 9-64 IU/L); total bilirubin, 4.1 mg/dL; direct bilirubin, 2.9 mg/dL and prothrombin time (PT), 13.5 s, with international normalized ratio (INR), 1.04. She was hospitalized, with immediate drug discontinuation. Her liver biopsy specimen showed mixed-type (both hepatocellular and cholestatic) hepatic injury, compatible with a diagnosis of drug-induced hepatitis. An objective causality assessment using the Naranjo probability scale suggested that clindamycin was the probable cause of the acute hepatitis. In susceptible individuals, clindamycin use may lead to acute mixed-type liver toxicity. Complete recovery may be possible if the drug is discontinued before severe liver injury is established.

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Key words: Clindamycin; Hepatic injury; Hepatitis

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INTRODUCTION

Liver injury may be caused by various drugs and chemicals. The severity of injury may vary from minor non-specific changes in hepatic structure and function to fulminant

hepatic failure, cirrhosis and even hepatocellular cancer. The term drug-induced liver disease should be confined to cases in which the nature of the liver injury has been characterized histologically, because biochemical parameters used to detect liver injury may also be elevated as an adaptive response to drugs.

The lincosamide antibiotics seem to be most useful against the bacteria classified as Gram-positive cocci. In addition, they are helpful against protozoa, such as *Toxoplasma* and *Mycoplasma*, as well as many anaerobic bacteria^[1]. Clindamycin (7-chlorolincomycin), a semisynthetic derivate of lincomycin, has been extensively used in the therapy of obstetric, gynecologic and oral infections for over 20 years. It is more active than erythromycin or clarithromycin against anaerobic bacteria. In humans, absorption of clindamycin is rapid and virtually complete (90%) following oral administration, and is widely distributed throughout the body^[2]. We describe herein a case of acute hepatitis following clindamycin use for dental infection.

CASE REPORT

A 42-year-old woman was admitted to hospital for pruritus, jaundice and asthenia. Her medical history included recurrent gingival infections treated with clindamycin on several previous occasions. Six days after the initiation of the last clindamycin (150 mg PO qid) treatment, the patient had fatigue, nausea, vomiting, anorexia, pruritus and jaundice. Emergency laboratory analysis showed the following results: Alanine aminotransferase (ALT), 1795 IU/L (normal range 0-40); aspartate aminotransferase (AST), 1337 IU/L (normal range 5-34); alkaline phosphatase (ALP), 339 IU/L (normal range 40-150); γ -glutamyl transpeptidase (GGT), 148 IU/L (normal range 9-64); total bilirubin, 4.1 mg/dL; direct bilirubin, 2.9 mg/dL and prothrombin time (PT), 13.5 s, with international normalized ratio (INR), 1.04. Blood tests for glucose, blood urea nitrogen (BUN), serum creatinine, total protein, albumin, uric acid, total cholesterol and triglycerides were within normal limits. There was no peripheral eosinophilia. The patient was hospitalized and clindamycin treatment was discontinued. Additional test results for hepatitis A, B and C viruses, cytomegalovirus, and Epstein-Barr virus were negative. Ferritin and ceruloplasmin levels were within normal limits. Autoantibodies (antinuclear, antimitochondrial, anti-smooth-muscle, anti-liver-kidney microsomal enzymes and anti-soluble liver antigen) were also negative. Hepatobiliary imaging with ultrasonography was normal. She denied taking any other medications,

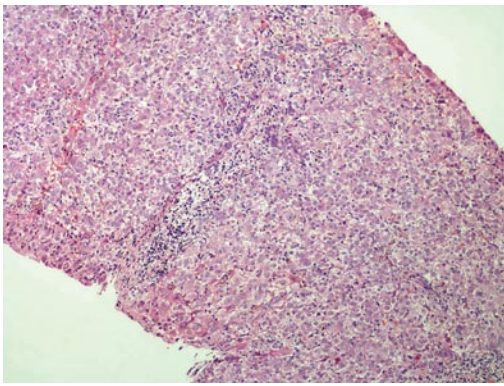


Figure 1 Extensive hepatocellular injury showing centrilobular and portal cholestatic hepatitis with inflammatory infiltration (HE, × 40).

