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AIM AND SCOPE

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Non-steroidal anti-inflammatory drugs: What is the actual risk of liver damage?

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) constitute a family of drugs, which taken as a group, represents one of the most frequently prescribed around the world. Thus, not surprisingly NSAIDs, along with anti-infectious agents, list on the top for causes of Drug-Induced Liver Injury (DILI). The incidence of liver disease induced by NSAIDs reported in clinical studies is fairly uniform ranging from 0.29/100 000 [95% confidence interval (CI): 0.17-0.51] to 9/100 000 (95% CI: 6-15). However, compared with these results, a higher risk of liver-related hospitalizations was reported (3-23 per 100 000 patients). NSAIDs exhibit a broad spectrum of liver damage ranging from asymptomatic, transient, hyper-transaminasemia to fulminant hepatic failure. However, under-reporting of asymptomatic, mild cases, as well as of those with transient liver-tests alteration, in conjunction with reports non-compliant with pharmacovigilance criteria to ascertain DILI and flawed epidemiological studies, jeopardize the chance to ascertain the actual risk of NSAIDs hepatotoxicity. Several NSAIDs, namely bromfenac, ibufenac and benoxaprofen, have been withdrawn from the market due to hepatotoxicity; others like nimesulide were never marketed in some countries and withdrawn in others. Indeed, the controversy concerning the actual risk of severe liver disease persists within NSAIDs research. The present work in-

tends (1) to provide a critical analysis of the dissimilar results currently available in the literature concerning the epidemiology of NSAIDs hepatotoxicity; and (2) to review the risk of hepatotoxicity for each one of the most commonly employed compounds of the NSAIDs family, based on past and recently published data.

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Key words: Non-steroidal anti-inflammatory drugs; Side effects; Fulminant hepatic failure; Cholestasis; Liver damage; Liver injury; Hepatitis; Hepatotoxicity

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INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) are consumed massively worldwide and, along with antimicrobial agents, are the most frequent causes of drug-induced liver injury (DILI)^[1-3]. Indeed, roughly 10% of total drug-induced hepatotoxicity is NSAIDs related. Recent data from England indicates that the relative frequency of NSAIDs prescription has changed, i.e. a 4% decline in the consumption of diclofenac and a parallel 5% increase in the use of naproxen are probably due to an increase in the awareness of both gastrointestinal and cardiovascular serious adverse events^[4]. The current policy concerning the use of NSAIDs recommends that patients take “the lowest effective dose for the shortest duration” needed to control symptoms. In this particular

setting evidence indicates that naproxen is associated with a lower thrombotic risk than coxibs, and that ibuprofen has a good thrombotic safety profile for daily doses of up to 1200 mg^[5]. Noticeably, 6% of the US population consumes at least one of these products in a month. Information regarding ibuprofen indicates that 24% is sold as over the counter medication^[6].

The paradox is that even when it has been known historically and widely accepted that the use of these NSAIDs is associated with a low rate of hepatic adverse events, it is severe liver damage that is the main adverse event through which these drugs are eventually withdrawn from the market^[7]. On the other hand, 50% of fulminant hepatic failure (FHF) in the USA is due to hepatotoxicity^[8]. Antimicrobial drugs are the most often involved^[9].

Hepatotoxicity is more frequently encountered in post-marketing studies or even after, once the drug has already been launched due to the fact that premarketing recruits a relatively low number of patients, frequently insufficient to fully judge the true incidence of hepatotoxicity^[10]. One of the most representative examples of this situation is that of bromfenac-related severe liver damage. Bromfenac was withdrawn from the market in 1998 as a result of multiple reports of FHF within the year of its approval (1 case for every 10 000 exposed individuals)^[11,12]. Frequent severe liver damage associated with nimesulide had also been reported^[13]. Although nimesulide is currently approved in 50 countries worldwide, national health authorities of several countries have withdrawn nimesulide from the market and others have never approved it^[14-16]. EMEA (European Medicines Evaluation Agency) currently recommends a restricted length of treatment (15 d) and a maximal drug dosage (100 mg/d) for nimesulide therapy, which in addition should be avoided in children.

Interestingly, NSAIDs-induced hepatotoxicity may be associated with liver injury six to nine times more frequently in patients who are taking other potentially hepatotoxic medications concomitantly. (i.e. amoxicillin-clavulanic acid, proton pump inhibitor, phenobarbital, isoniazid)^[17,18].

According to a recent study, hepatic steatosis and metabolic syndrome might predispose to an NSAIDs-induced liver toxicity^[19], a similar situation to that already proven for methotrexate and halothane.

Finally, chronic alcohol abuse during treatment with paracetamol was associated with an increase in liver injury induced by acetaminophen including those patients who were taking doses not higher than 4 g daily^[20].

Our attempt in the present work is two-fold (1) to analyze the clinical impact regarding severity of NSAIDs hepatotoxicity based on a critical analysis of the conflicting epidemiological approaches currently available in the literature; and (2) to review the individual expected risk of hepatotoxicity for NSAIDs most commonly employed in clinical practice based on past and recently published data.

EPIDEMIOLOGY

Epidemiological studies evaluating severe NSAID liver toxicity take into consideration hospitalization and

death of the exposed population. One important pitfall to demonstrate the risk of liver damage induced by NSAIDs resides in the epidemiological study design.

Generally speaking, the strengths of randomized control trials (RCT) are the close patient follow up, the comparison with a control group and an accepted design in order to prove specific therapeutic actions. However, RCT frequently represent just a sample of the general population seldom truly representative, particularly as evidence of the incidence of hepatotoxicity^[6]. In addition, trials frequently fail to be informative concerning populations under 18 years of age because this group of individuals is usually underrepresented when not openly excluded^[21]. On the other hand, data regarding incidence of liver toxicity from cohort, case-control studies and trials are not free from bias. Environmental factors, alcohol abuse, viral infections and metabolic factors coexist with DILI constituting confounding factors. Likewise, retrospective studies have important drawbacks such as researcher unawareness of concurrent drug intake other than the study drug. Other important limiting factors affect both pre- and post-marketing hepatotoxicity studies. Among these is the fact that mild and reversible cases are underreported. The other frequent source of error is the lack of a reliable “denominator” (defined as the number of patients potentially exposed) for calculations. We may also consider an additional source of underestimation of hepatotoxicity which is the report lag - the delay between the occurrence of adverse events and case communication. The latter should be taken into account when the alert is associated with severe liver damage. The incidence of liver disease induced by NSAIDs reported in most studies is fairly uniform ranging from 1 to 9 cases per 100 000 persons exposed^[21,22]. In spite of these results, epidemiologists analyzing the real risk of liver disease induced by different NSAIDs usually face difficulties, such as different study designs, different populations (ethnic groups, age and sex) and adjustment variable control methods that add complexity to the data analysis.

Traversa and co-workers analyzed a retrospective study from 1997 through 2001 in a region of Italy (850 000 inhabitants). About 2 million prescriptions corresponding to NSAIDs-treated patients through a 5-year period follow-up were analyzed^[23].

One of the main conclusions drawn from this study is that the risk of NSAIDs induced hepatotoxicity is very small (if the number of prescriptions is taken as the denominator, the incidence of liver injury was 1.7 per 100 000 exposed individuals). In contrast, a higher rate of hepatotoxicity was observed among people older than 75 years old (5.7-fold increased risk of liver disease when compared with people under 45 years). Interestingly, it was observed that nimesulide showed both a slightly higher incidence of liver damage and a higher hospitalization rate than that observed with the other NSAIDs (33 per 100 000 patient-years *vs* 22 per 100 000 patient-years respectively). The authors were unable to find severe liver damage and deaths related to NSAIDs. While the positive features of this study were the high number of patients

Table 1 Results of population-based studies showing liver toxicity induced by non-steroidal anti-inflammatory drugs

Authors	Study design	Incidence rate of hepatotoxicity (ALT \geq 3 ULN) per 100 000 patient-years (95% CI)
García Rodríguez <i>et al</i> ^[23] , 1992	Retrospective cohort study, cross over design	9/100 000 (6-15)
Traversa <i>et al</i> ^[23] , 2003	Retrospective cohort	1.4/100 000 (1.0-2.1)
de Abajo <i>et al</i> ^[24] , 2004	Retrospective population-based case-control study	2.4/100 000 (2.0-2.8)
Rubenstein <i>et al</i> ^[26] , 2004	Systematic review (hospitalization or death)	3.1 to 23.4/100 000
Rostom <i>et al</i> ^[21] , 2005	Systematic review	0.29/100 000 (0.17-0.51)
Laine <i>et al</i> ^[25] , 2009	Long-term prospective trial	2.1/100 000 (1.9-2.3)

ALT: Alanine transaminase; ULN: Upper limit of normal; CI: Confidence interval.

enrolled and the extended follow-up, the major limitations were that the database monitoring system neither included the reason for prescription nor NSAIDs doses.

In contrast, a case-control study that included prescriptions of all market-approved NSAIDs highlighted diclofenac as the only drug associated with an increased risk of liver damage (95% confidence interval: 1.9-8.8)^[24]. Laine and co-workers recently reported the largest prospective, randomized double-blind study comprising four times more patients than the largest previous trials. They evaluated the incidence of diclofenac-induced hepatotoxicity in 17 289 patients, showing that patients who suffered diclofenac-associated adverse drugs reactions seldom required hospital admission (23/100 000 patients). They also observed that symptoms of diclofenac-related liver disease developed either early or late after starting drug therapy^[25]. The conclusions were: a low rate of occurrence of diclofenac-related admissions, and a very low rate of diclofenac-associated FHF. Indeed, only one patient required liver hospitalization for every 132 patients with aminotransferase $> 3 \times$ upper limit of normal (ULN).

Rostom and co-workers investigated bibliographic databases MEDLINE and EMBASE and public FDA archives in order to identify randomized controlled trials of diclofenac, naproxen, ibuprofen, celecoxib, rofecoxib, valdecoxib, or meloxicam in adults with osteoarthritis or rheumatoid arthritis. The authors analyzed aminotransferase elevations > 3 ULN, liver-related drug discontinuation, serious hepatic adverse events, liver-related hospitalizations, and liver-related deaths^[21]. After analyzing 65 database articles and 67 FDA submitted studies, they concluded that diclofenac and rofecoxib had a higher level of transaminases both compared with placebo and with the other studied NSAIDs. Interestingly, none of these studies had a high rate of serious hepatic adverse events, hospitalizations or death.

The authors found only 1 hospital admission (naproxen) observed among 37 671 patients included in studies reporting hospitalization. This very low hospitalization rate represents 3 per 100 000 patients (0.5-15 per 100 000 patients). One patient died due to naproxen liver toxicity among 51 942 patients consuming NSAIDs, which in turn also represents a low death rate: 2 per 100 000 patients (0.3-11 per 100 000 patients).

These results are in concordance with those reported by Rubenstein and Laine who also analyzed several epi-

miologic studies designed to determine the incidence and risk of serious liver-related NSAIDs toxicity^[26]. Seven studies met the inclusion criteria proposed by the authors. They observed an incidence of liver toxicity associated with hospital admission ranging from 3.1-23.4/100 000 patient-years related to current use of NSAIDs, with an excess risk compared with past NSAIDs users of 4.8-8.6/100 000 patient-years of exposure. Moreover, these researchers documented zero mortality associated with NSAIDs when cumulative exposure of liver damage was analyzed in 396 392 patients/year.

Most of the information regarding the incidence and relative risk of hepatotoxicity associated with NSAIDs comes from cohort or case control studies and usually shows a low incidence of hepatotoxicity (Table 1).

Great efforts have been made to identify those clinical factors predictive of severe liver damage induced by drugs. Several years ago the FDA along with Representatives of Pharmaceutical Research and Manufacturers of America (PhRMA) and the American Association for the Study of Liver Disease (AASLD) constituted a working group to study how to minimize the risk of hepatotoxicity^[27]. Despite valuable effort, expert consensus could not be achieved concerning: (1) biochemical markers of liver injury to applied used in pre-marketing studies; and (2) clinical parameters able to predict severe liver injury. Yet today we still continue using transaminases level higher than three times the ULN as a marker of significant hepatocellular injury^[28].

CHARACTERISTICS OF NSAIDS INDUCED LIVER TOXICITY

The discovery of aspirin in 1946 followed by that of phenylbutazone was the beginning of the NSAIDs era. However, not until 1960 was indomethacin marketed. On the other hand, during the 1950s, ibuprofen was the second drug (along with aspirin) approved to be sold as over the counter medication. Interestingly, most of these substances were employed during the 60s, before the prostaglandin era.

The NSAIDs chemical classification recognizes four major groups of molecules: (1) carboxylic acids; (2) oxicams carboxamides; (3) sulphonanilides diaryl-substituted; and (4) pyrazole/furanones^[29]. From the clinical stand point NSAIDs induced hepatotoxicity is associated with

Table 2 Liver-related hepatotoxicity induced by non-steroidal anti-inflammatory drugs

Drug	Pattern of liver damage	Proposed mechanism	Incidence
Aspirin	Acute and chronic hepatitis Reye's syndrome	Dose dependent > with high dose	Low
Diclofenac	Acute and chronic hepatitis Mixed damage and pure cholestasis	Metabolic Immunologic	Low
Sulindac	Acute hepatitis and mixed injury	Hypersensitivity	Moderate
Ibuprofen	Acute hepatitis, ductopenia	Metabolic	Low
Naproxen	Cholestatic, mixed damage	Metabolic	Low
Coxibs	Acute hepatitis, mixed damage	Probably metabolic	Low
Oxicams	Acute hepatitis, massive and submassive necrosis, cholestasis and ductopenia	Metabolic	Low
Nimesulide	Acute hepatitis, pure cholestasis	Probably metabolic	Moderate

different patterns of clinical presentation, several mechanisms of liver damage and various pathological patterns.

We will only describe below the clinical characteristics of several NSAIDs that may be associated with a potential liver injury (Table 2).

Acetaminophen was not taken into account because most cases of liver damage are due to suicide attempts but a minority of cases are accidental and related to use of paracetamol as a therapeutic prescription. Acetaminophen-induced hepatotoxicity should be described separately and not within this topic.

ASPIRIN

Although liver toxicity induced by aspirin is considered to be dose-dependent, there is evidence that rheumatic patients may have predisposing conditions that may increase individual risk of liver damage. Hypoalbuminemia in patients with systemic lupus erythematosus and juvenile rheumatoid arthritis are two well documented risk factors for increased susceptibility to liver injury^[30,31]. In addition, studies in rats have shown that aspirin hepatotoxicity is more common in animals with experimentally-induced rheumatoid arthritis compared to that observed in those without arthritis^[32]. The clinical presentation of liver toxicity is often anicteric (jaundice at less than 5%) and transaminase levels correlate with serum salicylate levels ($> 25 \text{ mg}/100 \text{ mL}$)^[33]. Focal nonspecific necrosis, hepatocellular degeneration and hydropic changes are commonly seen in liver histology^[34]. Aspirin can also produce a mitochondrial dysfunction pattern that may lead to a liver free fatty acid accumulation and subsequently develop into a severe metabolic disorder associated with hepatic massive micro-steatosis. This syndrome, known as Reye's Syndrome, is characterized by metabolic acidosis, hepatic encephalopathy, hypoglycemia, coagulopathy and azotemia. Reye's disease induced by aspirin is a rarity since aspirin is currently avoided in pediatric patients and replaced by ibuprofen and paracetamol.

A recent experimental study in rats suggests that salicylic acid could trigger mitochondrial dysfunction causing a marked fall in intracellular ATP which in turn leads to a lethal hepatocellular injury mediated by a lipid peroxidation mechanism^[35].

DICLOFENAC

Diclofenac is the most widely used NSAID in the world^[36,37]. The vast majority of data related to hepatic reactions comes from retrospective studies. There were no more than 60 cases of diclofenac hepatotoxicity reported in the literature until Banks and co-workers in 1995 reported their analysis of 180 cases referred to FDA from 1988 through 1991. The authors observed evidence of liver disease in 85% of the patients within the first 6 mo after drug intake. Interestingly, a higher latency (after 6 mo) was observed in 12% of cases^[38]. The long latency period observed in a large number of cases in addition to the absence of hypersensitivity support a metabolic mechanism of hepatotoxicity. Jaundice was a very common sign present in 90 out of 120 patients. A total of 7 jaundiced patients died as a result of liver disease. In this analysis liver function tests (LFTs) showed a mixed (hepatocellular and cholestatic) pattern in 66% of cases, cholestatic in 8% and indeterminate in the remainder of the group. In contrast to Jick's point of view which suggests that hepatotoxicity induced by diclofenac is an uncommon event^[39], the study by Banks proposed for the first time that diclofenac is a much more common cause of liver damage than so far suspected^[38]. This concept was reinforced in a recent report from Laine who conducted the longest and largest liver-related diclofenac study so far ever reported [Multinational Etoricoxib and Diclofenac Arthritis Long-Term (MEDAL) program]^[25]. They conducted a prospective, randomized and double-blind study analyzing the frequency of diclofenac induced liver reactions in 17 289 patients. The authors randomized only those osteoarthritis or rheumatoid arthritis patients over 50 years of age who were going to receive diclofenac therapy (150 mg daily) or etoricoxib (60 or 90 mg daily). They also excluded cases with previous liver disease or more than 14 weekly alcohol drinks. Hypertransaminasemia $> 3 \times \text{ULN}$ was observed in over 3% of arthritic patients with a regular intake of diclofenac. It is interesting to point out that alanine transaminase (ALT) values higher than $10 \times \text{ULN}$ were only identified in 0.5% of cases^[25]. The incidence of diclofenac liver-related hospitalizations in this study was 16 per 100 000 patient-years. Through these results, the authors provide evidence that diclofenac is a very safe drug. The

small number of related hospitalizations (0.023%), and Hy's cases (transaminases $> \times 3$ ULN and bilirubin > 2 ULN or a fatal outcome or liver transplantation) (0.012%) are the strongest evidence showing that diclofenac bears a low liver toxicity rate.

These data are in concordance with those observed by Traversa and co-workers showing ibuprofen and naproxen also displayed a low hepatotoxicity rate. However, in this latter study nimesulide had a higher hospitalization rate when compared with a past control cohort (33 per 100 000 patient-years)^[23]. In a systematic analysis of several randomized studies, Rostom and co-workers also found a very low frequency of hospital admissions due to NSAIDs related-liver disease in 4261 patients (3 per 100 000 patient) and 0% when diclofenac was evaluated separately^[21].

Diclofenac is a typical example of the combination of factors resulting in hepatotoxicity (e.g. drug metabolism, reactive metabolite formation and clearance) determining the actual development and the severity of liver damage. Moreover, diclofenac might produce liver injury through either metabolic idiosyncrasy or an immunological mechanism generated by drug adduct formation^[8].

SULINDAC

Sulindac (SLD) is associated with an increased incidence of liver toxicity and serious hepatic reactions (5-10 times) when compared with other NSAIDs. Besides its recognized anti-inflammatory mechanism through the inhibition of cyclooxygenase (COX 1 and 2), SLD became very popular due to its antiapoptotic effect in colonic polyposis treatment^[40]. SLD induced hepatotoxicity was more frequently encountered among people over 50 years old. Regarding the mechanism of hepatotoxicity, SLD associated DILI is one other example of a combination of factors as judged by the conflicting results present in the literature. Indeed, in a series of 91 documented cases reported to FDA a predominantly cholestatic pattern was present in 43% while hepatocellular-related changes were found in 25% of patients^[41]. Moreover, a hypersensitivity mechanism of liver injury was present in 60% of cases. Zou *et al*^[42] recently reported an interesting pilot study showing that co-treatment of SLD and lipopolysaccharide (LPS) caused liver injury in rats. In this context they also found a selectively clotting system activation and fibrinolytic system inhibition in rats treated with SLD/LPS. These changes were also associated with tislular hypoxia and fibrin clot deposit in the hepatic sinusoids^[42]. Researchers suggest that these results may be extrapolated to humans with the disease. Furthermore, they hypothesize that hypoxia in the frame of the SLD/LPS association may underlie the idiosyncratic model where the sulfide metabolite probably plays a central role.

Another experimental study carried out by the same authors shows that tumor necrosis factor (TNF)- α augmented the cytotoxicity of SLD sulfide in primary hepatocytes and HepG2 cells. These results suggest that TNF- α can enhance SLD sulfide-induced hepatotoxicity, thereby

contributing to liver injury in SLD/LPS-cotreated rats^[43].

Regrettably the current coexistence of conflicting and heterogeneous results precludes us from reaching valid conclusions regarding SLD DILI.

IBUPROFEN

Ibuprofen has a recognized anti-inflammatory, analgesic and antipyretic property and is one of the most commonly NSAIDs used worldwide. It is characterized by a high safety profile and very low liver toxicity incidence. Along with paracetamol and aspirin, ibuprofen has become one of the largest ever selling over the counter drugs. It was first introduced to the UK market in 1969 and due to the low rate of gastrointestinal adverse events, it has ever since almost replaced aspirin, indometacin and phenylbutazone in arthritic patients^[44]. A scarce number of hepatotoxicity reports involving ibuprofen were published, associated to both hepatocellular and cholestatic liver damage. Indeed, one of the latter cases was linked to vanishing bile duct syndrome^[45,46]. It has also been suggested that ibuprofen may increase the risk of liver injury when administered to patients with chronic hepatitis C. An ibuprofen associated increase of transaminases $> 5 \times$ UNL was recently reported in three patients with chronic hepatitis C, eventually confirmed by re-challenge^[47].

Traversa *et al*^[23] in their cohort study that recruited thousands of patients receiving various NSAIDs confirmed that ibuprofen has a very low liver toxicity rate: Only two patients showed ibuprofen-associated liver injury (out of 126 cases that had NSAIDs). Despite the massive use of this drug worldwide, a low rate of ibuprofen liver toxicity along with a low incidence of gastrointestinal, renal and cardiovascular serious events characterizes the safety profile. This is probably based on the fact ibuprofen has a short plasma half life and does not form pathological metabolites. The absence of reports including ibuprofen induced liver injury in several studies and meta-analyses, strongly suggests that ibuprofen is an unlikely cause of liver disease^[48-50].

COXIBS

Coxibs are NSAIDs designed to selectively inhibit COX-2. Lately, this group of drugs has been gaining worldwide popularity due to a much better gastrointestinal safety profile when compared with nonselective NSAIDs^[25]. However, NSAIDs-induced cardiovascular adverse events have generated significant controversy^[51,52]. Due to increased risk of myocardial infarction and arterial hypertension, rofecoxib was removed from the market by Merck in 2004^[53]. Valdecoxib was also voluntarily discontinued by manufacturer Bextra, Pfizer Canada Inc and by FDA in 2005 because of severe allergic skin lesions including cases of Stevens-Johnson syndrome and also because of an increased risk of heart and stroke attack^[54]. On the other hand, celecoxib continues to be marketed in many countries. Laine and coworkers recently published a comprehensive evaluation of the literature up to 2007

analyzing controlled trials, meta-analyses and reviews related to the safety profile of selective inhibitors of COX-2 in patients with osteoarthritis (OA)^[55]. Their results are truly encouraging and show that coxibs have a therapeutic efficacy comparable to other NSAIDs and higher than that of acetaminophen. This was observed when patients with OA were treated for moderate to severe degree of pain. Through meta-analysis the authors documented that coxibs have a 74% lower risk of gastro-duodenal ulcer complications. In contrast, they found a double risk of myocardial infarction vs placebo and naproxen treated patients. There were no significant differences in cardiovascular risk between coxibs compared with non-naproxen NSAIDs. Despite these encouraging results, FDA reported an alert related to the increase of cardiovascular risk induced by coxibs.

Coxib-induced liver injury is an uncommon event and the annual reported incidence of hepatotoxicity is 1 in 100 000 exposed persons^[56]. A long term study evaluating the safety profile of celecoxib in arthritics patients (Celecoxib Long-term Arthritis Safety Study-CLASS) found increased transaminase level in 0.6 % patients^[57].

A rise of transaminase level was also reported and associated with rofecoxib (2%) and with higher doses of lumiracoxib (3%). In this study, a higher frequency of clinical hepatitis was found in patients receiving 400 mg of lumiracoxib when compared with ibuprofen and naproxen treated patients^[58]. In another survey, lumiracoxib was linked to severe hepatocellular necrosis^[59]. Health authorities from United Kingdom subsequently removed it from the market. Scottish authorities reported 20 cases of severe liver disease probably associated with lumiracoxib. FHF was documented in 14 out of 20 patients (two patients died and three patients needed liver transplantation). In addition, recent postmarketing reports from Australia of severe hepatic reactions with lumiracoxib at doses of at least 200 mg (two patients died and 2 underwent liver transplantation) led also to its withdrawal from the market in that country^[60].

On the other hand, rofecoxib was associated with a low rate of hepatic reactions and an increase of ALT $\geq 3 \times \text{UNL}$ was documented in 1.8/100 000 exposed persons^[55], but despite this excellent safety profile, Yan and colleagues reported two well-documented cases of cholestatic hepatitis induced by rofecoxib^[61]. The first patient showed a high level of alkaline phosphatase (APH) associated with hepatocellular injury in zone 1 whereas in the other case a significant increase in ALT associated with a minimum increase of APH was documented. In this latter patient mild liver damage in both acinar zones 1 and 3 was described. In contrast, four other published cases of rofecoxib induced liver toxicity were characterized by predominant cholestatic presentation^[62,63].

To our knowledge no case of etoricoxib severe hepatotoxicity has ever been published. However, transaminase elevation ($3 \times \text{ULN}$) has been reported in 1% of patients treated with etoricoxib for up to one year^[21,64]. The mechanism of liver damage induced by coxibs has not yet been completely elucidated. Kung and co-workers recently

suggested that the bioactivation of lumiracoxib and its metabolite [4'-hydroxylumiracoxib (M5)] may produce GSH depletion, covalent binding to proteins and oxidative stress, that in turn may lead to liver injury^[65].

Nevertheless, hepatotoxicity caused by non-selective NSAIDs has been more extensively studied. Furthermore, mitochondrial injury, cholestasis and oxidative stress induced by a reactive metabolite formation constitute the most conspicuous molecular reported disorders. An attractive hypothesis suggests that NSAIDs inhibition of COX-2, might cause liver damage through a prostaglandin (PGs) pathway. Regarding this concept the authors proposed that the inhibition of PGE2 could down regulate the antiapoptotic mitochondrial protein Bcl-2, which protects against bile acid induced apoptosis^[66].

OXICAMS

Oxicams induced hepatotoxicity is an uncommon clinical situation. Piroxicam induced severe hepatocellular necrosis was the most frequent reported clinical pattern^[35,67,68]. Uneventful recovery, death and need of liver transplantation have all been reported^[69,70]. On the other hand, a clinical and histological pattern of hepatocellular plus cholestatic (mixed) injury was also associated with piroxicam in one single case. LFTs normalization was observed in this patient within 4 mo after starting the clinical disease^[71]. Interestingly, a case of severe intrahepatic cholestasis linked to a long period of piroxicam intake, was originally described 20 years ago^[72].

Only two patients with piroxicam-induced liver toxicity were admitted to our liver unit during the last three decades (unpublished data). The first patient was a 44-year-old woman with clinical and histological evidence of submassive necrosis. The patient developed ascites and liver failure after 28 d of drug intake and uneventfully recovered without liver transplantation in 95 d from clinical onset. The other case was a 42-year-old man admitted with clinical and biochemical manifestations of severe cholestasis after piroxicam treatment as the single medication for 58 d. Liver histology showed a typical hepato-canalicular cholestasis associated with ductopenia (Figure 1). This latter case progressively evolved to prolonged cholestasis with asthenia, anorexia and intolerable itching. He underwent corticosteroids therapy for 30 d. We were able to document a complete biochemical recovery 120 d from the clinical onset.

Other oxicam derivatives were also occasionally implicated in cases of acute cholestatic hepatitis included isoxicam and droxicam^[73].

The mechanism of oxicams-induced hepatotoxicity appears to be idiosyncratic and dose independent. Due to the absence of immunoallergic features in most of the reported cases, it is very difficult to support an immune-mediated mechanism of liver injury^[74].

NIMESULIDE

Nimesulide has analgesic, anti-inflammatory and anti-

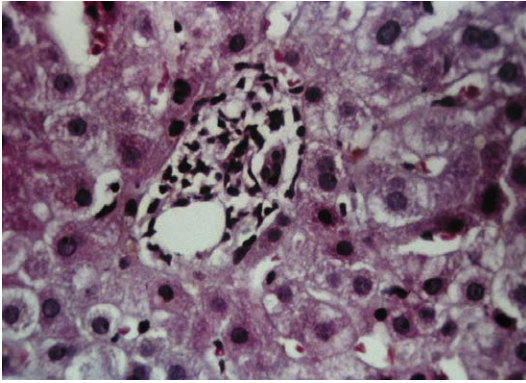


Figure 1 Piroxicam induced hepato-canalicular cholestasis associated with ductopenia (notice absence of bile duct in the portal tract).

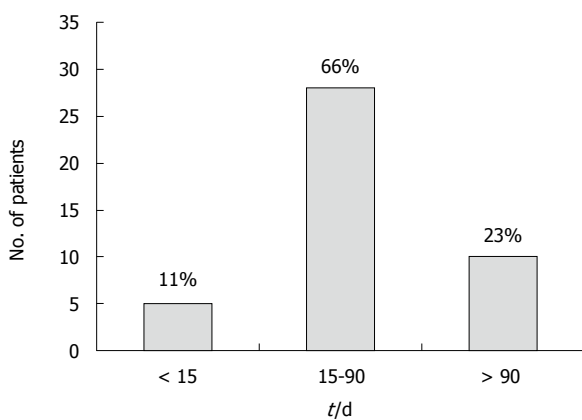


Figure 2 Latency (time from nimesulide intake to clinical onset) in 43 patients.

pyretic activity due to potent inhibitory effects on the COX-2 enzymes. Nimesulide bears a good gastro-intestinal tolerance. The mechanism of action has been attributed to a unique chemical structure of the sulphonanilides class of NSAIDs^[75].

Our group in Argentina reported the first observation linking nimesulide with liver toxicity in 1997^[76]. Since then, a steady flow of reports confirmed severe forms of hepatotoxicity, to the point that national health authorities of several countries withdrew nimesulide from the market^[77-88]. Despite this, nimesulide commercialization is still maintained in several European countries, although the EMEA reports recommend a length of therapy restricted to 15 d and maximal dosage of 100 mg/d^[13]. Controversy regarding nimesulide persists due to the fact that clinical series reports and epidemiological trials continue to involve nimesulide in severe liver damage^[19,89,90]. On the other hand, health institutions conclude that nimesulide-induced-liver injury is statistically comparable to that of the remainder of the NSAIDs^[91,92].

In our institution, 5 out of 30 cases (17%) had severe liver injury^[93]. In 2009 our series included 43 well documented cases of nimesulide-induced liver damage associated to a wide clinical and histological spectrum of hepatotoxicity^[94]. To our knowledge this constitutes the largest series of nimesulide hepatotoxicity ever reported. The

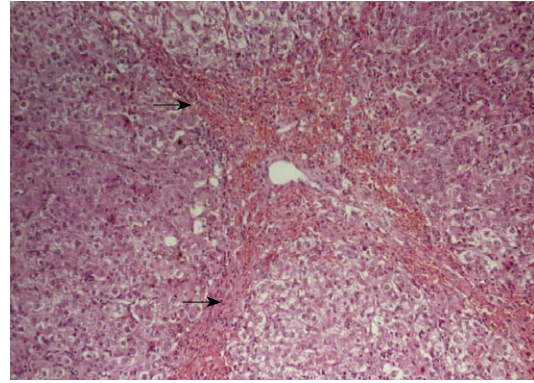


Figure 3 Acute hepatitis induced by nimesulide (hepatocellular collapsed areas are shown with arrows).

main clinical symptoms at presentation are jaundice (70%), malaise (65%) and pruritus (50%). Interestingly, two thirds of patients start liver toxicity 15 to 90 d after drug intake. Relevant to drug safety, in only 11% latency was shorter than 15 d (Figure 2). On the other hand, normalization exceeded 90 d in 27% of cases^[94]. In cholestatic liver injury, normalization of alkaline phosphatase serum level usually takes more time than transaminases (i.e. more than 1 year)^[95].

Nine patients in our series developed severe liver disease and FHF was observed in 6 cases. In agreement with the recent publication by Walker *et al*^[96], this subpopulation was composed predominantly of females older than 50 years. Two patients died before liver transplant due to multiorgan failure, while a 9-year-old girl successfully underwent orthotopic liver transplantation.

We observed a wide range of variations of ALT/aspartate transaminase level in concordance with Bjarnason who analyzed 33 case reports documenting an elevation of ALT of at least 2-fold in 100%, and a 5-fold elevation in 89% of patients^[97].

Nimesulide hepatotoxicity shows a wide spectrum of liver damage including acute hepatitis, cholestasis, mixed forms, massive and submassive hepatic necrosis. We found hepatocellular necrosis (Figure 3) in 64%, cholestatic hepatitis in 27% and pure cholestasis in 9%^[94].

The mechanism of nimesulide induced hepatotoxicity remains unknown. It has been suggested that it could be due to the formation of a reactive metabolite. On the other hand, individual genetic variations in drug metabolism have also been proposed.

Acknowledging the true impact of nimesulide on the liver is not an easy task. Despite the proliferation of reports describing nimesulide-induced severe liver injury (mainly Argentina, Ireland and Finland), the epidemiological studies have almost unanimously concluded that severe hepatotoxicity is of low incidence determining a positive risk-benefit ratio. Inquiring about nimesulide intake should be incorporated into standard anamnesis of liver disease, especially when acute liver damage is being investigated.

Addendum

Other than the previously analyzed drugs, indomethacin,

naproxen, meloxicam, tenoxicam and etodolac have also been associated with various hepatic reactions^[73].

CONCLUSION

Aspirin was the first discovered NSAID. Dose dependent liver injury is accepted as the prevalent mechanism. Liver toxicity rate is very low currently since aspirin has been replaced by paracetamol and ibuprofen in pediatric patients and in various rheumatic diseases.

Diclofenac is probably the most used NSAID in rheumatology. Severe liver reactions and diclofenac hospitalization rate are uncommon. An increase in ALT levels of $3\text{--}10 \times \text{ULN}$ is observed in 3% of cases.

Sulindac induced hepatotoxicity was documented more than fifty years ago. Liver damage occurrence was reported to be 5-10 times higher than that of other NSAIDs. A hypersensitivity mechanism of liver injury was the most prevalent liver reaction.

Ibuprofen has the highest liver safety profile among NSAIDs and showed no severe liver injury in larger studies. Along with paracetamol and aspirin, it is considered one of the most common over the counter NSAIDs sold in the world.

Coxibs have currently replaced several NSAIDs due to safer GI profile. However, the high rate of cardiovascular events associated to rofecoxib is the main drawback related to drug marketing. Despite liver damage being a rare clinical situation, lumiracoxib has been discontinued in several countries due to severe hepatotoxicity.

Oxicams are associated with a well-documented hepatic safety profile. Uncommonly, piroxicam may cause severe hepatocellular damage. The clinical and histological pattern may be mixed or associated to clinical and biochemical prolonged cholestasis with or without ductopenia. Isoxicam and droxicam were only linked to liver toxicity in sporadic reports. The mechanism of liver damage appears to be an idiosyncratic one.

Nimesulide was removed from the market in several countries due to severe liver damage described in clinical series, but various epidemiological surveys do not document these findings. EMEA recommends that nimesulide should only be used for short periods at daily doses not higher than 200 mg/d in adults.

In summary, neither documenting the possibility of the causative role of a drug when confronting liver damage in an individual patient nor determining the true incidence of NSAIDs induced hepatotoxicity in the general population, are easy tasks. Rigorous data collecting, caution and clinical commitment are required when judging potential hepatotoxicity. The clinician always needs to critically evaluate the possibility whether other factors may play a role in the actual findings.

Despite the shortage of well-designed epidemiological studies, there is evidence showing that most of the NSAIDs are safe drugs with low risk of liver injury (mostly ranging from 0.29-3.1/100 000 exposed individuals when recent based-population studies were analyzed).

Both sulindac and nimesulide have been linked to a higher frequency of liver damage. NSAIDs induced liver injury which might potentially lead to a fatal outcome or need liver transplantation. As in other forms of DILI, jaundice entails poor prognosis with 25% of jaundiced patients developing severe liver disease. Drugs with an increased risk of liver damage should undergo close LFTs monitoring in order to prompt drug withdrawal to avoid severe hepatotoxicity.

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Controversy of hand-assisted laparoscopic colorectal surgery

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Abstract

Laparoscopically assisted colorectal procedures are time-consuming and technically demanding and hence have a long steep learning curve. In the technical demand, surgeons need to handle a long mobile organ, the colon, and have to operate on multiple abdominal quadrants, most of the time with the need to secure multiple mesenteric vessels. Therefore, a new surgical innovation called hand-assisted laparoscopic surgery (HALS) was introduced in the mid 1990s as a useful alternative to totally laparoscopic procedures. This hybrid operation allows the surgeon to introduce the non-dominant hand into the abdominal cavity through a special hand port while maintaining the pneumoperitoneum. A hand in the abdomen can restore the tactile sensation which is usually lacking in laparoscopic procedures. It also improves the eye-to-hand coordination, allows the hand to be used for blunt dissection or retraction and also permits rapid control of unexpected bleeding. All of those factors can contribute tremendously to reducing the operative time. Moreover, this procedure is also considered as a hybrid procedure that combines the advantages of both minimally invasive and conventional open surgery. Nevertheless, the exact role of HALS in colorectal surgery has not been well defined during the advanced totally laparoscopic procedures. This article

reviews the current status of hand-assisted laparoscopic colorectal surgery as a minimally invasive procedure in the era of laparoscopic surgery.

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Key words: Colorectal surgery; Laparoscopic assisted colorectal surgery; Hand assisted laparoscopic colorectal surgery

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INTRODUCTION

Since its introduction in 1991, the number of performed laparoscopic colorectal surgery has remained a minority^[1-3]. This was attributed to the fact that such procedures are time-consuming, technically demanding and have a long steep learning curve^[4]. In the technical demand, surgeons need to handle a long mobile organ, the colon, and has to operate on multiple abdominal quadrants most of the time with the need to secure multiple mesenteric vessels. However, the most important deterring reason was the fear that laparoscopic colectomy was considered oncologically unsound in the management of colorectal cancers^[5]. Therefore, a new surgical innovation called hand-assisted laparoscopic surgery (HALS) was introduced in the mid 1990s as a useful alternative to totally laparoscopic procedures^[6-8]. This hybrid operation allows the surgeon to introduce the non-dominant hand into the abdominal cavity through a special hand port while maintaining the

pneumoperitoneum^[6,7]. This innovative technique met with fierce resistance and its validity was questioned. Nevertheless, there has been an upsurge in the performance of both laparoscopic and hand-assisted laparoscopic colectomy (HALC) over the past 5 years. The reasons are the introduction of more versatile laparoscopic instruments, the introduction of new vessel-sealing devices like the harmonic scalpel and Ligasure, and various laparoscopic endo-staplers. This has enabled surgeons to perform laparoscopic colorectal procedures without the need for a single intracorporeal knot or suture. Another important factor that has contributed tremendously to this upsurge is the emergence of a level I evidence confirming that the laparoscopic colorectal technique is as oncologically sound as the open procedure^[9,10]. The European Cooperation in Science and Technology (COST) multi-institutional study suggested that the laparoscopic approach is an acceptable alternative to open surgery for colon cancer with a similar rate of recurrent cancer after laparoscopically assisted colectomy (LAC) and open colectomy^[9]. However, the questions remain: is HALC an alternative to LAC? Is it second best? Or are they complementary to each other? This article reviews the current literature in an attempt to demonstrate the status of hand-assisted laparoscopic colorectal surgery as a minimally invasive procedure in the era of laparoscopic surgery.

ARGUMENT FOR HALC

The proponents of HALC claim that a hand in the abdomen will restore the tactile sensation which is usually lacking in laparoscopic procedures. It also improves the eye-to-hand coordination, allows the hand to be used for blunt dissection or retraction and also permits rapid control of unexpected bleeding^[6-8,11,12]. All of those factors can contribute tremendously in reducing the operative time. Moreover, HALC is also considered as a hybrid procedure that combines the advantages of both minimally invasive and conventional open surgeries. It is also strongly argued that if an incision is needed to extract the resected specimen at the end of the laparoscopic procedure, then such an incision may be inflicted earlier in the procedure and be utilized as a hand port. However, this new innovation was not quickly embraced and has been fiercely rejected by the surgical community.

ARGUMENT AGAINST HALC

The opponents argued that introducing a hand in the abdomen during any laparoscopic procedure violates the fundamental principles of minimally invasive surgery (MIS) and makes maintenance of pneumoperitoneum difficult. Furthermore, if this new surgical innovation is adopted, one may witness the birth of a new generation of surgeons who is reluctant to learn totally laparoscopic techniques; a generation who will be offering their patients a “second” best procedure^[13]. Also, this new innovation is more aggressive and traumatic as the incision for the hand insertion and specimen extraction is inflicted earlier

in the operation with persistent and continuous stretch and compression on the wound. It is also speculated that the increased handling and mobilization of the bowel will result in the development of postoperative ileus and intra-abdominal adhesions. Moreover, there is uncertainty about the long-term results such as development of adhesive small bowel obstruction and ventral hernias, *etc.* Other arguments focused on the cost incurred by the use of the hand port, the comparatively larger size of the hand port and the extraction incision, obstruction of the operative view by the inserted hand and the ergonomics of this technique as up to 20% of surgeons reported forearm fatigue and wrist pain at the end of the procedure^[14]. Hence, it is difficult to convince experienced laparoscopic surgeons to introduce a hand in the abdomen in order to speed up the procedure and it remains questionable whether there is actually a need for them to do so^[13].

INDICATIONS AND CONTRAINDICATIONS OF HALC

HALC can be offered to all patients who are undergoing any form of colorectal resection for benign as well as malignant conditions. The procedure is best suited for the obese especially those with body mass index (BMI) of 40 or more, as the conversion rate is high if the procedure is conducted laparoscopically^[13]. HALC is also indicated in cases where the pathology is bulky and whenever the laparoscopic surgeon is contemplating conversion of the laparoscopic procedure to an open technique due to unexpected difficulties during the procedure^[13]. Similarly, it can be utilized whenever the surgeon encounters difficulty or wants to speed up the operation pace in areas where there is laparoscopic technical difficulties such as taking down the splenic or hepatic flexures. It can also be considered in cases of total colectomy when an hour saving in the operating time can be gained^[13]. However, there is a doubtful advantage of HALC in low rectal surgery over the laparoscopic technique.

Therefore, generally speaking, HALC should be avoided in patients with low BMI, thin patients with a small abdomen, and in pediatric patients. It is also contraindicated when the pathology is non-bulky and the surgeon's hand is huge.

HAND PORTS

Hand ports facilitate the hand insertion; act as specimen retrieval site and also as a wound protector. They further serve as portals for construction of extracorporeal anastomoses and can also serve as laparoscopic trocar sites. The latter permits selective use of HAL and laparoscopically assisted (LA) techniques at various times during the same operation. The first generation of hand ports was cumbersome, and allows loss of pneumoperitoneum due to gas leak. This gave HALS a bad reputation in its early days. However, newer hand ports devices have better sealing mechanisms and are more user friendly abolishing



Figure 1 An operative picture showing the placement of the hand port and other trocars.

the initial criticism of the old hand ports. Currently, there are varieties of hand ports, but the most commonly used are LapDisc (Johnson and Johnson Endosurgery, USA) (Figure 1) and Gelport (Covidien, Autosuture, USA)^[14,15].

Some new special finger mounted surgical instruments that may help some delicate steps of surgical procedures such as intracorporeal dissection and cutting, have been designed for HALS. However, there have been few reports about their efficiency and usefulness in clinical practice.

HALS VS OPEN COLECTOMY

There is no doubt that HALC is far superior to open colectomy (OC) as it preserves the advantages of MIS. There are three randomized controlled trials (RCT) that compared HALC and OC surgery^[16-18]. The first RCT that compared two well matched groups: 41 patients with HALC *vs* 40 with OC undergoing elective management of right-sided colonic cancer^[16] found that HALC took significantly longer time to perform (110 min *vs* 97.5 min, $P = 0.003$), but resulted in significantly less blood loss (35 mL *vs* 50 mL, $P = 0.005$) and was associated with significantly less pain, less parenteral and enteral analgesia^[16]. Moreover, patients receiving HALC recovered faster, and had a shorter length of hospitalization (7 d *vs* 9 d, $P = 0.004$). The 5-year survival rate was similar between the two groups (83% *vs* 74%, $P = 0.90$)^[16].

The second RCT evaluated the postoperative recovery after HAL ($n = 30$) *vs* open ($n = 30$) restorative proctocolectomy with ileal pouch anal anastomosis for ulcerative colitis and familial adenomatous polyposis^[17]. The operating time was longer in the HAL group compared with the open group (210 and 133 min, respectively; $P < 0.001$). However, there were no significant differences in either narcotics requirement or morbidity or postoperative hospital stay (20% *vs* 17%, and 10 d *vs* 11 d, respectively). Moreover, there was no difference between the two procedures in quality of life (QOL) assessment score in the 3 mo after surgery. However, the HAL procedure was more costly than the open procedure^[17].

In the third RCT, Kang *et al*^[18] randomized 60 patients

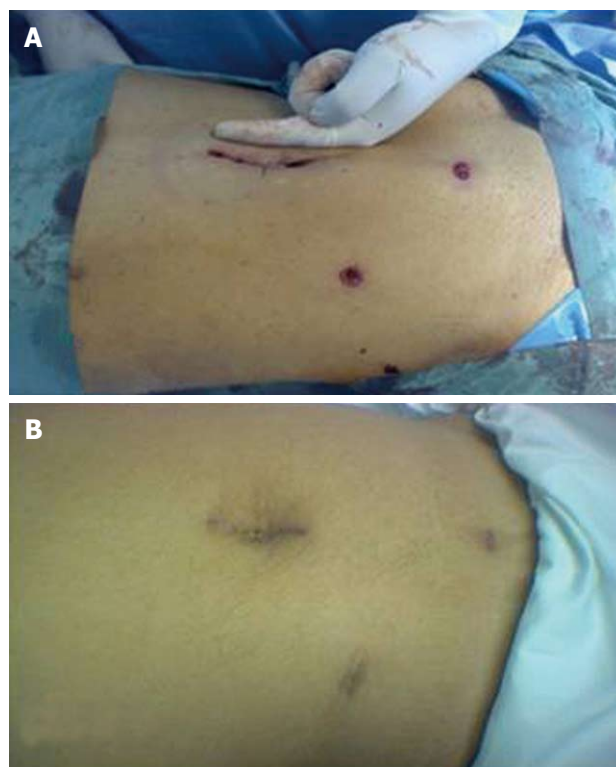


Figure 2 Cosmetic results after hand-assisted laparoscopic colectomy. A: It shows the hand port site closure and the trocar sites at the conclusion of hand-assisted laparoscopic sigmoid colectomy; B: It shows the operative wounds 2 mo after the procedure. Note the shrinkage of the port site scar and the acceptable cosmesis.

into two well-matched groups: HALC ($n = 30$) and OC ($n = 30$) for the management of benign or malignant colorectal diseases. The patients undergoing HALC had a significantly shorter hospital stay and incision length, faster recovery of gastrointestinal function, less analgesic use and blood loss, and lower pain scores on the 1st, 3rd and 14th postoperative days. Moreover, there were no significant differences in operating time, complications, or time to recover to normal^[18].

A fourth comparative study that aimed to compare the QOL, functional outcome, body image, and cosmesis after hand-assisted laparoscopic (HALRPC) *vs* open restorative proctocolectomy (ORPC) in 53 patients who completed the QOL and functional outcome questionnaires^[19] found no differences in the functional outcome, morbidity, or QOL between the two groups. However, at a median of 2.7 years after surgery, the body image and cosmesis scores of female patients were significantly higher in the HALRPC group^[19].

In summary of the above RCTs (Table 1), HALC takes longer time, but is associated with less blood loss, less pain, faster postoperative recovery with a shorter length of hospital stay and incision than OC. Furthermore, there is no difference in the complication occurrence, and HALC is associated with higher body image and cosmesis scores in female patients (Figure 2A and B), but is more costly than OC.

Table 1 Published studies comparing hand-assisted laparoscopic colorectal surgery with open colorectal surgery

Study, yr	Patients (HALC vs open)	Indication	Procedure	OT (min)	Blood loss (mL)	Analgesia narcotics or POD	Diet (POD)	Complications	LOS (d)
Maartense <i>et al</i> ^[17] , 2004	30 vs 30	UC and FAP	RPIPAA	214 vs 133	262 vs 300	30 mg vs 31 mg	6 vs 7	20% vs 17%	10 vs 11
Chung <i>et al</i> ^[16] , 2007	41 vs 40	Cancer	R. colectomy	110 vs 97	35 vs 50	19 mg vs 54 mg	3 vs 3	9.7% vs 22.5%	7 vs 9
Kang <i>et al</i> ^[18] , 2004	30 vs 30	Benign and malignant CR diseases	Colectomies (R, L and total), AP	169 vs 172	193 vs 84	2.6 d vs 3.3 d	3.7 vs 4.4	13% vs 30%	8 vs 10

UC: Ulcerative colitis; FAP: Familial adenomatous polyposis; RPIPAA: Restorative proctocolectomy and ileal pouch-anal anastomosis; OT: Operative time; POD: Postoperative day; HALC: Hand-assisted laparoscopic colectomy; R: Right; L: Left; AP: Anterior resection; CR: Colorectal.

HALC VS LAC

A review of the literature yielded 8 important studies which compare HALC and LAC; 4 randomized trials^[14,20-22], 2 prospective non-randomized^[23,24] and 2 large retrospective studies^[10,25]. The HALS study was conducted by 10 surgeons from Europe and America and included only 40 patients who were randomized into HALC (18 patients) and LAC (22 patients)^[14]. This study, though small in size, found no significant difference between the two groups in term of operating time (142 min vs 151 min), length of incision (7.4 cm vs 7.0 cm), rate of major complications and length of hospital stay (7 d vs 6 d). However, there were fewer conversions in the HALC group (14% vs 22%). The HALS study group concluded that HALC retains the benefits of MIS^[14]. The second study by Targarona *et al*^[20] included a larger number of patients: 54 patients randomized equally into HALC and LAC groups. The operating time and clinical outcome were similar. However, the conversion rate was much higher in the LAPC group (23% vs 7%). Of interest, 4 of 6 conversions in the laparoscopic group were completed with the hand-assisted technique. There is another interesting finding in this study that the inflammatory (tissue injury) markers such as interleukin-6 and C-reactive proteins were increased in the hand-assisted group. This may lead us to believe that HALC is a more aggressive procedure than LAC, but preserved the features of MIS. This has also opened the door for using HALC as a half-way house procedure and adjunct to LAC when difficulties are encountered and when conversion to the open procedure is contemplated during laparoscopic colectomy^[13].

The Minimally Invasive Therapy and Technology (MITT) group study^[21] consisted of a multi-centre (5 hospital, 11 surgeons), unblinded RCT which compared HALC and LAC for segmental (SC) and total colectomy/proctocolectomy (TC). The HALC group (47 patients: 33 SC and 14 TC) and LAC group (48 patients: 33 SC and 15 TC) were both matched for age, sex, diagnosis, BMI and previous surgery. There was no significant difference in the complication rates (19% vs 21%) and long-term clinical outcome, but the extraction incision was bigger (8.2 cm vs 6.1 cm) and the conversion rate was lower (2% vs 12.5%) in the HALC group. Moreover, there were no apparent differences in the time for bowel function recovery, tolerance of diet, length of hospital stay, postoperative pain scores, or narcotic use between the two groups. Another

interesting finding in this study is that the operating time can be reduced by more than 30 and 60 min in SC and TC, respectively if the procedure is conducted by HALC instead of LAC^[21]. The last RCT compared 35 HALRPC and 30 LARPC^[22]. There were neither conversions nor intraoperative complications, and the median operating time was longer in LAPRPC group (298 min vs 214 min, $P < 0.001$). Morbidity and reoperation rates were comparable (29% vs 20% and 17% vs 10%, respectively). The median hospital stay was 9 d in the laparoscopic group compared with 10 d in the HAL group. Moreover, there were no differences in QOL and the total costs^[22].

A prospective non-randomized case control study on ultra-low anterior resection was reported by Tjandra *et al*^[23] with an equal number of patients (32 HALC and 31 LAC). There were no conversions in both groups with similar oncological harvest in term of tumor clearance and number of lymph nodes retrieved. The length of hospital stay was the same (5.9 d vs 5.8 d). But, the operating time was significantly shorter in the HALC group (170 min vs 188 min). The duration needed for postoperative narcotics was significantly longer (3.0 d vs 1.5 d) and the bowel function recovery and flatus passage were delayed (3.4 d vs 1.9 d) in the HALC group. This study has confirmed some difference in recovery in favor of the laparoscopic group. This difference is, however, of doubtful clinical significance as the length of hospital stay is unaffected^[23].

Also a prospective comparative study analyzed 258 well-matched patients undergoing HALS ($n = 109$) or LAC ($n = 149$)^[24]. A significantly greater proportion of HALS patients underwent complex procedures and extensive resections. However, there were no differences in the conversion rates (15% vs 11%, $P = 0.44$), intraoperative complications (4% vs 1%, $P = 0.17$), the 30-d morbidity (18% vs 11%, $P = 0.12$) and surgical reinterventions (2% vs 1%, $P = 0.58$). There was no difference in the recovery judged by days to pass flatus (mean 3 d vs 3 d), however HALS took a longer operating time (276 min vs 211 min, $P < 0.0001$) and resulted in 1 d longer stay in hospital (6 d vs 5 d, $P = 0.0009$). It was concluded that HALS facilitates the expansion of a minimally invasive colectomy practice to include more challenging procedures while maintaining the short-term benefits of LAC^[24].

A large retrospective single institution study from the Lahey clinic^[25] comparing HAL sigmoid colectomy ($n = 66$) with LA sigmoid colectomy ($n = 85$) revealed no

Table 2 Published studies comparing hand-assisted laparoscopic colorectal surgery with laparoscopically assisted colorectal surgery

Study, yr	Patients (HALC vs LAC)	OT (min)	Incision length (cm)	Complications (%)	Conversion rate (%)	LOS (d)	Bowel function (d)	Comments and conclusion
HALS study ^[14] , 2000	18 vs 22	142 vs 151	7.4 vs 7.0	4.5 vs 5.5	14 vs 22	7 vs 6	NA	HALC retains the benefits of MIS
Targarona et al ^[20] , 2002	27 vs 27	120 vs 135	NA	26 vs 22	7 vs 23	6 vs 6	NA	Inflammatory markers such as interleukin-6 and C-reactive proteins were raised in HALC group
MITT study ^[21] , 2008	47 vs 48	163 vs 210	8.2 vs 6.1	19 vs 21	2 vs 12.5	5 vs 4	2.5 vs 3	The OT can be reduced by > 30 min and 60 min in SC and TC; respectively if conducted by HALC
Polle et al ^[22] , 2008	30 vs 35	214 vs 298	NA	Major: 16.5 vs 20	NA	10 vs 9	6 vs 5	No significant short-term benefits for total laparoscopic compared with HALRPC with respect to morbidity, OT, QOL, costs, and LOS
Tjandra et al ^[23] , 2008	32 vs 31	170 vs 188	NA	22 vs 25.8	0 vs 0	5.9 vs 5.8	3.4 vs 1.9	Some difference in recovery in favour of the laparoscopic group
Hassan et al ^[24] , 2008	109 vs 149	276 vs 211	NA	18 vs 11	15 vs 11	6 vs 5	3 vs 3	HALS facilitates expansion of a MIS colectomy to include challenging procedures while maintaining short-term benefits of LAC
Chang et al ^[25] , 2005	66 vs 85	189 vs 203	8.1 vs 6.2	21 vs 23	0 vs 13	5.2 vs 5	2.5 vs 2.8	No difference in return of bowel function, LOS or complications. Significant difference in the OT and conversion rate in favour of HALC group. The incision size was smaller in the LAC group
Ringley et al ^[10] , 2007	22 vs 18	120 vs 156	7 vs 5.5	Similar	NA	4 vs 4	NA	HALC is associated with shorter OT and greater lymph node harvest, but equal I.O blood loss, pedicle length and LOS

HALC: Hand-assisted laparoscopic colectomy; LAC: Laparoscopically assisted colectomy; OT: Operative time; LOS: Length of hospital stay; NA: Not available; MIS: Minimally invasive surgery; HALRPC: Hand-assisted laparoscopic restorative proctocolectomy; QOL: Quality of life; POD3: Postoperative day 3.

significant difference in bowel function recovery (2.5 d vs 2.8 d), length of hospital stay (5.2 d vs 5.0 d) or short-term complications such as anastomotic leak, ileus and wound infection (21% vs 23%) between the two groups. However, there was significant difference in the operating time (189 min vs 203 min) and conversion rate (0% vs 13%) in favor of the HALC group. The incision length was, however, significantly smaller (8.1 cm vs 6.2 cm) in the LAC group^[25].

Another retrospective review of 40 patients (22 HALC and 18 LAC) comparing conventional laparoscopic and hand-assisted oncological segmental colonic resection was reported by Ringley et al^[10]. HALC was found to be associated with a shorter operating time (120 min vs 156 min, $P < 0.05$) and greater lymph node harvest (16 vs 8, $P < 0.05$), but equal intraoperative blood loss, pedicle length and hospital stay (4 d)^[10]. LAC was completed with smaller incision length to retrieve specimen (7 cm vs 5.5 cm, $P < 0.05$), but this 1.5 cm difference is of doubtful clinical significance^[10]. Table 2 summarizes the above studies that compare HALC and LAC and their conclusions. Based on the reviewed literature, the following results were found between HALC and LAC: (1) HALC offers the same MIS benefits as LAC; (2) HALC has a shorter operative time and lower conversion rate; (3) They both have comparable complication rate and length of stay; (4) The incision length is bigger in HALC; (5) There an increased level of inflammatory markers in HALC; (6) There is an increased need for postoperative narcotics analgesia after HALC; (7) Bowel function recovery and passage of flatus are some-

what delayed in HALC, but this is of doubtful clinical significance; and (8) HALC is more suitable for the obese patients.

IMPACT OF HALC ON SURGICAL TRAINING

It has been postulated that "It takes 6-12 mo to teach fellows how to take down the splenic flexure independently using straight laparoscopic methods whereas most fellows become proficient at the same task about HAL method after performing 10-15 cases"^[13] and it was also claimed that "a surgeon who uses either straight laparoscopic or HAL methods extremely will be handicapped"^[13]. It was therefore advisable that trainees embrace and master both techniques. A recent comparative study measuring the percentage of left-sided HALC or straight LAC cases completed by a trainee surgical resident found that straight laparoscopy were more likely completed by the resident without the intervention of the attending physician than HALC (LAC, 88%; HALC, 72%; $P = 0.06$)^[26]. Differences in the mean operating time favoring LAC were noted (HALC, 142 min vs LAC, 133 min; $P = 0.04$)^[26]. However, the occurrence of complications was similar in the two groups (HALC, 19% vs LAC, 21%), so was the rate of conversions (HALC, 5.6% vs LAC, 4.5%). It was concluded that trainee surgical residents may be more successful in completing LAC than in adjusting to the novel HALC approach during training. This is somewhat surprising, but it may be explained by the hindrance of

the laparoscopic view by the intervening hand during HALC, especially in the early learning curve of the trainee surgeons.

