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Contents

Weekly Volume 19 Number 48 December 28, 2013

Contents

World Journal of Gastroenterology
Volume 19 Number 48 December 28, 2013

World Journal of Gastroenterology
Volume 19 Number 48 December 28, 2013

Contents

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EDITORIAL

Posterior tibial nerve stimulation for fecal incontinence: Where are we?

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Core tip: Posterior tibial nerve stimulation though in its infancy, holds promise to be an effective, patient friendly and cheap treatment for faecal incontinence refractory to available conservative options. However, several questions remain unanswered and pose dilemmas regarding the delivery of this treatment. Solving these dilemmas could hold the key for unlocking the pathway for this treatment to be brought into the limelight.

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Abstract

Neurostimulation remains the mainstay of treatment for patients with faecal incontinence who fails to respond to available conservative measures. Sacral nerve stimulation (SNS) is the main form of neurostimulation that is in use today. Posterior tibial nerve stimulation (PTNS) - both the percutaneous and the transcutaneous routes - remains a relatively new entry in neurostimulation. Though in its infancy, PTNS holds promise to be an effective, patient friendly, safe and cheap treatment. However, presently PTNS only appears to have a minor role with SNS having the limelight in treating patients with faecal incontinence. This seems to have arisen as the strong, uniform and evidence based data on SNS remains to have been unchallenged yet by the weak, disjointed and unsupported evidence for both percutaneous and transcutaneous PTNS. The use of PTNS is slowly gaining acceptance. However, several questions remain unanswered in the delivery of PTNS. These have raised dilemmas which as long as they remain unsolved can considerably weaken the argument that PTNS could offer a viable alternative to SNS. This paper reviews available information on PTNS and focuses on these dilemmas in the light of existing evidence.

INTRODUCTION

Neuromodulation is here to stay. Neurostimulation remains at present the first choice treatment for fecally incontinent patients who have failed to improve with biofeedback, except for the small minority in whom where there is an underlying surgically repairable sphincter defect[1-3]. The first reported use of the sacral nerve stimulation (SNS) for faecal incontinence (FI) was just under two decades ago[4]. However, over the past decade not only has the use of neurostimulation increased exponentially but the remit of neurostimulation has widened to include the stimulation of other nerves- primarily the posterior tibial nerve[5]. SNS for faecal incontinence remains a time tested treatment with more than 50 series reporting on its use. A large meta-analysis has confirmed

on its use in improving the symptoms of FI as well as improving the quality of life of the patients[6]. Posterior tibial nerve stimulation (PTNS) for faecal incontinence is relatively new with just under 20 studies being reported[7]. PTNS has been used mainly in the management of urinary incontinence[8,9]. Shafik *et al*[5] has been credited with attempting PTNS for faecal incontinence. PTNS can be performed either by using a more invasive percutaneous approach[5] where an inserted 34 gauge needle forms the route of stimulation or by the less invasive transcutaneous "Qualtero" approach[10] where cutaneous pads replace the needle. Studies that have been done looking at the efficacy of the percutaneous PTNS approach are far more than those which have looked at the less invasive transcutaneous approach. Though there have been no studies so far which have directly compared these two routes of stimulation, indirect evidence points to a better efficacy for the percutaneous approach[11].

PTNS is usually delivered unilaterally, at the nerve's most superficial position which lies just above and behind the medial malleolus. The area of the nerve stimulated is quite small as the grounding electrode is usually placed in the instep. No evidence exists as to any dominance of the left or right tibial nerve unlike the pudendal nerve[12].

DILEMMAS IN TREATMENT

Treatment protocols dilemmas

There remains a lack of an effective and standardised treatment protocol for both percutaneous and transcutaneous PTNS (Table 1).

Shafik *et al*[5] in 2003 reported giving 30 min of percutaneous PTNS stimulation on alternate days for a period of four weeks. Though there is now a general consensus that patients require 12 wk of continuous treatment and that each treatment episode should last 30 min, there is no uniformity on how this should be given. Studies have given a single 30 min session of PTNS once a week for 12 wk while others have given two 30 min sessions a week for 6 wk[13-15]. Three prospective studies of percutaneous PTNS from the same institution have used either once a week or twice a week patterns of treatment with no apparent differences in efficacy[16-18]. The superiority of one approach over the other remains yet remains to be demonstrated. The National Institute of Clinical Excellence (NICE) suggests both patterns could be adapted depending on patient response[19]. It is logical that the onset of symptom improvement for the patient will only occur later on into the treatment using the once a week regime compared to the twice a week regime. The once a week treatment can help alleviate hospital workloads and may be more acceptable to the patient. However, the onset of symptom improvement for the patient on a once a week regime could be delayed which may have a potential for more patient dropouts. All percutaneous PTNS studies so far have utilised unilateral stimulation. There remains the unexplored question as to whether bilateral percutaneous PTNS could be more effective-given that a recent pilot study on bilateral transcutaneous PTNS has shown better efficacy compared to unilateral stimulation[14,20].

The same treatment protocol dilemma exists for transcutaneous PTNS as well. Queralto provided patients with unilateral daily stimulation for 20 min for 4 wk and showed an 80% improvement in incontinence severity scores[10]. Eléouet *et al*[21] reported 63% improvement following a 20 min of unilateral twice daily stimulation for 1 mo. Vitton *et al*[22,23] attempted transcutaneous PTNS once daily for 3 mo on two groups of patients and reported a 41% and 54% improvement in symptoms. George at al attempted unilateral transcutaneous PTNS twice a week for 6 wk and reported a 45% improvement in symptoms[11]. Leroi *et al*[24] reported no improvements in the transcutaneous arm compared to the sham group following 20 min twice daily sessions for 3 mo. Thomas *et al*[25] suggested in a pilot study that daily stimulation may offer a better response compared to a twice weekly regime. A more recent variation has been the application of transcutaneous PTNS as a daily bilateral stimulation for 6 wk which has been reported to be more effective than the unilateral approach[14,20]. Only in one study was the transcutaneous PTNS stimulation provided in a hospital setting[11] while all the other studies required patients to apply the stimulation themselves at home after being trained.

Stimulation endpoint dilemmas

The stimulation end point for the transcutaneous PTNS was to look for a motor response which was visualization of rhythmic flexion of toes during stimulation[10]. Intensity of stimulation was then turned down to just below the threshold required for motor contraction. This seems to be a common end point for stimulation in most of the transcutaneous PTNS studies except the published RCT[11] where a sensory and a motor response was sought and a study by Vitton *et al*[23] where a sensory response was looked for.

However, the end point for stimulation for percutaneous PTNS remains uncharted with no specific end points described to confirm effective stimulation. Percutaneous PTNS can cause both a sensory and a motor response. The motor response is flexion of the big toe or fanning of all toes; the sensory response is a tingling sensation felt on the foot radiating to all of the toes[26]. The original paper by Shafik *et al*[5] looked for a motor response following stimulation. However, subsequent studies introduced a sensory response as an endpoint for stimulation[16-18]. The voltage used and the intensity of stimulation to achieve a sensory response remains lower than the intensity required to achieve a motor response[26]. This could imply that the voltage used for eliciting a sensory response alone could be sub-optimal without the full potential of the treatment being realised. This could in turn be reflected in lower treatment response rates.

Using the presence of either a motor or a sensory response could imply different treatment levels for differ-

Table 1 Posterior tibial nerve stimulation evidence summary *n* (%)

Ref.	Patient (*n*)	Type of PTNS	Time, frequency and duration of therapy	Follow-up	Stimulation endpoints	Efficacy	Study classification
Shafik *et al*[5]	32	Pct	30 min, alternate days 4 wk	22 mo	Motor	27 (84)	Nonrandomised controlled
Queralto *et al*[10]	10	Tct	20 min, daily 4 wk	4 mo	Motor	8 (80)	Prospective uncontrolled
Mentes *et al*[43]	2[1] (spinal)	Pct	30 min, alternate days 4 wk	3 mo	Motor	2 (100)	Prospective uncontrolled
Vitton *et al*[22]	12[2] (IBD)	Tct	20 min, daily 12 wk	3 mo	Sub sensory	5 (42)	Prospective uncontrolled
Babber *et al*[44]	8	Pct	30 min, weekly 12 wk	3 mo	Not specified	7 (87)	Prospective uncontrolled
De La Portilla *et al*[41]	16	Pct	30 min, weekly 12 wk	6 mo	Motor and sensory	10 (62)	Prospective uncontrolled
Vitton *et al*[23]	24	Tct	20 min, daily 12 wk	15 mo	Sub sensory	13 (54)	Prospective uncontrolled
Govaert *et al*[42]	22	Pct	30 min, twice weekly 6 wk	12 mo	Motor and/or sensory	18 (82)	Prospective uncontrolled
[3]Boyle *et al*[18]	31	Pct	30 min, weekly 12 wk	14 mo	Motor or sensory	21 (68)	Prospective uncontrolled
Findlay *et al*[45]	13	Pct	30 min, weekly 12 wk	4 mo	Sub motor	12 (92)	Retrospective uncontrolled
Eléouet *et al*[21]	32	Tct	20 min, twice daily 4 wk	6 mo	Motor	20 (63)	Prospective uncontrolled
[3]Allison[17]	90	Pct	30 min, twice weekly or weekly; 6 or 12 wk	21 mo	Motor or sensory	69 (77)	Prospective uncontrolled
[3]Hotouras *et al*[16]	100	Pct	30 min, twice weekly or weekly; 6 or 12 wk	6 mo	Motor or sensory	85 (85)	Prospective uncontrolled
Leroi *et al*[24]	144	Tct	20 min, twice daily 3 mo	3 mo	Sub motor	34 (47)	Randomised controlled trial
George *et al*[11]	11	Pct	30 min, twice weekly 6 wk	6 mo	Motor and sensory	9 (82)	Randomised controlled trial
	11	Tct	30 min, twice weekly 6 wk	6 mo	Motor and sensory	5 (45)	
Thomas *et al*[25]	15	Tct	30 min, daily 6 wk	6 wk	Sensory	3 (20)	Prospective randomised
	15	Tct	30 min, twice weekly 6 wk	6 wk	Sensory	0 (0)	
Moreira *et al*[46]	10	Pct	30 min, weekly 12 wk	3 mo	Not specified	6 (60)	Prospective uncontrolled
[3]Hotouras *et al*[30]	150	Pct	30 min, twice weekly or weekly; 3 mo	26 mo	Motor or sensory	60 (52)	Prospective uncontrolled

[1]Study included spinal injury patients; [2]Study included patients with inflammatory bowel disease (IBD); [3]Studies from the same institution - possibility of duplication of results. PTNS: Posterior tibial nerve stimulation; Pct: Percutaneous posterior tibial nerve stimulation; Tct: Transcutaneous posterior tibial nerve stimulation.

ent patients. In addition, patients with diabetes mellitus or with peripheral neuropathy could have an impaired sensory response or none at all. The published RCT used the presence of both a motor and sensory response as the end point for effective stimulation[11]. The presence of a combined motor and sensory response on PTNS has been reported to be better associated with a successful outcome than the presence of either a motor or a sensory response alone[27]. However, this could cause patient discomfort as higher voltages required for achieving a motor response may have the potential to cause discomforting sensory stimulations in some patients. The CONFIDENT multicentre randomised controlled trial (ISRCTN 88559475) presently underway in the United Kingdom utilises either a sensory or a motor response as an endpoint for stimulation.

Efficacy dilemmas

Percutaneous PTNS for FI remains a relatively new and untested treatment with only 12 studies, one randomised controlled trial[11] and one review[28] having been published to date on its use. The only published RCT on PTNS only reports on a 6 mo follow-up[11]. There remains no doubt regarding the short term efficacy of PTNS which are comparable to that of SNS. However, the true test of the effectiveness of PTNS would be its efficacy in the medium and long term. This is crucial as this could validate its effectiveness as a treatment option for faecal incontinence rather than a stepping stone towards SNS. There is a dearth of information on such results though

early reports from Hotouras *et al*[15,16] who has published on the largest group of PTNS patients so far (n = 100) reports a possible sustained efficacy for PTNS after 42 mo of follow-up[29]. However, this group[16-18] provided percutaneous PTNS as the first line therapy for fecally incontinent patients without assessing whether they were refractory to other non-interventional treatments[19]. This could perhaps imply that some of their patients would have had improvement in symptoms with other less invasive treatments had this been attempted. The CONFIDENT multicentre randomised controlled trial (ISRCTN 8855947) which is presently underway across 14 centres in the United Kingdom may shed more light on the true short term efficacy of PTNS though only the percutaneous approach is compared to a sham route of stimulation. Though this study recruits patients who have been refractory to other less invasive therapies, the lack of any form of standardisation nationally for such therapies nationally remains notable.

The efficacy of transcutaneous PTNS remains even more untested with only a handful of studies which have looked at this approach to PTNS. Though several studies have reported symptoms improvements in patients a recent multicentre trial reported no improvements following stimulation and concluded that unilateral transcutaneous PTNS was no more effective than sham stimulation[24]. Patients were exposed to stimulation for 20 min twice daily for 3 mo[24]. However, a new pilot study has looked at bilateral transcutaneous PTNS and found it to be effective compared to unilateral stimulation[20].

FOLLOW-UP DILEMMAS

There remain no standardised follow-up and top-up regimes that can be used for percutaneous and transcutaneous PTNS. Most studies report efficacy only at the end of the 6 or 12 wk treatment period. The first percutaneous PTNS study reported a relapse of symptoms in 29% of patients with the majority of patients improving with further treatment though the exact regime for such follow up treatment was not reported[5]. Almost all studies on PTNS mention the need for "top-up" treatments. However there remains no clarity as to whether such top-up sessions should be offered only when patients report back due to recurrence of symptoms or whether such sessions should be offered at lengthening intermittent intervals after the intense initial treatment period. One study on percutaneous PTNS reported good efficacy with a median of one 12 monthly top-up session[15]. Regular percutaneous PTNS top-ups at lengthening intermittent intervals resulted in a sustained therapeutic effect for urological dysfunction[13]. New studies on PTNS make inroads into this aspect though this has to be verified through more independent trials[30].

The same dilemmas exist for transcutaneous PTNS as well. The efficacy following transcutaneous PTNS lasts for about 3 wk post treatment[20]. Though there is no definite top-up regimes recommended there remains the advantage that such treatments can be undertaken by the patient in the comfort of their own homes as well as the fact that the costs for such top-ups will be very low[20].

In comparison to SNS where the treatment effects are short-lived following the withdrawal of treatment, PTNS appears to confer a slightly longer lasting effect (albeit with a declining efficacy). However, a recent study on SNS has shown persisting efficacy even after the device was switched off which may bring it to par with the longer effects of PTNS[31].

The heterogeneity of follow-up regimes for PTNS makes it difficult to assess exactly the long-term effects of its treatment. Furthermore, only a few studies have performed rigorous assessment of "top-up" regimes to maintain efficacy. Further work needs to be done on the follow-up of patients who benefit from PTNS to accurately assess the duration of efficacy.

COST IMPLICATIONS

The present worldwide financial crisis has thrown into stark view the cost implications of neurostimulation. The direct medical costs for PTNS remain nearly ten times cheaper compared than those for SNS[17,32,33]. In PTNS itself the costs between percutaneous and transcutaneous PTNS also varies significantly. Percutaneous PTNS requires a re-usable stimulator 9V stimulator (Urgent PC®, Uroplasty Inc., United States) along with 12 disposable single-use leads. The disposable kits with 12 individually packed sterile stimulation units and a disposable battery for the Urgent PC stimulator unit costs £480 and are sufficient for the full treatment of 12 sessions[26,34]. The cost for the Urgent PC stimulator unit (Uroplasty, Berkshire, United Kingdom) is £1000. However, the reusable nature of the stimulator unit can reduce the costs of multiple treatments.

The costs for transcutaneous PTNS remain even smaller with the 50 mm × 50 mm self-re-usable adhesive surface electrode stimulation pads (Model VS.5050; Premier Medical Products, Bedford, United Kingdom) costing £1 per pair. The stimulator unit used is the NeuroTrac Continence Neurostimulator (Verity Medical Ltd, United Kingdom) costs $80 and can be re-used as the percutaneous stimulator[20].

SNS involves the *in-vivo* implantation of highly advanced technological devices and both the temporary and permanent wires were implanted under general anaesthesia. The higher costs for SNS arise due to the two-stage procedure along with associated pre- and post-operative care. The equipment only costs of SNS (2008 tariffs) were $526 for the temporary implant and $13500 for the permanent implant[35]. However, the actual charges levied for these procedures vary. Reports of costs for the initial temporary procedure for SNS vary from $1300[35] to about $5300[33]. Costs for the permanent implant procedure also varies from $14500[35] to about $21200[33]. Performing the initial stage of SNS under local anaesthesia appears to be more patient friendly and cheaper[36-38].

One of the underlying concerns regarding PTNS remains on the follow up post treatment and the hidden costs for these which may outweigh the initial costs savings. Running an SNS service is expensive[39]. However, there remains the possibility that the costs for maintaining the efficacy of PTNS in patients may be higher as they remain yet unknown. Conflicting reports on the cost effectiveness of both procedures are available[7]. A two-year follow up of percutaneous PTNS in patients with faecal incontinence from one center reported that PTNS became cost effective after the first year of treatment[17]. However, another study which compared SNS to PTNS at 5 years post treatment for urological dysfunction reported that SNS therapy became much more cost efficient compared to PTNS[40]. Unlike SNS, running costs and long term follow up expenses for PTNS lacks clarity given the absence of a uniform and universally accepted follow up protocol along with the dearth of independent medium and long term follow up data on "successfully" treated PTNS patients.

Future for PTNS?

There remains no question that SNS is less patient friendly and more expensive than PTNS in the short term[33]. Early attempts to make SNS more patient friendly have experimented at less invasive forms of SNS administration using a transcutaneous Percutaneous PTNS though minimally invasive does not require any operative procedures or a hospital inpatient stay. Patients also do not require a 3 wk trial phase which presently exists for SNS with insertion of a temporary SNS wire and a permanent implant subsequently if successful. Percutaneous PTNS has the potential to be delivered through a primary care setting using perhaps the abilities of specialist nurses who could provide these services on an outpatient basis. This could drive the costs of PTNS down even further.

Transcutaneous PTNS has the unique potential of being a treatment which is truly "by the patient, for the patient". FI can be socially crippling with patients sometimes being unwilling to leave the safety of their own homes for fear of incontinent episodes[41]. Transcutaneous PTNS may hold promise as a treatment which patients can self-administer safely, cheaply and effectively in the comfort of their own homes[20].

Presently PTNS appears to have the role as a stepping stone towards SNS in patients with faecal incontinence. Efficacy of transcutaneous PTNS has been used as a predictor for suggesting efficacy of SNS[42]. However, the question remains as to why patients should choose a potentially less patient friendly and clinicians should offer a more expensive and invasive treatment in the form of SNS when PTNS is available-albeit, in its infancy. This seems to have arisen as the strong, coherent, uniform and evidence based data on SNS remains to have been unchallenged yet by the weak, incoherent, disjointed and unsupported evidence for PTNS. A pilot study comparing SNS and percutaneous PTNS (UKCRN ID 10479/MREC ID 10/H 0808/38) may help shed more light on direct comparison between the two treatments.

The true role for PTNS remains yet to be validated and time tested - as SNS has been. However, the question as to whether SNS and PTNS become "brothers in arms" in treating FI or whether this may yet turn out to be the "David *vs* Goliath" battle will be answered only once PTNS has come into its prime.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (7): Liver transplant

Non-alcoholic fatty liver disease and liver transplantation: Outcomes and advances

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is one of the most prevalent causes of chronic liver disease worldwide. In the last decade it has become the third most common indication for liver transplantation in the United States. Increasing prevalence of NAFLD in the general population also poses a risk to organ donation, as allograft steatosis can be associated with non-function of the graft. Post-transplant survival is comparable between NAFLD and non-NAFLD causes of liver disease, although long term outcomes beyond 10 year are lacking. NAFLD can recur in the allograft frequently although thus far post transplant survival has not been impacted. *De novo* NAFLD can also occur in the allograft of patients transplanted for non-NAFLD liver disease. Predictors for NAFLD post-transplant recurrence include obesity, hyperlipidemia and diabetes as well as steroid dose after liver transplantation. A polymorphism in PNPLA3 that mediates triglyceride hydrolysis and is linked to pre-transplant risk of obesity and NAFLD has also been linked to post transplant NAFLD risk. Although immunosuppression side effects potentiate obesity and the metabolic syndrome, studies of immunosuppression modulation and trials of specific immunosuppression regimens post-transplant are lacking in this patient population. Based on pre-transplant data, sustained weight loss through diet and exercise is the most effective therapy for NAFLD. Other agents occasionally utilized in NAFLD prior to transplantation include vitamin E and insulin-sensitizing agents. Studies of these therapies are lacking in the post-transplant population. A multimodality and multidisciplinary approach to treatment should be utilized in management of post-transplant NAFLD.

Key words: Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis; Liver transplantation; Metabolic syndrome; Outcomes; Management

Core tip: Non-alcoholic fatty liver disease (NAFLD) is a prevalent indication for liver transplantation. It also poses a risk to organ donation, with decreasing rates of suitable allografts. NAFLD frequently recurs in the allograft or develops *de novo*. Post-transplant recurrence is related to obesity and immunosuppression associated metabolic derangements. A polymorphism in PNPLA3 also increases recurrence risk. Pre-transplant data favors sustained weight loss through diet and exercise as the most effective therapy for NAFLD. Vitamin E and insulin-sensitizing agents are occasionally used. Trials on immune-suppression regimens in this population are sorely needed. A multimodality approach to treatment should be utilized in management of post-transplant NAFLD.

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EPIDEMIOLOGY OF NON-ALCOHOLIC FATTY LIVER DISEASE AND ASSOCIATED ADVANCED LIVER DISEASE

Non-alcoholic fatty liver disease (NAFLD) is the most prevalent chronic liver disease in the developed world with a prevalence averaging 20% in the ulcerative colitis[1,2]. Its incidence in the developing world is also increasing sharply[3]. Prevalent in adults, it has also become the most common chronic liver disease in children[4]. Mirroring the epidemic of obesity, it is closely related to the metabolic syndrome particularly diabetes and dyslipidemia in association with truncal obesity[5]. Prior to the widespread recognition of NAFLD which was first described as a separate clinic-pathologic entity in 1980[6], many cases of NAFLD were likely classified as cryptogenic liver disease and cryptogenic cirrhosis (CRC). In a study where 39 liver transplant candidates diagnosed with CRC were carefully re-evaluated, 44% had prior biopsy consistent with NAFLD or clinical features of the metabolic syndrome[7].