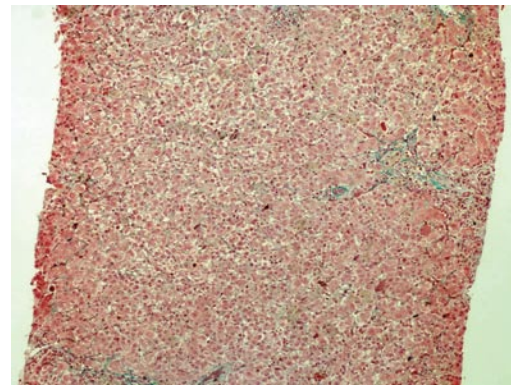


Figure 2 Intense inflammatory reaction in liver without evidence of fibrosis (Mason's Trichrome, × 40).

or using alcohol or any herbal or folk remedies at any time. There were no known contributing environmental issues. One week after hospitalization, her liver function tests were as follows: ALT, 1579 IU/L; AST, 1031 IU/L; ALP, 345 IU/L; GGT, 112 IU/L; total bilirubin, 3.34 mg/dL; and direct bilirubin, 2.4 mg/dL. Her medical history and laboratory results suggested severe acute hepatitis of mixed-type, with hepatocellular and cholestatic hepatic injury, and liver biopsy was performed. The histopathological samples revealed centrilobular and portal cholestatic hepatitis, without fibrosis or necrosis, highly suggestive of drug-related hepatotoxicity (Figures 1 and 2). Within 3 wk of hospitalization, the patient had significant subjective improvement, and showed improvement of liver function tests (ALT, 754 IU/L; AST, 429 IU/L; ALP, 175 IU/L; GGT, 82 IU/L; total bilirubin, 1.8 mg/dL; and direct bilirubin, 1.26 mg/dL). After 8 wk hospitalization, her liver function test results were within normal limits (ALT, 28 IU/L; AST, 23 IU/L; and total bilirubin, 0.74 mg/dL). The patient was discharged in good condition. The probability that the symptoms of hepatotoxicity that occurred after clindamycin treatment were an adverse drug reaction (ADR) was assessed using the Naranjo ADR probability scale. The total Naranjo score for the patient was 7, which is in the "probable" range^[3].

DISCUSSION

Six days after the start of treatment with clindamycin, our 42-year-old female patient experienced signs of hepatotoxicity with jaundice and asthenia. Laboratory testing showed marked elevations in serum ALT, AST, ALP, GGT and total bilirubin concentrations. Her symptoms, as well as the liver tests, resolved on discontinuation of the drug treatment. The subtype of the hepatotoxicity observed in the patient was considered as mixed hepatocellular and cholestatic. The diagnosis of severe clindamycin-induced hepatotoxicity in our case was suggested by: (1) symptoms such as fatigue, nausea, vomiting, anorexia, pruritis and jaundice 6 d after clindamycin initiation; (2) the chronology between clindamycin introduction and liver test abnormalities; (3) histological findings that were highly suggestive of a toxic mechanism; (4) absence of any other clear etiology;

and (5) recovery after clindamycin withdrawal.

Cholestasis with hepatitis is a frequent type of hepatic drug reaction and it is characterized by conspicuous cholestasis with hepatocellular injury. Histological lesions may show lobular and portal tract inflammation, often with neutrophils, eosinophils or mononuclear cells^[4]. The clinical spectrum of cholestatic hepatitis was indicated in our case by pruritis and jaundice. It was also reflected by the markedly elevated liver tests, with increased serum bilirubin, GGT and ALP. Cases of mixed cholestasis and hepatitis are considered highly suggestive of a drug reaction^[5].

To exclude dilation of large bile ducts produced by biliary obstruction, and hepatic or pancreatic masses, hepatobiliary imaging is essential. Ultrasound is the most preferred method to rule out possible bile duct obstruction. Endoscopic retrograde cholangiopancreatography, percutaneous transhepatic cholangiography or computed tomography may be necessary in difficult cases. In the absence of the symptoms mentioned earlier, drug-induced cholestasis is more likely, and a liver biopsy is often advisable. Certain histological findings, such as lobular and portal tract inflammation, suggest a hepatic drug reaction, whereas others such as edema of the portal tracts suggest biliary obstruction. When the temporal relationship to drug ingestion indicates a high probability of a drug reaction, particularly when the agent is known to be potentially hepatotoxic, it is appropriate to discontinue the incriminated drug and observe whether improvement occurs. Recovery may be seen rapidly if the drug is discontinued before severe liver injury is established^[6].