A 25-question survey organized by The American Society of Colon and Rectal Surgeons about hands-on training cadaver courses, found that a laparoscopic colon resection was performed within 1 wk of the course by 52% of participants and within 1 mo by 90%^[27]. Hand-assisted technologies have lowered the threshold for performing the first LAC in 62% of participants. Most participants (77%) declared that the most important factor in the course selection was a cadaver model. It was therefore concluded that cadaver courses enabled rapid integration of laparoscopic colon resection into clinical practice and that hand-assisted technologies promoted technique acquisition^[27]. The author conducted HALC workshops on live animal (sheep) which made consultant surgeons with no experience in laparoscopic colectomy more confident in using the HALS technology they obtained in the animal workshop as a bridge towards totally laparoscopic procedures in humans^[15].

COST ANALYSIS

A concern has been raised regarding the higher direct cost of HALC, however, the results are inconsistent. In a RCT that evaluated postoperative recovery after HAL *vs* open restorative proctocolectomy with ileal pouch anal anastomosis for ulcerative colitis and familial adenomatous polyposis^[17], the HAL procedure was found more costly than the open procedure (the median overall cost was \$16,728 for HAL procedure and \$13,406 for the open procedure; $P = 0.095$)^[17]. In a retrospective analysis of 73 patients undergoing LAC *vs* 101 undergoing HALC^[28], it was found that the operative cost and cost of consumables were higher for HALC (US\$4024.2 *vs* US\$3568.1, $P = 0.01$ and US\$1724.7 *vs* US\$1302.7, $P < 0.001$, respectively). However, the total costs were not significantly different between the two procedures (HALC US\$8999.8, LC US\$7910.7, $P = 0.11$). In a more recent US study that looked at direct costs for the operating room, nursing care, intensive care, anesthesia, laboratory, pharmacy, radiology, emergency services and consultation, and professional and ancillary services related to the initial hospitalization and readmissions associated with 100 HALC *vs* 100 matched LAC cases which were performed concurrently^[29], there were no differences in the operating time (168 and 163 min, respectively), length of hospital stay (4 d), readmission (6% and 11%, respectively), or reoperation rates (5% and 9%). The overall morbidity was 16% and 32% for HAL and LAC, respectively ($P = 0.009$). The major morbidities, including abscess, hemorrhage and anastomotic leak, were also similar in the two procedures. However, operating room costs were increased for HALC (US\$3476 *vs* US\$3167); the total costs were similar (US\$8521 *vs* US\$8373). Therefore, it can be concluded that the total costs for HALC and LAC are similar^[29] and HALC reserves the benefits of LAC at no extra cost^[30].

LONG-TERM COMPLICATIONS OF HALC

As HALS requires a larger incision than that used in totally laparoscopic procedures, it has been postulated that HALS may be associated with more long-term complications such as incisional hernias and adhesive small bowel obstruction. This has been addressed by Sonoda *et al*^[31] who compared HALS ($n = 270$) and LAS ($n = 270$) over a median follow-up of 27 mo (1-72 mo). Despite the larger wound in the HALS group (median 75 mm *vs* 45 mm), the incidence of incisional hernia was similar in both groups and the rate of small bowel obstruction was also comparable (4.1% *vs* 7.4%, $P = 0.11$)^[31]. Moreover, the incidence of wound infections was also comparable (HALS 6.8% *vs* LAS 4.8%, $P = 0.33$). Interestingly, the converted cases had a significantly higher incidence of incisional hernia than that of the non-converted patients (25% *vs* 5%), although the rate of small bowel obstruction was the same. It was therefore concluded that HALS does not lead to more long-term complications of incisional hernia and small bowel obstruction than totally laparoscopic procedures^[31].

CONCLUSION

Based on the available evidence, hand-assisted laparoscopic colorectal resection offers similar short and long-term MIS benefits to that of totally laparoscopically assisted procedures. It combines the advantages of both laparoscopic (minimally invasive) and conventional open surgery. It is safe and feasible in benign as well as malignant colorectal tumors. Furthermore, it is easy to learn, easy to teach and most useful in complex colorectal procedures. Hence, hand-assisted colorectal surgery is advocated first as a 'bridge' and later as an adjunct to laparoscopically assisted colorectal procedures. Moreover, it can be used as an alternative to laparoscopic colectomy in the complex colorectal procedures.

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Biomarkers in Barrett's esophagus and esophageal adenocarcinoma: Predictors of progression and prognosis

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Abstract

Barrett's esophagus is a well-known premalignant lesion of the lower esophagus that is characterized by intestinal metaplasia of the squamous epithelium. It is clinically important due to the increased risk (0.5% per annum) of progression to esophageal adenocarcinoma (EA), which has a poor outcome unless diagnosed early. The current clinical management of Barrett's esophagus is hampered by the lack of accurate predictors of progression. In addition, when patients develop EA, the current staging modalities are limited in stratifying patients into different prognostic groups in order to guide the optimal therapy for an individual patient. Biomarkers have the potential to improve radically the clinical management of patients with Barrett's esophagus and EA but have not yet entered mainstream clinical practice. This is in contrast to other cancers like breast and prostate for which biomarkers are utilized routinely to inform clinical decisions. This review aims to highlight the most promising predictive and prognostic biomarkers in Barrett's esophagus and EA and to discuss what is required to move the field forward towards clinical application.

Key words: Barrett's esophagus; Esophageal adenocarcinoma; Esophageal dysplasia; Prognosis

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INTRODUCTION

Barrett's esophagus is defined as an esophagus in which the distal portion of the normal squamous lining has been replaced by a metaplastic columnar epithelium. In order to make a diagnosis of Barrett's esophagus, a segment of columnar metaplasia of any length must be visible endoscopically above the esophagogastric junction and be confirmed or corroborated histologically^[1]. This condition usually develops in the context of longstanding, severe gastroesophageal reflux disease (GERD)^[2], and is the only recognized precursor lesion for development of esophageal adenocarcinoma (EA). The incidence of EA arising from Barrett's esophagus is variable, depending on the grade of dysplasia associated with it. The risk of progression to cancer increases gradually from 0.5% per year for non-dysplastic Barrett's, to 13% in low-grade dysplasia (LGD) and 40% in high-grade dysplasia (HGD)^[3,4].

In Barrett's esophagus, it is widely accepted that there are three main histological subtypes. They include epithelium that comprises mainly a gastric fundus subtype with parietal and chief cells; a junctional (cardia) subtype with mucus-secreting glands; and the distinctive metaplastic columnar epithelium with intestinal-type goblet cells^[1,5]. These three histological subtypes occupy different zones in the esophagus. The intestinal-type metaplasia with goblet cells is found most proximally next to the squamous epithelium, followed by the junctional (cardia) subtype in the middle, and the gastric fundus subtype most distally. The relevance of this subgrouping of the histological subtypes of Barrett's esophagus lies in the potential to develop malignancy. The fundic subtype has a very low risk of developing EA malignant potential, whereas the metaplastic columnar epithelium with intestinal-type goblet cells and the junctional (cardia) type have a more significant risk of malignant transformation^[6,7]. This concept is important as this together with the problem of defining Barrett's esophagus based on location and length of metaplastic epithelium has led to a detailed discussion in the American Gastroenterological Association Institute technical review on Barrett's esophagus. This meeting redefined Barrett's esophagus as "the condition in which any extent of metaplastic columnar epithelium that predisposes to cancer development replaces the stratified squamous epithelium that normally lines the distal esophagus"^[8]. However, it is slowly becoming apparent that the risk for development of EA is not solely limited to the intestinal type and that better designed and powered studies are required to assess properly the true risk of progression in each subtype^[9].

During the development of EA, the epithelium accumulates multiple molecular abnormalities and becomes increasingly dysplastic^[10]. The diagnosis of dysplasia allows the progression from Barrett's esophagus to EA to be monitored by endoscopic surveillance biopsies with the aim of intervening prior to the development of invasive adenocarcinoma. Although randomized controlled evidence is lacking, EA detected *via* this strategy appears to confer a much better prognosis, as surveillance detected disease is often at an early stage prior to lymph node involvement^[11,12].

There are a number of problems with this current clinical algorithm. First of all, a significant proportion of patients with Barrett's esophagus are undiagnosed^[13-16], and therefore, will not benefit from any cancer prediction strategies. Second, surveillance is not proven to reduce population mortality and is based on the subjective assessment of dysplasia, which has inter and intra-observer error^[17-19]. Lastly, because most patients with Barrett's esophagus are at extremely low risk of developing EA^[20], the majority are having unnecessary surveillance, which is cumbersome both for the clinician and the patient, and poses a strain on the healthcare system. A recent review to assess the cost-effectiveness of surveillance of Barrett's esophagus based on a Markov model has revealed that surveillance of Barrett's esophagus for all grades of dysplasia does more harm than good when compared to

no surveillance^[21]. This report has suggested that surveillance does not produce more quality-adjusted life years than no surveillance, and that there is no apparent survival advantage of cancer detected by surveillance due to a high recurrence rate and increased mortality from surgical interventions. It is hoped that biomarkers assayed in readily obtainable biological samples, such as blood or endoscopic biopsies, can be identified to improve the clinical management at each stage in the disease. Screening biomarkers could enable unidentified cases of Barrett's esophagus to be diagnosed in the population (Figure 1, green arrow), whereas predictive biomarkers could be used as adjuncts or to replace the current surveillance program for the detection of dysplasia, as well as potentially being able to predict which patients are at high risk of developing cancer in the future (Figure 1, blue arrow). For patients presenting *de novo* with EA, prognostic biomarkers could be useful to determine the best therapeutic approach and prognosis (Figure 1, red arrow). In addition, biomarkers might have a role in determining response to treatment including chemopreventive agents, endoscopic treatments for patients with Barrett's, and the use of molecular targeted therapy for those with cancer.

CLINICAL BIOMARKERS

Clinical biomarkers can be defined as a characteristic that can be objectively measured or evaluated as an indicator of normal biological processes, pathological processes or a response to a therapeutic intervention^[22]. Importantly, the quantification of biomarkers should aid, improve or alter clinical management. The criteria required for adoption of biomarkers into clinical use are not well defined. Therefore, the Early Detection Research Network (EDRN) has defined five stages for development of biomarkers for risk of progression^[23] and similarly, McShane *et al*^[24] have recently published recommendations for prognostic tumor marker development (Figure 2). Despite the recommendation of different robust algorithms for biomarker development, fewer than 12 biomarkers have been approved by the US Food and Drug Administration for monitoring response, surveillance and recurrence of cancer at the current time^[25]. This is alarming as thousands of biomarkers have been declared to be useful for diagnosis, surveillance or therapeutic markers for diseases. Most of these biomarkers do not progress to clinical practice either due to problems developing accurate assays or because the biomarker lacks sufficient sensitivity and specificity in validation studies^[26]. Clearly, a large concerted effort is needed to advance the field of biomarker discovery and clinical implementation.

Biomarkers in Barrett's esophagus and EA are mostly selected due to their role in carcinogenesis. It is clear that during the transition from metaplasia to carcinoma, many molecular alterations take place and they operate together to influence the pathogenesis of dysplasia and EA. Biomarkers can be identified and investigated for their clinical applicability using two different complementary

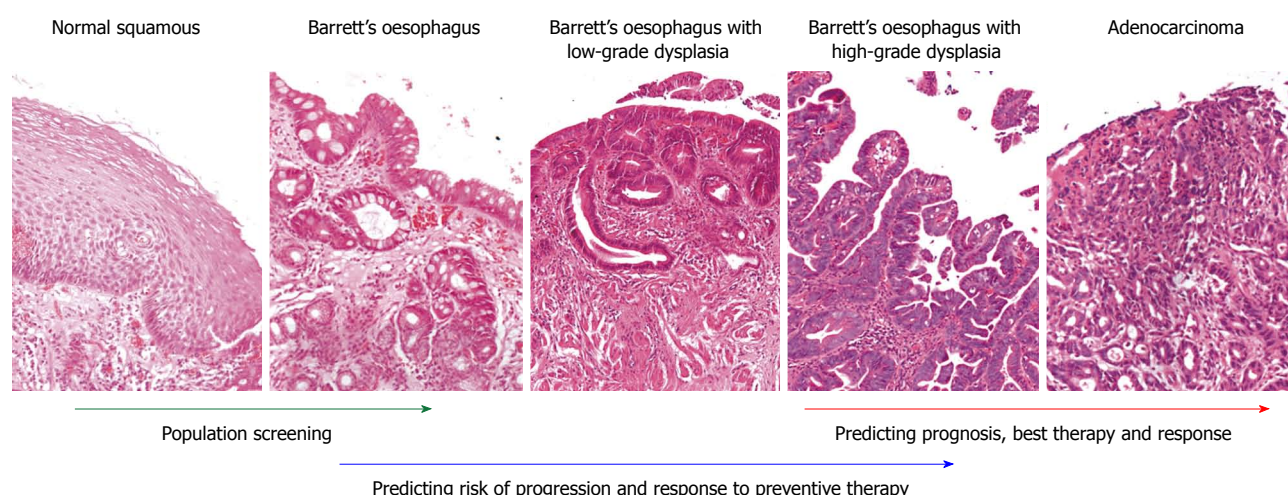


Figure 1 Transition of squamous epithelium to intestinal metaplasia, dysplasia and adenocarcinoma, with potential useful biomarkers at each stage of the disease. The left-most panel shows normal stratified squamous epithelium. The second panel shows Barrett's esophagus without dysplasia, with the presence of goblet cells. The third and fourth panels show Barrett's esophagus with low-grade dysplasia and high-grade dysplasia, whereas the last panel shows adenocarcinoma.

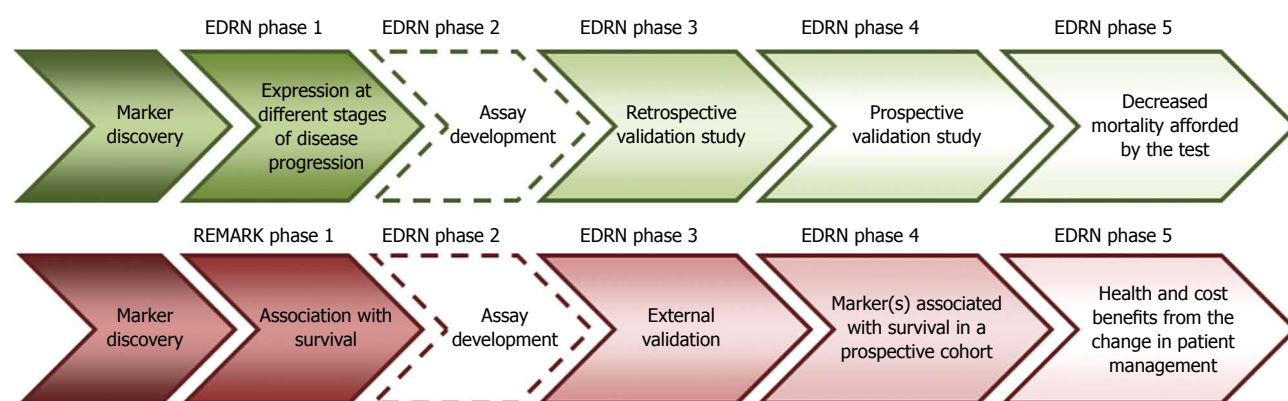


Figure 2 Phases of diagnostic and prognostic biomarker development proposed by the Early Detection Research Network and reporting recommendations for tumor marker prognostic studies before clinical implementation^[23,24]. EDRN: Early Detection Research Network; REMARK: Reporting recommendations for tumor marker prognostic studies.

approaches^[27]. The first approach is to identify candidate biomarkers from what is currently understood about the disease process. This is a comparatively inexpensive way to identify putative biomarkers and possibly allow for faster clinical implementation of the biomarker. The second method is to use a global screening approach without an *a priori* hypothesis. This has become possible due to the rapid expansion of “omics” technologies, including gene expression analysis, epigenetics, proteomics and single nucleotide polymorphism (SNP)-based platforms. The availability of microarray databases and other datasets on the internet also allows for the interrogation of multiple datasets to identify potential biomarkers. For example, Lao-Sirieix *et al.*^[28] have identified trefoil factor 3 (TFF3) as a promising biomarker to screen asymptomatic patients for Barrett's esophagus by comparing three publically available microarray databases. However, this approach requires an intensive validation process due to the potential for false discovery and can potentially be expensive and not reproducible between laboratories.

This review focuses on two main areas: (1) biomark-

ers predictive of progression in Barrett's esophagus, which it is hoped could transform the current surveillance program; and (2) prognostic biomarkers in EA.

PROMISING BIOMARKERS IN SURVEILLANCE OF BARRETT'S PATIENTS

Many biomarkers aimed at predicting progression in Barrett's patients have emerged over several years of research because it is appreciated that current clinical and endoscopic criteria are unable to predict which patients are likely to progress to EA. Biomarkers in Barrett's esophagus can be used for population screening and early detection of disease, confirmation of diagnosis of disease and prediction of risk of progression, which determine the prognosis of patients once adenocarcinoma develops and predict the effectiveness of therapy. Table 1 shows a summary of the biomarkers that have been most extensively investigated and their potential as clinical biomarkers. In studies evaluating the efficacy of the proposed biomarkers to determine the risk of progression from Barrett's

Table 1 Summary of the most promising biomarkers for identifying patients with Barrett's esophagus at high risk of developing esophageal adenocarcinoma

Surveillance biomarker	Highest EDRN stage	Study size (n) ¹	Findings	Statistical significance	Ref.
HGD	4	15	Progression to EA in 4 out of 15 patients with unifocal HGD	RR not available	[29]
		485	20 patients with HGD treated with omeprazole only developed EA	RR not available	[30]
		327	33 out of 76 patients with HGD developed EA	RR 28 (95% CI: 13-63)	[31]
		1099	12 out of 75 patients with HGD developed EA	RR 12.1 (95% CI: 5-29.4)	[32]
Aneuploidy and LOH (Reid Panel)	4	243	Panel of biomarkers (LOH of 17p and 9p and DNA abnormalities) can best predict progression to EA	RR 38.7 (95% CI: 10.8-138.5)	
			LOH of 17p alone	RR 10.6 (95% CI: 5.2-21.3)	[33]
			LOH of 9p alone	RR 2.6 (95% CI: 1.1- 6.0)	
			Aneuploidy alone	RR 8.5 (95% CI: 4.3-17.0)	
p53 positivity by immunohistochemistry	3		Tetraploidy alone	RR 8.8 (95% CI: 4.3-17.7)	
		164	Diffuse or intense TP53 staining elevated in patients who developed EA compared to controls	OR 11.7 (95% CI: 1.93-71.4)	[34]
		48	3 out of 5 patients with low grade dysplasia who progressed to high grade dysplasia had positive p53	RR not available	[35]
Mcm2	3	27	Ectopic luminal surface expression predictive of progression to HGD or EA	OR 136 (95% CI: 7.5-2464)	[36]
Cyclin A	3	48	Ectopic luminal surface expression predictive of progression to HGD or EA	OR 7.6 (95% CI: 1.6-37)	[37]
Methylation markers	3	53	Hypermethylation of <i>p16</i> (cyclin-dependent kinase inhibitor 2A), <i>RUNX3</i> (Runt-related transcription factor 3) and <i>HPP1</i> (transmembrane protein with EGF-like and two follistatin-like domain 2) associated with an increased risk of progression to high grade dysplasia or EA	OR 1.74 (95% CI: 1.33-2.2), 1.80 (95% CI: 1.08-2.81) and 1.77 (95% CI: 1.06-2.81), respectively	[38]
		195	A 8 gene methylation panel in combination with age could predict half of progressors to HGD or EA who would not have been diagnosed without the use of the panel	RR not available	[39]

¹Study size includes all patients in study and findings are extracted when relevant. Mcm2: Minichromosome maintenance protein 2; EA: Esophageal adenocarcinoma; HGD: High-grade dysplasia; LOH: Loss of heterozygosity; EDRN: Early Detection Research Network; RR: Relative risk; OR: Odds ratio; CI: Confidence interval.

esophagus to dysplasia and cancer, the odds ratio and relative risks are included whenever data were available in order to give a representation of the usefulness of the biomarkers.

DYSPLASIA

Dysplasia has been assessed as part of routine clinical practice for > 20 years. Although the assessment of dysplasia cannot be measured objectively, it is still considered a biomarker by most institutions, and is the current gold standard for determining the risk for cancer progression. The current dysplasia grading system is the Vienna classification, which divides patients into no dysplasia, LGD and HGD^[40]. Due to its routine use, very few studies have been performed to document formally its predictive power. A recent meta-analysis has shown that the incidence of EA in patients undergoing surveillance for Barrett's esophagus rises in a stepwise manner using dysplasia as a biomarker. The incidence of EA was reported to be 5.98 per 1000 patient years, 16.98 per 1000 patient years and 65.8 per 1000 patient years in Barrett's patients without dysplasia, and with LGD and HGD, respectively^[4]. However, histological differentiation of the different grades of dysplasia in Barrett's patients presents one of the most difficult tasks for the pathologist. In one study, 50% of Barrett's patients who were identified to have LGD by general pa-

thologists were misdiagnosed. Forty-two percent of these misdiagnosed cases had only Barrett's esophagus without dysplasia, and 8% had HGD^[41]. It is clear that histological differentiation between non-dysplastic Barrett's esophagus and LGD in particular is fraught with difficulties with poor intra- and inter-observer agreement.

HGD is known to be a surrogate marker for the high likelihood of progression to EA. Following diagnosis of HGD, endoscopic or surgical intervention is usually considered. Therefore, confirmation by two independent pathologists is a pre-requisite. As a result of the practice for intervention once HGD is detected, data on progression to EA have become much harder to obtain. Studies have shown that the risk of progression to EA ranges from 16% to 59%^[31,32] and a proportion of patients in whom HGD is detected will already harbor invasive adenocarcinoma^[29,32], although with intensive biopsy protocols and high definition endoscopes, this should no longer be so likely. A more ideal biomarker would be one that is less subjective and that appears earlier in the pathogenetic process, so that intervention could be considered for the highest risk patients earlier in the course of their disease. The evaluation of dysplasia is now well established and it has been suggested that other promising biomarkers are more likely to be used in conjunction with the current system than to replace the histopathological assessment of dysplasia^[42].

DNA CONTENT ABNORMALITIES AND LOSS OF HETEROZYGOSITY

The use of DNA content abnormalities (aneuploidy and tetraploidy) and loss of heterozygosity (LOH) as biomarkers to predict progression of Barrett's esophagus to EA has been intensively studied by the Reid group. DNA content abnormalities are a well-known phenomenon in cancer biology. A normal cell contains 46 chromosomes, commonly referred to as 2N, and aneuploidy refers to the state in which cells have an abnormal number of chromosomes. Tetraploidy, on the other hand, specifically refers to cells that have double the number of chromosomes compared to normal cells (4N). In Barrett's esophagus, numerous studies have correlated aneuploidy and specific DNA abnormalities with the progression of Barrett's esophagus to EA^[31,43-45], with Reid *et al.*^[31] producing the best results by combining DNA content abnormalities with LOH. Galipeau *et al.*^[44] have demonstrated that increased 4N (G2/tetraploid) cell populations predict progression to aneuploidy, and that the development of 4N abnormalities is interdependent with inactivation of the *p53* gene. Using flow cytometry and histology in a systematic endoscopic biopsy protocol, Reid *et al.*^[31] first described the use of aneuploidy and increased 4N fractions as biomarkers to identify subsets of patients with Barrett's esophagus at low and high risk of developing EA. Using a cut-off for 4N fractions of > 6% as abnormal, Reid has reported that the relative risk of cancer for these patients compared to those below this cut-off value was 7.5 (95% CI: 4-14). In addition, patients who had baseline aneuploidy had a relative risk of cancer of 5 (95% CI: 2.7-9.4) compared to patients who did not have baseline aneuploidy.

p16 and *p53* are two commonly studied tumor suppressor genes that reside on chromosome 9p and 17p, respectively. These two tumor suppressor genes can be silenced *via* LOH, mutations and DNA methylation. Silencing of the *p16* allele is thought to be one of the earliest events in Barrett's esophagus, which results in clonal expansion^[46]. However, a recent study by Leedham *et al.*^[47] has demonstrated that Barrett's esophagus can arise from multiple independent clones, which results in clonal heterogeneity. This study was performed by investigating individual crypts microdissected from esophagectomy specimens that contained adenocarcinoma and associated dysplasia, to detect clonal heterogeneity not detected by whole biopsy analysis. Overall, *p16* by itself is unlikely to be an ideal biomarker to predict progression because it appears too early in the pathogenesis, and it has been shown that there is no evidence of association between silencing of *p16* and grade of dysplasia^[46]. *p53* LOH, on the other hand, provides one of the most promising biomarkers to predict progression of Barrett's esophagus, as part of the Reid panel. *p53* is a nuclear tumor suppressor protein that is responsible for the integrity of the genetic sequence. Any damage to DNA should result in increased expression of *p53*, which causes cells to arrest at the G1 phase to allow for DNA repair, and if this is not possible, then apoptosis ensues. Silencing of *p53* can occur *via* LOH or

mutation of the genetic sequence, thus removing the self repair mechanism. Reid *et al.*^[48] have performed a prospective cohort study in 325 patients with Barrett's esophagus, and have demonstrated that LOH of chromosome 17p(*p53*) significantly increased the risk of progression to cancer (relative risk of 16, 95% CI: 6.2-39). In addition, Galipeau *et al.*^[33] have demonstrated that LOH of 17p can be combined with LOH at 9p, DNA content abnormalities and aneuploidy to form a panel of biomarkers to predict better progression of Barrett's esophagus. This panel of biomarkers provides the best predictor of progression to EA to date (relative risk of 38.7, 95% CI: 10.8-138.5). Each individual marker in the panel could in itself predict progression to EA with varying RR (Table 1), but when combined together in the Reid panel, they can most accurately predict progression to EA.

The panel of biomarkers that incorporate DNA abnormalities and LOH, which have been developed by the Reid group, are not easy to apply to the clinical setting. Efforts have therefore been made to develop alternatives. Fang *et al.*^[45] and Vogt *et al.*^[49] have tried to circumvent the problem of a high level technical expertise being required and the laboratory variability associated with flow cytometry, by using image cytometric DNA analysis in smaller studies. In these studies, they have concluded that image cytometry can provide a more sensitive marker than using HGD to identify groups of patients who are likely to progress to EA, and have highlighted that image cytometry has significant advantage over flow cytometry in terms of costs and practicality. These findings, while promising, still require validation with a much larger sample size. The development of high-fidelity DNA histograms generated by automated software to measure aneuploidy further strengthens the role of DNA abnormalities as a biomarker to predict progression in Barrett's patients^[50,51]. Other interesting novel techniques to measure aneuploidy and other chromosomal aberrations have also been described in the literature. Li *et al.*^[52] have demonstrated that the number of SNPs was highly correlated with chromosomal abnormalities in Barrett's esophagus and EA, and have suggested that SNP-based genotyping could possibly be used to stratify the cancer risk in patients with Barrett's esophagus.

As mentioned previously, the use LOH as biomarkers is not without its own problems. The detection of LOH is complex and requires the collection of snap frozen samples, followed by extraction of DNA and an amplification step prior to polymerase chain reaction analysis^[53]. This is in addition to the high costs needed to build and maintain facilities to enable the use of this panel of biomarkers in routine medical institutions. An alternative method would be to use fluorescence *in situ* hybridization (FISH) to detect LOH, but this method is limited by poor sensitivity (68.4%) when compared to genotyping^[54].

Immunostaining for *p53* provides another alternative to genotyping of chromosome 17p to predict progression of Barrett's esophagus because the presence of *p53* mutations can often cause protein accumulation, which allows for detection by immunohistochemistry^[34,35]. Although

the use of immunostaining of *p53* allows easy clinical implementation, its efficacy as a biomarker is limited, and positive staining was only seen in one third of patients in a nested case-control study to evaluate the efficacy of immunostaining for *p53* as a marker to predict progression^[34]. This is because staining for *p53* does not always correlate with mutations. In instances in which mutations result in deletion or truncation of *p53*, it will not be detected by immunostaining.

In summary, the detection of aneuploidy and DNA content abnormalities in the Reid panel appears to be one of the most promising biomarker panels to detect the progression of Barrett's esophagus to EA. However, technical difficulties that have hindered the use of analysis of DNA content abnormalities in the Reid panel need to be addressed. SNP analysis or image cytometry are other alternative techniques used to measure aneuploidy and other chromosomal aberrations but remains to be validated in larger studies.

PROLIFERATION MARKERS

Dysplasia is typically described as being associated with abnormal cellular proliferation and differentiation^[55,56]. Our laboratory and others have demonstrated abnormal surface staining of markers of proliferation [minichromosome maintenance protein (Mcm) 2, 5 and Ki67] in dysplastic Barrett's mucosa^[36,55,56]. This finding has served as the basis for the use of aberrant surface expression of Mcm2, together with a brushing technique to predict progression in patients with Barrett's esophagus^[36]. However, large prospective studies are needed before they can be used in routine clinical practice.

CELL CYCLE MARKERS

Members of the cyclin family such as cyclin A and D are also interesting biomarkers for Barrett's esophagus. Cyclin D is a proto-oncogene protein and overexpression in Barrett's esophagus results in inappropriate phosphorylation and inactivation of p105-Rb. Increased expression of cyclin D has been implicated in the predisposition to transform from metaplastic epithelium to cancer, and can possibly be a useful biomarker in identifying patients with Barrett's esophagus at high risk of developing EA^[57,58]. Bani-Hani *et al.*^[58] have performed a case-control study and have shown that Barrett's patients who are positive for cyclin D detected *via* immunohistochemistry were more likely to develop EA (OR: 6.85, 95% CI: 1.57-29.91). These findings were however not replicated in a larger population-based case-control study performed by Murray *et al.*^[34]. In that study, only immunohistochemical detection of *p53* has been shown to be a useful biomarker for malignant progression in Barrett's esophagus. Cyclin A is expressed just before the beginning of DNA synthesis and is an important check mechanism in the G1-S transition of the cell cycle. In a case-control study, surface expression of cyclin A in Barrett's esophagus samples has been shown to be correlated with the degree of dysplasia, and

patients with biopsies that express cyclin A at the surface were more likely to progress to EA than those who did not (OR: 7.5, 95% CI: 1.8-30.7)^[37]. Prospective studies are required to determine properly the usefulness of cyclins as predictive biomarkers.

EPIGENETIC CHANGES

Epigenetic changes (or non-DNA sequence changes) in the form of hypomethylation, hypermethylation and alteration to histone complexes have also been found to be implicated in the pathogenesis of Barrett's esophagus and EA^[38,59]. Hypermethylation of promoter CpG island is thought to be the cause of transcriptional silencing of tumor suppressor genes such as *CDKN2A* (*p16*), *APC*, *CDH1* (E-cadherin), and *ESR1* (ER, estrogen receptor α)^[59]. Hypermethylation of these genes is usually found in a large contiguous field, which suggests possible clonal expansion of hypermethylated cells or hypermethylation of a field of metaplastic cells^[59]. Further work on the methylation status of promoter regions of genes has revealed that methylation of *p16* (OR: 1.74, 95% CI: 1.33-2.20), *RUNX3* (OR: 1.80, 95% CI: 1.08-2.81) and *HPP1* (OR: 1.77, 95% CI: 1.06-2.81) in patients with non-dysplastic Barrett's esophagus and LGD were independent risk factors for progression to HGD and EA^[38]. More recently, Jin *et al.*^[60] have demonstrated that a methylation biomarker panel that comprises eight genes could accurately determine the risk of progression in patients with Barrett's esophagus in a retrospective, multicenter validation study. In that study, promoter methylation levels of eight genes were quantified by methylation-specific PCR in patients who did not progress ($n = 145$) compared to those who did progress ($n = 50$) to HGD or EA. Receiver operating characteristics curves were constructed to evaluate the usefulness of the eight-gene methylation panel and the authors have concluded that, with specificity set at 0.9, the eight-gene methylation panel in combination with age predicted half the progressors who would not have been diagnosed without using these biomarkers. Similarly, a recent study by Wang *et al.*^[61] has shown that hypermethylation of *p16* and *APC* was a good predictor of progression to HGD or EA [OR: 14.97, 95% CI (1.73, inf)]. The fact that methylation changes in DNA occur early in the progression from Barrett's esophagus to dysplasia suggest that they could potentially be used as biomarkers to predict which groups of patients are likely to progress to dysplasia and EA^[59,62]. However, the main problem of the utility of hypermethylation as biomarkers lies in the fact that techniques that have been applied for detection of epigenetic changes require enzyme digestion, affinity enrichment or bisulfite treatment before probe hybridization or sequencing can be done to detect methylation in samples. These arrays of techniques are far too technically demanding and time consuming for routine utilization in the clinic^[63-72].

PROGNOSTIC BIOMARKERS IN EA

The overall 5-year survival for EA remains < 14%^[73].

The current staging of EA is the internationally recognized TNM system^[74], which is based exclusively on the anatomical extent of the disease. This is assessed using a combination of tumor depth (T), number of lymph nodes involved (N), and presence or absence of metastasis (M). The TNM system remains useful for staging of esophageal tumors because patients with more advanced stage disease clearly do worse than those in the early stage of the disease. For patients deemed to have potentially curative disease (T3N1 or less), surgical treatment with or without chemotherapy provides the only chance of cure, but it is highly invasive and has a high morbidity rate. Biomarkers that can accurately predict the prognosis of patients with this disease could aid in the selection of patients most likely to benefit from surgery. In addition, it is also hoped that biomarkers can identify different subgroups of tumors that will benefit from specific treatment, including molecularly targeted treatments.

Prognostic biomarkers in patient with EA have commonly been studied to determine the association with the following outcome and tumor characteristics: (1) survival; (2) lymphovascular invasion and metastasis; and (3) response to chemotherapy and radiotherapy.

Traditional candidate approaches for analyzing gene and protein expression in cancer have identified a large number of biomarkers that have important prognostic value. These biomarkers can be considered in terms of the six classical hallmarks described by Hanahan *et al.*^[75], with inflammation added as the seventh hallmark recently. They include: (1) self sufficiency in growth signals; (2) insensitivity to growth inhibitory (antigrowth) signals; (3) evasion of programmed cell death (apoptosis); (4) limitless replicative potential; (5) sustained angiogenesis; (6) invasion and metastasis; and (7) cancer-related inflammation.

Table 2 gives an overview of the biomarkers in each category and their association with survival or surrogate measures of prognosis. This list is not exhaustive but it highlights the important biomarkers that have been investigated and reported to be prognostic. A recent review by Lagarde *et al.*^[76] has described in greater detail many of these biomarkers and their molecular basis. It is well known that many of these molecular alterations occur in tandem during the progression of Barrett's esophagus to EA and are present to varying degrees. These biomarkers have been shown to be associated with survival or tumor characteristics, but subsequent replication of findings, as required for the EDRN validation of biomarkers, is often lacking. It is highly unlikely that any of these markers by itself can predict survival accurately because several molecular alterations can operate together to influence the pathogenesis of EA. Again, generating panels of biomarkers to create a molecular signature in EA could be useful in determining the prognosis of patients with EA.

MOLECULAR SIGNATURE OF EA

Several studies have used microarray technologies to generate molecular signatures that correlate with overall

survival, lymph node involvement or response to chemotherapy. The advantage of using these methods is that they allow the hypothesis-free interrogation of many targets simultaneously. Table 3 gives a summary of the molecular signatures discovered by microarray technology, including the methodology used. However, despite the number of studies, none of these molecular signatures or techniques to stratify patients with EA has yet reached clinical utility. This is in contrast to other cancers for which prognostic signatures are starting to be used in the clinical setting^[101-103]. In EA, molecular signatures have usually been generated from underpowered cohorts and many studies have combined molecular profiling of both EA and squamous cell carcinoma of the esophagus in the same study. It is known that the molecular profile of squamous cell carcinoma and EA is different^[104,105], and for accurate prognosis, studies should differentiate between these two types of tumors. An additional problem, which is not dissimilar to biomarkers discovered for the transition of Barrett's esophagus to EA, is that the technique used might not be applicable to routine laboratories and will therefore be expensive. It is therefore important that researchers also consider how best to apply molecular biomarkers to the clinic, and they should consider validation using methods such as immunohistochemistry. Whichever method is used, data reproducibility and validation in independent samples are perhaps the most important factors to determine whether molecular signatures are adopted for clinical application. This problem is becoming increasingly recognized, and many reviews have reiterated the need for validation of molecular signatures and the development of assays that have general clinical applicability^[106-112].

CONCLUSION

The pathogenesis from Barrett's esophagus to EA is highly complex. Multiple molecular alterations occur during this process, which leads to a heterogeneous tumor by the time that EA develops. Biomarkers can complement the current clinical management of Barrett's esophagus and its transition to EA in three main ways. They can be used to: identify patients not previously diagnosed with Barrett's esophagus *via* population screening; improve the surveillance of patients with Barrett's esophagus; and identify prognostic groups and best therapy once EA develops.

There has already been a tremendous amount of research done to create an ideal biomarker or panel of biomarkers to predict accurately progression of Barrett's esophagus to dysplasia or EA. This is in conjunction with large amounts of resources and money spent in laboratories and in clinical trials as the research is being conducted. Although no biomarkers have been able to replace the current gold standard of dysplasia as a biomarker in routine clinical practice, it is reassuring to know that certain biomarkers hold great promise to transit from the bench to the bedside. It is becoming increasingly clear that one biomarker by itself is highly unlikely to predict progression with high sensitivity and

Table 2 Summary of the biomarkers and the prognostic impact in esophageal adenocarcinoma

Category of cell alteration	Biomarker	Sample size (n)	Endpoint	Findings	Statistical significance	Ref.
Self sufficiency in growth signals	Cyclin D EGFR	124	Survival	2 of 3 genotypes confers a poorer overall survival	$P = 0.0003$	[77]
		103	Survival	Expression showed a trend towards a correlation with poorer overall survival	$P = 0.07$	[78]
		75	Survival	Decreased expression correlated with poorer survival on univariate analysis only	$P = 0.034$	[79]
	Ki-67	59	Survival	Low levels (< 10%) of staining correlated with poorer survival	HR: 3.9, $P = 0.02$	[80]
	Her2/neu	63	Survival	Amplification detected by FISH correlated with poorer survival	$P = 0.03$	[81,82]
	TGF- α	61	Survival	Low levels significantly correlated with cancer specific death	$P = 0.03$	[83]
		87	Tumor progression, lymph node metastasis	High levels significantly correlated with: Tumor progression Lymph node metastasis	$P = 0.025$ $P < 0.05$	[84]
Insensitivity to growth inhibitory (antigrowth) signals	TGF- β 1	123	Survival	Overexpression correlated with poorer survival on univariate analysis only	$P = 0.0255$	[85]
	APC	57	Survival	High plasma levels correlated with poorer overall survival	$P = 0.0317$	[86]
		52	Survival	High plasma levels of methylation of APC associated with poorer survival	$P = 0.016$	[87]
	P21	30	Survival	Alteration in expression after chemotherapy correlated with better survival	$P = 0.011$	[88,89]
Evasion of programmed cell death (apoptosis)	P53	30	Survival	Alteration in expression after chemotherapy correlated with better survival	$P = 0.011$	[88]
	Bcl-2	35	Survival	Expression correlated with poorer survival	$P = 0.03$	[90]
	COX-2	100	T-stage, N-stage, tumor recurrence and survival	Higher levels expression correlated with: Higher T-stage, Higher N-stage, Increased risk of tumor recurrence Poor survival	$P = 0.008$ $P = 0.049$ $P = 0.01$ $P < 0.001$	[91]
		20	Survival	Strong staining correlated with poorer survival	$P = 0.03$	[92]
		145	Distant metastasis, local recurrence and survival	Strong staining correlated with: Distant metastasis Local recurrence Poorer survival	$P = 0.02$ $P = 0.05$ $P = 0.002$	[93]
		43	Survival	Activated NF- κ B predictive of: Poorer disease free survival Poorer overall survival	$P = 0.010$ $P = 0.015$	[94]
	NF- κ B	46	Survival	Higher telomere-length ratio shown to be an independent poor prognostic factor	$P < 0.02$	[95]
				Significant correlation between expression and: Poorer survival	$P < 0.01$	[96]
				Presence of angiolymphatic invasion More lymph node metastasis Higher tumor stage More distant metastasis	$P < 0.05$ $P < 0.01$ $P < 0.001$ $P < 0.01$	
Limitless replicative potential Sustained angiogenesis	Telomerase	46	Survival	Higher telomere-length ratio shown to be an independent poor prognostic factor	$P < 0.02$	[95]
	CD105	75	Survival, angiolymphatic invasion, lymph node metastasis and tumor stage and distant metastasis	Significant correlation between expression and: Poorer survival Presence of angiolymphatic invasion More lymph node metastasis Higher tumor stage More distant metastasis	$P < 0.01$ $P < 0.05$ $P < 0.01$ $P < 0.001$ $P < 0.01$	[96]
		75	Survival, angiolymphatic invasion, lymph node metastasis, stage of tumor and distant metastasis	Significant correlation between high expression and: Poorer survival Presence of angiolymphatic invasion More lymph node metastasis Higher stage of tumor More distant metastasis	$P < 0.01$ $P < 0.05$ $P < 0.01$ $P < 0.01$ $P < 0.01$	
	VEGF	75	Survival, angiolymphatic invasion, lymph node metastasis, stage of tumor and distant metastasis	Significant correlation between high expression and: Poorer survival Presence of angiolymphatic invasion More lymph node metastasis Higher stage of tumor More distant metastasis	$P < 0.01$ $P < 0.05$ $P < 0.01$ $P < 0.01$ $P < 0.01$	
Tissue invasion and metastasis	Cadherin	59	Survival	Reduced level correlated with poorer overall survival	HR: 3.3, $P = 0.05$	[80]
	uPA	54	Survival	High uPA correlated with poorer survival	$P = 0.0002$	[97]
	TIMP	24	Survival and disease stage	Reduction of expression correlated with poorer overall survival and higher disease stage	$P = 0.007$ $P = 0.046$	[98]
Others	Promoter hypermethylation	41	Survival and tumor recurrence	Earlier tumor recurrence and poorer overall survival if > 50% of gene profile methylated	$P = 0.05$	[99]
		84	Differentiation	Hypermethylation of MGMT (Methylated-DNA-protein-cysteine methyltransferase) gene correlated with: Higher tumor differentiation	$P = 0.0079$	[100]

EGFR: Epidermal growth factor receptor; Her2/neu: Human EGFR2; TGF: Transforming growth factor; APC: Adenomatous polyposis coli; P21: Cyclin-dependent kinase inhibitor 1; Bcl-2: B-cell lymphoma 2; COX-2: Cyclooxygenase-2; NF- κ B: Nuclear factor- κ B; CD105: Endoglin; VEGF: Vascular endothelial growth factor; uPA: Urokinase-type plasminogen activator; TIMP: Tissue inhibitor of metalloproteinase.

specificity. Panels of biomarkers such as the eight-gene methylation panel or the Reid panel, which combine LOH at various loci and DNA content abnormalities to

predict progression, seem to provide the most accurate predictor of progression based on statistics. Unfortunately, the common theme in these panels of markers is

Table 3 Summary of the molecular signatures discovered by microarray technology and latest methods used to correlate molecular alterations and prognosis in patients with esophageal adenocarcinoma

Method	Sample size (n)	Outcome	Findings	Statistical significance	External validation	Ref.
Oligonucleotide cRNA microarray	75	Survival	A 4-gene signature prognosticated patients	$P = 0.0001$	Yes	[113]
	77	Lymphatic spread	Created a gene signature predicting lymph node metastasis	Argininosuccinate synthetase expression (ASS) ($P = 0.048$)	No	[114]
	19	Chemotherapy response	Unsupervised hierarchical clustering divided patients into 2 groups, one of which responded to preoperative chemotherapy	Not statistically significant	No	[115,116]
	47	Chemotherapy response	86 genes dysregulated Ephrin B3 expression associated with chemotherapy response, tumor grading and stage	$P < 0.001$	No	[117]
Oligonucleotide cDNA microarray	46	Chemotherapy response	Gene signature not predictive in adenocarcinoma of esophagus	Not statistically significant	No	[118]
Proteomic analysis	34	Chemotherapy response	HSP27 expression associated with response to chemotherapy	$P < 0.05$	No	[119]
Single nucleotide polymorphism	210	Survival and recurrence	5 polymorphisms in 3 genes associated with longer recurrence free survival and reduced recurrence	$P = 0.004$	No	[120]
microRNAs analysis	96	Survival	Low miR-375 levels associated with worse survival	$P = 0.002$	No	[121]
Multiplex ligation-dependent probe amplification	33	Survival	Patients with more than 12 chromosomal aberrations had a poorer outcome than patients with < 12	$P = 0.014$	No	[122]

that they are far too expensive to be applied in routine clinical use, and technical expertise is not available in all centers to utilize these panels of biomarkers. The issue of costs and practicality of biomarkers should be one of the principle considerations before research and resources are channeled into it.

Although traditional methods of identifying biomarkers in Barrett's esophagus and its transition to dysplasia and EA have helped greatly in the understanding of the disease process, new technologies to create molecular signatures have also helped by identifying many important biomarkers not previously thought to be involved in its pathogenesis. A few biomarkers identified from both traditional methods and new technological platforms have shown great potential in predicting the progression from Barrett's esophagus to EA. However, a concerted effort is still needed to validate these biomarkers or molecular signatures in independent, large-scale prospective cohorts and to develop inexpensive, practical assays to allow for clinical applicability. Realistically, this can only be achieved by a multicenter collaboration to tackle the challenges of the large amount of resources, scientific and clinical input required to advance the field of biomarkers in Barrett's esophagus. There are a few major collaborations in the United Kingdom to date, and they include the Chemoprevention of Premalignant Intestinal neoplasia trial (CHO-PIN) and Oesophageal Cancer Clinical and Molecular Stratification Study (OCCAMS). This is also mirrored in the international arena with Barrett's Esophagus and Adenocarcinoma Consortium (BEACON) and Asian Barrett's Consortium as two examples of collaborative work on Barrett's esophagus. These initiatives allow for the pooling of resources, expertise and knowledge between centers and allow for the recruitment of large numbers of patients that are necessary to advance the field of biomarkers in Barrett's esophagus and EA. Although each study

has a slightly different focus, much could be gained these collaborative efforts if a proportion of the resources and patient samples could be used to validate biomarkers in Barrett's or tumor samples.

Lastly, biomarkers should be seen as adjuncts to aid clinical management of patients with Barrett's esophagus and EA rather than in isolation in predicting the risk of progression, prognosis or response to therapy. As such, clinical factors in conjunction with biomarkers should be incorporated into a model that can accurately determine the desired outcome. Such models have been used in other cancers and diseases such as the MELD score for liver disease or the Nottingham prognostic index for breast cancer. Upon generation and validation of the model, it should then be rigorously validated in an independent large cohort of patients in a prospective fashion. In future, patients can then be risk stratified based on a score to determine the treatment strategy, hence individualizing treatment to improve patient care and outcome.

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Management of mucinous cystic neoplasms of the pancreas

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Abstract

The purpose of this study was to investigate the actual management of mucinous cystic neoplasm (MCN) of the pancreas. A systematic review was performed in December 2009 by consulting PubMed MEDLINE for publications and matching the key words "pancreatic mucinous cystic neoplasm", "pancreatic mucinous cystic tumour", "pancreatic mucinous cystic mass", "pancreatic cyst", and "pancreatic cystic neoplasm" to identify English language articles describing the diagnosis and treatment of the mucinous cystic neoplasm of the pancreas. In total, 16322 references ranging from January 1969 to December 2009 were analysed and 77 articles were identified. No articles published before 1996 were selected because MCNs were not previously considered to be a completely autonomous disease. Definition, epidemiology, anatomopathological findings, clinical presentation, preoperative evaluation, treatment and prognosis were reviewed. MCNs are pancreatic mucin-producing cysts with a distinctive ovarian-type stroma localized in the body-tail of the gland and occurring in middle-aged females. The majority of MCNs are slow

growing and asymptomatic. The prevalence of invasive carcinoma varies between 6% and 55%. Preoperative diagnosis depends on a combination of clinical features, tumor markers, computed tomography (CT), magnetic resonance imaging, endoscopic ultrasound with cyst fluid analysis, and positron emission tomography-CT. Surgery is indicated for all MCNs.

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Key words: Pancreatic cystic lesion; Pancreatic mucinous cystic neoplasm; Pancreatic mucin-producing cysts; Pancreatic cystic neoplasm; Pancreatic ovarian-type stroma

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INTRODUCTION

Becourt first described cystic lesions of the pancreas in 1824^[1]. In 1978, Compagno *et al*^[2] first classified cystic tumors into serous cystic neoplasms (SCNs) and mucinous cystic neoplasms (MCNs) of the pancreas and identified MCN as a distinct disease occurring almost exclusively in the pancreas body and tail of middle-aged women^[2,3]. Until 1996, when the World Health Organization distinguished between intraductal papillary mucinous neoplasms (IPMNs) and MCNs, emphasizing the presence of ovarian stroma in the latter, and until 1997 when the Armed Forces Institute of Pathology confirmed this distinction, MCN and IPMNs were frequently confused^[3-7]. Nowadays, they represent two distinct neoplasms with different biologic behaviour, pathologic features, and prognosis^[8-11].

Although until 1987, Warshaw *et al*^[12] considered that pseudocysts account for the majority of pancreatic cys-

tic lesions, nowadays mucinous and serous cystic tumors represent 50%-60% of all cystic lesions^[13]. Nevertheless pancreatic cystic neoplasms occur with less frequency than solid ones^[4,14,15], but are now found with increasing frequency compared to the past due to the improvement and refining of modern imaging techniques like multidetector, three-dimensional computed tomography (CT) or magnetic resonance imaging (MRI), or endoscopic ultrasound (EUS)^[16].

The aim of this study was to review the literature to clarify the management of cystic mucinous neoplasm of the pancreas.

LITERATURE SEARCH

A comprehensive literature review was performed in December 2009 by consulting PubMed MEDLINE for publications, matching the key words of “pancreatic mucinous cystic neoplasm”, “pancreatic mucinous cystic tumor”, “pancreatic mucinous cystic mass”, “pancreatic cyst” and “pancreatic cystic neoplasm” to identify English language articles on MCNs.

Only studies including series with more than four patients affected by MCNs were included. Articles reporting reviews, case reports, abstracts and studies on only IPMNs, SCNs or pancreatic pseudocysts were excluded. Definition, epidemiology, anatomopathological findings, clinical presentation, preoperative evaluation, treatment and prognosis were analyzed.

A total of 16 322 references ranging from January 1969 to December 2009 were analyzed (“pancreatic mucinous cystic neoplasm”, *n* = 930; “pancreatic mucinous cystic tumor”, *n* = 924; “pancreatic mucinous cystic mass”, *n* = 143; “pancreatic cyst”, *n* = 6215; “pancreatic cystic neoplasm”, *n* = 8110) and 77 articles were selected^[10,14,17-89]. No articles before 1996 were usable because MCNs were not previously considered as a completely autonomous disease^[9-11,17,77,86,89,90].

DEFINITION AND EPIDEMIOLOGY

MCNs are defined as mucin-producing and septated cyst-forming epithelial neoplasia of the pancreas with a distinctive ovarian-type stroma. Usually solitary, their size ranges between 5 and 35 cm with a thick fibrotic wall and without communication with the ductal system^[11]. MCNs are rare and, in most series, less common than IPMNs and SCNs^[73]. MCNs show a female to male ratio of 20 to 1 and a mean age at diagnosis of between 40 and 50 years (range 14-95 years)^[6,7,10,11,91-93]. The site of the neoplasm is in the body and tail of the pancreas in 95%-98% of cases^[3,7,9,34,35,89,94,95]. When localized in the pancreatic head, mucinous cystadenocarcinoma is more prevalent^[7,10].

Invasive carcinoma incidence in MCN varies between 6% and 36%^[8-11,14,34,35,86]. The Ulm series reported on 39 patients with MCNs and a malignant histology in 51%, including carcinoma *in situ* and advanced cancer^[11]. The explanation of this wide range may be the difficulty in interpreting the data on the prevalence of carcinoma because

the majority of series have only indicated the advanced form.

ANATOMOPATHOLOGICAL FINDINGS

Macroscopically, MCNs usually appear as solitary, multilocular or unilocular lesions with a mean size of 7-8 cm (range 0.5-35 cm) with a thick fibrotic wall and containing mucin, even when hemorrhagic, watery or necrotic content is observed^[8].

In 2004, the consensus conference of the International Association of Pancreatology in Sendai (Japan)^[8,9] established that the histological presence of unique ovarian-type stroma was mandatory to diagnose MCN and that this was not found in other pancreatic neoplasms^[10,73,93]. MCNs display no communication with the pancreatic ductal system, although some studies suggested that a small proportion of MCNs may show microscopic communication with the pancreatic ducts^[68,96,97].

Under light microscopy, the cysts are lined by a columnar mucin-producing epithelium with different grade of dysplasia: mild (MCN adenoma), moderate (MCN borderline), and severe (MCN carcinoma *in situ*)^[98]. The epithelial lining is positive for CKs (CK7, CK8, CK18, CK19), EMA and, less frequently, CK20, CEA, DUPAN-2 and CA 19-9^[8,10,67]. An invasive adenocarcinoma of the tubular or ductal type is associated in about one-third of cases^[6]. The immunophenotype of ovarian-type stroma is similar to the normal ovarian one with positivity for vimentin, calretinin, tyrosine hydroxylase, SMA, α -inhibin, Melan-A, CD99 and Bcl-2 and frequently for PR and ER. The origin of ovarian stroma of the pancreas is still being debated^[99]. A stimulation of endodermal immature stroma by female hormones or primary yolk cell implantation in the pancreas has been suggested in literature^[10] because buds of the genital tract and dorsal pancreas are adjacent to each other during embryogenesis. Moreover, dorsal pancreatic enlargement mainly gives rise to the pancreatic body and tail, and this could explain the predilection of MCNs for the distal pancreas^[17].

Although the pathologic diagnosis of malignancy is based on invasion of the pancreatic parenchyma or metastases^[5], MCNs that do not have conclusive evidence of carcinoma are considered premalignant^[7].

A thickened wall with peripheral calcification and papillary proliferations, vascular involvement and hypervascular pattern should be considered as suggestive of MCN with malignant changes^[68,95]. Although the invasive MCN (mucinouscystadenocarcinoma or mucinous cystic neoplasm with associated invasive carcinoma) is generally a tubular/ductal carcinoma^[8], rare histological variants are represented by undifferentiated carcinoma with osteoclast-like giant cells^[100], adenosquamous or colloid cells^[101], or sarcomatoid carcinoma^[99], carcinosarcoma and choriocarcinoma^[8,102].

The increasing degree of dysplasia and tendency for invasion have been correlated with activating point mutations in the *k-ras* gene and mutations in the *TP53* gene^[8,103,104]; moreover, the discovery that the inactiva-

tion of SMAD4/DPC4 in the epithelium of the invasive MCNs, but not in the ovarian-like stroma, could suggest that the ovarian-type stroma is not neoplastic^[105].

CLINICAL PRESENTATION

The majority of MCNs are slow growing and asymptomatic^[95]. In a series of 212 consecutive patients with cystic pancreatic lesions, 36.7% were asymptomatic and among them 28% had MCNs; in the symptomatic group, 16% had MCN^[106]. In spite of these lesions being occasionally discovered in patients scanned for other indications^[16,35,107], the typical clinical appearance is characterized by epigastric heaviness and fullness (60%-90%) or by an abdominal mass (30%-60%)^[7,10,12,35,89,106,108]. Nausea, vomiting (20%-30%) and back pain (7%-40%) can also be present.

No specific symptom was significantly associated with a likelihood of malignancy^[35] although increasing anorexia and weight loss (10%-40%) may be associated with malignant changes^[7,12,35,89,95,106,108,109].

PREOPERATIVE EVALUATION

MCNs main differential diagnosis includes other neoplastic cystic lesions (serous cystic neoplasm and the intraductal papillary mucinous neoplasms) and non-neoplastic cystic lesions (pancreatic pseudocysts). There is no single discriminating test, but preoperative diagnosis depends on a combination of modes, including clinical features, tumor markers, CT and MRI, EUS with cyst fluid analysis, and positron emission tomography (PET).

High values of CEA and CA 19-9 show a high positive predictive value for pancreatic malignancy or premalignancy in the preoperative assessment of pancreatic cystic mass (70%-100%)^[9,95,110]. A CEA level of more than 400 ng/mL is a good predictor of malignancy in MCNs (sensitivity 45%-50%, specificity 95%-100%, accuracy 75%-80%)^[107,111,112].

Trans-abdominal ultrasound examination has a low accuracy (50%) for cystic neoplasms of the pancreas^[89].

EUS improves that accuracy and allows better evaluation of the wall as it may show separation or nodules within the cyst. Furthermore, EUS can be used to obtain aspiration of the cyst contents and to perform a biopsy of the wall. Cyst fluid amylase concentration of < 250 U/L has been considered capable of excluding pseudocysts of the pancreas (sensitivity 40%-45%, specificity 95%-100%, accuracy 60%-65%), while CEA < 5 ng/mL could suggest a benign etiology (sensitivity 45%-50%, specificity 95%-100%, accuracy 65%-70%)^[31,111]. EUS-FNA cytology and cyst fluid CEA greater than 192 ng/mL show the highest accuracy (79%) for differentiating mucinous cystic from non-mucinous cystic neoplasms^[113]. On the contrary EUS morphology alone cannot distinguish between the two groups^[47,50,113].

In any case, the main differential diagnosis of MCNs is with SCNs which have a low CEA in the fluid and an equal distribution throughout the pancreas, with pancreatic pseudocysts (PC) that usually show necrotic debris

Table 1 Imaging-based classification system of cystic pancreatic lesions

Type of lesions	Morphologic features	Pancreatic cystic lesions
Unilocular cyst	Without internal septation and solid component or wall calcification	Serous cystic neoplasm, intraductal papillary mucinous neoplasm ¹ , pancreatic pseudocyst
Microcystic lesion	Six or more cysts with diameter 0.2 mm-2 cm, external lobulation, fibrous central scar with or without stellate calcifications	Serous cystic neoplasm
Macrocystic lesions	Diameter > 2 cm, with internal septation and solid component or wall calcification	Intraductal papillary mucinous neoplasm ¹ , mucinous cystic neoplasm ²
Cyst with a solid component	Unilocular or multilocular	Intraductal papillary mucinous neoplasm ¹ , mucinous cystic neoplasm ²

¹With or ²without communication with main duct, respectively.

within the cyst cavity, and with branch duct IPMNs communicating with the ductal pancreatic system and consequently showing elevated cystic fluid amylase^[3].

Although pancreatitis may be present in the history of patients with pancreatic cystic neoplasms, when a cyst arises in a patient with chronic pancreatitis, the most frequent diagnosis is PC^[109]. On the other hand, when pancreatitis is unexpected and occurs for the first time, the cyst could be a tumor, determining the development of pancreatitis due to compression of the pancreatic duct^[13]. This is a crucial problem, because the risk of managing cystic mucinous neoplasms in patients with a prior history of pancreatitis, like pseudocysts by a pseudocyst-jejunal anastomosis or pseudocyst-gastrostomy, is higher than usual, with disastrous long-term prognosis^[12,83]. Proper sampling of pseudocysts is essential and should consist of sampling of the cyst wall during surgery or analysis of cyst content during minimal access drainage procedures. Although the clinical context, radiological imaging and biochemical findings may help differentiate PC from cystic neoplasms, small lesions may be problematic.