Although NAFLD has been associated with excess mortality compared to the general population (Hazard ratio 1.34)[8], the natural history of NAFLD is often one of slow progression. In patients with isolated steatosis (fatty liver) the course of liver disease can be frequently benign[9,10]. The progressive form of NAFLD known as Non-alcoholic steatohepatitis (NASH) is associated with hepatocyte damage and consequently can lead to fibrosis as well as cirrhosis and end-stage liver disease[11]. Recently data about the natural history of NAFLD related cirrhosis was reported from four international referral centers. In this study, patients with NAFLD or hepatitis C virus (HCV) associated compensated (Childs A) cirrhosis were enrolled. Over the long term (mean follow up 86 mo for NAFLD and 75 mo for HCV), the incidence of liver related complications and hepatocellular carcinoma (HCC) was lower for NAFLD than for HCV. The probability of remaining free from liver related decompensation was 81.5% in the NAFLD cohort and 76.5% in the HCV cohort at 120 mo of follow up with a higher incidence of complications in HCV when adjusted for age, sex, body mass index and diabetes ($P = 0.03$). The incidence of HCC over follow up was 2.4% in the NAFLD cohort and 6.8% in HCV. Despite these differences, the incidence of cardio-vascular disease and overall mortality were similar between NAFLD and HCV patients (82% survival at 120 mo in both cohorts)[12].

LIVER TRANSPLANTATION INCIDENCE FOR NAFLD

The incidence of liver transplantation related to NAFLD has exploded in the last decade[13]. Although some of the reported increase in incidence of NAFLD related liver transplantation is due to increased recognition of patients previously classified as CRC, the increased incidence of NAFLD related liver transplantation is real. Even if the majority of CRC related liver transplants in prior eras were due to unrecognized NAFLD, the magnitude of increase in transplants for NAFLD far outweighs any classification bias[13]. In an audit of United States national transplant data (SRTR), liver transplants attributed to NAFLD related liver disease increased from 1.2% in 2001 to 9.7% by 2009 and this is now the third most common indication for liver transplantation in the United States[13]. In this study patients with NAFLD receiving a liver transplant were older, more likely to be females, had higher body mass index (BMI) and were less likely to have HCC at transplant compared to all other recipients.

There have been concerns about bias in transplant evaluation and listing of patients with NAFLD related cirrhosis. NAFLD patients are on average older at presentation and have higher rates of obesity and metabolic syndrome raising concerns about worse outcomes of transplant in these patients including increased risks of cardiovascular disease and chronic kidney disease. In a study from a single liver transplant center, the cohort of NAFLD patients with MELD less than 15 at listing were found to progress more slowly compared to patients with HCV and were more likely to die on the waiting list or be taken off the transplant list due to becoming "too sick"[14]. However for patients who were listed with MELD scores over 15 there were no differences in rate of progression of end-stage liver disease, listing rate and receipt of liver transplantation. In another study, patients with NAFLD were equally likely than non-NAFLD patients to undergo liver transplant evaluation, listing and transplantation. In this single center study, NASH patients were older, had similar rates of HCC but increased rates of other prior cancers by history. In addition diabetes and complications of metabolic syndrome were more prevalent in NASH patients. NAFLD patients also had higher creatinine levels at transplant listing than non-NAFLD patients[15]. Routine audits of multicenter and national data will have to be done to see if NAFLD patients are indeed at a disadvantage for evaluation and listing due to these concerns.

OUTCOMES AFTER LIVER TRANSPLANTATION FOR NAFLD

Survival after liver transplantation for NAFLD

Outcomes after liver transplantation in patients with NAFLD have been reported in both large national database audits as well as from single center studies. These studies have been restricted to adult recipients (> 18 years) of liver transplants. In the pediatric population although NAFLD is common, it is a rare indication for liver transplantation[16] (Table 1).

The national databases (UNOS and SRTR) studies have looked at outcomes at 1 year and beyond after liver transplantation (Table 1). Overall 1-year, 3-year and 5-year survival has been comparable between NAFLD and non-NAFLD recipients[13]. In more specific sub-analyses of the same databases post-transplant survival for NAFLD

Table 1 Liver transplantation for non-alcoholic fatty liver disease

Ref.	Patient	Population	Follow up	Graft survival	Patient survival	NAFLD recurrence in graft	Predictors of NAFLD recurrence	Predictors of survival
National registry data								
Charlton *et al*[13]	35781 adults adult liver transplant recipient NASH primary or secondary indication for 1959 recipient	SRTR (US national data) of liver transplant recipients from 2001 to 2009 Included NASH plus 50% of CRC and NASH plus CRC with BMI > 30 kg/m^2)	3 yr post-transplant survival reported	NASH 3-yr survival 76% (similar to other indications)	NASH 1-yr survival 84% and 3-yr 78% CRC 1 -yr survival 86% and 3 -yr 79%Other Diagnoses 1-yr survival 87% and 3-yr 78% (P = 0.67)	Not reported	Not reported	Not reported
Singal *et al*[18]	54687 adult liver transplant recipient NASH 1368 recipients	UNOS adult liver transplant recipients from 1994 to 2009	10-yr survival reported	1-yr, 3-yr, 50-yr and 10-yr survival NASH: 86%, 82%, 80% and 80%NAFLD post-transplant survival similar to cholestatic liver disease, HBV and better than ALD, CRC, HCV and HCC	1-yr, 3-yr, 5-yr and 10-yr survival NASH: 89%, 85%, 84% and 84% NAFLD post-transplant survival similar to cholestatic liver disease, HBV and better than ALD, CRC, HCV and HCC	Not reported	Not reported	For all recipients, age of recipient, male recipient black race, ventilator support pre transplant and MELD score as well as donor risk index associated with worse patient survival
Afzali *et al*[17]	53738 adult liver transplant recipients NASH 1810 recipients	UNOS adult liver transplant recipients from 1997 to 2010	5-yr survival reported	Not reported	1-yr, 3-yr and 5-yr survival NASH: 88%, 82% and 77% Overall adjusted HR for NASH post-transplant mortality compared to other etiologies was 0.75 (95%CI: 0.66-0.85) Adjusted survival was better for NASH than for ALD, HCV, and HCC. NASH survival was worse than cholestatic liver disease, AIH, HBV	Not reported	Not reported	Not specified- although state survival adjusted for several donor, recipient characteristics (individual Hazards ratios not reported)
Single center studies								
Tanaka *et al*[57]	7 patient with NAFLD (425 total LDLT recipients)	Patients with NAFLD that underwent Live donor liver transplant at a single center in Japan between 1996 and 2013	Median follow up 5.3 yr	100% at last follow up	100% at last follow up	1/7 (14%) had recurrent NASH	Not reported	Not reported
El Atrache *et al*[27]	83 recipient, NALFD[46] and CRC[37]	Liver transplant recipients at a single US center between 1996 and 2008	Mean follow up 46 mo	12/83 underwent re-transplantation	12 recipients died. Overall survival not reported	NAFLD recurrence in 20/83 recipients (15 with NASH pre-transplant and 5 with CRC pre-transplant	Predictors of recurrence were metabolic syndrome, hypertension and Insulin use as well as hyperlipidemia after transplant	Five year survival worse for those with metabolic syndrome, hypertension and insulin use No difference in survival between those with NASH recurrence and those without

Dureja *et al*[26]	88 recipients with NAFLD	Liver transplant recipients at a single US center between 1993 and 2007	Mean follow up 82 mo	Not reported	5-yr patient survival similar between those with NAFLD recurrence and those without NAFLD recurrence ($P = 0.78$)	NAFLD Disease Recurrence in 34/88 (39%)	Pre and post-transplant BMI. Triglyceride levels and prednisone dose was higher in those with NAFLD recurrence	Post-transplant survival was worse in NAFLD patients with post-transplant cardiac disease (HR 3.2, 95%CI: 1.3-7.7)
Agopian *et al*[20]	144 recipients with NAFLD (total 1294 transplants)	Liver transplant recipients at a single US center between 1993 and 2011	Mean follow up 2.3 yr	Graft survival similar between NAFLD and non NAFLD (90 d survival 86% for NASH) and lower only than PBC/PSC (90 d graft survival of 94%) 5-yr graft survival for NASH 63% similar to ALD, HBV, CC and PBC/PSC but better than HCV	Patient survival similar between NASH and non-NASH. 90 d survival 90% for NASH 5-yr patient survival for NASH (70%) similar to ALD, HBV, CC and PBC/PSC but better than HCV	NASH recurrence in 23 (16%)	Not reported	Post-transplant BMI > 35 kg/m^2 independent factor for mortality in NAFLD recipients only. Pretransplant dialysis also had worse survival in NASH patients
Kennedy *et al*[21]	129 recipients with NAFLD and 775 with other liver disease	Liver transplant recipients at a single US center between 1999 and 2009	5-yr survival reported	Graft survival not reported	1-yr, 3 -yr, and 5-yr survival NASH: 90%, 88% and 85% Non-NASH: 92%, 86% and 80% (P = NS) Mortality within 4 mo higher in NASH -8.5% *vs* non –NASH 4.2%, $P = 0.04$	Not reported	Not reported	No predictors of survival found
Barritt *et al*[24]	21 recipients with NAFLD and 97 with other liver disease	Liver transplant recipients at a single US center between 2004 and 2007	3-yr survival reported	30-d graft survival worse in NAFLD (81%) *vs* non-NAFLD (95%), $P = 0.02$ 1-yr survival for NAFLD and non-NAFLD patients was 76% *vs* 83%, $P = 0.32$ 3-yr survival was 76% for NAFLD *vs* 73% for non-NAFLD, $P = 0.78$	30-d patient survival worse in NAFLD (81%) *vs* non-NAFLD (97%), (81% *vs* 97%, $P = 0.001$) 1-yr survival for NAFLD and non-NAFLD patients was 76% *vs* 90%, $P = 0.06$ 3-yr survival was 76% for NAFLD *vs* 84% for non-NAFLD, $P = 0.23$	Not reported	Not reported	3-yr survival was significantly worse for diabetic patients compared to non-diabetics (63% *vs* 89%, $P = 0.006$)
Yalamanchili *et al*[22]	40 recipients with NAFLD	Liver transplant recipients at a single German center between 2007 and 2011	1-yr survival reported	Not reported	30 d mortality for NAFLD patients was 25% and 1-yr mortality was 35%	Not reported	Not reported	Patients with BMI > 35 kg/m^2 had worse graft survival (1-yr graft failure 55%) than those with lower BMI

Contos *et al*[32]	30 recipients with CRC and NASH compared to patients with ALD[16] and PBC/PSC[12]	Liver transplant recipients at a single US center between 2004 and 2007	Median follow up 3.5 yr	Graft survival similar between NAFLD and non-NAFLD patients (P = 0.32)	Patient survival similar between NAFLD and non-NAFLD patients (P = 0.32)	100% of the 30 NAFLD patients had steatosis in the graft by 5-yr post-transplant compared to 25% in the ALD and PBC/PSC groups	Steroid dose post-transplant associated with NAFLD recurrence	Not reported
Ong *et al*[31]	51 recipients with CRC	Liver transplant recipients at a single US center between 2004 and 2007	> 6 mo post-transplant, not reported	Not reported	Not reported	Post-transplant NAFLD developed in 25% and NASH in 16%	Predictors of post-transplant NAFLD was pre or post-transplant diabetes and triglyceride levels	Not reported
Bhagat *et al*[19]	71 NAFLD patients compared to 83 ALD patients	Liver transplant recipients at a single US center between 1997 and 2007	Median follow up 1517 d in NAFLD group and 1686 d in ALD group	Graft survival similar between NAFLD (76%) and ALD (82%)	1-yr, 3-yr, 5-yr, and 9-yr survival NASH: 82%, 79%, 75%, and 62% ALD: 92%, 86%, 86%, and 76% (P = 0.17)	NAFLD recurrence was 33% (21/64 NASH patients)	None reported	No predictive factors in NAFLD
Malik *et al*[23]	98 NAFLD recipients compared to 196 with PBC/PSC 196 with ALD 196, with HCV 98, with CR	Liver transplant recipients at a single US center between 2004 and 2007	Mean follow-up was 994 d	Not reported	Survival similar between NAFLD and non-NAFLD recipients 30-d mortality in NAFLD 6.1% 1-yr mortality 21.4% in NAFLD (similar to controls) 3-yr mortality in NAFLD 25% similar to controls, less in PBC (15%) 5-yr mortality (28%) similar in NAFLD patients and controls	Not reported	Not reported	Sepsis accounted for more deaths in NAFLD transplant recipients Liver recipients transplanted for NASH cirrhosis who died within the first post-transplant year were more likely to be older (≥ 60 yr), more obese (BMI ≥ 30 kg/m^2) and have both pretransplant diabetes and Hypertension

NAFLD: Non-alcoholic fatty liver disease; NASH: Non-alcoholic steatohepatitis; ALD: Alcoholic liver disease; PBC: Primary biliary cirrhosis; CRC: Cryptogenic cirrhosis; HCV: Hepatitis C virus; HCC: Hepatocellular carcinoma; PSC: Primary sclerosing cholangitis; CR: Cryptogenic cirrhosis; LDLT: Living donor liver transplantation.

was better as compared to HCV, alcohol, CRC, and HCC related liver disease[17,18]. When compared to primary biliary cirrhosis (PBC) one study showed similar survival[18] and another study showed worse survival for NAFLD[17].

In single center studies, post transplantation outcomes were also similar between NAFLD and non-NAFLD patients[19-22]. Survival at 1, 3, 5 and 10-year was reported as similar in the studies, although some have demonstrated higher early mortality (30 d) after transplantation in NAFLD than in non-NAFLD patients. Kennedy *at al*[21] reported a twofold higher mortality for NAFLD patients at 4 mo after transplantation than for non-NAFLD patients (8.5% mortality *vs* 4.2% for others). The commonest causes of mortality in NAFLD patients were infectious and cardiac disease. Another study confirmed higher 30-d mortality and 1-year mortality in NAFLD patients, although by 3 years survival were comparable in NAFLD and non-NAFLD patients[23]. In this study infections accounted for the majority of deaths. Factors associated with decreased survival in the cohort of NAFLD patients have included age of recipient post-transplant, diabetes[24], obesity and post-transplant metabolic syndrome[25]; and post-transplant cardiovascular disease[19,23,26]. Close monitoring and critical analysis of early and late outcomes after liver transplantation for NAFLD is thus necessary to further refine criteria and improve outcomes for liver transplantation in NAFLD.

NAFLD recurrence after liver transplantation

Recurrent NAFLD is common[22,26,27]. The recurrence rate depends to some extent on the methodology chosen for detection, (*i.e.*, evaluation of abnormal liver enzymes, liver biopsy, imaging techniques). Use of liver enzymes alone is fairly insensitive as a significant proportion of patients with NAFLD recurrence have normal liver enzymes.

Metabolic syndrome including obesity, diabetes, hyperlipidemia and hypertension are all increased in prevalence after transplantation linked largely to immunesuppression use, particularly steroid use and calcineurin inhibitors. Other factors include post-transplant weight gain due to reduced mobility, at least in the early period and these factors all contribute to recurrence of NAFLD in the allograft[28].

In some studies the risk of allograft steatosis was increased by the presence of the rs738409 single nucleotide polymorphism (SNP) in the *PNPLA3* gene in the recipient[29] as well as post-transplant obesity and diabetes[28]. This polymorphism (rs738409:I148M) in *PNPLA3* has been associated with reduced triglyceride hydrolysis in the adipocyte and increases the risk of developing NAFLD and NASH in the general population[30]. The presence of this SNP in *PNPLA3* in the donor has not been associated with development of allograft steatosis, obesity and diabetes. Thus the role of peripherally mediated triglyceride hydrolysis (in extrahepatic adipose tissue) seems to account for risk of NAFLD recurrence rather than liver related triglyceride hydrolysis, at least in post-transplant NAFLD[28,29].

In a study that systematically re-examined post-transplant biopsies and imaging, recurrent NAFLD was seen in 39% (34/88), with NASH in 25 and isolated steatosis in 9 of these 34 patients within 5 years post-transplant. Severe recurrence (NAS score $\geqslant$ 5) or advanced fibrosis was seen in 6 of the 34 with recurrent NAFLD[26]. NAFLD recurrence was correlated with pre and post-transplant BMI and post-transplant triglyceride levels and prednisone dose at 6 mo post-transplant. In this study post-transplant survival was similar between those with NAFLD recurrence *vs* those without.

Other studies have showed similar rates of NAFLD recurrence with one study showing recurrent NAFLD in 20 of 83 (24%). The metabolic syndrome and insulin use were linked to recurrent NAFLD in this study[27].

Yalamanchili *et al*[22] reported long term outcomes with post-transplant NAFLD recurrence. In this study, recurrent steatosis was reported in 45% of NAFLD transplant recipients and NASH was less common occurring in 4%. Advanced allograft fibrosis or cirrhosis was reported in 5% by 5 years and 10% by 10 years post transplantation and was more common in those with recurrent NASH (31%) *vs* those with steatosis alone (6%) or no steatosis (3%). In this study survival was similar at 1, 5 and 10 years in those with NAFLD and those with other liver diseases at transplant. Death from cardiovascular disease was more common than due to recurrent liver disease attesting to the strong link between the factors that predict development of NAFLD (Metabolic syndrome) and cardiac disease[22].

Other studies have also not shown reduced survival with NAFLD recurrence so far[26], although studies have been limited by a dearth of long term follow up (10 years or more) for large number of patients.

In patients transplanted for CRC, NAFLD has been reported to occur post transplantation and may be due to recurrent disease in a significant number of these patients who likely had undiagnosed NAFLD prior to transplantation. In one study steatosis alone developed in 25% and NASH in 16% of patients transplanted for CRC[31]. Predictors for post-transplant NAFLD in this population included pre or post-transplant diabetes, hypertriglyceridemia and higher BMI. In another study of thirty CRC patients who had the NAFLD phenotype (metabolic syndrome) prior to liver transplantation, recurrent steatosis was seen in 100% by 5 years post-transplant. Steroid dose was correlated with development of post-transplant NAFLD[32].

Very few if any data exist on risk of HCC in NAFLD and outcomes for these patients after transplantation. In a single center study, 17% of NASH cirrhosis patients referred for liver transplantation had HCC (6 noted incidentally on explant) which was higher than the number of patients with PBC/PSC with HCC and similar to ALD and HCV with HCC. Survival in NASH and HCC patients was good after liver transplant with 88% survival at a mean follow-up of 2.5 years[33].

DEVELOPMENT OF *DE NOVO* NAFLD AFTER LIVER TRANSPLANTATION

De novo NAFLD has been reported after liver transplantation in recipients who did not carry the diagnosis of NAFLD prior to liver transplantation. The incidence of *de novo* NAFLD after liver transplantation has ranged from 18% to 33%[34-36] with the progressive form NASH reported in 9% in one report[34]. In a study with liver biopsies done as protocol at 1, 5 and 10 years post-transplantation, as well as for clinical indications, the incidence of *de novo* NAFLD (defined as steatosis greater than 5% after more than 6 mo post liver transplantation) was 31% in 599 recipients with an average follow up of 40 mo. Histological NASH was present in only 3.8%, but perisinusoidal fibrosis was present in 29% and advanced fibrosis/cirrhosis in 2.25%[37]. The increased incidence of perisinusoidal fibrosis without steatohepatitis has not been well described in non-transplant populations and may represent a modified presentation in immunesuppressed individuals who may not present with brisk inflammatory response. In addition 51% of the recipients with *de novo* NAFLD had normal liver enzymes in this study attesting to the importance of liver biopsies and possibly imaging in accurately diagnosing NAFLD.

Factors associated with *de novo* NAFLD include post-transplant obesity, post-transplant diabetes, hyperlipidemia and hypertension[37]. In addition tacrolimus was also associated with recurrent NAFLD and this drug has

been well described as having an increased risk for developing diabetes[38].

In addition in this study a pretransplant diagnosis of alcoholic cirrhosis was associated with an increased risk of *de novo* NAFLD. In this study patients with recurrent alcoholism and recurrent hepatitis C or hepatitis B were excluded from the analysis as these conditions can lead to steatosis. The increased risk of *de novo* NAFLD in patients with prior ALD may reflect an underlying predisposition to NAFLD that could not be diagnosed prior to transplantation due to the concomitant alcoholic steatohepatitis. Donor allograft steatosis was also more prevalent in the group that developed *de novo* NAFLD (30%) as compared to the group that did not develop NAFLD (12.65%). This study did not quantify the degree of hepatic steatosis and nor were any genetic polymorphisms tested for in the donor. Other studies have suggested that donor polymorphisms that regulate cytokine release, inflammation and microsomal triglyceride transfer may be important in risk of developing NAFLD[39]. Protective factors against *de novo* NAFLD may include use of Angiotensin converting enzyme inhibitors[40], although this approach has not been tested in a trial.

The consequences of *de novo* NAFLD are not well known. In the study mentioned above complete regression occurred in 13 % (all with grade 1 steatosis initially), reduction of steatosis was seen in 35%, stability in 22%, and exacerbation in 30%. Higher prevalence of obesity was present in those with progression of histological liver disease[34].

In patients with hepatitis C the risk of developing *de novo* NAFLD is higher and can be linked to recurrence of hepatitis C[35]. Development of *de novo* NAFLD in the allograft can reduce the response rate to current antiviral therapy for hepatitis C and thus impact graft and patient outcomes[35].

MANAGEMENT OF NAFLD AFTER LIVER TRANSPLANTATION

There have been no published trials of pharmacotherapy specifically for post-transplant NAFLD. Analysis of the predictors of post-transplant NAFLD recurrence and data from non-transplant therapeutic studies on NAFLD suggest that sustained weight loss through a combination of dietary changes and exercise are most successful in reversing the histological findings of NAFLD[40], and improving biochemical and metabolic parameters including liver enzymes, insulin resistance, lipid levels and blood pressure in this condition[41].

Studies on pharmacotherapeutic agents in non-transplant patients suggest a role for vitamin E in selected individuals. In non-diabetics a large randomized controlled trial over 48 wk improved the histological features and liver enzymes in NAFLD[42]. Recent concerns about risk of prostate cancer[43] and risk of cardiac disease in susceptible individuals[44], as well as lack of long term data on sustained efficacy and safety may limit its usefulness in the post-transplant population.

The use of PPAR-gamma agonists (*e.g.*, Pioglitazone) improves insulin resistance and has shown some promise in reversing NAFLD in non-transplant patients[45,46]. In a large randomized controlled trial however it was not superior to placebo and inferior to vitamin E in reversing NAFLD[42]. This class of agents is also associated with weight gain and this also limits its utility in treatment of NAFLD[45,46].

Pharmacologic treatment of clinically overt diabetes, dyslipidemia and hypertension should be carried out as per best practice guidelines for managing these conditions[46] and in multidisciplinary teams involving the transplant team, primary care providers[47], diabetes specialists and preventive cardiologists.

Given that to a large extent immune-suppression exacerbates or promotes the development of the metabolic syndrome, immunosuppression modulation should be considered in patients with recurrent NAFLD or at risk of developing recurrent or *de novo* NAFLD. In particular minimization or avoidance of steroids, minimization of calcineurin inhibitor dose and levels and avoiding sirolimus in patients with hyperlipidemia is important in the management of NAFLD, obesity and metabolic syndrome post liver transplantation.

Bariatric surgery for obesity and morbid obesity has shown promising results in non-transplant patients and can reverse some of the metabolic consequences related to obesity such as diabetes[48]. Limited series have reported successful bariatric surgery specifically in patients with NAFLD[49], and in case reports in patients with NAFLD with compensated cirrhosis[50].