Hepatocytes, because of their capability to metabolize drugs, form minute amounts of drug-protein adducts, for which the immune system normally shows tolerance. Hypersensitivity reactions occur when this tolerance is impaired. Additional signals, such as a concomitant inflammatory reaction, may eventually be needed to break this tolerance. The allergic hepatitis induced by drugs is generally a type IV hypersensitivity reaction involving CD4+, CD8+ cytotoxic lymphocytes, as well as natural killer cells. Antibodies directed to the drug are much less common. Antibodies against cellular components may, however, occur when the sensitization process evolves towards an autoimmune reaction^[7,8]. In our patient, autoantibodies were negative and several features, such as the absence of

predictable dose-dependent toxicity of clindamycin and pruritis (hypersensitivity manifestations), were consistent with a metabolic type of idiosyncratic toxicity.

Clindamycin is a widely used antibiotic that is available in many countries. Previously, another case of hepatotoxicity associated with use of clindamycin has been reported, in which the clinicopathological spectrum encompassed cholestatic liver disease with portal inflammation, bile duct injury and bile duct paucity (ductopenia). In the same patient, a second biopsy after clinical recovery showed resolution of cholestasis but persistence of duct paucity, whereupon the authors concluded that early onset hepatic injury with cholestasis and duct paucity may occur after clindamycin use, and long term duct paucity may persist on repeat biopsy^[9]. Bile duct paucity as a biopsy finding was not significant in our patient.

In summary, with this report, we presented a case of hepatotoxicity after 6 d of oral clindamycin treatment for dental infection. The toxicity appeared to be associated with drug use. In conclusion, although rare, mixed-type (hepatocellular and cholestatic) hepatic injury might be associated with clindamycin use in some cases. Patients may present with fatigue, nausea, vomiting, anorexia, pruritis and jaundice. Complete recovery may be possible if the drug is discontinued before critical liver injury is established.

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Sudden hearing loss associated with peginterferon and ribavirin combination therapy during hepatitis C treatment

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Abstract

Adverse effects associated with peginterferon and ribavirin during hepatitis C treatment are well known. Sudden hearing loss has rarely been reported. Possible mechanisms involved include direct ototoxicity of interferon, autoimmunity, and hematological changes. Hearing loss is frequently fully resolved after discontinuation of antiviral therapy. We report a 47-year-old man with chronic hepatitis C, genotype 2 ac who developed sudden hearing loss 22 wk after starting therapy with peginterferon alpha 2a at a dose of 180 µg per week and ribavirin 800 mg per day. Since symptoms did not worsen, antiviral therapy was continued for 2 wk, according to the patient's wish. Hearing loss resolved within 2 wk after the end of treatment. Serum liver alanine aminotransferase remained normal during and after the end of antiviral therapy. HCV RNA was undetectable at the end of therapy and remained negative 24 wk later. Thus, patients should be aware that hearing loss may occur with peginterferon therapy, but the decision whether to continue or to stop the treatment is based on the clinical judgment of the physician and the wishes of the patient.

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Key words: Adverse effects; Hearing loss; Hepatitis C; Interferon; Ribavirin

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INTRODUCTION

Chronic hepatitis C affects 250-300 million people worldwide, and in Tunisia, the prevalence is estimated to be 0.4%-1.6% of the general population^[1-3]. The higher prevalence is in the north which corresponds to the south side of the Mediterranean basin. The most frequent genotype in Tunisia is 1b^[4].

Treatment of hepatitis C virus infection with the combination of peginterferon alpha (2a or 2b) and ribavirin therapy is now recommended^[5,6]. Both peginterferon and ribavirin are well known to be associated with significant adverse effects^[7]. Hearing loss has been reported as a consequence of therapy with pegylated and non-pegylated interferon^[8-10]. However, we still pay little attention to auditory acuity. Moreover, contrary to standard interferon, hearing loss seems to not be fully resolved after discontinuation of peginterferon treatment^[8,10].

We report a case of sudden hearing loss in a patient with HCV infection treated with a combination of peginterferon and ribavirin. The interesting aspect of our case is that peginterferon therapy was continued during the last month and hearing loss was fully resolved after the end of treatment.