The image based classification system proposed by Sahani *et al.*^[107], in which cystic pancreatic lesions are classified in four subtypes, is reported in Table 1.

The demonstration of a solid component, invasion outside the confines of the pancreas, or pancreatic duct obstruction through EUS is highly indicative of malignancy with sensitivity, specificity and accuracy of 70%, 100% and 60%, respectively^[107]. However, in the absence of these findings the ability of EUS to diagnose malignancy is limited with an overall sensitivity, specificity and accuracy of 56%, 45% and 51%, respectively^[113]. The added advantage of EUS in performing aspiration of cyst content and sampling of the cyst wall and septa or mural nodules is that it allows small lesions as well as suspicious areas to be analysed. Laparoscopic and intraoperative ultrasounds are highly operator dependent with an accuracy

ranging from 40% to 90%^[3,114-120].

Multidetector computed tomography and magnetic resonance cholangiopancreatography (MRCP) play a critical role in assessment, defining size, septation, calcifications, nodules of the wall, and communication with the ductal system of the pancreatic cyst.

At cross-sectional imaging, the MCN appears as a unilocular or multilocular single macrocyst with a solid component, with no communication with the main duct^[95,107,121]. The internal architecture of the cyst, including septa and internal wall, is best appreciated with MR imaging^[122,123].

Recently, Kim *et al.*^[60] defined some significant CT features for differentiating MCNs from SCNs and IPMNs: the shape is smooth in MCNs, multicystic and lobulated in SCNs, and pleomorphic and clubbed finger-like in IPMNs; the main pancreatic duct is not dilated or proximally only in SCNs, and if dilated, whole in IPMN.

In spite of the improvement in pancreatic tumor visualization resulting from CT and MRI, the ability to perform diagnosis of these techniques individually - as well as EUS - remains poor (25%-30%)^[6,124,125]. In a multivariate analysis by Visser *et al.*^[14] in 2008, the combination of CT and MRI data showed an accuracy ranging from 44% to 83%.

Cross-sectional imaging generally shows peripheral calcification, a thickened wall, papillary proliferations, vascular involvement and hypervascular pattern in the cases of malignant MCNs^[36,95,126]. Although peripheral eggshell calcification is not easily detected by CT, this is a specific feature of the MCNs and is highly predictive of malignancy^[126].

The clinical value of MRCP is similar to endoscopic retrograde cholangiopancreatography or percutaneous transhepatic cholangiography^[127] but an MR multi-imaging protocol, which includes MR cross-sectional imaging, MRCP and dynamic contrast-enhanced MR angiography, integrates the advantages of multiple imaging techniques without morbidity^[128-131].

The role of PET in managing pancreatic cystic lesions is currently limited but recent studies report detection of malignant pancreatic cysts with sensitivity and positive predictive values above 90%^[132,133].

In spite of a complete diagnostic assessment, the surgeon's preoperative diagnosis is correct in one-third of cases, incorrect in another third, and non-specific in the remainder^[6,14].

TREATMENT

Surgical excision is indicated for all MCNs considered pre-malignant. Factors influencing treatment include tumor histological features, the patient's age and surgical risk, and tumor size and location.

Left pancreatectomy

Because mucinous cystic adenoma of the pancreas are usually localized at the level of the body and tail of the pancreas, the most common operation performed to cure

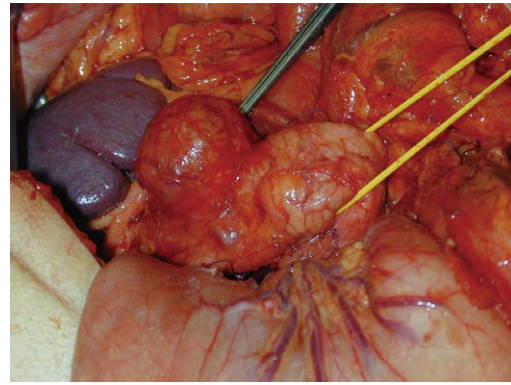


Figure 1 Distal pancreatectomy. Dissection of the pancreas in a patient with mucinous cystic neoplasm.

these neoplasms is distal pancreatectomy (Figure 1), which is a safe procedure in high volume centres (overall postoperative morbidity ranging from 5% to 50% and a mortality rate of 0%)^[3,7,20,35,134,135]. The main complication, pancreatic fistula, occurs in 15%-20% of cases^[136].

The distal pancreatectomy technique was first described in 1913 by Mayo^[137] and the spleen-preserving distal pancreatectomy was outlined in 1943 by Mallet-Guy *et al.*^[138]. Preservation of the spleen can be performed with or without preservation of the splenic artery and vein. In 1988, Warshaw described a technique without the preservation of the splenic artery and vein, ligating the splenic vessels at the hilum^[139]. Although this method appears technically less difficult and can be performed in a shorter operating time, it has been associated with a higher incidence of spleen vascular insufficiency^[140]. However, this procedure should be considered in the event of an inflamed or fibrosed splenic artery and vein^[139]. Spleen-preserving techniques must be avoided when in the presence of the largest tumors or risk factors for invasive malignancy, such as the size of the lesion, eggshell calcifications and mural nodules, in order to perform the complete oncological lymph node dissection^[9,27,31]. However, these techniques are preferred in all other cases to avoid long term infectious and haematological complications^[3,20,27,139].

Studies comparing patients undergoing distal pancreatectomy with or without splenectomy show no significant differences compared to perioperative complications, mean operating time, pancreatic fistula rate, length of hospital stay and mortality^[20,108,135,141,142].

MCNs affecting the pancreatic neck or the proximal body could be managed either by an extended right or, more frequently, by an extended left pancreatectomy. These extended resections of normal pancreatic tissue may induce endocrine and exocrine insufficiency respectively in 30%-35% and 15%-20%, which in a benign or premalignant disease could be discussable^[143-147].

Middle pancreatectomy

Middle pancreatectomy can be considered in the surgical management of MCNs located at the level of the pancreatic proximal body or neck, preserving endocrine and

exocrine function with respect to extended left pancreatectomy or pancreaticoduodenectomy, and also preserving the spleen.

The main pitfalls of this technique are the technical difficulty, the higher incidence of postoperative complications and the risk of recurrence from potentially residual neoplasm^[9,146-153].

Different techniques have been proposed for gastrointestinal reconstruction, including jejunal anastomosis of the stump or the distal stump, with pancreaticoduodenal or pancreaticogastric anastomosis^[143,148-156].

In the literature, mortality after middle pancreatectomy was none and the overall morbidity was 25%-35%^[147-149,155-157]. The incidence of overall pancreatic fistula was 22%-45% and the type of reconstruction through Roux-en-Y pancreatojejunostomy or pancreaticogastrostomy did not affect the rate of any complication^[147-149,155-157]. Moreover, the incidence of endocrine and exocrine insufficiency after middle pancreatectomy was 4%-7% and 5%-8%, respectively^[147-149,155-157].

Enucleation

Because the probability of malignancy in patients with MCNs smaller than 2 cm without nodules is very low, enucleation could be performed to avoid post-operative pancreatic insufficiency^[35]. This procedure is proposed for patients with MCNs smaller than 2 cm with benign features and superficially located^[146,155,156]. Enucleation can be performed without risk of recurrence but has been associated with a higher incidence of pancreatic fistula (30%-50%)^[158-160].

Whipple procedure

A major oncologic resection, applying a Kausch-Whipple or pylorus-preserving technique, is recommended for MCNs that are localized monocentrically in the head.

The operative mortality ranges from 0% to 5% and is generally related to pancreatic anastomosis complications^[136,161]. The most common complications following the Whipple procedure are delayed gastric emptying and the pancreatic fistula occurring in 5%-10% and 6%-20% of operations, respectively^[136,161-163].

When an enucleation is impossible or contraindicated, MCNs localized monocentrically in the pancreatic head that do not have an association with an invasive pancreatic cancer could be treated by duodenum-preserving total pancreatic head resection^[34,94,164-167].

This procedure shows significant advantages when compared to Traverso-Longmire or Whipple pancreaticoduodenectomy, as regards the postoperative rate of morbidity and mortality, glucose metabolism, hospitalization and costs^[11,168].

Lymphadenectomy

Pancreatectomy with lymph node dissection is necessary when an invasive carcinoma is suspected. Although the preoperative and intraoperative assessment of the grade of invasiveness is often difficult, whenever any doubt exists typical resection with lymph node dissection must be

pursued^[9]. There is no evidence in literature of invasive mucinous cystic adenocarcinoma with distant lymph node metastases, so only a loco-regional lymphadenectomy is justified^[3,35]. Because the probability of malignancy is very low in the cases of small MCNs without nodules, lymphadenectomy can be avoided^[3,146].

Laparoscopy

In the cases of benign-appearing and small malignant lesions (< 5 cm), a minimally invasive approach may be considered^[35]. Recent experiences from high-volume centers demonstrate that the laparoscopic approach for distal pancreatectomy for MCNs of the body and tail of the pancreas is feasible and safe^[169]. The complication rate of laparoscopic distal splenopancreatectomy (Lap SDP) ranges between 15% and 20%^[30,46,170-173] with a mortality rate of 0%. In spleen-preserving laparoscopic pancreatic (Lap SPDP) resection, the overall morbidity ranges from 25% to 40% with a mortality rate of 0%^[30,46,169-173]. The overall reported pancreatic fistula was 5%-8% and 10%-15% after Lap SPDP and Lap SDP, respectively^[30,46,170-173]. This laparoscopic approach decreases the hospital stay and minimizes the cosmetic impact of the surgical wound^[30,169,174,175].

Chemotherapy

Gemcitabine (GEM) is the standard therapy for advanced pancreatic cancer^[176]. Its effectiveness against advanced MCNs has been reported^[177,178].

Recently, some combinations have been reported to be superior to GEM alone^[177,179-181]. GEM-oxaliplatin treatment has been proposed to be more effective in terms of clinical progression-free survival^[177].

Discordant results on survival were reported by phase II and III trials combining GEM and inhibitors of epidermal growth factor receptor (cetuximab) and vascular endothelial growth factor (bevacizumab)^[182-184].

Other modest but interesting advances have been provided by combinations such as GEM-capecitabine and GEM plus a platinum salt^[185]. In spite of this, survival results remain disappointing.

Conservative treatment

A conservative management with regular follow-up has been proposed in the presence of asymptomatic cystic lesions of the pancreas smaller than 3 cm without mural nodules, because the reported risk of malignancy in these cysts was found to be 3%^[11,35,63,186]. The suggested follow-up consisting of cross-sectional imaging and FNA cytology should be performed every 6 mo for a period of 2 years and yearly after that. This should be continued for at least 4 years and then the interval of follow-up can be lengthened after 6 years of no change^[31,52,186]. When the cyst enlarges or when symptoms occur (in up to 20% of patients after follow-up), surgery is mandatory. The reported incidence of the subsequent resection due to change of the clinical, radiological and biochemical features of the lesions after initial conservative treatment was 4%-10% and malignancy rate in these cases was 3%^[23,63].

PROGNOSIS

After resection, in the absence of invasive carcinoma, prognosis of MCNs is excellent, with an overall survival rate of 100%^[5-7,10,35] and patients do not need follow-up, since several studies have shown that the risk of recurrence following resection is 0%^[11,18]. Patients with invasive mucinous cystadenocarcinoma, show a 5-year survival rate of 20%-60%, which is much better than that for non-MCN-associated ductal adenocarcinoma^[5-8,11,17,35,73,86]. When an anaplastic carcinoma of the pancreas associated with MCN is reported, the prognosis is obviously extremely poor, with a 3-year survival rate lower than 30%^[5-8,11,17,35,73,86].

CONCLUSION

Although the histological distinction between MCNs and IPMNs, through the identification of ovarian stroma initially, is very important in clinical practice, the management of MCNs has not yet been standardized and continues to evolve.

The approach to patients with suspected MCN is based on EUS and cross-sectional imaging in association with FNA cytology, detecting an incidence of correct differentiation between mucinous cystic and non-mucinous cystic neoplasms of 75%.

Because at present we are unable to identify the benign MCNs that will progress into invasive carcinoma, all MCNs should be resected, regardless of size, in patients who are fit candidates for surgery, because surgery is routinely curative in the cases of non-invasive tumor. Moreover, the non-operative management based on periodic CT or MRI requires years of careful follow-up with a high cost of imaging and the enucleation technique carries the risk of non-oncological radicality. In patients with non-invasive MCN after complete anatomic resection, postoperative surveillance is unnecessary.

In order to obtain more benefit by applying adjuvant chemotherapy for the treatment of the advanced MCNs, further research focused on sequential cellular transformation from benign to malign tumor and on new combinations, incorporating the new targeted therapies and identifying potential predictive factors of response, is required to be able to offer effective tailored treatment to these patients.

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Insulin like growth factor-1 increases fatty liver preservation in IGL-1 solution

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factor-1 (IGF-1) supplementation to serum-free institut georges lopez-1 (IGL-1)[®] solution to protect fatty liver against cold ischemia reperfusion injury.

METHODS: Steatotic livers were preserved for 24 h in IGL-1[®] solution supplemented with or without IGF-1 and then perfused "ex vivo" for 2 h at 37°C. We examined the effects of IGF-1 on hepatic damage and function (transaminases, percentage of sulfobromophthalein clearance in bile and vascular resistance). We also studied other factors associated with the poor tolerance of fatty livers to cold ischemia reperfusion injury such as mitochondrial damage, oxidative stress, nitric oxide, tumor necrosis factor- α (TNF- α) and mitogen-activated protein kinases.

RESULTS: Steatotic livers preserved in IGL-1[®] solution supplemented with IGF-1 showed lower transaminase levels, increased bile clearance and a reduction in vascular resistance when compared to those preserved in IGL-1[®] solution alone. These benefits are mediated by activation of AKT and constitutive endothelial nitric oxide synthase (eNOS), as well as the inhibition of inflammatory cytokines such as TNF- α . Mitochondrial damage and oxidative stress were also prevented.

CONCLUSION: IGL-1[®] enrichment with IGF-1 increased fatty liver graft preservation through AKT and eNOS activation, and prevented TNF- α release during normothermic reperfusion.

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Key words: AKT; Institut georges lopez-1[®] solution; Insulin like growth factor-1; Ischemia reperfusion injury; Nitric oxide; Oxidative stress; Steatotic graft preservation

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Abstract

AIM: To investigate the benefits of insulin like growth

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Zaouali MA, Padriša-Altés S, Ben Mosbah I, Ben Abdennebi H, Boillot O, Rimola A, Saidane-Mosbahi D, Roselló-Catafau J. Insulin like growth factor-1 increases fatty liver preservation in IGL-1 solution. *World J Gastroenterol* 2010; 16(45): 5693-5700 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i45/5693.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i45.5693>

INTRODUCTION

One of the major challenges in liver transplantation is to increase the use of marginal organs. Steatotic, or fatty, livers are increasingly transplanted, in spite of the associated risk of graft dysfunction or non-function as a result of cold ischemia reperfusion injury (IRI)^[1,2]. Steatotic livers show poor tolerance to IRI due to severe mitochondrial damage, impaired energy metabolism^[3], increased reactive oxygen species (ROS) and release of inflammatory cytokines such as tumour necrosis factor- α (TNF- α), which impairs microcirculation^[4,5]. All these factors render steatotic livers more vulnerable to cold IRI.

Liver preservation is a crucial step in maintaining graft quality after prolonged ischemic periods, especially steatotic livers. University of Wisconsin (UW) is the most widely used serum-free preservation solution for transplantation but it does not fully protect liver grafts during prolonged storage^[6-8]. Recently, the new Institut Georges Lopez-1 (IGL-1)[®] solution has been proposed as an effective alternative to UW in clinical kidney transplantation^[9,10], and in experimental orthotopic liver transplantation models^[11,12]. IGL-1[®] solution is characterized by inversion of K⁺ and Na⁺ concentrations in the UW solution and contains polyethylene glycol as osmotic support instead of HES. In addition, we have previously demonstrated that IGL-1[®] is more suitable than UW solution for fatty liver preservation^[13]. Its benefits are associated in part, with the prevention of oxidative stress and its capacity to generate nitric oxide (NO)^[13]. NO is a vasodilator with anti-inflammatory properties that prevents microcirculatory alterations^[5,13] and the release of pro-inflammatory cytokines, such as TNF- α , during IRI^[14-16].

Insulin like growth factor-1 (IGF-1) is a vascular protective factor that is mainly synthesized and released by the liver^[17,18]. We have recently reported impaired synthesis of IGF-1 in steatotic livers subjected to warm IRI, and that exogenous administration of recombinant IGF-1 reduced IRI in steatotic livers^[18]. IGF-1 prevents oxidative stress^[17] and induces NO generation by eNOS activation, as a consequence of AKT kinase phosphorylation^[19,20].

Several trophic factors (TF), including IGF-1, have been added to UW solution in an attempt to improve the survival of pig orthotopic liver allografts after 18 h of cold storage^[21]. In vascular endothelial cells, the benefits of TF supplementation are associated with limitation of mitogen-activated protein kinase (MAPK) activities after cold ischemia/rewarming injury^[22].

Taking this into account, we explored the effects of the addition of IGF-1 to IGL-1[®] solution on fatty liver preservation during cold IRI. We examined the mechanisms responsible for such effects, including AKT phosphorylation and NO generation, and the prevention of ROS production and TNF- α release after reperfusion. These latter factors have been implicated in the poor tolerance of steatotic livers to cold IRI.

MATERIALS AND METHODS

Animals and liver procurement

Isolated perfused rat liver was used to evaluate hepatic function in isolation from the influence of other organ systems (undefined plasma constituents and neural/hormonal effects). Hepatic architecture, microcirculation and bile production were preserved in this experimental model, as previously reported^[23]. Homozygous obese (Ob) Zucker rats, aged 16-18 wk were purchased from Iffa-Credo (L'Abresle, France) and were housed at 22°C^[23]. All procedures were performed under isoflurane inhalation anaesthesia. Experiments were conducted according to European Union regulations for animal experiments (Directive 86/609 CEE).

Liver procurement and experimental groups

The surgical technique was performed as previously reported^[23,24]. After cannulation of the common bile duct, the portal vein was isolated and the splenic and gastroduodenal veins were ligated. All animals were randomly distributed into groups as described below. The steatotic livers were flushed and preserved in cold IGL-1[®] solution for 24 h with or without the addition of IGF-1 (10 μ g/L), as described elsewhere^[21,22,25].

All animals were randomized according to the experimental protocols, as follows.

Cold storage: After 24 h of cold storage at 4°C, livers from 8 Zucker rats preserved in IGL-1 solution with (IGL-1 + IGF-1) or without IGF-1 were flushed with Ringer's lactate solution. Control livers (Cont 1) from 8 Zucker rats (Ob) were flushed with Ringer's lactate solution at room temperature immediately after laparotomy *via* the portal vein without cold storage. Aliquots of the effluent flush were sampled for measurements of cumulative AST and ALT after 24 h cold storage.

Cold storage and reperfusion: Briefly, after 24 h cold storage at 4°C, livers from 8 Zucker rats (Ob) preserved in IGL-1 and IGL-1 + IGF-1 solutions were subjected to 2 h reperfusion at 37°C, as previously reported^[13,24]. Control livers (Cont 2) from 8 Zucker rats (Ob) were flushed with Ringer's lactate at room temperature and immediately perfused *ex vivo* without ischemic preservation.

Transaminase assay

Hepatic injury was assessed in terms of transaminase levels with commercial kits from RAL (Barcelona, Spain)^[26].

Briefly, 200 μ L of effluent perfusate were added to the substrate provided by the commercial kit and then alanine aminotransferase (ALT)/aspartate aminotransferase (AST) levels were determined at 365 nm with a UV spectrometer and calculated following the supplier's instructions.

Hepatic clearance

As with bile output, hepatic clearance was considered as another parameter of hepatic function. Thirty minutes after the onset of perfusion (t_{30}), 1 mg of sulfobromophthalein (BSP) (Sigma, Madrid, Spain) was added to the perfusate. The concentration of BSP in bile samples (t_{120}) was measured at 580 nm with a UV-visible spectrometer. Bile BSP excretion was expressed as a percentage of perfusate content (t_{120} bile/ t_{30} perfusate*100)^[23].

Vascular resistance

Liver circulation was assessed by measuring perfusion flow rate and vascular resistance. Perfusion flow rate was assessed continuously throughout the reperfusion period and expressed as mL/min per gram of liver. Vascular resistance was defined as the ratio of portal venous pressure to flow rate and expressed in mmHg/min per gram of liver/mL^[26].

Glutamate dehydrogenase activity

Glutamate dehydrogenase (GLDH) was used as an indirect measure of mitochondrial damage. GLDH was measured in the perfusate as described elsewhere^[26].

Lipid peroxidation assay

Lipid peroxidation in liver was used as an indirect measure of the oxidative injury induced by ROS. Lipid peroxidation was determined by measuring the formation of malondialdehyde (MDA) with the thiobarbiturate reaction^[13].

Determination of nitrite and nitrate

NO production in liver was determined by tissue accumulation of nitrite and nitrate^[13].

Western blotting analysis of eNOS, AKT, P38 and ERK 1/2

Liver tissue was homogenized as previously described and proteins were separated by SDS-PAGE and transferred to PVDF membranes^[27-29]. Membranes were immunoblotted using the following antibodies: eNOS (Transduction Laboratories, Lexington, KY, USA), total and phosphorylated AKT, total and phosphorylated P38, and total and phosphorylated ERK 1/2 (Cell Signaling, Beverly, MA, USA) and β -actin (Sigma Chemical, St. Louis, MO, USA). Signals were detected by the enhanced chemiluminescence kit (Bio-Rad Laboratories, Hercules, CA, USA) and quantified by scanning densitometry as previously described^[26,28].

TNF- α determination

Perfusate TNF- α levels were measured after 120 min of reperfusion using a commercial immunoassay kit for rat TNF- α from Biosource (Camarillo, CA, USA)^[30,31].

Statistical analysis

Data are expressed as means \pm SE, and were compared statistically by variance analysis, followed by the Student-Newman-Keuls test (Graph Pad Prism software). $P < 0.05$ was considered significant.

RESULTS

Protective effect of IGF-1 against liver injury and function

As shown in Figure 1A and B, the AST and ALT levels observed for steatotic livers preserved for 24 h at 4°C in IGL-1[®] solution confirmed their poor tolerance to cold ischemia injury. IGF-1 addition prevented AST/ALT release from steatotic livers when compared with IGL-1 alone.

Similar AST/ALT profiles were obtained when fatty livers were subjected to normothermic reperfusion for 30 and 120 min, respectively. Reduced AST/ALT levels were observed when IGL-1 was enriched with IGF-1 (Figure 1C and D). Similar profiles were seen for 60 and 90 min of reperfusion (data not shown).

The mechanisms by which IGF-1 protects fatty livers against the harmful consequences of cold I/R damage were also investigated. For this reason, liver function was evaluated by the alterations in BSP clearance and vascular resistance, respectively. A reduction in BSP clearance was found in IGL-1 solution when compared to Cont 2. The highest BSP clearance in bile was observed after 2 h of normothermic reperfusion when IGF-1 was present (Figure 2A).

Vascular resistance was increased by IGL-1 solution (Figure 2B), but this was reversed by the addition of IGF-1. These alterations in vascular resistance were also seen in all groups at 30 and 120 min of reperfusion (Figure 2B).

Beneficial effects of IGF-1 on AKT and NO after reperfusion

In order to explore whether the beneficial effects of IGF-1 in IGL-1 solution are associated with other protective cell signalling involved in the protective mechanisms against IRI, we evaluated the changes in AKT and NO levels. As shown in Figure 3, IGF-1 increased AKT phosphorylation when compared to IGL-1 alone and Cont 2, respectively (Figure 3A). This was concomitant with a significant generation of NO, as revealed by the nitrites/nitrates levels, as well as eNOS activation (Figure 3B and C).

Role of IGF-1 on liver oxidative stress, mitochondrial damage and TNF- α release after reperfusion

In order to examine the relationship between the parameters studied and others associated with the poor tolerance of fatty livers to reperfusion injury, we evaluated lipid peroxidation (MDA) and mitochondrial damage (GLDH). Steatotic livers preserved in IGL-1 showed significant MDA augments when compared to Cont 2. These increases were significantly prevented by the addition of IGF-1 (Figure 4A). In accordance with this, IGF-1 also diminished liver mitochondrial damage, as shown by the reduction in GLDH (Figure 4B). These beneficial effects of IGF-1 were

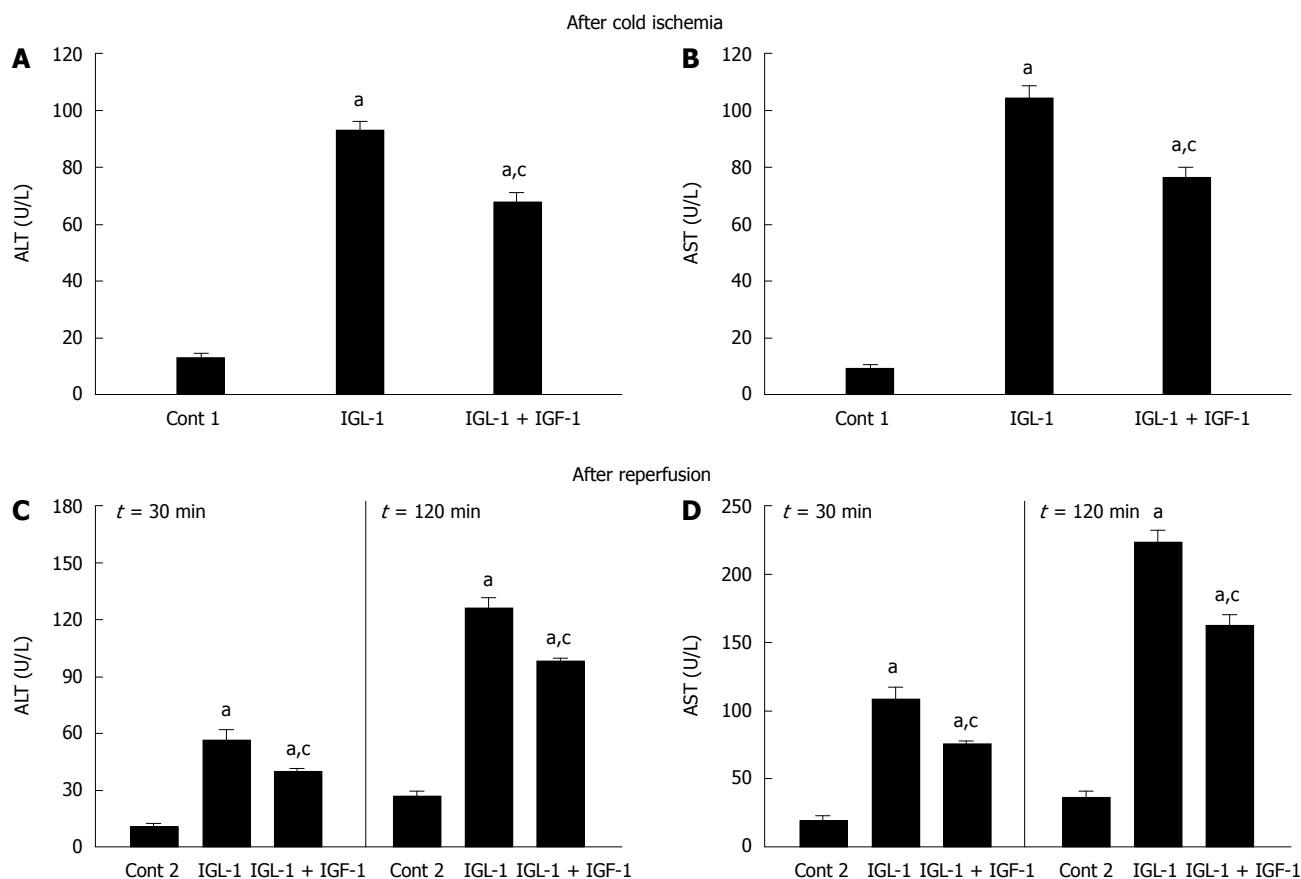


Figure 1 Alanine aminotransferase (A) and aspartate aminotransferase (B) levels in perfusate after 24 h cold storage, alanine aminotransferase (C) and aspartate aminotransferase (D) levels in perfusate after 30 and 120 min of normothermic reperfusion. Cont 1: Liver flushed without cold preservation; IGL-1: Livers preserved in IGL-1 solution; IGL-1 + IGF-1: Livers preserved in IGL-1 with IGF-1. ^a*P* < 0.05 vs Cont 1, ^b*P* < 0.05 vs IGL-1. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; IGF-1: Insulin like growth factor-1; IGL-1: Institut georges lopez-1.

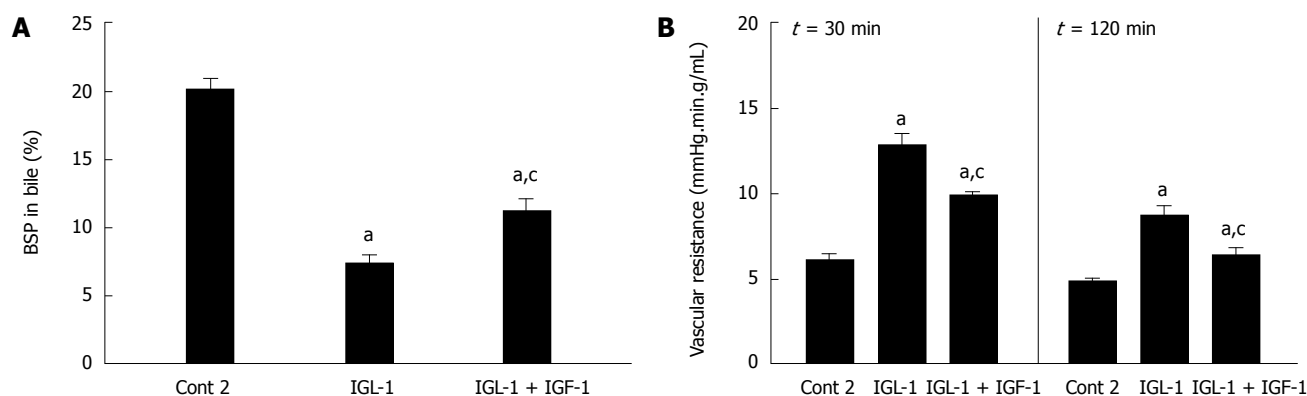


Figure 2 Percentage of sulfobromophthalein in bile (A) and vascular resistance (B) of steatotic livers after 30 and 120 min of normothermic reperfusion. Cont 2: Liver flushed and perfused *ex vivo* without cold preservation; IGL-1: Livers preserved in IGL-1 solution; IGL-1 + IGF-1: Livers preserved in IGL-1 with IGF-1. ^a*P* < 0.05 vs Cont 2, ^b*P* < 0.05 vs IGL-1. BSP: Sulfobromophthalein; IGF-1: Insulin like growth factor-1; IGL-1: Institut georges lopez-1.

also accompanied by a significant reduction in pro-inflammatory cytokine TNF- α level when compared to IGL-1 alone after 2 h normothermic reperfusion (Figure 4C).

Effects of IGF-1 on p-P38 and p-ERK MAPKs after reperfusion

Finally, we examined the effects of IGL-1 with and without IGF-1 on P38 and ERK 1/2 MAPKs, whose activation is closely related with hypothermic conditions. Figure 5 shows

increased phosphorylation of ERK 1/2 and P38 MAPK in IGL-1 solution when compared to Cont 2, but again this increase was offset by the addition of IGF-1.

DISCUSSION

IGF-1 is a 70-amino-acid polypeptide, mainly synthesized by the liver, with protective effects against IRI in a variety of tissues^[32-35], including the liver^[18]. We recently reported

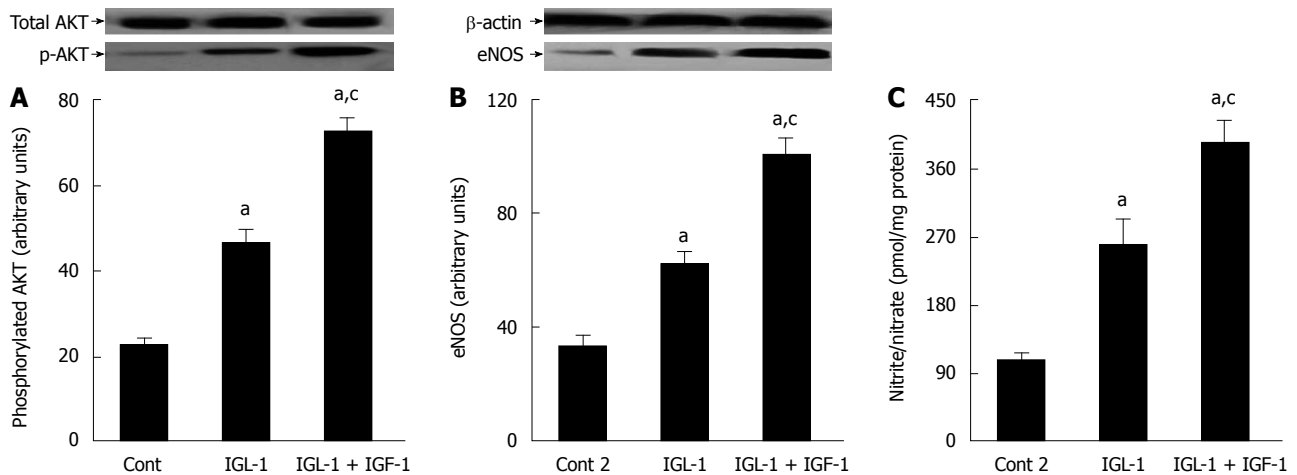


Figure 3 Beneficial effects of insulin like growth factor-1 on AKT and nitric oxide in steatotic liver graft preservation. A: Representative Western blottings of total and p-AKT at the top and densitometric analysis at the bottom after 24 h of cold storage and 120 min of normothermic reperfusion; B: Endothelial nitric oxide synthase (eNOS) protein levels in liver after 120 min of normothermic reperfusion. Representative Western blottings at the top and densitometric analysis at the bottom; C: Nitrite and nitrate levels after 120 min of normothermic reperfusion. Cont 2: Liver flushed and perfused *ex vivo* without cold preservation; IGL-1: Liver preserved in IGL-1 solution; IGL-1 + IGF-1: Livers preserved in IGL-1 with IGF-1. ^a $P < 0.05$ vs Cont 2, ^c $P < 0.05$ vs IGL-1. IGF-1: Insulin like growth factor-1; IGL-1: Institut georges lopez-1.

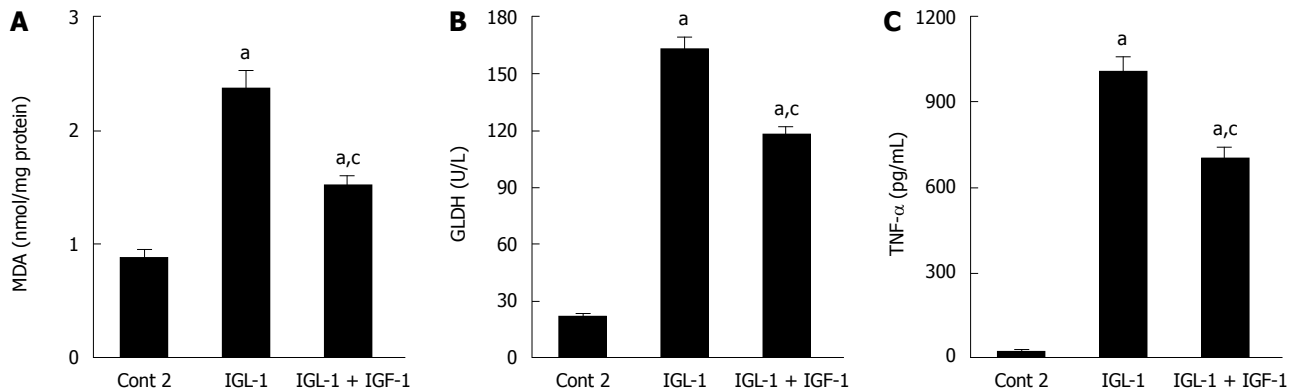


Figure 4 Role of insulin like growth factor-1 on oxidative stress, mitochondrial damage and tumor necrosis factor- α release in steatotic liver. A: Hepatic malondialdehyde (MDA) levels after 120 min of reperfusion; B: Glutamate dehydrogenase (GLDH) activity levels after 120 min of normothermic reperfusion; C: Tumor necrosis factor- α (TNF- α) levels in perfusate after 120 min of normothermic reperfusion. Cont 2: Liver flushed and perfused *ex vivo* without cold preservation; IGL-1: Livers preserved in IGL-1 solution; IGL-1 + IGF-1: Livers preserved in IGL-1 with IGF-1. ^a $P < 0.05$ vs Cont 2, ^c $P < 0.05$ vs IGL-1. IGF-1: Insulin like growth factor-1; IGL-1: Institut georges lopez-1.

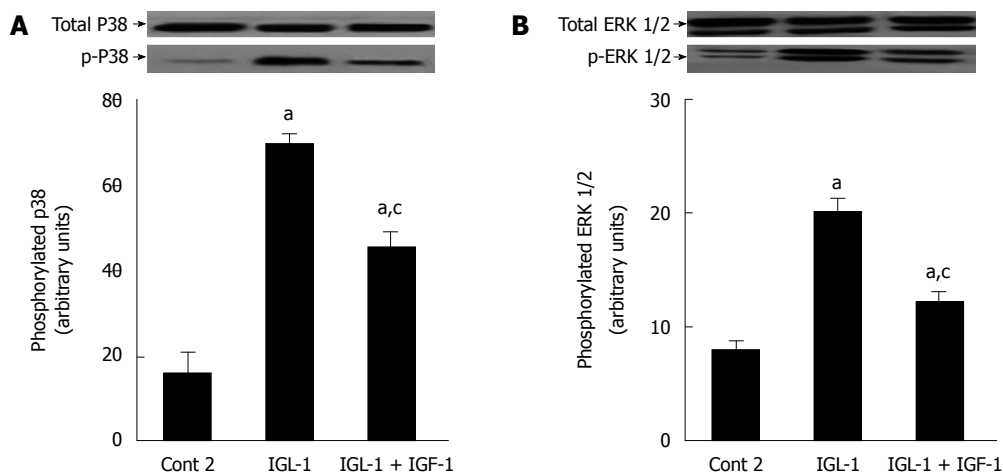


Figure 5 Effects of insulin like growth factor-1 on p-P38 and p-ERK in steatotic livers subjected to cold ischemia reperfusion. A: P38 protein levels in liver after 120 min of normothermic reperfusion. Representative Western blottings of total and p-P38 at the top and densitometric analysis at the bottom after 24 h of cold storage and 120 min of normothermic reperfusion; B: ERK 1/2 protein levels in liver after 120 min of normothermic reperfusion. Representative Western blottings of total and p-ERK 1/2 at the top and densitometric analysis at the bottom. Cont 2: Liver flushed and perfused *ex vivo* without cold preservation; IGL-1: Liver preserved in IGL-1 solution; IGL-1 + IGF-1: Livers preserved in IGL-1 with IGF-1. ^a $P < 0.05$ vs Cont 2, ^c $P < 0.05$ vs IGL-1. IGF-1: Insulin like growth factor-1; IGL-1: Institut georges lopez-1.

that IGF-1 protects steatotic livers subjected to warm IRI, but its role in fatty liver preservation has been poorly investigated^[18]. For this reason, we explored the effect of IGF-1 supplementation of serum free IGL-1® solution, which has been shown to protect fatty livers against cold IRI^[13]. We used the concentration of IGF-I (10 µg/L) according to previous studies carried out in different experimental models. In these studies, IGF-I it was used to supplement UW solution in dog kidney transplantation, as well as, primary canine kidney tubule and human umbilical vein endothelial cell cultures^[21,22,25,36]. The results reported here show that the addition of IGF-1 to IGL-1® solution significantly improved fatty liver preservation, as evidenced by the reduction in AST/ALT levels. These results are consistent with previous reports by other authors, who demonstrated that the addition of TF to UW solution improves the survival of orthotopic liver allografts^[21].

BSP clearance in bile is a useful tool for assessing liver function after prolonged cold ischemia^[23,24]. It is well established that complications in biliary structures appear in more than 25% of liver transplant recipients. The increases in BSP clearance observed in IGL-1 + IGF-1 solution revealed that IGF-1 supplementation enhances steatotic graft function.

Steatosis is the result of intracytoplasmic fat accumulation, which is associated with an increase in hepatocellular volume, induced distortion and narrowing of sinusoids with a reduction in the luminal diameter by up to 50% when compared to normal liver^[5]. This provokes severe alterations in hepatic blood flow and microcirculation, and prevents appropriate revascularization of the graft. The benefits of the use of IGL-1® solution for fatty liver preservation are associated with its capacity to generate NO^[13], a potent vasodilator involved in the regulation of hepatic microcirculation, through eNOS activation, as previously reported by us^[24]. Moreover, it has been established that IGF-1 up-regulates eNOS activity by interacting with a tyrosine kinase membrane receptor which activates the AKT signalling pathway^[19,37,38]. The results of the present study show that IGF-1 increases AKT phosphorylation and enhance eNOS activation induced by IGL-1® alone. This in turn induces NO generation, which reduces vascular resistance following reperfusion. This is in line with several reported studies demonstrating that a low concentration of eNOS-derived NO maximizes blood perfusion, promotes cell survival and protects the liver against IRI^[39]. In contrast, the sustained presence of iNOS-derived NO might become detrimental by increasing toxic reactive oxygen species, thus leading to liver injury^[39-41]. In our experimental model, the benefits of NO were not associated with iNOS activation (data not show). Moreover, other studies demonstrate that while increasing e-NOS, IGF-1 inhibits inducible NO^[19,42].

It is clear that fatty accumulation resulting from steatosis induces ultra-structural and biochemical changes in liver mitochondria^[43,44], which may render these organelles intrinsically more susceptible to IRI injury. Given that mitochondria are the main sites of ROS production in IRI, the increased oxidative stress observed in steatotic livers after

cold IRI could be attributed to mitochondrial damage. It is well known that IGF-1 prevents mitochondrial damage and oxidative stress following IRI^[17,33,34]. Our results indicate that the addition of IGF-1 to IGL-1® preservation solution increases liver mitochondria protection, and prevents ROS generation associated with reperfusion injury.

Vairetti *et al.*^[45] using the same “*ex vivo*” experimental model, have shown that TNF-α is released when fatty livers are subjected to cold ischemia reperfusion. In our conditions, TNF-α release was also evidenced in response to cold ischemia-reperfusion insult.

IGF-1 supplementation prevented the release of TNF-α^[46], which has a pivotal role in the progression of liver reperfusion damage. This finding is consistent with the accumulating evidence of crosstalk between IGF-1 and TNF-α during ischemia reperfusion injury^[47]. NO generated by IGF-1 could decrease TNF-α release, as occurs in liver ischemic preconditioning, in which the induced hepatoprotection is also mediated by the inhibitory action of NO on TNF-α release through eNOS activation^[48].

In addition, several intracellular signalling pathways, including the extracellular signal-regulated kinases 1/2 and P38 mitogen-activated protein kinase, are activated during hypothermia^[49-52]. In these conditions, a mutual activation effect between TNF-α and MAPKs may also occur thus exacerbating the liver damage^[53]. Our results indicate that cold storage in IGL-1® solution alone activates P38 and ERK 1/2, and that IGF-1 addition prevented this activation, which is consistent with a TNF-α reduction that leads to an improvement in liver injury and function. This is in line with previous reports that the addition of TF to UW improved endothelial cell preservation by reducing ERK 1/2 and P38 activation^[22].

In conclusion, we have demonstrated that IGF-1 addition to IGL-1® solution protects fatty liver against cold IRI. The beneficial action of IGF-1 appears to be mediated by AKT activation and NO generation, with concomitant prevention of pro-inflammatory cytokines, such as TNF-α. However, further studies will be required to examine the underlying mechanisms. This may increase the use of steatotic livers, which partially compensates the shortage of organs for transplant.

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COMMENTS

Background

Static preservation solution is crucial for graft viability; especially when steatosis is present. Serum-free preservation solutions deprive the donor organ of essential trophic factors such as insulin like growth factor-1 (IGF-1) which is associated with deleterious effects including cell cycle arrest and death. The addition of IGF-1 to University of Wisconsin (UW) solution improves the capacity of this preservation solution for protecting liver grafts subjected to a prolonged ischemic period. Serum-free institut georges lopez-1 (IGL-1) solution has been proposed as an effective alternative to UW for steatotic liver preservation.

Research frontiers

In this study, the authors focused on the importance of IGF-1 as a biological additive to IGL-1 preservation solution. The induction of nitric oxide (NO) synthesis by the modified IGL-1 solution improves microcirculatory disorders in fatty livers. IGL-1 supplementation with IGF-1 increases graft protection against ischemia reperfusion injury through the activation of protein kinase AKT and a concomitant reduction in tumour necrosis factor (TNF) release and P38/ERK 1/2 MAPKs activation which play an important cryoprotective role in liver graft preservation.

Innovations and breakthroughs

The enrichment of IGL-1 solution with IGF-1 increases NO generation through AKT activation and prevents pro-inflammatory cytokine release such as TNF- α during steatotic liver graft reperfusion.

Applications

The use of modified IGL-1 solutions should be a useful strategy for increasing steatotic liver graft preservation.

Peer review

This manuscript describes the beneficial effects of IGF-1 when added to IGL-1 solution in the preservation of (fatty) liver.

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Redefining the properties of an osmotic agent in an intestinal-specific preservation solution

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Abstract

AIM: To investigate the effects of dextrans of various molecular weights (Mw) during a 12 h cold storage time-course on energetics, histology and mucosal infiltration of fluorescein isothiocyanate (FITC)-dextran.

METHODS: Rodent intestines were isolated and received a standard University of Wisconsin vascular flush followed by intraluminal administration of a nutrient-rich preservation solution containing dextrans of varying Mw: Group D1, 73 kdal; Group D2, 276 kdal; Group D3, 534 kdal; Group D4, 1185 kdal; Group D5, 2400 kdal.

RESULTS: Using FITC-labeled dextrans, fluorescent

micrographs demonstrated varying degrees of mucosal infiltration; lower Mw (groups D1-D3: 73-534 kdal) dextrans penetrated the mucosa as early as 2 h, whereas the largest dextran (D5: 2400 kdal) remained captive within the lumen and exhibited no permeability even after 12 h. After 12 h, median injury grades ranged from 6.5 to 7.5 in groups D1-D4 (73-1185 kdal) representing injury of the regenerative cryptal regions and sub-mucosa; this was in contrast to group D5 (2400 kdal) which exhibited villus denudation (with intact crypts) corresponding to a median injury grade of 4 ($P < 0.05$). Analysis of tissue energetics reflected a strong positive correlation between Mw and adenosine triphosphate ($r^2 = 0.809$), total adenylates ($r^2 = 0.865$) and energy charge ($r^2 = 0.667$).

CONCLUSION: Our data indicate that dextrans of Mw > 2400 kdal act as true impermeant agents during 12 h ischemic storage when incorporated into an intraluminal preservation solution.

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Key words: Intraluminal preservation solution; Intestinal-specific; Osmotic impermeant; Organ preservation; Cold storage

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INTRODUCTION

Small bowel transplantation (SBT) has become an essential treatment for patients with irreversible intestinal failure who do not succeed on parenteral supplementation^[1]. The global frequency and success rates of such procedures have seen steady increases over the last decade^[1]. While only 11 intestinal transplants were performed in 1990, 140 cases were reported in 2003; currently approximate 200 are performed annually across the world^[1]. For those individuals receiving antibody-based induction therapy and tacrolimus-based maintenance immunosuppression, one year post-transplant survival rates are comparable to those of liver (> 80%)^[1]. The majority of individuals do not receive these therapies, hence 5-year graft survival rates for SBT remain low (31%-69%; 48% weighted average for 2005 data) compared to other commonly transplanted organs^[2].

Among the obstacles to successful SBT is the extreme susceptibility of the mucosal epithelium to even brief periods of ischemia^[3]. For this reason, the ability to successfully preserve graft viability during the period of ischemic cold storage is critical. Preventing hypothermia-induced cellular swelling is a basic principle of successful organ preservation^[4]. One major advancement in preservation solution technology and design was the development of the "gold standard" preservation solution, the University of Wisconsin (UW) solution. Improvements in organ quality and safe cold ischemic times can be largely attributed to the control of cellular edema. This was accomplished by including cell-impermeant molecules lactobionate, raffinose, and hydroxyethylstarch (HES)^[5]. With respect to the intestine, studies have shown that net fluid shifts contributing to mucosal injury can originate from the vasculature or lumen^[6,7]. Since standard intestinal procurement involves a common intra-aortic flush of all abdominal organs, current preservation strategies do not address fluid shifts of a luminal origin.

Throughout the last several years, our laboratory has developed a novel nutrient-rich preservation solution (containing a large amino acid component) tailored to the specific metabolic requirements of the small intestine; termed AA solution. Numerous *in vitro* and *in vivo* models have documented superior maintenance of energetics, a reduction in oxidative stress, and a preservation of mucosal morphology and barrier function following intraluminal administration of the nutrient-rich solution^[8-10]. We have demonstrated the benefits of using a high molecular weight (Mw) HES (2200 kdal) as an impermeant molecule in our novel solution^[11,12], with no direct evidence that the starch molecule does not penetrate the mucosal barrier during ischemia. Recent data from our lab has demonstrated that dextran (Mw = 70 kdal) is not osmotically active when delivered as part of an intraluminal preservation solution; direct visualization of a fluorescently-labeled dextran-70 clearly showed the rapid migration of the dextran into the mucosa. This has raised the question as to the exact Mw characteristic of an effective impermeant agent in the realm of intestinal ischemia as it relates to a novel strategy of intraluminal preservation.

Although the UW solution contains HES on the basis of preventing interstitial edema incurred during cold ischemia^[4], there exists controversy over its effectiveness for static organ storage. Some studies report that HES may be omitted from the UW solution without detrimental effects on overall graft quality^[13-15], while others suggest a protective role of HES in stored tissues^[16-20]. Because these previous studies deal solely with an intravascular delivery of UW solution, they do not provide information about the role of colloids in preservation solutions administered intraluminally for intestinal grafts. We suspect that during cold storage, alterations in the permeability characteristics of the mucosa will result in the infiltration of relatively large osmotic agents, thereby failing to effectively control tissue edema and mucosal viability. We hypothesized that there is a critical Mw for an effective osmotic agent to be used in an intraluminal preservation solution during cold ischemic storage.

MATERIALS AND METHODS

Summary of experimental design

Briefly, small intestines from rats were flushed intravascularly with UW solution, isolated and flushed intraluminally with a nutrient-rich preservation solution containing 5% dextran of varying Mw (73, 276, 534, 1185, 2200 kdal). Fluorescein isothiocyanate (FITC)-labeled dextrans to a final fluorescence of 10×10^6 fluorescence units/mL were incorporated into the solutions to aid in direct visualization of dextran infiltration. Intestines were stored at 4°C and samples were taken over a 12 h time-course for subsequent analysis.

Surgical procedure and intestinal procurement

Male Sprague-Dawley rats (200-250 g) were obtained from the University of Alberta and used as organ donors. All experiments were conducted in accordance with Canadian Council on Animal Care policies. Animals were fasted overnight and provided water *ad libitum*. Rats were induced with pentobarbital (65 mg/250 g; IP), followed by inhalational isoflurane (0.5%-2%) to maintain anesthesia. Following a midline laparotomy, the aorta was exposed infrarenally and at the celiac trunk. The supraceliac aorta was clamped and 2-4 mL modified UW solution was administered *via* the infrarenal aorta. The vena cava was transected to facilitate the outflow of blood and perfusate. The entire jejunum and ileum was subsequently harvested. A nutrient-rich preservation solution (AA solution), developed in our lab, was used to flush and clear the lumen of its contents (40 mL; approximate 2.0 mL/g). Sixteen centimeter-long sections of ileum were measured out. The sections were filled at the proximal end with 4-5 mL nutrient-rich preservation solution containing 5% dextran of varying Mw (fluorescence was standardized to 10×10^6 fluorescence units/mL and each end ligated with 3-0 silk). The preservation solution contained (values in brackets are mmol/L): Glutamine (35), Lactobionate (20), Glutamate (20), Aspartate (20), Glucose (20), BES [N,N-Bis(2-hydroxyethyl)taurine] (15), Arginine (10), Glycine

Table 1 Characteristics of high-purity dextrans

Group	Mp	Mw	Mn	PDI	IV
D1	62900	72700	50700	1.43	0.263
D2	238000	275900	204600	1.35	0.463
D3	490000	534000	371000	1.43	0.633
D4	1050000	1185000	705000	1.67	0.862
D5	2000000	2400000	1600000	1.50	0.803

Mp: Peak average molecular weight; Mw: Weight average molecular weight; Mn: Number average molecular weight; PDI: Polydispersity index ($PDI = Mw/Mn$); IV: Intrinsic viscosity (dL/g).

(10), Asparagine (10), Threonine (10), Lysine (10), Valine (10), Serine (10), Methionine (5), Leucine (5), Isoleucine (5), Histidine (5), Ornithine (5), Proline (5), Adenosine (5), Cysteine (5), β -Hydroxybutyrate (3), Tyrosine (1), Tryptophan (1), Trolox (1), 3-Aminobenzamide (1), Allopurinol (1); plus the inclusion of 5% Dextran. The pH of each solution was adjusted to 7.40 with sodium hydroxide; measured osmolarity was 320 mOsm.

Group designations were as described in Table 1; briefly the dextran Mw for the 5 groups (D1-D5) were: 73 (D1); 276 (D2); 534 (D3); 1185 (D4); 2400 (D5) kdal.

Tissues were stored on ice at 4°C in standard AA solution and sampled at 2, 4, and 12 h; at each time-point a 4 cm length of intestine was sampled; half was used for histology and half for metabolite analysis. To arrest metabolic activity, samples were snap frozen in liquid nitrogen, and stored at -65°C until processed.

FITC-labeling of dextrans

Dextrans used in this study were obtained from American Polymer Standards Corporation, Ohio, US; these dextrans were of the highest purity available and characteristics are provided in Table 1. Briefly, peak average molecular weight (Mp), weight average molecular weight (Mw), number average molecular weight (Mn), and polydispersity index (PDI) describe molecular distribution curves. The key parameter defining dextran purity is the PDI with a value of 1.0 representing absolute purity. PDI values of less than 3 represent high purity fractions; hence values in the current study of 1.35 to 1.67 are of extremely pure dextran fractions. The procedure used to FITC-label the dextrans from the above solutions was based on the original procedure used by De Belder and Granath^[21]. The dextran of interest together with fluorescein 5-isothiocyanate (FITC) were dissolved in dimethyl sulphoxide, pyridine and dibutyltin dilaurate and heated to 95°C for 2 h. The mixture was then put on ice and the dextran was precipitated out with cold 100% ethanol, filtered and dried in an oven at 40°C. Labeling efficiency was 0.015-0.020; this corresponds to 1 fluorescein molecule per 50-67 glucose units. FITC-labeled dextran accounted for $8.5\% \pm 0.6\%$ of total dextran in each solution; fluorescence was standardized. Fluorescence for FITC-dextrans was evaluated at an excitation wavelength = 485 nm/emission wavelength = 535 nm.

Stability of the FITC label

When labeling fluorescent moieties to target molecules, one of the key concerns that must be considered for subsequent experimentation to be valid is that the fluorescent label must remain bound to the target and does not spontaneously detach over time when in aqueous solution. In this study, a dilute solution (approximate 0.1%) of each dextran was incubated at 4°C for 24 h. Following the incubation period, FITC-dextrans were precipitated with cold 100% ethanol, centrifuged, and the supernatant (containing free FITC) was assessed for fluorescence. When solutions were incubated at 4°C, there were no statistically significant decreases in FITC-bound dextrans; this indicated a stable conjugation for all high-purity dextrans used in this study.

Sample preparation and metabolite assay

Frozen small bowel samples were weighed and then extracted 1:5 weight/volume in perchloric acid containing 1 mmol/L ethylenediamine tetra-acetic acid. The precipitated protein was removed by centrifugation (20 min at $20000 \times g$). Acid extracts were neutralized by the addition of 3 mol/L KOH/0.4 mol/L Tris/0.3 mol/L KCl and then recentrifuged (20 min at $14000 \times g$). Aliquots of neutralized extracts were immediately processed *via* standard enzyme-linked metabolite assays^[22]. Spectrophotometric analysis was then performed to measure the absorbance of NADH at 340 nm, providing quantification of adenosine triphosphate (ATP), total adenylates [ATP + adenosine diphosphate (ADP) + adenosine monophosphate (AMP)] and energy charge [(ATP + 0.5 ADP)/total adenylates] and malondialdehyde^[22,23]. Values are reported as μmol per gram protein. Protein was measured according to the method of Lowry *et al.*^[24].

Histology

Bowel samples were fixed in alcohol formalin solution, processed to paraffin wax, embedded, and sections cut at 5 μm . Sections were dewaxed then mounted with Prolong Gold anti-fade reagent with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) and sealed with nail polish. Fluorescent microscopy was used to view FITC and DAPI fluorescence in tissues; wavelengths were Ex = 485/Em = 535 and Ex = 360/Em = 460, respectively. Fluorescent images were digitally captured using AxioVision software. All photos shown reflect representative findings. A second set of sections were stained with hematoxylin and eosin and graded according to a modified Park's classification for intestinal injury^[25] as follows.

Grade 0: Normal mucosa; grade 1: Subepithelial space at villus tip; grade 2: Moderate subepithelial space; grade 3: Epithelial lifting along villus sides; grade 4: Denuded villi; grade 5: Loss of villus tissue; grade 6: Crypt layer injury; grade 7: Transmucosal injury; and grade 8: Transmural injury.

Statistical analysis

Metabolite data were reported as mean \pm SE for each

Table 2 Intestinal morphology following cold ischemic storage

Time (h)	Group	Grade	Median	Significance	Event
2	D1	0, 2, 6, 7	4	D3, D4, D5 vs D1, D2, $P = 0.06$	Denuded villi
	D2	3, 4, 6, 6	5		Loss of villi
	D3	1, 2, 2, 3	2		Moderate clefting
	D4	1, 2, 3, 5	2.5		Moderate-extensive clefting
	D5	1, 2, 3, 4	2.5		Moderate-extensive clefting
12	D1	0, 6, 7, 8	6.5	D5 vs D1-D4, $P < 0.05$	Injury to crypts and submucosa
	D2	2, 6, 7, 7	6.5		Injury to crypts and submucosa
	D3	3, 7, 8, 8	7.5		Injury to crypts, submucosa and muscularis
	D4	3, 6, 8, 8	7		Injury to crypts and submucosa
	D5	0, 4, 4, 5	4		Clefting and denuded villi

A modified Park's classification for intestinal injury was used to assess mucosal morphology as follows: Grade 0: Normal mucosa; Grade 1: Subepithelial space at villus tip; Grade 2: Moderate subepithelial space; Grade 3: Epithelial lifting along villus sides; Grade 4: Denuded villi; Grade 5: Loss of villus tissue; Grade 6: Crypt layer injury; Grade 7: Transmucosal injury; Grade 8: Transmural injury.

group. Statistical differences between groups were determined using analysis of variance, followed by Student-Newman-Keuls'. Analysis of relationships between Mw and ATP, total adenylates, or energy charge was performed with a linear regression analysis for parametric data. Differences in histology grades were assessed by a non-parametric Kruskal-Wallis test.