For NAFLD patients undergoing liver transplantation there are limited case reports of the utility of bariatric surgery after recurrence of NAFLD post transplantation[51]. There are also risks of exacerbation of NASH after bariatric surgery due to excessive weight loss as well as risks of impaired drug absorption and bacterial overgrowth that can impact post-transplant outcomes. At this point more evidence is needed before advocating bariatric surgery in transplant recipients.

DONORS WITH NAFLD

An adverse consequence of the epidemic of obesity and fatty liver in the population is the impact on suitable donors for liver transplantation. There is an increased risk of primary non-function of the allograft with fatty donors[52]. This data suggest that greater than 30% steatosis in the donor organ increases the risk of primary non-function. As NAFLD in the populations increases, the pool of potentially suitable organs for liver transplantation may diminish as a consequence.

In a Korean paper that evaluated steatosis in potential donors over a year, NAFLD (> 5% steatosis) was present in 51% and greater than 30% steatosis was present in 10.4% with NASH in 2.2%. The prevalence of steatosis was higher in donor over the age of 30, and those donor

with obesity and elevated triglyceride levels. In this study ultrasonography and CT both had limitations in diagnosis of NAFLD (> 30% steatosis in donors) with sensitivity of 92% for ultrasound but positive predictive value of only 34.5% and for CT a sensitivity of 64% and PPV of 45%. More recently the use of MRI Quantification methods for steatosis have been developed and validated independently against liver biopsy showing excellent correlation with histological steatosis grading[53,54]. Although donor biopsies should still be considered before excluding donors as unsuitable due to steatosis, utilization of MRI, particularly for liver donors may in the near future supplant the need for liver biopsies[55].

Although patient and graft survival can be diminished due to use of steatotic grafts, this is possibly not a risk factor for diminished graft survival if it exists in isolation[56]. Selection bias also confounds the picture as grafts that are not utilized due to steatosis may have different outcomes than steatotic grafts that are transplanted[57].

FUTURE DIRECTIONS

With increasing numbers of transplants in patients with NAFLD, current data support a careful audit of both short and long term post-transplant outcomes. Rigorous studies on immune-suppression regimens designed to decrease the incidence of metabolic complications for this population are needed. In addition post-transplant therapy for NAFLD including diet and exercise regimens, pharmacologic agents and bariatric surgery all warrant prospective study. With increasing numbers of donors with fatty livers, outcomes with these grafts should be tracked in prospective databases that include both donor and recipient variables.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (7): Liver transplant

Pharmacogenetic considerations for optimizing tacrolimus dosing in liver and kidney transplant patients

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Abstract

The introduction of tacrolimus in clinical practice has improved patient survival after organ transplant. However, despite the long use of tacrolimus in clinical practice, the best way to use this agent is still a matter of intense debate. The start of the genomic era has generated new research areas, such as pharmacogenetics, which studies the variability of drug response in relation to the genetic factors involved in the processes responsible for the pharmacokinetics and/or the action mechanism of a drug in the body. This variability seems to be correlated with the presence of genetic polymorphisms. Genotyping is an attractive option especially for the initiation of the dosing of tacrolimus; also, unlike phenotypic tests, the genotype is a stable characteristic that needs to be determined only once for any given gene. However, prospective clinical studies must show that genotype determination before transplantation allows for better use of a given drug and improves the safety and clinical efficacy of that medication. At present, research has been able to reliably show that the *CYP3A5* genotype, but not the *CYP3A4* or *ABCB1* ones, can modify the pharmacokinetics of tacrolimus. However, it has not been possible to incontrovertibly show that the corresponding changes in the pharmacokinetic profile are linked with different patient outcomes regarding tacrolimus efficacy and toxicity. For these reasons, pharmacogenetics and individualized medicine remain a fascinating area for further study and may ultimately become the face of future medical practice and drug dosing.

Key words: Pharmacogenetics; Calcineurin inhibitors; Tacrolimus; Liver transplant; Kidney transplant; Single nucleotide polymorphisms; CYP3A4; CYP3A5; ABCB1

Core tip: As researchers continue to evaluate the influence of single nucleotide polymorphisms on tacrolimus dosing and on the response to the drug, the challenge now becomes to assess the potential clinical implications of this research for medical practice. Sufficient data have been accumulated to be certain that the liver donor and kidney recipient *CYP3A5* genotype has an important influence on tacrolimus dosing and on the

observed blood trough levels of the drug. However, the question remains, should genotyping become a standard of practice in transplantation?

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INTRODUCTION

Transplantation is typically the standard of therapy for all patients with end-stage liver or kidney disease. Almost sixty years have passed since the first kidney transplant between identical twins was successfully performed, in 1954[1]. The first human liver transplant was performed almost 10 years later, in 1963. Another decade would pass before it was performed again. Since then, a significant effort has been made to improve the graft survival, as well as the patient outcome.

Despite the significant advances in terms of surgical techniques, tissue typing and patient care, most of the progress in organ transplantation is largely attributable to the recognized importance of immunosuppressive therapy[2,3].

Since the success of the transplant depends on a delicate balance between immunosuppression and rejection, reaching and maintaining an adequate therapeutic level by giving appropriate doses of immunosuppressive drugs is extremely important, especially in the first phases after the transplant.

The introduction of tacrolimus into clinical practice has undoubtedly improved patient survival after organ transplant. However, this drug is characterized by a restricted therapeutic index, a high inter- and intra-individual pharmacokinetic variability, including irregular oral bioavailability, and a series of severe adverse effects[4,5].

Given the high variability in blood levels and clinical response after administering fixed doses of tacrolimus, several studies have recently been conducted to find the optimal dosage of tacrolimus and thus to minimize its toxicity and to improve its risk/benefit ratio[6].

A number of studies have found a close correlation between the pharmacokinetic parameters of tacrolimus and the clinical outcome[7,8]. However, despite the long use of the drug in clinical practice, the best way to use tacrolimus is still a matter of intense debate[9,10].

The start of the genomic era has generated new research areas, such as pharmacogenetics, which studies the variability of drug response in relation to the genetic factors involved in the processes responsible for the pharmacokinetics and/or action mechanisms of a drug in the body[11,12].

This variability seems to be correlated with the presence of genetic polymorphisms, where, for example, some of the genes of the enzymes of phase I and II drug metabolic processes present, in at least 1%-2% of the population, allelic variants[13,14].

These variants can encode for different molecular isoforms of the same protein and, in most cases, consist of single nucleotide polymorphisms (SNPs), which may determine the production of isoforms differing by a single amino acid[14].

The variations in the DNA sequence of genes encoding for drug metabolizing enzymes can cause significant phenotypic differences in their expressivity and activity[15-17].

The clinical implications of genetic polymorphisms can include other aspects of drug bioavailability and elimination, as well as the pharmacodynamics of the drug and/or its metabolites and the therapeutic index. Despite the many genetic polymorphisms, only a small number of them have clinically significant consequences in terms of drug metabolism. This occurs mainly when two conditions coexist: the concerned metabolic pathway is the only pathway for the biotransformation of the drug and the drug has a low therapeutic index.

For all this reasons, pharmacogenetics research has begun to delve into genotyping approaches which may help to optimize the initiation and maintenance dosing of tacrolimus, to attain faster its target concentrations and to limit its dose-related adverse reactions[18]. This paper will review various studies that highlight the current genetic considerations in the dosing of tacrolimus and the future implications that this data may have for best individualizing the treatment with this drug.

TACROLIMUS PHARMACODYNAMICS AND PHARMACOKINETIC CHARACTERISTICS AND CONSIDERATIONS

The two calcineurin inhibitors utilized in transplantation are cyclosporine and tacrolimus.

Tacrolimus is a macrolide containing a 23-membered lactone ring produced by the *Streptomyces tsukubaensis* fungus (Figure 1). Its molecular weight is 822.05 Da[19]. Tacrolimus is now preferred to cyclosporine for its potency (10-100 times higher in *in vitro* and *in vivo* immunosuppression models) and for the reduction in episodes of rejection; it allows the use of lower doses of combination corticosteroids, thus reducing the possibility of adverse effects associated with such drugs[20,21].

Tacrolimus becomes biologically active only when it forms a complex with the immunophilin FK binding protein 12 (FKBP-12), that is different from the immunophilin (cyclophilin) to which cyclosporine binds. The complex FKBP-12-tacrolimus interferes with the transduction pathway of the intracellular calcium-dependent signal, which is a fundamental processes for the activation of T lymphocytes. The biological target of the complex

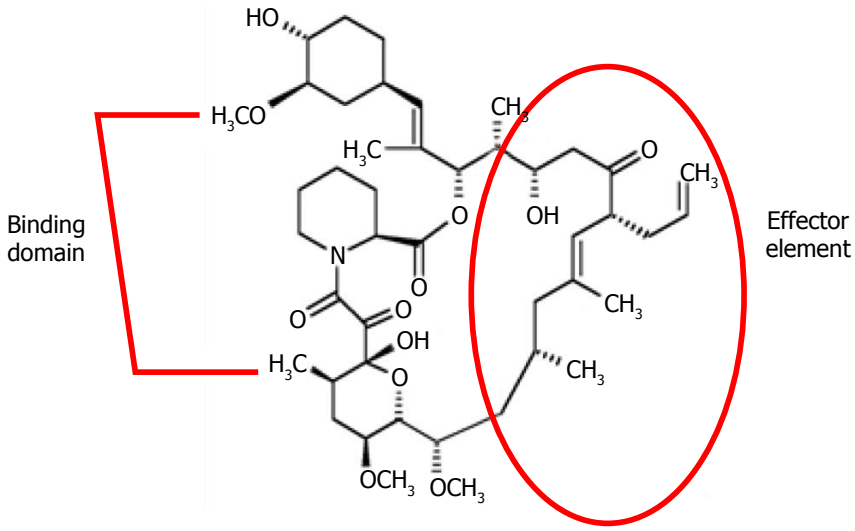

Figure 1 Functional active groups of tacrolimus.

is the calcium/calmodulin-dependent protein phosphatase calcineurin, a fundamental molecule for the reactions necessary to the synthesis of various cytokines, including IL-2.

Tacrolimus acts as a molecular linker between the calcineurin/calmodulin complex and immunophilin, which are molecules that in normal conditions would not interact. The tacrolimus-FKBP-12 complex has a strong inhibitory dose-related effect on calcineurin phosphatase activity and consequently on IL-2 expression. The passage of the signal from the cytoplasm to the nucleus to activate the transcription of the *IL-2* gene involves in fact a protein named nuclear factor of activated T-cell (NF-ATc). This protein is a T lymphocyte-specific transcription factor, the activity of which is correlated with the level of transcription of IL-2 after the T-cell receptor is activated[20,22]. The NF-ATc has two subunits, one of which is confined to the cytoplasm, while the other is mostly nuclear. An increase in intracellular calcium allows the cytoplasmic unit to move into the nucleus, where it combines with the nuclear component and allows the formation of the IL-2 transcription factor. The immunophilin/drug complexes would inhibit NF-ATc transcription activities, hindering the formation of the functional transcription factor. The signal transduction cascade starts at the presentation of the antigen to the T-cell receptor, which induces an increase in intracellular calcium, the activation of the calcium/calmodulin complex and the formation of the competent T-cell transcription factor (NF-ATc). The specific role of calcineurin is not entirely clear, but it is widely accepted that dephosphorylation induces the translocation into the nucleus of cytoplasmic NF-ATc, a process that can be blocked by the immunophilin/drug complexes. The liaison of DNA and the genetic transcription of IL-2 require both nucleic and cytoplasmic subunits of NF-ATc. Tacrolimus stops transduction pathways of the signal and therefore hinders the IL-2 production by means of the intracellular action of the drug-FKBP-12 complex. The tacrolimus molecule can therefore be divided into two separate functional groups[22]: a binding group for the drug-FKBP-12 complex, and an effector group to bind to calcineurin (Figure 1).

Tacrolimus, a lipophilic drug, exhibits variable absorption and first pass metabolism when administered orally and this can influence its efficacy and toxicity. P-glycoprotein (P-gp, also known as ABCB1), an ATP-dependent membranous transporter which helps to protect the body against toxic xenobiotics by extruding these compounds out of cells and into the intestinal lumen and bile[23], can limit the oral bioavailability and influence the disposition of the calcineurin inhibitors[24-28]. In particular, the presence of P-gp in the intestine can limit tacrolimus absorption. Also, its presence in liver and kidney promotes tacrolimus efflux into bile and urine, respectively.

However, the conclusions drawn so far on the actual influence of P-gp SNPs on tacrolimus pharmacokinetics are highly controversial[29-31].

Additionally, CYP3A4 and CYP3A5, which exhibit variable levels of activity among transplant patients, are the primary enzymes responsible for the metabolism of the calcineurin inhibitors. The same drugs are also known inhibitors of P-gp and of the CYP enzyme system, so that they inhibit their own metabolism and excretion[32]. Other factors that can influence the pharmacokinetics of the calcineurin inhibitors include, but are not limited to, transplant type, baseline renal and hepatic function, concomitant use of corticosteroids, which induce both CYP3A and P-gp activity, patient age and race, time after transplantation, albumin and hematocrit concentration, trauma and food administration[33-40].

As a result, therapeutic drug monitoring is typically initiated after transplantation to facilitate the choice of the dosage of the calcineurin inhibitors and ensure appropriate levels of exposure to these medications. To date the most widely used parameter for the therapeutic monitor-

ing of tacrolimus is its trough whole blood concentration (C_0), which is measured 12 h after the dose administration and correlates well with the area under the concentration-time curve (AUC_{0-12})[41,42].

In practice, target trough levels for tacrolimus are typically set at around 10 ng/mL, but this can vary depending on individual patient characteristics, type of transplant and time after transplantation. Pharmacogenetics is estimated to account for between 20%-95% of drug variability in patients and this has prompted research to assess the feasibility of genotyping as a mean to more rapidly and accurately determine the appropriate starting and maintenance dosages of the immunosuppressant[43].

Also to assess the economic advantage of the genotypic determinations, a number of pharmacodynamics studies have been undertaken to define the overall impact of a more delayed optimization of the drug dosage on patient outcomes. Typically these studies evaluate the effects of sub- or supra-therapeutic calcineurin inhibitor drug levels on graft life, patient mortality and development of various drug toxicities. The calcineurin inhibitors are known to be endowed with a number of possible deleterious effects including seizures, tremors, nephrotoxicity, malignancy, hyperglycemia, hypertension, insomnia, hyperesthesia and hyperlipidemia[4]. They are also expensive and potentially life-long medications that can impose a heavy economic burden on patients and on the health care system in general. As a result, it would be beneficial to rapidly attain target blood trough drug levels in order to avoid side-effects, limit costs and assure appropriate level of immunosuppression.

GENETIC POLYMORPHISMS

To date, a number of SNPs have been studied in relation to the dosing of tacrolimus. However, alleles relating to the following three genes have been the most frequently studied and shown to be the most promising.

CYP3A4

CYP3A4, located in the liver, jejunum, colon, and pancreas, is polymorphically expressed, with at least 42 SNPs identified to date[44]. The most known *CYP3A4* polymorphisms are *CYP3A4*1B* (A392G)[45], *CYP3A4*2* (Ser 222 Pro), and *CYP3A4*3* (Met 445 Thr)[46].

The primary polymorphism implicated and studied in the metabolism of the calcineurin inhibitors occurs at position 392 and is an A>G substitution that produces a variant allele with diminished enzymatic activity, referred to as CYP3A4*1B[47-51]. On the other hand, researchers have demonstrated that CYP3A4 expression is higher in carriers of the mutant allele due to reduced binding of a transcriptional repressor[52,53]. Consequently, the functional significance of this SNP is controversial and *in vivo* studies have generally failed to evidence an association between this polymorphism and the metabolism of various drugs[54-56]. This allele has been shown to occur in 2%-10% of Caucasians, 4.2%-11% of Hispanics, 35%-67% of African-Americans, and about 0% of Asians[57-60].

CYP3A5

CYP3A5 in the liver, small intestine, stomach and kidney shows polymorphic expression, which is currently known to occur with at least 11 different SNPs. The most important polymorphism is that of the CYP3A5*3, which, in homozygous condition, determines the absence of the enzyme, since the variant sequences A→G at nucleotide 6986 in intron 3 of the *CYP3A5* gene cause alternative splicing and the formation of a truncated protein that is not functional[61,62]. On the contrary, the G6986A (CYP3A5*1) allele is correlated with a high expression of the protein[63]. Consequently, individuals that exhibit homozygous expression of the variant allele CYP3A5*3 are often referred to as "CYP3A5 non-expressers". Patients with at least one CYP3A5*1 wild type allele are able to produce functional CYP3A5 enzymes and are known as "CYP3A5 expressers"; they have a different pattern of metabolite formation compared with the non-expressers, resulting also in the belief that CYP3A5 expression in the kidney may play a protective role against the development of nephrotoxicity by limiting the exposure of the organ to toxic metabolites[64-66]. Several studies have also suggested a link between CYP3A4*1B and the CYP3A5*1 wild type allele, as these two allelic variants appear generally to be inherited together[59,61,67-69]. Again the CYP3A5*1 wild type allele is differently distributed among the races and occurs in 5%-15% of Caucasians, 15%-35% of Asians, 25% of Mexicans, and 45%-73% of African-Americans[57].

ABCB-1 (MDR-1, P-gp)

The multidrug resistance-1 (MDR-1) gene, which encodes for the P-gp (ABCB-1) efflux pump in many organs and tissues (*e.g.*, liver, kidney, hematoencephalic barrier, blood testis barrier, maternal side of the placenta, adrenal glands and small intestines), is also polymorphically expressed, with at least 50 currently known SNPs. Its name derives from the fact that it was first found in tumor cell lines where it enhanced the resistance to antineoplastic drugs[23,70-74].

The most commonly studied *ABCB1* polymorphisms include a C to T substitution at position 3435 on exon 26, a C to T substitution at position 1236 on exon 12, and a G to T/A substitution at position 2677 on exon 21[24].

These three variant alleles have been shown to typically occur together, exhibiting a linkage disequilibrium that suggests that they may be further genetically linked[74-78].

Several studies have also suggested that this haplotype results in diminished P-gp expression *in vivo* and, in turn, in lower drug efflux activity. Theoretically this could result in tacrolimus accumulation in the blood stream and nervous system and, as a result, in symptoms of neurotoxicity[79,80].

In addition, recent data have suggested that 3435C>T may reduce MDR-1 mRNA stability in the liver[81] or affect the insertion and folding of P-gp into the membrane, resulting in an altered substrate specificity of the transporter[82].

This haplotype occurs in 5% of African-Americans, 27%

Table 1 Effect of *CYP3A4*1B* single nucleotide polymorphism on tacrolimus pharmacokinetics

Ref.	Study population	Transplant type/analysis of recipients, donors or both	Findings
Cho *et al*[84]	70 Korean	Kidney recipients	No association between *CYP3A4*1B* genotype and tacrolimus dose requirements up to 6 mo after transplantation
Roy *et al*[85]	38 Caucasian, 4 Black, 2 Asian	Kidney recipients	No correlation between the *CYP3A4*1B* SNP and tacrolimus pharmacokinetic at first week and third month after transplantation
Hesselink *et al*[67]	37 Caucasian, 9 Black, 18 Asian	Kidney recipients	*CYP3A4*1B* allele carriers had lower tacrolimus dose-adjusted trough levels with respect to patients carrying the wild-type (*1/*1) genotype at third and 12th month after transplantation This effect was not observed when analyzing only the Caucasian population.
Hesselink *et al*[87]	120 Caucasian, 7 Black, 8 Asian, 1 other	Kidney recipients	No significant correlation observed between *CYP3A4*1B* SNP and tacrolimus pharmacokinetics when *CYP3A5* and *ABCB1* SNPs were taken into account
Gervasini *et al*[33]	103 Spanish	Kidney recipients	Carriers of the *CYP3A4*1B* variant allele had 59% lower tacrolimus concentrations than those with *CYP3A4*1/*1* wild type genotype All CYP3A4*1B carriers were also carriers of *CYP3A5*1* allele (linkage disequilibrium)

of Asians, 32% of Caucasians and 35% of Mexicans[77].

GENETIC INFLUENCE ON TACROLIMUS PHARMACOKINETICS

A number of clinical studies have begun to evaluate the actual impact of the previously described polymorphisms on tacrolimus dosing, efficacy and toxicity. We will now review a number of these studies and summarize their findings before analyzing the potential clinical implications of their data.

*CYP3A4*1B*

Data regarding the influence of *CYP3A4* polymorphisms on tacrolimus pharmacokinetics are often inconsistent and confounded by the highly frequent linkage disequilibrium found between the CYP3A4*1B variant allele and the CYP3A5*1 wild-type allele[59,61,67-69,83]. The overall impact of the *CYP3A4* genotype on tacrolimus dose requirements appears uncertain and should be further studied.

A study by Cho *et al*[84] on 70 Korean renal transplant patients found no association between *CYP3A4* genotype and tacrolimus dose requirements up to 6 mo after transplantation (Table 1).

Another study, by Roy *et al*[85], confirmed these results, showing no correlation between the CYP3A4*1B (392A>G) SNP and tacrolimus pharmacokinetics (Table 1). However, as other authors have pointed out, due to the limited data available it is not possible to understand if these results were influenced by the ethnicity or by a genetic linkage with the CYP3A5 6986A>G SNP[86].

In a study on 64 kidney transplant patients, Hesselink *et al*[67] showed that patients carrying the *CYP3A4*1B* allele had lower tacrolimus dose-adjusted trough levels with respect to patients carrying two copies of the wild-type *1 allele. This effect was not observed when the analysis was made only in the Caucasian population (Table 1).

However, in a further study carried out in a more consistent population composed of 136 renal transplant patients the same authors found that there was no significant correlation between the CYP3A4*1B (392A>G) SNP and tacrolimus pharmacokinetics (dose and C_0/Dose) when the influences of the CYP3A5 6986A>G SNP and ABCB1 polymorphisms were taken into account[87] (Table 1).

In another study on 103 Spanish renal transplant patients, Gervasini *et al*[33] found that carriers of the CYP3A4*1B variant allele displayed tacrolimus concentrations that were on average 59% lower than those of patients with the *CYP3A4*1/*1* genotype. The dose-adjusted trough levels observed were 145.59, 86.89, and 58.21 ng/mL per mg/kg per day for the 3A4*1-3A5*3, 3A4*1-3A5*1 and 3A4*1B-3A5*1 haplotypes, respectively, suggesting that the CYP3A4*1B-CYP3A5*1 haplotype may have a more profound impact on tacrolimus pharmacokinetics than the *CYP3A5*1* allele alone (Table 1). However, because of the linkage disequilibrium between the CYP3A4 and *CYP3A5* polymorphisms, all *CYP3A4*1B* carriers were also carriers of the *CYP3A5*1* allele.

*CYP3A5*3*

Many studies have confirmed that *CYP3A5* polymorphisms have a major influence on the pharmacokinetics of tacrolimus. Consistently, patients homozygous for the *CYP3A5*3* allele have shown lower dose requirements and higher whole blood trough levels of tacrolimus after transplantation, as well as clearances of the drug 25%-45% lower than patients expressing the *CYP3A5*1* allele. In liver transplant patients, donor genotype has also generally been shown to have more important consequences on tacrolimus pharmacokinetics and dose requirements than recipient genetics[38,63,88-93]. However, it still remains to be seen whether these alterations in the drug pharmacokinetics correlate or not to the patient clinical outcomes.