CASE REPORT

A 47-year-old Tunisian man with chronic hepatitis C, with 10⁶ UI/L HCV RNA and genotype 2a/2c, attended the Gastroenterology Department for follow-up. He did not have any pathological history and he had not taken any drugs prior to his visit. His initial physical and neurological examinations were normal. Serum levels of glucose, urea, creatinine, lipids, electrolytes, alkaline phosphatase, bilirubin, gammaglutamyl transferase, blood cell count, ferritin and cryoglobulin were within normal limits. Alanine aminotransferase (ALT) was 62 UI/L (upper limit of normal < 45), aspartate aminotransferase (AST) was 39 UI/L (upper limit of normal < 45 UI/L). Antinuclear, smooth muscle, anti-liver-kidney microsome 1 and anti-mitochondrial antibodies were negative. He was treated with peginterferon alpha 2a (180 µg/wk) and ribavirin (800 mg/d) for a predetermined period of 24 wk. Twenty-two weeks after beginning therapy, he developed otalgia and sudden onset of left-sided hearing loss. He was seen in consultation by an otolaryngologist who reported that ear examination was normal. Since

symptoms did not worsen, and in accord with the patient's wishes, antiviral therapy was continued for 2 wk. Hearing loss resolved within 2 wk after the end of treatment. Serum liver ALT remained normal during and after the end of antiviral therapy, and no sign of thrombocytopenia was detected during blood cell count monitoring. HCV RNA was undetectable at the end of therapy and remained negative 24 wk later.

DISCUSSION

This is another report on sudden hearing loss after treatment with peginterferon alpha, which adds to the observations made in patients treated with peginterferon and standard interferon for chronic hepatitis C^[8-11]. Such hearing loss is mostly unilateral and sensorineural^[12]. In one prospective study of 73 patients receiving interferon, hearing impairment (tinnitus and/or hearing loss) occurred in 32 patients (43.8%), among whom audiometry documented hearing loss in 27 cases (36.9%)^[13].

Several factors may cause sensorineural hearing loss in patients treated with interferon. First, the drug itself may be directly toxic to the auditory nerve hairy cells^[9]. Also, an indirect effect of interferon due to its immunoregulatory and antiviral activity can explain this sensorineural pathology. Cadoni has detected anti-endothelial-cell antibodies in an HCV patient with sudden hearing loss during interferon therapy^[11]. The finding of these antibodies suggests microvascular damage during therapy for vasculitis. In addition, interferon-induced thrombocytopenia may cause a microvascular accident in the inner ear.

Some authors have reported that hearing loss fully resolves 5 d to 1 mo after discontinuation of antiviral therapy with interferon^[9,14]; whereas others have demonstrated that hearing impairment does not completely recover after cessation of peginterferon, and worsens with continued treatment^[8,10].

In the case reported here, hearing loss fully resolved after the end of treatment with peginterferon. Auditory disability frequently develops in the later stages of treatment^[15]; in our case, it developed 2 wk before the end of the treatment (i.e., after 22 wk).

The average cumulative dose until development of auditory disability is about 176.9 mV^[13]. In a recent study, hearing loss was detected in nine of 27 patients after 7 d, and the degree of hearing loss increased until 21 d of treatment^[14]. Therefore, it is recommended that patients on interferon therapy should be monitored during treatment for changes in hearing perception. It remains possible that those who develop hearing loss can continue with inter-

feron therapy. The decision whether to continue or to stop the treatment when signs of ototoxicity appear is based on the clinical judgement of the treating physician and the wishes of the patient.

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Positron emission tomography/computer tomography in guidance of extrahepatic hepatocellular carcinoma metastasis management

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INTRODUCTION

Recurrence and metastasis are the major obstacles to further improve the prognosis of hepatocellular carcinoma (HCC). Tumor recurrence is common after hepatic resection of huge HCC greater than 10 cm in diameter. The overall 1- and 3-year survival rates are 66% and 44%, respectively. The overall 5-year survival ranges from 11% to 76%, with a median around 30% after hepatectomy^[1]. Extrahepatic recurrence occurs in 30% of the patients. Resection of isolated extrahepatic HCC metastasis has been advocated to obtain a possibility of long-term survival^[2,3]. Accurate re-staging plays the most important role in making a decision on extrahepatic metastasis resection. Nowadays, PET/CT is more and more widely available, and its application with 18F-FDG in oncology has become one of the standard imaging modalities in diagnosing, staging and re-staging HCC^[4]. We present here a patient with huge HCC resected six years ago and a new omental lymph node metastasis recently identified by 18 F-FDG PET/CT. Long-term survival was achieved in this patient after resection of the omental lymph node metastasis.