RESULTS

Infiltration of FITC-labeled dextrans during cold ischemic storage

The lower Mw dextrans tested (D1, D2, D3) consistently demonstrated infiltration of the fluorescent label at all time-points. At the earlier time-points, 2 and 4 h, fluorescence intensity was greatest in the epithelium or in sloughed epithelial cells. After 12 h storage, there was significant infiltration into the cryptal regions, the lamina propria, as well as the vascular epithelium. Mucosal injury was greatest in these groups (D1, D2, D3), ranging from development of subepithelial clefts at 2 h to complete denudation at the later time-points, leaving the underlying tissue vulnerable to further dextran penetration.

Interestingly, the intermediate Mw dextran, D4, exhibited no extensive penetration of label into the epithelial layer and largely remained within the layer of mucous coating the surface of the villi. Several goblet cells and the apices of some villi did have a minor amount of fluorescence. However, by 4 h, there was considerable infiltration of the dextran label throughout the underlying tissue. Limited mucosal injury was apparent even after 12 h storage, at which time epithelial clefting had developed. Treatment with the highest Mw tested, D5, resulted in no observable infiltration into the epithelium or lamina propria; this was evident at all time-points (Figure 1).

Evidence of mucosal injury

Histologic injury was evident early on during cold storage, particularly for the lower Mw dextrans after only 2 h. Most notably, in groups D1 and D2, 50% of specimens exhibited a significant degree of crypt infarction, compared to no evidence of crypt infarction in groups D3,

D4, and D5 ($P = 0.06$).

By 12 h, 75% (12/16) of specimens in groups D1-D4 had an injury grade of 6 or greater (damage to the crypts, mucosa, or transmural injury). Conversely, 75% of specimens in Group D5 exhibited clefting where the epithelium had lifted off the underlying lamina propria (grade 3) and at times had dissociated from the villus proper (grade 4) ($P < 0.05$). A summary of injury grading and median grades are presented in Table 2. Representative fields of injury are presented in Figure 2 micrographs.

ATP, total adenylate, energy charge levels

Following 12 h cold storage, ATP levels ranged from 5.3 to 9.6 $\mu\text{mol/g}$, with D1 and D2 having the lowest levels (5.3-5.6 $\mu\text{mol/g}$). Intermediate Mw groups, D3 and D4, exhibited intermediate ATP levels, both significantly different from D1 and D2 ($P < 0.05$). ATP progressively increased to a maximum of 9.6 $\mu\text{mol/g}$ in D5; this group had significantly greater levels than all other groups ($P < 0.05$). Levels of total adenylates (ATP + ADP + AMP) closely resembled those of ATP. Levels in groups D2 and D3 were significantly higher than in D1 and D2, $P < 0.05$. Similarly, D5 level was significantly greater than all groups, $P < 0.05$. Atkinson described another useful measure of tissue energetics, "energy charge" as: $\text{EC} = (\text{ATP} + \text{ADP}/2)/\text{total adenylates}$ ^[26]. In illustrating the significance of this measurement, Pegg used the analogy of a battery in describing the energy charge as being a measure of the "charged up" state of the adenylate pool^[27]. As ATP levels decline relative to ADP and AMP levels, energy charge also drops, indicating that less of the total adenylate pool exists in a form that is immediately available for cellular work. Energy charge ratios showed similar trends to those of ATP and total adenylates. Values increased as Mw of the dextran increased, starting at 0.45 in D1 and reaching 0.62 in D5; D5 value was significantly different than all values in groups D1-D4 ($P < 0.05$) (Figure 3).

Correlation between energetics and dextran Mw

With respect to dextran Mw, there was a strong positive and statistically significant correlation with ATP ($r^2 = 0.809$); total adenylates ($r^2 = 0.865$); and energy charge

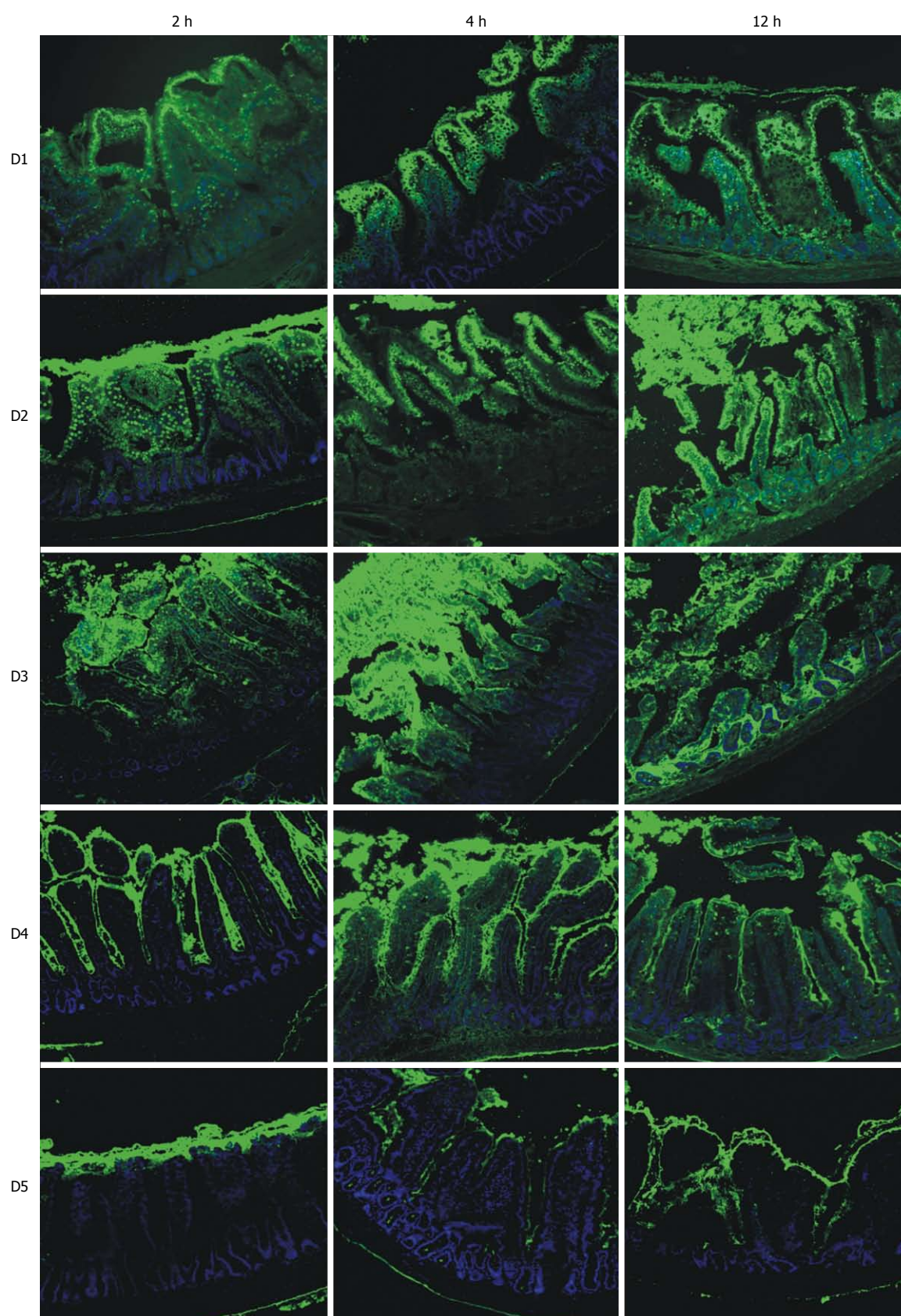


Figure 1 Infiltration of fluorescein isothiocyanate-labeled dextrans during cold ischemic storage. All magnifications ($\times 10$ objective) are of representative fluorescent intensities and mucosal injury. All photos have a standardized exposure time of 250 ms for the fluorescein isothiocyanate channel; exposure times for 4',6-diamidino-2-phenylindole, a common nuclear stain, are 2-20 ms and are included for contrast.

($r^2 = 0.667$). Overall, group D5 was the most effective in preserving the three parameters of tissue energetics over

the 12 h period of cold ischemic storage, while lower Mw groups exhibited poor conservation of energetics. Supe-

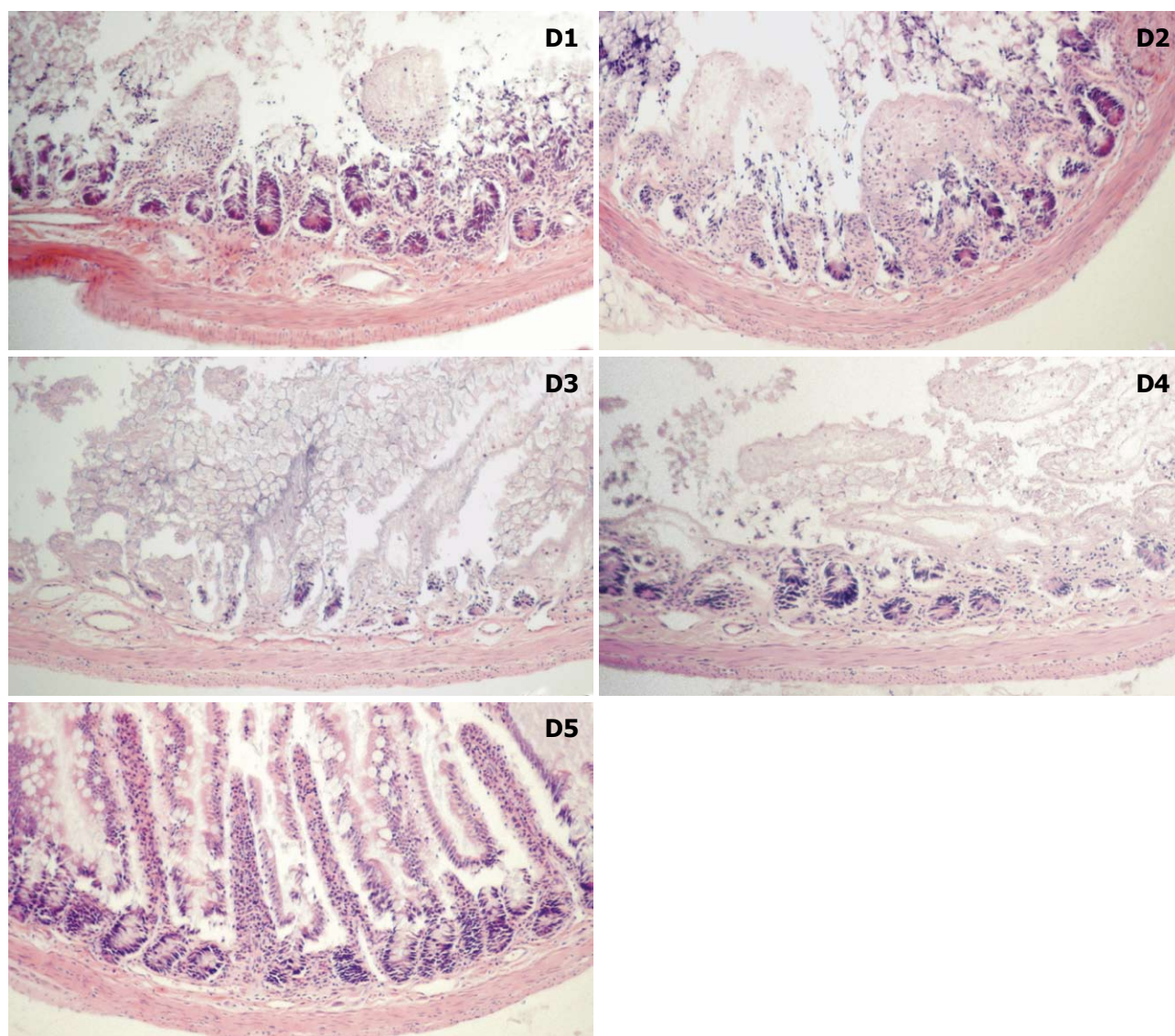


Figure 2 Histology after 12 h cold ischemic storage. Photos are of representative median grades of histologic injury according to a modified Park's classification of intestinal injury^[25]. Magnification with 10 × objective. HE staining, 5 μm sections. D1: Grade 6.5; D2: Grade 6.5; D3: Grade 7.5; D4: Grade 7; D5: Grade 4.

rior maintenance of energetic parameters supported the preservation of mucosal viability as determined by direct histologic observation of mucosal morphology as well as penetration of the FITC-labeled dextrans (Figure 4).

Oxidative stress

No significant differences were detected among groups D1-D5 with respect to the parameter of oxidative stress (malondialdehyde) after 12 h storage; values varied between 236 and 274 nmol/g protein in all 5 experimental groups (data not shown).

DISCUSSION

The major clinical application of dextrans, in addition to other starches and albumin, has been for their plasma volume expansion properties. These molecules exert their osmotic effects by binding water within the vasculature and creating an osmotic force to extract water from edematous tissues. The Mw of a molecule required

to remain captive within the vasculature is very low as demonstrated by the effectiveness of an intravascular flush during organ procurement with the UW solution (osmotic agents, lactobionate and raffinose; Mw = 358 and 504, respectively). With respect to osmotic support and the application of an intraluminal preservation solution, the Mw permeability limit of the epithelial barrier during ischemic storage has not been clearly defined.

In the realm of organ preservation, osmotic agents are required to counteract the metabolic events that result in the influx of water into the cells/tissue. Under normal circumstances, cells have an extracellular environment rich in Na⁺ and low in K⁺^[5]. The Na⁺/K⁺ transporter maintains the respective gradients of these ions, relying on the use of ATP as its energy source. Essentially, this pump makes Na⁺ an impermeant outside of the cell, creating an osmotic force that counteracts the osmotic pressure exerted by proteins and impermeable anions in the cell interior. Together, these intracellular proteins and impermeable ions require an osmotic force of 110-140 mOsm/kg to

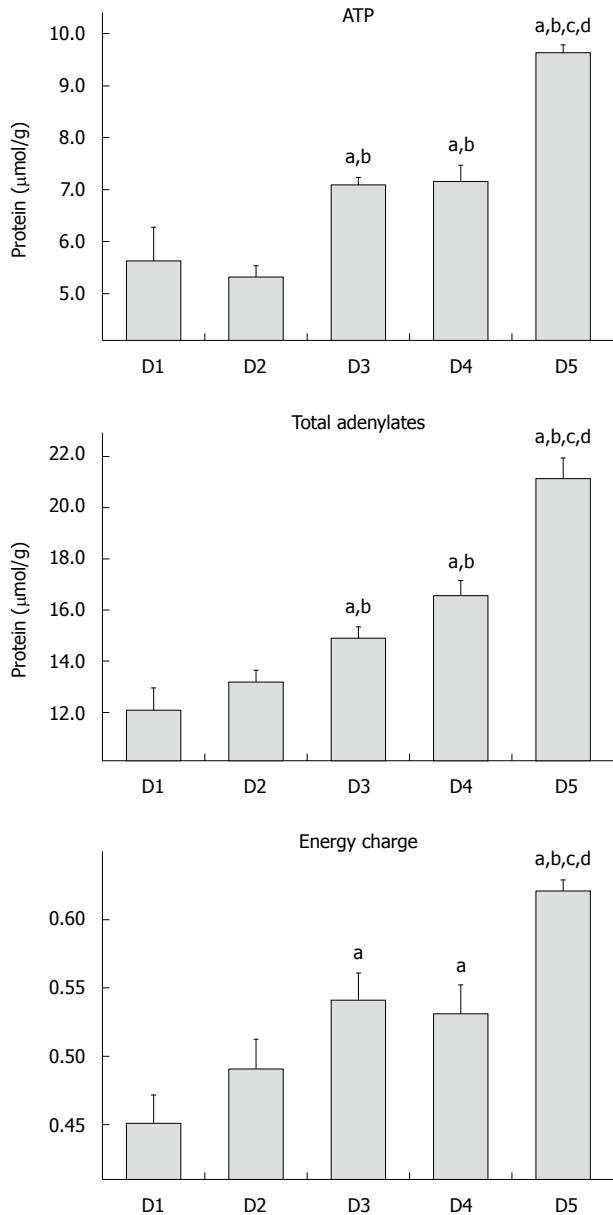


Figure 3 Parameters of energy metabolism during cold ischemic storage. Adenosine triphosphate (ATP), total adenylates and energy charge levels are presented. ^{a,b,c,d} $P < 0.05$, significantly different vs D1, D2, D3, D4, respectively.

offset fluid flow into the cell^[5]. In ischemic tissue under cold storage, the above situation is quite different. Hypothermic conditions coupled with aerobic metabolism collectively work to inhibit Na^+/K^+ ATPase activity, decreasing the cell membrane potential. As a result, Na^+ and Cl^- flow down their concentration gradients into the cell, and water follows, causing the cell to swell; cells eventually become damaged and may lyse^[5,19]. Cell impermeant molecules are critically important in regulating fluid movement into the cell; there is a fundamental requirement of 110-140 mmol/L of osmotic agents in order to counteract cell swelling^[5].

When utilizing an intraluminal preservation solution, inadequate osmotic support will culminate in direct injury to the epithelium. This leads to the compromise of cellular integrity and consumption of essential energy reserves

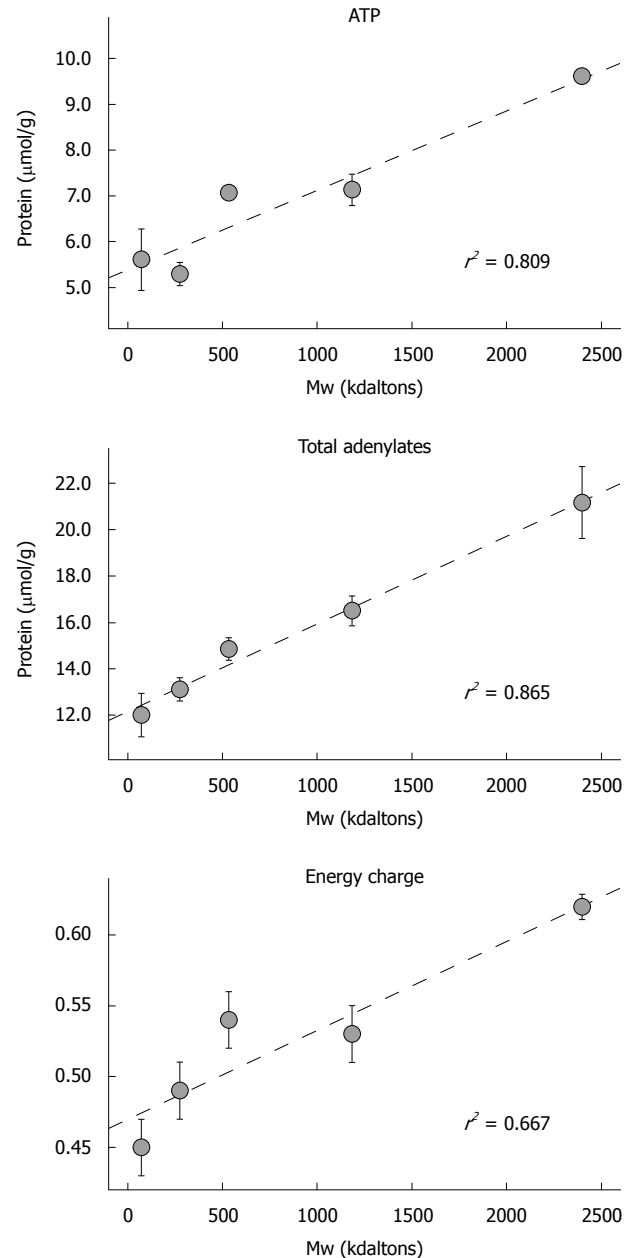


Figure 4 Correlation between dextran size and energy metabolism. Linear regression analysis was performed; correlation coefficients (r^2) are presented. ATP: Adenosine triphosphate; Mw: Molecular weight.

in an attempt to repair damaged tissue. Depletion of cellular energy reserves (ATP and total adenylates) results in an inability of the mucosal epithelium to preserve its barrier function, compromising epithelial integrity during organ storage^[20,28]. The barrier consists of numerous tight junctions located toward the apical surface of intestinal epithelial cells^[8]. A number of proteins form the tight junctions, which are active, energy-consuming structures^[8]. The transepithelial flow of both macromolecules and enteric bacteria are thought to be caused by tight junction dilation^[3], and the consequences are twofold: (1) an increased likelihood of sepsis and (2) a need to consider the permeability of molecules used for osmotic support during preservation. Sepsis may occur due to bacterial translocation^[29,30], and continues to account for the majority

of deaths after intestinal transplantation (46.0% between April 1985 and May 2003)^[1]. Increased permeability of the intestine during ischemic conditions should influence the critical Mw limit for molecules added to the intraluminal preservation solution for impermeant support. Inclusion of the appropriate agent capable of acting as a true osmotic will improve the quality of the intestinal graft and ultimately influence success of the transplant.

Hydrostatic and impermeant forces regulate the movement of fluids between intracellular and extracellular compartments. Hydrostatic forces have a predominant role in continuous perfusion methods; whereas, impermeant forces are the sole contributors governing net fluid flux in the setting of static cold storage, the current standard for most intra-abdominal organs. Shifts in net fluid flow during cold storage are proposed to originate from the intestinal vasculature and/or lumen^[6,7]. In the current study, fluid shifts of vascular origin were controlled by delivering a standard intra-aortic vascular flush with UW solution to all treatment groups, as would happen in the clinical situation. Keeping such fluid shifts constant was essential, allowing us to attribute any inter-group differences of luminal permeability to the specific dextran size of interest. Based on the data presented in the current communication, there was a definitive inverse relationship between dextran Mw and its ability to cross the epithelial barrier during cold storage. Several relatively large Mw dextrans (73-534 kdal) exhibited a definitive pattern of penetration of the mucosal layer within 2 h cold storage, eventually infiltrating the submucosa and muscularis propria after 12 h. The only effective dextran fraction tested that exhibited impermeant characteristics by remaining captive within the lumen even after 12 h cold ischemia was one of Mw = 2400 kdal. This study is the first to establish the critical permeability limit of the mucosal layer during intestinal preservation and to delineate the requisite Mw of a true osmotic impermeant in the lumen.

The relationship between Mw (size) and permeability determined in this study was somewhat expected based on the results from various animal and human intestinal permeability studies^[15,17,18]. An inverse relationship between Mw and mucosal-to-serosal permeability has been established previously for rodents^[17]. In a comprehensive report addressing comparative intestinal permeability in pig, rat, and human models, Nejdfor *et al.*^[15] found an inverse relationship between the Mw of marker molecules and their mucosal permeability across all species, irrespective of location in the gastrointestinal tract. The power of such a study comes from the fact that identical standardized methods of evaluating molecular permeability and a wide range of Mw were used for each species; this included ¹⁴C-mannitol, FITC-dextrans 4.4/70 kdal and several proteinaceous macromolecules (α -lactalbumin, 65 kdal and ovalbumin, 45 kdal).

In an animal study assessing the uptake of FITC-dextran (Mw 70 kdal) by the small intestinal epithelium, Ekström *et al.*^[29] found that during neonatal development (< 30-d old) there were negligible amounts of FITC-dextran in the serum with no enterocyte infiltration. One

should note that this experiment was conducted in healthy fully oxygenated tissues without any prior ischemic insult. Based on such reports, our nutrient-rich solution has in the past contained a dextran fraction with similar Mw for impermeant support^[8,30,31]. There is no reason to suspect that a macromolecule which is impermeant in the lumen of a healthy bowel will do so during ischemic conditions, even at reduced temperatures. As the number of tight junctions and the magnitude of the dilatations increase, the potential for infiltration and translocation of large macromolecules and eventually bacteria also increases. As a consequence of these impermeant characteristics, the lack of osmotic support contributes directly to tissue edema and its negative sequelae. In tissues lacking sufficient impermeant support during hypothermic storage, edema becomes a major concern.

In the current study, parameters of cellular energetics (ATP, total adenylates and energy charge) consistently reflected strong and significant positive correlations with respect to dextran Mw. Although the value of ATP to the cell is obvious, higher Total Adenylate levels reflect reduced rates of purine catabolism, potentially leading to the accumulation of hypoxanthine and the production of uric acid and superoxide (a highly reactive oxygen free radical)^[32]. A second consequence is that greater amounts of purines are available for ATP regeneration (once carbon exits the total adenylate pool, reconversion is not enzymatically possible in this pathway). These biochemical parameters of tissue integrity all indicate that the 2400 kdal dextran was maximally effective in protecting the mucosa from ischemic insult during cold storage.

In conclusion, the permeability characteristics of the rat small bowel during cold ischemia are very different than those of intestinal tissue that is not limited with respect to oxygen and nutrients. In order for a molecule to behave as a true osmotic impermeant, the Mw must be much greater than that established for intravascular preservation solutions. From the data presented in this study, the size of dextran showing the lowest degree of epithelial or mucosal infiltration, superior morphology and minimal disruption to cellular energetics was 2400 kdal. These findings clearly delineate the critical Mw limit that must be addressed in the development of an effective intraluminal preservation solution.

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COMMENTS

Background

An effective osmotic agent is one of the fundamental requirements of organ preservation for transplantation. Commonly used preservation solutions that are typically administered *via* an intravascular route utilize low molecular weight (Mw) impermeant compounds to maintain tissue water balance. Our laboratory has developed a novel strategy for intestinal preservation involving the intraluminal delivery of a nutrient-rich preservation solution; however, an appropriate impermeant molecule has not been clearly defined.

Research frontiers

Although organ preservation methods have existed for decades, luminal administration of a preservation solution tailored to the metabolic requirements of the small bowel is a novel concept.

Innovations and breakthroughs

This study reveals for the first time that a large Mw impermeant molecule is a fundamental requirement of an intraluminal preservation solution during cold, static storage of the small intestine. Interestingly, only the highest Mw dextran (2400 kdal) remained within the intestinal lumen throughout the entire 12 h period of cold storage, resulting in superior graft energy status and tissue morphology.

Applications

The current study focused on defining a suitable osmotic agent for use in our intraluminal preservation solution, a key factor affecting intestinal viability during organ storage. As a back-table luminal flush that does not interfere with the clinical vascular preservation method, our AA solution has the potential to revolutionize intestinal preservation, and therefore improve patient outcomes following transplantation.

Peer review

The manuscript submitted by Schlachter *et al* represents a methodical and well written study investigating the protective effects of different Mw dextrans in a novel intestinal preservation solution. The findings of the study are potentially important and widely applicable to the intestinal transplantation field.

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Role of simple biomarkers in predicting fibrosis progression in HCV infection

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Abstract

AIM: To examine the accuracy of the aspartate aminotransferase (AST)/Platelet Ratio Index (APRI) and FIB-4, in predicting longitudinal changes in liver histology in hepatitis C virus (HCV) patients.

METHODS: Patients that underwent repeat liver biopsies at least 1 year apart from 1999 to 2007 were identified. Liver fibrosis was staged on needle core biopsies evaluated by a single expert liver pathologist. Only laboratory values within 3 mo of the liver biopsies were used.

RESULTS: Thirty-six patients met the inclusion criteria with 50% stage 1 on initial biopsy, 25% stage 2, and 22% stage 3. Nineteen of 36 (53%) had progression of fibrosis on repeat biopsies, while 16 (44%) showed no change in stage, and one (3%) showed improvement. Patients that showed progression of fibrosis had significantly higher alanine aminotransferase and aspartate aminotransferase levels than the group that did not show progression. A significant correlation was seen between change in stage of fibrosis and change in APRI ($r^2 = 0.39$, $P = 0.00001$) and a change in FIB-4 ($r^2 = 0.31$, $P = 0.00004$). A change in APRI (Δ APRI) of 0.18 had 80% positive predictive value (PPV) and 67% negative predictive value (NPV) for progression of fibrosis. A change in FIB-4 (Δ FIB-4) of 0.39 had 75% PPV and 75% NPV for predicting progression of fibrosis.

CONCLUSION: Δ APRI and Δ FIB-4 parallel changes in fibrosis progression, and could be useful tools for clinicians in following patients with active chronic HCV infection.

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Key words: Hepatitis C; Liver fibrosis; Liver biopsy; Biomarkers

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DOI: <http://dx.doi.org/10.3748/wjg.v16.i45.5710>

INTRODUCTION

Hepatitis C virus (HCV) infection is one of the most common causes of chronic liver disease, and affects ap-

proximately 3% of the world's population^[1]. Current treatment decisions in chronic HCV are guided by the histopathological findings on liver biopsy. Although it is still considered the gold standard for assessment of fibrosis in chronic hepatitis C, liver biopsy has limitations. It is an expensive and invasive procedure with complications that range from pain (0.06%-22%) to death (0.0088%-0.3%)^[2]. Liver biopsy is also limited by both inter-observer and sampling variability. Although inter-observer variability has been improved by standardized scoring systems for fibrosis and inflammation, discordance rates for the stage of fibrosis of approximately 33% have been reported^[3,4]. The frequency of sampling errors is not surprising given the fact that a standard percutaneous liver biopsy specimen has been calculated to sample only 1/50 000 part of the liver^[5]. Considering the expense and limitations of liver biopsy, there is great interest in the use of non-invasive markers of liver fibrosis that can replace liver biopsy both as a guide to therapy and as a prognostic indicator. Current studies have shown that non-invasive markers can predict the absence of significant disease or the presence of advanced disease at the time of liver biopsy, but it is not clear that they can predict step-wise progression of fibrosis^[6]. It is also not clear if they have prognostic value regarding long-term clinical outcomes. If they can be proven to be useful longitudinally, this would be of great benefit to clinicians and their patients.

Non-invasive markers can be broadly divided into two major groups: radiological and serum-based markers. Transient elastography using ultrasound waves to measure stiffness of liver is an example of a radiological method but is limited in terms of cost, technical complexity and availability. It currently is not approved for clinical use in the United States by the Food and Drug Administration. Serum biomarkers have been proposed as an inexpensive and effective alternative to replace liver biopsy and can be subdivide into two categories: (1) indirect markers that are composed of simple routine biochemical and/or hematological tests; and (2) markers composed of substances that are part of the extracellular matrix (ECM). Although simple markers are inexpensive and available universally, the markers of ECM are expensive and available only in reference or research laboratories. Two indirect marker panels, aspartate aminotransferase (AST)/Platelet Ratio Index (APRI) and FIB-4, are composed of panels that are routinely preformed in patients with liver disease, and therefore, could be a practical and convenient way to follow patients^[7,8].

Most studies of biomarkers in fibrosis have compared their diagnostic accuracy in predicting the presence of mild vs significant fibrosis, mild/moderate vs advanced fibrosis, or cirrhosis, using a liver biopsy obtained at the same time as the gold standard^[6]. This approach has limitations because liver biopsy is a less than ideal reference standard and it is not clear if these biomarkers are useful in identifying patients in whom fibrosis is likely to progress. It is important to develop a non-invasive tool for predicting progression of fibrosis, because these patients are the ones who might benefit from clinical interventions like antiviral therapy and/or more frequent and intense monitoring.

In this study, we examined APRI and FIB-4 in a group of patients with paired liver biopsies to determine if longitudinal changes in the markers correlated with changes in the histological stage of fibrosis, and also examined whether the initial or follow-up APRI and FIB-4 was useful in predicting those patients who showed an increase in stage of fibrosis on liver biopsy.

MATERIALS AND METHODS

Patients studied

Following approval by the Institutional Review Board, we searched a database available in the Department of Pathology for all patients who had undergone at least two liver biopsies for staging fibrosis in mono-infected HCV-positive patients between 1999 and 2007. Patients were selected for inclusion if the liver biopsies were at least 1 year apart. Patients were excluded if they had cirrhosis, were co-infected with hepatitis B virus (HBV) or human immunodeficiency virus, had an organ transplant, daily alcohol intake of > 30 g/d, hepatocellular carcinoma or primary metabolic or autoimmune liver disease. The laboratory values used for the calculations of the APRI and FIB-4 were those that were closest to the liver biopsy date. Our hepatology service has had an ongoing prospective study of hepatic fibrosis markers since 2003, and 20 of the patients were enrolled in that study for one or both of their liver biopsies. Those patients had their blood drawn on the day of the liver biopsy. All other patients had blood work within 3 mo of the biopsy, or they were excluded from the study.

Laboratory studies

The platelets were measured using the Sysmex SE 9500 (Sysmex, Mundelein, IL, USA) and the AST and the alanine aminotransferase (ALT) were measured using either the Vitros 950 or 5,1 FS (Ortho Clinical diagnostics, Raitan, NJ, USA).

Biomarker panels

The APRI is a numerical value that is calculated using the following formula: $APRI = [AST \text{ (U/L)} / \text{upper limit of normal (U/L)}] \times 100 / \text{platelets (} 10^9/\text{L)}^{[7]}$. The FIB-4 index is also a numerical value that is calculated using the following formula $FIB\ 4 = \text{Age} \times AST \text{ (U/L)} / [\text{platelets (} 10^9/\text{L)} \times ALT^{1/2} \text{ (U/L)}]^{[8]}$.

Liver histology

All liver biopsies were read by a single pathologist (S.X.), without prior knowledge of the study status or the value of laboratory parameters being tested. Fibrosis was staged as described below. The fibrosis score is based on a five-point scale: stage 0 = no fibrosis; 1 = either mild pericellular fibrosis in the lobules, or mild portal fibrosis; 2 = periportal fibrosis, or portal fibrosis plus lobular pericellular fibrosis; 3 = septal or bridging fibrosis without evident parenchymal remodeling; and 4 = cirrhosis (with architectural remodeling and nodule formation). The rationales of including lobular pericellular fibrosis in our staging system are: (1) that this type of fibrosis is frequently observed among pa-

tients with chronic HCV infection; and (2) that zone 3 (or centrilobular) hepatic stellate cell activation is common in hepatitis C liver biopsies without other co-morbidity^[9].

Statistical analysis

In the initial analysis the median AST, ALT, platelets, APRI and FIB-4 scores were compared between patients who had an increase in at least one stage on liver biopsies (progressors) and those who did not progress (non-progressors) using a non-parametric test (Mann-Whitney *U* test). Logistic regression was used to calculate the accuracy of the ALT, AST, platelets, APRI and FIB-4 in predicting progression of fibrosis. Receiver operating characteristic (ROC) curves were calculated for the biomarkers at the time of initial and repeat biopsies. Area under the ROC curve (AUROC), sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated. Non-parametric tests were performed using Stats Direct statistical software (Sale, Cheshire, UK) and ROC curves were constructed using MedCalc statistical software (Marialterke, Belgium).

Calculation of sample size

Assuming that the AUROC for APRI and FIB-4 would be 0.8 if the null hypothesis is rejected and 0.5 if the null hypothesis is valid, a sample size of 29 is needed to detect the difference at an α value of 0.05 and β value of 0.8.

RESULTS

Patient characteristics

A total of 36 patients met the inclusion criteria. The baseline characteristics of the subjects are described in Table 1. The median age at the time of initial biopsies was 47 years (range: 25-68 years). Seventy-five percent (27/36) of patients were male, with 50% (18/36) Caucasians, 31% (11/36) African Americans and 19% (7/36) Hispanics. The majority (50%) of the patients were stage 1 on biopsy followed by stage 2 (25%) and stage 3 (22%). One patient had stage 0 fibrosis on biopsy, and there were no patients with cirrhosis because they were excluded from the study. The median duration between biopsies was 4 years (range: 2-9 years).

Relationship of baseline parameters and progression

Out of a total of 36 patients, 19 (53%) had progression of fibrosis on repeat biopsies, 16 patients (44%) showed no change in stage, and one (3%) showed improvement in fibrosis on repeat biopsy. Among the patient factors examined (Table 2), the group that showed progression had significantly higher ALT and AST at baseline when compared to the group that did not show progression ($P = 0.003$ and 0.0001 , respectively). There was no statistically significant difference between baseline APRI, FIB-4 index, stage at initial biopsy, age, or duration between biopsies between progressors and non-progressors.

Parameters at time of repeat biopsy

Progressors vs non-progressors: Differences in param-

Table 1 Baseline characteristics of subjects included in the study

<i>n</i> = 36	
No. of males, <i>n</i> (%)	27 (75)
Age (yr, range)	47 (25-68)
Race, <i>n</i> (%)	
Caucasian	18 (50)
African American	11 (31)
Hispanic	7 (19)
Stage at time of initial biopsy, <i>n</i> (%)	
Stage 0	1 (3)
Stage 1	18 (50)
Stage 2	9 (25)
Stage 3	8 (22)
ALT (IU/mL, range)	73 (14-322)
AST (IU/mL, range)	49 (22-266)
Platelets ($\times 10^9/L$, range)	235 (185-362)
Median duration between biopsies (yr, range)	4 (2-9)

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase.

Table 2 Differences in parameters between progressors and non-progressors at initial biopsy

	Progressors	Non-progressors	<i>P</i> value
No.	19 (14 with a 1-stage change; 5 with a 2-stage change)	17	0.999
Age (yr)	50	52	0.621
Sex	Male 13 Female 6	Male 14 Female 3	> 0.050 (NS)
APRI	0.66	0.56	0.173
FIB-4	1.307	1.347	0.268
ALT (IU/mL)	92	47	0.003
AST (IU/mL)	100	38	0.001
Platelets ($\times 10^9/L$)	235	243	0.956
Stage	2	1	0.477
Duration between biopsies (yr)	4	4	0.200

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; APRI: AST/Platelet Ratio Index; NS: Not significant.

eters were explored between the two groups at the time of repeat biopsies. ALT, AST, APRI and FIB-4 were significantly higher in patients who showed progression of fibrosis (Table 3). The APRI and FIB-4 at the time of the second biopsy had excellent predictive value for progression in fibrosis with an APRI ≥ 0.69 having a 79% PPV and a 78% NPV for progression of at least one stage of fibrosis. A FIB-4 ≥ 1.65 had an 81% PPV and 70% NPV for prediction of fibrosis by at least one stage. A one-stage change in fibrosis on repeat biopsies might reflect sampling variability, therefore, we examined the predictive value of the APRI and FIB-4 for a change of two stages of fibrosis. We found that the APRI and FIB-4 had excellent predictive values for a two-stage progression of fibrosis with an APRI ≥ 1.94 and FIB-4 ≥ 3.01 having a 100% PPV and NPV and 62% PPV and 100% NPV, respectively. In addition the APRI had an AUROC of 1.0 [95% confidence interval (CI): 0.85-1.00] and FIB-4 had an AUROC of 0.911 (95% CI: 0.717-0.986, $P = 0.0001$) for those who

Table 3 Differences between progressors and non-progressors on repeat biopsies

	Progressors	Non-progressors	P value
APRI	1.293	0.413	0.0006
FIB-4	2.816	1.36	0.0038
ALT (IU/mL)	92	47	0.0030
AST (IU/mL)	100	38	0.0001
Platelets ($\times 10^9/L$)	184	221	0.1160

ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; APRI: AST/Platelet Ratio Index.

had a two-stage change in fibrosis. The number of patients who progressed by two stages was low ($n = 5$), and caution should be exercised in interpreting this particular subset of the results.

Change in APRI and FIB-4 and progression vs non-progression: Changes in APRI and FIB-4 were correlated with change in stage of fibrosis. There was a significant correlation between change in stage and change in APRI ($r^2 = 0.39$, $P = 0.00001$) (Figure 1A) with the regression equation for a change in stage = $0.50 \times (\Delta\text{APRI}) + 0.44$. AUROCs were constructed for predictive value of change in APRI for change in stage with a ΔAPRI of 0.18 having an 80% PPV and 67% NPV in predicting progression of fibrosis. Similarly, the correlation between change in stage and $\Delta\text{FIB-4}$ was also significant ($r^2 = 0.31$, $P = 0.00004$) (Figure 1B). The regression equation for a change in stage = $0.34 \times (\Delta\text{FIB-4}) + 0.32$. An analysis of ROC curve revealed that a $\Delta\text{FIB-4}$ of 0.39 had 75% PPV and NPV for predicting progression of fibrosis.

Accuracy of individual tests: AST proved to be the most useful of the tests. There was a significant correlation between change in stage of fibrosis and change in AST ($r^2 = 0.33$, $P = 0.002$). There was a less but still significant correlation of ALT between change in fibrosis and change in ALT ($r^2 = 0.21$, $P = 0.0048$). Changes in the platelets alone did not correlate well with changes in fibrosis ($r^2 = 0.07$).

DISCUSSION

Liver biopsy is the gold standard for estimating severity of fibrosis in patients with liver disease and is used as a guide for therapy of HCV infection. However, because antiviral therapy with pegylated interferon and ribavirin carries significant toxicity, and fibrosis progresses in only 20%-30% of patients with chronic HCV infection^[10], therapy is ideally offered to patients whose risk of progressive liver damage is high. Current strategy involves using the stage of fibrosis on initial liver biopsy to determine the risk of progressive liver damage. However, the stage at the time of the biopsy might not predict the risk of progression, especially in a younger patient and/or someone with a shorter duration of infection.

Apart from the complications associated with the invasive nature of liver biopsy, issues like sampling variability,

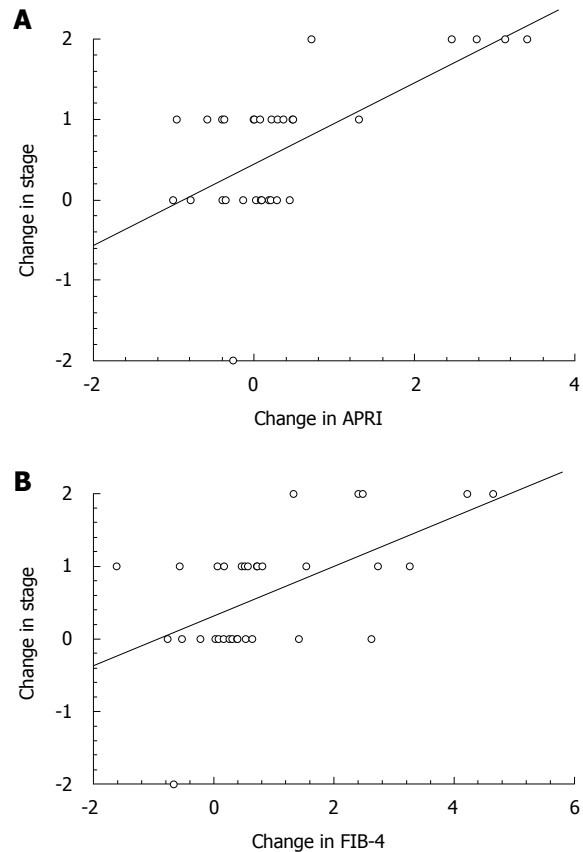


Figure 1 Linear regression plot. A: Correlation between changes in stage of fibrosis vs change in aspartate aminotransferase/Platelet Ratio Index (APRI); B: Correlation between stages of fibrosis vs FIB-4.

inter-observer variability, and expense limit its utility and diagnostic accuracy^[9,11]. Factors that determine the varying rates of fibrosis progression are poorly understood, partially because of the logistical difficulties for studies in performing serial liver biopsies. Non-invasive biomarkers that reflect hepatic fibrosis have been proposed to overcome these limitations with the ideal biomarker of hepatic fibrosis being inexpensive, easily available, and accurately reflecting changes in fibrosis. The APRI and FIB-4 are two biomarker panels that incorporate readily available and routinely performed tests for all patients with liver disease. Both have been confirmed to be accurate in predicting fibrosis stage on liver biopsies^[1,7,8,12-23]. However, few studies have examined the longitudinal behavior of fibrosis biomarkers. Patel *et al*^[24] have evaluated a single direct hepatic fibrosis marker, hyaluronic acid (HA), in patients undergoing liver biopsies before, and 6 mo following antiviral therapy for chronic HCV. Although HA has some association with the stage of fibrosis, it does not predict histological changes over the treatment period. Our study addressed the longitudinal behavior of these markers in individuals who underwent serial biopsies, and examined the role of these biomarkers in predicting progression.

There is accumulating evidence that hepatic fibrosis markers can be used for prognosis. Following 6 mo of interferon-based therapy, an APRI > 1.5 has been shown to be associated with significant short-term mortality and risk of hepatocellular carcinoma in patients with chronic

HCV^[21]. A commercially available test, FibroTest, has been found at baseline to predict outcomes in chronic HCV^[25]. In addition, two other commercially available tests, Fibrometer and Hepascore, have been shown to be as good as liver biopsy in the prediction of survival in alcoholic liver disease^[26]. Recently, the European Liver Fibrosis Test has been shown to be superior to several other parameters in the prediction of outcomes of primary biliary cirrhosis^[27].

One finding of our study that is potentially useful for clinicians is that serial changes in APRI and FIB-4, which are easily calculated using routine laboratory tests, correlated well with changes in fibrosis staging. There was a significant correlation between change in stage and change in APRI and FIB-4 of 0.627 ($r^2 = 0.39$, $P = 0.00001$) and 0.56 ($r^2 = 0.31$, $P = 0.00004$), respectively. A Δ APRI of 0.18 had 80% PPV and 67% NPV in predicting progression of fibrosis, whereas a Δ FIB-4 of 0.39 had 75% PPV and NPV for predicting progression of fibrosis. These results are significant as they suggest that longitudinal changes of APRI and FIB-4 markers can be useful in predicting progression of fibrosis, which could lead to changes in clinical management.

The baseline characteristics of the study population did not differ between progressors and non-progressors, except for aminotransferases, which were significantly higher in the group that later showed progression of fibrosis, which has been shown previously^[28]. This was independent of the initial stage of fibrosis and did not differ significantly between groups. It seems likely that, even with the same stage of fibrosis, higher enzyme levels are a marker for subsequent progression. The effect of timing between the biopsies is unlikely to have an impact on the conclusions because there was no significant difference between duration of biopsies between the progressor and non-progressor groups. As expected, the APRI and FIB-4 were significantly higher at the time of the second liver biopsy in the patients with progression of fibrosis, which reflected the accuracy of these tests at a single point in time. Therefore, not unexpectedly, the APRI and FIB-4 were predictive of progression at the time of the second biopsy. Although the number of patients was small, both tests were able to predict a two-stage change in fibrosis at the second biopsy.

This study had several limitations, mainly due to the largely retrospective nature of the study. Previously, we have found that the APRI was less accurate retrospectively than prospectively^[16]. This might have been because the retrospective patient group was more heterogeneous, as well as the fact that laboratory values were not always performed close to the time of the biopsy. In addition, concurrent illnesses that could influence aminotransferases and platelets could not be easily determined in some of the patients. Another significant limitation was the relatively small sample size. A larger and well-designed prospective trial that could confirm the role of biomarkers, including those from the ECM, in predicting fibrosis progression would be very useful.

In summary, the APRI and FIB-4 at initial liver biopsy were not useful in predicting patients whose fibrosis would

be progressive. However, as previously reported^[28], patients with higher aminotransferases appear to have an increased risk of progression of fibrosis. Importantly, the longitudinal use of the APRI and FIB-4 is accurate in predicting progression of fibrosis, and could be useful to clinicians that follow patients with chronic HCV. Based on our data, a Δ APRI of 0.18 or a Δ FIB-4 of 0.39 in a patient with chronic HCV suggests progression in fibrosis of at least one stage, and could be used to trigger a reconsideration of antiviral therapy and/or liver biopsy. Larger, prospective studies are needed to validate the findings of our study.

COMMENTS

Background

The complications of chronic liver disease are largely related to the development of fibrosis. Medical treatments are aimed at prevention of progression of cirrhosis. A liver biopsy has been the usual way to estimate fibrosis. However, it is invasive and expensive, and can be inaccurate because of variability in the degree of fibrosis in parts of the liver that can be sampled. The use of certain serum biomarkers to estimate fibrosis has been found to be useful, especially in chronic hepatitis C. These biomarkers can be composed of a combination of simple tests that are used to assess liver function, as well as more complex indices that measure substances in the blood that can originate in the extracellular matrix of the liver itself.

Research frontiers

Although several simple and complex biomarkers have been found to be accurate at differentiating mild from significant fibrosis, there has been little evaluation of them in a longitudinal manner. In other words, it is not clear whether changes over time in the value of the markers reflect actual changes in the degree of fibrosis. If these markers can accurately predict changes in fibrosis, then this would be useful to clinicians. Not only could this lead to fewer liver biopsies, but it could lead to changes or initiation of treatment. This study evaluated two simple biomarkers, the aspartate aminotransferase (AST)/Platelet Ratio Index (APRI) and the FIB4, longitudinally in a group of patients with chronic hepatitis who had two liver biopsies that were at least 1 year apart. It found that both biomarkers could accurately predict changes in the stage of fibrosis.

Innovations and breakthroughs

The APRI utilizes AST and platelets, which are simple tests obtained in all liver patients. It was originally described by Wai *et al* in 2003 (*Hepatology* 38: 518-526). Its usefulness has been confirmed in multiple studies including that of Snyder *et al* (*J Clin Gastroenterol* 2006; 40: 535-542). The FIB4 utilizes AST, platelets, alanine aminotransferase (ALT), and age. It was originally described by Sterling *et al* in 2006 (*Hepatology* 43: 1317-1325). A high APRI score at 6 mo following treatment has been shown to correlate with poor survival, as well as the development of hepatocellular carcinoma.

Applications

This article shows that physicians can accurately follow patients with chronic hepatic C that have failed previous antiviral therapy or that have not been treated. Treatment decisions can be based on changes in these indices. These findings should be confirmed in larger studies from other institutions. Also, further research should be done with the longitudinal use of the more complex biomarkers, as well as the longitudinal use of simple and complex biomarkers in diseases other than chronic hepatitis C.

Terminology

Liver fibrosis refers to the amount of scar tissue in the liver. This impairs blood flow and eventually can lead to cirrhosis. AST is an enzyme that is in liver cells and is released when there is active liver damage. ALT is an enzyme specific to liver cells, which is released when there is liver damage. The platelets are a component of the blood that helps with clotting. Platelets decrease with advancing liver disease and fibrosis because some of them are sequestered in the spleen, which enlarges with liver disease, and also a hormone called thrombopoietin that stimulates platelets production is released in smaller quantities with advancing disease. Biomarkers are substances that can be objectively measured that are used as an indicator of a biological state. They are being increasingly used in medicine as non-invasive ways to screen for and evaluate various diseases including cancer.

Peer review

The authors show that evolutionary changes in simple scores (APRI and FIB-4) based on routine biochemical parameters can be of value in predicting the evolution of liver fibrosis in chronic hepatitis C. The data provided in this study have important clinical implications that pertain to the management of patients with chronic hepatitis C.

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Differential changes in intrinsic innervation and interstitial cells of Cajal in small bowel atresia in newborns

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Abstract

AIM: To investigate morphological changes of the enteric nervous system (ENS) and the interstitial cells of Cajal (ICCs) in small bowel atresia.

METHODS: Resected small bowel specimens from affected patients ($n = 7$) were divided into three parts (proximal, atretic, distal). Standard histology and enzyme immunohistochemistry anti-S100, anti-protein gene product (PGP) 9.5, anti-neurofilament (NF), anti-kit-receptor (CD117) was carried out on conventional paraffin sections of the proximal and distal part.

RESULTS: The neuronal and glial markers (PGP 9.5, NF, S-100) were expressed in hypertrophied ganglia and nerve fibres within the myenteric and submucosal plexuses. Furthermore, the submucous plexus contained typical giant ganglia. The innervation pattern of the proximal bowel resembled intestinal neuronal dyspla-

sia. The density of myenteric ICCs was clearly reduced in the proximal bowel, whereas a moderate number of muscular ICCs were found. The anti-CD117 immunoreaction revealed additional numerous mast cells. The distal bowel demonstrated normal morphology and density of the ENS, the ICCs and the mast cells.

CONCLUSION: The proximal and distal bowel in small bowel atresia revealed clear changes in morphology and density of the ENS and ICCs.

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Key words: Small bowel atresia; Enteric nervous system; Gastrointestinal motility; Interstitial cells of Cajal

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INTRODUCTION

Small bowel atresia is a congenital disorder that carries a substantial morbidity^[1,2]. The etiology of bowel atresia remains unclear. One of two accepted theories of its pathogenesis is the concept of a lack of recanalization of the solid organ cord during the late stage of intestinal development^[3]. Another concept is the occurrence of a late intrauterine mesenteric vascular accident^[4]. The lack of revacuolization is the probable cause for most cases of duodenal atresia. Further studies have demonstrated that

jejunoileal atresias occur as a result of intestinal volvulus, intussusception, internal hernia, or strangulation in a tight gastroschisis or omphalocele defect^[5-11].

Newborns with small bowel atresia are operated on soon after birth. Owing to the severity of the dilatation of the proximal bowel and the hypoplasia of the distal bowel, various postoperative gastrointestinal motility problems may occur; such problems include prolonged adynamic ileus (11%) and the need for total parenteral nutrition (30%-70%)^[12]. The underlying cause of this postoperative intestinal motility disorder is still unclear.

Basically, normal gastrointestinal motility depends on the coordinated function of the enteric nervous system (ENS), the intestinal smooth muscle and the interstitial cells of Cajal (ICCs). Previous studies revealed histological changes within the wall of the atretic and adjacent bowel in small bowel atresia. Hypertrophy of the bowel muscle proximal to the atresia was found in clinical and experimental studies on small bowel atresia. Various changes have been reported within the ENS in small bowel atresia^[13]. Nevertheless, the relationship between the macroscopic and histological changes of the affected bowel and the postoperative motility disorder are still under investigation. Furthermore, the role of the ICCs in small bowel atresia needs to be elucidated further.

ICCs play a major role in gastrointestinal motility. ICCs express the tyrosine kinase receptor c-kit. Therefore specifically designed c-kit antibodies have been developed which stain ICCs but also other cell groups such as stem cells and mast cells. However, c-kit positive ICCs can be identified by clear morphological features.

The aim of this study was to investigate the morphology of the ENS and the ICCs in resection specimens of small bowel atresia.

MATERIALS AND METHODS

Patients and tissues

Resected small bowel specimens (ileum) from affected newborn patients ($n = 7$) were included in the investigation after parental consent. The resected ileal specimens were divided into three parts (proximal, atretic, distal).

Tissue processing

The specimens were fixed in 4% paraformaldehyde and processed into paraffin blocks. Paraffin-embedded tissues were sectioned at 2-4 μ m (Leica SM 2000 R) followed by drying at 37°C in an incubator overnight. Before immunohistochemical staining, the paraffin sections were dewaxed for 10 min in xylene, followed by 10 min in acetone and 10 min in acetone/Tris-buffered saline (TBS; 1:1). After this treatment, the slides were washed in TBS.

Antigen demasking

If heat antigen retrieval was required, dewaxed paraffin sections were placed in microwave-proof tubes containing target retrieval solution (Dako). The slides were treated in the tubes for 5 min at 600 W in a microwave (SS 566H;

Table 1 Primary antibodies

Antibody	Clone	Company	Dilution
S-100	Polyclonal (Z0311)	Dako, Glostrup, Denmark	1:500
PGP-9.5	Polyclonal (Z5116)	Dako, Glostrup, Denmark	1:25
Neurofilament	2F11 (M0762)	Dako, Glostrup, Denmark	1:50
c-kit	Polyclonal (A4502)	Dako, Glostrup, Denmark	1:50

PGP: Protein gene product.

Bosch, Munich, Germany). The evaporated volume was replaced by distilled water, and the procedure was repeated twice. After microwave treatment, the slides were left to cool down, then were washed in TBS.

Histochemistry and immunohistochemistry

Standard histology [hematoxylin/eosin (HE)] was performed on the sections. For immunohistochemistry, an alkaline phosphatase-anti-alkaline phosphatase (APAAP) staining kit (Dako Real™ Detection System, APAAP, Mouse) using anti-S100, anti-protein gene product (PGP) 9.5, anti-neurofilament (NF) and anti-c-kit-receptor (CD117) antibodies was employed. A non-sense mAb (clone: MR 12/53) served as negative control by omitting the primary antibody. The final concentrations are given in Table 1.

Evaluation

The evaluation of the immunohistochemical staining results was focused on the proximal and distal parts of the resected ileum.

The sections were evaluated by two independent investigators using light microscopy (magnification, $\times 40$). HE staining was used to determine the overall histology of the investigated specimen. The distribution and density of immunoreactive ganglion cells, glial cells, nerve fibers and c-kit positive cells (ICCs, mast cells) were studied in each part of the resected bowel (proximal, atretic, distal). Since quantitative analysis of immunohistochemical staining is not possible, semi-quantitative scoring was performed as follows; - no expression (no staining), + low expression (few neuronal fibers and cells/few ICCs), ++ moderate expression (numerous neuronal fibers and cells/numerous ICCs), +++ high expression (dense networks of neuronal fibers and cells/dense network of ICCs).

RESULTS

Patients

The study included resected ileal segments from seven newborn patients (gestational age 38-40 wk). Six patients presented with type IIIa ileal atresia and one patient presented with multiple ileal atresia.

Neuronal markers (PGP 9.5, NF)

The neuronal markers PGP 9.5 and NF were strongly expressed within the ganglion cells and nerve fibers of the myenteric plexus (and submucosal plexus in the proxi-

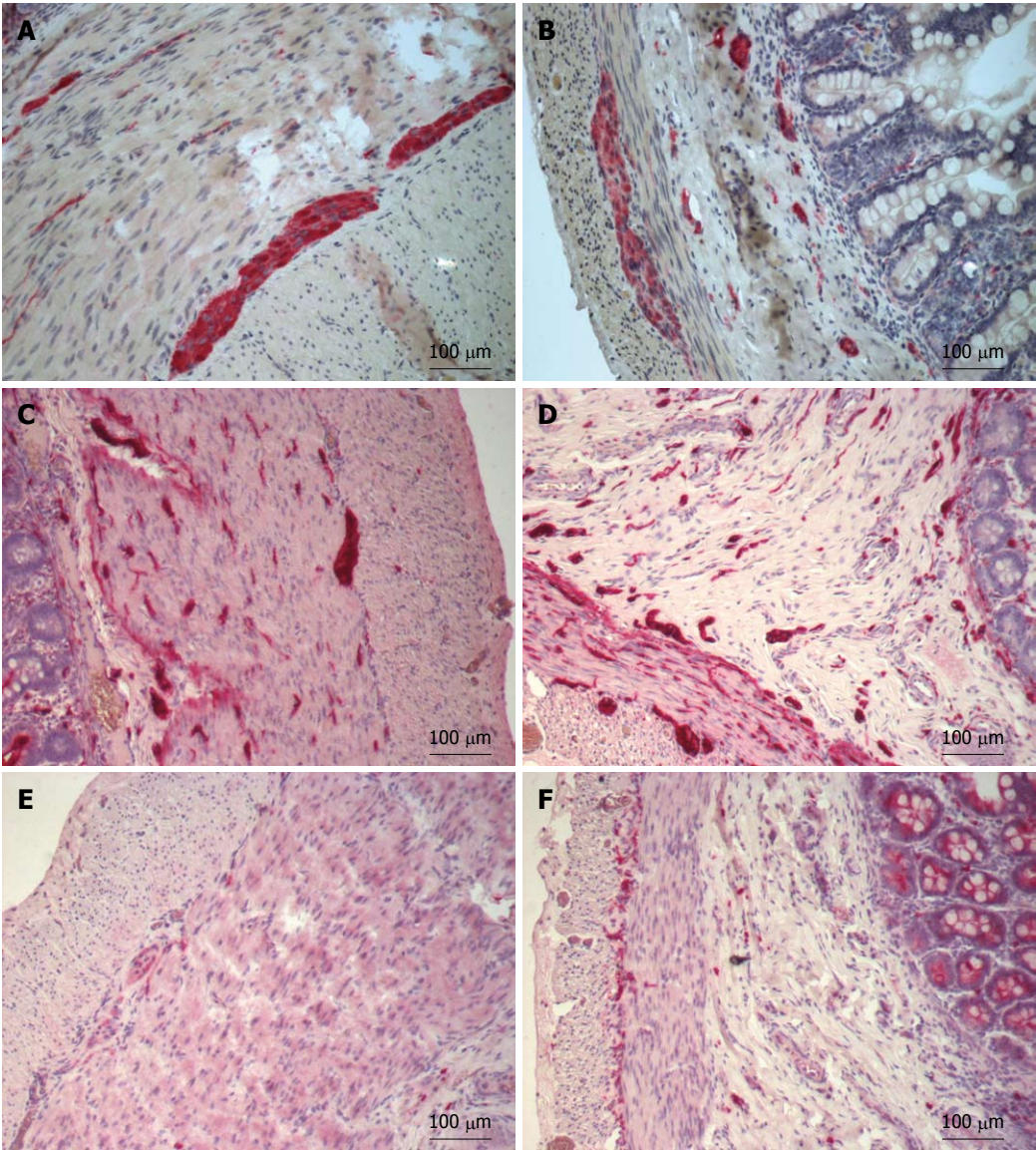


Figure 1 Immunohistochemistry of a full-thickness section of the ileum. A: Protein gene product (PGP) 9.5 in the ileum proximal to the atresia shows a dense innervation within the submucosal and myenteric plexus; B: PGP 9.5 in the ileum distal to the atresia shows a normal innervation pattern of the submucosal and myenteric plexuses; C: S-100 in the ileum proximal to the atresia shows numerous fibers and large ganglia (> 20 µm) within the submucosal plexus and normal innervation within the myenteric plexus; D: S-100 in the ileum distal to the atresia shows a normal innervation pattern within the submucosal and myenteric plexuses; E: c-kit in the ileum proximal to the atresia shows that myenteric and muscular interstitial cells of Cajal (ICCs) are almost absent; F: c-kit in the ileum distal to the atresia shows normal distribution of myenteric and muscular ICCs.