A study by Barrera-Pulido *et al*[94] on 53 liver transplant recipients found that recipients with the *CYP3A5*1/*3* genotype receiving organs from *1/*3 donors failed to

achieve minimum blood tacrolimus levels at one month post-transplant (Table 2). Between days 30 and 60 post-transplant *3/*3 recipients from *1/*3 donors also had significantly greater tacrolimus dose requirements than recipients from *3/*3 donors.

These results also occurred in a study on 24 Native American kidney transplant recipients where it was observed that after 1 mo from transplant the patients required a significantly lower daily tacrolimus dose than a control group of Caucasian kidney transplant patients (0.03 mg/kg per day *vs* 0.5 mg/kg per day). To explain these data, many of these Native Americans, but not the Caucasians, were found to express the *CYP3A5*3/*3* genotype, associated with diminished CYP3A5 enzymatic activity (Table 2). However, despite the differences in tacrolimus dose requirements, there were no differences in the drug trough levels or the incidence of nephropathy between the two study groups[95].

A study on 32 Caucasian liver transplant patients by Provenzani *et al*[91] found that dose requirements were significantly higher in patients receiving a liver with the CYP3A5*1 allele compared with donors who were homozygous for the *3 polymorphism (0.111 mg/kg per day *vs* 0.057 mg/kg per day). In the organ recipients, the *CYP3A5*1* genotype tended to increase tacrolimus doses, though not to a statistically significant degree (Table 2).

In a case report, the same research group found that a 53-year-old Caucasian male who was homozygous for the *CYP3A5*3* allele and had received a liver from a donor expressing the *CYP3A5*1/*1* genotype required a dose two-fold higher than that reported in the literature for adult liver transplant patients. During the first, second and third week of therapy the patient received tacrolimus doses of 0.219, 0.287, and 0.273 mg/kg per day, respectively, while the trough drug levels obtained remained below the target of 10-12 ng/mL (4.6, 5.6 and 6.1 ng/mL at the first, second and third week of therapy, respectively). The patient reached a target level of 10.4 ng/mL only after one month of therapy. This corroborates that the *CYP3A5*1* allele may be associated with increased hepatic metabolic capacity for tacrolimus and, consequently, delayed response to drug therapy[93].

The authors further confirmed these results when they looked at 51 Caucasian liver and 50 Caucasian kidney transplant recipients at 1, 3, and 6 mo post-transplant and again found that the presence of the *CYP3A5*1* allele in liver donors, but not in recipients, had a statistically significant effect of decrease on the tacrolimus dose-adjusted trough levels. A similar result was also observed in the kidney transplant recipients, where the dose required to achieve and maintain target trough blood levels at 1, 3, and 6 mo was statistically lower in patients homozygous for the *CYP3A5*3* allele compared with the patients expressing at least one copy of the wild type allele *CYP3A5*1*[92] (Table 2).

Another study by Cho *et al*[84] on 70 Korean renal transplant patients found that patients expressing either the *CYP3A5*1/*3* or *CYP3A5*1/*1* genotype, and thus a functional CYP3A5 protein, had tacrolimus dose requirements up to 80% greater than patients homozygous for the *3 allele up to 6 mo post-transplant (Table 2).

Glowacki *et al*[96] in a study on 209 French kidney transplant patients, also found that patients with at least one *CYP3A5*1* allele had significantly higher tacrolimus dose requirements and lower trough drug levels than *3 homozygotes. However, these pharmacokinetic findings appeared to have no influence on the incidence of biopsy-proven acute rejection or on delayed graft function (Table 2). Patients were followed for a mean period of 21.8 mo, with no data suggesting that alterations in tacrolimus pharmacokinetics might have any significant impact on long-term clinical outcomes.

Another study, in 181 Japanese liver transplant recipients and 114 donors, showed that the level of CYP3A5 mRNA was significantly reduced in patients with livers carrying the *CYP3A5*3/*3* genotype (0.41 amol/μg total RNA) *vs* the *1/*1 and *1/*3 genotypes (4.85 and 2.99 amol/μg total RNA, respectively). As a result, the dose-adjusted tacrolimus trough levels were significantly decreased, due to increased metabolism, in patients receiving a liver carrying the *CYP3A5*1/*1* genotype[63] (Table 2).

Wei-lin *et al*[88], in a study on 50 Chinese liver transplant donors as well as recipients, found again that at one month after transplantation, recipients who received organs from *CYP3A5*3/*3* donors had significantly higher dose-adjusted tacrolimus trough levels than the patients receiving livers from *CYP3A5*1* expressers (Table 2). However, neither the donors' ABCB1 genotype nor the recipients' CYP3A5 genotype had any impact on the recipients' tacrolimus pharmacokinetic profile, suggesting once more that in liver transplantation the donors' CYP3A5 genetics, rather than that of the recipient, has a more important effect on tacrolimus dosing.

López-Montenegro Soria *et al*[97] studied 35 kidney transplant patients and found that during the first six weeks after transplant the tacrolimus concentration/dose ratios were remarkably lower for patients expressing at least one *CYP3A5*1* allele compared with those homozygous for the *CYP3A5*3* genotype (0.65 *vs* 1.45), due to higher drug clearances in *CYP3A5*1* expressers (Table 2).

Another trial, by Shi *et al*[66], involving 216 Chinese liver transplant recipients concluded that daily tacrolimus dose requirements were higher for recipients with the *CYP3A5*1/*1* genotype than patients expressing the *3/*3 genotype (3.0 mg per day *vs* 2.0 mg per day). Dose-adjusted tacrolimus trough levels were also lower in the *1/*1 genotype than *1/*3 expressers and in the *3 homozygotes (97.5, 124.8, and 144.4, respectively), suggesting in particular that CYP3A5 enzymatic activity is increased proportionally by the presence of one or two copies of the *1 allele (Table 2).

These results were supported by Jun *et al*[98] in a study of 506 Korean solid organ transplant recipients and 62 corresponding liver transplant donors, which concluded that the blood tacrolimus concentrations per adjusted

Table 2 Effect of *CYP3A5*3* single nucleotide polymorphism on tacrolimus pharmacokinetics

Ref.	Study population	Transplant type/analysis of recipients, donors or both	Findings
Barrera-Pulido *et al*[94]	53 Caucasian	Liver recipients and donors	*CYP3A5*1/*3* recipients with *1/*3 donor livers had lower than minimum required blood tacrolimus levels at 1 mo after transplantation *3/*3 recipients with *1/*3 donors had significantly greater tacrolimus dose requirements at 1 and 2 mo after transplantation
Chakkera *et al*[95]	24 native American and Caucasian control group	Kidney recipients	Native Americans had lower tacrolimus dose requirements than Caucasians at 1 mo after transplantation Native Americans more commonly expressed *CYP3A5*3/*3* No difference in blood trough levels or nephropathy between the two groups
Provenzani *et al*[91]	32 Caucasian	Liver recipients and donors	Dose requirements significantly higher in the case of donors with the *CYP3A5*1* allele at 1, 3 and 6 mo after transplantation No statistically significant difference in dose requirements considering recipient's genotypes
Provenzani *et al*[92]	101 Caucasian	Kidney (n = 50, recipients) and liver (n = 51, recipients and donors)	*CYP3A5*1* allele in liver donors (n = 51) had a significant effect of decrease on tacrolimus dose-adjusted trough levels at 1, 3 and 6 mo after transplantation. No statistically significant difference in dose requirements considering recipient's genotype Tacrolimus dose in kidney recipients (n = 50) with CYP3A5*3/*3 genotype was significantly lower than in patients with at least one copy of the wild type allele
Cho *et al*[84]	70 Korean	Kidney recipients	Those patients who had *CYP3A5*1/*3* or **1/*1* genotypes had 80% higher tacrolimus dose requirements than patients homozygotes for *3 allele (up to 6 mo after transplantation)
Glowacki *et al*[96]	209 French	Kidney recipients	Patients with at least one copy of the CYP3A5*1 allele had significantly higher dose requirements and lower blood trough levels than patients homozygous for the *3 allele No influence of this SNP on rejection or graft dysfunction rates.
Goto *et al*[63]	181 Japanese	Liver recipients and donors	Patients with the *CYP3A5*3/*3* genotype had reduced levels of CYP3A5 mRNA Dose-adjusted tacrolimus trough levels decreased in patients receiving a liver with the *1/*1 genotype
Wei-Lin *et al*[88]	50 Chinese	Liver recipients and donors	Those patients receiving a liver with the *3/*3 genotype had, at first month after transplantation, significantly higher tacrolimus dose-adjusted trough levels than those with at least one copy of the *1 allele
López-Montenegro Soria *et al*[97]	35 Spanish	Kidney recipients	Concentration/dose ratios were remarkably lower in patients with at least one copy of the *1 allele than in patients homozygous for the *3 allele
Shi *et al*[66]	216 Chinese	Liver recipients	Recipients with *1/*1 genotype had higher dosage requirements than those with *3/*3 genotype The study suggested also that CYP3A5 enzymatic activity is increased proportionally by the presence of the *1 allele
Jun *et al*[98]	568 Korean	Kidney and liver recipients (n = 506), and liver donors (n = 62)	Patients with the *3 alleles had higher tacrolimus dose-adjusted trough levels than patients with the *1 allele *1/*1 patients may be more rapid metabolizers than *1 heterozygous patients
Elens *et al*[99]	150 Belgian	Liver donors	Those patients with at least one *1 allele had at least 67% higher tacrolimus dose requirements No influence of CYP3A5 expression on tacrolimus hepatic concentrations
Macphee *et al*[100]	119 White, 23 Black, 26 South Asian, 12 Middle Eastern	Kidney recipients	Patients with at least one copy of the wild-type *1 allele achieved twofold lower dose-normalized tacrolimus blood concentrations compared with *CYP3A5*3/*3* homozygote patients
Thervet *et al*[101]	168 Caucasian, 8 Black, 12 other	Kidney recipients	Pre-transplant dose adaptation, according to *CYP3A5* genotype, is associated with improved achievement of the target blood trough levels
Spierings *et al*[102]	81 Caucasian, 12 Black, 20 South Asian, 5 other	Kidney recipients	Tacrolimus dose requirements were significantly higher in patients expressing the wild type *CYP3A5* genotype Intra-patient variability of tacrolimus clearance was not associated with the same genotype
Chen *et al*[103]	120 Chinese	Kidney recipients	CYP3A5 expressers not receiving diltiazem required significantly higher tacrolimus doses than those who received the CYP inhibitor. In non-expressers, no significant difference in tacrolimus dose requirements was observed between the subjects treated with diltiazem and those who were not

dose ratio was significantly higher in recipients with the *1/*3 genotype than in those with the *1/*1 one, and again higher in *3/*3 patients rather than in heterozygous *1/*3 recipients, suggesting that *1 homozygous patients may be even more rapid metabolizers than heterozygous patients expressing only one *1 allele (Table 2).

A study by Elens *et al*[99] on 150 liver donors found that tacrolimus dose requirements were at least 67% higher among patients with at least one *CYP3A5*1* allele and expressing hepatic CYP3A5 (Table 2). However, though hepatic CYP3A5 expression reduced blood tacrolimus levels and increased dose requirements, it failed to influence hepatic tacrolimus concentrations, which may be better related to liver graft outcome[99].

Another study, by Macphee *et al*[100], in white and South Asian renal transplant patients, suggested that patients with at least one copy of the wild-type *1 allele achieved twofold lower dose-normalized tacrolimus blood concentrations compared with *CYP3A5*3/*3* homozygote patients (Table 2).

In a prospective study involving 280 kidney transplant patients, Thervet *et al*[101] found that a pre-transplant tacrolimus dose adaptation according to the *CYP3A5* genotype is associated with fewer successive dose modifications and with a rapid achievement of target trough levels (Table 2).

In a more recent study by the Macphee's group on 118 renal transplant patients, Spierings *et al*[102] confirmed that the tacrolimus dose requirements were significantly higher in patients with the wild type *CYP3A5* genotype (Table 2). However, they also found that intra-patient variability of tacrolimus clearance was not associated with the wild type *CYP3A5* genotype.

Finally, in a 42-mo, prospective, randomized, parallel-controlled, open-label, single-center study, 62 Chinese CYP3A5 expressers and 58 non-expressers who had received kidney transplants were randomized to receive 30 mg of diltiazem (a known CYP inhibitor) three times daily in order to assess the efficacy of the drug as a calcineurin sparing agent. Patients who were known to be CYP3A5 expressers and did not receive diltiazem required significantly higher tacrolimus doses than the other groups (P = 0.017). Among the CYP3A5 non-expressers, there was not a significant difference in tacrolimus dose requirements between the subjects treated with diltiazem and those who were not. This was expected, as the proposed mechanism for diltiazem as a calcineurin sparing agent involves the inhibition of the metabolism of tacrolimus through the CYP3A5 pathway (Table 2). This suggests that CYP3A5 expressers are more susceptible to diltiazem-induced tacrolimus dose reductions and may possibly provide the prescribers with a mechanism able to limit the cost of immunosuppressive therapy as well as to treat concomitant hypertension in transplant patients[103].

ABCB1

Data showing a link between a patient's ABCB1 genotype and tacrolimus pharmacokinetics have been inconsistent. Though most studies have failed to find any association, some clinical trials have found a significant relation between the *ABCB1* genotype and tacrolimus dosing. These results are often confounded by the linkage disequilibrium expressed among genetic variants, underscoring the need for further research on *ABCB1* genetics before a definitive conclusion can be reached.

Provenzani *et al*[91], in a study on 32 Caucasian liver transplant patients, found no influence of the 3435C>T and 2677G>T SNPs on tacrolimus dose requirements (Table 3). A study by Cho *et al*[84] on 70 Korean renal transplant patients also found no association between the ABCB1 genotype and tacrolimus dose requirements up to 6 mo after transplantation (Table 3).

Further supporting these results, Shi *et al*[66] found that in 216 Chinese liver transplant patients, there was no significant association between any of the *ABCB1* polymorphisms and daily tacrolimus dose requirements or trough levels (Table 3).

This was again confirmed by Jun *et al*[98], who studied 506 Korean solid organ transplant recipients and 62 corresponding liver transplant donors. They found no correlation between the *ABCB1* patient genotype and tacrolimus concentration to adjusted dose ratios (Table 3).

Gervasini *et al*[33] also found that, in 103 renal transplant patients, none of the *ABCB1* polymorphisms were associated with altered dose-adjusted trough levels or increased dose requirements. This study also found no association between the *ABCB1* genotype and tacrolimus-induced toxicity (Table 3).

Another study by Kuypers *et al*[104] found that in 304 kidney transplant patients the *ABCB1* genotype had no significant impact on tacrolimus exposure parameters or dosing requirements (Table 3).

A study by Provenzani *et al*[92] on 51 liver and 50 kidney transplant patients found no association between the *ABCB1* polymorphisms and tacrolimus dosing among liver transplant patients, but did observe that kidney transplant patients carrying the 2677T/A allele required a significantly higher daily tacrolimus dose than patients homozygous for the wild type allele (Table 3).

Another study on 181 liver transplant recipients and 114 donors found that, in the first week post-transplantation, the recipients who displayed the wild type *MDR-1* allele and thus high *ABCB-1* activity in the intestine, had lower dose-adjusted tacrolimus trough levels than patients who displayed *MDR-1* variant alleles and were low ABCB-1 expressers, even among patients with the same liver *CYP3A5* genotype. However, this difference was not observed after two weeks, suggesting that *MDR-1* expression in the intestine may contribute to tacrolimus trough levels in the first week post-transplantation; afterwards the transplanted liver would achieve a greater metabolic capacity and becomes the main organ that influences tacrolimus pharmacokinetics[63] (Table 3).

This was supported by Herrero *et al*[43] in a study on 71 renal transplant patients, in which it was found that patients with the wild type *ABCB1* genotype tended to have more stable tacrolimus concentrations within the

Table 3 Effect of *ABCB1* single nucleotide polymorphism on tacrolimus pharmacokinetics

Ref.	Study population	Transplant type/analysis of recipients, donors or both	Findings
Provenzani *et al*[91]	32 Caucasian	Liver recipients and donors	No influence of 3435C>T and 2677G>T SNPs on tacrolimus dose requirements
Cho *et al*[84]	70 Korean	Kidney recipients	No association between *ABCB1* genotype and tacrolimus dose requirements
Shi *et al*[66]	216 Chinese	Liver recipients	No association between any ABCB1 SNPs and tacrolimus dose requirements or blood trough levels
Jun *et al*[98]	568 Korean	Kidney and liver recipients (n = 506), and liver donors (n = 62)	No correlation between *ABCB1* genotype and tacrolimus dose-adjusted blood trough levels
Gervasini *et al*[33]	103 Spanish	Kidney recipients	None of the *ABCB1* polymorphisms were associated with changes in dose-adjusted blood trough levels and in dose requirements No association between *ABCB1* genotype and tacrolimus-induced toxicity
Kuypers *et al*[104]	304 Belgian	Kidney recipients	No significant impact of *ABCB1* genotype on tacrolimus exposure parameters or dosing requirements
Provenzani *et al*[92]	101 Caucasian	Kidney (n = 50) and liver (n = 51, recipients and donors)	No ABCB1 influence on dosing in liver transplant patients Those patients receiving kidney transplant carrying the 2677T/A allele required significantly higher doses than those patients with the wild type allele
Goto *et al*[63]	181 Japanese	Liver recipients and donors	In the first week after transplantation, the recipients with wild type ABCB1 allele had lower tacrolimus dose-adjusted blood trough levels No difference observed after 2 wk
Herrero *et al*[43]	71 Spanish	Kidney recipients	Patients with wild type *ABCB1* alleles had more stable tacrolimus concentrations within the therapeutic range during the first 3 mo On the contrary, patients carrying the polymorphic *ABCB1* alleles showed a mean increase in tacrolimus blood concentration of more than 60%
Wei-Lin *et al*[88]	50 Chinese	Liver recipients and donors	Recipients with the wild type *ABCB1-3435CC* allele had significantly higher tacrolimus dose requirements than those with C3435T at 1 and 2 wk and 1 mo after transplantation
López-Montenegro Soria *et al*[97]	35 Spanish	Kidney recipients	Wild type ABCB1 3435CC patients had 40% lower concentration/dose ratios than those patients with variant alleles
Elens *et al*[99]	150 Belgian	Liver donors	*ABCB1* genetic polymorphisms significantly influence tacrolimus hepatic concentrations, but have no effect on tacrolimus blood levels Patients with *ABCB1 1236C>T* polymorphism showed significantly better liver functions and lower Banff scores with respect to patients with the wild-type allele

therapeutic range during the first 3 mo after transplantation, while patients expressing polymorphic ABCB1 alleles showed a mean increase in the drug blood concentrations greater than 60% due to a diminished elimination capacity by the body (Table 3).

A study on 50 Chinese liver transplant donors and recipients also evidenced that daily tacrolimus dose requirements were significantly higher in recipients carrying the wild type ABCB1-3435CC rather than the *C3435T* allele at the weeks 1 and 2 and at 1 mo post-transplantation (Table 3). These data suggested that in Chinese people the *ABCB1* genotype plays a dominant role in the intestinal tacrolimus pharmacokinetics[88]: in fact patients with the wild type *MDR-1* genotype are more likely to extrude tacrolimus from enterocytes and therefore need a higher daily dose to achieve adequate blood tacrolimus levels.

López-Montenegro Soria *et al*[97], in a study on 35 renal transplant patients, also found that patients expressing the wild type *ABCB1-3435CC* genotype showed up to 40% lower concentration/dose ratios compared with patients carrying variant alleles (Table 3).

Finally, a study by Elens *et al*[99] on 150 liver transplant patients found that *ABCB1* genetic polymorphisms in the donors significantly influenced tacrolimus concentrations in the liver, but failed to influence the drug mean blood levels. The *ABCB1-1236C>T* polymorphism was also associated with improved liver function and significantly lower Banff scores compared with the situation of patients with the wild type allele (Table 3). These data suggest that *ABCB1* polymorphisms may be important in liver transplant patients due to their effects on tacrolimus levels in the liver, which, as already said, may be a good marker to predict the liver graft rejection.

INFLUENCE OF GENETICS ON TACROLIMUS PHARMACODYNAMICS

Despite many studies have demonstrated a strong association between *CYP3A5* genotype and alterations in tacrolimus pharmacokinetics, the results do not provide consistent evidence of organ rejection or drug-related toxicity as a consequence of genotype-related sub- or supra-therapeutic immunosuppression. This is likely due to the fact that the

patients are closely monitored in the first period following transplantation and undergo dose adjustments to more rapidly achieve target trough drug levels. However, different clinical trials have begun to explore the practical pharmacodynamics implications of genetic alterations in tacrolimus pharmacokinetics, and some of them have found clinically significant results.

Jun *et al*[98] found no significant difference in the incidence of organ rejection in 506 Korean solid organ transplant recipients and 62 liver transplant donors after comparing both patients' genotypes and mean tacrolimus concentration per an adjusted dose ratios (Table 4).

Another study by Chen *et al*[103] on 120 Chinese kidney transplant patients who were a mix of CYP3A5 expressers and non-expressers, found that patients who received genotype-guided initial tacrolimus dosing achieved target drug levels more rapidly than the patients who received a standard protocol dose of tacrolimus (90.9% *vs* 27.3% of patients in target range, respectively). However, no differences were observed between the two groups with respect to the incidence of leukocytopenia, nephropathy, abnormal liver function, hyperlipidemia, diarrhea or hyperglycemia (Table 4).

Jacobson *et al*[105], in a prospective study on 945 kidney transplant patients, found that every increase in tacrolimus trough level of 1 ng/mL increased the hazard of early calcineurin-inhibitor-associated nephrotoxicity by 22%, even after adjusting for clinical factors. Nine SNPs of the *XPC*, *CYP2C9*, *PAX4*, *MTRR* and *GAN* genes exhibited an association with cyclosporine, but not with tacrolimus, nephrotoxicity (Table 4).

In a prospective, open-label, observational cohort study, Kuypers *et al*[106] found that among 304 kidney transplant patients, the proportion of patients who developed new-onset diabetes after transplant (NODAT) was significantly higher in patients with delayed graft function and who displayed trough tacrolimus levels greater than 15 ng/mL on the first day post-transplantation. In this study, the presence of the *CYP3A5*1* allele and a functional *CYP3A5* enzyme appeared to attenuate the effects of delayed graft function on initial tacrolimus exposure and dose requirements, suggesting that CYP3A5 expressers may be at lower risk of NODAT following kidney transplantation due to diminished exposure to potentially toxic levels of tacrolimus (Table 4).