CASE REPORT

A 60-year old woman with a medical history of HBV infection and no cirrhosis was referred to our hospital for a liver tumor found by coincidence. The AFP level was 13.4 ng/mL. HCC was suspected (Figure 1) and a left hemihepatectomy was performed in December 2000. Histology showed a HCC (Edmondson-Steiner's grade I) with a diameter of 13.0 cm with central necrosis. No microscopic vascular invasion was found. After partial liver resection, AFP values returned to normal. The patient accepted transcatheter arterial chemo-embolization (TACE) treatment 1 mo after hepatectomy and no special change was found during angiography. After 78 mo of follow-up, her AFP increased to 597.8 ng/mL. Conventional chest and abdominal CT did not find any evidence of intrahepatic recurrence and extrahepatic metastasis. To find out the reason for AFP increasing, the patient was referred to our center for a whole body PET/CT examination. 18F-FDG PET/CT detected a high metabolic lymph node in the omental position (Figure 2). Due to the maximum stan-

Abstract

Hepatocellular carcinoma (HCC) is one of the most common primary cancers in the world. Surgery is the gold standard for treatment of patients with HCC. Recurrence and metastasis are the major obstacles to further improve the prognosis of HCC. Most recurrences are intrahepatic. However, 30% of the recurrences are extrahepatic. The role of resection in intrahepatic recurrences is widely accepted. The role of resection in extrahepatic HCC recurrence and metastasis is not well established. 18F fluorodeoxyglucose (18F-FDG) positron emission tomography/computer tomography (PET/CT) is useful in detecting distant metastasis from a variety of malignancies and shows superior accuracy to conventional imaging modalities in identification of intrahepatic and extrahepatic metastasis. We present one patient with one new isolated omental lymph node metastasis, who had a history of huge HCC resected six years ago. The metastatic focus was identified with 18 F-FDG PET/CT and resected. The follow-up revealed good prognosis with a long-term survival potential after resection of the omental lymphatic metastasis.

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Key words: Fluorodeoxyglucose; Positron emission tomography/computer tomography; Huge hepatocellular carcinoma; Omental node metastasis; Extrahepatic metastasis

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Figure 1 Contrast CT demonstrating a huge lesion at the left lobe of liver (black arrows, portal phase).

standard uptake value (SUVmax), lymph node metastasis was considered. The high metabolic lymph node was excised. Histopathological examination of the resected nodule revealed a metastatic hepatocellular carcinoma. After excision of the tumor, her AFP level decreased to normal. Ten months after resection of the extrahepatic metastasis and 88 mo after partial liver resection, the patient was in good condition with no recurrence at the time when we wrote this paper.

DISCUSSION

HCC over 10 cm in diameter is found in a number of patients undergoing hepatic resection. A large HCC has its specific clinicopathological features. Multiple tumor nodules, venous invasion and impaired liver function are factors associated with its recurrence^[5]. Hepatic resection of HCC with a diameter ≥ 10 cm at stage II or III is safe and effective for selected patients without liver cirrhosis. The overall incidence of postoperative complications is 39%. The 3-, 5- and 9-year overall survival rates after hepatic resection are 32%, 27%, and 17%, respectively^[6]. Appropriate selection of candidates for partial hepatectomy based on the above prognostic factors may play an important role in decreasing the high mortality rate and poor long-term survival of such patients^[7].

Approximately 50% of patients with resected HCC may have tumor recurrence. The overall 1-, 3- and 5-year disease-free survival rates are reported to be 43%, 26% and 20%, respectively^[1]. Thus, it is of importance to predict its relapse and treat it with other modalities^[8]. The role of resection in case of intrahepatic recurrences is widely accepted. If the distribution and localization of extrahepatic metastasis are fully confirmed, these lesions may be resected. In our case, conventional CT did not find any intrahepatic and extrahepatic metastasis. However, the whole body PET/CT detected isolated extrahepatic metastasis which we treated with surgery. 18F-FDG PET/CT is not only a useful tool for preoperative staging, but also a follow-up means better than conventional diagnostic modalities, especially for staging and re-staging after hepatectomy.