Table 2 Distribution and density of immunostaining (proximal vs distal)								
	Proximal				Distal			
	SP	CM	MP	LM	SP	CM	MP	LM
PGP 9.5	++	+	++	+	++	+	++	+
S-100	+++	+	+++	+	++	+	++	+
NF	++	+	++	+	++	+	++	+
c-Kit (ICCs)	-	-/+	+/-	-	-	+	++	+

PGP: Protein gene product; ICCs: Interstitial cells of Cajal; SP: Submucosal plexus; CM: Circular muscle; MP: Myenteric plexus; LM: Longitudinal muscle; NF: Neurofilament; -: No expression (no staining); +: Low expression (few neuronal fibers and cells/few ICCs); ++: Moderate expression (numerous neuronal fibers and cells/numerous ICCs); +++: High expression (dense networks of neuronal fibers and cells/dense ICCs networks).

mal ileum (Figure 1A). The submucosal plexus contained some giant ganglia ($n > 10$ ganglion cells) and numerous thick nerve fibers. The overall numbers of ganglia were not increased within the myenteric and submucosal plexuses (Table 2). The immunohistochemical staining revealed features of intestinal neuronal dysplasia (giant ganglia, hypertrophied nerve fibers). The distal bowel had a normally expressed ENS within the submucosal and myenteric plexuses (Figure 1B).

Glial marker (S-100)
Numerous S-100 immunoreactive glial cells were found in the two plexuses of the proximal ileum (Figure 1C). The expression of glial cells was increased within the proximal dilated bowel compared to the atretic and distal bowel

(Table 2). Additional giant ganglia (> 10 ganglion cells/ganglion) were stained by S-100 within the submucosal plexus of the proximal bowel. Regular distribution and morphology of S-100 immunoreactive cells and fibers was found within the myenteric and submucosal plexuses of the distal ileum (Figure 1D).

c-kit (CD-117) staining (ICC, mast cells)

The density of ICCs within the myenteric plexus was clearly reduced in the proximal bowel (Figure 1E), whereas some ICCs were found in the circular and longitudinal muscle layer (Table 2). The anti-CD117 immunoreaction revealed numerous mast cells within the proximal bowel. The distal bowel had a normal distribution of ICCs within the myenteric plexus and muscular layers and a normal number of mast cells (Figure 1F).

DISCUSSION

This study revealed distinct changes in the morphology of the ENS and ICCs in parts of the resected bowel proximal to small bowel atresia. In general, our findings are not unique since previous studies described changes within the ENS in small bowel atresia. Ozguner *et al.*^[14] reported that the proximal segment of the atretic intestine showed structural deficits. Abnormal ganglia cells and defects in the intestinal musculature were prominent, but the intestinal mucosa remained intact. They found abnormalities in both the antimesenteric side and mesenteric side. Their interpretation supported a vascular accident as a causative factor.

Di Nardo *et al.*^[15] presented an interesting case report of small bowel atresia that was initially diagnosed as hyper-ganglionosis and later changed to an enteric degenerative neuropathy. Watanabe *et al.*^[16] contributed an elegant study investigating the myenteric plexus in small bowel atresia using whole-mount preparations. This study showed mild hypoplasia of the ENS plexuses in the proximal segments^[16].

In our study the expression of nerve fibers and glial cells appeared to be slightly increased, and some submucosal giant ganglia were found, which resembled intestinal neuronal dysplasia within the proximal dilated bowel. The expression of c-kit positive ICCs was clearly decreased in the dilated proximal bowel. The reduction of ICCs within the proximal dilated bowel has also been described before. In contrast to our study, some previous investigators have shown a reduction of the innervation density within the proximal bowel segments^[13]. The innervation pattern and the expression of ICCs within the distal small bowel were normal in our study, similar to comparable investigations^[13].

The crucial problem of investigating the developing ENS or pathological changes within the ENS in order to define hyper-ganglionosis or hypoganglionosis is the uncertainty in the normal numbers and distribution of enteric ganglia. The normal neuron density of the human myenteric plexus was investigated in several studies and resulted in a huge variation of more than 200-fold^[17-20].

Schuffler *et al.*^[17] found 27.8 nerve cells per 100 mm of smooth muscle, Ikeda *et al.*^[19] described more than 30 nerve cells per 10 mm of smooth muscle as normal, whereas Smith^[18] estimated 7 nerve cells per mm of smooth muscle. Finally Meier-Ruge *et al.*^[20] found 756 ganglion cells per 10 mm of smooth muscle. All these investigations were performed on conventional paraffin sections^[17-20], which usually display only a two-dimensional image of the investigated structures. Since these profound differences have already been found within normal bowel specimens, conflicting results on numerical changes of ganglia and nerve fibers within the changed ENS in bowel atresia must be expected. Another major aspect is the widely accepted concept of ongoing postnatal plasticity of the ENS, which has to be taken into consideration.

A previous animal study which created a partial obstruction in dog ileum revealed that the ganglion cells increased in size and the smooth muscle of the dilated bowel became thicker^[21]. The number of ganglion cells related to the muscle mass was decreased above the constriction. Although some ganglion cells increased in size, there was no evidence of any increase in number. Experimentally, a similar increase in ganglion size was observed proximal to a stenosis in another study^[22]. In a chicken embryonic model of bowel obstruction, a marked reduction of the submucous plexus ganglia with intact muscular plexus ganglia was noted in the dilated gut^[23].

Our study also showed an increase in size of ganglia (hypertrophy). These hypertrophic myenteric and submucosal ganglia in the proximal resected bowel could be considered the result of adaptive phenomena to the long-lasting prenatal bowel obstruction. Several experimental models have been instrumental in supporting the concept that adaptive changes to the ENS occur proximal to the obstructed gut^[24-31]. These studies revealed an increased density and size in both myenteric^[26,28,29,31] and submucosal neurons^[29-31], along with neurochemical^[28,30] and cytoskeletal abnormalities of the myenteric neurons^[31].

Eklblad *et al.*^[28] showed that, in the hypertrophic ileum, several neuronal populations showed changes in the expression of neuro-messengers. Myenteric neurons expressing vasoactive intestinal peptide (VIP), pituitary adenylate cyclase activating peptide, and galanin were notably increased in number. In submucosal ganglia, the number of VIP-immunoreactive neurons decreased, while those expressing VIP mRNA increased. NADPH diaphorase-positive submucosal neurons increased dramatically, while the number of neuronal-type nitric oxide synthase expressing neurons was unchanged. The number of ICCs decreased markedly in the hypertrophic ileum.

Another observation is the delayed maturation of ENS within the distal bowel during fetal development in cases with small bowel atresia^[32]. Distortion of the polygonal architecture of the myenteric plexus has been shown postnatally in atretic parts of ileal type IIIa atresia^[33]. It could only be speculated that after the restoration of bowel continuity does maturation of the ENS occur.

A previous animal study using a partial small obstruc-

tion model in mice revealed that 2 wk following the onset of a partial obstruction, the bowel increased in diameter, and hypertrophy of the tunica muscularis was observed oral to the obstruction site^[27]. Networks of ICCs were disrupted oral to the obstruction, and this disruption was accompanied by the loss of electrical slow waves and responses to enteric nerve stimulation. These defects were not observed aboral to the obstruction. Furthermore, it was shown that removal of the obstruction led to the re-development of ICC networks and the recovery of slow wave activity within 30 d. Neural responses were partially restored in 30 d^[27]. Similar repair mechanisms may occur after surgical correction of small bowel atresia. It seems obvious that decreases in ICCs in small bowel atresia and their restoration after removal of the obstruction contributes to the regulation of gastrointestinal motility.

Masumoto *et al.*^[34] showed muscular alterations in both segments of IA cases, which further contributes to the postoperative motility disorder. Another case report recently revealed long lasting chronological changes within the ENS, muscle components and ICCs in small bowel atresia^[35].

The ENS and ICCs are altered in the proximal and dilated bowel in small bowel atresia. The innervation pattern of the proximal bowel resembles intestinal neuronal dysplasia. These changes might be the result of long-lasting bowel obstruction and stasis of bowel contents. The presented histological features do not contribute to the search for the pathogenesis of small bowel atresia. Nevertheless, the confirmed innervation abnormalities and defective expression of CD117-positive cells of the proximal bowel may have an influence on the postoperative gastrointestinal motility of the affected patients.

COMMENTS

Background

Small bowel atresia is a congenital anomaly of unknown cause. Despite early corrective surgery, patients carry a substantial morbidity because of postoperative gastrointestinal motility problems. Normal gastrointestinal motility is generated by the complex interaction of the enteric nervous system (ENS), the intestinal smooth muscle and the interstitial cells of Cajal (ICCs). Alterations in the ENS and ICCs may contribute to the motility problems in patients with small bowel atresia after surgery.

Research frontiers

Previous studies revealed histological changes within the wall of the atretic and adjacent bowel in small bowel atresia. Nevertheless the relationship between the macroscopic and histological changes of the affected bowel and the postoperative motility disorder are still under investigation. Furthermore, the role of the ICCs in small bowel atresia needs to be elucidated further.

Innovations and breakthroughs

This study showed that the ENS and ICCs are altered in the proximal and dilated bowel in small bowel atresia. The innervation pattern of the proximal bowel resembles intestinal neuronal dysplasia.

Applications

The changes within the ENS and the ICCs may be the result of a long-lasting bowel obstruction and stasis of bowel contents. The presented histological features do not contribute to the search for the pathogenesis of small bowel atresia. However, the confirmed innervation abnormalities and defective expression of CD117-positive cells of the proximal bowel may have an influence on postoperative gastrointestinal motility in the affected patients.

Terminology

Bowel atresia is a congenital defect in the continuity of the bowel. The incidence of small bowel atresia is higher than that of large bowel atresia and varies between 1:300 to 1:3000.

Peer review

The authors studied the ileum of seven patients with ileal atresia. They reported that the proximal dilated intestine had an increased expression of nerve fibers and glial cells, with a decrease in ICCs, and an increase in ganglia size, whereas the distal intestine displayed normal features. They conclude that these alterations may influence on postoperative intestinal motility. The authors performed a well designed study in a very uncommon congenital disease, which is very interesting for the readers.

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Esophagogastric anastomosis with invagination into stomach: New technique to reduce fistula formation

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Abstract

AIM: To present a new technique of cervical esophagogastric anastomosis to reduce the frequency of fistula formation.

METHODS: A group of 31 patients with thoracic and abdominal esophageal cancer underwent cervical esophagogastric anastomosis with invagination of the proximal esophageal stump into the stomach tube. In the region elected for anastomosis, a transverse myotomy of the esophagus was carried out around the entire circumference of the esophagus. Afterwards, a 4-cm long segment of esophagus was invaginated into the stomach and anastomosed to the anterior and the posterior walls.

RESULTS: Postoperative minor complications occurred in 22 (70.9%) patients. Four (12.9%) patients had seri-

ous complications that led to death. The discharge of saliva was at a lower region, while attempting to leave the anastomosis site out of the alimentary transit. Three (9.7%) patients had fistula at the esophagogastric anastomosis, with minimal leakage of air or saliva and with mild clinical repercussions. No patients had esophagogastric fistula with intense saliva leakage from either the cervical incision or the thoracic drain. Fibrotic stenosis of anastomoses occurred in seven (22.6%) patients. All these patients obtained relief from their dysphagia with endoscopic dilatation of the anastomosis.

CONCLUSION: Cervical esophagogastric anastomosis with invagination of the proximal esophageal stump into the stomach tube presented a low rate of esophagogastric fistula with mild clinical repercussions.

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Key words: Esophageal cancer; Esophagectomy; Constriction; Pathologic; Fistula; Gastropasty

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INTRODUCTION

Definitive curative treatment for cancer of the esophagus remains a challenge for surgeons^[1-8]. The approach involves major surgery that has high morbidity and mortality rates, mainly due to pulmonary complications,

cervical fistulas, stenosis of anastomosis, necrosis of the tubularized stomach, and mediastinitis^[5,9-12].

Among these possible complications, fistula of the esophagogastric anastomosis represents one of the principal problems of esophagectomy. In several studies, the incidence has ranged from 0% to 50%, with most authors reporting a high incidence of this complication^[4,6,13-18].

Although these fistulas usually have a favorable course, about 2% of cases can have a catastrophic outcome^[5,16]. In cases in which the fistula does not lead directly to death, it can compromise quality of life, interfere with resumption of feeding, require laborious local care, and prolong hospital stay. Additionally, 30%-50% of those patients who present with fistula go on to develop stenosis^[15,19-22].

Given this scenario and personal experience of a high incidence of cervical esophagogastric fistula in treatment of carcinoma of the esophagus^[23], we decided to perform cervical esophagogastric anastomosis with invagination of the proximal esophageal stump into the stomach tube, and to analyze the incidence of fistula and stenosis formation following this procedure.

MATERIALS AND METHODS

This study conformed to the regulations of The Human Ethics Research Committee at our Institution and with the Helsinki Declaration, revised in 1983.

Our study group consisted of 31 patients with thoracic or abdominal carcinoma of the esophagus, who underwent open access with subtotal esophagectomy and esophagogastric anastomosis with invagination of the proximal esophageal stump into the stomach tube. The study group included 27 (87.1%) men and four (12.9%) women, with a mean age of 60.2 ± 8.5 years (range: 44-74 years). Lesions were located in the medial third of the esophagus in 15 cases (48.3%) and the inferior third in 16 (51.6%).

The inclusion criteria for operation were: esophagogram with no abnormal axis deviation, lesions up to 5.0 cm long, absence of signs of invasion of the respiratory tree on bronchoscopy, and absence of signs of irresectability of the esophageal lesion or neoplastic dissemination on thoracic and abdominal helicoidal tomography. Cases in which an anesthetic or surgical procedure was contraindicated due to compromised clinical state and/or concurrent serious systemic disease were excluded from the study. The diagnosis was confirmed by upper esophageal endoscopy and biopsy: 25 (80.6%) patients had squamous cell carcinoma and the remaining six (19.3%) had adenocarcinoma.

All patients underwent preoperative clinical evaluation. Thirteen (41.9%) had serious clinical malnutrition as shown by weight loss of greater than 20% of normal weight. Tumor staging was performed using physical examination, thoracic radiography, barium esophography, thoracic and abdominal tomography, and bronchoscopy in patients whose lesions were situated in the medial third of the esophagus. Clinicopathological staging using the TNM classification by the UICC^[24] was: stage I in two (6.4%) patients; stage II A in five (16.1%); stage II B in

four (12.9%); stage III in 16 (51.6%); and stage IV A in four (12.9%).

Surgical technique

In the absence of contraindications, a transhiatal esophagectomy followed by a cervical esophagogastric anastomosis was performed. All surgeries were carried out in parallel during the same operating period by two teams, with one team operating in the abdominal region and the other in the cervical region. Lymph node resection was done in both the abdominal and inferior mediastinal fields. In all cases, the tubularized stomach was placed into the cervical region by the posterior mediastinum.

The esophagus was dissected and separated from its neighboring structures in the cervical, thoracic and abdominal areas. In distally located tumors, the esophagus was sectioned in the cervical region, with care taken to preserve enough of the proximal end to allow 4.0 cm of esophagus to be inserted into the stomach, with a safe margin ≥ 5.0 cm. The esophagus was then pulled to the abdominal region, and the stomach sectioned with a linear stapler that released the surgical specimen.

To guarantee a sufficient margin for lesions that involved the middle third, the stomach was initially sectioned and tubularized, and the piece pulled up to the cervical region, where it was very carefully examined, and the surgical section site was chosen with a safe margin. If the margin was judged to be inadequate, end-to-end anastomosis was performed instead of invagination, and that patient was excluded from the study.

In the region that was selected for anastomosis, a transverse myotomy was carried out around the entire circumference of the esophagus (Figure 1A). The proximal border of the myotomy was anastomosed, with the tip of the tubularized stomach placed in the cervical region. The anastomosis of the posterior wall was performed first using interrupted sutures of 4-0 polydioxanone (Figure 1B). Subsequently, the 4-cm segment of esophagus was introduced or invaginated into the stomach and sutured to the anterior wall as per the posterior wall (Figure 1C). In all patients, extra-mucosal pyloroplasty was carried out, a nasogastric tube was also inserted, and the cervical region was drained by a laminar drain.

Oral feeding was typically started on the postoperative day 10, in the absence of signs of esophagogastric fistula. If a fistula was present, the affected site was treated, to maintain feeding by nasogastric tube. In this case, oral diet was begun following closure of the fistula.

RESULTS

No patients died intraoperatively. Postoperative minor complications occurred in 22 (70.9%) patients. Four (12.9%) patients had serious complications that led to death: two (6.4%) as a result of bronchopneumonia, one due to multiple organ failure after acute cholecystitis, and the other from sepsis following ischemic necrosis of the stomach; all of them with no relationship to the esophagogastric anastomosis.

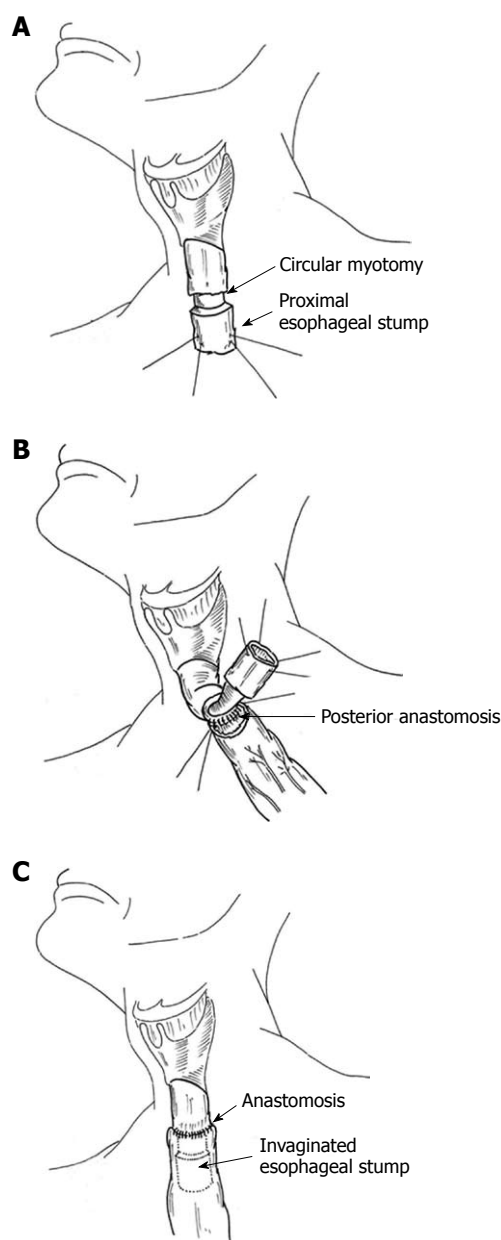


Figure 1 Esophagogastric anastomosis with stomach invagination. A: Diagram showing the circular myotomy (long arrow) in the section of the proximal esophageal stump (short arrow), which created a 4.0 cm segment of extension to be invaginated into the stomach (the illustration of the trachea was omitted); B: Diagram showing the anastomosis of the posterior wall of the esophagus performed first using interrupted sutures (the illustration of the trachea was omitted); C: Diagram showing the sectioned esophagus protruding into the stomach (the illustration of the trachea was omitted).

Three (9.7%) patients had fistula at the esophagogastric anastomosis with minimal leakage of air or saliva; all of them with mild clinical repercussions. Two of these had a fistula on postoperative days 7 and 10, with the leak of a small quantity of air or saliva from the cervical incision and consequent formation of a bubble during swallowing. In these two patients, spontaneous closure occurred after 10 and 5 d, respectively. The third case had a seropurulent pleural effusion on postoperative day 13, which was later drained. There was a negligible quantity of secretion from the pleural drain, which indicated a mild

Table 1 Postoperative complications in 31 patients with thoracic and abdominal esophagus cancer, who underwent cervical esophagogastric anastomosis with invagination of the proximal esophageal stump into the stomach tube

Complication	n (%)
Dysphonia	15 (48.4)
Anastomosis stricture	7 (22.6)
Bronchopneumonia	6 (19.3)
Anastomosis fistula	3 (9.7)
Atelectasis	2 (6.4)
Renal failure	2 (6.4)
Gastric ischemic necrosis	1 (3.2)
Acute cholecystitis	1 (3.2)
Wound infection	1 (3.2)

blocked esophago-pleurocutaneous fistula. This patient had no adverse effects from this and was discharged from hospital on postoperative day 23. No patients had esophagogastric fistula with intense saliva leakage from either the cervical incision or the thoracic drain.

Postoperative stricture of the anastomosis occurred in seven (22.6%) patients; in six of these, this appeared within 16-60 d, and the last appeared at 12 mo after surgery. All of these patients obtained relief from their dysphagia with endoscopic dilatation of the anastomosis, with the number of sessions required ranging from one to seven (mean = 3). One (3.2%) patient with a lesion located in the inferior third of the esophagus had recurrence of the cancer in the area of the anastomosis and required a nasogastric tube. The other complications were successfully treated: dysphonia in 15 (48.4%), bronchopneumonia in four (12.9%), atelectasis in two (6.4%), renal failure in two (6.4%), and wound infection in one (3.2%). Dysphonia was temporary and resolved after a few weeks in all cases. Patients with pre-renal renal failure responded well to expansion with fluids. Atelectasis was reversed with respiratory physiotherapy. The mean length of hospital stay was 15.2 d, with a range of 13-35 d (Table 1).

DISCUSSION

Esophagogastric anastomosis with invagination is a modification of a technique that is performed to reduce fistula formation at the anastomosis site^[22]. Szücs *et al*^[22] have reported 108 patients that underwent esophagectomy with esophagogastric anastomosis and telescoping of a 10-15-mm length of the esophageal end into the stomach. Twelve (11.1%) of these patients developed fistula at the anastomotic site. We chose to invaginate a 4.0-cm segment made up of all the layers of the esophagus wall, a much longer segment than that suggested by Szücs *et al*^[22] and we added a transverse myotomy around the circumference of the esophagus. Our intention was not only to cover the entire site of the anastomosis, but also to encourage the discharge of saliva at a lower region, while attempting to leave the anastomosis site out of alimentary transit. To this end, it was necessary to invaginate a longer segment that consisted of all the layers of the wall of the esophagus.

gus, such that the inserted portion remained in the shape of a tube in the interior of the stomach.

To execute the anastomosis, we elected a region at the proximal esophagus where the suture would be placed, and preserved 4.0 cm of esophagus to be invaginated into the stomach. At this point, a transverse myotomy was done around the circumference of the esophagus. We sutured the proximal border of the myotomy together with the seromuscular layer of the stomach. The purpose of the myotomy was to create a border with viability in the muscular layer of the esophagus, to be sutured with the seromuscular layer of the stomach, and also to elongate the esophageal tube to be inserted into the stomach.

The point of esophageal section must be chosen to allow for a safe margin, because carcinoma of the esophagus can disseminate within the wall to sites distal from the principal lesion^[8,25-28]. To perform an esophagogastric anastomosis with invagination, it is necessary to save 4.0 cm more of proximal esophagus than for anastomosis without invagination. If it is not possible to achieve an adequate margin, the invagination procedure should be abandoned.

In our view, the fact that this technique conserves 4.0 cm more of the esophagus does not detract from the radical nature of the operation. Upon constructing a cervical esophagogastric anastomosis with invagination, the amount of remaining esophagus is no greater than that usually left when anastomosis is done in the thoracic apex. Walther *et al*^[6], in a prospective randomized study, have compared cervical with intrathoracic esophagogastric anastomosis. They have concluded that the withdrawal of an extra 5.0 cm of esophagus to perform anastomosis in the neck does not affect the 5-year survival rate. Consequently, we believe that following all the recommendations and leaving a secure margin, esophagogastric anastomosis with invagination does not breach any radical oncological principles.

The diagnosis of fistula of the esophagogastric anastomosis was made based exclusively on clinical criteria, given that a radiological study with water-soluble contrast medium has low sensitivity and a high incidence of false-negative results^[29]. None of our cases operated upon by esophagectomy with esophagogastric anastomosis and invagination developed fistula with heavy egress of saliva from the cervical incision. Compared with results from the literature^[4,6,13-18,30] which show an incidence of fistulas of 0%-50%, cervical esophagogastric anastomosis with invagination had a low incidence of fistula formation, with only one case (3.4%) having clinical repercussions.

It is possible that esophagogastric anastomosis with invagination did not influence the factors responsible for the formation of the fistula. Moreover, it is likely that points of dehiscence could occur along the suture line similarly when we perform the end-to-end technique. However, as the saliva flows to an area below the anastomosis, these points of dehiscence probably can undergo rapid regeneration. On the other hand, in cases without invagination, the saliva discharges directly into the area of the suture with dehiscence, which provokes local inflammation and infection, thereby delaying the healing process of the suture line and enlarging this area.

In view of this mechanism, we believe that the three cases observed of fistula formation in esophagogastric anastomosis presented with mild clinical repercussions, even when the fistula was directed toward the pleural space. We also believe that, despite fistula with minimal clinical repercussions, the technique of esophagogastric anastomosis with invagination can still prove advantageous over the method without invagination.

In the present study, seven (24.1%) cases developed postoperative strictures of anastomosis; this rate lies within the 5%-45% limit described by other authors^[15,19,20]. We believe that this result could have been due to the fact that anastomosis with invagination did not influence the factors that might predispose the formation of fistulas, such as ischemia in the proximal portion of the gastropasty. In this situation, the points of dehiscence would have occurred along the suture line at a similar rate to anastomosis without invagination. However, the presence of a fistula was not always identified using clinical criteria, possibly due to the fact that saliva discharges below the point of the dehiscence. These events could possibly trigger a fibrotic reaction and scarring, with subsequent stenosis formation in the anastomosis.

We conclude that performing cervical esophagogastric anastomosis with invagination of the proximal esophageal stump into the stomach in subtotal esophagogastric resection with gastropasty in patients with carcinoma of the thoracic and abdominal regions of the esophagus represents a potential real advantage of this technique over other conventional techniques without invagination.

However, prospective, randomized, and controlled studies that involve esophagogastric anastomosis with invagination of the proximal esophageal stump into the tip of the stomach placed to the cervical region are needed to confirm the initial results obtained in this study.

COMMENTS

Background

Definitive curative treatment for cancer of the esophagus remains a challenge for surgeons. The approach involves major surgery that has a high morbidity and mortality rate, mainly due to pulmonary complications, cervical fistulas, stenosis of anastomosis, necrosis of the tubularized stomach, and mediastinitis. Among these possible complications, fistula of the esophagogastric anastomosis represents one of the principal problems of esophagectomy. Incidence in several studies has ranged from 0% to 50%, with most authors reporting a high incidence of this complication.

Research frontiers

In view of the high incidence of esophagogastric fistulas associated with significant levels of mortality and morbidity, several surgical techniques have been tried to reduce the frequency of fistula formation. These approaches include protection of the anastomosis with fibrin glue, anastomosis in two stages, gastric fundus rotation, microsurgical revascularization of the transposed viscera, mechanical anastomosis, laparoscopic construction of the gastric tube 5 d before esophagectomy, preservation of the vascular arcade of the splenic hilum, administration of prostaglandin E1, and anastomosis with invagination.

Innovations and breakthroughs

Esophagogastric anastomosis with invagination is a modification of a technique that is performed to reduce fistula formation at the anastomosis site. We chose to invaginate a 4.0-cm segment made up of all the layers of the esophagus wall, a much longer segment than that suggested by other authors, and we added transverse myotomy around the circumference of the esophagus. Our intention was not only to cover the entire site of the anastomosis, but also to

encourage the discharge of saliva at a lower region, while attempting to leave the anastomosis site without contact with saliva. To this end, it was necessary to invaginate a longer segment that consisted of all the layers of the wall of the esophagus, such that the inserted portion remained in the shape of a tube in the interior of the stomach.

Applications

Cervical esophagogastric anastomosis with invagination of the proximal esophageal stump into the stomach in subtotal esophagogastric resection with gastroplasty in patients with carcinoma of the thoracic and abdominal regions of the esophagus is associated with a low incidence of esophagogastric fistula, while having similar stenosis rates to anastomosis without invagination.

Terminology

Cervical esophagogastric anastomosis with invagination of the proximal esophageal stump into the stomach tube is a new technique of cervical esophagogastric anastomosis to reduce the frequency of fistula formation.

Peer review

This is an interesting report on a novel technique for cervical esophagogastric anastomosis after esophagectomy and gastric replacement.

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***SLC11A1* polymorphisms in inflammatory bowel disease and *Mycobacterium avium* subspecies *paratuberculosis* status**

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Abstract

AIM: To test for association of *SLC11A1* with inflammatory bowel disease (IBD) and *Mycobacterium avium* subspecies *paratuberculosis* (MAP) status in a Caucasian cohort.

METHODS: Five hundred and seven Crohn's disease (CD) patients, 474 ulcerative colitis (UC) patients, and 569 healthy controls were genotyped for *SLC11A1*

1730G>A and *SLC11A1 469+14G>C* using pre-designed TaqMan® SNP assays. χ^2 tests were applied to test for association of single nucleotide polymorphisms (SNPs) with disease, and the presence of MAP DNA.

RESULTS: *SLC11A1 1730G>A* and *SLC11A 1469+14G>C* were not associated with CD, UC, or IBD. The *SLC11A1 1730A* minor allele was over-represented in patients who did not require immunomodulator therapy ($P = 0.002$, OR: 0.29, 95% CI: 0.13-0.66). The frequency of the *SLC11A1 469+14C* allele was higher in the subset of study participants who tested positive for MAP DNA ($P = 0.02$, OR: 1.56, 95% CI: 1.06-2.29). No association of *SLC11A1 1730G>A* with MAP was observed.

CONCLUSION: Although *SLC11A1* was not associated with IBD, association with MAP suggests that *SLC11A1* is important in determining susceptibility to bacteria implicated in the etiology of CD.

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Key words: *NRAMP1*; Crohn's disease; Ulcerative colitis; IS900 polymerase chain reaction

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INTRODUCTION

The solute carrier family 11 (*SLC11A1*) gene (also known

as *natural resistance associated macrophage protein 1*, *NRAMP1*)^[1] has been associated with susceptibility to intracellular pathogens since its initial identification in mice^[2]. *SLC11A1* encodes a divalent cation transporter that is located in endosome and phagosome membranes^[3] of macrophages and monocytes within the liver, spleen and lungs^[2,4]. This transporter plays a key role in mounting an effective immune response against intracellular pathogens^[1,5] through its involvement in the acidification of the phagosomes^[6], as well as the regulation of nitric oxide, interleukin-10^[7] and vacuolar iron concentrations^[8].

Given the pivotal roles that *SLC11A1* plays in innate immunity, it is not surprising that the relationship between polymorphisms in *SLC11A1* and a number of autoimmune and mycobacterial diseases has been explored. Associations have been found with leprosy^[9], tuberculosis^[10], rheumatoid arthritis^[11], visceral leishmaniasis^[12], multiple sclerosis^[13], type 1 diabetes mellitus^[14], and inflammatory bowel disease (IBD)^[15–18]. Most of these disease associations have been with a promoter dinucleotide microsatellite (GT)_n that is known to affect *SLC11A1* expression levels^[19]. However, *SLC11A1* also contains a number of single nucleotide polymorphisms (SNPs), including *SLC11A1* 1730G>A (*rs17235409*; D543N) and *SLC11A1* 469+14G>C (*rs3731865*; INT4G>C). The non-synonymous SNP 1730G>A is thought to alter the protein function^[18], whereas the intronic SNP 469+14G>C has no known functional effect, but has been suggested to be in linkage disequilibrium with functional promoter polymorphisms^[12].

SLC11A1 1730G>A and *SLC11A1* 469+14G>C have been tested for association with Crohn's disease (CD) in two European cohorts. Although the smaller of the two studies found no association with CD, Gazouli *et al.*^[18] have reported a significant association of both SNPs with disease (*SLC11A1* 1730G>A $P_{\text{genotypic}} = 0.0001$, OR: 3.43, 95% CI: 1.95–5.93, *SLC11A1* 469+14G>C $P_{\text{genotypic}} = 0.006$, OR: 15.91, 95% CI: 0.92–273.46). The involvement of *SLC11A1* in the handling and elimination of intracellular pathogens, as well as its association with mycobacterial diseases makes it a biologically plausible candidate risk gene for CD. The results of recent genome-wide association studies strongly suggest defects in genes involved in bacterial detection, handling, and elimination are central to CD pathogenesis. Furthermore the assertion, albeit controversial, that *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is an initial trigger for CD provides an additional rationale to investigate *SLC11A1* as a candidate risk gene for IBD. As a result, this study had two aims. The first was to attempt the first independent replication of the association of *SLC11A1* 1730G>A and *SLC11A1* 469+14G>C with IBD. The second aim was to use previously collected MAP IS900 data^[20] to test for association of *SLC11A1* genotypes with occurrence of MAP DNA in peripheral blood.

MATERIALS AND METHODS

Study participants

Patients were selected from a New Zealand Caucasian IBD

cohort that had been recruited to investigate genetic and environmental factors that contribute to CD and UC etiology^[20–24]. Detailed phenotypic data were available for members of this cohort including ancestry, location of disease, family history of IBD, age of onset, presence of extra-intestinal manifestations, and requirement for surgery. The MAP status of the CD patients in this cohort had been determined previously using IS900 polymerase chain reaction^[20]. Randomly selected blood donors ($n = 501$) from Christchurch (New Zealand), including 180 who had been previously tested for MAP status^[20] served as controls.

Genotyping

Genotyping of *SLC11A1* 1730G>A (*rs17235409*) and *SLC11A1* 469+14G>C (*rs3731865*) was performed in 384-well plates using the pre-designed Taqman[®] SNP genotyping assays C_256352269_10 and C_1659793_10 (Applied Biosystems, Foster City, CA, USA) in a LightCycler[®] 480 II (Hoffmann La Roche, Basel, Switzerland). Cycling conditions for *rs17235409* were 10 min at 95°C, 40 cycles of 15 s at 92°C and 1 min at 60°C, and 30 s of cooling at 40°C. Conditions were the same for *rs3731865*, but annealing was at 66°C rather than 60°C. Results were analyzed using Lightcycler[®] 480 software version 1.5.0. The accuracy of the genotyping assays was confirmed by repeat analysis of 13% of samples. Concordance between original and repeat genotype calls was 99%.

Statistical analysis

A web-based calculator (<http://ihg2.helmholtz-muenchen.de/cgi-bin/hw/hwa1.pl>) was used to test for deviations from Hardy-Weinberg Equilibrium (HWE). The χ^2 and OR analyses were performed using SPSS for Windows, version 13.0 (SPSS Inc., Chicago, IL, USA). Associations were considered significant if P was < 0.05. *Post hoc* power analysis demonstrated that our cohort had 90% power to detect a relative risk of 2.15 for *SLC11A1* 1730G>A ($\text{MAF}_{\text{controls}} = 0.02$, $\alpha = 0.05$) and 99.8% power to detect a relative risk of 1.5 for *SLC11A1* 469+14G>C ($\text{MAF}_{\text{controls}} = 0.30$, $\alpha = 0.05$).

Ethical considerations

All study participants provided written informed consent to be involved in ongoing IBD research, and ethical approval for this study was given by the Upper South Regional Ethics Committee (Canterbury, New Zealand).

RESULTS

Genotyping for *SLC11A1* 1730A>G and 469+14G>C was successful in 1468 (94.7%) and 1432 (92.4%) of study participants, respectively. No deviations from HWE were detected in cases or controls for either SNP ($P > 0.05$). The percentage minor allele frequency (MAF) of *SLC11A1* 1730G>A and *SLC11A1* 469+14G>C in our controls was 2% and 30%, respectively. We found no evidence of association of either *SLC11A1* SNP with overall CD, UC or IBD susceptibility (Table 1). Similarly, the minor allele and genotype frequencies of *SLC11A1*

Table 1 Genotype and allele frequencies of *SLC11A1* 1730G>A and 469+14G>C in New Zealand Crohn's disease and ulcerative colitis patients, and healthy controls *n* (%)

Phenotype	Genotype			MAF	Allelic <i>P</i> value	Allelic OR (95% CI)
1730G>A	GG	GA	AA	A		
CD (<i>n</i> = 495)	474 (96)	21 (4)	0	21 (2)	0.832	1.07 (0.57-2.00)
UC (<i>n</i> = 470)	450 (96)	20 (4)	0	20 (2)	0.827	1.07 (0.57-2.02)
HC (<i>n</i> = 503)	483 (96)	20 (4)	0	20 (2)		
469+14G>C	GG	GC	CC	C		
CD (<i>n</i> = 495)	265 (54)	192 (39)	38 (8)	268 (27)	0.153	0.83 (0.65-1.07)
UC (<i>n</i> = 451)	245 (54)	171 (38)	35 (8)	241 (27)	0.101	0.81 (0.62-1.04)
HC (<i>n</i> = 486)	238 (49)	204 (42)	44 (9)	292 (30)		

MAF: Minor allele frequency; OR: Odds ratio; CI: Confidence interval; CD: Crohn's disease; UC: Ulcerative colitis; HC: Healthy controls.

Table 2 Genotype frequencies of *SLC11A1* 1730G>A (*rs17235409*) in inflammatory bowel disease patients who have used/not used immunomodulators *n* (%)

Phenotype/immunomodulator status	Genotype			<i>P</i> value	OR (95% CI)
	GG	GA	AA		
CD/never used IM (<i>n</i> = 217)	203 (94)	14 (6)	0	0.031	0.38 (0.15-0.95)
CD/have used IM (<i>n</i> = 278)	271 (98)	7 (2)	0		
UC/never used IM (<i>n</i> = 356)	336 (94)	20 (6)	0	0.010	0.75 (0.71-0.79)
UC/have used IM (<i>n</i> = 114)	114 (100)	0	0		
IBD/never used IM (<i>n</i> = 573)	539 (94)	34 (6)	0	0.002	0.29 (0.13-0.66)
IBD/have used IM (<i>n</i> = 392)	385 (98)	7 (2)	0		

OR: Odds ratio; CI: Confidence interval; CD: Crohn's disease; UC: Ulcerative colitis; IBD: Inflammatory bowel disease; IM: Immunomodulator.

Table 3 Distribution of *SLC11A1* 469+14G>C genotype by *Mycobacterium avium* subspecies *paratuberculosis* status¹ in New Zealand Caucasians *n* (%)

MAP DNA in blood	Genotype frequency		<i>P</i> value	OR (95% CI)
	GG	GC + CC		
Present (<i>n</i> = 150)	66 (44)	84 (56)	0.02	1.56 (1.06-2.29)
Absent (<i>n</i> = 351)	193 (55)	158 (45)		

¹Tested by IS900 polymerase chain reaction to detect the presence of *Mycobacterium avium* subspecies *paratuberculosis* (MAP) DNA in peripheral blood^[20]. OR: Odds ratio; CI: Confidence interval.

1730G>A and 469+14G>C did not associate with age of disease onset, disease behavior, disease location, or requirement for resectional surgery (all *P* values > 0.1, data not shown). A significantly higher frequency of the *SLC11A1* 1730A allele was seen in IBD patients who did not require immunomodulator therapy, compared to those who did require this treatment approach (*P*_{IBD} = 0.002, OR: 0.29, 95% CI: 0.13-0.66, *P*_{CD} = 0.03, OR: 0.38, 95% CI: 0.15-0.95, *P*_{UC} = 0.01, OR: 0.75, 95% CI: 0.71-0.79) (Table 2). There was no significant association of *SLC11A1* 1730G>A with MAP status, whereas the *SLC11A1* 469+14C allele was associated with increased incidence of MAP DNA in peripheral blood (*P* = 0.02, OR: 1.56, 95% CI: 1.06-2.23) in our cohort (Table 3).

DISCUSSION

Previous association of *SLC11A1* 1730G>A and 469+

14G>C with mycobacterial infections and preliminary evidence of association with CD^[10-12,25] suggest that *SLC11A1* alters susceptibility to IBD. The primary aim of our study was to conduct the first independent replication of the association of *SLC11A1* with CD. In contrast to the original study of Gazouli *et al.*^[18], we found no evidence of *SLC11A1* 1730G>A or 469+14G>C as risk factors for IBD, CD or UC (all *P* values > 0.8) (Table 1). Comparison of the MAFs for the two *SLC11A1* SNPs revealed the existence of significant heterogeneity between Gazouli *et al.*^[18] and other studies for *SLC11A1* 1730A, and between populations of Northern versus Southern European ancestry for *SLC11A1* 469+14C. Our cohort and the cohort of Liu *et al.*^[26], which were composed primarily of individuals of Northern European ancestry, had *SLC11A1* 469+14C frequencies of 30% and 27% respectively. In contrast, the cohorts drawn from Southern European populations (Italian, Greek, and Turkish) exhibited significantly lower MAFs for this SNP. These differences in MAF distribution hint at the existence of a North-South gradient for *SLC11A1*, which could in turn explain the discordance between our study and that of Gazouli *et al.*^[18]. The occurrence of such gradients is not without precedence. The frequency of the CD-associated SNPs, *R702W*, *G908R* and *1007fs*, within the nucleotide oligomerization binding domain 2 gene (*NOD2*, also known as *CARD15*) exhibits a strong North-South gradient within Europe. A recent meta-analysis of *NOD2* association studies performed on European IBD cohorts has found that the MAFs and thus the contribution of these SNPs to CD risk increased significantly with decreasing latitude^[27].

The minor allele of *SLC11A1* 1730G>A was found to be significantly over-represented in the subset of our IBD patients who had never used immunomodulators, and by inference had less severe disease (Table 2). However, we saw no association with other markers of disease severity in our cohort. Due to the very low minor allele frequency (no minor allele homozygotes were observed), this result requires replication in other large cohorts to rule out a type 1 error.

The second aim of this study was to test for association of *SLC11A1* with MAP. The MAP status of 321 CD patients and 180 controls has been determined previously^[20]. Combining these patients and controls, we found no association between MAP status and *SLC11A1* 1730G>A, but did find an association with *SLC11A1* 469+14G>C ($P = 0.02$, OR: 1.56, 95% CI: 1.06-2.29) (Table 3). Earlier studies^[14,16] on smaller CD cohorts ($n = 37$ or 59) did not find any evidence of association of MAP status with *SLC11A1* 469+14G>C. However, this polymorphism has been associated with susceptibility to *Mycobacterium tuberculosis*^[10], and additional variation within *SLC11A1* has been associated with susceptibility to other mycobacterial diseases such as leprosy^[9]. Our results provide preliminary evidence of an association of the *SLC11A1* 469+14C allele with susceptibility to MAP.

We conclude that although *SLC11A1* could be a risk factor for IBD in some Southern European populations, we did not find an association of *SLC11A1* 469+14G>C or *SLC11A1* 1730G>A with IBD in our cohort that comprised primarily patients of Northern European ancestry. However, the significantly higher incidence of MAP DNA in the peripheral blood of *SLC11A1* 469+14C heterozygotes and homozygotes compared to *SLC11A1* 469+14G within our cohort suggests that this *SLC11A1* SNP, although not directly influencing disease risk, might modify susceptibility to potential CD-causing bacteria.

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COMMENTS

Background

The involvement of *SLC11A1* in the handling and elimination of intracellular pathogens, as well as its association with mycobacterial diseases makes it a biologically plausible candidate risk gene for Crohn's disease (CD). The suggestion that *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is an initial trigger for CD provides an additional rationale to investigate *SLC11A1* as a candidate risk gene for inflammatory bowel disease (IBD).

Research frontiers

A previous genetic association study has indicated that *SLC11A1* is a susceptibility gene for IBD. The authors performed an independent replication of this study in a large population-based cohort of Northern European origin. They also tested for the association of these polymorphisms with MAP status.

Innovations and breakthroughs

This is believed to be the first study to examine the association of *SLC11A1* polymorphisms in a well-powered cohort of Northern European origin. These findings indicate that *SLC11A1* polymorphisms do not modify disease risk for IBD, but might influence disease behavior (through indirect markers of severity) and susceptibility to MAP, a putative pathogen in CD. The authors also note the disparity of allele frequency between populations of Northern and Southern European origin.

Applications

By understanding how *SLC11A1* genotype influences the risk of colonization/infection with MAP, the authors might gain some insight into the contribution of this bacterium to IBD, and how defective clearance of MAP and other intracellular bacteria might be associated with modified disease risk.

Terminology

SLC11A1, solute carrier family 11 gene (also known as Natural Resistance Associated Macrophage Protein 1, *NRAMP1*) plays a key role in an effective innate immune response against intracellular pathogens. MAP is an intracellular bacterium that has been cited in several studies as a putative causal agent of CD.

Peer review

This paper provides interesting new results regarding the possible relationship between *SLC11A1* polymorphisms and IBD risk. The study has been done carefully and thoroughly, and the paper is very well written. The lack of association of *SLC11A1* and IBD risk in the study population (New Zealand Caucasians primarily of Northern European descent) is an important finding. The positive result that shows an association of an *SLC11A1* allele and MAP status is novel and interesting.

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Anti-pancreatic antibody in Turkish patients with inflammatory bowel disease and first-degree relatives

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RESULTS: In terms of PAB positivity, no difference was found between patients with CD (14.1%) and UC (7.9%) however, significant difference was observed between patients with CD and subjects in the control group ($P < 0.05$). No difference was found between patients with CD and their relatives in terms of ASCA positivity, whereas a significant difference was found between other groups ($P < 0.001$). Compared to ASCA, the sensitivity of the PAB was 19% (7/37), its specificity was 93% (25/27), positive predictive value was 77% (7/9) and negative predictive value was 45% (25/55). ASCA was found with significantly higher prevalence in patients with CD activity index > 150 ($P < 0.05$).

CONCLUSION: PAB is valuable in the diagnosis of IBD rather than CD, but cannot be used alone for diagnostic purposes. PAB is not superior to ASCA in CD diagnosis and in detecting CD among relatives of patients with CD.

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Key words: Anti-pancreatic antibody; Anti-Saccharomyces cerevisiae antibody; Crohn's disease; Ulcerative colitis; Inflammatory bowel disease

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Abstract

AIM: To identify the role of anti-pancreatic antibody (PAB) in the diagnosis of inflammatory bowel diseases (IBD) among Turkish patients, and its frequency in first-degree relatives.

METHODS: PAB and anti-Saccharomyces cerevisiae (ASCA) were examined in serum samples of 214 subjects including patients with Crohn's disease (CD, $n = 64$), ulcerative colitis (UC, $n = 63$), first-degree relatives of patients with CD ($n = 25$), first-degree relatives of patients with UC ($n = 28$), and a control group with gastrointestinal symptoms other than (IBD) ($n = 34$) by indirect immunofluorescence. Positivity of PAB and ASCA was compared in terms of Vienna classification, disease activity and medications used.

Demirsoy H, Ozdil K, Ersoy O, Kesici B, Karaca C, Alkim C, Akbayir N, Erdem LK, Onuk MD, Beyzadeoglu HT. Anti-pancreatic antibody in Turkish patients with inflammatory bowel disease and first-degree relatives. *World J Gastroenterol* 2010; 16(45): 5732-5738 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i45/5732.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i45.5732>

INTRODUCTION

The incidence of Crohn's disease (CD) and ulcerative colitis (UC) is gradually increasing. Despite clinical, endoscopic, radiological and histopathological findings, about 10% of patients with CD and UC are misclassified^[1]. Moreover, 10% of the cases are not classified and referred to as indeterminate colitis. New treatment options for inflammatory bowel disease (IBD) are available today. Medical treatment and surgical operations to be implemented vary depending on the type of the disease.

Several serological indicators can be used for differential diagnosis^[2]. The most commonly used two indicators are anti-Saccharomyces *cerevisiae* (ASCA) and perinuclear anti-neutrophil cytoplasmic antibodies (pANCA)^[3]. Anti-pancreatic antibody (PAB) is another indicator that is currently under investigation in this regard. Serological indicators are not sensitive enough in IBD screening. Therefore, depending on merely serological indicators, diagnosis and treatment of IBD are not possible. Several studies that are examining the serological indicators in diagnosis and treatment are underway.

The familial occurrence of IBD is well known. Around 5.5%-22.5% of patients with IBD have another family member also affected with the disease^[1-3]. In fact, the most important risk factor for IBD is having a family member with the disease. The relative risk for a sibling of a CD patient to also become affected is 13-36, and for a sibling of a UC patient this risk is 7-17^[4], therefore first-degree relatives with no complaints are at risk of developing the disease. Early diagnosis is considered to decrease the disease complications, as well as possible surgical treatment in the long term. Although the search for predictive markers that could identify family members at risk for IBD has been intensive, no such markers have been identified to date. Studies with auto antibodies are being carried out, as these are non-invasive assays. Positive serum antibody findings in first-degree relatives of patients with IBD will, in most cases, indicate potential disease.

To the best of our knowledge, there are no studies that are investigating PAB in CD in Turkey. In our study, PAB was compared with ASCA, which is the most commonly used serological indicator in CD. The five groups included in the study were: patients with CD, patients with UC, first-degree relatives of patients with CD, first-degree relatives of patients with UC, and control subjects. The study aimed to: identify whether PAB plays a role in the differential diagnosis of CD; compare PAB with ASCA; determine the frequency of PAB in first-degree relatives of CD patients who carry potential risk for the disease; and determine whether PAB can contribute to early diagnosis in first-degree relatives.

MATERIALS AND METHODS

Patient enrollment

Among outpatients and inpatients who presented to Sisli Etfal Education and Research Hospital Department of Gastroenterology; we enrolled 64 patients with CD, 63

with UC, 25 first-degree relatives of patients with CD, 28 first-degree relatives of patients with UC, and 34 control patients with gastrointestinal symptoms other than IBD. Diagnosis of CD and UC was established by means of clinical, endoscopic and histopathological examinations. Exclusion criteria were; infective enterocolitis (excluded *via* feces microscopy, culture, serological examination for bacterial and ameba infection, staining of the biopsy with acid-resistant dye and bacterial culture), Behcet's disease, microscopic colitis and indeterminate colitis. Patients with a diagnosis of IBD for > 6 mo were enrolled. Enrolled patients were categorized according to age, sex, disease type, disease activation, clinical picture and involvement site of the disease.

Patients with UC were classified with proctitis, distal involved colitis, left colon involved colitis, diffuse colitis, and pancolitis. First-degree relatives involved either the siblings or the parents of the patients with IBD. First-degree relatives who had suspected complaints and histories of IBD were excluded. The control group consisted of patients from outpatient clinics who presented with gastrointestinal symptoms other than IBD, and who had no first-degree relative with IBD. Table 1 summarizes the demographical characteristics, disease duration, disease type and sites involvement, and medication being taken for all the enrolled groups.

For the activity of the diseases, CD activity index (CDAI) for CD and Trulove and Witts clinical activity index for UC were used. Medication was classified into seven groups: no medication; 5-aminosalicylic acid (ASA); azathioprine (AZA); 5-ASA + corticosteroid (CS); 5-ASA + AZA; 5-ASA + antibiotics and 5-ASA + CS + AZA.

Conduction of antibody testing

All tests were conducted by product specialists from Euroimmun AG, Turkey, who were trained at Euroimmun Laboratories in Germany, and were confirmed by a second specialist. Both specialists were blinded to the diagnoses. After being collected from patients, relatives and control group, venous blood samples were centrifuged within 3 h, and serum was separated and were maintained at 80°C until the time of testing. Serum samples to be studied were transported to laboratories in ice boxes.

Determination of PAB

The presence of PAB was determined by indirect immunofluorescence staining in primate pancreas tissue (Figure 1). Kits prepared by Euroimmun AG were used. Substrates that were developed from pancreatic tissue from primates were divided into thin sections (biochips), transferred to slides, and utilized in the kit. The materials in the kits were kept under suitable conditions (4-8°C) until use. For dilution and washing steps, we used a solution that was prepared from 10.2 g phosphate buffer, pH 7.2 and 2 mL Tween 20 (organic detergent). Antibodies tend to precipitate after the serum is dissolved, therefore serum samples of each patient were subjected to a string step (vortexing) to ensure homogeneous dispersion in the serum. Dilutions of 1/10 with PBS were prepared for

Table 1 Characteristics of patients, relatives and control group subjects *n* (%)

	CD (<i>n</i> = 64)	CD relatives (<i>n</i> = 25)	UC (<i>n</i> = 63)	UC relatives (<i>n</i> = 28)	Control (<i>n</i> = 34)
Sex					
Male	28 (43.8)	9 (36.0)	32 (50.8)	11 (39.3)	15 (44.1)
Female	36 (56.2)	16 (64.0)	31 (49.2)	17 (60.7)	19 (55.9)
Age (yr, mean \pm SD)	37.93 \pm 14.01	32.12 \pm 14.31	38.74 \pm 13.13	35.35 \pm 17.07	38.58 \pm 15.09
Disease duration (yr, mean \pm SD)	4.37 \pm 3.61		4.03 \pm 3.66		
Disease location (CD)					
Colon	2 (3.1)				
Ileum	26 (40.6)				
Ileocolonic	36 (56.3)				
Disease location (UC)					
Proctitis			9 (14.3)		
Distal involvement			24 (38.1)		
Left colon invol			9 (14.3)		
Diffuse			2 (3.2)		
Pancolitis			19 (30.2)		
Disease type (CD)					
Stricturing	7 (10.9)				
Penetrating	6 (9.4)				
Inflammatory	51 (79.7)				
Medications (for CD and UC)					
No medication	5 (7.8)		5 (7.9)		
5-ASA	57 (89.06)		57 (90.4)		
Steroids	5 (7.8)		5 (7.9)		
AZA	13 (20.3)		8 (12.6)		
Antibiotics	1 (1.6)		3 (4.7)		

CD: Crohn's disease; UC: Ulcerative colitis; 5-ASA: 5-aminosalicylic acid; AZA: Azathioprine.

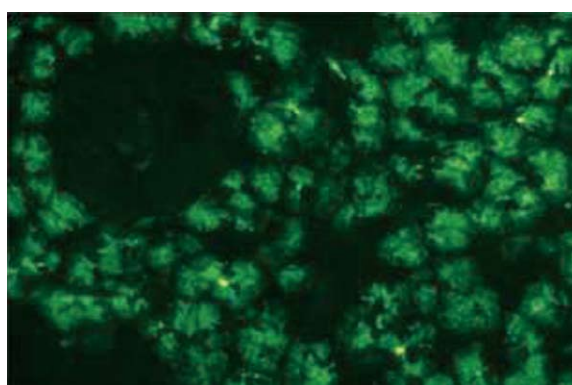


Figure 1 Pancreatic antibodies were determined by indirect immunofluorescence staining in primate pancreas tissue.

each patient. Prepared dilutions were incubated at room temperature (18–25°C) for 30 min, followed by 5 min washing with PBS. Later, incubation with fluorescence-marked anti-human globulin IgA and IgG conjugates was performed for each patient. After being washed with PBS for 5 min, the slides were prepared for examinations by specialists by adding glycerol, pH 8.4, included in the kit and closing with lamella.

Determination of ASCA

Kits prepared by Euroimmun AG that contained antibodies against *S. cerevisiae* were used. Storage conditions of this kit were the same as for the PAB kit. The same solutions were used for dilutions and washing steps. However, we used 1/100 dilutions for ASCA rather than 1/10 as in the

previous preparation. Dilutions were incubated at room temperature (18–25°C) for 30 min, followed by 5 min washing with PBS. Later, incubation with fluorescence-marked anti-human globulin IgA and IgG conjugates was performed for each patient. After washing with PBS for 5 min, the slides were prepared for examination by specialists by adding glycerol, pH 8.4, included in the kit, and closing with lamella.

Statistical analysis

Data were evaluated using (SPSS) for Windows, version 9.05 statistical software. Research findings were converted into numeric and percentage distributions and Fischer's exact χ^2 test (used when the number of the object was < 20, or between 20 and 40 but if the least expectant value was < 5) and Pearson's χ^2 test were used to determine the level of significance of correlations between dependent (PAB and ASCA), independent (disease duration, disease onset age, disease type, site of involvement, medication) variables. $P < 0.05$ was considered statistically significant.

RESULTS

Demographic features of the involved patients are summarized in Table 1.

PAB was found to be positive in nine patients with CD (9/64; 14.06%) and five with UC (5/63; 7.93%) (Table 2, Figure 2), but the difference was not statistically significant.

ASCA was found to be positive in 57.80% (37/64) of patients with CD, and 20.60% (13/63) of patients with UC, 36% (9/25) of CD relatives, 17.90% (5/28) of UC

Table 2 Pancreatic antibody and anti-Saccharomyces cerevisiae positivity results in study groups *n* (%)

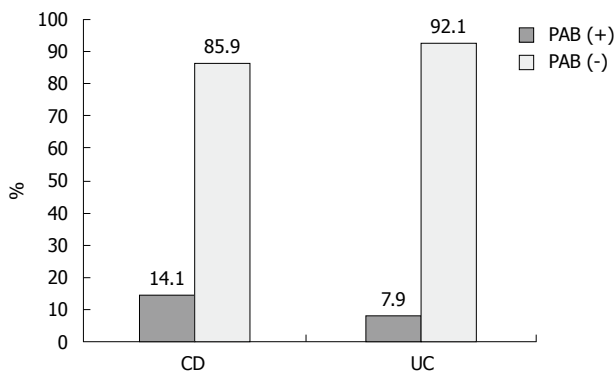
	CD (<i>n</i> = 64)	UC (<i>n</i> = 63)	CD relatives (<i>n</i> = 25)	UC relatives (<i>n</i> = 28)	Control (<i>n</i> = 34)
PAB	9 (14.1)	5 (7.9)	0 (0)	0 (0)	0 (0)
ASCA	37 (57.8)	13 (20.6)	9 (36)	5 (17.9)	6 (17.6)

CD: Crohn's disease; UC: Ulcerative colitis; PAB: Anti-pancreatic antibody; ASCA: Anti-Saccharomyces cerevisiae.