In a separate study, but in the same population of 304 kidney transplant patients, Kuypers *et al*[104] found that calcineurin-inhibitor-associated nephrotoxicity (CNIT) was more common in patients carrying the CYP3A5*1 allele than in patients who did not (32.4% *vs* 15.2%). Additionally, these researchers observed that CNIT developed in 25% of patients with dose requirements exceeding 0.2 mg/kg per day, 16.2% of patients with doses between 0.1-0.2 mg/kg per day and 4.5% of patients needing less than 0.1 mg/kg per day; the carriers of the *CYP3A5*1* allele predominantly comprised the higher tacrolimus dose ranges. These results suggest that patients expressing the *CYP3A5*1* allele and a functional CYP3A5 enzyme may be predisposed to developing CNIT following transplantation due to greater daily tacrolimus dose requirements. This was observed especially in patients who continued corticosteroid therapy (Table 4). However, the incidence of delayed graft function and post-transplant diabetes mellitus was not different between CYP3A5 expressers and non-expressers.

In a more recent study, on 319 Hispanic kidney transplant patients, other authors found that the SNPs in the cytoplasmic nuclear factor of activated T cells 4 (NFATc4) gene, which is expressed in pancreatic islets, may confer a certain protection or also a predisposition with regard to NODAT; in particular, the patients carrying the SNP (rs10141896) T allele (T-T-T-T-G haplotype) showed a protection from NODAT, while patients homozygous for the C-C-C-G-G haplotype were associated with increased risk of NODAT. Furthermore, the authors found that the use of sirolimus and tacrolimus and a more advanced age (> 45 years) were also possibly correlated to the development of NODAT[107] (Table 4).

Cho *et al*[84] found that in 70 Korean renal transplant patients tacrolimus toxicity was more frequent in the subjects with *CYP3A5*1* alleles, who had significantly higher dose requirements of the drug than patients expressing the *3 polymorphism (Table 4). Despite these findings, the study found no difference in the rate of graft survival between the various genotype-differentiated study groups.

A study by Barrera-Pulido *et al*[94] on 53 liver transplant recipients found that patients with the *CYP3A5*3/*3* genotype receiving the organs from donors with an *ABCB1* polymorphism had a lower frequency of renal dysfunction, the same rejection rate and a higher rate of diabetes than the other groups studied (Table 4).

However, Shi *et al*[66] found that in 216 Chinese liver transplant patients, carriers of the *CYP3A5*3* allele had an increased risk of early renal injury compared with expressers of the *CYP3A5*1* allele, possibly due to decreased enzymatic activity and higher dose-adjusted trough concentrations (Table 4).

CONSIDERATIONS FOR FURTHER GENETIC RESEARCH

In addition to the previously discussed genetic polymorphisms, a number of other variants that may potentially influence the pharmacokinetics and pharmacodynamics of tacrolimus and transplant outcomes have been proposed for further study.

*P450 oxidoreductase*28*

Cytochrome P450 oxidoreductase (POR) is essential for the electron donation in the microsomal-CYP450-mediated mono-oxygenation that catalyzes the metabolism of approximately 85%-90% of therapeutic drugs. More than 40 SNPs have been identified in the *POR* gene, and it has been suggested that several of these mutations, specifically the *POR*28-C>T* polymorphism, can increase this

Table 4 Effect of various single nucleotide polymorphisms on tacrolimus pharmacodynamics

Ref.	Study population	Transplant type/analysis of recipients, donors or both	Findings
Jun *et al*[98]	568 Korean	Kidney and liver recipients (n = 506), and liver donors (n = 62)	No difference in incidence of organ rejection between different genotypes
Chen *et al*[103]	120 Chinese	Kidney recipients	Patients that received genotype-guided initial tacrolimus dosing vs standard protocol dose were more likely to achieve target drug levels No influence on incidence of adverse effects between CYP3A5 expressers and non-expressers
Jacobson *et al*[105]	945 (different ethnicities)	Kidney recipients	Every increase in tacrolimus blood trough level of 1 ng/mL increased the risk of early tacrolimus nephrotoxicity by 22% Polymorphism was not associated with an increased or decreased risk of tacrolimus-related nephrotoxicity
Kuypers *et al*[106]	273 White, 3 Hispanic, 24 North African, 2 African, 2 Asian	Kidney recipients	Delayed graft function was associated with higher initial mean tacrolimus blood trough levels and lower tacrolimus daily dose requirements, especially in CYP3A5 non-expressers CYP3A5 expressers may be at lower risk of new-onset diabetes after transplant (NODAT) due to diminished exposure to potentially toxic tacrolimus levels
Kuypers *et al*[104]	273 White, 3 Hispanic, 24 North African, 2 African, 2 Asian	Kidney recipients	Patients expressing the *CYP3A5*1* allele and a functional CYP3A5 enzyme may be predisposed to developing calcineurin-inhibitor-associated nephrotoxicity (CNIT) following transplantation due to greater daily tacrolimus dose requirements This was observed especially in patients continuing corticosteroid therapy The incidence of delayed graft function and post-transplant diabetes mellitus was not different between CYP3A5 expressers and non-expressers
Chen *et al*[107]	319 Hispanic	Kidney recipients	SNPs in the cytoplasmic *NFATc4* gene may confer a certain protection or also predisposition for NODAT. Patients carrying the T allele and the T-T-T-T-G haplotype showed a trend of protection from NODAT while patients with the C-C-C-G-G haplotype were associated with an increased risk of NODAT The use of sirolimus and tacrolimus and advanced age were also possibly correlated in development of NODAT
Cho *et al*[84]	70 Korean	Kidney recipients	Higher drug-related toxicity in patients with the *CYP3A5*1* allele than in those with the CYP3A5*3 SNP No difference in graft survival between the two genotypes
Barrera-Pulido *et al*[94]	53 Spanish	Liver recipients and donors	Patients with *CYP3A5*3/*3* allele receiving livers with an ABCB1 SNP had lower frequency of renal dysfunction, same rejection rate and higher diabetes rate
Shi *et al*[66]	216 Chinese	Liver recipients	Patients with the *CPY3A5*3* allele had greater risk of early renal injury than the patients with the *1 allele

activity and alter the baseline metabolic capacity of several CYP isoforms. The *28 allelic variant has been found to be expressed in 19.1% of African-Americans, 26.4% of Caucasian Americans, 31.0% of Mexican Americans, and 36.7% of Chinese Americans[108].

A study by Zhang *et al*[109] on 71 healthy Chinese volunteers found that the mean tacrolimus $AUC_{(0\text{-}24)}$ and C_{max} (71.5 and 17.6 ng/mL, respectively) for patients who were CYP3A5 expressers as well as carriers of the wild type CC POR genotype were 1.53 and 1.57 fold higher than those (46.7 and 11.2 ng/mL) observed in patients carrying POR allelic variants. No significant differences were observed between POR*28-CC homozygotes and POR*28-T carriers in CYP3A5 non-expressers, suggesting that the POR genotype is important in altering tacrolimus metabolism only in CYP3A5 expressing patients.

These results were supported by a cohort study of de Jonge *et al*[110] on 298 renal transplant recipients, which it was found that in CYP3A5 expressers, *POR*28T* allele carriers had lower trough tacrolimus levels in the first three days post-transplant and took longer to reach the target trough levels when compared with *POR*28CC* homozygous patients. These patients with the variant POR genotype ultimately had 25% higher tacrolimus dose requirements than patients expressing the wild type allele. Again, POR*28 polymorphisms were found to have no influence on tacrolimus pharmacokinetics in CYP3A5 non-expressers, and no differences in transplant outcomes were observed between the study groups.

CYP3A7

Previously thought to be confined to the fetal liver, CYP3A7 has been found to be expressed in up to 54-88% of adult livers, but with a diminished metabolic capacity compared with that observed in children[111,112]. The role of CYP3A7 in the biotransformation of the CYP3A substrates in the adult liver and intestine is unknown. However, it was observed that CYP3A7 expression in the adult liver and intestine is increased in the carriers of the *CYP3A7*1C* allele[112,113]. This allele has a very low frequency (3%) both

in Caucasians and African-Americans[61].

Although tacrolimus is believed to be a substrate for the CYP3A7 enzyme, the influence of CYP3A7 metabolism on the pharmacokinetics of tacrolimus requires further study, especially in pediatric patients[99].

*CYP3A4*18B*

This *CYP3A4* polymorphism appears only in Asian (25%-30%)[114], primarily Korean, populations, but has been linked with a potentially increased metabolic capacity of the CYP3A4 enzyme. It has also been shown that carriers of the *CYP3A5*1* allele are more likely to possess the *CYP3A4*18B* allele. As a result, further study is required to determine whether linkage disequilibrium with the *CYP3A5*1* allele may confound the observed metabolic effects of the *CYP3A4*18B* polymorphism. One study, by Jun *et al*[98], found no correlation between the *CYP3A4*18* allele and tacrolimus concentration to adjusted dose ratios in 506 Korean solid organ transplant recipients. A study of 22 healthy Chinese people showed a higher tacrolimus clearance in patients carrying the *CYP3A4*18B* allele with respect to those carrying the *CYP3A4*1* allele[115]. A more recent study, by Li *et al*[116], on 83 Chinese renal transplant recipients confirmed the results of the previous study. It found that the tacrolimus-dose-adjusted trough concentration was significantly lower in patients carrying the *CYP3A4*18B* allele compared with patients with the *CYP3A4*1* allele.

*CYP3A4*22*

A new *CYP3A4* allele (*CYP3A4*22*; rs35599367 C>T in intron 6) was recently discovered and also investigated in transplant patients[117,118].

In particular, a study on 185 renal transplant patients, mostly Caucasians, evaluated the impact of this new SNP on tacrolimus pharmacokinetics. It showed that in the first year after transplantation, patients carrying one or two T alleles required significantly lower tacrolimus doses (33%) compared with patients homozygous for the wild-type C allele[118]. The authors attributed the result to the fact that this *CYP3A4*22* SNP is significantly linked to reductions in CYP3A4 mRNA production and enzyme activity in human livers[118-120]. This SNP is relatively frequent in Caucasians (2.5%-6.9%). The authors also suggested that, though further studies are necessary, that pre-transplant genotyping of the CYP3A4 C>T could reduce the risk of achieving supra-therapeutic tacrolimus levels[118].

However, in a study done on Brazilian renal transplant patients, CYP3A4*22 was not associated with changes in tacrolimus dose requirements[121].

CYP2C8 and CYP2J2

These enzymes, which are polymorphically expressed in the kidney, are involved in the synthesis of epoxyeicosatrienoic acids that play a protective role against acute rejection and toxicity by acting as vasodilators to maintain adequate renal perfusion and limit hypertension.

In a study on 163 liver transplant patients the authors found that patients with the *CYP2C8*3* variant genotype appeared to be at higher risk of tacrolimus-induced kidney disease, possibly because of reduced formation of the kidney protecting epoxyeicosatrienoic acids[122].

In another study, on 103 renal transplant patients, the authors found a higher incidence of delayed graft function and nephrotoxicity in patients homozygous for the *CYP2C8*3* genotype, associated with reduced epoxyeicosatrienoic acid production and, consequently, less vasodilator activity[33].

In a more recent study the same research group could associate both *CYP2C8*3* and donor age (> 48 years) with a higher incidence of delayed graft function and poorer creatinine clearance[123].

SLC01B1

This gene is responsible for expressing the organic anion transporting polypeptides *OATP1B1* and *OATP1B3*. These transporters play a role in the transport of multiple compounds from the portal vein to hepatocytes and in the biliary excretion of many drugs. Recently, Elens *et al*[99] found that the 388A>G and 521T>C polymorphisms in the *SLCO1B1* gene influenced tacrolimus trough blood concentrations after the administration of the first dose in 150 liver transplant patients. In this study, patients expressing the 388 polymorphism showed a lower mean tacrolimus blood level, while alterations of the 521 allele resulted in significantly greater trough drug levels. It was also recently demonstrated that cyclosporine and tacrolimus are inhibitors of the organic anion transporters, so that one cannot exclude the possibility that these drugs may be substrates of OATP1B1 and OATP1B3 as well.

Angiotensinogen C3889T (rs4762) gene polymorphism

It is well known that tacrolimus has a negative effect on pancreatic beta islet cells and can cause glucose intolerance and diabetes mellitus[124]. However, new studies have suggested that post-transplant diabetes mellitus can also be related to other factors and, consequently, not only to tacrolimus administration[124,125]. Angiotensinogen (AGT) is the initial component of the renin-angiotensin system (RAS) and a precursor of both angiotensin I and Ⅱ. In a study on 302 subjects, the authors found that the *AGT* gene polymorphism (rs4762) is associated with post-transplant diabetes mellitus, due to insulin resistance, in Korean renal transplant patients[126]. Molecular and genetic studies demonstrate a relationship between variants of the *AGT* gene, *AGT* gene expression and plasma AGT levels[127,128]. However, the association between this gene and glucose metabolism remain controversial.

DISCUSSION

As clinical trials continue to evaluate the influence of genetics on drug dosing and response, the challenge now becomes to assess the potential clinical implications of this research for medical practice. Sufficient data has been accumulated to be certain that the liver donors and renal re-

cipients *CYP3A5* genotype has important influences on tacrolimus dosing and on its blood through levels. However, it remains the question whether genotyping should become a standard practice in transplantation.

This question is difficult to answer because of the multi-factorial approach needed to assess the pharmacokinetic profile of a drug. Wide variability of tacrolimus dosing requirements to reach target blood levels has been observed even among patients carrying the same genotype. This underlies the fact that genetic polymorphisms are only one of the possible factors that can influence tacrolimus pharmacokinetics. Patient age, race, metabolic level, concomitant medications and a variety of other environmental factors appear to play an even more significant role than genotype in altering drug pharmacokinetics. Specifically in liver transplant patients, time after transplantation also plays a critical role in altering drug metabolism and distribution. The intestine may play a more important role soon after liver transplantation, before the liver recovers from the trauma of surgery and resumes a higher level of metabolic capacity. As liver function improves, hepatic synthesis of albumin also increases, which, in turn, decreases the unbound fraction of tacrolimus and lowers drug clearance. This is just one example of the many considerations that can ultimately impact the pharmacokinetics of an agent and highlights the difficulties in basing drug dosing on just one parameter.

To further complicate the issue, studies have yet to demonstrate a clear association between tacrolimus blood trough levels, genotype and transplant outcomes. Organ rejection and drug toxicities have been seen to develop in patients without any notable difference in tacrolimus blood concentration, making difficult to predict the optimal trough drug targets in relationship to the characteristics of the individual patient. Toxicities associated with tacrolimus are also often difficult to study because of their insidious onset. Hypertension, hyperlipidemia and NODAT develop slowly over a period of many years, making the length of a trial an issue when one wants to monitor these chronic medication effects. The mechanisms of such adverse effects are, again, not fully understood and require further research to determine the need to genotype patients, not only as a way of lowering the incidence of organ rejection, but also of preventing drug toxicity after transplantation.

Studies in transplantation are also often difficult to conduct because of the limited patient population. Many studies involve fewer than 100 patients, which may help explain some of the variable results. A number of these studies also differ in their pharmacokinetic methods, dosing strategies, times when blood drug concentrations are assessed and patient's characteristics. Differences between donor and recipient organ genotypes may also have confounded the results of some studies, as the genetics of both the recipient and of the donor were not always taken into account.

Genotyping is an attractive option for starting the dosing of tacrolimus; also, unlike phenotypic tests, the results of which may vary with environmental factors, the genotype is a stable characteristic that needs to be determined only once for any given gene. However, to ultimately prove the usefulness of genotyping, prospective clinical studies must show that genotype determination before transplantation allows the better use of a given drug and improves the safety and clinical efficacy of that medication. Currently Amplichip, a genetic test manufactured by Roche Pharmaceuticals, can determine a patient's *CYP2D6* and *CYP2C19* polymorphisms for between United States $350 and $400, not including the mark up and other costs associated with the test. As a result, to offset the cost of genetic testing, genotypic analyses must demonstrate the ability to significantly improve transplant patient outcomes, in particular, graft life and patient survival, and show a cost saving for patients and for the health care system as a whole.

CONCLUSION

At present, research has been able to reliably show that the CYP3A5, but not the CYP3A4 or ABCB1, genotype modifies the pharmacokinetics of tacrolimus. However, it has not been possible to incontrovertibly show that the corresponding changes in the pharmacokinetic profile are linked with different patient outcomes regarding tacrolimus efficacy and toxicity. Additionally, given the high cost of genotypic tests and the wide availability and utility of therapeutic drug monitoring, genotyping all transplant patients is not convenient for many individuals or Institutions. This may change in the near future as further studies on pharmacogenetics will produce new data and the improvements in the genotyping analyses will drive down the costs associated with this type of tests. For these reasons, pharmacogenetics and individualized medicine remain a fascinating area for further study and may ultimately become the face of future medical practice and drug dosing.

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TOPIC HIGHLIGHT

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Liver transplantation for hepatocellular carcinoma: Role of inflammatory and immunological state on recurrence and prognosis

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Abstract

Criteria for liver transplantation (LT) for hepatocellular carcinoma (HCC) and post-LT indicators of prognosis are historically based on the measurement of the tumor mass. Recently, high throughput technologies have increased the prediction of recurrence, but these tools are not yet routinely available. The interaction between HCC and the immune system has revealed an imbalance of lymphocyte phenotypes in the peritumoral tissue, and the increase of regulatory T cells with respect to cytotoxic lymphocytes has been linked to a higher rate of post-LT HCC recurrence. Moreover, some inflammatory markers have shown good reliability in predicting cancer reappearance after surgery, as a result of either a systemic inflammatory response or a decreased capacity of the organism to control the tumor growth. Among these markers, the neutrophil-to-lymphocyte ratio appears to be the most promising and easily available serum parameter able to predict HCC recurrence after LT and following other types of treatment, although the exact mechanisms determining its elevation have not been clarified. Post-LT immunosuppression may impact on cancer control, and the exposure to high levels of calcineurin inhibitors or other immunusuppressants has recently emerged as a negative prognostic factor for HCC recurrence and patient survival. Despite the absence of prospective randomized trials, inhibitors of the mammalian target of rapamycin have been shown to be associated with lower rates of tumor recurrence compared to other immunosuppressors, suggesting their use especially in patients with HCC exceeding the conventional indication criteria for LT.

Key words: Liver transplantation; Hepatocellular carcinoma; Inflammation; Immunosuppression; Recurrence

Core tip: This review focuses on inflammatory markers recently emerged as indicators of tumor biological behavior and on immune state of patients submitted to liver transplantation for hepatocellular carcinoma (HCC), with a particular reference to the role of neutrophil-to-lymphocyte ratio. The impact of post-transplant immunosuppression on HCC recurrence is also analyzed according to the most relevant evidences published so far, which outline the importance of minimization of the use of calcineurin inhibitors and the protective role of inhibitors of the mammalian target of rapamycin.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, and its incidence is increasing in Western countries[1]. For patients with HCC and cirrhosis, liver transplantation (LT) represents the treatment of choice and provides excellent oncological results and a cure for cirrhosis.

Prognostic factors for tumor recurrence and patient outcome have mainly been recognized as an expression of tumor burden and of its biological aggressiveness. Among these factors, the number and size of HCC nodules, the degree of differentiation, the presence of hepatic vascular invasion and elevated serum levels of alpha-fetoprotein (AFP) are the ones most widely utilized to define the indications for LT and to predict the outcome[2-8]. Since it is often difficult to safely and/or reliably obtain histological parameters before LT[9,10], radiological tumor criteria and AFP levels are the main preoperative indicators of prognosis.

The role of markers of inflammation and of the patient's immunological state have recently emerged as predictors of outcome, providing information on the environment in which the tumor grows and on the systemic response to its expansion[11-20]. These markers are often correlated with dimensional and histological factors determining a high risk of recurrence, but the mechanisms by which they are expressed are still largely unexplored. While waiting for more precise molecular markers[21-23] to become of routine use in defining the indications for and the prognosis of LT, the above parameters of inflammation may help to predict the biological behavior of HCC.

Since post-LT pharmacological immunosuppression can ideally impact on the ability to control tumor reappearance, the type, duration and total load of immunosuppressors have also been investigated in recent years as predictors of HCC recurrence[7,8,24-33].

The role of inflammatory markers and of post-LT immunosuppression on tumor recurrence and patient prognosis after LT for HCC are the subject of the present review. For this purpose, an extensive review of the English literature using the PubMed database was performed independently by two authors (Cescon M, Bertuzzo VR), separately selecting papers pertinent to the key terms "liver transplantation", "hepatocellular carcinoma", "recurrence" and "inflammation" for the investigation of the impact of inflammatory markers, and to the terms "liver transplantation", "hepatocellular carcinoma", "recurrence" and "immunosuppression" to assess the post-LT impact of pharmacological immunosuppression.

RELATIONSHIP BETWEEN INFLAMMATORY AND IMMUNOLOGICAL MARKERS, AND OUTCOME AFTER LIVER TRANSPLANTATION FOR HCC

In the last two decades, Virchow's hypothesis, which postulates that a relationship exists between inflammation and cancer, has permitted new insights into the phenomenon of carcinogenesis[34]. Given the importance of the peritumoral (micro)-environment, researchers have focused on markers that could be an expression of the relationship between liver cancer and surrounding tissue, with a possible consequent change of systemic inflammatory response.

Infiltration of pro-inflammatory macrophages, cytokines and chemokines in the tumor microenvironment has been shown to enhance tumor growth, invasion and metastases[34-36], allowing the use of inflammation parameters as tumor markers[37,38] and the development of new therapeutic strategies[35,36].

C-reactive protein (CRP)[37-41] and erythrocyte sedimentation rate (ESR)[42-45] were the first serum inflammation indicators used as tumor markers. Elevated preoperative CRP, an acute-phase reactant synthesized by hepatocytes in response to systemic inflammation, has been recognized as a risk factor for incidental colorectal cancer[39] and as an adverse prognostic factor in patients undergoing hepatectomy for HCC[40], whereas ESR has been identified as an indicator of poor prognosis in patients with clear cell renal cell carcinoma and in children with Hodgkin's lymphoma[42,45].

Inflammatory cytokines such as interleukin-6 (IL-6) and IL-1b are linked to transcriptional signaling pathways associated with carcinogenesis, tumor growth, and invasion[36,46]. IL-6 is known as one of the main regulators of CRP production.

The neutrophil-to-lymphocyte ratio (NLR) is another inflammation index that has been evaluated as a tumor marker[47-53]. Originally used as a systemic inflammatory response index in critically ill patients, it is obtained by dividing the absolute neutrophil count by the absolute lymphocyte count. According to published literature, an NLR $\geqslant$ 5 can be considered a valid cut-off[48,50,51].

Some studies have demonstrated the relationship between NLR and tumor progression in patients with colon cancer, liver metastases from colorectal cancer, pancreatic cancer, breast cancer, esophageal cancer, cholangiocarcinoma, and HCC; in addition, a higher incidence of HCC recurrence has been observed in patients with high NLR and undergoing hepatic resection[47-53].

An elevation of NLR could be related to a relative increase of neutrophils - as a consequence of some sort of inflammatory response - to a decrease of lymphocyte count - reflecting a lower immunological control of tumor growth - or to both phenomena, with several studies supporting each of these hypotheses.

LT for HCC represents a particular field of investiga-

tion of inflammatory markers and local immunological activation as possible expressions of tumor invasiveness and biological behavior. Although the visible tumor mass is usually treated preoperatively with neoadjuvant treatments, and then entirely removed with hepatectomy, some parameters detected in the serum may help in recognizing a systemic response to cancer relapse due to viable cancer cells still in the patient's circulation or in remote organs, at any time during the waiting time to LT, and following the procedure.