In case of an AFP producing HCC, unexplained rise of serum AFP after treatment is an early indicator of tu-

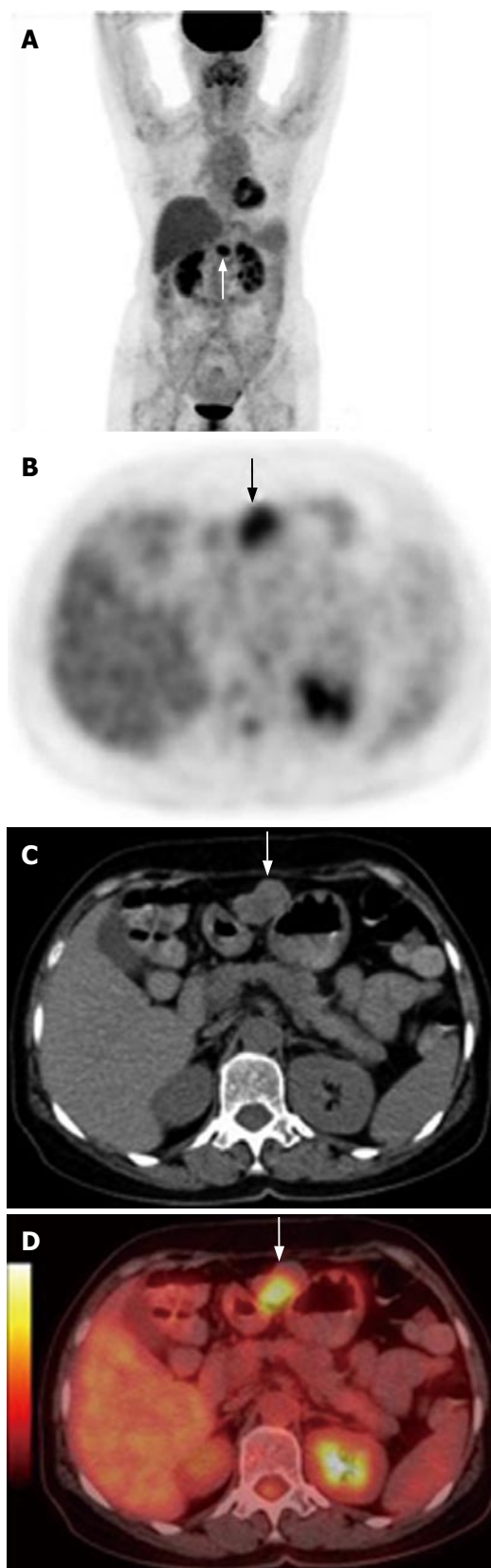


Figure 2 Whole body PET showing an isolated highly metabolic focus in the upper abdomen (white arrow) (A), non-enhanced CT (C) detecting a median density lesion in the omentum (white arrow), ¹⁸F-FDG PET (B) and fused imaging of PET/CT (D) revealing a highly metabolic lesion at the same position.

mor recurrence or extrahepatic metastasis. However, the sensitivity of conventional imaging techniques is limited in detecting recurrent diseases in such patients^[9]. 18F-FDG

PET/CT has proved to be an effective whole-body imaging technique that detects metabolic changes preceding structural findings. It was reported that tumor restaging with PET/CT can detect and localize disease recurrence among patients with no or mild symptoms and an elevated tumor marker level^[10]. PET/CT also can provide information about whether the detected disease is resectable. 18F-FDG PET/CT provides fused images that demonstrate the complementary role of functional and anatomic assessments in the diagnosis of cancer recurrence through the precise localization of suspected 18F-FDG uptake foci and their characterization as malignant or benign^[11].