Table 3 Positivity rates of pancreatic antibody according to Vienna classification

		A1 (<i>n</i> = 43, PAB = 7, 16.27%)			A2 (<i>n</i> = 21, PAB = 2, 9.52%)		
		B1 (<i>n</i> = 31, PAB = 5, 16.12%)	B2 (<i>n</i> = 6, PAB = 1, 16.66%)	B3 (<i>n</i> = 6, PAB = 1, 16.66%)	B1 (<i>n</i> = 20, PAB = 2, 10%)	B2 (<i>n</i> = 1, PAB = 0, 0%)	B3 (<i>n</i> = 0, PAB = 0, 0%)
L1 (<i>n</i> = 26, 3.84%)	A1 (<i>n</i> = 18, PAB = 1, 5.55%)	<i>n</i> = 15, PAB = 1, 6.66%	<i>n</i> = 0, PAB = 0, 0%	<i>n</i> = 3, PAB = 0, 0%	A2 (<i>n</i> = 8, PAB = 0, 0%)	<i>n</i> = 7, PAB = 0, 0%	<i>n</i> = 1, PAB = 0, 0%
L2 (<i>n</i> = 2, 0%)	A1 (<i>n</i> = 1, PAB = 0, 0%)	<i>n</i> = 1, PAB = 0, 0%	<i>n</i> = 0, PAB = 0, 0%	<i>n</i> = 0, PAB = 0, 0%	A2 (<i>n</i> = 1, PAB = 0, 0%)	<i>n</i> = 1, PAB = 0, 0%	<i>n</i> = 0, PAB = 0, 0%
L3 (<i>n</i> = 36, 22.22%)	A1 (<i>n</i> = 24, PAB = 6, 25%)	<i>n</i> = 15, PAB = 4, 25%	<i>n</i> = 6, PAB = 1, 16.66%	<i>n</i> = 3, PAB = 1, 33.33%	A2 (<i>n</i> = 12, PAB = 2, 16.66%)	<i>n</i> = 12, PAB = 2, 16.66%	<i>n</i> = 0, PAB = 0, 0%

A1: Disease onset age < 40 yr; A2: Disease onset age ≥ 40 yr; B1: Inflammatory type; B2: Stricture type; B3: Fistulizing type; L1: Ileal involvement; L2: Colonic involvement; L3: Ileocolonic involvement. PAB: Pancreatic antibody.

**Figure 2** Pancreatic antibody frequency in Crohn's disease and ulcerative colitis patients. CD: Crohn's disease; UC: Ulcerative colitis; PAB: Pancreatic antibody.

relatives, and 17.60% (6/34) of the control subjects. A statistically significant difference was found between the patients with CD and UC and between the relatives of patients with UC and the control group ($P < 0.001$ for both), but there was no significant difference between the patients with CD and their relatives.

Sensitivity and specificity of PAB was 19% (7/37) and 93% (25/27), respectively. Positive predictive value was 77% (7/9) and negative predictive value was 45% (25/55). The likelihood ratio was 2.7 (0.19/10.93) and coherence was 0.50 (7+25/64).

Although frequencies of PAB and ASCA were found to be higher among the patients aged < 40 years than in the older group (Tables 3 and 4), the differences were not statistically significant for PAB or ASCA. The positivity ratios of ASCA and PAB were not significantly dif-

ferent between the patients with UC and CD.

When CD activity was classified as mild and severe, no difference was found in PAB positivity (Table 5). The same assessment performed for ASCA yielded no statistically significant difference.

CD patients were grouped according to medication and classified according to PAB and ASCA positivity. No significant difference was found in patients using corticosteroids or immunosuppressive drugs in terms of PAB and ASCA levels.

DISCUSSION

Tissue damage in IBD is caused by multiple mechanisms that are mediated by the immune system. Although several studies have supported the argument that autoimmune mechanisms are the primary responsible mechanism, the consensus is that autoimmune stimulation does not have direct significance. Therefore, IBD is not actually an autoimmune disease. However, because the pathogenesis is mediated by the immune system, determination of auto-antibodies and other immunological indicators is of clinical importance and warrants further research^[5].

PAB frequency in patients with CD was found as 14.06% (9/64) and as 7.93% (5/63) for UC patients in the present study. No PAB positivity was noted in the relatives of both CD and UC patients. Although PAB was more positive in CD patients, the difference was not statistically significant. Related studies in the literature have reported PAB frequencies of 15%-40% in CD, 1%-4% in UC, and 1%-4% in control subjects^[6-15]. The results of the present study can be considered consistent with those of Greek

Table 4 Positivity rates of anti-Saccharomyces cerevisiae according to Vienna classification

		A1 (n = 43, ASCA = 27, 62.79%)			A2 (n = 21, ASCA = 10, 47.61%)		
		B1 (n = 31, ASCA = 18, 58.06%)	B2 (n = 6, ASCA = 3, 50%)	B3 (n = 6, ASCA = 6, 100%)	B1 (n = 20, ASCA = 9, 45%)	B2 (n = 1, ASCA = 1, 100%)	B3 (n = 0, ASCA = 0, 0%)
L1 (18/26, 69.23%)	A1 (n = 18, ASCA = 13, 72.22%)	n = 15, ASCA = 10, 66.66%	n = 0, ASCA = 0, 0%	n = 3, ASCA = 3, 100%	A2 (n = 8, ASCA = 5, 62.5%)	n = 7, ASCA = 4, 57.14%	n = 1, ASCA = 1, 100%
L2 (0/2, 0%)	A1 (n = 1, ASCA = 0, 0%)	n = 1, ASCA = 0, 0%	n = 0, ASCA = 0, 0%	n = 0, ASCA = 0, 0%	A2 (n = 1, ASCA = 0, 0%)	n = 1, ASCA = 0, 0%	n = 0, ASCA = 0, 0%
L3 (19/36, 52.77%)	A1 (n = 24, ASCA = 14, 58.33%)	n = 15, ASCA = 8, 53.33%	n = 6, ASCA = 3, 50%	n = 3, ASCA = 3, 100%	A2 (n = 12, ASCA = 5, 41.66%)	n = 12, ASCA = 5, 41.66%	n = 0, ASCA = 0, 0%

A1: Disease onset age < 40 yr; A2: Disease onset age ≥ 40 yr; B1: Inflammatory type; B2: Stricture type; B3: Fistulizing type; L1: Ileal involvement; L2: Colonic involvement; L3: Ileocolonic involvement. ASCA: Anti-Saccharomyces cerevisiae.

Table 5 Pancreatic antibody and anti-Saccharomyces cerevisiae positivity according to Crohn's disease activity index n (%)

Disease activity according to CDAI	PAB		ASCA		Total
	Negative	Positive	Negative	Positive	
Mild (< 150 points)	49 (84.5)	9 (15.5)	27 (46.6)	31 (53.4)	58 (100)
Moderate (150-450 points)	5 (100)	0 (0)	0 (0)	5 (100)	5 (100)
Severe (> 450 points)	1 (100)	0 (0)	0 (0)	1 (100)	1 (100)
Total	55	9	27	37	64

CDAI: Crohn's disease activity index; PAB: Pancreatic antibody; ASCA: Anti-Saccharomyces cerevisiae.

and Belgian studies that have demonstrated that PAB is present not only in patients with CD, but also in those with UC. Frequency of antibody in patients with UC was reported to be 23.3% in a Belgian study and 24.7% in a Greek study^[11-14]. As PAB positivity is also seen in patients with UC, and because the frequency in patients with UC is not significantly different from that in CD patients, PAB seems to be a more suitable indicator for IBD rather than CD. In the light of these findings, PAB cannot be regarded as a screening test for IBD. Nishimori *et al.*^[13], Desplat-Jégo *et al.*^[15] and Koutroubakis *et al.*^[11] have arrived at the same conclusion.

Several serological indicators that are useful in differential diagnosis of UC and CD have been studied recently, and utilization of multiple indicators has been shown to improve differential diagnosis in cases of indeterminate colitis. Some of these indicators are pANCA, ASCA, PAB, OmpC antibody and I-2 and anaerobic coccoid rods antibodies^[8-19]. This is why ASCA, together with PAB, was examined in the present study. As shown in Table 2, ASCA positivity was 57.8% (37/64) in CD patients, 20.6% (13/63) in UC patients, 36% (9/25) in the first-degree relatives of patients with CD, 17.9% (5/28) in the first-degree relatives of patients with UC, and 17.6% (6/34) in the control group. These values are much higher than the PAB positivity rates. In terms of these positivity rates, a statistically significant difference was identified between CD and UC, and relatives of UC patients and control subjects ($P < 0.001$); however, no significant difference was noted be-

tween CD patients and their relatives between UC patients and their relatives, or between UC patients and the control group. PAB seems to be a less desirable serological indicator compared to pANCA and ASCA in patients with IBD, as our findings indicate a lower prevalence. However, it can improve diagnosis and the predictive value when used in combination with common indicators.

ASCA, which is found with higher rates of positivity in patients with CD, is reported to be positive also in spondyloarthropathy associated with HLA-B27, which is its disadvantage^[8]. In contrast, studies of PAB, have emphasized that it has a high specificity for CD and is positive only in patients with CD^[8,17,18].

In a study by Stöcker *et al.*^[9], higher rates of PAB positivity were noted in patients with disease duration > 2.5 years compared to those with duration < 2.5 years. Similarly, Klebl *et al.*^[16] have also identified a significant relationship between PAB positivity and disease duration ($P = 0.04$). Koutroubakis *et al.*^[11] have compared patients with disease duration less and more than 2 years, and have noted a tendency towards increased PAB positivity in the latter; however, the result was not statistically significant. When PAB frequency was assessed in terms of disease duration in our study, positive findings were identified for 14.3% (5/35) of the patients with disease duration < 3 years compared to 13.8% (4/29) in those with disease duration > 3 years. The results are very close to each other and no difference was detected with the statistical methods used in this study (Fisher's exact χ^2 test).

Another research topic in studies with PAB is the relationship of PAB with disease activity. Most of the earlier studies^[7,11,19,20] have failed to identify a correlation between PAB positivity and disease activity, and a parallel was found only by Goischke *et al.*^[12]. There were no significant relationships between disease activity and PAB positivity in our study (Fisher's exact χ^2 test).

According to the disease type of CD, PAB was positive in 13.72% of patients with inflammatory type, in 14.28% of patients with stricturing type, and in 16.66% of patients with fistulizing type disease. The differences between the groups mentioned above were not found to be statistically significant, although a higher frequency was noted for the fistulizing type. Antibody frequency has been shown to be higher in fistulizing and stricturing type diseases^[11,16]. Klebl *et al.*^[16] have found that PAB frequency was 31.5% in stricturing and non-penetrating type, 41.7% in stricturing type, and 31.5% in penetrating type disease. Koutroubakis *et al.*^[11], on the other hand, have reported the frequency as 60% in stenotic type, 28.6% in inflammatory type, and 41.2% in fistulizing type disease, with a statistically significant result for stenotic type compared to the other types.

The most problematic cases in differential diagnosis are CD patients with isolated colon involvement, as the sites of involvement are the same as with UC. Sites of involvement in CD patients in this study were ileum for 26, ileocolon for 36 and colon for two patients. There were very few subjects with colon involvement, therefore, it was not possible to perform a comparison with subjects with ileum involvement. PAB positivity of patients with ileocolon involvement was higher than for other groups, but without statistical significance. There were also no significant differences in ASCA results according to site of involvement. These results were in accordance with several other relevant studies. In their studies from 1991 and 1996, Seibold *et al.* did not find a relationship between PAB and site of bowel involvement^[7-21]. Klebl *et al.*^[16] noted no relationship between PAB positivity and L category by the Vienna classification system. Koutroubakis *et al.*^[11] did not detect a significant relationship between disease localization and PAB positivity, but found less frequent PAB positivity in CD with colon involvement as compared to ileum or ileocolon involvement ($P = 0.1$). Similarly, no relation between PAB and disease localization was identified by Lawrance *et al.*^[22].

Another issue investigated in PAB studies is the relationship between PAB and concomitant drugs. Despite the studies by Stöcker *et al.*^[9] and Folwaczny *et al.*^[10] that have reported a high antibody frequency in patients not using glucocorticosteroids, Seibold *et al.*^[7,21], Goischke *et al.*^[12] and Klebl *et al.*^[16] did not detect any relationship between PAB frequency and drug use, including glucocorticosteroids. In our study, PAB positivity rates showed no difference between CD patients who were and were not taking glucocorticosteroids. Similarly, no statistically significant difference was noted in patients who were and were not using AZA.

Of the patients with IBD in the present study, 5%-10%

had a positive familial history. Farmer *et al.*^[23] observed that one third of IBD patients had family histories positive for IBD. It is known that the incidence of CD is 14-times higher in complaint-free first-degree relatives of CD patients compared to the general population, and that these individuals are at risk of developing the disease^[24] whereas Monsén *et al.*^[25] found that the prevalence of CD among first degree relatives 21 times higher than among non-relatives. Some studies have indicated that PAB positivity has a low frequency in the first-degree relatives of patients with CD. Seibold *et al.*^[21] have investigated 606 patients and arrived at the conclusion that PAB was a specific indicator for CD, and that PAB was rarely positive in family members of CD patients (2.5%). They concluded that most of the PAB-positive family members were CD patients^[5,19]. Folwaczny *et al.*^[10] have calculated PAB positivity in first-degree relatives of CD patients as 4%. The same study has reported that there were no significant differences between the incidence of PAB-positivity in the relatives of CD and patients and healthy controls^[6]. On the other hand, the findings of Joossens *et al.*^[14] are inconsistent with those reported in the above studies; they have found PAB prevalence of 32% in CD, 23.3% in UC and 22.2% in family members of patients with IBD. One of the purposes of the present study was to investigate PAB and ASCA positivity in first-degree relatives of patients with IBD, and to determine whether these indicators would contribute to early detection of the disease in these risk groups. However, PAB was detected in none of the first-degree relatives of IBD patients, nor in any subjects in the control group. ASCA results for the relatives of patients with CD, relatives of UC patients and the control group did not differ significantly, either. Shanahan *et al.*^[19] had summarized that autoantibodies were unlikely to have a direct, primary pathogenic role in CD and viewed that autoantibodies as important bridge between clinical and basic science. Müller-Ladner *et al.*^[20] had also found a link between pancreatic antibodies and Crohn's disease. Therefore, we believe it would not be appropriate to use PAB or ASCA alone for determining the potential of CD patients' relatives to develop CD.

In conclusion, in our study based on the absence of a significant difference between positivity in CD and UC, PAB has a diagnostic value for detecting patients with IBD rather than CD, and it should not be used alone for diagnosis. Also, we can say that investigating PAB and ASCA in first-degree relatives of IBD patients does not offer much benefit in early detection of the disease, and there is no superiority of PAB over ASCA in clinical practice in terms of CD diagnosis and early detection of the disease in patients' relatives. Further studies with novel indicators are still needed in the diagnosis of CD, and early diagnosis in relatives of CD patients.

COMMENTS

Background

Several antibodies have been associated with inflammatory bowel disease (IBD), with one of the most comprehensively studied being antibodies against

anti-Saccharomyces cerevisiae (ASCA). Pancreatic antibodies (PABs) are also newly studied antibodies that are specific for Crohn's disease (CD) and ulcerative colitis (UC), but their sensitivity alone is low.

Research frontiers

PAB in combination with ASCA might increase the sensitivity for detecting CD, especially isolated colonic CD. This study focused on the value of PAB alone or in combination with ASCA for diagnosing IBD, and differentiating CD from UC.

Innovations and breakthroughs

Several serological indicators can be used for differential diagnosis of IBD. PAB is another indicator that is currently under investigation in this regard. Serological indicators are not sensitive enough for IBD screening. Therefore, depending on serological indicators alone, diagnosis and treatment of IBD will not be possible. Several studies that are examining serological indicators for diagnosis and treatment are underway. There are no studies that are investigating PAB in CD in Turkey. The present study aimed to: identify whether PAB plays a role in the diagnosis and differential diagnosis of CD; compare it with ASCA; determine its frequency in first-degree relatives of CD patients who carry a potential risk for the disease; and determine whether it can contribute to early diagnosis.

Applications

This study suggest that PAB is a novel indicator that is still needed in the diagnosis of CD, and early diagnosis in relatives of CD patients.

Peer review

This study aimed to identify the role of PAB in the diagnosis of CD, and determine its frequency in first-degree relatives. The main focus of this work was to measure the frequency of PAB and ASCA in Turkish IBD patients.

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Palliation of malignant esophageal obstruction and fistulas with self expandable metallic stents

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Abstract

AIM: To evaluate the efficacy of self expandable metallic stents (SEMS) in patients with malignant esophageal obstruction and fistulas.

METHODS: SEMS were implanted in the presence of fluoroscopic guidance in patients suffering from advanced and non-resectable esophageal, cardiac and invasive lung cancer between 2002 and 2009. All procedures were performed under conscious sedation. All patients had esophagus obstruction and/or fistula. In all patients who required reintervention, recurrence of dysphagia, hemorrhage, and fistula formation were indications for further endoscopy. Patients' files were scanned retrospectively and the obtained data were analyzed using SPSS 13.0 for Windows. The χ^2 test was used for categorical data and was analysis of variance for non-categorical data. Patients' long-term survival was assessed using the Kaplan-Meier method.

RESULTS: Stents were successfully implanted in 90 patients using fluoroscopic guidance. Reasons for stent implantation in these patients were esophageal stricture (77/90, 85.5%), external pressure (8/90, 8.8%) and tra-

cheo-esophageal fistula (5/90, 5.5%). Dysphagia scores (mean \pm SD) were 3.37 ± 0.52 before and 0.90 ± 0.43 after stent implantation ($P = 0.002$). Intermittent, non-massive hemorrhage due to the erosion caused by the distal end of the stent in the stomach occurred in only one patient who received implementation at cardio-esophageal junction. Mean survival following stenting was 134.14 d (95% confidence interval: 94.06-174.21).

CONCLUSION: SEMS placement is safe and effective in the palliation of dysphagia in selected patients with malignant esophageal strictures.

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Key words: Esophagus cancer; Stenosis; Stents; Complication; Dysphagia

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INTRODUCTION

Patients with esophageal cancer and cancer of the gastro-esophageal junction commonly present with an advanced disease mainly because the esophagus is quite distensible and patients may not experience dysphagia until almost half of the luminal diameter is compromised^[1]. At the time of diagnosis, a high percentage of patients with esophageal cancer have an advanced stage of disease and when the tumor is not operable, only palliative treatment is applicable, primarily to manage dysphagia^[2]. Dysphagia is the most devastating symptom in malign stricture of the

esophagus. Aspiration is another frequent symptom requiring palliative therapy. This is either aspiration of saliva or food as a result of complete dysphagia or a tracheo-esophageal fistula. Similar problems may occur due to local pressure, particularly in patients with advanced lung cancer or mediastinal tumors. Early palliation of dysphagia and other symptoms is important in terms of nutritional status and a good quality of life. Among palliative treatments, radiotherapy, laser therapy and conventional plastic endoprostheses have a limited effect in preventing rapid weight loss due to malnutrition^[3].

Several self expandable metallic stents (SEMS) are now available and have been used widely to provide immediate symptomatic relief of malignant dysphagia. They are useful for patients with poor functional status who cannot tolerate radiotherapy or chemotherapy, who have advanced metastatic disease, or in whom previous therapy has failed^[3]. The aim of this report was to summarize our experience with expandable metal stents for palliation of malignant dysphagia in our 90 patients.

MATERIALS AND METHODS

From September 2002 to December 2009, 90 patients (65 men, 25 women; mean age 61.57 years, range 38-85 years) with malignant inoperable esophageal obstruction and high grade dysphagia or fistula were treated using flexible self-expanding metallic stents. Surgery was considered to be contraindicated in all patients due to the patients' poor general condition, the advanced stage of tumors, untreatable tumor recurrence or distant metastases. The ability to swallow was expressed as a dysphagia score. The scoring system was modified from that reported by Mellow and Pinkas; a score of 0 denoted the ability to eat a normal diet; 1, the ability to eat some solid food; 2, the ability to eat semisolid only; 3, the ability to swallow liquids only; and 4, complete dysphagia^[4]. The data were compared with that published in the literature. All patients gave their informed consent for the procedure.

Stent material

We employed self-expandable covered metallic esophageal stents from different companies (Boston Scientific, Watertown, MA, USA and Micro-Tech, Nanjing, China) in 76 patients. A covered self expanding stent was used in 5 patients with esophagotracheal fistulas. Diameters and length of covered stents varied between 18 and 20 mm, and 6 and 15 cm, respectively. Uncovered metallic stents (Ultraflex Boston Scientific, Watertown, MA, USA) were used in 14 patients. Diameters and length of uncovered stents varied between 18 and 23 mm, and 10 and 15 cm, respectively.

Implantation procedure

Before stent implantation, patients were prepared by administering conscious sedation analgesia (2.5-5 mg midazolam and 25-50 mg meperidine iv) The entire length of tumor stenosis and the location of the esophagotracheal fistula, if available, were precisely determined by an endoscope with an 11 mm diameter. High-grade strictures that

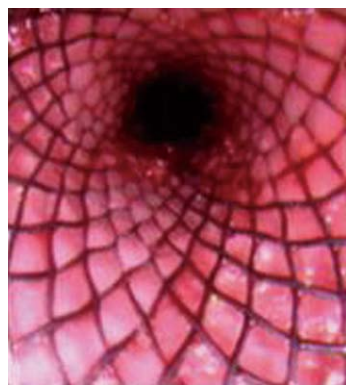


Figure 1 Proximal view of a fully opened stent.

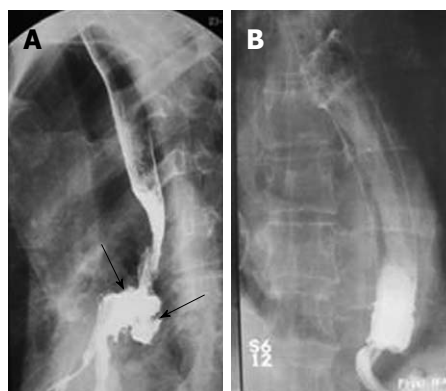


Figure 2 Palliation of tracheoesophageal fistula with stenting. A: Barium esophagogram showing a tracheo-esophageal fistula resulting from lung cancer; B: Complete occlusion of the fistula after stenting. Arrows show a large tracheoesophageal fistula.

could not pass an endoscope were opened prior to stenting to a diameter of at least 8-10 mm using a balloon dilator. After the preparatory treatment, the upper gastrointestinal tract was inspected endoscopically and the tumor margins on both sides were marked on the patients' skin with metallic markers. After the placement of a guidewire into the stomach and the removal of the endoscope, the stent introducer was inserted over the guidewire into the esophagus under fluoroscopic guidance. The stent was positioned under fluoroscopic control with guidance of radio-opaque skin markers and then released from the delivery system. The stents expanded by themselves within 10-60 s. After another minute, the delivery system and the guidewire were carefully removed. After retraction of the delivery system, an immediate endoscopic check was made (Figure 1). A plain chest radiograph and a contrast study using swallowed water-soluble contrast medium were performed to ensure the correct positioning and expansion of the stent and to exclude perforation. In cases of esophago-tracheal fistula, a radiographic control using a water-soluble contrast medium was carried out (Figure 2). These patients were advised to consume only liquids until the stent position was checked. Then they had semisolid or solid food, as individually tolerated. Most patients were discharged on an outpatient basis and were instructed to

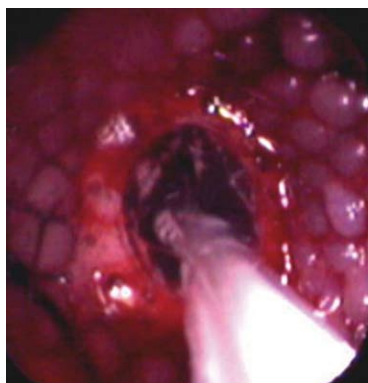


Figure 3 In some cases with insufficient stent expansion due to tight stricture, endoscopic balloon dilatation was performed through the opened stent.

start oral ingestion the same day. Anti reflux measures (proton pump inhibitors and/or prokinetic agents) were administered to patients whose prosthesis extended beyond the gastroesophageal junction. We did not use anti-reflux stents.

In some cases, because of the kinking of the esophageal lumen due to malign stricture, insertion of the stent introducer was difficult. In such cases, an endoscope was inserted after the introducer to help push it through the narrowed malign segment. In 3 cases with distal esophageal polypoid tumors, debulking was performed with a polypectomy snare because of extensive vegetation in the lumen preventing stent positioning and expansion. In 17 cases with insufficient stent expansion due to tight stricture, an endoscopic balloon dilatation through the opened stent was performed immediately after stenting to obtain an appropriate passageway (Figure 3).

An exploratory analysis was performed by using SPSS 13.0. Analysis of variance was used for non-categorical (continuous) data and the χ^2 test was used for categorical data. Survival data were assessed by the Kaplan-Meier method.

RESULTS

A total of 100 expandable metal stents were placed in 90 patients for malignant dysphagia caused by esophageal cancer or extrinsic compression. Fifteen uncovered SEMS were placed in 14 patients and 85 covered stents were placed in 76 patients. Our series consisted of 25 women and 65 men with a mean \pm SD age of 61.57 ± 12.05 years (range, 38–85 years). The mean size of the strictured segment of the esophagus where the stent was implanted was 7.14 ± 2.67 cm. The primary reason for stenting was esophageal stenosis alone in 77 patients (85.5%), followed by esophageal extrinsic compression in 8 patients (8.8%) and trachea-esophageal fistula in 5 patients (5.5%). In one patient stenting was performed in both his esophagus and the bronchus. In 4 cases, when the stent was opened in an inappropriate position, the stent was removed by pulling the attached string and a new stent was reinserted. Endoscopic balloon dilatation was performed before stenting in

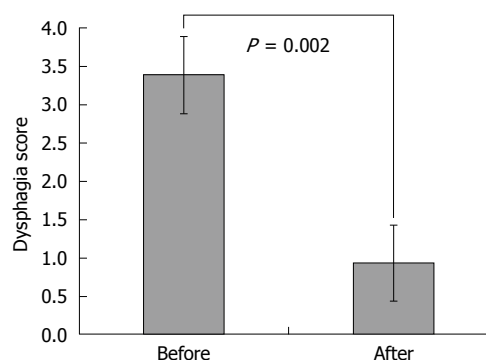


Figure 4 Comparison of oral alimentation status before and after placement of self expandable metallic stents. Figure shows the change in dysphagia score on day 3 after stenting. For the scoring system, see Materials and Methods section.

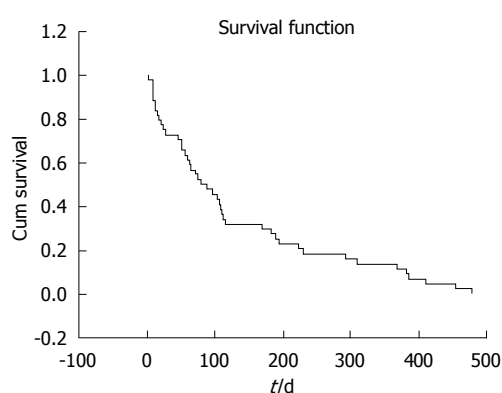


Figure 5 Kaplan-Meier survival curve of 90 patients following stenting.

27 patients (30%). Almost all patients improved in terms of oral intake. After the procedure, 84.4% of the patients (76/90) did not report any dysphagia during follow-up. Before stent placement, mean dysphagia score was 3.37 ± 0.52 ; after stent placement, mean dysphagia score was 0.90 ± 0.43 ($P = 0.002$) (Figure 4). There were no clinically significant complications during the insertion of stents. With respect to complications associated with stents, migration was noted in 4 patients (5%). Intermittent, non-massive hemorrhage due to the erosion caused by the distal end of the stent in the proximal stomach occurred in one patient who had received stent implantation in the cardio-esophageal junction. Migration was noted after 140 d on average (after 419 d in the first patient, after 69 d in the second patient, after 45 d in the third patient and after 27 d in the fourth patient). Migrations occurred following chemotherapy in 3 of the patients. Proximal tumor overgrowth was observed after 165 d on average following stenting in 6 patients (8.1%). Tumor overgrowth was observed within the first month following stenting only in one patient (at day 13). A second extendable stent was implanted in all of these patients. Minimal tissue ingrowth was detected in 3 patients (3.3%) treated with the uncovered stent and none had overt dysphagia.

Mean survival following stenting was 134.14 d [95% confidence interval: 20.45 (94.06–174.21)] (Figure 5). Restenting was needed in 10 patients (Table 1). No patient

Table 1 Characteristics of restented patients

Age (yr)	Sex	Reason for restenting	Tumor location (stage)	Stenosis location	Dilatation	Stent type
62	M	Proximal overgrowth	Gastric (T4)	Distal	No	6 cm, 20 mm CVRD
52	M	Proximal overgrowth	Lung Tm	Middle	No	6 cm, 20 mm CVRD
54	M	Proximal overgrowth	Gastric (T4)	Distal	APC	6 cm, 20 mm CVRD
71	M	Proximal overgrowth	Esophagus (T4)	Distal	No	6 cm, 20 mm CVRD
75	M	Migration	Lung Tm	Middle	No	10 cm, 20 mm CVRD
53	M	Proximal overgrowth	Esophagus (T4)	Proximal	No	6 cm, 18 mm CVRD
64	M	Migration	Esophagus (T3)	Middle	No	10 cm, 18 mm CVRD
67	M	Migration	Esophagus (T4)	Distal	No	10 cm, 20 mm UCVRD
55	F	Proximal overgrowth	Gastric (T4)	Distal	No	15 cm, 20 mm CVRD
49	F	Migration	Esophagus (T4)	Distal	No	6 cm, 20 mm CVRD

CVRD: Covered; UCVRD: Uncovered; APC: Argon plasma coagulation.

Table 2 Patient demographics

	<i>n</i> (%)
Total No. of patients	90
Mean age (yr, range)	61.57 (38-85)
Male/female	65/25 (72.2/27.8)
Present illness	
Esophagus carcinoma	54 (60.0)
Squamous cell carcinoma	47 (52.2)
Adenocarcinoma	7 (7.7)
Gastric carcinoma (and cardiac)	20 (22.2)
Lung carcinoma	16 (17.7)
Location of obstruction	
Proximal	5 (5.6)
Middle	31 (34.4)
Distal	54 (60.0)
Restenting	10 (11.1)

had esophageal perforation or procedure-related death. Dilatation was performed in 27 patients pre-operatively *via* a 12-16 mm balloon dilator for high grade strictures. Argon plasma coagulation was performed for one patient because of proximal tumor overgrowth.

Table 2 illustrates the localizations of the stents, the reasons for stenting and the patients' demographic data.

DISCUSSION

Our results suggest that SEMs provide a rapid and effective palliation for dysphagia in malignant stenosis, and low morbidity is associated with the procedure. In our study, all patients had significant relief of dysphagia. The frequency of the common conditions associated with stenting as identified in our study are presented in Table 3, in comparison with data reported from other studies^[5-15].

Palliation is often difficult to achieve in patients with esophageal obstruction as a result of cancer. Among many endoscopic and nonendoscopic treatment alternatives for palliation of cancer-related dysphagia, stenting with SEMs is one of the main options. It is useful for patients with poor functional status who cannot tolerate radiation or chemotherapy, who have advanced metastatic disease, or in whom previous therapy has failed^[16]. It can be concluded that stents provide better oral intake and quality of life compared to surgical palliation techniques. Despite

the substantially higher cost of expandable metal stents as compared to traditional rigid plastic esophageal stents, there are substantial overall cost savings resulting from the reduction in the number of days of hospitalization for surgery and possible complications^[7]. The majority of our subjects were patients who were referred to our clinic for dysphagia palliation from different centers and none were hospitalized after stenting. Our experience supported that SEMs could be inserted in outpatients, reducing the cost of treatment.

It has been shown that dysphagia was relieved in approximately 90% of patients who received an expandable metal stent^[7,12,17]. Compared to other palliative methods, the most significant and fastest improvement in swallowing is achieved in patients undergoing implantation of SEMs^[6,14,18,19]. Improvements in dysphagia were achieved in almost all of our patients following stenting. While the mean dysphagia score was 3.37 before stenting, the score was 0.90 following SEMs ($P = 0.002$). This decrease in dysphagia score is consistent with the literature. Recurrent dysphagia due to device migration and proximal overgrowth occurred in 10/90 (11.1%) of those who received SEMs. No significant difference was found between mean decreases in dysphagia scores in patients who were implanted with covered and uncovered stents (mean decreases in dysphagia scores in the 2 groups were 2.48 ± 0.53 , 2.42 ± 0.51 , respectively; $P = 0.487$). Covered and uncovered stents are equally good for dysphagia palliation^[20].

Esophageal expandable metal stents are also used to treat tracheo-esophageal fistulas due to cancer. Tracheo-esophageal fistulas develop in patients with advanced esophageal and lung cancer and lead to devastating symptoms as a result of continuous aspiration of saliva and food. Survival over 30 d is rare in these patients, unless they undergo an occluding procedure using an endoprosthesis^[18,21-25]. Noncovered stents are not suitable for the treatment of fistulas, because the esophageal contents can pass easily through the mesh and into the esophageal defect. The covered types of metal stents are considered as the primary choice of treatment since treatment of fistula with SEMs improves survival^[18,26,27]. In our study group, fistulas were successfully closed with covered stents in all patients with a fistula. All 5 of these patients had primary lung tumors.

Table 3 Outcome of published series of self-expandable metallic stent insertion: with or without fluoroscopy for self-expandable metallic stent insertion

Series	n	Dilation rate (%)	Stents uncovered	Perforation rate (%)	Migration rate (%)	Overgrowth rate (%)	Ingrowth rate (%)	Reintervention rate (%)	Median survival (d)
Present series	90	30.0	15/85	0	4.4	6.6	3.3	15.5	134
Wilkes <i>et al</i> ^[5]	98	8	95/5	0	3.1	25.5	-	39.8	100
Adam <i>et al</i> ^[6]	42	100	45/55	0	19	2.3	9.5	36	53
Knyrim <i>et al</i> ^[7]	21	0	100/0	0	0	9.5	14.2	3.8	167
Sarper <i>et al</i> ^[8]	41	100	19/81	4.9	2.4	2.4	2.4	-	94
Siersema <i>et al</i> ^[9]	100	7	0/100	6	13	10	0	49	109
Christie <i>et al</i> ^[10]	100	77	76/24	1	8.7	4	29.1	51	-
Wengrower <i>et al</i> ^[11]	81	-	100/0	3.6	5.95	2.4	0	-	120
De Palma <i>et al</i> ^[12]	19	0	100/0	0	0	0	10.5	-	198
Kozarek <i>et al</i> ^[13]	38	100	5/95	3	18.4	23.6	5.2	80	90
White <i>et al</i> ^[14]	70	100	57/43	2.8	0	1.4	2.8	-	-
Maroju <i>et al</i> ^[15]	30	-	9/91	0	3	7	10	-	161

¹Stent was placed under direct visualization in the study; ²Study of self-expandable metallic stent (SEMS) vs plastic esophageal prostheses treatment, but only data from SEMS arm was included; ³Only esophageal stents were used.

Stents were implanted in 5 of our patients because of proximal constriction within 4 cm. The Ultraflex type of covered stent was implanted in all these patients. Stent intolerance due to foreign body sensation, aspiration, perforation, proximal migration or pain was not observed in any of these patients. Verschuur *et al*^[28] reported 44 patients had a malignant stricture within 4 cm of the upper esophageal sphincter. In this study, dysphagia improved in most patients, and the occurrence of complications and recurrent dysphagia was comparable to that in patients who underwent stent placement in the mid and distal esophagus. It has been reported that 5%-15% of patients had a foreign body sensation; however, in none of the patients was stent removal indicated^[29]. There is a continuing debate about the advisability of SEMS placement for proximal esophageal cancer. If placed, it is frequently recommended that a distance of 2 cm below the upper esophageal sphincter should be maintained while placing a stent.

Intraprocedural complications of endoscopic stenting include those associated with sedation, aspiration, malpositioning of the stent, and esophageal perforation. Immediate postprocedural complications may include chest pain, bleeding, and tracheal compression, with resultant airway compromise and respiratory arrest. Late complications include distal or proximal stent migration, formation of an esophageal fistula, bleeding, perforation, and stent occlusion. Approximately 0.5%-2% of patients who undergo the procedure die as a direct result of placement of an expandable metal stent^[30]. No patients died during the procedure in our patient group. Perforation of the esophageal wall is potentially related to the device itself and prior chemoradiotherapy^[31]. This complication is presumably due to stent-induced pressure necrosis within devitalized esophageal tissue. The majority of stenting complications are reported in the literature to occur with plastic endoprotheses. In a study by Knyrim *et al*^[7], 42 patients with metallic stent and plastic endoprosthesis were compared and the frequency of complications was reported to be less in patients who were implanted with metallic stents (0 and 9 patients, respectively). Two prospective, random-

ized, controlled trials have shown a significantly lower rate of procedural complications using expanding metal stents^[7,12]. Perforation as a direct complication of stent placement was not seen in our patients. This result of perforation compares favorably with other studies^[18,32-37]. In contrast to placement of conventional esophageal stents, placement of expandable metal stents do not require a large-bore bougienage, thereby minimizing the risk of perforation and facilitating the insertion procedure. However, in our series balloon dilatation was performed in 27 patients before stenting. The frequency of dilatation has been reported to be in the range of 0%-100% in the literature^[7,12,13].

A tracheoesophageal (TE) fistula is one of the late complications that may occur after SEMS. The incidence of fistulas following stenting has been reported as 0%-10% in the literature. The type of the implanted stent was found to have no role in fistula development^[6,18,38,39]. Fistulas should be treated with placement of additional, overlapping covered metallic stents. Retrospective analysis of our patients did not reveal any patient developing fistulas after stenting. Kozarek *et al*^[13] did not find an association between perforation, bleeding or TE fistula development in patients who had received previous chemotherapy/irradiation. Consistent with the findings reported by Kozarek *et al*^[13], TE fistulas did not develop in any of our patients who were receiving treatment.

Stent migration may be a bothersome problem in SEMS. Studies have reported the incidence of late migration from 0% to 58% for different types of covered stents. A higher incidence of late migration of all types of covered stents was observed compared to the uncovered types^[6,18,21,40]. Stent migration was noted in 4 (4.4%) of our patients; 3 of these patients had covered and one had an uncovered type of stent.

Another ongoing issue with stents is the occurrence of recurrent dysphagia because of stent migration, tumoral or nontumoral tissue growth and food impaction. Frequencies of recurrent dysphagia associated with overgrowth, ingrowth and obstruction due to food impaction

reported in the literature varies between 17% and 33%, somewhat higher than those observed in our study^[2,7]. As the malignant tumor continues to grow after stent implantation, the growth of tumor tissue through the stent lumen (ingrowth) and extension over the stent borders (overgrowth) are 2 major late complications^[10,41,42]. The disadvantages of uncovered stents are tumor ingrowth and recurrent obstruction. Covered stents have been designed to prevent ingrowth. Ingrowth in uncovered stents has been reported in literature to be in the range of 0%-100%^[6,10,11,38,39]. Authors who used partially covered stents reported the incidence of growth in the uncovered ends of stents between 2% and 25% in different series^[10,41,42]. The frequency in covered stents has been reported as 0%-53%^[2,6]. In our study, tumor ingrowth was found in 3 patients (3.3%) treated with uncovered stents.

Overgrowth is the result of progression of malignancy rather than a failure or a complication of the stent. It is usually seen in 2.3%-30% of patients after a mean period of 2-4 mo after stent replacement^[2,6,9,39,43]. Overgrowth may not always be due to the tumor but may also result from benign epithelial hyperplasia or granulation tissue. The most frequent symptom in cases of overgrowth was dysphagia in our group. Following stenting, 6 patients (6.6%) were restented due to restenosis associated with proximal overgrowth. Restenosis associated with early proximal overgrowth occurred in only one of our patients (at day 13). Stent migration may facilitate development of overgrowth formation though it may not be possible to confirm this complication endoscopically. The use of covered stents may help decrease tumor ingrowth, although it will not affect tumor overgrowth. The most frequently used method for the treatment of tumor overgrowth is the placement of a second stent. Argon plasma coagulation was performed additionally in one of our patients with proximal tumor overgrowth. Stent obstruction was observed in 4 of our patients as a result of food impaction, and stents were cleaned endoscopically in all patients.

Hematemesis is also a possible complication after SEMS insertion, and the incidence in our study was 1.1% (1/90). This complication could have been the result of pressure necrosis, the natural progress of the disease, or esophageal or gastric trauma from the sharp end of the stent.

Mean survival after stenting, reported in the literature, varies between 53 and 198 d^[6,7,12]. In our study mean survival after stenting was 134 d. Overall survival time in our study was not significantly different from others in the literature. It has been shown that survival is influenced by the type and stage of the underlying disease.

It has been reported that SEMS are being implanted in the absence of fluoroscopic guidance in some centers and that this is a safe approach^[5,6,15]. We believe that esophageal stenting under fluoroscopic guidance with endoscopic control is safer.

In summary, in our retrospective screening, we noted that all of our patients had been stented with covered or uncovered metallic stents and that the use of these stents was safe and effective in rapid alleviation of dysphagia. The placement of a self-expandable metallic stent can

improve the oral alimentation status. Esophageal fistulas can be completely and rapidly closed with placement of covered metallic endoprostheses. Esophageal stents are a means to an end: in the setting of malignant disease, they occlude TE fistulae or alleviate dysphagia, but do not affect the natural course of the disease process except by virtue of inadequate palliation or subsequent stent-related complications.

COMMENTS

Background

Self expandable metallic stents (SEMS) introduction plays an important role in the management of esophageal obstruction and fistula secondary to malignancy. Early palliation of dysphagia is important for the maintaining of nutritional status and life quality. SEMS provides a considerable palliation with low mortality and morbidity especially in patients with advanced disease.

Research frontiers

This research is regarding to the efficacy and safety of SEMS in patients with esophageal obstruction or fistula due to advanced cancer.

Innovations and breakthroughs

The authors found that the covered and uncovered SEMS are equally effective for dysphagia palliation in malignant esophageal obstruction. This study is the most comprehensive retrospective review from Turkey about the palliation of malignant esophageal obstruction and fistula with SEMS. Also this study showed that it is possible to minimize the complications which may arise from SEMS placement.

Applications

The advantages of SEMS over the other palliative methods are rapidly improving of dysphagia, nutritional status and life quality. Stenting with covered SEMS is the most effective method in patients with malignant esophageal fistula.

Peer review

Palliation of malign obstruction of esophagus via SEMS is a rapid and effective treatment. The authors summarized their own experience retrospectively and the manuscript is generally well written.

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Antimicrobial susceptibility of *Helicobacter pylori* strains isolated from patients in Shiraz, Southern Iran

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Abstract

AIM: To improve our understanding of Iranian regional variation in *Helicobacter pylori* (*H. pylori*) antibiotic resistance rates to find the best antibiotic therapy for eradication of *H. pylori* infections.

METHODS: A total of 266 patients undergoing endoscopy in Shiraz, Southern Iran, were included in this study. *H. pylori* strains were isolated from antral biopsies by culture and confirmed by the rapid urease-test and gram staining. Antibiotic susceptibility of *H. pylori* isolates was determined by E-test.

RESULTS: A total of 121 *H. pylori* strains were isolated, 50 from male and 71 from female patients. Data showed that 44% ($n = 53$), 20% ($n = 24$), 5% ($n = 6$), and 3% ($n = 4$) of all strains were resistant to the antibiotics metronidazole, amoxicillin, clarithromycin, and tetracycline, respectively. When the antibiotics were considered together we found 11 sensitivity patterns for the strains. Resistance to metronidazole was significantly higher in female than in male patients ($P < 0.05$). In about 71% of the metronidazole-resistant isolates, the minimum inhibitory concentrations (MICs) exceeded 256 $\mu\text{g/mL}$.

CONCLUSION: We found a moderate rate of primary resistance to metronidazole. However, a high MIC ($> 256 \text{ mg/L}$) which was found in 71% of the isolates is considerable. In the case of amoxicillin, an increased resistance rate of 20% is worrying. Resistance to clarithromycin and tetracycline is also emerging among the *H. pylori* strains in our region.

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Key words: Gastric disorders; *Helicobacter pylori*; Iran; Sensitivity; Treatment

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INTRODUCTION

Helicobacter pylori (*H. pylori*) is involved in the pathogenesis

of a number of gastrointestinal diseases, including acute and chronic gastritis, peptic ulceration, gastric carcinoma and gastric lymphoma^[1]. Eradication treatment of *H. pylori* infection usually consists of various combinations of drugs. Most commonly, an acid suppressor (usually a proton pump inhibitor) or an H₂-receptor antagonist (e.g. ranitidine) is prescribed in combination with two antibiotics usually amoxicillin, metronidazole or clarithromycin^[1]. The combination of two antibiotics can increase the success of eradication therapy and decrease the possibility of secondary antibiotic resistance^[2]. Antibiotic resistance in *H. pylori* is the major cause of eradication failure. Growing resistance often parallels the patterns of antibiotic consumption, and may vary within patient groups according to the geographic region, patient age and sex, type of disease, birthplace, other infections and other factors. The geographic map and the process of primary *H. pylori* resistance are clinically important, and should be considered when choosing eradication regimens, as is constant monitoring at both national and global level in an attempt to reach the recently recommended goal of eradicating more than 95% of resistant cases^[3]. The prevalence of clarithromycin, metronidazole and amoxicillin resistance varies between countries and is highest for metronidazole^[4,5]. Resistance to tetracycline and ciprofloxacin has been reported but appears uncommon^[6-8].

There are several problems with antimicrobial susceptibility testing of *H. pylori*^[9,10]. Agar or broth dilution methods are difficult to perform routinely^[11], thus, disk-diffusion testing is often used because it is simple, easy to perform, and economical^[12]. However, the E-test has proved to be an accurate method for assaying the susceptibility of fastidious organisms, including *H. pylori*, to antibiotics. The E-test has a more stable pattern of antibiotic release and has been found to tolerate prolonged incubation^[13]. This is the main reason why the E-test rather than the disk diffusion method, has been recommended for *H. pylori*.

Pre-treatment resistance rates in *H. pylori* vary markedly between countries and regions. In Europe, mean resistance rates of 27% for metronidazole and 10% for clarithromycin are typical^[14,15]. There is no systematic surveillance of primary antibiotic resistance rates in Shiraz, and widely divergent rates have been reported in Iran, depending on the local population.

The present study aimed to improve our understanding of the Iranian regional variation in *H. pylori* antibiotic resistance rates in relation to gender, and to find the best antibiotic therapy for the eradication of *H. pylori* infections.

MATERIALS AND METHODS

The patient groups and sample collection

In this study, 266 patients attending the endoscopy ward of Motahhary Clinic of Shiraz University of Medical Sciences during the period between October 2008 and October 2009 were enrolled. Exclusion criteria for patient recruitment to the study were: previous attempts to eradicate *H. pylori*, use of antibiotics or proton pump inhibitors within the last 2 wk prior to endoscopy, and previous gastric surgery. The diagnosis of *H. pylori* infec-

tion and confirmation of gastric disease by histology were established by a central study pathologist. Antral biopsies taken from each patient were transferred to the lab in an appropriate transfer medium (brain heart infusion broth, supplemented with 20% glucose) for *H. pylori* isolation and identification.

Isolation and identification of *H. pylori*

Biopsy samples were gently homogenized and cultured on rapid urease-test media and colombia agar base (Merck, Germany), supplemented with 10% lysed horse blood and 7% fetal calf serum and the antibiotics amphotericin B (5 µg/L), trimethoprim (5 µg/L) and vancomycin (10 µg/L). The cultures were kept in a microaerophilic atmosphere (7% O₂, 7.1% CO₂, 7.1% H₂, 79.8% N₂), provided by Anoxomate (Mark II, Mart Microbiology BV, Netherlands) at 37°C for 2-4 d. The isolates were then confirmed as *H. pylori* by positive oxides, catalase and rapid urease-tests. The samples were also evaluated for the presence of *H. pylori* by the modified gram staining and rapid urease-tests. If any of the two tests were positive simultaneously, the sample was considered *H. pylori* positive.

Antibiotic susceptibility test

For *in vitro* susceptibility testing of the *H. pylori* strains, a suspension equal to the McFarland tube no. 3 was prepared for each isolate. We used only one colony from each patient for the analysis. Brain heart infusion broth (Merck, Germany) plates, supplemented with fetal calf serum (Gibco, USA) were inoculated by confluent swabbing of the surface with the adjusted inoculum suspensions. The E-test strips (Biomérieux, France) for the antibiotics amoxicillin, metronidazole, tetracycline, and clarithromycin, were aseptically placed onto the dried surface of inoculated agar plates. The plates were then incubated at 37°C under microaerophilic conditions. The minimum inhibitory concentrations (MICs) were read after 48-72 h of incubation on the basis of the intersection of the elliptical zone of growth inhibition using the MIC scale on the E-test strip, as per the manufacturer's instructions^[16]. Susceptibility results were recorded as resistant according to the following interpretive criteria; for metronidazole, clarithromycin, tetracycline and amoxicillin, MIC break-points of ≥ 8 mg/L, ≥ 1 mg/L, ≥ 4 mg/L and ≥ 0.5 mg/L, respectively^[17-19].

Statistical analysis

Fisher's exact test and *P* values were determined. A *P* value of < 0.05 was considered significant.

RESULTS

A total of 121 *H. pylori* strains were isolated from the patients under study, 50 from males and 71 from females. The antimicrobial susceptibility results of the *H. pylori* strains are presented in Table 1. According to the data, 44% ($n = 53$), 20% ($n = 24$), 5% ($n = 6$), and 3% ($n = 4$) of the strains were resistant to metronidazole, amoxicillin, clarithromycin, and tetracycline, respectively. Fifty isolates were

Table 1 Rates of antibiotic resistance in *Helicobacter pylori* isolates in relation to patient gender

Sex No.	No. of isolates (% of resistance)			
	MTZ	AMX	CLA	TET
Male (<i>n</i> = 50)	17 (34)	9 (18)	1 (2)	1 (2)
Female (<i>n</i> = 71)	36 (50)	15 (21)	5 (7)	3 (4)
Total (<i>n</i> = 121)	53 (43)	24 (19)	6 (5)	4 (3)

MTZ: Metronidazole; AMX: Amoxicillin; CLA: Clarithromycin; TET: Tetracycline.

Table 2 Antibiotic resistance patterns of the *Helicobacter pylori* strains

Antibiotic resistance patterns	<i>n</i>	Male	Female
MTZ	44	15	29
AMX	14	7	7
CLA	1	0	1
TET	1	1	0
MTZ-AMX	3	1	2
MTZ-TET	1	0	1
AMX-TET	1	0	1
AMX-CLA	1	0	1
MTZ-AMX-TET	1	0	1
MTZ-AMX-CLA	4	1	3
Sensitive	50	25	25
Total	121	50	71

MTZ: Metronidazole; AMX: Amoxicillin; CLA: Clarithromycin; TET: Tetracycline.

sensitive to all the tested antibiotics. When the antibiotics were considered together, we found 11 sensitivity patterns for drug sensitivity among the strains (Table 2). When the data were analyzed on the basis of patient gender (Table 1), 32% (*n* = 16), and 51% (*n* = 36) of the strains isolated from males and females, respectively, were resistant to metronidazole. Statistical analysis showed that resistance to metronidazole was significantly higher in female than in male patients (*P* < 0.05). With regard to clarithromycin and amoxicillin, the percentage of resistance in female patients was 7% and 20%, respectively and in male patients was 2% and 20%, respectively. The differences in resistance to these two antibiotics among the strains isolated from both genders were not significant (*P* > 0.05). We found that 3 (4%) and 1 (2%) strains isolated from female and male patients were resistant to tetracycline, respectively.

In about 71% of the metronidazole-resistant isolates, the MICs exceeded 256 µg/mL. The MIC ranges for the antibiotics tested in both genders are shown in Table 3.

DISCUSSION

Resistance to antimicrobials is of particular concern to practitioners in this field, and is a major cause of the failure to eradicate *H. pylori* infections^[20,21]. It has also been shown that resistance to different antibiotics develops in *H. pylori* strains by acquiring chromosomal mutations at the site where the drug acts^[22]. However, many reports have

Table 3 Range of minimum inhibitory concentrations for antibiotics tested against *Helicobacter pylori* strains in relation to patient gender

Sex	Range of MICs for antibiotics (mg/L)			
	MTZ	AMX	CLA	TET
Male	0.064 to > 256	< 0.016 to > 256	< 0.016 to 64	0.016 to 8
Female	0.047 to > 256	< 0.016 to > 256	< 0.016 to > 256	0.016 to 8

MIC: Minimum inhibitory concentration; MTZ: Metronidazole; AMX: Amoxicillin; CLA: Clarithromycin; TET: Tetracycline.

indicated that the prevalence of resistance varies geographically and that there is a broad range of resistance variability depending on the drug used^[23]. The special nutritional and atmospheric conditions required by these organisms make susceptibility testing relatively difficult; however, the E-test technique developed to determine the minimum inhibitory concentration (MIC) has remained valid^[24]. Accordingly, in the present study we evaluated the sensitivity of *H. pylori* strains isolated from patients with gastric disorders to 4 antibiotics using the E-test to find the resistance pattern in these strains in our region.

It was observed that 44% of the isolates in this study were resistant to metronidazole with a MIC range of 0.064 to > 256 µg/mL. This resistance rate was consistent with reports from some developed countries, where it has been reported that 15.8%-40% of *H. pylori* strains were resistant to metronidazole^[25-27]. However, most reports from developing countries describe a high level of resistance to metronidazole, which varies from 66.2% to 100%^[28,29]. Resistance rates to metronidazole may also vary within a country. For example, in India, the resistance rate to metronidazole was high in Lucknow, Chennai and Hyderabad (68%, 88.2% and 100%, respectively), whereas a moderate rate was observed in Delhi (37.5%) and Chandigarh (38.2%)^[30]. Similarly, the resistance rate was high (78%) in Tehran, Iran in one study carried out by Falsafi *et al*, while in another study it was reported to be 34%^[31,32]. In Europe, according to studies conducted between 1989-2001 and 1990-2002, respectively, the resistance rate varied between 16.0% and 43% in pediatric patients and between 14.9% and 40.3% in adult patients^[22]. It seems that primary resistance to nitro-imidazole has been attributed to frequent use of the drug, which is commonly prescribed for other diseases, especially parasitic conditions, and periodontal or gynecological infections. The higher resistance rate to metronidazole in females reported in this and other studies could be due to the treatment of gynecological infections using this drug which is also used in the treatment of bacterial vaginosis. Moreover, the use or abuse of this inexpensive drug may contribute to the increased metronidazole resistance seen in developing countries^[33]. For this reason, metronidazole has been excluded from first-line empirical therapy plans in some countries^[23]. However, it has been reported that the results of *in vitro* resistance to this drug are also poorly correlated with the outcome of therapy, and consequently, susceptibility testing is not rou-

tinely indicated^[34]. Therefore, assessment of the drug concentration in blood samples could be recommended to assess the correlation with *in vitro* results. Another important finding from the present study was that in about 71% of the metronidazole-resistant isolates, the MICs exceeded 256 µg/mL, which has rarely been reported^[17].

In contrast to most studies, we found a high resistance (20%) to amoxicillin among *H. pylori* isolates. Most studies have shown that *H. pylori* resistance to this drug is either very rare or non-existent^[25,33,35]. Usually, the MIC of amoxicillin for *H. pylori* is very low (0.03 µg/mL); nevertheless, in our study, we found a few sensitive isolates with reduced susceptibility (MICs: < 0.016 µg/mL)^[33]. However, high resistance rates have been reported in some studies from other parts of the world: 18.5% in South Korea^[36], 19.4% in Indonesia^[37], 32.8% in India^[38], and 38% in Brazil^[39]. In a study conducted in Ile-Ife, southwest of Nigeria, 100% of the 32 isolates were resistant^[28]. It has been shown that resistance to amoxicillin could have emerged by genomic mutation in the *pbp1A* gene^[39]. When comparing different sets of data, it is important to note that variations in rates may arise due to the effects of inter-laboratory reproducibility, caused by the lack of standardized testing protocols or regional prescribing practice. This may be the reason why the resistance rates for amoxicillin in *H. pylori* isolates have been reported to be 1.6% and 27% in different studies conducted in Iran^[31,32]. Moreover, high resistance to amoxicillin observed in the present study reflects the importance of its use in our country.

Clarithromycin is a macrolide used frequently in combination with other antimicrobial agents for the treatment of *H. pylori* infection^[40]. However, resistance to clarithromycin has become one of the major reasons for treatment failure^[41]. The prevalence of *H. pylori* resistance to clarithromycin varies in different countries, and was 12% in Japan, 1.7%-23.4% in Europe and 10.6%-25% in North America^[22]. Resistance in 5% of our isolates resembles data from the Northern regions of Europe^[27]. In contrast, two other studies from Tehran, Iran reported a high rate of resistance to clarithromycin ranging from 16.7% to 21%^[31,32]. Since clarithromycin is not currently used in Iran, emerging resistance to this antibiotic is unexpected. On the other hand, it has been shown that there is cross-reactivity between clarithromycin and other macrolides such as erythromycin, which implies that resistance to one macrolide could cause the emergence of resistance to other macrolides^[42]. Genetic studies have revealed that clarithromycin resistance is often associated with point mutation of the 23S rRNA^[2].

We observed a low resistance (3%) to tetracycline among the isolates, which is consistent with most studies which have reported no resistance or low resistance to this antibiotic in *H. pylori* strains^[2,31,35,43]. In contrast, a high resistance rate (20%) to tetracycline was reported by Falsafi *et al.*^[32]. Similarly, these variations could be due to the effects of inter-laboratory reproducibility caused by the lack of standardized testing protocols or regional prescribing practice. However, resistance to tetracycline, mainly caused by mutations in the 16S rRNA gene, is

emerging and can impair the efficacy of such second-line regimens^[44]. Thus, it seems that molecular methods can help verify the exact rate of resistance to this antibiotic. Recently a novel real-time PCR has been described which is able to detect the strains carrying the mutant genes for tetracycline resistance^[44].

In conclusion, this study showed a moderate rate of primary resistance to metronidazole which is included in the guidelines for the empirical therapy of *H. pylori* infections. However, a high MIC (> 256 mg/L) observed in about 71% of the isolates is considerable. On the other hand, in the case of amoxicillin, there was an increased resistance which is worrying. In particular, it is important to determine whether the increased resistance to amoxicillin is a result of its increased use or due to the ethnic differences of the populations described herein. The data also indicate that resistance to clarithromycin and tetracycline is emerging among the *H. pylori* strains in our region. Therefore, considering the increasing resistance rate in many countries, monitoring of susceptibility of *H. pylori* to these antibiotics appears to be necessary in order to choose effective therapy to eradicate *H. pylori* infections and to optimize the regimen in case of treatment failure. Finally, taking into account the present findings along with other reported findings, continued surveillance of the resistance profiles and the resistance mechanisms present in *H. pylori* strains isolated in Iran is essential, if therapeutic plans are to satisfy the country's needs.