The role of CRP has been analyzed for prediction of post-LT outcomes of HCC patients[14]. In a series of 85 patients, those with high CRP levels (≥ 1 mg/dL) at the time of LT had higher total bilirubin levels, Child-Pugh grade, Model for End-Stage Liver Disease score, maximal tumor size, and frequency of intrahepatic metastasis compared to patients with low CRP levels (< 1 mg/dL).

By multivariate analyses, HCC beyond the Milan criteria, a high CRP level, and microvascular invasion were associated with tumor recurrence, while a high CRP level and microvascular invasion were related to lower overall survival. In addition, high CRP level was an independent factor for predicting poor outcomes in patients with HCC beyond the Milan criteria, but not in patients with HCC within the criteria[14]. Taken together, these findings suggest that CRP is related to poor liver function and higher tumor invasiveness, but the precise molecular mechanisms for its increase in such circumstances are not clarified. Moreover, another study[16] failed to detect any relationship between CRP (and ESR) and post-LT HCC recurrence.

Unitt *et al*[11] studied the tumor $CD4^+$, $CD8^+$, $CD25^+$ and $Foxp3^+$ lymphocyte infiltrate in the explant tissue of 69 patients transplanted due to HCC. On multivariate analysis, CD4:CD8 ratio, vascular invasion, tumor size, and reduced lymphocyte infiltration were significant independent predictors of recurrence. The presence of regulatory T cells (Tregs; $CD4^+$, $CD25^+$, $Foxp3^+$ T-lymphocytes) was not predictive of recurrence, but was associated with tumor vascular invasion. These data suggest that a reduced immunological response against cancer expressed as prevalence of Tregs and a lower expression of cytotoxic lymphocytes is associated with poor prognosis.

The above findings were partly supported by another study by Mathai *et al*[12], who assessed the phenotype of tumor-infiltrating lymphocytes in 131 histology sections of patients undergoing LT or liver resection for HCC. An increased Foxp3:CD3 ratio was associated with poorly differentiated HCC and higher Edmonson-Steiner nuclear grade. An increased Foxp3:CD8 ratio was also associated with poorer differentiation, higher Edmonson-Steiner nuclear grade, tumor recurrence, decreased overall survival, and decreased disease-free survival.

Although not focused on LT recipients, other studies showed that patients with HCC have increased numbers of $CD4^+$ $CD25^+$ Tregs not only among tumor-infiltrating lymphocytes, but also in the peripheral blood; furthermore, the abundance of this cell population correlated with tumor progression. These cells were anergic toward T-cell receptor stimulation and, when cocultured with activated $CD4^+$ $CD25^-$ cells, potently suppressed their proliferation and cytokine secretion. Concomitantly, the expression of granzyme A, granzyme B, and perforin was decreased dramatically in tumor-infiltrating CD8(+) T cells, confirming their inefficacy in controlling tumor expansion[54,55].

In summary, an imbalance between Tregs and CD8 lymphocytes, with a prevalence of the former and a defective function of the latter, does reflect an aggressive behavior of HCC and the inability of the organism to control the disease. While these findings potentially pave the way to new treatments, they cannot be unequivocally correlated with markers easily available by means of common lab tests, such as NLR (see below).

Nevertheless, novel methods for assessing the immune function of transplanted patients could be useful in the future. The Immu-Know assay, which measures the amount of adenosine triphosphate (ATP) produced by activated $CD4^+$ T cells, has been used to evaluate the global immune status, and thus the tendency to develop rejection or, on the contrary, post-LT infections[56].

This tool has also proven to be reliable in predicting post-LT HCC recurrence, with recipients diagnosed with recurrent tumors having significantly lower values of ATP compared to those without recurrence[13]. This refined measurement of the immune state of LT recipients could replace the more indirect evaluation allowed by systemic exposure to immunosuppressive agents.

Several studies have demonstrated that an increased NLR is an independent factor for lower recurrence-free survival and/or overall survival in LT HCC patients[15-20]. These studies are reported in Table 1. A total of 892 patients were included. The chosen cutoff value of NLR ranged from 3 to 5, with most studies using the value of 5[15,16,18], while others identified lower values[17,19,20].

In the groups of patients with NLR above the selected risk thresholds, overall survival ranged between 14% and 57%, and recurrence-free survival was between 6% and 42%. Only one study reported both the NLR at diagnosis of HCC and NLR at transplant, showing that this variable had a similar negative impact on outcome at the two chosen time points[18].

High NLR was an independent predictor of outcome in all studies, in most cases together with other commonly recognized risk factors. Interestingly, in two studies NLR was not correlated with histological, serological and dimensional features with a recognized, negative impact on recurrence[15,18].

In the above reports, different explanations for the alteration of NLR were provided but, though reasonable, most of them were speculative. Only one group, which produced two different analyses on this topic, investigated the correlation between NLR and the alterations of phenotype/function of leucocytes or other cells in tissues surrounding neoplastic nodules[19]. Interestingly, the Authors found that serum and peritumoral IL-17 levels were significantly higher in patients with high NLR, and that the density of peritumoral CD163-positive tumor

Table 1 Studies reporting the negative impact of increased neutrophil-to-lymphocyte ratio measured at transplant on the outcome of liver transplantation for hepatocellular carcinoma

Ref.	Patients (*n*)	Type of LT	NLR cut-off level for poor prognosis	Other factors associated with worse outcome	5-yr RFS with high *vs* low NLR	5-yr OS with high *vs* low NLR	Parameters positively correlated with increased NLR
Halazun *et al*[15]	150	NA	5	Tumor size AFP	25% *vs* 75%[1]	28% *vs* 64%	None
Bertuzzo *et al*[16]	219	DDLT	5	Microvascular invasion	6% *vs* 89%	14% *vs* 73%	Micro/macro vascular invasion Tumor grading AFP CRP Outside MC
Wang *et al*[17]	101	DDLT	3	Tumor number Macrovascular invasion	28% *vs* 65%[1]	19% *vs* 62%	Macrovascular invasion AFP Tumor size Outside MC Outside UCSF criteria Outside Hangzhou criteria
Limaye *et al*[18]	160	NA	5	Microvascular invasion AFP	27% *vs* 79%	38% *vs* 68%	None
Motomura *et al*[19]	158	LDLT	4	Outside MC	30% *vs* 89%	57% *vs* 84%	Serum/peritumoral IL-17 Density of peritumoral CD163 CRP Tacrolimus *vs* cyclosporine
Yoshizumi *et al*[20][2]	104	LDLT	4	Nodule size + number ⩾ 8.0	42% *vs* 86%	Not reported	Microvascular invasion Tumor grading

[1]In these studies, disease-free survival instead of recurrence-free survival rates were reported (and displayed in the present table); [2]This study was performed by the same authors as the previous one[19], and included only patients with surgical and/or locoregional treatment preceding living donor liver transplantation (LDLT). Thus, the patient population is probably at least partly included in the population of the previous study from the same Institution. NLR: Neutrophil-to-lymphocyte ratio; LT: Liver transplantation; HCC: Hepatocellular carcinoma; RFS: Recurrence-free survival; OS: Overall survival; NA: Not assessable; AFP: Alpha-fetoprotein; CRP: C-reactive protein; MC: Milan criteria; DDLT: Deceased donor liver transplantation; UCSF: University of California at San Francisco.

associated macrophages (TAM) was both correlated with the density of peritumoral IL-17-producing cells, and significantly higher in subjects with elevated NLR. Conversely, tumor, peritumoral and serum expression of vascular endothelial growth factor (VEGF) and of IL-8, *i.e.*, two recognized angiogenesis and tumor growth factors, was similar between high and low NLR groups. Tumor expression of IL-17, CD68, and CD163 was also comparable in patients with elevated or normal NLR.

A positive correlation between CRP and NLR, the absence of correlation between NLR and tumor markers, number and size of nodules, and microvascular invasion, the association between high NLR and an increased serum neutrophil count, and the absence of correlation between NLR and total serum lymphocytes were other important findings[19].

Consistently with previous studies[57-61], the authors came to the following conclusions: (1) contrary to other investigations, the elevation of NLR seems correlated with an increase of neutrophil number rather than of lymphocytes, suggesting a dependence of tumor relapse on the inflammatory state rather than on an impaired host immune response; (2) elevated neutrophils are thought to be a reservoir of VEGF, but the expression of VEGF and of IL-8 did not have any impact on NLR, suggesting that NLR elevation is not directly responsible for augmented HCC-related neo-angiogenesis; (3) IL-17 is a pro-inflammatory cytokine that promotes HCC growth and neutrophil recruitment, thus it could be a key molecule in the relationship between NLR (which is supposed to increase due to expansion of neutrophils following recruitment) and HCC recurrence; and (4) the authors' results are consistent with the demonstrated relationship between IL-7-producing T cells and TAMs. IL-7-producing T cells promote the differentiation of tissue macrophages in peritumoral tissue into TAMs, which in turn promote tumor proliferation and angiogenesis. In fact, monocytes are recruited from the circulation into local tissue or malignant sites, where they are recognized by CD68-positive residential macrophages. Under the effect of inflammatory cytokines released by tumors, some of these macrophages differentiate into CD163-positive TAMs that, contrary to $CD68^+$ macrophages, are suppressors of the anti-tumor immune response.

IL-17-producing cells interact with TAMs in patients with HCC, and both IL-17-producing cells and $CD163^+$ TAMs generate the same family of chemokines promoting the recruitment of monocytes and neutrophils[19,57-61].

Finally, it should be considered that in the authors' series splenectomy was performed during LT in patients with hepatitis C virus-positive or significant portal hypertension, and splenectomy itself could have had a role in the balance between neutrophil and lymphocyte count. Moreover, TAMs have been demonstrated to originate from splenic monocytes. However, splenectomy itself was not associated with HCC recurrence in this study,

Table 2 Studies reporting the effect of different basal immunosuppression schedules on the outcome of liver transplantation for hepatocellular carcinoma

Ref.	Evaluated immunosuppressor	Evaluated parameter	Patients (*n*)	Overall recurrence rate	Outcome parameters	*P* value
Vivarelli *et al*[24]	CsA cumulative dosage 1st yr	Low dosage 1st yr *vs* high dosage 1st yr	39 *vs* 30	12.20%	5 yr RFS: 93% *vs* 5 yr RFS: 76%	0.0100
Kneteman *et al*[25]	SRL	in MC *vs* out MC	19 *vs* 21	12.50%	4 yr RFS: 81.1% *vs* 4 yr RFS: 76.8%	0.4800
Vivarelli *et al*[26]	CsA	Low exposure *vs* high exposure	49 *vs* 21	10.00%	RR: 0% *vs* RR: 33.3%	< 0.0010
Decaens *et al*[27]	CNI	CsA *vs* TAC	264 *vs* 119	31.80%	5 yr RFS: 52.5% *vs* 5 yr RFS: 70.8%	0.0030
Decaens *et al*[27]	ATG/OKT3	Not administered *vs* administered	356 *vs* 55	31.80%	5 yr RFS: 58.8% *vs* 5 yr RFS: 45.4%	0.0200
Vivarelli *et al*[7]	TAC	Low exposure *vs* high exposure	44 *vs* 16	20.00%	RR: 9.1% *vs* RR: 50%	0.0010
Zhou *et al*[28]	TAC and SRL in patients outMC	TAC *vs* SRL	46 *vs* 27	27.40%	2 yr OS: 50.9% *vs* 2 yr OS: 80.6%	0.0110
Zimmerman *et al*[29]	TAC and SRL	TAC + MMF *vs* TAC + SRL	52 *vs* 45	12.40%	5 yr RFS: 54.0% *vs* 5 yr RFS: 78.8%	-
Chinnakotla *et al*[8]	TAC and SRL	TAC + MMF *vs* SRL	106 *vs* 121	11.00%	5 yr RFS: 60% *vs* 5 yr RFS: 80%	0.0001
Vivarelli *et al*[30]	TAC and SRL	TAC *vs* TAC + SRL	31 *vs* 31	25.80%	3 yr RFS: 56% *vs* 3 yr RFS: 86%	0.0400
Toso *et al*[31]	SRL	Not administered *vs* administered	2382 *vs* 109	-	5 yr OS: 68.7% *vs* 5 yr OS: 83.1%	≤ 0.0500
Xing *et al*[32]	Basiliximab and steroids in patients in MC	TAC + MMF + basiliximab *vs* TAC + MMF + steroids	28 *vs* 36	-	5 yr OS: 88.9% *vs* 5 yr OS: 57.4%	0.0220
Rodríguez-Perálvarez *et al*[33]	CNI	Low exposure 1st mo *vs* high exposure 1st mo	171 *vs* 48	16.40%	5 yr RR: 14.7% *vs* 5 yr RR: 27%	0.0070

LT: Liver transplantation; HCC: Hepatocellular carcinoma; CsA: Cyclosporine A; RFS: Recurrence free survival; SRL: Sirolimus; MC: Milan criteria; RR: Recurrence rate; CNI: Calcineurin inhibitors; TAC: Tacrolimus; ATG: Anti-thymocyte globulins; OS: Overall survival; MMF: Mycophenolate mofetil.

even though in the group of patients with elevated NLR, splenectomy led to significantly better recurrence-free survival than the abstention from this procedure, suggesting the supply of splenic TAMs with high IL-17 concentrations after LT[19].

The same authors confirmed the relevant role of NLR on HCC recurrence in patients undergoing living donor liver transplantation for tumor recurrence after surgical resection and/or locoregional treatment[20], and in those submitted to liver resection[62].

By evaluating 958 patients who underwent hepatectomy without preoperative therapy for HCC, multivariate analysis showed that NLR was an independent prognostic factor of lower overall and recurrence-free survival, the best cutoff being 2.81. Again, CD163-positive cell counts were significantly higher in tumors of patients with high NLR than in those with low NLR[62].

Finally, one of the advantages of an easily obtainable serum marker is to assess the response to pre-LT treatments of HCC and the probability of dropout from the waiting list. NLR has been shown to be a good predictor of the risk of dropout, while platelet-to-lymphocyte ratio has been related to post-LT HCC recurrence[63]. On the other hand, since multimodal treatments are usually adopted while on the waiting list for LT, it has also been shown that NLR, or NLR postoperative changes, correlate with HCC recurrence and patient outcome after radiofrequency ablation[64,65].

EFFECT OF IMMUNOSUPPRESSION ON HCC RECURRENCE AFTER LIVER TRANSPLANTATION

At present, there is a general consensus on the negative impact of pharmacological immunosuppression on the outcome of LT for HCC[7,8,24-33]. Specifically, two clinical pieces of evidence have emerged: (1) the higher the exposure to calcineurin inhibitors (CNI), *i.e.*, cyclosporine and tacrolimus, the higher the risk of post-LT HCC recurrence; and (2) one specific class of immunosuppressors, *i.e.*, inhibitors of the mammalian target of rapamycin (mTORi), have a favorable effect in reducing the incidence of post-LT HCC recurrence compared to standard immunosuppressors (CNI). Everolimus and sirolimus, the two mTORi currently in use in solid organ transplantation, interfere with hepatocarcinogenesis through the inhibition of the PI3K/Akt/mTOR pathway, which is a key regulator of cellular proliferation and angiogenesis[66,67].

Several studies led to the above conclusions[7,8,24-33], although it is of relevance that none of these is a prospective, randomized trial. Table 2 depicts the retrospective clinical studies published so far on this topic, with the exclusion of reports with less than 20 patients and previous reviews or meta-analyses.

Overall recurrence rates ranged between 12% and 32%. Four out of 13 reported studies showed that among patients immunosuppressed with CNI, those exposed to higher dosages had unfavorable outcomes, with significantly higher HCC recurrence rates or lower recurrence-free survival rates compared to patients receiving lower dosages[7,24,26,33]. One study reported a lower recurrence-free survival in patients treated with cyclosporine *vs* those treated with tacrolimus[27].

In 5 studies, patients treated with sirolimus (most frequently in combination with low dosages of tacrolimus) showed higher overall or recurrence-free survival rates compared to patients receiving standard CNI-based immunosuppression[8,28-31]. In one study[25], patients treated

with sirolimus had similar recurrence-free survival rates, irrespective of fulfillment of the Milan criteria.

One study showed a detrimental effect of the use of monoclonal antibodies (anti-thymocite globulins or OKT3), with a lower recurrence-free survival in patients receiving these drugs compared to those not administered them[27]. Another study revealed that the use of steroids *vs* basiliximab led to significantly lower overall survival rates[32].

A definitive validation of the benefit of mTORi in LT for HCC is expected to be provided in 2014 by an international multicenter, prospective, randomized trial comparing the outcomes of patients administered or not administered sirolimus following post-LT histological confirmation of HCC[68]. However, at present the use of mTORi in LT for HCC seems justified on the basis of the above reported results and according to a recent meta-analysis conducted on 5 studies and 474 patients, which showed a lower recurrence rate, longer recurrence-free survival and overall survival, and lower recurrence-related mortality in sirolimus-treated patients in comparison with CNI-treated patients[69].

CONCLUSION

Recent insights into the interactions between tumor, peritumoral tissue, and systemic inflammatory and immune response have offered new indicators for prognosis of patients with HCC undergoing various types of treatment, including LT. NLR has proven to be a reliable and easily available inflammatory marker of tumor biological aggressiveness, making its use advisable along with common dimensional indexes in assessing the response to treatments and the indication for LT, and to predict the outcomes. Although recent reports provided a reasonable molecular basis for the alteration of NLR and, more in general, for the tumor-related imbalance between immune cells in terms of number and function, much remains to be explored to expand targeted diagnostic and therapeutic tools. On the other hand, despite the lack of prospective, randomized studies, there is sufficient evidence for the minimization of immunosuppression and for the use of mTORi in LT for HCC, especially in the case of extended indications for transplant.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (7): Liver transplant

Transplant benefit for patients with hepatocellular carcinoma

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Abstract

Although liver transplantation is theoretically the best treatment for hepatocellular carcinoma (HCC), it is limited by the realities of perioperative complications, and the shortage of donor organs. Furthermore, in many cases there are available alternative treatments such as resection or locoregional therapy. Deciding upon the best option for a patient with HCC is complicated, involving numerous ethical principles including: urgency, utility, intention-to-treat survival, transplant benefit, harm to candidates on waiting list, and harm to living donors. The potential contrast between different principles is particularly relevant for patients with HCC for several reasons: (1) HCC candidates to liver transplantation are increasing; (2) the great prognostic heterogeneity within the HCC population; (3) in HCC patients tumor progression before liver transplantation may significantly impair post transplant outcome; and (4) effective alternative therapies are often available for HCC candidates to liver transplantation. In this paper we suggest that allocating organs by transplant benefit could help balance these competing principles, and also introduce equity between patients with HCC and non-malignant liver disease. We also propose a triangular equipoise model to help decide between deceased donor liver transplantation, living donor liver transplantation, or alternative therapies.

Key words: Hepatocellular carcinoma; Deceased donor liver transplantation; Living donor liver transplantation; Transplant benefit; Utility; Urgency; Intention-to-treat survival; Harm

Core tip: Deciding upon the best option for a patient with hepatocellular carcinoma is complicated, involving numerous ethical principles including: urgency, utility, intention-to-treat survival, transplant benefit, harm to candidates on waiting list, and harm to living donors. In this paper we suggest that allocating organs by transplant benefit could help balance these competing principles, and also introduce equity between patients with hepatocellular carcinoma and those with nonmalignant liver disease. We also propose a triangular equipoise model to help decide between deceased donor liver transplantation, living donor liver transplantation, or alternative therapies.

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GENERAL PRINCIPLES REGULATING PATIENT SELECTION AND ORGAN ALLOCATION IN LIVER TRANSPLANTATION

Urgency, utility, and equity

Liver transplantation (LT) is theoretically the best treatment for patients with end-stage liver disease but its effectiveness is limited by intrinsic characteristics with important ethical implications: (1) LT remains a technical demanding procedure with a well-established short-term mortality and morbidity[1]; (2) a persistent shortage of deceased donors corresponds to an increasing demand of deceased donor liver transplantation (DDLT)[2]; and (3) the application of living donor liver transplantation (LDLT) is limited by ethical and legal issues related to the risk of harming the living donor[3]. Specific selection policies have consequently been developed over the last two decades to identify good candidates for this complex therapeutic option[4,5].

For patients with non-malignant (NM) liver cirrhosis, scores have been developed to measure disease severity, such as the Child Pugh and the model for end-stage liver disease (MELD) scores[4], which support a selection policy based on the urgency principle. Under a medical urgency-based selection system, patients with worse outcomes while on the waiting list (WL) are given higher priority for transplantation[6]. Use of the MELD score, for example, has significantly reduced waiting list times and in the United States system in recent years[4,7]. If we consider the development of hepatocellular carcinoma (HCC) as a complication of liver cirrhosis, and therefore as a sign of disease severity, assigning a high priority to HCC patients would also comply with this principle of urgency. This viewpoint is reflected in the United Network for Organ Sharing (UNOS) allocation system, where an arbitrary high MELD score is assigned to patients with T2-HCC[7].

The limit of this approach is that it fails to consider the extremely relevant prognostic heterogeneity of patients with HCC and the potential effectiveness of alternative therapies[8]. It is also only reasonable to consider HCC as a complication of liver cirrhosis if this condition is maintained within certain proportions of candidates on the WL (*e.g.*, < 20%), as in the US[9]. In some geographical LT settings, however, there has been a significant increase in the proportion of LT candidates on the WL with liver tumors in recent years and this has given rise to similar proportions of liver transplants for HCC and NM disease[10]. In these modern LT realities, it is probably more reasonable to consider HCC patients as a separate LT population and analyze the prognostic heterogeneity of this particular medical condition more deeply[11].

If we observe the issues of patient selection and organ allocation from the HCC population point of view, therefore, current LT selection policies for HCC patients (*e.g.*, the UNOS allocation system) appear to be based mainly on a utility principle for two main reasons. First, a utility-based system is one that gives priority according to expected post-transplant outcomes[6]. For patients with HCC, the poor results achieved in early experiences with patients transplanted for advanced tumors have favored the introduction of strict selection criteria focusing mainly on post-LT outcome[5]. Therefore, patients beyond Milan criteria have limited probability of receiving a transplant.

Second, in the current system all T2 HCC patients receive the same priority regardless of their likelihood of death on the waiting list.

If we consider LT candidates with and without cancer as two separate populations, therefore, apparently opposite allocation principles are currently used at the majority of LT centers around the world. This diversity in patient selection policy intrinsically creates an ethical paradox, in that donated organs are allocated to the "sickest patient first" among the candidates with NM hepatic disease, but to the "earliest patient first" among candidates for LT who have HCC, irrespective of their survival prospects with therapies other than transplantation.

Aristotle defined justice as "treating equal cases equally, and unequal cases unequally". One of the fundamental challenges of organ allocation science is maintaining equity among the heterogeneous groups of patients on the waiting list. In the specific organ allocation context, equity means treating all patients according to a common endpoint. From this perspective, the principle of equity is hierarchically more important than all others, whether we decide to favour urgency, or utility or benefit as endpoints for our allocation system.