TACE is a procedure involving injection of chemotherapeutic agents into the hepatic artery supplying the tumor, followed by embolization with lipiodol or gelfoam pieces. It appears that postoperative adjuvant hepatic arterial chemotherapy is more likely beneficial for preventing recurrence than preoperative TACE. To date, convincing evidence supporting the effectiveness of adjuvant therapy in preventing HCC recurrence is lacking. Validated information in the literature is limited in a paucity of randomized studies. It was reported that meaningful meta-analysis is impossible because of different therapeutic regimens used and the lack of stratification of patients according to the risk of recurrence^[12]. Better understanding of biological characteristics of HCC can lead to better outcomes of adjuvant surgical treatment^[13]. For example, if patients at a high risk of HCC recurrence could be identified before the resection, they would benefit from additional treatment modalities, such as neoadjuvant therapy, adjuvant therapy and biotherapy. It is not necessary for patients at a low risk of tumor recurrence to receive the above therapies^[14]. Molecular imaging in oncology is the noninvasive imaging of the key molecules and molecule-based events characterized by the genotype and phenotype of human cancer. Tumors with increased 18F-FDG uptake are more metabolically active and biologically aggressive. PET/CT with 18F-FDG would play a key role in evaluating recurrence risk of HCC before hepatectomy^[15]. Further research is warranted because of the limited number of reports and the absence of randomized trials.

Aggressive management with combined resection of isolated extrahepatic recurrence and re-resection or local therapy for intrahepatic recurrence may offer long-term survival in selected HCC patients. Our case demonstrates that 18F-FDG PET/CT provides the most significant additional information related to the accurate detection of extrahepatic spread of tumors and additional important information in patients with presumed resectable extrahepatic metastases. This test should be used for patients at a high risk of extrahepatic disease and should be evaluated prospectively for all patients under consideration for intra-

hepatic recurrence and extrahepatic metastasis resection.

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Meetings

MAJOR MEETINGS COMING UP

Meeting Falk Research Workshop: Morphogenesis and Cancerogenesis of the Liver
25-26 January 2007
Goettingen
symposia@falkfoundation.de

Meeting Canadian Digestive Diseases Week (CDDW)
16-20 February 2007
Banff-AB
cagoffice@cag-acg.org
www.cag-acg.org/cddw/cddw2007.htm

Meeting Falk Symposium 158: Intestinal Inflammation and Colorectal Cancer
23-24 March 2007
Sevilla
symposia@falkfoundation.de

Meeting BSG Annual Meeting
26-29 March 2007
Glasgow
www.bsg.org.uk/

NEXT 6 MONTHS

Meeting 42nd Annual Meeting of the European Association for the Study of the Liver
11-15 April 2007
Barcelona
easl2007@easl.ch
www.easl.ch/liver-meeting/

Meeting Falk Symposium 159: IBD 2007 - Achievements in Research and Clinical Practice
4-5 May 2007
Istanbul
symposia@falkfoundation.de

Meeting European Society for Paediatric Gastroenterology, Hepatology and Nutrition Congress 2007
9-12 May 2007
Barcelona
espghan2007@colloquium.fr

Digestive Disease Week
19-24 May 2007
Washington Convention Center, Washington DC

Meeting Gastrointestinal Endoscopy Best Practices: Today and Tomorrow, ASGE Annual Postgraduate Course at DDW
23-24 May 2007
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Meeting ESGAR 2007 18th Annual Meeting and Postgraduate Course
12-15 June 2007
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Meeting Falk Symposium 160: Pathogenesis and Clinical Practice in

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Meeting ILTS 13th Annual International Congress
20-23 June 2007
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www.ils.org

Meeting 9th World Congress on Gastrointestinal Cancer
27-30 June 2007
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Meeting 39th Meeting of the European Pancreatic Club
4-7 July 2007
Newcastle
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Meeting XXth International Workshop on Helicobacter and related bacteria in cronic degistive inflammation
20-22 September 2007
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Meeting Falk Workshop: Mechanisms of Intestinal Inflammation
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www.apdw2007.org

15th United European Gastroenterology Week, UEGW
27-31 October 2007
Le Palais des Congrès de Paris, Paris, France

Meeting The Liver Meeting® 2007 - 57th Annual Meeting of the American Association for the Study of Liver Diseases

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- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

In press

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci U S A* 2006; In press

Organization as author

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

Both personal authors and an organization as author

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

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- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; **42** Suppl 2: S93-99 [PMID: 12028325]

Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900]

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- 9 Outreach: bringing HIV-positive individuals into care. *HRS/A Careaction* 2002; 1-6 [PMID: 12154804]

Books

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- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

Chapter in a book (list all authors)

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

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- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ Cell Tumour Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

Conference paper

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

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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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