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COMMENTS

Background

Antibiotic resistance in *Helicobacter pylori* (*H. pylori*) is the major cause of eradication failure. Growing resistance often parallels the patterns of antibiotic consumption, and may vary within patient groups according to the geographic region, patient age and sex, type of disease, birthplace, other infections and other factors.

Research frontiers

Geographic mapping and the process of primary *H. pylori* resistance are clinically important, and should be considered when choosing eradication regimens. These should also be constantly monitored both at national and global level in an attempt to reach the recently recommended goal of eradicating the highest rate of resistance. The present study aimed to improve our understanding of Iranian regional variation in *H. pylori* antibiotic resistance rates in relation to gender and to find the best antibiotic therapy for the eradication of *H. pylori* infections.

Innovations and breakthroughs

The E-test has proven to be an accurate method of assaying the susceptibility of fastidious organisms, including *H. pylori*, to antibiotics. Using this method and improved culture conditions, we found a moderate rate of primary resistance to metronidazole which is included in the guidelines for the empirical therapy of *H. pylori* infections. However, a high minimum inhibitory concentration (MIC) (> 256 mg/L) observed in 71% of the isolates is considerable. On the other hand, in the case of amoxicillin, there was an increased resistance (20%) which is worrying. The data also indicate that resistance to clarithromycin and tetracycline is emerging among the *H. pylori* strains in our region.

Applications

Considering the increasing rate of resistance in many countries and based on the varied results from different studies, even in the same regions, the results of this study can improve the monitoring of *H. pylori* susceptibility to antibiotics, which is necessary in order to choose effective therapy to eradicate *H. pylori* infections and to optimize the regimen in case of treatment failure in our region.

Terminology

MIC is minimum inhibitory concentration. E test is the epsilometry test, a test to determine the MIC of antimicrobial agents using strips with epsilometric concentrations of antimicrobials.

Peer review

It is an interesting publication showing the prevalence of antibiotic resistance of *H. pylori* in southern part of Iran (Shiraz).

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Expression and purification of a functional human hepatitis B virus polymerase

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Abstract

AIM: To identify a method for efficient large-scale purification of functional hepatitis B virus polymerase (HBV-Pol) without addition of cellular factors.

METHODS: Full-length HBV-Pol (843 amino acids) tagged with 5' end Polyhistidine was expressed at a high level in an *Escherichia coli* (*E. coli*) system. Sodium dodecyl sulfate lysis buffer was utilized to dissolve insoluble HBV-Pol, and Ni-NTA resin affinity chromatography was utilized for HBV-Pol purification. Most recombinant HBV-Pol was eluted with 100 mmol/L imidazole in the presence of NP-40, a weak detergent that keeps HBV-Pol in solution. A reducing agent was utilized throughout the purification steps to keep soluble HBV-Pol from redundant disulfide bond formation.

RESULTS: The large-scale production of functional in-

tact human HBV-Pol was achieved in an *E. coli* expression system. Purified HBV-Pol showed stable reverse transcriptase activity and DNA polymerase activity. The purified protein was of high purity and had stable reverse transcriptase activity.

CONCLUSION: Large-scale production of HBV-Pol in pure form should facilitate crystallization and detailed analysis of the structure and mechanism of HBV-Pol. Ability of this purification approach to obtain human HBV-Pol in an enzymatically active form should be helpful for development of drugs for treatment of chronic hepatitis B.

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Key words: Hepatitis B Virus; Virus polymerase; Reverse transcriptase; Detergent

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INTRODUCTION

Hepatitis B virus (HBV) infection is a global public health problem. It is estimated that between 350 and 400 million people worldwide are chronically infected, and a significant proportion of chronic infection patients ultimately develop life-threatening liver disease such as cirrhosis, hepatocellular carcinoma (HCC) and other complications^[1]. HBV replicates *via* a reverse transcription step, using the polymerase (HBV-Pol) that is encoded by its own genome. HBV-Pol is a multifunctional protein, with protein-priming activity^[2-4],

DNA polymerase, reverse transcriptase^[2,5] and RNase H activity, but it is short of proofreading activity^[6]. Most approved medications for chronic hepatitis B (CHB) infection are nucleotide reverse transcriptase inhibitors (NRTIs) that target HBV-Pol^[7]. Although NRTIs have been used in CHB infection for several decades, their therapeutic efficacy has been limited by high frequency appearance of mutants during treatment^[8]. Therefore, a quick and easy way to obtain a large quantity of functionally intact human HBV-Pol is required for selection of sensitive CHB medications, mutated HBV strains research, and mass high-throughput screening.

It is known that expression of an enzymatically active HBV-Pol in heterologous systems, or purification of useful quantities of human HBV-Pol from virions is difficult to achieve. Due to these problems, drug development for HBV infection has not progressed satisfactorily, and biological studies of hepadnaviral polymerase have been conducted by using duck HBV^[9]. Several groups have succeeded in achieving heterologous *in vitro* expression of full-length polymerase proteins of duck HBV that exhibit DNA-dependent DNA polymerase (DDDP) activity and RNA-dependent DNA polymerase (RDDP) activity. However human HBV-Pol is expressed by *in vitro* translation with a rabbit reticulocyte lysate system^[10], and *in-vitro*-translated human HBV-Pol shows only DDDP activity and fails to show RDDP activity. RDDP activity of human HBV-Pol has been observed in *Escherichia coli* (*E. coli*) as a fusion protein in frame with maltose-binding protein^[11]. The enzymatically active HBV-Pol has also been obtained in *E. coli* by co-expression of the polymerase with the chaperone GRP94^[12]. However, the stable and large-scale heterologous expression of intact human HBV-Pol without co-expression of molecular chaperon in common hosts such as *E. coli* or yeast has not been reported.

In this study, a full-length HBV-Pol with a 6 × His tag was expressed in *E. coli*. HBV-Pol is a large molecule with approximately 2.5% cysteine residues^[2], therefore, the protein is expected to be present as inclusion bodies. For this reason, the total lysate was dissolved by applying high concentration of sodium dodecyl sulfate (SDS) and reducing agents to dissolve inclusion bodies, and then SDS was replaced by weak detergent for renaturation during washing. Finally, the target protein was purified with nickel-based chromatography. Purified HBV-Pol showed RDDP and DDDP activity. This is believed to be the first time that functional intact human HBV-Pol has been expressed in *E. coli* without co-expression molecules or in the presence of certain helper chaperons. The functional HBV-Pol might be helpful for development of potential pharmaceutical agents for CHB treatment.

MATERIALS AND METHODS

Plasmid construction

Liver tissues were obtained from a chronic hepatitis B surface antigen carrier who developed HCC and underwent surgical resection. The tumor tissues were dissected and immediately cut into small pieces and stored into liquid nitrogen until use. The cellular DNA was isolated

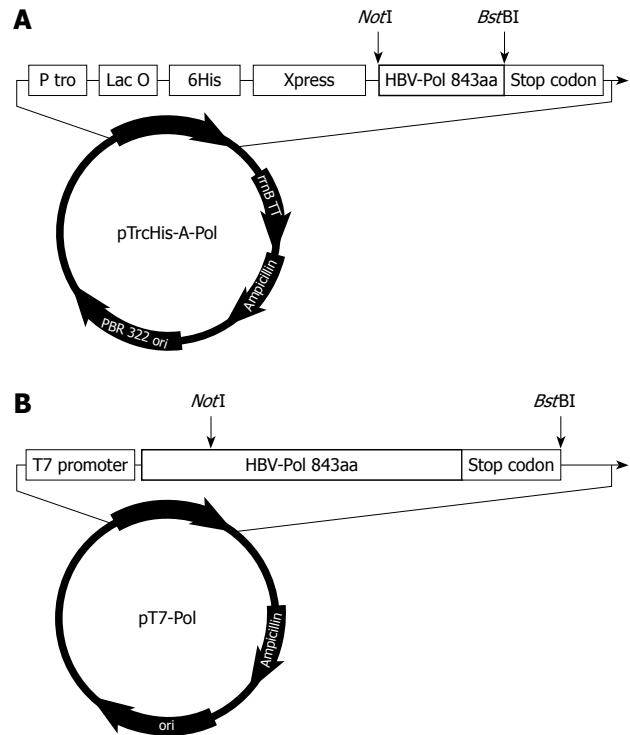


Figure 1 Organization of pTrcHis-A-Pol and pT7-Pol recombinant plasmids. A: Structural arrangement of pTrcHis-A-Pol. A full-length hepatitis B virus polymerase (HBV-Pol) sequence was fused into pTrcHis-A between *NotI* and *BstBI* sites under the control of Trc promoter and Lac operator. This polyhistidine tag plays a role in rapid purification using a nickel-based resin. To determine the expression level under different conditions, an Xpress antigen was fused to the 5'-end of the HBV-Pol sequence; B: Structure of pT7-Pol. A full-length HBV-Pol sequence was fused to P-T7 between *NotI* and *BstBI* sites. Full-length HBV-Pol sequence was under the control of the T7 promoter for expression in the TNT T7 transcription-translation-coupled rabbit reticulocyte lysate expression system.

from tissues by SDS-protease K digestion and phenol-chloroform extraction as described previously^[13]. HBV-Pol sequence [spanning 2307 to 1623 bp, 843 amino acids] were amplified by PrimeSTARTM high fidelity polymerase using CPNotIF01: GTTGC GCGCCGCATA-ATGGCCCTATCTTATC and CPBstBIR01: ATTTTC-GAATTCTCACGGTGGTTTCCA for complete P gene. The vectors were designed as follows (Figure 1).

The pTrcHis-A-Pol and pT7-Pol were constructed by the following procedure. HBV-Pol full-length sequence was cloned into *NotI* and *BstBI* sites of the two expression vector pTrcHis-A (Invitrogen, Carlsbad, CA, USA) and pT7 vector (Promega, Madison, WI, USA). For expression of HBV-Pol in *E. coli*, the plasmid pTrcHis-A-Pol was constructed as showed in Figure 1A. The HBV-Pol frame was under the control of the Trc promoter and Lac operator. His-tag and Xpress antigen were fused at the 5' end of the HBV-Pol sequence. For expression of HBV-Pol in the rabbit reticulocyte lysate system, the plasmid pT7-Pol was constructed as showed in Figure 1B. The HBV-Pol frame was driven by T7-promoter.

E. coli transformation

Plasmid pTrcHis-A-Pol was chemically transformed in competent DH5α (*F*⁻, ϕ 80*dlacZ*Δ*M15*, Δ(*lacZYA*-

argF)U169,*deoR*,*recA1*,*endA1*,*hsdR17*(*rk⁻*,*mk⁺*),*phoA*,*supE44*,*λ*,*thi-1*,*gyrA96*,*relA1*) cells following the manual supplied by Real Biotech Corporation (Taipei, Taiwan, China). Isopropylthiogalactopyranoside (IPTG, Sigma, St Louis, MO, USA) was added into 5 mL overnight-cultured *E. coli*, to a final concentration of 1 mmol/L, and incubated for 4, 8, 12 and 16 h, respectively, at 18°C, 24°C, 30°C, 37°C and 42°C, as indicated. Cells were harvested by centrifugation and disrupted with lysis-buffer (50 mmol/L phosphate buffer, pH 8.0; 0.5 mmol/L NaCl; 1% SDS; 10 mmol/L imidazole; 0.1 mg/mL lysozyme; 20 mmol/L 2-mercaptoethanol; 1 × Roche protein inhibitor cocktail). Protein expression level was determined with dot blot using anti-Xpress antibody (Invitrogen).

Expression and purification of histidine-tagged HBV-Pol

Transformed cells were grown in 100 mL LB broth until the culture reached OD₆₀₀ 0.6-0.8. IPTG was added to a final concentration of 1 mmol/L and incubated for 8-10 h at 18°C with shaking. Induced cells were harvested by centrifugation at 2000 *g* for 20 min at 4°C. The ratio of lysis-buffer volume and wet weight of cell pellet was about 4 to 1. The lysate was incubated at room temperature for 30 min and sonicated for 10 × 10 s. The samples were cooled on ice for 5-10 s between each sonication. The suspension was centrifuged at 20000 *g* for 30 min at room temperature. The supernatant was transferred to an Ni-NTA resin (Invitrogen) column (bed volume: 2 mL), which had been equilibrated with 16 mL equilibration buffer (same components of previous lysis buffer without lysozyme). Resin and lysate supernatant were mixed thoroughly but gently for 45-60 min at room temperature. The resin was washed with 8 mL washing buffer (50 mmol/L phosphate buffer, pH 8.0, 0.5 mmol/L NaCl, 1% NP-40, 10 mmol/L imidazole, 20 mmol/L 2-mercaptoethanol, and 1 × Roche protein inhibitor cocktail) for 6-8 times. The HBV-Pol fractions were eluted with 6 mL E-50, E-75, and E-100 Elution buffer (50 mmol/L phosphate buffer, pH 8.0, 0.5 mmol/L NaCl, 1% NP-40, 1 × Roche protein inhibitor cocktail), and dithiothreitol (DTT) was added to the harvest tube to achieve a final concentration of 5 mmol/L. The concentration of imidazole in E-50, E-75, and E-100 buffer was 50 mmol/L, 75 mmol/L and 100 mmol/L, respectively. HBV-Pol elution fractions were harvested and stored at -70°C. The concentration of purified protein samples was determined using a BCA assay with bovine serum albumin as a standard.

Anti-reverse transcriptase polyclonal antibody preparation

Anti-reverse transcriptase (RT) polyclonal antibody was prepared before full-length polymerase purification (data not shown). A recombinant RT domain (spanning 304-693 amino acids) was purified from *E. coli*. Fifty micrograms purified RT and Freund's complete adjuvant were injected subcutaneously into C57BL/6 mice (Macrogen, Seoul, South Korea). Another 50 µg purified RT and Freund's incomplete adjuvant were injected 2 wk after the first injection. The mice were sacrificed 2 wk after the sec-

ond injection for serum extraction of anti-RT polyclonal antibody. The antibody titers against the RT peptides, monitored with ELISA (Sigma), were > 1:32000.

SDS-PAGE, dot blot and Western blotting analysis

Total lysate and other washing/elution fractions were heated at 95°C for 5 min for denaturation. Proteins were separated by SDS-PAGE. Gels were stained with Coomassie Blue. For dot blot analysis, 1 µL of each sample was dropped on nitrocellulose membranes (Amersham, Bucks, UK), and the membranes were treated first with a 1/4000 dilution of anti-Xpress antibody and then goat-anti-mouse conjugated alkaline phosphatase (AP; Invitrogen). For Western blotting analysis, proteins were electrophoretically transferred to a polyvinylidene difluoride blotting membrane (Amersham, Piscataway, NY, USA), and membranes were treated first with a 1/4000 dilution of anti-RT antibody and then goat-anti-mouse conjugated AP.

Expression of HBV-Pol in *in vitro* transcription and translation system

The recombinant plasmid pT7-Pol was purified with Qia-gen Midiprep DNA purification kit. *In vitro* transcription and translation reactions were performed using the TNT T7 coupled reticulocyte lysate system (Promega). Two micrograms of the plasmid DNA template was transcribed and the protein was translated in each 50-mL reaction in the presence or absence of 40 mCi of [³⁵S]-methionine (1000 Ci/mmol) (Amersham) at 30°C for 75 min^[10]. The *in vitro* translation reaction was stopped by the addition of 0.1 mg/mL cycloheximide for the polymerase activity assay, or SDS sample buffer for checking the efficiency of translation. The *in vitro* translated proteins were separated by 4%-12% SDS-PAGE and dried prior to autoradiography.

DNA polymerase activity and RT activity assays

DDDP and RT/RDDP were monitored by synthesis of DNA using poly (dA) • oligo (dT)₁₂₋₁₈ and poly (rA) • oligo (dT)₁₂₋₁₈ as template primer (Amersham Biosciences), respectively. The standard enzyme reaction (50 mL) contained 50 mmol/L Tris-HCl pH 7.4, 50 mmol/L KCl, 10 mmol/L MgCl₂, 1 mmol/L DTT, 0.01% Nonidet P-40, 50 ng homopolymer template [poly(dA) • oligo(dT)₁₂₋₁₈ for DDDP activity assay and poly(rA) • oligo(dT)₁₂₋₁₈ for RDDP activity assay], and 2 mCi of [α-³²P]dTTP (3000 Ci/mmol), (PerkinElmer, USA). For RDDP activity assay, RNase inhibitor and RNase-free water were employed in the reaction^[10,11]. Reactions were started by the addition of 0.5 mg of the purified HBV-Pol or 5 µL products from the TNT-T7 coupled reticulocyte lysate system into the reaction buffer. The endogenous DNA polymerase activity from the reticulocyte lysate was suppressed by the addition of 60 mmol/L aphidicolin and 1 mmol/L NEM. After incubation at 37°C for 75 min, reactions were stopped by the addition of 0.2 mg/mL protease K in the presence of 0.5% SDS. Incubation was continued for another 20 min, followed by spotting on Whatman DE81 filter paper. Filters were washed three times with 0.5 mol/L Na₂HPO₄, and once with distilled

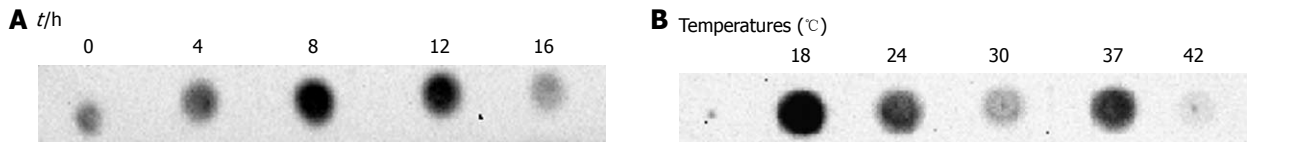


Figure 2 Induction optimization of hepatitis B virus polymerase expressed in *Escherichia coli*. Total lysate samples of *Escherichia coli* transformants after different induction times were blotted on to nitrocellulose membranes. Hepatitis B virus polymerase (HBV-Pol) expression level was determined by anti-Xpress antibody. A: 8-h induction sample showed the strongest signal; B: HBV-Pol expression level at different temperatures was determined by anti-Xpress. Sample from 18 °C showed the highest expression level. Preliminary expression data showed that no obvious signal was detected in samples induced at temperatures < 18 °C, therefore, previous values for duration and temperature for induction were selected for this study.

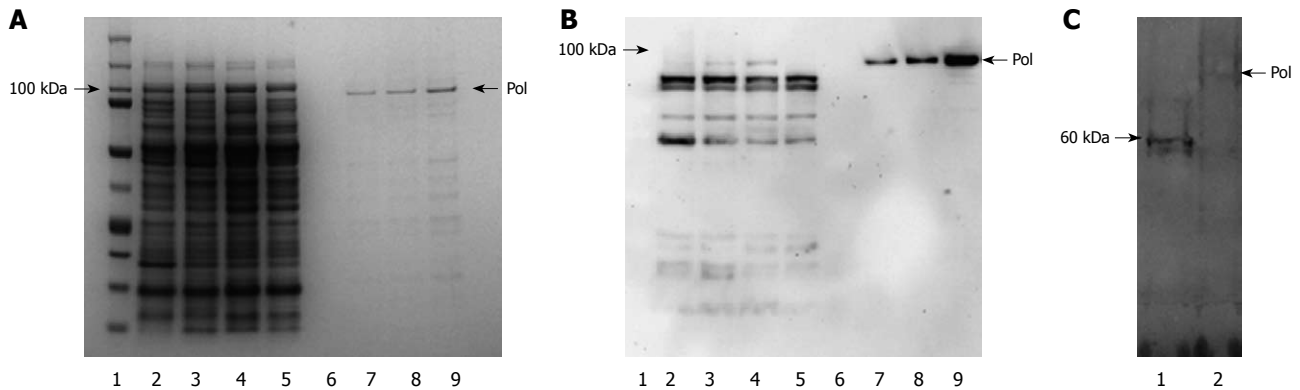


Figure 3 Expression and purification of hepatitis B virus polymerase. Recombinant human hepatitis B virus polymerase (HBV-Pol) was produced in *Escherichia coli* (*E. coli*) cells transformed by pTrcHis-A-Pol and purified with nickel-based resin. Protein samples were analyzed by 4%-12% sodium dodecyl sulfate (SDS)-PAGE, and gels were stained with Coomassie Blue (A). HBV-Pol bands were located at the expected molecular mass, approximate 98 kDa (lane 1). Although HBV-Pol bands were also detected in 50 mmol/L (lane 7) and 75 mmol/L (lane 8) imidazole washing fractions, most HBV-Pol was eluted by 100 mmol/L imidazole (lane 9). Purified recombinant human HBV-Pol was also analyzed by immunoblotting with an anti-reverse transcriptase (RT) antibody (B). HBV-Pol bands were detected in total lysate of uninduced transformant (lane 3) and total lysate of induced transformant (lane 4), but not in untransformed *E. coli* (lane 2), unbound fraction (lane 5) and fifth washing fraction with 10 mmol/L imidazole (lane 6). Some positive small bands were also detected in lanes 2-5, which was mainly because some components from total lysate of *E. coli* were recognized by anti-RT polyclonal antibody. Expression of human HBV-Pol in TNT T7 transcription-translation-coupled rabbit reticulocyte lysate expression system is shown in C. PT7-pol and pT7-luciferase (1650 bp, ORF size approximate 61 kDa) were added to *in vitro* transcription-translation reactions for 75 min at 30 °C in the presence of [³⁵S]-methionine. Five microliters of each sample was incubated in SDS sample buffer at 95 °C for 3 min, and electrophoresed through 4%-12% SDS-PAGE. A band of pT7-luciferase (lane 1) and pT7-pol (lane 2) were detected by autoradiography.

H₂O^[11]. Incorporation of radioactivity was determined by liquid scintillation counting in a Packard Tri-Carb Series 2300 liquid scintillation counter.

RESULTS

Expression, induction optimization and purification of HBV-Pol in *E. coli*

A high expression level of target protein is crucial to obtain an ideal elution result in affinity chromatography. To achieve the highest expression level, expression level was examined at various conditions. Recombinant HBV-Pol is a large molecule of 95 kDa full-length HBV-Pol plus a 5-kDa fused tag, and a low induction temperature and short or long induction time were considered for expression. It is known that heterologous protein was stabilized at low temperature^[14] and formation of inclusion bodies was restrained^[15]. Decreasing the temperature of induction medium should lower the rate of association of folding intermediates, which allows unfolded proteins and partially folded proteins to have more time to refold into native, soluble tertiary structures. Appropriate induction time is another important factor to obtain sufficient recombinant polymerase. The quantity of expressed protein varies with

different induction times.

HBV-Pol level was monitored in the total lysate of *E. coli* transformed with HBV-Pol recombinant plasmid, pTrcHis-A-Pol (Figure 2). HBV-Pol expression level was the highest at 8 h induction compared to shorter or longer incubation (Figure 2A). In terms of temperature, the lowest temperature tested, 18 °C, showed the highest level of expression compared to other samples expressed at higher temperatures (Figure 2B). Based on these data, incubation of HBV-Pol expression was carried out under these conditions throughout the experiment. For HBV-Pol purification, a denaturing-renaturing purification protocol was applied. According to previous lysis data under native conditions, inclusion bodies do not dissolve in the presence of weak detergent at 4 °C, which decreased the quantity of target protein in the binding step. A strong detergent (SDS) was employed in the lysis step to dissolve overexpressed HBV-Pol in inclusion bodies, and to denature some protease present in the total lysate.

HBV-Pol fractions were eluted with elution buffers (50, 75, 100 mmol/mL imidazole). Figure 3A shows that a prominent HBV-Pol band was detected with SDS-PAGE, and analysis by Western blotting confirmed the purification results (Figure 3B). DTT, a stronger reducing agent

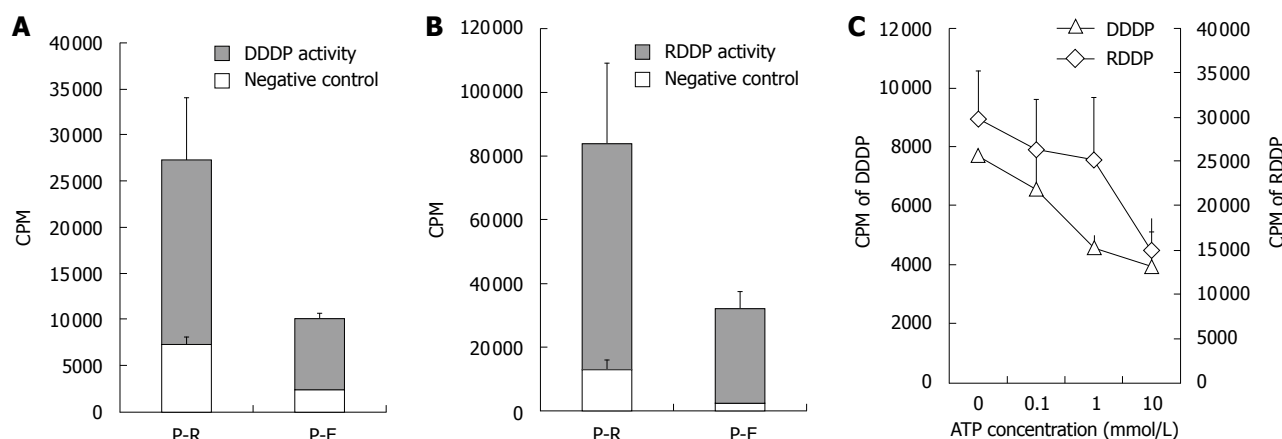


Figure 4 Enzyme activity assay of recombinant hepatitis B virus polymerase. Comparison of enzyme activities between hepatitis B virus polymerase (HBV-Pol) from *Escherichia coli* expression system (P-E) and *in vitro* expression system (P-R). DNA-dependent DNA polymerase (DDDP) activity and RNA-dependent DNA polymerase (RDDP) activity of the P-E and P-R were monitored under standard conditions. All endogenous DDDP activities were suppressed by 60 mmol/L aphidicolin and 1 mmol/L NEM (A and B). The effects of increasing concentrations of adenosine triphosphate (ATP) are shown in C. DDDP activity using poly (dA) • oligo (dT)₁₂₋₁₈ and RDDP activity using poly (rA) • oligo (dT)₁₂₋₁₈ were used as the substrate for DDDP activity and RDDP activity assays, respectively. CPM: Counts per minute.

than 2-mercaptoethanol, is suitable for long-term protein storage and is used in many experiments for HBV-Pol purification at a high concentration^[2,5,6,16]. Although nickel resin is easily reduced by strong detergent, in the present study, 2-mercaptoethanol was employed in all steps except elution. DTT was finally added into HBV-Pol fractions for long-term storage. SDS-PAGE and Western blotting clearly showed high efficiency of purification. Involvement of the denaturing-renaturing purification process also suggested that purified recombinant HBV-Pol is a stable, single-component product. HBV-Pol was purified up to 5 mg/mL.

There have been many reports about purified or partially purified active HBV-Pol in eukaryotic systems. In the present study, recombinant HBV-Pol was also expressed in a reticulocyte lysate system as a positive control in the activity assay. The protein products from *in vitro* expression labeled with [³⁵S]-methionine are shown in Figure 3C. Approximately 95-kDa HBV-Pol protein was detected as predicted from the nucleotide sequence of the HBV-Pol-ORF.

Characterization of the purified HBV-Pol

To measure the enzymatic activity of purified HBV-Pol, the enzymatic activity assays for DDDP and RDDP were performed by using purified HBV-Pol (expressed in recombinant *E. coli*, named P-E) and HBV-Pol (expressed in reticulocyte system, named P-R). pT7 plasmid was expressed in the reticulocyte lysate system as a negative control for P-R enzyme activity assays. The solvent of P-E was regarded as a negative control for P-E enzyme activity assays. Enzyme activities of P-R and P-E were compared (Figure 4A-C). P-R showed medium DDDP activity and much higher RDDP activity under these reaction conditions. Homopolymer primer-template has been used for many groups^[10,11] in HBV-Pol activity assays. Activity assay results showed that P-E and P-R have high RDDP activity and relatively weak DDDP activity compared to the negative controls. This pattern had been observed previously with partially purified HBV-Pol^[9].

DDDP and RDDP activities affected with adenosine triphosphate

It has been shown that adenosine triphosphate (ATP) participates in HBV-Pol activation during duck HBV replication^[17]. To study the effect of ATP on purified HBV-Pol, different concentrations of ATP were added to the enzyme activity assays. As shown in Figure 4B, RDDP and DDDP activities of P-R were affected by ATP concentration. In the presence of ATP (0.1, 1 and 10 mmol/L), RDDP and DDDP activities of P-R showed a declining trend in a dose-dependent manner.

DISCUSSION

Human HBV-Pol is crucial for HBV genome DNA replication. It shows RDDP activity in minus strand DNA synthesis and DDDP activity in plus strand synthesis. Among six medications approved by the United States Food and Drug Administration for the treatment of CHB, lamivudine, adefovir dipivoxil, entecavir and telbivudine are HBV-Pol inhibitors^[7]. Blocking HBV DNA replication is still the main target for CHB therapy. However, stable and large-scale heterologous expression of intact human HBV-Pol in common hosts such as *E. coli* or yeast has not been successfully achieved^[9]. Although recombinant HBV-Pol has been expressed in different systems and purified in various ways, obtaining high-purity functional HBV-Pol without coexpression of a chaperon is still problematic^[2,5,9,12]. Therefore, we developed a reliable purification method for further investigation of HBV-Pol activities.

The major obstacle for a large multi-functional protein such as HBV-Pol is its instability in heterologous expression systems and during purification^[9]. It is known that inclusion bodies are formed during expression of human HBV-Pol, not only in *E. coli* expression systems, but also in eukaryotic expression systems^[2]. In accordance with this, the target protein was present in total lysate and pellet rather than supernatant in detergent-free lysis buffer. Our preliminary study showed that expressed human HBV-Pol was degraded easily

under native purification conditions, even with the presence of protease inhibitor. Choi *et al.*^[9] also have observed this phenomenon in a yeast expression system. Therefore, the key points of successful large-scale purification of human HBV-Pol are the solubilization and stabilization of the target protein. So far, nobody has reported a high level of expression and purification of HBV-Pol by using an *E. coli* expression system, in which we purified the protein up to 5 mg/mL. This is far higher than the earlier studies done by Choi *et al.*^[9], who used a *Pichia methanolica* expression system and Qadri *et al.*^[5] who used *Saccharomyces cerevisiae*.

In this study, we tried to achieve a high level of recombinant HBV-Pol with application of the following strategies. First, an amino-terminal 6 × His tag was used to facilitate rapid purification. Also, a sequence that encoded the Xpress epitope was fused to the N terminus of HBV-Pol so that it could be recognized rapidly by anti-Xpress primary antibody. Second, induction temperatures were lowered to maximize target protein expression. Third, for cell lysate purification, strong detergent lysis was followed by weak detergent washing and elution. Fourth, a high-concentration reducing agent was present during all the purification steps to keep HBV-Pol in soluble form by inhibiting disulfide bond formation, because approximately 2.5% of amino acid residues of HBV-Pol are cysteine.

It has been reported that structural and biochemical investigations of HBV-Pol have been complicated by the requirement of cellular factors such as HSP90^[18], HSP60^[3] and other cofactors^[19] during purification. However, we developed a new method for large-scale purification of fully active HBV-Pol without addition of cellular factors by adjustment of salts during the purification steps. A binding step under denaturation conditions was used to isolate target protein from total lysate. With the presence of 1% SDS in the lysate, insoluble human HBV-Pol was dissolved rapidly. Proteases released from *E. coli* were also inactive in this SDS denaturing step. Soluble HBV-Pol was washed and eluted from nickel resin using the buffer in the presence of weak detergent and reducing agent. SDS was replaced by NP-40 during washing and elution. To prevent target protein from oxidization and to keep cysteine residues in a reduced state, a high concentration of reducing agents was employed throughout the purification steps.

The large-scale production of functionally intact human HBV-Pol was achieved in the *E. coli* expression system in this study. The availability of this recombinant protein in pure form should facilitate the crystallization and detailed analysis of the structure and mechanism of HBV-Pol. The availability of a large quantity of functional human HBV-Pol will help in high-throughput screening assays for development of potential pharmaceutical agents for CHB treatment.

COMMENTS

Background

Hepatitis B virus polymerase (HBV-Pol) is a multifunctional protein, which has intrinsic RNA-dependent reverse transcriptase (RT), DNA-dependent DNA poly-

merase, and RNase H activity. HBV-Pol is limited by difficulties in expressing and purifying the proteins in a heterologous system. This is believed to be the first time that functionally intact human HBV-Pol was expressed in *Escherichia coli* (*E. coli*) without co-expression of other molecules or in the presence of certain helper chaperons. Purified HBV-Pol showed stable RT activity and DNA polymerase activity. Functional HBV-Pol might be helpful for development of potential pharmaceutical agents for chronic hepatitis B (CHB) treatment.

Research frontiers

HBV is a small DNA virus that replicates by reverse transcription of pre-genomic RNA, and is a major threat to human health. Most approved medications for CHB infection are nucleotide reverse transcriptase inhibitors that target HBV-Pol. Analysis of HBV-Pol has been hampered by the inability to express the functional enzyme in a recombinant system. For this reason, we applied various strategies to the production of functionally intact human HBV-Pol on a large scale by using an *E. coli* expression system.

Innovations and breakthroughs

This is believed to be the first time that functionally intact human HBV-Pol has been expressed in *E. coli* without co-expression of other molecules or in the presence of certain helper chaperons. To achieve high levels of recombinant HBV-Pol, an N-terminal 6×His tag and Xpress epitope were fused to the N terminus of HBV-Pol. Second, the induction temperature was lowered to maximize target protein expression, and a high concentration of reducing agent was present during all the purification steps to keep HBV-Pol in a soluble form. The availability of this recombinant protein in pure form should facilitate the crystallization and detailed analysis of the structure and mechanism of HBV-Pol.

Applications

The availability of a large quantity of functional human HBV-Pol can be helpful in high-throughput screening assay for potential pharmaceutical agents for CHB treatment.

Peer review

This is a well-written paper and documents the expression and purification of full-length HBV-Pol in an *E. coli* system with promising results that could be the basis of forthcoming research on potential pharmaceutical agents for CHB treatment.

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Protective effects of *Lactobacillus plantarum* against epithelial barrier dysfunction of human colon cell line NCM460

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monolayers against the disruption caused by enteropathogenic *Escherichia coli* (*E. coli*) or enteroinvasive *E. coli*. *L. plantarum* also prevented the decrease in the expression of TJ proteins and F-actin in NCM460 cells.

CONCLUSION: *L. plantarum* can protect against dysfunction of NCM460 intestinal epithelial barrier caused by enteropathogenic *E. coli* or enteroinvasive *E. coli*, and thus can be a potential candidate of therapeutic agents for the treatment of intestinal diseases.

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Key words: *Lactobacillus plantarum*; NCM460; Tight junction; Intestinal barrier

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Abstract

AIM: To investigate the effects of *Lactobacillus plantarum* (*L. plantarum*) in the intestinal permeability and expression of tight junction (TJ) using the normal human colon cell line NCM460.

METHODS: Paracellular permeability of NCM460 monolayers was determined by transepithelial electrical resistance and dextran permeability. Expression of TJ proteins in NCM460 cell monolayers was detected by Western blotting and quantitative real-time polymerase chain reaction.

RESULTS: *L. plantarum* played an important role in increasing transepithelial electrical resistance and decreasing the permeability to macromolecules of NCM460

INTRODUCTION

The human intestinal system included a group of viable microorganisms, which exceed the total number of somatic and germ cells^[1-3]. Therefore, the human colon is confronted with the highest bacterial load in the digestive tract with enormous bacteria per gram of feces. Growing evidence showed that bacteria closely adherent to the mucosa are more relevant to human body, compared with those evacuated in the feces^[4,5]. There is a homeostasis between probiotics and pathogens in the intestinal

systems of healthy individuals^[6,7], which, if broken, may lead to an imbalanced ecological microenvironment and subsequent intestinal barrier dysfunction^[8,9]. Thereafter, the accumulation of pathogens and secretory products, such as the exotoxins and secretory antigens, can also directly or indirectly initiate and amplify the local and systemic inflammatory responses^[10,11]. Probiotics of the genus *Lactobacillus* that reside in the human intestine play an important part in maintaining the homeostasis of gut flora by adhering and colonizing to the intestinal mucosa and competing with pathogenic bacteria, such as pathogenic *Escherichia coli* (*E. coli*)^[12,13]. Enteric diseases with flora disequilibrium have been treated with *Lactobacillus* over the past decades^[14-16]. There is evidence indicating that the modulation of the gut flora by *Lactobacillus* can improve the intestinal epithelial barrier function^[17].

Adhesion of *Lactobacillus* to the intestinal epithelium initially involves the activation of specific binding between bacterial ligands and their corresponding surface receptors on the intestinal cells of the host, following the non-specific physical interactions^[18,19]. Generally, these ligands are adhesive molecules either existing on the surface layer of the bacteria or secreting from the mycelium of the bacteria. Furthermore, these ligands could interact with the corresponding receptors on the surface of the intestinal epithelial cells. Thereafter, these adhesins activate specific signal transduction pathways in both the bacteria and host cells. The interaction between *Lactobacillus* and the intestinal epithelial cells can also block the adhesion of other pathogenic bacteria to the receptors of the intestinal epithelial cells, such as enteropathogenic *E. coli* (EPEC) and enteroinvasive *E. coli* (EIEC). As a widespread member of the genus *Lactobacillus*, *Lactobacillus plantarum* (*L. plantarum*) is commonly found in many fermented food and anaerobic plant products. Our previous studies demonstrated that *L. plantarum* was able to prevent colonic damage caused by EIEC or inflammation *in vitro*, *in vivo* and in patients with acute pancreatitis^[20-24]. The normal human colon cell line NCM460, which is derived from the normal human colon mucosal epithelium and expresses colonic epithelial cell-associated antigens such as cytokeratins and villin, has been applied exclusively in various intestinal research areas, including the infectious diseases^[25-27].

Our previous studies indicated that *L. plantarum* exerted its therapeutic effects by adhering to the intestinal epithelial cells, restoring tight junction (TJ) structure and function, and reducing paracellular permeability. However, studies about the interaction between *Lactobacillus* and the human intestine were limited in the cancer cell line and the animal models, and further researches based on the normal human intestinal cells are still needed. Therefore, our study aims to investigate the protective effects of *L. plantarum* against epithelial barrier dysfunction of the normal human colon cell line NCM460 caused by EIEC and EPEC.

MATERIALS AND METHODS

Bacterial strains and culture conditions

L. plantarum CGMCC 1258 (generously provided by Dr.

Xiao-Min Hang, the Onlly Institute of Life Science, Shanghai Jiao Tong University, Shanghai, China) was inoculated in 5% fresh De Man, Rogosa and Sharpe broth at 37°C for 24 h, harvested by centrifugation (3500 × *g*) at 4°C for 20 min, and washed with 50 mL 0.01 mol/L phosphate buffered saline (PBS) (pH 7.4). The EIEC strain ATCC 43893 (O124:NM) and EPEC strain ATCC 43887 (O111:NM) (both from Shanghai Municipal Center for Disease Control and Prevention, Shanghai, China) were grown in static Dulbecco's modified eagle media (DMEM) at 37°C for 24 h. Quantification of bacterial density was measured at 600 nm (Beckman DU-50 spectrophotometer) with the colony forming units.

NCM460 cells were purchased from INCELL Corporation (San Antonio, TX, USA) and cultured in M3 media supplemented with 10% FBS, 100 U/mL penicillin and 100 g/mL streptomycin at 37°C in a 95% humidified atmosphere with 5% CO₂, as previously described^[25].

Measurement of transepithelial electrical resistance in NCM460 cell monolayers

NCM460 cells were grown on filters (Millicell culture plate inserts; 0.4 µm pore size; 0.6 cm² surface area) at 37°C in a 95% humidified atmosphere, with 5% CO₂. At full confluence (10-14 d) (i.e. a monolayer was formed), a transepithelial electrical resistance (TER) of > 450 Ω·cm² monolayer was achieved as measured using a voltmeter (Millicell-ERS; Millipore, MA, USA). The intestinal epithelial monolayers were treated with EIEC or EPEC in the presence or absence of *L. plantarum*. In infection groups, 100 µL EIEC ATCC43893 (O124:NM) and EPEC ATCC43887 (O111:NM) at 1.0 × 10⁸/mL were, respectively, added to the apical side of the cell culture insert for rapid infection of the monolayer, with an inoculation ratio of EIEC/EPEC to NCM460 cells of 100:1, and the insert was placed in a 50-mL tube and centrifuged at 200 × *g* for 4 min. In *L. plantarum* groups, *L. plantarum* (100 µL of 1.0 × 10⁸/mL) was added onto the monolayer of NCM460 cells simultaneously with the EIEC/EPEC infection. NCM460 cells cultured under the same conditions but without the infection of EIEC/EPEC, and addition of *L. plantarum* served as the control group. Two experiments were performed separately for EIEC and EPEC.

The integrity of the confluent polarized monolayers was verified by measuring TER at different time intervals. TER (Ω·cm² monolayer) = (Total resistance - Blank resistance) (Ω) × Area (cm² monolayer). Because TER values often vary among individual NCM460 cultures, the electrical resistance value was recorded for each monolayer before and after the treatment, and the percentage in the decrease of TER from the baseline (%TER) was calculated.

Determination of dextran permeability in NCM460 monolayers

DMEM (0.2 mL) containing conjugated dextran was added to the apical compartment of Transwells (Corning Costar Co., MA, USA), and 0.4 mL DMEM alone added to the basolateral compartment. After treatment as described above, samples (0.5 mL) collected from the basolateral

Table 1 Primers used for real-time polymerase chain reaction amplification for genes encoding tight junction proteins in NCM460 cells

Gene	Upstream primer	Downstream primer
Occluding	GCAGCTACTGGACTCTACG	ATGGGACTGTCAACTCTTTC
Claudin-1	GTGCCTTGATGGTGGTTG	TGTTGGGTAAGAGGTTGT
JAM-1	GATGTGCCTGTGGTGCTG	GCTCTGCCTTGAGATAAGAA
ZO-1	AAGAGTGAACACGAGAC	TCCGTGCTATACATTGAG

compartment were placed into a 96-well plate (Corning Costar Co., MA, USA) and analyzed to determine their fluorescent intensity using the Odyssey infrared imaging system (LI-COR Biosciences, NE, USA) at a wavelength of 700 nm. Relative intensity (RI; the integrated intensities of treated samples relative to the integrated intensity of untreated samples) was calculated to indicate the effect of the treatment.

Western blotting for determining the distribution and expression of TJ proteins in NCM460 cell monolayers

For Western blotting, NCM460 cells were cultured and the monolayers were treated as described above, and the protein samples from NCM460 cells was prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the previous studies^[20]. SDS-PAGE was performed using the standard laboratory techniques with a discontinuous gradient, 5% (w/v) stacking gel and a 10% (w/v) separating gel, in a Mini-PROTEAN II (Bio-Rad Laboratories, CA, USA). Briefly, samples were mixed with loading buffer containing SDS and mercaptoethanol, boiled for 3 min, centrifuged, and loaded onto the SDS-PAGE gel for separation. Molecular weights of samples were determined by comparing mobility with known marker proteins. Gel was then transferred to PVDF membrane (Millipore, MA, USA) in a semidry electroblotter (Bio-Rad Laboratories) for 120 min at 100 V. The membrane was washed three times (20 min each) with PBS containing 0.1% Tween-20 (PBS-T buffer). After blocking overnight in Tris-buffered saline containing 0.05% Tween-20 (TBS-T) and 5% dry powdered milk, membranes were washed three times for 5 min each with TBS-T and incubated with corresponding primary antibodies against TJ proteins (claudin-1, occludin, JAM-1, and ZO-1) and a cell cytoskeleton element F-actin (all from Abcam, MA, USA) for 2 h at room temperature. After three washes with TBS-T, the membranes were incubated for 1 h with corresponding HRP-conjugated secondary antibodies. The membrane was washed three times (60 min each) with PBS-T buffer. The TJ proteins were tested using enhanced chemiluminescence (ECL kit; Pierce, IL, USA) according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction for detecting the mRNA expression of TJ proteins in NCM460 cells

mRNA expression of TJ proteins, including claudin-1, occludin, JAM-1, and ZO-1, was determined by quantitative real-time polymerase chain reaction (RT-PCR). After the treatment as described above, total RNA was isolated

from NCM460 cells using the Trizol reagent (Gibco Brl, USA)^[28], followed by DNase I treatment. The quantity and quality of RNA were verified with the ratio of absorbance values at 260 and 280 nm, and by visualization of the bands on agarose gels. For each sample, 600 ng mRNA was used in reverse transcription reaction (iScript kit from BioRad Laboratories) according to the manufacturer's specifications. Further analysis of mRNA levels of each group was performed by RT-PCR with a light-cycling system (LightCycler; Roche Diagnostics GmbH, Mannheim, Germany). Sequences of the primers used are listed in Table 1. The mRNA expression level was described as the ratio of the mean reading of the experimental group over that of the control group for NCM460 cells.

RESULTS

***L. plantarum* prevented EIEC/EPEC-induced decrease of TER in NCM460 cells**

TER in the NCM460 cell monolayers was decreased significantly in response to infection with EIEC/EPEC compared with uninfected control cells. However, decrease of TER induced by EIEC/EPEC was prevented by the simultaneous treatment of *L. plantarum* (Figure 1A and B).

***L. plantarum* inhibited increased macromolecular permeability of NCM460 cell monolayers in response to EIEC/EPEC**

EIEC/EPEC had an obvious enhancing effect on permeability of NCM460 cell monolayers, as compared with the uninfected control cells. However, this effect was inhibited by the co-treatment of *L. plantarum* (Figure 2A and B).

***L. plantarum* prevented the decreased expression of TJ proteins and a cell cytoskeleton element F-actin detected by Western blotting**

The expression of TJ proteins, including claudin-1, occludin, JAM-1 and ZO-1, and the cytoskeleton element F-actin, was decreased in NCM460 cells infected with EIEC or EPEC (Figure 3A and B) compared with the control cells, as detected by Western blotting of epithelial whole cell protein extracts of NCM460 cells ($P < 0.001$). However, after the pre-treatment of *L. plantarum*, the expression of TJ proteins and F-actin remained at similar levels to the control cells.

***L. plantarum* prevented the decreased expression of TJ proteins as detected by quantitative RT-PCR**

mRNA expression of TJ proteins, including claudin-1, oc-

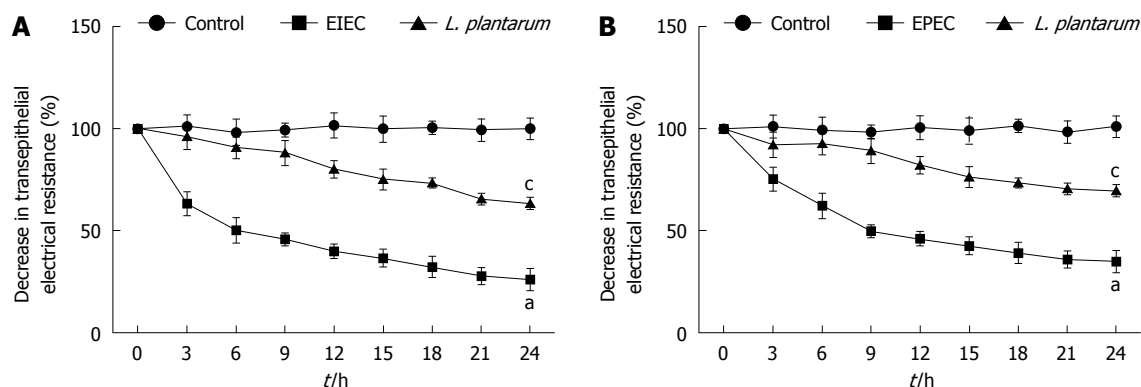


Figure 1 *Lactobacillus plantarum* inhibited the decreased transepithelial electrical resistance through NCM460 cells induced by enteroinvasive *Escherichia coli* (A) or enteropathogenic *Escherichia coli* (B). A: After infection with enteroinvasive *Escherichia coli* (EIEC) for 24 h, transepithelial electrical resistance (TER) of NCM460 monolayers was decreased significantly compared with the value in the control group. However, TER of EIEC-infected NCM460 monolayers, when simultaneously co-incubated with *Lactobacillus plantarum* (*L. plantarum*), was significantly higher than those in the EIEC-infected NCM460 monolayers; B: Similar results were obtained in the experiments with enteropathogenic *Escherichia coli* (EPEC). The data at each time point represent the mean \pm SD obtained from four individual NCM460 monolayers. ^a $P < 0.05$ vs control group; ^c $P < 0.05$ vs corresponding EIEC or EPEC group.

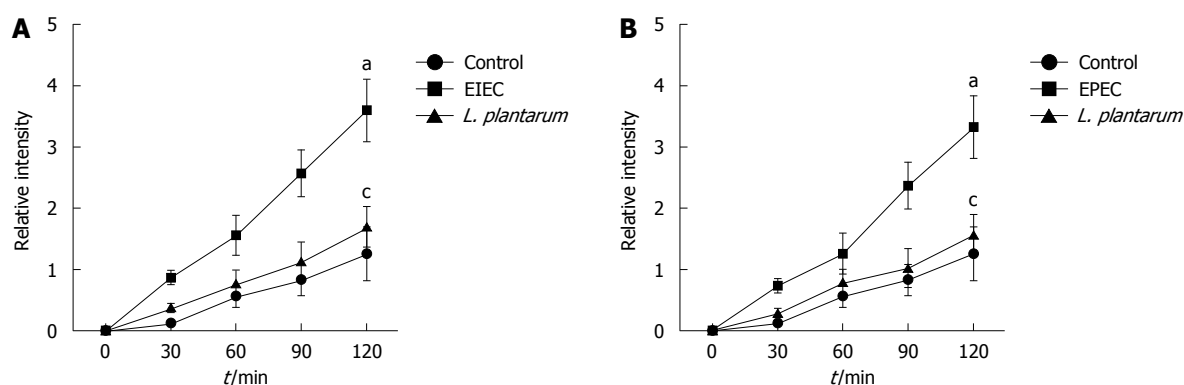


Figure 2 *Lactobacillus plantarum* inhibited the increased macromolecular permeability through NCM460 cells induced by enteroinvasive *Escherichia coli* (A) or enteropathogenic *Escherichia coli* (B). A: After infection with enteroinvasive *Escherichia coli* (EIEC) for 120 min, the relative intensity (RI) was significantly increased in the EIEC group compared with the control group. However, the RI was decreased significantly in *Lactobacillus plantarum* (*L. plantarum*) groups compared with the EIEC group; B: Similar results were obtained in the experiments with enteropathogenic *Escherichia coli* (EPEC). The data at each time point represent the mean \pm SD obtained from four individual NCM460 monolayers. ^a $P < 0.05$ vs control group; ^c $P < 0.05$ vs corresponding EIEC or EPEC group.

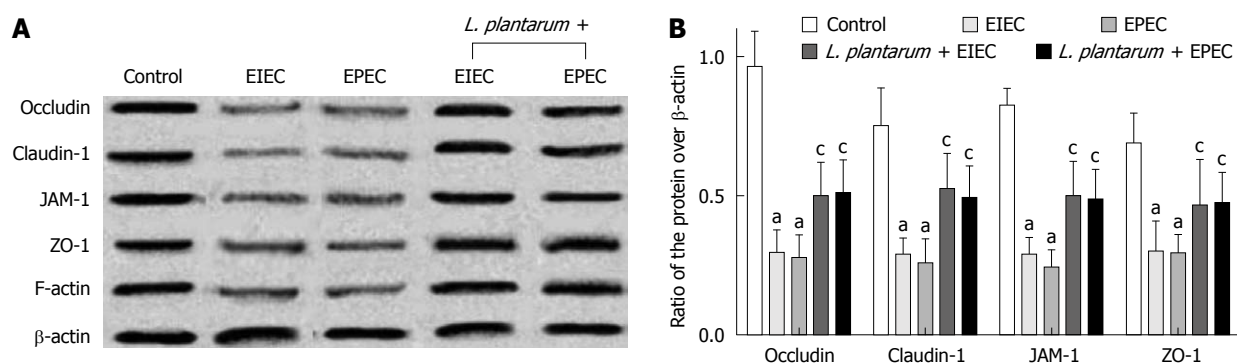


Figure 3 *Lactobacillus plantarum* prevented the decrease in the expression of tight junction proteins in NCM460 cells induced by enteroinvasive *Escherichia coli* or enteropathogenic *Escherichia coli* detected by Western blotting. A: The expression level of tight junction (TJ) proteins was high, including claudin-1, occludin, JAM-1 and ZO-1, in the control group. However, in the enteroinvasive *Escherichia coli* (EIEC) or enteropathogenic *Escherichia coli* (EPEC) group, TJ proteins were significantly decreased compared with the control group, which was not observed in the *Lactobacillus plantarum* (*L. plantarum*) group; B: Semi-quantitative analysis of Western blotting showed similar results. The data at each time point represent the mean \pm SD obtained from four individual NCM460 monolayers. ^a $P < 0.05$ vs control group; ^c $P < 0.05$ vs corresponding EIEC or EPEC group.

cludin, JAM-1 and ZO-1, was significantly decreased in the NCM460 cells infected with EIEC or EPEC, as compared

with the uninfected control cells (Figure 4A and B). However, treatment with *L. plantarum* raised mRNA expres-

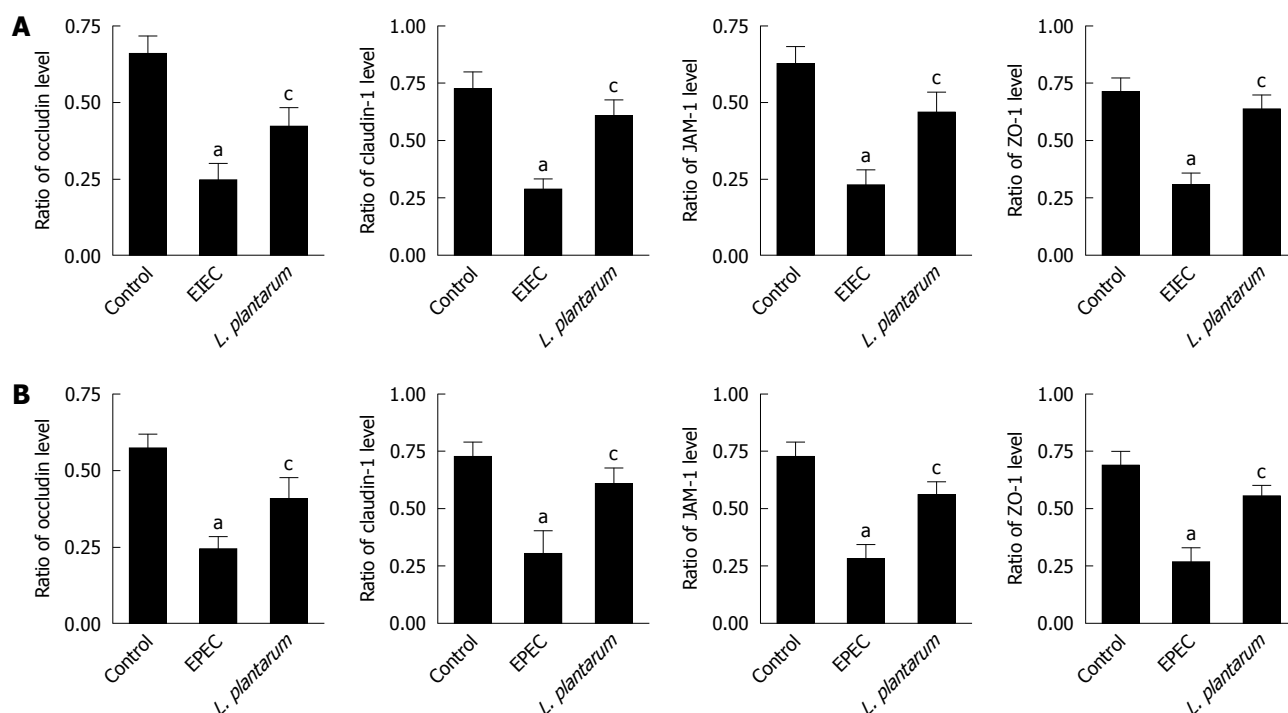


Figure 4 Protective effects of *Lactobacillus plantarum* and MIMP in mRNA expression of tight junction proteins in NCM460 cells detected by real-time polymerase chain reaction. A: The mRNA expression of tight junction (TJ) proteins, including occluding, claudin-1, JAM-1 and ZO-1, was decreased in enteroinvasive *Escherichia coli* (EIEC) group compared with the control group. However, in *Lactobacillus plantarum* (*L. plantarum*) group, the mRNA expression levels of the TJ proteins were similar to those in the control group; B: Similar results were obtained with enteropathogenic *Escherichia coli* (EPEC). The data represent the mean \pm SD obtained from three individual NCM460 monolayers. ^a $P < 0.05$ vs control group; ^c $P < 0.05$ vs corresponding EIEC or EPEC group.

sion levels similar to those in the uninfected control cells (Figure 4A and B).

DISCUSSION

It has been reported that probiotics, such as *L. plantarum*, have beneficial effects on the human intestinal barrier function in patients with intestinal diseases^[29]. Our previous studies also found that *L. plantarum* adhered to the intestinal epithelial cells, restored TJ structure and function, reduced paracellular permeability, and then showed the therapeutic effects^[20,23]. However, the studies about the interaction between lactobacillus and the human intestine were only limited in the cancer cell line and the animal models. The present study investigated the protective effects of *L. plantarum* against epithelial barrier dysfunction of the normal human colon cell line NCM460.

L. plantarum played an important role in increasing TER and decreasing the permeability to macromolecules of NCM460 monolayers against the disruption caused by EIEC or EPEC. EIEC and EPEC had the ability to decrease TER and increase the permeability to macromolecules^[20]. In the present study, we further observed that *L. plantarum* protected the epithelial barrier of NCM460 monolayers against the disruption caused by EIEC or EPEC. In other words, *L. plantarum* is able to attenuate the pathogen-induced decrease in TER, and inhibit the increase in the macromolecular permeability of dextran. Similar results were also found in other studies. Johnson-Henry reported that probiotics attenuated enterohemorrhagic *E. coli*

O157:H7-induced drop in electrical resistance, and increased the corresponding intestinal barrier permeability^[30].

Furthermore, we found that *L. plantarum* prevented the decrease in the expression of TJ proteins and F-actin in NCM460 cells. The expression of TJ proteins, including claudin-1, occludin, JAM-1 and ZO-1, and the cytoskeleton element F-actin were decreased in NCM460 cells infected with EIEC or EPEC compared with the control cells, as detected by Western blotting of epithelial whole cell protein extracts of NCM460 cells. However, after the pretreatment of *L. plantarum*, the expression of TJ proteins and F-actin remained at similar levels to the control cells. Other studies also found that *Lactobacillus rhamnosus* GG protected epithelial monolayers against EHEC-induced redistribution of the claudin-1 and ZO-1 TJ proteins. Resta-Lenert suggested that probiotics and/or commensals also reversed the epithelial damage produced by cytokines, and prevented the deleterious effects of tumor necrosis factor- α and interferon- γ in epithelial function^[31].