Based on these considerations (*i.e.*, the increasing proportion of HCC patients enlisted, and an excess of priority for HCC patients with a low urgency for LT), recent proposals have tried to resolve the unbalance in the access to transplantation between HCC and non-HCC patients. One attempt involved developing risk models within the HCC population for 3-mo drop-out risk as common urgency endpoint[12,13]. However, this approach (*i.e.*, to equate the drop-out risk of different patients) carries the risk of prioritizing HCC patients with higher biological aggressiveness in terms of nodule size and AFP levels, and consequently dramatically increasing the risk of post-LT tumour recurrence or death[14]. Thus, methods are needed which balance the principles of urgency and utility when attempting to reach equity between HCC and non-HCC patients.

Intention-to-treat survival

To describe the effect of long waiting times on the effectiveness of LT as curative therapy for HCC[15], some years ago the concept of intention-to-treat (ITT) survival was introduced. Interestingly, analyzing the survival figures of HCC patients from the day of enlisting and not from that of transplant, the overall results of LT for HCC became worse than resection[16] due to the high dropout rate of HCC patients from the WL for tumor progression.

However, ITT survival is strongly related to the specific local/regional WL characteristics and in particular to the patient median waiting time: assuming as a constant the post-LT outcome, the lower the pre- LT mortality, the higher the intention-to-treat survival. For this reason, in a clinical scenario where HCC patients receive high priority for LT (*i.e.*, low waiting time and low risk of dropout) the intention-to-treat survival of LT for HCC patients may exceed that of liver resection[17].

For these reasons, survival analysis in LT should use the ITT principle because it accounts for all the complex LT processes from the day that LT is first considered.

Transplant benefit

The concept of transplant benefit expresses the survival gain offered by LT by comparison with the best alternative therapy. Transplant benefit can be calculated from the time of transplant, or from the time a patient is first evaluated for transplant-the latter would make it an ITT endpoint. On an individual basis, the main advantage of this principle is that it covers the overall LT process, simultaneously considering post- and pre-LT outcome. The transplant benefit principle applied to the individual LT candidate thus has the potential to create an ideal balance between the concepts of urgency and utility. As suggested by Schaubel *et al*[6], moreover, by prioritizing patients based on life-years gained thanks to transplantation, the transplant benefit principle performs better than urgency and utility schemes from a population perspective too. This is because an urgency-based system would assign donor organs to patients who are most likely to die while on the WL, but this approach may be to the detriment of utility because patients at the greatest risk of death while on the WL may also be patients with the highest post-LT mortality risk. A utility-based allocation system would ensure that transplanted organs go to patients with the lowest post-LT mortality risk, but patients with the best post-LT outcomes may also have the best outcomes while on the WL. The transplant benefit principle is consequently the one best able to maximize the total life-years gained by the patient population.

In recent years, the transplant benefit principle has been proposed for LT candidates based on studies using data from the Scientific Registry of Transplant Recipients (SRTR)[2,6,18], but these studies did not consider the transplant benefit for the HCC population of LT candidates, because they either focused only on NM candidates[2,11] or they considered HCC as a complication[6] and not as a separate, prognostically heterogeneous medical condition.

The concept of transplant benefit has the intrinsic potential for being especially useful for HCC patients since a particular feature of the approach lies in that it is calculated by subtracting the area under the survival curve after alternative therapies from the area under the survival curve after transplantation[9], a definition that coincides with the gain in life expectancy (LE). This gives a relevant weight not only to the crude post-LT outcome, but also to the alternative therapies available and to the patient's

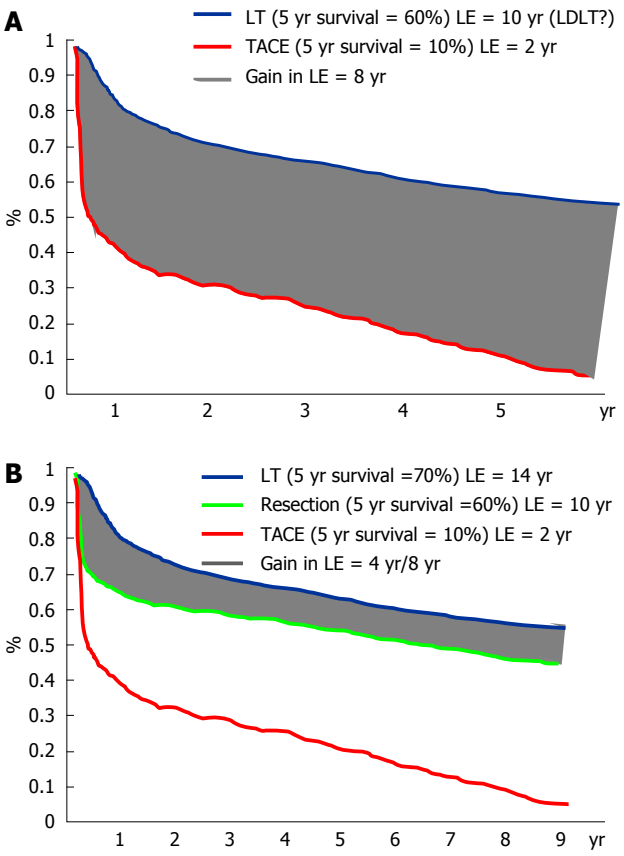

Figure 1 Clinical examples of the transplant benefit principle applied to hepatocellular carcinoma patients. A: Man 40-year-old, HBV with 2 HCC nodules, the largest of 6 cm, Child B (Milan out, University of California San Francisco out); B: Man 65-year-old, HCV, with 1 HCC (diameter = 4 cm), Child A. HCC: Hepatocellular carcinoma; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

age, which are extremely important prognostic variables for HCC patients[19]. Figure 1 shows two different clinical scenarios. The first (Figure 1A) concerns the case of a young patient (40 years old) with a tumor beyond the Milan criteria (calculated 5-year post-transplant survival = 60%). The lack of any effective alternative therapies makes the benefit of LT extremely high (8 years). The second scenario (Figure 1B) considers an older patient (65-year-old) within the accepted indications for LT (5-year post-transplant survival = 70%), but with an effective alternative treatment option, *i.e.*, liver resection, which makes the benefit of LT much lower (4 years) than in the first case, although the post-LT outcome would be better.

The recent publication of important studies on the survival prospects of patients with more advanced tumors after LT[20] and other therapies[21,22] makes it potentially feasible now to evaluate transplant benefit across different stages of HCC disease. This could be extremely important because, from a utility perspective, adopting extended criteria for HCC patients would mean allocating more donated organs to HCC patients than to NM patients[16]; taking a transplant benefit perspective, on the other hand, would mean reallocating the same number of organs to different groups of patients with a greater

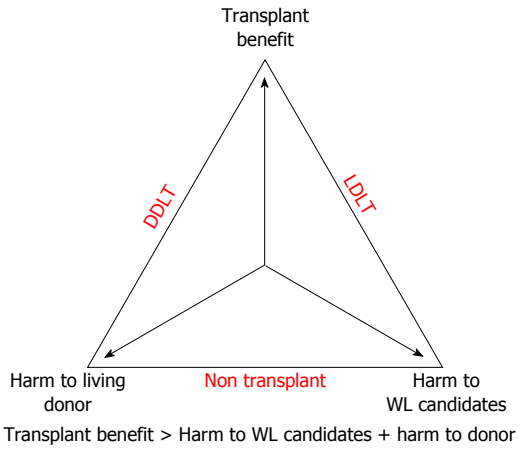

Figure 2 Ethical equipoise between benefit and harm of deceased-donor liver transplantation and living donor liver transplantation. WL: Waiting list; DDLT: Deceased donor liver transplantation; LDLT: Living donor liver transplantation.

benefit. In other words, the transplant benefit principle would be able to maximize the total life-years of both the HCC and NM population.

These concepts have been recently incorporated in three papers[23-25] evaluating the transplant benefit principle in the HCC population. These studies underline three main points: (1) Liver transplantation results in the highest survival benefit for HCC patients with advanced liver cirrhosis (BCLC stage D); (2) Patients with intermediate tumours (BCLC stages B-C) without effective alternative therapies receive a relevant benefit from LT, regardless of the nodule number-size criteria (*i.e.*, Milan criteria), provided that macroscopic vascular invasion and extra-hepatic disease are absent; and (3) Patients with early tumors and compensated cirrhosis have the lowest benefit from LT when effective alternative therapies are available[23-25].

Harm-benefit to other patients on the waiting list

When patients on a given WL receive an organ, they harm the rest of the candidates on the WL because it is as if they were taking that organ away from other potential candidates. The entity of this harm depends on the extra time the other patients on the WL have to wait for another organ. We can also see this concept from the opposite point of view: if we find an alternative treatment for a patient on a WL for LT (*e.g.*, if we perform a LDLT or a liver resection), we create a benefit for the people on said WL that can be calculated from the further waiting time they spare. Knowing the characteristics of a WL in detail (death probabilities according to disease severity, median waiting time for LT, mean number of organs per year, patient stratification according to MELD score and HCC stage), we can calculate this harm/benefit to candidates on the WL[9,26,27]. This is very important because it is the only allocation principle that takes the characteristics of a specific WL into account (WL size, donor resources and proportions of patients with severe disease).

Harm to the living donor

The crucial element limiting the general applicability of LDLT is the risk of harming a healthy living donor. In the literature, the overall mortality attributed to living donor procedures is lower than 1%, but the risk of morbidity is significant, being around 38% in some experiences as a whole, and < 10% when severe complications are considered alone[28].

A recent worldwide survey[29] has brought more evidence about this field. Overall donor morbidity rate was 24%, but only 0.2% of them died, and 0.04% required transplantation. If harm to donors is only considered in terms of mortality, its impact on the therapeutic decision (between LDLT, DDLT, or no LT) would be minimal compared to the recipient's risk of death on the WL[30]. Quantifying morbidity could be done by determining the impact of complications on quality of life, but limited data is currently available to derive such estimates.

Furthermore, it is controversial whether donor morbidity and mortality should be weighted equally to that of the recipient[31]. Currently the transplant community takes a protective approach (paternalist principle) to the living donor, and tends to assign greater ethical weight to the donor's risk of death than to the recipient's risk of death. This approach, however, comes at the expense of donor autonomy. Further thought is needed on this subject, including input from donors themselves.

One interesting proposal is to define a cut-off for acceptable morbidity and mortality from the perspective of the donor[32,33].

REPRESENTATION OF THE POTENTIAL EQUIPOISE BETWEEN BENEFITS AND HARMS OF TRANSPLANTATION FOR HCC PATIENTS

An ideal selection/allocation process for patients with HCC should consider all aspects of the benefits and harms of LT, and the aim of allocation systems should be to reach a balance between the different principles involved in the selection process.

We have represented this equipoise using a triangle containing vectors (Figure 2): the transplant benefit (life expectancy with LT minus life expectancy without LT) is at the top vertex and the potential harm to the rest of the WL and to the living donor at the bottom vertices. According to this model, transplantation is generally indicated when the transplant benefit exceeds the harm. Then, according to the relative weights of the harm to the WL and donor, the decision will be oriented towards LDLT or DDLT.

The first advantage of this conceptual model is that it includes all ethical principles involved in the LT decision process. The use of transplant benefit satisfies both utility and urgency principles, while the relationship between benefit and harm to the waiting list satisfies equity-the first principle aims to maximize the need of the single

patient, while the second maximizes population total life years[26].

The second advantage of this model is that it considers as different therapeutic procedures DDLT and LDLT. Whenever we used urgency, utility or benefit, these principles taken alone do not distinguish between LDLT and DDLT, so they cannot be used to decide between these different strategies. The indication for LDLT is therefore inevitably the same as for DDLT[32,33], so choosing between the two is difficult. This may partially explain why LDLT has had a limited development in Western countries, especially since the introduction of the MELD[33].

Some authors[32] have recently stressed the possibility to consider different indications between LDLT and DDLT based on the consideration that living donor recipients don't compete with other patients on the WL. The same authors proposed a sort of double equipoise model specific for LDLT to balance the donor risk and the recipient benefit[32]. Our model has the advantage to be used for both DDLT and LDLT. LDLT has a potentially relevant advantage over DDLT because it only minimally harms the other candidates on the WL: this harm is limited to the risk of the patient needing re-LT after LDLT, which is estimated to be approximately 7%[34], while the risk of liver failure requiring transplantation of the donor is estimated to be 0.04%[29].

This model helps the selection of HCC patients for LT and the choice of the more appropriate transplant procedure (DDLT *vs* LDLT). However, it can not consider some crucial aspects. First of all, in some countries religiosity or cultural aspects are barriers to DDLT[31]. As second point, in some recipients of a partial liver from a living donor insufficient liver volume can not be avoided to maintain an adequate donor safety. A small-for-size graft easily causes perioperative complications and results in poor outcomes[31]. In summary, although LT is theoretically the best treatment for HCC, it is limited by the realities of perioperative complications, and the shortage of donor organs. Furthermore, the benefit of transplantation is not uniform among patients with HCC; rather, it depends upon the severity of liver disease and the available alternative treatment options. Current systems allocate organs to HCC patients primarily based upon the utility principle, as opposed to the urgency principle which governs allocation to patients with nonmalignant liver disease. Allocating organs by transplant benefit could introduce equity between these patient groups. We propose a triangular equipoise model to help decide between DDLT, LDLT, or alternative therapies.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (7): Liver transplant

Review of the pharmacological management of hepatitis B viral infection before and after liver transplantation

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Abstract

The progress in treatment against hepatitis B virus (HBV) with the development of effective and well tolerated nucleotide analogues (NAs) has improved the outcome of patients with HBV decompensated cirrhosis and has prevented post-transplant HBV recurrence. This review summarizes updated issues related to the management of patients with HBV infection before and after liver transplantation (LT). A literature search using the PubMed/Medline databases and consensus documents was performed. Pre-transplant therapy has been initially based on lamivudine, but entecavir and tenofovir represent the currently recommended first-line NAs for the treatment of patients with HBV decompensated cirrhosis. After LT, the combination of HBV immunoglobulin (HBIG) and NA is considered as the standard of care for prophylaxis against HBV recurrence. The combination of HBIG and lamivudine is related to higher rates of HBV recurrence, compared to the HBIG and entecavir or tenofovir combination. In HBIG-free prophylactic regimens, entecavir and tenofovir should be the first-line options. The choice of treatment for HBV recurrence depends on prior prophylactic therapy, but entecavir and tenofovir seem to be the most attractive options. Finally, liver grafts from hepatitis B core antibody (anti-HBc) positive donors can be safely used in hepatitis B surface antigen negative, preferentially anti-HBc/anti-hepatitis B surface antibody positive recipients.

Key words: Hepatitis B virus; Liver transplantation; Hepatitis B virus immunoglobulin; Antivirals; Lamivudine; Adefovir; Entecavir; Tenofovir; Telbivudine; Resistance

Core tip: In the present review the current knowledge on the management of hepatitis B virus (HBV) infection before and after liver transplantation is updated. There is no doubt that all HBV patients with decompensated cirrhosis should be treated with potent anti-HBV agents with high genetic barrier (*i.e.*, entecavir or tenofovir). After liver transplantation, the combination of HBV immunoglobulin (HBIG) (at least for a certain period) and entecavir or tenofovir currently appears to be the most reasonable approach, while HBIG-free antiviral prophylaxis cannot be excluded in the future, particularly in patients with low risk of recurrence.

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INTRODUCTION

The development of effective, well tolerated and relatively safe oral antiviral agents [nucleos(t)ide analogues (NAs)] has offered the opportunity for successful management of hepatitis B virus (HBV) related chronic liver disease. However, chronic hepatitis B (CHB) is still associated with increased morbidity and mortality. Currently, it is estimated that more than half a million people die every year due to complications of liver decompensation and hepatocellular carcinoma (HCC)[1,2]. Liver transplantation (LT) remains the only hope for many patients with complications of end-stage CHB, mostly HCC[2,3].

The introduction of passive immunoprophylaxis using long-term hepatitis B immune globulin (HBIG) in early 1990s significantly decreased the rates of post-LT HBV recurrence[4]. During the last 15 years, the use of NAs has decreased the need for LT due to HBV decompensated cirrhosis and has further improved the outcome of HBV transplant patients[5]. NAs have been used either in combination with HBIG or as monotherapy in an effort to further improve the rates of HBV recurrence after LT and/or reduce the need for expensive HBIG preparations[5]. The management of hepatitis B surface antigen (HBsAg) positive transplant patients can be divided into the pre-transplant, prophylactic post-transplant and therapeutic post-transplant approach[6]. HBV prophylaxis is also required for recipients who receive grafts from anti-hepatitis B core (HBc) positive donors, as they are at risk for *de novo* HBV infection.

PRE-TRANSPLANT APPROACH

Anti-HBV therapy in HBV decompensated cirrhosis

The aim of antiviral therapy is to reverse or delay complications of cirrhosis and the need for LT, and to decrease the risk of HBV re-infection in those who eventually undergo LT. Currently, there are five oral NAs that have been licensed for the treatment of CHB: three nucleoside (lamivudine, telbivudine, entecavir) and two nucleotide (adefovir dipivoxil and tenofovir disoproxil fumarate) analogues[7-9]. NAs target the reverse transcriptase of HBV and achieve inhibition of HBV replication via their incorporation in viral HBV DNA causing DNA chain termination[7-9]. Antiviral therapy should be started immediately in patients with HBV decompensated cirrhosis and any level of detectable serum HBV DNA regardless of ALT activity.

Lamivudine was the first NA approved for treatment of CHB and probably remains the most widely used NA worldwide due to its low cost. Its efficacy, at a daily dose of 100 mg, has been confirmed in randomized controlled trials and cohort studies showing stabilization or even improvement of liver function and reduction in the incidence of HCC[10] and the need for LT[11-13]. However, long-term lamivudine monotherapy is associated with progressively increasing rates of viral resistance due to *YMDD* mutations (15%-25% at year 1, 65%-80% at year 5), which can lead to clinical deterioration with development of liver failure and even death[13-15]. Importantly, patients with detectable HBV DNA at LT have increased rates of post-transplant recurrence of HBV[16,17] and even of pre-existing HCC[18]. Thus, lamivudine monotherapy is not currently recommended for patients with HBV decompensated cirrhosis[7-9].

Adefovir was the second NA approved for the treatment of CHB. It is effective against both wild type and lamivudine resistant HBV strains[3]. Adefovir at the daily licensed dose of 10 mg improves liver function in patients with HBV decompensated cirrhosis[19]. However, its weak potency[20], the moderate risk of resistance during long-term therapy in naive patients (29% at year 5)[21-23] and its higher cost have resulted in its replacement by the newer, more effective and cheaper nucleotide analogue, tenofovir, in all countries with tenofovir availability[9,21]. Finally, adefovir has been associated with renal adverse events including decline of glomerular filtration rate (GFR) and proximal tubular dysfunction resulting occasionally in Fanconi syndrome[24,25]. The potential nephrotoxicity, which seems to be dose dependent[26], is of particular concern in difficult-to-manage patients with decompensated cirrhosis.

Telbivudine is a potent nucleoside analogue[27] which achieves satisfactory virological remission rates in CHB patients with undetectable HBV DNA at 24 wk of therapy[28]. However, it also selects for mutations in the YMDD motif, but at a lower rate compared to lamivudine [25% *vs* 40% after 2 years of treatment in hepatitis B e antigen (HBeAg) positive CHB patients][3,8,9]. In a recent randomized trial[29] including 232 naïve patients with HBV decompensated cirrhosis, telbivudine was well tolerated. In addition, telbivudine, compared to lamivudine, achieved greater viral suppression, similar stabilization of liver function and significant improvement in the estimated GFR[29]. The place of telbivudine monotherapy in the treatment of patients with HBV decompensated cirrhosis is unclear due to its unfavourable resistance profile, compared to the newer NAs with high genetic barrier [*i.e.,* entecavir (ETV) and tenofovir (TDF)]. However, its use in a combined regimen may need further evaluation in patients with HBV decompensated cirrhosis due to the potentially favourable effect of telbivudine on renal function[30].

ETV (0.5 mg daily) is a selective anti-HBV agent with potent activity against wild type HBV[7,31]. ETV has a high genetic barrier to resistance in naïve patients (< 1.5% cumulative rate of viral resistance after 6 years of treatment)[32,33] including those with advanced fibrosis or histological cirrhosis[34]. Regarding safety, lactic acidosis has been occasionally reported in small cohorts patients with severe liver dysfunction receiving ETV[35]. However, its true incidence is unclear, since studies with larger cohorts did not confirm this lethal complication[2,31]. In any case, close monitoring is advised for ETV and perhaps any NA treated patient with MELD score ⩾ 20. The high efficacy and the minimal resistance rates combined with the lack of significant nephrotoxicity make ETV a first-line option for the treatment of naive patients with HBV decompensated cirrhosis[36]. On the other hand, ETV monotherapy even at the licensed dosage of 1 mg daily

Table 1 Studies of nucleos/tide analogues in patients with hepatitis B related decompensated cirrhosis

Ref.	Fontana *et al*[47]	Schiff *et al*[19]	Shim *et al*[31]	Liaw *et al*[44]	Chan *et al*[29]	Hyun *et al*[49]
Number of patients	154	226	70	45/45/22	114/114	45/41
NA(s) used	LAM	ADV	ETV	TDF/TDF + FTC/ETV	LdT/LAM	ETV/LAM
Baseline data						
LAM resistance (%)	0	100	0	18/22/14	0/0	0/0
CTP score	9	NR	8.4	7/7/7	8.1/8.5	9.6/9.5
MELD score	NR	NR	11.5	11/13/10.5	14.7/15.5	16.7/16.1
1-yr data						
↓ CTP score ⩾ 2 (%)	NR	NR	49	26/48/42	32/39	NR/NR
MELD score ↓	NR	-2	-2.2	-2/-2/-2	-1.0/-2.0	-4.9/-3.7
1-yr survival (%)	84	86	87	96/96/91	94/88	90.7/92.4
Prognostic factors of the outcome	Serum bilirubin and creatinine levels at baseline	NR	NR	NR	NR	Baseline CTP and MELD at 3 mo

ADV: Adefovir; CTP: Child-Turcotte-Pugh; ETV: Entecavir; TDF: Tenofovir; FTC: Emtricitabine; LAM: Lamivudine; LdT: Telbivudine; MELD: Model for end stage liver disease; NR: Not reported; NAs: Nucleostide analogues.

taken ⩾ 2 h away from food is not a good option for patients with lamivudine resistance, as HBV resistance develops in approximately 50% of lamivudine resistant patients after five years of ETV treatment[37,38].

TDF is the most recently approved agent for the treatment of CHB. Although it is structurally similar to adefovir, it is more potent with activity against both wild type and nucleoside-resistant HBV strains[21,39-41]. It is also active in patients with primary non-response to adefovir[2]. To date, there has been no confirmed case of drug resistance in CHB patients treated with TDF for 6 years, although most patients remaining viremic after 72 wk and being therefore at the highest risk for drug resistance received additional treatment with emtricitabine[42]. Due to its great potency and high genetic barrier, TDF has a beneficial effect on regression of advanced liver fibrosis[43]. Although TDF may be potentially nephrotoxic, similar rates of renal adverse events were observed after one year of therapy with TDF, TDF plus emtricitabine or ETV in patients with HBV decompensated cirrhosis[44].