Lactobacillus is reported to exert its beneficial effects by either producing bacteriostatic or bactericidal agents^[32,33], competitively excluding pathogenic bacteria^[34], or regulating immunomodulatory effects^[13,31]. Furthermore, special signal transduction pathway is involved in the protective effects of *L. plantarum* on the intestinal epithelial barrier. Janus kinase inhibitor synergistically potentiated the effects of lactobacillus acidophilus on TER and permeability, while p38, ERK1, 2, or PI3K had no effects. After treated by lactobacillus, epithelial cells exposed to cytokines reduced the activation of SOCS3 and STAT1, 3.

We believe that our study broadens our knowledge of effects of *L. plantarum* in intestinal epithelial function and its therapeutic effects in the cellular and molecular mechanisms of intestinal barrier dysfunction and intestinal inflammation and justifies the use in inflammatory disorders, which is significant to both biotechnical and clinical fields. *L. plantarum* can protect against intestinal epithelial barrier dysfunction of NCM460 caused by EIEC or EPEC. However, the bacterial protein and its exact mechanisms of action remain unknown. We are conducting a study in an attempt to identify the protein and the smallest active domain within the protein from *L. plantarum* strain CGMCC1258 that is responsible for the adhesion of the bacterium to the intestinal epithelium. And further functional characterization by determining the effects of smallest active domain on the intestinal barrier function and immune responses is also in progress.

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COMMENTS

Background

Lactobacillus plantarum (*L. plantarum*) that resides in the human intestine plays an important part in maintaining the homeostasis of gut flora by adhering and colonizing to the intestinal mucosa and competing with pathogenic bacteria, which contributes to the protection of the human intestinal barrier function.

Research frontiers

Although *L. plantarum* exerted its therapeutic effects by adhering to the intestinal epithelial cells, restoring tight junction structure and function, and reducing paracellular permeability, the studies about interaction between lactobacillus and the human intestine were just limited in the cancer cell line and the animal models, and further studies based on the normal human intestinal cell had been unavailable.

Innovations and breakthroughs

Using the normal human colon cell line NCM460, this study investigated the protective effects of *L. plantarum* against epithelial barrier dysfunction caused by enteropathogenic *Escherichia coli* (*E. coli*) or enteroinvasive *E. coli*.

Applications

L. plantarum can be a potential candidate of therapeutic agents for the treatment of intestinal diseases.

Terminology

NCM460 cell line is a normal human colon cell line, which is derived from the normal human colon mucosal epithelium and expresses colonic epithelial cell-associated antigens such as cytokeratins and villin.

Peer review

This is a straightforward study extending previous work of the authors showing that *L. plantarum* maintains a high resistance to permeability to enteropathogenic and enteroinvasive *E. coli*. The work is extended to NCM460 colon cell cells in culture. Transepithelial electrical resistance was maintained high, dextran permeability was low and TJ protein expression was normal.

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Transarterial embolization ablation of hepatocellular carcinoma with a lipiodol-ethanol mixture

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Abstract

AIM: To determine the safety and effectiveness of transarterial embolization ablation (TEA) of hepatocellular carcinoma (HCC) with a lipiodol-ethanol mixture.

METHODS: Between January 1 and December 31, 2009, 15 patients with HCC (13 men/two women, aged 38-75 years) accepted TEA treatment and were enrolled in this study, including five newly diagnosed patients and 10 with refractory disease. Two months after TEA, angiography and contrast computed tomography (CT) were performed, and responses were assessed using a modified version of Response Evaluation Criteria in Solid Tumors (RECIST version 1.1). The follow-up period was to June 30, 2010.

RESULTS: Every new case was treated once. Angiogra-

phy was performed immediately after TEA, and showed that the tumor-feeding vessels were completely embolized and that lipiodol was densely deposited inside tumors. Two months after treatment, contrast CT showed no enhanced lesions. Alpha fetoprotein levels returned to normal in four patients and markedly decreased in another. mean \pm SD survival after treatment was 10.8 \pm 4.5 mo. All five patients survived during the follow-up period. Ten patients with refractory disease were treated a total of 14 times. Angiography immediately after TEA showed that blood flow to the tumors was obviously decreased in all cases, and contrast CT showed obvious depositions of lipiodol. Two months after treatment, the tumors had shrunk (6/10) or were stable (3/10). One had progressed after 2 mo and died of tumor rupture 3 mo after TEA. mean \pm SD survival after treatment was 8.6 \pm 4.3 mo; two patients survived during the follow-up period. Adverse effects included reversible hepatic decompensation, upper abdominal pain, and fever.

CONCLUSION: TEA is an effective therapy for patients with HCC and might be more effective than transcatheter arterial chemoembolization for treating refractory disease.

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Key words: Transarterial embolization ablation; Lipiodol-ethanol mixture; Hepatocellular carcinoma

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Gu YK, Luo RG, Huang JH, Si Tu QJ, Li XX, Gao F. Transarterial embolization ablation of hepatocellular carcinoma with a lipiodol-ethanol mixture. *World J Gastroenterol* 2010; 16(45): 5766-5772 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v16/i45/5766.htm> DOI: <http://dx.doi.org/10.3748/wjg.v16.i45.5766>

INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common tumor worldwide and the third most common cause of tumor-related death^[1-3]. HCC is not sensitive to radiotherapy or chemotherapy, therefore, surgery is still the treatment of choice. Unfortunately, < 30% of patients with HCC benefit from surgical resection because of unfavorable tumor location, stage or extent of disease, limited liver functional reserve, or high operative risk^[4,5].

Minimal invasive ablation, a new treatment for HCC, achieves complete responses in > 80% of tumors < 3 cm in diameter. However, in tumors of 3-5 cm in diameter, the complete response rate is only 50%^[6]. Therefore, ablation is not recommended for tumors > 5 cm. HCC is a hypervascular tumor that receives mostly hepatic arterial blood^[7]. Since its introduction by Yamada *et al.*^[8,9] in the 1980s, transcatheter arterial chemoembolization (TACE) has been widely used to treat patients with inoperable liver tumors. The theoretical basis of TACE for treating HCC is that chemotherapeutic agents mixed with embolic material and injected into the hepatic artery will embolize the arteries that supply the tumor and kill the tumor cells^[8,9]. However, Liu *et al.*^[7] also have reported that many HCC tumors, especially those > 5 cm in diameter, receive blood from both the hepatic artery and the portal vein. Therefore, TACE must be administered more than once, and tumor necrosis might still be incomplete^[10-12].

Ethanol can kill tumor cells, and percutaneous ethanol injections have been used to treat unresectable small HCC tumors^[13]. Ethanol can also produce permanent micro-circulatory embolization that inhibits tumor growth by denaturing protein, coagulating platelets, and dehydrating vascular endothelial cells in tumors^[14,15].

Transarterial embolization ablation (TEA) has been used to treat HCC with lipiodol-ethanol mixture (LEM)^[16-18]. Compared with TACE, TEA has the following potential advantages: (1) LEM can permanently embolize tumor-feeding vessels that come from the hepatic artery and portal vein^[15,17,19]; therefore, TEA might have a stronger antitumor effect than TACE; (2) after TACE, tumor and peritumoral normal tissues are ischemic, hypoxic, and secrete angiogenic substances that provide new conditions for tumor proliferation and recurrence^[20,21], whereas TEA leads to infarction of the entire tumor and of the peritumoral normal liver tissue without collateral circulation; and (3) embolization of tumor vessels by LEM helps ethanol diffuse into the tumor^[18,22-26]. It has been reported that the mean \pm SD lipiodol retention rate ($89.5\% \pm 10.7\%$) after TEA was significantly higher than after TACE ($47.5\% \pm 21.2\%$) in liver lesions, and 1- and 2-year survival after TEA (93.3% and 80.0%, respectively) was also significantly higher than that after TACE (73.3% and 43.3%, respectively). Furthermore, the 1- and 2-year incidence of extrahepatic metastasis after TEA (both 0%) was substantially lower than it was after TACE (35.5% and 39.2%, respectively)^[18].

In the present small pilot study, we sought to determine further the safety and effectiveness of TEA in

treating patients with newly diagnosed and refractory HCC tumors.

MATERIALS AND METHODS

Patient recruitment

The protocol was approved by the Institutional Review Board of the Department of Medical Imaging and Interventional Radiology, Cancer Center, Sun Yat-sen University, Guangzhou, Guangdong, China. A total of 15 HCC patients who provided written informed consent before being enrolled in the study were treated with TEA from January 1 to December 31, 2009. Inclusion criteria included accurate proof of HCC, refusal of or contraindication to surgical resection (five cases), refractory disease (10 cases, progressed after TACE), Eastern Cooperative Oncology Group performance status not greater than 2. Exclusion criteria included total serum bilirubin ≥ 50 $\mu\text{mol/L}$, serum albumin level < 28 g/L, and evidence of extrahepatic disease at presentation.

The demographic data of these patients are shown in Table 1. There were 13 men and two women with an average age of 55.7 ± 10.3 years (range: 38-75 years). All 15 patients were positive for hepatitis B and had concomitant cirrhosis. With regard to liver function status, 13 had Child-Pugh classification grade A and two had grade B. Of the 15, five had been recently diagnosed with single-nodule HCC (patients with new tumors and tumor diameter ≤ 5 cm) and 10 patients with refractory tumors, including five with huge lesions (> 5 cm) and five with diffuse lesions. The five patients recently diagnosed with single-nodule HCC were all α fetoprotein (AFP)-positive and the lesion diameters ranged from 2.6 to 4.8 cm (mean \pm SD, 3.7 ± 1.0 cm). The lesions were in the right lobule in two patients and in the left in three patients. Three of the five patients with huge HCC were AFP-positive, and all lesions were in the right lobule and the lesion diameters ranged from 8 to 15 cm (10.8 ± 3.1 cm). The five patients with diffuse lesions were all AFP-positive.

Treatment protocol

After puncturing the right femoral artery with the Seldinger technique, a Yashiro (5F, Terumo Heart, Inc., Japan) or R-H (5F, Terumo Heart) catheter was inserted into the common hepatic or superior mesenteric artery through a 5-F catheter sheath. Angiography was then performed to evaluate tumor blood supply and the surrounding vascular anatomy. Next, a 3F microcatheter (Terumo Heart) was inserted into the catheter (which remained in the proximal hepatic artery) and was advanced into the tumor-feeding arteries. For diffuse tumors, the microcatheter was inserted into segmental or subsegmental liver arteries to avoid the gastroduodenal and cystic arteries.

One percent lidocaine (from 2% lidocaine with saline, 1:1 by volume), 5-10 mL, was injected through a 2.5-mL syringe and followed by injection of a mixture of lipiodol (Guerbert S.A., Villepinte, France) and absolute ethanol (Department of Pharmacy, General Hospital of Guangzhou Military Command, China), 1:1 by volume. The in-

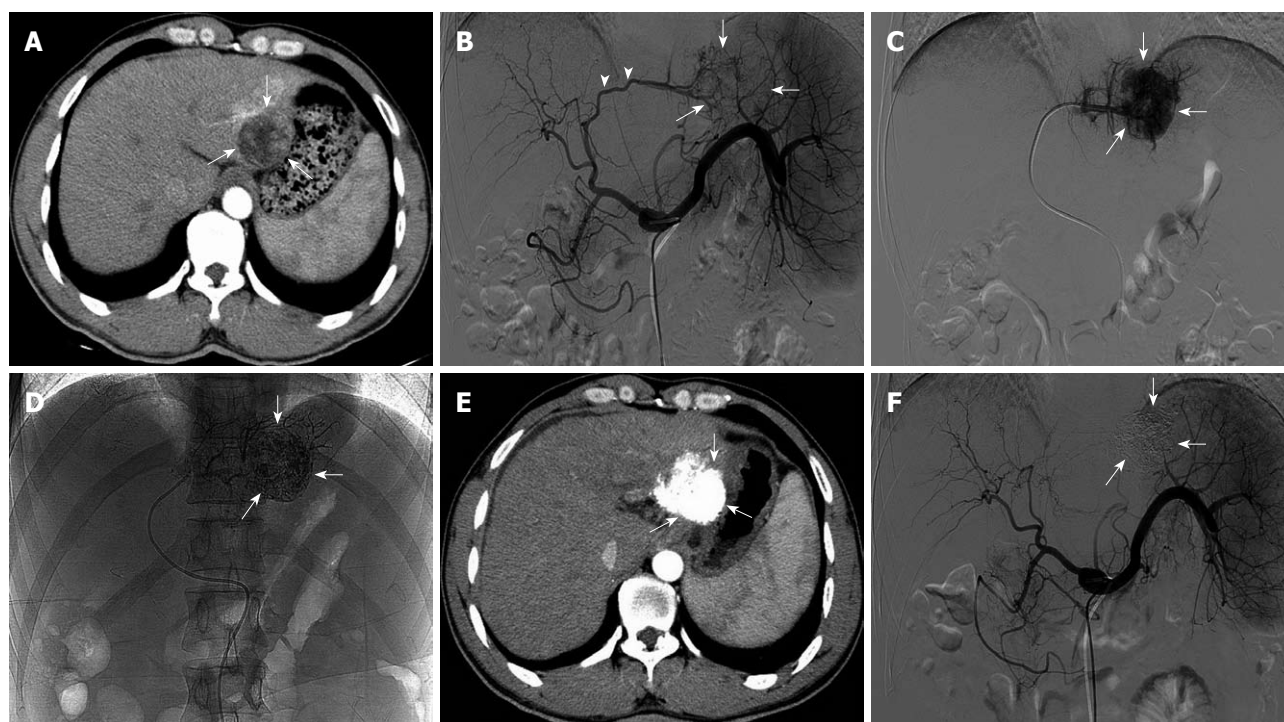


Figure 1 A newly diagnosed patient with single-nodule treated by transarterial embolization ablation. A: An enhanced computed tomography (CT) scan of hepatocellular carcinoma tumor before treatment. The tumor measured 3.5 cm × 3.3 cm (arrows); B: Hepatic artery angiography showed the thick blood vessels of the tumor (arrowheads) and the abnormal vascular group (arrows) before treatment; C: Supraselective angiography clearly showed tumor staining (arrows); D: After transarterial embolization ablation with an lipiodol-ethanol mixture, lipiodol accumulated in the tumor (arrows); E: An enhanced CT scan 2 mo after treatment showed dense deposition of lipiodol in the tumor without enhancement (arrows); F: Hepatic artery angiography showed the absence of tumor blood vessels and tumor staining at 2 mo after treatment (arrows).

Table 1 Demographic data

	<i>n</i>
Features	
Sex (M/F)	13/2
Age (yr)	55.7 ± 10.3
Seropositive for HBsAg/HCV	15/0
Underlying cirrhosis (+/-)	13/2
Radiological/histological evidence of HCC	14/1
Newly diagnosed/refractory cases	5/10
Serum AFP level (μg/L)	
≤ 200	2
> 200	13
Liver function status	
Grade A/B	13/2
Feature of tumors	
Single nodule ¹ (≤ 5 cm)	5
Huge lesion ² (> 5 cm)	5
Diffused lesion ³	5

¹Patient with one tumor that was ≤ 5 cm in diameter; ²Patient with one tumor that was > 5 cm in diameter; ³Patient with multiple different size tumors. HBsAg: Hepatitis B surface antigen; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; AFP: α fetoprotein.

jection of LEM was stopped when the tumor-feeding arteries were occluded, or when injection reflux was noted, or when the amount injected reached 60 mL.

Follow-up was to June 30, 2010 and measurements consisted of the degree of tumor embolization as determined visually immediately after TEA by angiography, judged by two skilled radiologists, liver function [alanine

aminotransferase (ALT) and aspartate aminotransferase (AST)] for 1-7 d after TEA, and AFP levels, computed tomography (CT) or magnetic resonance imaging, and changes of postoperative symptoms and signs up to 8 wk after treatment. Responses were assessed using a modified version of Response Evaluation Criteria in Solid Tumors (RECIST version 1.1)^[27].

RESULTS

Every newly diagnosed patient was treated once. The maximum total dose of LEM was 54 mL (10.8 ± 5.4 mL). Angiography immediately after TEA showed that all tumor-feeding arteries were completely embolized and that lipiodol had been densely deposited inside tumors. CT images 2 mo after treatment showed lesions with non-enhancement that had been replaced by deposition of lipiodol (Figure 1). AFP levels returned to normal in four patients (before TEA: 2947.6 ± 3724.5 μg/mL; post-TEA: 13.7 ± 8.2 μg/mL; *P* = 0.04) and decreased substantially in another patient (from 37 625 μg/mL to 26 376 μg/mL). Mean survival after treatment was 10.8 ± 4.5 mo. All five patients survived during the follow-up period (Table 2).

All refractory patients were treated a total of 14 times. The total dose of LEM was 256 mL (mean: 18.3 ± 13.5 mL). Angiography immediately after TEA showed that blood flow to the tumors was obviously decreased. Two months after treatment, CT showed that lipiodol was deposited more obviously than before and the tumors were smaller

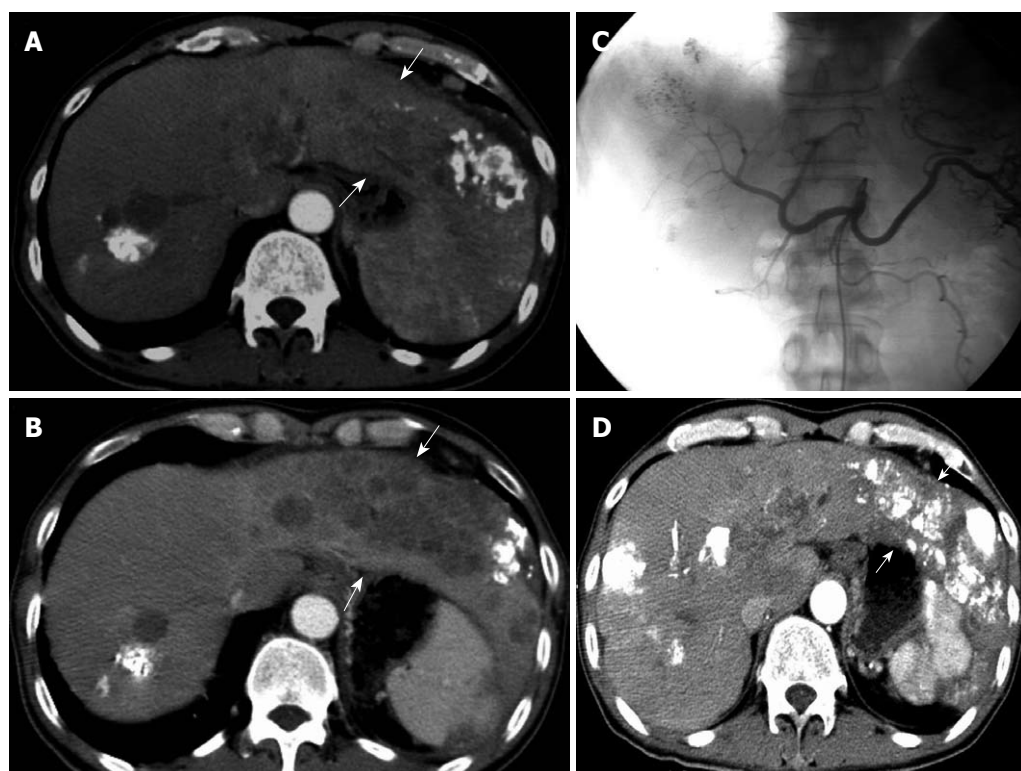


Figure 2 A patient with refractory tumors treated by transarterial embolization ablation. A: Limited lipiodol accumulation in a patient with a diffuse hepatocellular carcinoma tumor after transcatheter arterial chemoembolization (TACE) (arrows); B: After a second treatment with TACE, the tumor had progressed and showed less lipiodol accumulation (arrows); C: Hepatic artery angiography showed that tumor staining was not obvious. According to computed tomography images before transarterial embolization ablation (TEA), lipiodol-ethanol mixture was injected through the left and right hepatic arteries; D: Two months after TEA, the tumor had shrunk and lipiodol accumulation had increased (arrows).

Table 2 Clinical efficiency of newly diagnosed case

Newly diagnosed cases	Tumor response evaluation ¹		Follow-up period (mo)	Survival ²
	Target lesion 2 mo post-TEA	Time to progress (mo)		
1	CR	9.9	16.8	Yes
2	CR	6.1	14.1	Yes
3	PR	6.3	9.5	Yes
4	PR	5.1	7.5	Yes
5	PR	0	6.3	Yes

¹RECIST version 1.1; ²Survival (yes), death (no). TEA: Transarterial embolization ablation; CR: Complete response; PR: Partial response.

(6/10) or stable (3/10) in nine patients (Figure 2). For eight AFP-positive patients, AFP levels returned to normal in one patient, were decreased in six after a mean 2.5 ± 1.1 mo, and continued to rise in one. The mean survival was 8.6 ± 4.3 mo after treatment. Two patients survived during the follow-up period (Table 3).

Adverse events

Adverse events post-TEA are listed in Table 4. Of the 15 patients, 10 had transient elevations in transaminase after TEA, all of whom returned to normal after drug treatment to protect liver function. All patients had intraoperative or postoperative upper abdominal pain, which was relieved or eliminated after symptomatic treatment. Eleven patients had fever at 2-7 d after TEA but recovered with

Table 3 Clinical efficiency of refractory cases

Refractory cases	Tumor response evaluation ¹		Follow-up period (mo)	Survival ²
	Target lesion ⁴ 2 mo post-TEA	Time to progress (mo)		
1	PR	6.5	8.1	No
2	PR	7.8	14.3	Yes
3	PR	4.2	7.1	No
4	SD	3.9	6.1	No
5	SD	3.7	4.9	No
6	PR	6.2	10.1	No
7	SD	3.0	5.1	No
8	PR	6.5	16.7	Yes
9	PR	5.0	9.9	No
10	PD	³	3.5	No

¹RECIST version 1.1; ²Survival (yes), death (no); ³Continued to progress;

⁴Percentage of response evaluation: CR (0%), PR (60%), SD (30%), PD (10%). TEA: Transarterial embolization ablation; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease.

the administration of antipyretic drugs or dexamethasone (5-10 mg). There were no other serious adverse effects.

DISCUSSION

Currently, TACE has become an important palliative treatment for patients with inoperable HCC^[5,11,12,28-30]. Embolization of tumor-feeding arteries also prolongs the exposure and concentration of tumor drugs and chemo-

Table 4 Adverse events

Adverse events	<i>n</i>
Pain	15
Fever	11
Hepatic decompensation	10
Liver abscess	0
Biliary stricture or obstruction	0

therapeutic agents in the tumor microvascular bed, rather than allowing the drugs to reach the systemic circulation, which would reduce their effectiveness^[5,8,9]. However, HCC tumors are occasionally partially supplied by the portal vein, especially when capsule formation around the tumor is incomplete^[7,17,31]. After TACE, tumor blood supply from the portal vein increases, which makes achieving complete tumor necrosis difficult. In addition, embolization after TACE creates ischemia and hypoxia in the tumor and surrounding normal tissue, which leads tumor cells and peritumoral normal liver cells to secrete angiogenic factors, such as vascular endothelial growth factor and fibroblast growth factors, which promote collateral circulation and restoration of the tumor blood supply, which leads to tumor proliferation and recurrence^[20,21]. To achieve complete tumor necrosis, the blood supply to the tumor from the liver artery and portal vein and fistula between them needs to be stopped, and the formation of collateral circulation needs to be prevented^[32,33].

In animal experiments, Kan *et al*^[15] have proved that transarterial injection of LEM achieves lobar ablation effects because it simultaneously embolizes the hepatic artery and portal vein. Ethanol is a strong protein coagulant that can inactivate tumor cells directly *in situ* and cause vascular endothelial injury and platelet cohesion, which results in permanent occlusion of tumor-feeding vessels and tumor infarction without causing collateral circulation^[14,15].

In 1993, Matsui *et al*^[17] described transarterial ethanol embolization of HCC. However, ethanol is not radio-opaque, and its flow and speed are difficult to visualize when it is administered. In contrast, LEM traces not only the ethanol, which is necessary to avoid regurgitation and ectopic embolization, but also enhances the effects of embolization. Therefore, we call the transarterial injection of LEM TEA to distinguish it from TACE by injection of gelatin sponge or other embolic materials.

Optimal lipiodol:ethanol ratios

We treated 15 HCC patients with a mixture of equal volumes of lipiodol and ethanol. The results showed that TEA could completely embolize tumor-feeding vessels, reduce the size of single-nodule HCC tumors, and decrease levels of AFP. Enhanced CT examinations of the liver showed no enhancement of lesions. For refractory HCC, TEA can also shrink tumors and decrease AFP levels.

Reports were varied about the effectiveness of different ratios of lipiodol to ethanol. Matsui *et al*^[17] have treated 100 patients with 124 HCC tumors < 4 cm with equal volumes of lipiodol and ethanol through hepatic

artery injection. Survival rates for 82 patients with Child class A or B disease at 1 and 4 years were 100% and 67%, respectively. Kan *et al*^[15] have reported that embolic effects were better when the lipiodol:ethanol ratio was 5:1, 4:1, or 3:1 than if the ratio was 1:1. However, several studies have reported good results in treating HCC with transarterial injections with lipiodol:ethanol ratios between 3:1 and about 1:3^[16,17,19,34-36].

We think that the lipiodol:ethanol ratio can be adjusted according to the type of HCC. For single-nodule HCC, a relatively high lipiodol ratio (such as 2-3:1) is appropriate because the tumors are relatively small, have a complete capsule, and have a blood supply that comes mainly from the hepatic artery, with few fistulae between the hepatic artery and the portal vein. The injury to small arteries is relatively slight because of the lower percentage of ethanol in the mixture, and accordingly, more LEM is deposited inside the tumor. Meanwhile, the uptake of lipiodol by liver cancer cells means that the mixture is retained longer in tumor cells and has more time to produce antitumor effects^[15,37].

For refractory HCC, a relatively high ethanol ratio is appropriate to inhibit the double blood supply from the hepatic artery and the portal vein with fistulae between them. Ethanol can permanently embolize tumor-feeding vessels and block the fistulae, as well as reducing the loss of lipiodol and extending the exposure time in the tumor cells, which achieves a better therapeutic effect. However, further research is needed to determine the optimal lipiodol:ethanol ratios for different types of HCC.

Treating adverse events

The most common adverse events after TEA were liver dysfunction, fever, and upper abdominal pain. In this study, 10 of 15 patients experienced liver dysfunction, and mean levels of ALT and AST rose to 600-1200 U/L in serious cases; therefore, liver function needs to be monitored closely and protected after TEA. We usually review patient's liver function the day after TEA and then every 2-3 d until it returns to normal or nearly normal. Antipyretic drugs or dexamethasone are usually effective in treating fever. Damage to the vascular wall caused by ethanol results in upper abdominal pain in all patients. We administer intramuscular injections of 10 mg diazepam at 10-30 min before treatment and 10 mg intramuscular morphine after catheterization of tumor-feeding arteries. We also inject 5-10 mL 1% lidocaine into the arteries before LEM. Patients generally tolerate TEA given this preparation. We stop injection of LEM for 1-2 min if patients feel pain and resume injection when the pain is relieved after injection of lidocaine through the catheter. For those who still feel pain after TEA, a fentanyl patch (4.2 mg from beginning) can be applied.

Potential problems and study limitations

Ethanol is a strong protein coagulant and a permanent embolic material, but it can also injure normal liver tissue and vessels^[4,16,27,35,36,38]. Therefore, tumor blood vessels must be selected and treated carefully to avoid severe liver damage,

and even failure, and other serious complications caused by shallow intubation. On the other hand, excessively deep intubation can lead to incomplete embolization.

In addition, the speed of injection can also affect the efficacy of TEA. Blood dilutes ethanol and therefore will weaken the effect of ethanol on tumor cells if it is injected slowly, yet rapid injection might destroy blood vessel walls and form a vessel-casting mold. Injection of too much mixture can lead to reflux into normal liver tissues or to the blood vessels that supply the gastrointestinal tract. Therefore, research is needed into the effect of injection speed and on the dose of LEM.

Our study had some limitations. The sample size was small, but it was large enough to prove the concept. The follow-up after TEA was also short, and long-term efficacy needs to be studied further. In addition, some huge tumors had some necrosis before TEA, and the degree of tumor shrinkage was difficult to determine. Finally, we determined efficacy only on the basis of angiographic and CT imaging, without a postoperative pathological examination. These limitations can easily be avoided in larger, longer, and better-funded studies.

On the basis of our findings, we conclude that TEA is effective therapy for patients with HCC and might be better than TACE for treating refractory disease. Further studies, including randomized controlled trials, are warranted to confirm its role.

COMMENTS

Background

Transarterial embolization ablation (TEA) is a new treatment strategy for hepatocellular carcinoma (HCC). Some researchers have reported its effectiveness in HCC, especially in single-nodule HCC. However, its clinical effect for refractory HCC [not suitable for surgery and local ablation and with disease progression after transcatheter arterial chemoembolization (TACE)] remains unknown.

Research frontiers

TACE is accepted widely in treating advanced HCC because of its confirmed clinical effect. Advanced HCC tumors often receive blood from the hepatic artery and portal vein. Therefore, TACE must be administered more than once, and tumor necrosis can still be incomplete. In the present study, the authors demonstrated that TEA with lipiodol-ethanol mixture was an effective therapy for patients with HCC and might be more effective than TACE for treating refractory disease.

Innovations and breakthroughs

TACE with a mixture of lipiodol and ethanol has been shown to be an effective treatment for intrahepatic lesions of HCC, although it has not been widely used or described. Recent reports have highlighted the importance of this new method for its good results in nodular or capsulated HCC. Furthermore, this study suggests that this new method can also be applied in advanced refractory HCC.

Applications

Due to the exciting results and acceptable adverse events, TEA could represent a future treatment strategy for advanced HCC.

Terminology

Lipiodol is a very important embolization agent in TACE of HCC. Ethanol can kill tumor cells, and percutaneous ethanol injections have been used to treat unresectable small HCC tumors (chemical ablation). Ethanol can also produce permanent microcirculatory embolization that inhibits tumor growth by denaturing proteins, coagulating platelets, and dehydrating vascular endothelial cells in tumors. Transarterial lipiodol-ethanol mixture injection is called TEA.

Peer review

The authors report on the prospective follow-up of 15 patients treated with lipiodol-ethanol injection. The results are interesting. They reveal that TEA is a effective

and safe treatment for advanced HCC. A further comparative study with TACE is needed to re-evaluate this new method.

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Mitochondrial uncoupling protein 2 expression in colon cancer and its clinical significance

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Abstract

AIM: To detect the expression of mitochondrial uncoupling protein 2 (UCP2) in colon cancer and analyze the relation between UCP2 expression and clinical pathological features of colon cancer.

METHODS: Fifteen colon tissue samples and 15 its adjacent tissue samples were obtained from colon cancer patients during surgical interventions. UCP2 expression was detected with immunohistochemical method in 10 normal controls, 10 hyperplastic polyp patients, 20 tubular adenoma patients and 78 colon cancer patients. Patients with rectal cancer were excluded. Quantitative reverse transcription polymerase chain reaction and Western blotting were used to detect UCP2 expressions in colon cancer tissue samples and its adjacent tissue samples. Relation between UCP2 expression and clinical pathological features of colon cancer was also analyzed.

RESULTS: The UCP2 mRNA expression level was four-fold higher in colon cancer tissue samples than in its ad-

jacent tissue samples. The UCP2 protein expression level was three-fold higher in colon cancer tissue samples than in its adjacent normal tissue samples. The UCP2 was mainly expressed in cytoplasm. The UCP2 was not expressed in normal colon mucosa. Strong positive staining for UCP2 with a diffuse distribution pattern was identified throughout the mucosa in colon cancer tissue samples with a positive expression rate of 85.9%. The UCP2 expression level was higher in colon cancer tissue samples at clinical stages III and IV than in those at stage I + II. Univariate analysis showed that the high UCP2 expression level was significantly correlated to colon cancer metastasis (hazard ratio = 4.321, confidence interval = 0.035-0.682, $P = 0.046$).

CONCLUSION: UCP2 is highly expressed in human colon cancer tissue and may be involved in colon cancer metastasis.

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Key words: Mitochondrial uncoupling protein 2; Colon cancer; Uncoupling protein 2; Clinicopathologic characteristics

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INTRODUCTION

Colon cancer is one of the malignant tumors threatening to human health, and the mortality of colon cancer patients ranks second among all malignant tumors in developed countries. In recent years, its incidence has been increas-

ing in China, particularly in the economically developed coastal cities^[1,2]. Its etiology and pathogenesis remain unclear. Great achievements have been made in studies on changes in tumor suppressor gene or proto-oncogene, but some problems cannot be explained. Recent studies showed that mitochondrial dysfunction is involved in the occurrence and development of tumor^[3-5]. Uncoupling protein-2 (UCP2) is a mitochondrial membrane protein, which negatively regulates the production of reactive oxygen species (ROS)^[6-8]. Adaptive mechanisms of cancer cells include resistance to tumor growth inhibition and evasion of apoptosis, and cellular events that are appreciably affected by oxidative stress^[9,10]. The UCP2 expression level is significantly higher in colon cancer tissue than in its adjacent tissue and UCP2 may play a role in intestinal epithelial cells from benign to malignant transformation^[11]. However, the role of UCP2 in development of colon cancer is unclear. In this study, the expression of UCP2 in normal human colon tissue and colon cancer tissue was detected, and the relation between UCP2 expression in colon cancer tissue and clinical pathological features of colon cancer was also analyzed.

MATERIALS AND METHODS

Patients and tissue samples

Fifteen colon cancer tissue samples and 15 its adjacent tissue samples were obtained from the First Affiliated Hospital of Nanjing Medical University, snap-frozen and stored at -70°C. UCP2 expression was detected with immunohistochemical method in 10 normal controls, 10 hyperplastic polyp patients, 20 tubular adenoma patients, and 78 patients (45 males and 33 females) with colon cancer at different stages. Rectal cancer patients were excluded. Clinical pathological characteristics of the 78 colon cancer patients are listed in Table 1.

Immunohistochemistry

Tissue sections were stained with rabbit polyclonal antibody against human UCP2 (LS-C41270, LifeSpan BioSciences, Seattle, WA, USA), horseradish peroxidase (HRP)-labeled goat anti-rabbit secondary antibodies (Santa Cruz Biotechnology, Inc, USA) and visualized using peroxidase. Negative control sections were treated in PBS instead of primary antibodies. Intensity of UCP2 staining was scored as negative (0), weak (+1), moderate (+2), and strong (+3).

Quantitative reverse transcription polymerase chain reaction

After extraction of total RNA from snap-frozen colonic surgical samples with TRIzol reagent (Invitrogen, Carlsbad, CA) and removal of contaminating genomic DNA with DNase I and RNasefree (Roche Diagnostics Corp., Indianapolis, IN), reverse transcription polymerase chain reaction (RT-PCR) was performed using a first-strand cDNA synthesis kit (Roche Diagnostics Corp., Indianapolis, IN) following its manufacturer's instructions. Quantitative RT-PCR was performed using an ABI Prism 7300 real-time PCR detection system (Bio-Rad, Hercules, CA) following its manufacturer's instruc-

Table 1 Clinical and histological features of colon cancer patients

Characteristics	n (%)
Gender	
Female	33 (42.3)
Male	45 (57.7)
Age (yr): median 60.7, range 31-78	
< 60	36 (46.2)
≥ 60	42 (53.8)
Primary sites	
Left colon	44 (56.4)
Right colon	34 (43.6)
Clinical stage	
I (T1N0M0)	10
II (T2N0M0)	17
III	37
IV	14
Tumor differentiation	
Well	16
Moderately	38
Poorly	24

tions. The sequences of UCP2 and internal control GAPDH primers used in this study are as follows: Ucp2 gene: R: 5'-TCAGAATGGTGTCCCATCACA-3', F: 5'-CCGGTTACAGATCCAAGGAGAA-3', GAPDH: R: 5'-ACCCTGTTGCTGTAGCCA-3', F: 5'-CCACTCCTCCACCTTTGAC-3'. The PCR amplification conditions were 95°C for 5 min, followed by 40 cycles at 95°C for 30 s, at 60°C for 1 min, and a final extension at 72°C for 3 min. The relative amount of gene expression = $2^{-\Delta\text{Ct}} \times 100$ ($\Delta\text{Ct} = \text{ct target gene} - \text{ct internal reference}$).

Western blotting for UCP2

Sample was prepared for Western blotting using a mitochondria isolation kit following its manufacturer's instructions (AR0156, Wuhan Boster Biological Technology, LTD, Wuhan, China). An equal amount of protein was size-fractionated with 12.5% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Perkin-Elmer Life Sciences, Boston, MA). Immunoblots were developed using anti-UCP2 antibody (C-20; Santa Cruz Biotechnology, Inc, USA). Mitochondrial protein obtained from wild-type mouse spleen was used as a positive control. An equal loading was confirmed using cytochrome C (sc-13561, Santa Cruz Biotechnology, Inc, USA). Membranes were incubated with secondary antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology, Inc, USA), detected with enhanced chemiluminescence (ECL detection system, NEN, Boston, MA, USA), and used for visualization.

Statistical analysis

Data are expressed as mean \pm SE. Correlation between categorical groups was evaluated by χ^2 test or Fisher's exact test, when appropriate. Univariate analysis of hazard model was employed to detect independent predictors of colon cancer metastasis. When appropriate, linear regression and Pearson r correlation were calculated. Two-tailed *P* value of ≤ 0.05 was considered statistically significant.

Table 2 Uncoupling protein 2 mRNA expression in human colon cancer and peritumoral tissue samples detected by quantitative real-time polymerase chain reaction

Patient No.	Tissue	UCP2 mRNA relative abundance	T/P
1	T	8.12	3.45
	P	2.35	
2	T	19.68	5.70
	P	3.45	
3	T	13.6	2.96
	P	4.6	
4	T	21.4	2.32
	P	9.21	
5	T	26.2	3.67
	P	12.7	
6	T	78.6	2.51
	P	31.2	
7	T	86.5	3.59
	P	24.1	
8	T	12.31	6.00
	P	20.5	
9	T	45.2	8.85
	P	5.1	
10	T	36.1	0.84
	P	42.6	
11	T	9.12	2.37
	P	3.85	
12	T	3.67	1.78
	P	2.05	
13	T	10.34	4.40
	P	2.35	
14	T	17.63	5.86
	P	3.01	
15	T	6.54	4.47
	P	1.46	
Average T/P ratio			3.92

UCP2: Uncoupling protein 2; T: Tumour; P: Peritumoral.

RESULTS

Expression of UCP2 mRNA in colon cancer and its adjacent tissue samples

The expression of UCP2 mRNA in 15 colon cancer tissue samples and 15 its adjacent tissue samples was detected by quantitative RT-PCR. The expression level of UCP2 mRNA was about four-fold higher in colon cancer tissue samples than in its adjacent tissue samples (Table 2).

Expressions of UCP2 protein in colon cancer and its adjacent tissue samples

The expression of UCP2 protein in 15 colon cancer tissue samples and 15 its adjacent tissue samples was detected by Western blotting. The expression level of UCP2 protein was three-fold higher in colon cancer tissue samples than in its adjacent tissue samples (Figure 1, Table 3).

Correlation between UCP2 protein and mRNA expressions in human colon cancer tissue samples

The correlation between UCP2 protein and UCP2 mRNA expressions in human colon cancer tissue samples was observed. A strong linear correlation was found between the T/P ratio of UCP2 mRNA and protein expression ($r = 0.7442$, $P < 0.05$), suggesting that increased UCP2 expression in co-

Table 3 Uncoupling protein 2 protein expression in human colon cancer and peritumoral tissue samples detected by Western blotting

Patient No.	Tissue	UCP2 protein (total gray)	T/P
1	T	2.10	2.14
	P	0.98	
2	T	4.16	5.33
	P	0.78	
3	T	2.45	2.82
	P	0.87	
4	T	5.14	4.25
	P	1.21	
5	T	3.02	3.70
	P	0.82	
6	T	6.24	2.70
	P	2.31	
7	T	5.55	5.00
	P	1.09	
8	T	8.27	3.93
	P	2.1	
9	T	9.43	8.65
	P	1.08	
10	T	1.96	0.78
	P	2.51	
11	T	3.15	3.80
	P	0.83	
12	T	7.12	2.13
	P	3.34	
13	T	4.51	3.72
	P	1.21	
14	T	7.12	5.45
	P	1.31	
15	T	2.54	4.17
	P	0.62	
Average T/P			3.27

UCP2: Uncoupling protein 2; T: Tumour; P: Peritumoral.

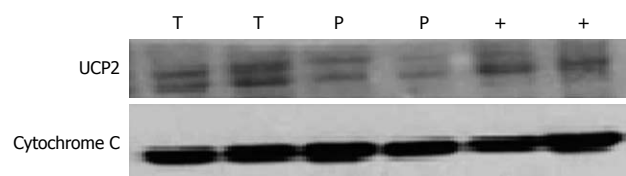


Figure 1 Western blotting analysis showing uncoupling protein 2 expression in human colon cancer tissue samples. T: Colon cancer tissue sample, P: Peritumor tissue sample; +: Positive control, spleen from wild type mouse. Cytochrome C was used as a loading control. UCP2: Uncoupling protein 2.

lon cancer tissue is largely determined at the transcriptional level (Figure 2).

UCP2 expression in colon tissue samples detected by immunohistochemistry

The expression of UCP2 was detected by immunohistochemistry in colon tissue samples from 78 colon cancer patients, 20 adenoma patients, 10 hyperplastic polyp patients and 10 normal controls. UCP2 was mainly expressed in cytoplasm but not expressed in epithelium of normal colon. In contrast, strongly positive staining for UCP2 with a diffuse distribution pattern was identified throughout the mucosa in most tubular adenomas and adenocarcinomas. The positive staining rate was 85.9% and 55% for

Table 4 Uncoupling protein 2 expression in different diseases detected by immunohistochemistry

Tissue	<i>n</i>	Negative	Mild	Moderate	Strong	Rate of positive (%)
Normal colon	10	10	0	0	0	0
Hyperplastic polyps	10	9	2	0	0	20
adenoma	20	9	3	6	2	55
Colon cancer	78	10	15	30	22	85.9

Positive cells < 15% in negative control and ≥ 15% positive cells were counted as positive. Staining intensity was classified as negative (0), mildly positive (+1), moderately positive (+2), and strongly positive (+3).

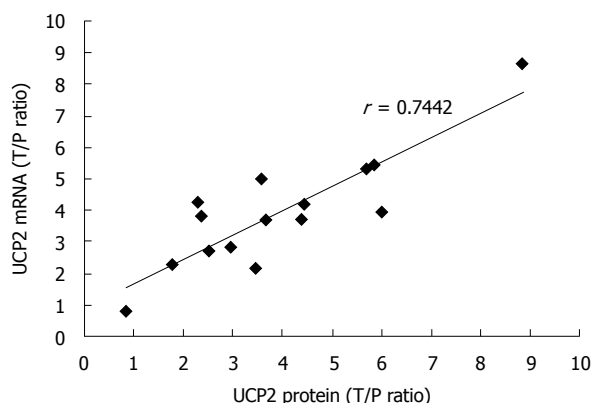


Figure 2 Correlation between uncoupling protein 2 and uncoupling protein 2 mRNA expressions in human colon cancer. UCP2: Uncoupling protein 2; T: Tumour; P: Peritumoral.

colon cancer tissue and colon adenoma tissue, respectively (Figure 3, Table 4).

Correlation between UCP2 expression and clinical pathological features of colon cancer

The correlation between UCP2 expression and clinical pathological features of colon cancer (including tumor stage and cell differentiation) was analyzed. The expression level of UCP2 was significantly higher in patients with colon cancer at clinical stages III and IV than in those at stage I + II. However, the UCP2 expression seemed to be irrelevant to cell differentiation status, indicating that further study is needed (Table 5).

Analysis of colon cancer metastasis-related factors

The relation of colon cancer metastasis with sex and age of patients, tumor location, UCP2 expression and other features was assessed by univariate analysis, showing that cell differentiation and UCP2 expression were involved in colon cancer metastasis. However, colon cancer metastasis seemed to be irrelevant to age and sex of patients and tumor location (Table 6).

DISCUSSION

In this study, different UCP2 expressions in colon cancer tissue and normal peritumoral tissue samples were observed. The expression of UCP2 mRNA in colonic mucosa samples from patients undergoing colon cancer resection was detected by quantitative PCR. As shown in Table 2, the UCP2 mRNA expression level was 4-fold higher in colon cancer mucosal tissue samples than in grossly normal peritumoral colonic mucosal

Table 5 Relation between uncoupling protein 2 expression and clinical pathological features of colon cancer

Tumor differentiation/clinical stage	Positive staining rate of UCP2 (%)	<i>P</i>
Tumor differentiation		
Well differentiation	72.8	0.072
Poor differentiation	91.4	
Clinical stage		
Stage I + II	65.1	0.032 ¹ , 0.0248 ²
Stage III	80.4	0.248 ³
Stage IV	92.6	

¹*P* = 0.032, uncoupling protein 2 (UCP2) expression in colon tissue from patients with clinical stage III *vs* that in colon tissue from patients with clinical stage I + II; ²*P* = 0.0248, UCP2 expression in colon tissue from patients with clinical stage IV *vs* that in colon tissue from patients with clinical stage I + II; ³*P* = 0.248, UCP2 expression in patients with clinical stage IV *vs* that in colon tissue from patients with stage III.

Table 6 Univariate Cox proportional hazards analysis of metastasis variables in colon cancer patients

Variable	Hazard ratio	95% CI	<i>P</i> -value
Age (≥ 60 yr)	0.098	0.081-33.058	0.915
Gender	0.895	0.078-41.221	0.389
Primary site	2.135	0.610-18.281	0.078
Tumor differentiation	1.270	0.031-1.672	0.049
UCP2 high expression	4.321	0.035-0.682	0.046

UCP2: Uncoupling protein 2; CI: Confidence interval.

tissue samples (3.92 ± 0.84 , *n* = 15). The expression level of UCP2 protein in colon cancer tissue samples was measured by Western blotting and densitometry, respectively (3.27 ± 0.78 , *n* = 15). A strong linear correlation was found between the T/P ratio of UCP2 mRNA and protein expressions (*r* = 0.744, *P* < 0.05), suggesting that increased UCP2 expression in colon cancer tissue is largely determined at the transcriptional level, which is consistent with the reported findings^[11].

To characterize the UCP2 protein expression in different human colonic lesions, immunohistochemistry staining was performed for colon tissue sections (including normal colon tissue, non-neoplastic hyperplastic polyp tissue, tubular adenoma tissue, and colon cancer tissue). UCP2 in epithelium of normal colon was not stained. In contrast, strongly positive staining for UCP2 with a diffuse distribution pattern was identified throughout the mucosa from most tubular adenomas and adenocarcinomas. These findings indicate that colonic epithelium is the primary source of increased UCP2

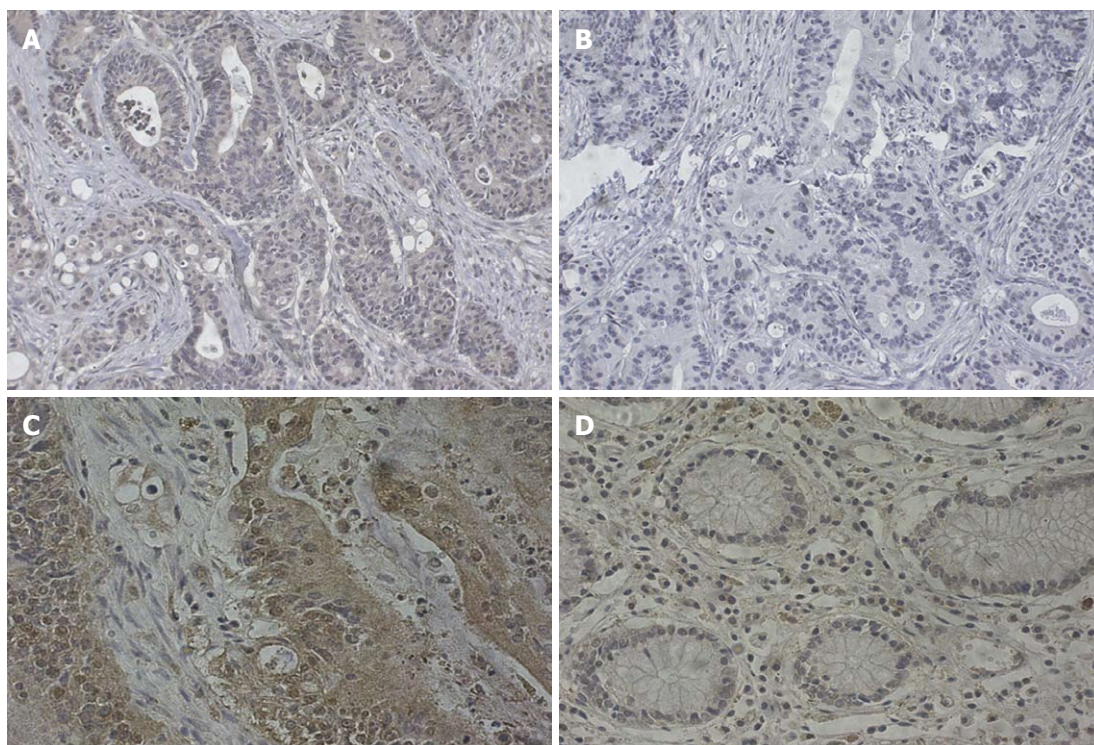


Figure 3 Immunohistological analysis showing uncoupling protein 2 expression in positively stained colon cancer tissue sample (A, C), negative control sample (B), and normal colonic tissue sample (D). Magnification: $\times 200$ (A and B), $\times 400$ (C and D).

expression in colon cancer. The positive UCP2 expression rate was 20% in hyperplastic polyp tissue samples, and over 50% in tubular adenoma samples, and 85.9% in colon cancer tissue samples, respectively, suggesting that UCP2 expression may intensify along the adenoma-carcinoma sequence.

The relation between UCP2 expression and clinical pathological features of colon cancer was analyzed. The expression level of UCP2 was significantly higher in patients with colon cancer at clinical stages III and IV than in those at stage I + II. However, the UCP2 expression seemed to be irrelevant to cell differentiation status, indicating that further study is needed (Table 5). Furthermore, the relation of colon cancer metastasis with sex and age of patients, tumor location, UCP2 expression and other features was also assessed by univariate analysis, showing that highly expressed UCP2 was significantly correlated to colon cancer metastasis (hazard ratio = 4.321, confidence interval = 0.035-0.682, $P = 0.046$).

Very few studies have reported the potential role of UCP2 in carcinogenesis^[12]. Limited literature showed that experimental data are somewhat controversial^[13,14]. It has been shown that the UCP2 expression level is moderately higher in human colon cancer cells (LoVo) treated with ionized radiation than in controls not treated with ionized radiation^[12,15], and the amount of UCP2 transcripts is greater in apoptosis-sensitive lymphoma cell line after radiation than in apoptosis-resistant cell line^[16], suggesting that the decreased mitochondrial membrane potential mediated by UCP2 may activate the cell death pathways^[17,18]. Recent reports from multiple laboratories offer a different conclusion^[14]. It was reported that drug-resistant tumor cell sub-lines increase the UCP2 expression with a lower mitochondrial membrane potential and a diminished susceptibility to oxidative stress^[19-21],

indicating that tumor cells may use UCP2 in their metabolic adaptation to avoid ROS-mediated apoptosis.

Over-expression of UCP2 reduces neuronal cell death in transgenic mice and in cell culture exposed to hypoxia and glucose deprivation, coinciding with a decrease in mitochondrial ROS formation^[21], and over-expression of UCP2 in cultured cardiomyocytes limits mitochondrial ROS production and suppresses loss of mitochondrial membrane potential elicited by H_2O_2 treatment^[22], suggesting that UCP2 over-expression may protect different normal cells against apoptosis, and the cyto-protective role of UCP2 is likely involved in reduction of mitochondrial ROS production.

It has been shown that glycolysis is the preferred energy-producing pathway in rapidly growing cancer cells, while their mitochondrial respiration is diminished^[23]. Changes in cancer cell bioenergetics are often associated with more aggressive tumor growth and drug resistance, resulting in worse prognosis^[24]. This metabolic switch in cancer cells is to steer away reducing equivalents from the mitochondria to limit ROS generation.

In conclusion, UCP2 is over-expressed in human colon cancer *in vivo*. Increased UCP2 expression in colon cancer is largely determined at the transcriptional level. Highly expressed UCP2 is associated with colon cancer metastasis. Over-expression of UCP2 may be involved in tumor aggressiveness and UCP2 may be a novel target therapy for colon cancer.

COMMENTS

Background

Colon cancer is one of the malignant tumors threatening to human health, and

the mortality of colon cancer patients ranks second among all malignant tumors in developed countries. Its etiology and pathogenesis remains unclear. Adaptive mechanisms in cancer cells include resistance to tumor growth inhibition and evasion of apoptosis, cellular events that are appreciably affected by oxidative stress. Uncoupling protein-2 (UCP2) negatively regulates the production of reactive oxygen species (ROS).

Research frontiers

The etiology and pathogenesis of colon cancer remain unclear. Recent studies showed that mitochondrial dysfunction is involved in the occurrence and development of tumor. UCP2 is a mitochondrial membrane protein, which negatively regulates the production of ROS. The role of UCP2 in development of colon cancer is unclear. In this study, the expression of UCP2 in normal human colon tissue samples and colon cancer tissue samples was detected and the relation between UCP2 expression and clinic-pathological features of colon cancer was analyzed.

Innovations and breakthroughs

Recent studies showed that mitochondrial dysfunction is involved in the occurrence and development of tumor. UCP2 is a member of the inner mitochondrial membrane anion-carrier protein super family and negatively regulates the production of ROS. This is the first study to report the relation between high expression of UCP2 and clinical pathological features of colon cancer.

Applications

By understanding how the expression of UCP2 in colon cancer and analyzing the relation between UCP2 expression and clinical pathological features of colon cancer, we showed that UCP2 might be a novel target therapy for colon cancer.

Terminology

UCP2 is a member of the inner mitochondrial membrane anion-carrier protein super family and negatively regulates the production of ROS. Cancer cells acquire drug resistance as a result of selection pressure dictated by unfavorable microenvironments. This survival process is facilitated through efficient control of oxidative stress originating from mitochondria that typically initiates programmed cell death. This critical adaptive response in cancer cells is linked to UCP2, a mitochondrial suppressor of reactive oxygen species.

Peer review

The authors detected the expression of UCP2 and the relation between UCP2 expression and clinical pathological features of colon cancer. The authors showed that UCP2 expression was increased in colon cancer, and UCP2 might be involved in colon cancer metastasis.

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Meetings

Events Calendar 2010

January 25-26
Tamilnadu, India
International Conference on Medical
Negligence and Litigation in Medical
Practice

January 25-29
Waikoloa, HI, United States
Selected Topics in Internal Medicine

January 26-27
Dubai, United Arab Emirates
2nd Middle East Gastroenterology
Conference

January 28-30
Hong Kong, China
The 1st International Congress on
Abdominal Obesity

February 11-13
Fort Lauderdale, FL, United States
21th Annual International Colorectal
Disease Symposium

February 26-28
Carolina, United States
First Symposium of GI Oncology at
The Caribbean

March 04-06
Bethesda, MD, United States
8th International Symposium on
Targeted Anticancer Therapies

March 05-07
Peshawar, Pakistan
26th Pakistan Society of
Gastroenterology & Endoscopy
Meeting

March 09-12
Brussels, Belgium
30th International Symposium on
Intensive Care and Emergency
Medicine

March 12-14
Bhubaneswar, India
18th Annual Meeting of Indian
National Association for Study of
the Liver

March 23-26
Cairo, Egypt
14th Pan Arab Conference on
Diabetes PACD14

March 25-28
Beijing, China
The 20th Conference of the Asian

Pacific Association for the Study of
the Liver

March 27-28
San Diego, California, United States
25th Annual New Treatments in
Chronic Liver Disease

April 07-09
Dubai, United Arab Emirates
The 6th Emirates Gastroenterology
and Hepatology Conference, EGHG
2010

April 14-17
Landover, Maryland, United States
12th World Congress of Endoscopic
Surgery

April 14-18
Vienna, Austria
The International Liver Congress™
2010

April 28-May 01
Dubrovnik, Croatia
3rd Central European Congress
of surgery and the 5th Croatian
Congress of Surgery

May 01-05
New Orleans, LA, United States
Digestive Disease Week Annual
Meeting

May 06-08
Munich, Germany
The Power of Programming:
International Conference on
Developmental Origins of Health
and Disease

May 15-19
Minneapolis, MN, United States
American Society of Colon and
Rectal Surgeons Annual Meeting

June 04-06
Chicago, IL, United States
American Society of Clinical
Oncologists Annual Meeting

June 09-12
Singapore, Singapore
13th International Conference on
Emergency Medicine

June 14
Kosice, Slovakia
Gastro-intestinal Models in
the Research of Probiotics and
Prebiotics-Scientific Symposium

June 16-19
Hong Kong, China
ILTS: International Liver
Transplantation Society ILTS Annual
International Congress

June 20-23
Mannheim, Germany
16th World Congress for
Bronchoesophagology-WCBE

June 25-29
Orlando, FL, United States
70th ADA Diabetes Scientific
Sessions

August 28-31
Boston, Massachusetts, United States
10th OESO World Congress on
Diseases of the Oesophagus 2010

September 10-12
Montreal, Canada
International Liver Association's
Fourth Annual Conference

September 11-12
La Jolla, CA, United States
New Advances in Inflammatory
Bowel Disease

September 12-15
Boston, MA, United States
ICAAC: Interscience Conference
on Antimicrobial Agents and
Chemotherapy Annual Meeting

September 16-18
Prague, Czech Republic
Prague Hepatology Meeting 2010

September 23-26
Prague, Czech Republic
The 1st World Congress on
Controversies in Gastroenterology &
Liver Diseases

October 07-09
Belgrade, Serbia
The 7th Biannual International
Symposium of Society of
Coloproctology

October 15-20
San Antonio, TX, United States
ACG 2010: American College of
Gastroenterology Annual Scientific
Meeting

October 23-27
Barcelona, Spain
18th United European
Gastroenterology Week

October 29-November 02
Boston, Massachusetts, United States
The Liver Meeting® 2010--AASLD's
61st Annual Meeting

November 13-14
San Francisco, CA, United States
Case-Based Approach to the
Management of Inflammatory Bowel
Disease

December 02-04
San Francisco, CA, United States
The Medical Management of HIV/
AIDS



Instructions to authors

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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 USA* 2006; In press

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- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462 PMID:2516377 DOI:10.1161/01.HYP.00000035706.28494.09]

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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 DOI:10.1097/01.ju.0000067940.76090.73]

No author given

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303 DOI:10.1136/bmj.325.7357.184]

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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 DOI:10.1046/j.1526-4610.42.s2.7.x]

Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; **(401)**: 230-238 [PMID: 12151900 DOI:10.1097/00003086-200208000-00026]

No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRSA Careaction* 2002; 1-6 [PMID: 12154804]

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Personal author(s)

- 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

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- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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

Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Write as mean \pm SD or mean \pm SE.

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