In conclusion, ETV and TDF are potent antiviral agents with a minimal or even no risk of resistance and therefore they represent the currently recommended first-line NAs for the treatment of patients with HBV decompensated cirrhosis[5]. In addition, TDF is the preferred option for patients with lamivudine, ETV or telbivudine resistance, while the use of ETV (even at a higher daily dose of 1.0 mg) is a less attractive option for the long-term treatment of patients with known lamivudine resistant strains[9]. Whether a combination of antivirals could offer additional benefits is unknown. Given the current cost of anti-HBV agents, the combination that might have a reasonable cost is that of TDF plus lamivudine or emtricitabine[5]. The combination of TDF with emtricitabine was reported not to be significantly superior to TDF or ETV monotherapy[44], but the small numbers of patients in each group of this study cannot allow strong conclusions. Thus, whether any NA combination therapy would confer benefits in patients with impaired renal function who need NA dose reductions or in patients with very high baseline viral load has not been completely clarified yet. Telbivudine (alone or in a combined regimen) with its potentially favorable effect on glomerular filtration seems to be an attractive option in patients with HBV decompensated cirrhosis and renal dysfunction[30].

Referral for liver transplantation

Patients with HBV decompensated cirrhosis should be referred for LT, since the relevant criteria are fulfilled in most of these patients with hepatic dysfunction (Child-Pugh score ⩾ 7 or MELD score ⩾ 10) and/or at least one major complication (ascites, variceal bleeding, hepatic encephalopathy)[45]. While waiting for LT, the patients should be monitored carefully at least every 3 mo for virologic response and possible virologic breakthrough in order to achieve serum HBV DNA undetectability using a sensitive polymerase chain reaction assay[36,46]. Interestingly, the liver function of patients with HBV decompensated cirrhosis may substantially improve under effective antiviral therapy and LT candidates may be eventually withdrawn from the transplant lists[47,48] (Table 1). However, the most important parameters affecting the outcome of patients with HBV decompensated cirrhosis under antiviral agents have not been completely elucidated.

Previous studies using lamivudine monotherapy showed that baseline HBV DNA levels are independently associated with the outcome[47], but in a recent study using a quantitative PCR technique, neither HBV DNA at baseline nor its changes from baseline to 3 mo of treatment were associated with death or LT[49]. Most of the studies including patients with HBV decompensated cirrhosis under oral antivirals have shown that the baseline severity of liver disease, expressed by the Child-Pugh score or the baseline bilirubin and creatinine levels, are critical for the outcome[47,49] (Table 1). In a prospective multicenter study[47] including 154 lamivudine treated patients with HBV decompensated cirrhosis, most of the deaths (78%) occurred within the first 6 mo suggesting that lamivudine may not be able to reduce the short-term mortality or the need for LT in patients with very

advanced liver failure. In contrast, initiation of antiviral therapy at earlier stages is associated with better chances of liver function recovery, since clinical benefit may take 3-6 mo. Whether these results are still valid with the current more potent anti-HBV agents is not clear, but this might be still the case as patients with very advanced liver failure may not benefit from antiviral therapy regardless of the rapidity of the inhibition of viral replication[2]. Nevertheless, further well designed large studies with longer follow-up are needed for final conclusions (Table 1).

PROPHYLACTIC POST-TRANSPLANT APPROACH

Hepatitis B immune globulin

HBIG is a polyclonal antibody to HBsAg derived from pooled human plasma[50]. Its mechanism of action is not completely understood, but it possibly acts by binding with circulating viral particles preventing hepatocyte infection[50]. It also seems to undergo endocytosis by hepatocytes decreasing HBsAg secretion[50]. HBIG was introduced in the early nineties leading to reduction in the rates of post-transplant HBV recurrence[4]. In the landmark study by Samuel *et al*[4] in 1991, it was shown that HBV recurrence could be prevented in 80% of transplant patients treated with HBIG. Prior to the availability of NAs, the initial anti-HBV prophylaxis included administration of high dosage HBIG monoprophylaxis at the anhepatic phase followed by daily doses and then monthly at a fixed dose or according to anti-HBs titers (usually aiming to maintain anti-HBs titers > 100-500 IU/L)[51-53]. However, protocols that use high doses of HBIG are expensive (estimated cost at least $50000-70000 for the first year and $25-40000 for each additional year post-transplant)[54]. Additional limitations of HBIG include the unreliable supply, the parenteral administration, the local or systemic side effects and the risk of infection from HBV mutants that escaped from neutralization[50].

The use of HBIG monoprophylaxis was abandoned after the introduction of lamivudine and the more recent and potent NAs[55]. Nowadays, the most commonly used protocol includes the combination of a NA with a low dose of HBIG[5,55]. Several efforts have tried to reduce the cost using HBIG in lower dosage or preparations for intramuscular administration, which have similar pharmacokinetic properties with intravenous preparations[56], or subcutaneous HBIG[57]. Another strategy has been the substitution of HBIG with HBV vaccination. However, results on the efficacy of active vaccination using new vaccines and adjuvants are rather conflicting[58-60], and therefore, further studies with greater numbers of patients and longer follow-up periods are required before definite conclusions can be drawn.

Prophylactic post-transplant combined approach

Currently, the combination of HBIG and NA is considered the standard of care against HBV recurrence after LT[5]. This combined regimen relies on the complimentary mechanisms of action of HBIG and NA[55]. A recent meta-analysis of 6 studies showed that HBIG plus lamivudine, compared to HBIG alone, was associated with 12-fold, 12-fold and 5-fold reduction of HBV recurrence, HBV-related death and all-cause post-transplant mortality, respectively[61]. A second meta-analysis also showed that the combination of HBIG and lamivudine was superior in preventing only serum HBsAg re-appearance, compared to lamivudine alone[62]. However, lamivudine is not considered an optimal first-line option because of the progressively increasing rates of viral resistance[5,63]. This was confirmed in our systematic review[55] including 2162 HBV liver transplant recipients from 46 studies. In this review, we found that the patients under HBIG and lamivudine, compared to those under HBIG and adefovir (with or without lamivudine) had HBV recurrence more frequently (6.1% *vs* 2%, $P = 0.024$), although they had detectable HBV DNA less frequently at the time of LT (39% *vs* 70%, $P < 0.001$).

Although several questions about the ideal duration, dosage, frequency and mode of HBIG administration remain unanswered[55], we found that patients under HBIG and lamivudine who received high (≥ 10000 IU/d) dosage of HBIG, compared to those who received low HBIG dosage (< 10000 IU/d) during the 1st wk post-LT, had significantly less frequent HBV recurrences (3.3% *vs* 6.5%, $P = 0.016$). On the other hand, HBIG administration had no impact on HBV recurrence in patients under HBIG and adefovir. Based on these findings[55], we concluded that the patients under HBIG and lamivudine combination prophylaxis should receive high HBIG dosage (10000 IU IV) for the first week after LT, while the characteristics of the HBIG protocol do not seem to have any impact on the efficacy of HBIG and adefovir combination prophylaxis against HBV recurrence.

Adefovir has several drawbacks in the post-transplant setting including high cost, relatively low potency in the licensed 10 mg daily dose, risk of viral resistance and risk of nephrotoxicity[5]. The latter is of particular concern in liver transplant recipients because most of them receive nephrotoxic calcineurin inhibitors as part of an immunosuppressive regimen and frequently suffer from diabetes mellitus and arterial hypertension.

Newer and more potent NAs with a higher genetic barrier, such as ETV and TDF, are currently used in the post-transplant period in many transplant centers, mainly in an effort to increase the efficacy of post-LT prophylaxis and/or reduce the need for the expensive HBIG preparations at least after the initial post-operative period[5]. The efficacy of ETV and TDF was evaluated in our recently published systematic review including 519 HBV liver transplant recipients from 17 studies[64]. We found that patients under HBIG and lamivudine developed HBV recurrence significantly more frequently, compared to patients under HBIG and ETV or TDF combination (6.1% *vs* 1.0%, $P < 0.001$) (Figure 1), although they received a more intense HBIG protocol after LT[64]. In addition, ETV and TDF had similar antiviral efficacy when

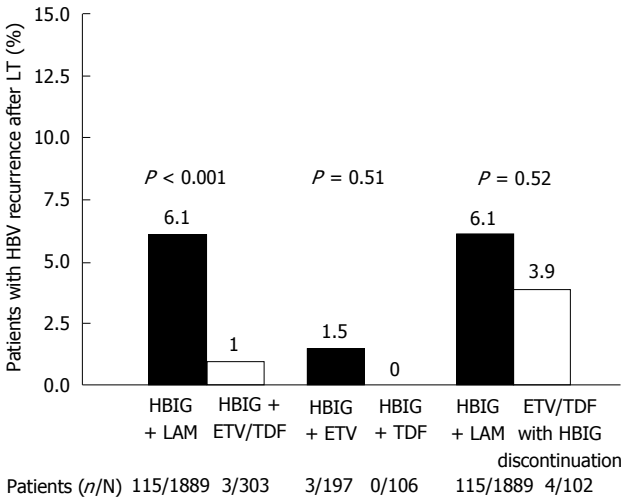

Figure 1 Risk of recurrence of hepatitis B virus infection after liver transplantation in relation to the type of post-transplant hepatitis B virus prophylaxis[64]. HBIG: Hepatitis B immunoglobulin; LAM: Lamivudine; ETV: Entecavir; TDF: Tenofovir; LT: Liver transplantation.

they combined with HBIG (1.5% *vs* 0%, respectively, $P > 0.05$)[64] (Figure 1).

Given the several limitations of HBIG and the fact that waiting list patients are more likely to undergo LT with undetectable HBV DNA, one relatively recent strategy has been the use of HBIG for a limited post-transplant period followed by long-term NA therapy alone[64]. The first results published with lamivudine monoprophylaxis after HBIG withdrawal were encouraging[65,66], but longer follow-up showed that 20% of patients eventually experienced recurrence of HBV[65,67]. ETV and TDF, however, may allow early and safe discontinuation of HBIG. Strong data are not available, but our systematic review[64] showed that ETV or TDF monoprophylaxis after HBIG discontinuation does not seem to be inferior to the combination of a newer NA with HBIG or the combination of HBIG plus lamivudine (3.9% *vs* 1.0%, 3.9% *vs* 6.1%, $P > 0.05$) (Figure 1). Although larger studies with longer follow-up are needed for definitive conclusions, this approach has been already used in several transplant centres, particularly in patients with relatively low risk of HBV recurrence[68].

Prophylactic post-transplant monotherapy with nucleos(t)ides analogues

The high efficacy of antiviral prophylaxis using a shorter course of HBIG with continuation of NA without HBIG, and the availability of NAs without cross-resistance in cases of prophylaxis failure, led to the consideration of HBIG-free prophylactic regimens. This approach, which is challenging and controversial, started with lamivudine, but the unacceptably high rates of HBV recurrence (up to 35%-50% of cases at 2 years post-transplant)[69-74] has rendered this approach suboptimal. However, recent studies have renewed the interest in HBIG-free prophylactic regimens using the more potent regimens with high genetic barrier ETV and TDF. Recently, Fung *et al*[75] evaluated 80 consecutive patients transplanted for HBV-related liver disease. Fifty nine (74%) of the patients had detectable HBV DNA at the time of LT, and all patients received ETV monoprophylaxis without HBIG at any time point after LT. After a median follow-up of 26 mo, 18 (22.5%) patients were HBsAg positive, but only one of them had detectable HBV DNA[75]. In their subsequent study[76] including 362 transplant recipients under HBIG-free prophylaxis, none of the patients who receive ETV had HBV recurrence, compared to 17% of those who received lamivudine, highlighting the importance of using potent regimens with a high genetic barrier (ETV or TDF) in HBIG-free prophylaxis protocols.

In our recent systematic review[64], HBV recurrence was observed significantly more frequently in patients who received ETV or TDF HBIG-free prophylaxis, compared to patients under combination of HBIG and lamivudine prophylaxis, if the definition of HBV recurrence was based on HBsAg positivity (26% *vs* 5.9%, $P < 0.0001$). However, if the definition of HBV recurrence was based on HBV DNA detectability, the rates of HBV recurrence were similar between the two groups (0.9% *vs* 3.8%, $P = 0.11$)[64]. Given the current availability of potent NAs with negligible risk of long-term viral resistance, the clinical significance of HBsAg seropositivity in HBV transplant patients is unclear[68]. The prognosis of non-transplant CHB patients who maintain HBV DNA undetectability under NA(s) is excellent, particularly if they had not developed cirrhosis before treatment[77], but the long-term outcome of HBsAg-positive, HBV DNA negative transplant patients under NAs needs further evaluation. In a recent study[78], 5 (20%) of 25 HBV transplant patients who discontinued anti-HBV prophylaxis became HBsAg-positive, but none of them experienced any clinically relevant event and three eventually cleared HBsAg and achieved seroconversion to anti-HBs without any therapeutic intervention.

Currently, ETV and TDF should be the first-line options for HBIG-free prophylaxis. ETV may be avoided in patients with previous lamivudine resistance, who should be preferably treated with TDF. Compliance is always an issue with long-term oral antiviral therapy, particularly in prophylaxis after LT when patients feel well but remain at life-long risk of HBV recurrence[64]. Until well designed studies determine the optimal monoprophylaxis approach, the combination of HBIG (at least for a short period) and one nucleos(t)ide appears to be the most reasonable post-transplant approach. Monoprophylaxis with the new nucleos(t)sides analogues cannot be excluded in the future, particularly in patients with low risk of recurrence[68].

THERAPEUTIC POST-TRANSPLANT APPROACH

Recurrence of HBV infection after LT is usually characterized by reappearance of serum HBsAg and/or serum

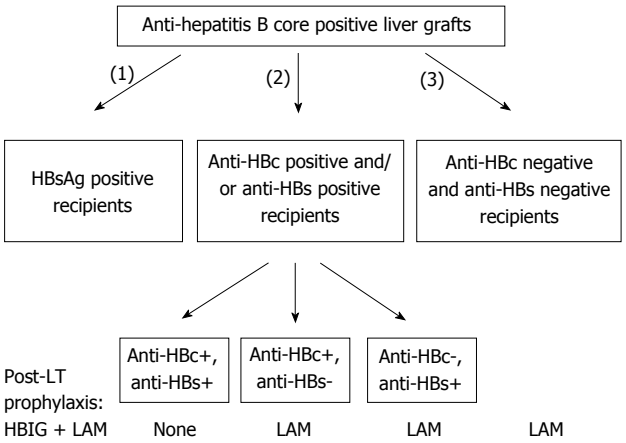

Figure 2 Proposed algorithm for allocation and management of anti-hepatitis B core positive liver grafts. Such grafts should be first offered to hepatitis B surface antigen positive, then to anti-hepatitis B core (HBc) and/or anti-hepatitis B surface (HBs) positive and lastly to hepatitis B virus naive (both anti-HBc and anti-HBs negative) recipients[79]. LT: Liver transplantation; HBIG: Hepatitis B immunoglobulin; LAM: Lamivudine.

HBV DNA, which is frequently accompanied with biochemical or clinical evidence of recurrent liver disease. As mentioned before, particularly in patients under HBIG-free post-LT HBV prophylaxis, the definition of HBV recurrence might be reconsidered, as HBsAg seropositivity, usually in low titers, with undetectable HBV DNA, normal liver enzymes and no clinical manifestations of HBV recurrence may not have any clinical impact on the long-term graft and patient survival.

The choice of treatment for HBV recurrence depends on prior prophylactic therapy. In general, the principles of treatment in post-transplant HBV recurrence resemble those in the pre-transplant setting. ETV may be preferred in NA-naïve patients because of the lack of nephrotoxicity, although in a recent study there was no difference in renal complications between ETV and TDF in liver transplant recipients[68]. In patients with prior lamivudine resistance, TDF is the best choice[37]. Little is known about the efficacy and safety of the combination of TDF and ETV which might be used in patients with multidrug resistant HBV strains.

ANTI-HBC POSITIVE DONORS

The current efforts to overcome the organ shortage include the use of marginal liver grafts, such as those from anti-HBc positive donors. This source of organs can be of particular importance in countries with high prevalence of HBV infection, such as the Mediterranean area and Asia. HBsAg positive liver patients are the optimal recipients to receive liver grafts from anti-HBc positive donors. Unfortunately, the "occult" HBV infection in the donor liver may be reactivated in the HBsAg negative recipient due to post-LT immunosuppressive therapy leading to *de novo* HBV infection. In our systematic review[79] including 903 recipients of anti-HBc positive liver grafts, *de novo* HBV infection developed in 19% of HBsAg negative recipients being less frequent in anti-HBc/anti-HBs positive than HBV naive cases without prophylaxis (15% *vs* 48%, $P < 0.001$). Anti-HBV prophylaxis reduced *de novo* infection rates in both anti-HBc/anti-HBs positive (3%) and HBV naive recipients (12%)[79]. *De novo* HBV infection rates were 19%, 2.6% and 2.8% in HBsAg-negative recipients under HBIG, lamivudine and their combination, respectively. Based on these findings[79], we concluded that liver grafts from anti-HBc positive donors can be safely used in HBsAg negative recipients, preferentially in anti-HBc/anti-HBs positive recipients who may need no prophylaxis at all, while the anti-HBc and/or anti-HBs negative recipients should receive long-term prophylaxis with lamivudine (Figure 2).

CONCLUSION

Over the last two decade, the progress in anti-HBV therapy has led to great improvements in the management of HBV patients before and after LT. There is no doubt that all HBV patients with decompensated cirrhosis should be treated with a potent antiviral agent with minimal or no risk of resistance, *i.e.* ETV or TDF. In addition, TDF is the preferred option for patients with prior lamivudine, ETV or telbivudine resistance. An effective pre-transplant anti-HBV therapy often stabilizes or even improves the underlying liver disease resulting sometimes in withdrawals from the transplant list. In addition, achievement of serum HBV DNA undetectability prevents post-transplant HBV recurrence. After LT, the combination of HBIG (at least for a certain period) and one NA (ETV or TDF) currently appears to be the most reasonable prophylaxis, while monoprophylaxis with ETV or TDF cannot be excluded in the future, particularly in patients with low risk of recurrence. Depending on previous drug exposure and possible pre-existing resistance mutations, ETV or TDF seem to be the most attractive options for post-LT HBV recurrence as well. Finally, liver grafts from anti-HBc positive donors can be safely used in HBsAg negative, preferentially anti-HBc/anti-HBs positive recipients.

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TOPIC HIGHLIGHT

WJG 20th Anniversary Special Issues (7): Liver transplant

Long-term survival after liver transplantation for alcoholic liver disease

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Abstract

Currently, alcoholic cirrhosis is the second leading indication for liver transplantation in the United States and Europe. The quality of life and survival after a liver transplantation (LT) in patients with alcoholic liver disease (ALD) are similar to those in patients with other cirrhosis etiologies. The alcoholic relapse rate after a LT varies from 10%-50%, and these relapse patients are the ones who present a reduced long-term survival, mainly due to cardiovascular diseases and the onset of *de novo* neoplasms, including lung and upper aerodigestive tract. Nearly 40% of ALD recipients resume smoking and resume it early post-LT. Therefore, our pre-and post-LT follow-up efforts regarding ALD should be focused not only on alcoholic relapse but also on treating and avoiding other modifiable risk factors such as tobacco. The psychiatric and psychosocial pre-LT evaluation and the post-LT follow-up with physicians, psychiatrists and addiction specialists are important for reversing these problems because these professionals help to identify patients at risk for relapse as well as those patients who have relapsed, thus enabling responsive actions.

Key words: Alcoholic liver disease; Alcohol recidivism; Alcohol relapse prevention; Long term survival; Liver transplantation

Core tip: Transplanted alcoholic liver disease (ALD) patients who relapse have an increased long-term mortality due to cardiovascular pathologies and the onset of *de novo* neoplasms, including lung and upper aerodigestive tract cancer. Nearly 40% of ALD recipients resume smoking and resume it early post-liver transplantation (LT). Therefore, our pre-and post-LT follow-up efforts regarding alcoholic liver disease should be focused not only on alcoholic relapse but also on treating and avoiding other modifiable risk factors such as tobacco. The psychiatric and psychosocial pre-LT evaluation and the post-LT follow-up with physicians, psychiatrists and addiction specialists are important for reversing these problems.

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INTRODUCTION

Excessive alcohol consumption causes approximately 2.5 million deaths per year and is responsible for almost 4% of mortality worldwide. Alcohol has been associated with nearly 60 types of diseases and is the third leading risk factor for disease and disability worldwide. Furthermore, excessive alcohol consumption contributes to multiple social problems, including violence, child neglect and ab-

Table 1 Primary indication for liver transplantation in Europe and the corresponding survival

Indication for LT	Patients (*n*)	From 1988 to 2009		
		1 yr	5 yr	10 yr
Alcoholic cirrhosis	15019	86%	73%	59%
Acute hepatic failure	6507	70%	64%	58%
Cirrhosis virus C	10753	80%	65%	53%
Cirrhosis viral C and alcoholic	1790	85%	69%	54%
Cirrhosis virus B	4187	83%	74%	68%
Hepatocarcinoma and cirrhosis	9122	83%	62%	49%
Cholestatic disease	9114	87%	78%	70%
Autoimmune cirrhosis	1892	85%	76%	67%
Hemochromatosis	468	76%	66%	53%

Adapted from the European Liver Transplant Registry[3]. LT: Liver transplantation.

senteeism[1].

Alcoholic liver disease (ALD) is the main cause of cirrhosis in Western countries and contributes to one third of the mortality associated with liver cirrhosis. Furthermore, ALD is the second most common indication for liver transplantation (LT) in the United States and Western Europe[2-7], accounting for about 40% of transplants in Europe and 20% of transplants in the United States[2-7].

If we analyze the medium- and long-term survival of a transplant patient, there is no doubt that the recipients with an alcoholic etiology have great results, with a European global 5-year survival rate of 73% and a 10-year survival rate of 59%[2,3], rates that are superior to those for recipients with other etiologies (Table 1)[3]. Therefore, we can infer that ALD is a good indication for LT[7]. However, these excellent results are diminished when a harmful alcohol consumption relapse occurs. These relapses and their possible consequences on the transplant and on the survival and quality of life of the patient, as well as possible actions to avoid relapses, are discussed below.

An evidence-based approach was used for this review. MEDLINE search was performed to September 2013 using the following MeSH terms: liver transplantation, alcohol-related disorders, alcohol-induced disorders, drug abuse, substance abuse, tobacco, and neoplasm. Searches were limited to English language articles. References of suitable articles were searched for other appropriate articles.

QUALITY OF LIFE AFTER LT

Quality of life involves physical, mental and social well-being, including working life, and is considered a survival indicator that even surpasses traditional indicators[8]. In the cirrhotic patient, a decrease in physical, psychological and intellectual capabilities occurs alongside liver function impairment[9]. Therefore, it is logical to believe that those patients with advanced liver disease might have a significant reduction in their quality of life. Therefore, the question we might ask is the following: does LT improve the quality of life in these patients? To answer this question, numerous studies that evaluated the quality of life after LT have been performed[10-17]. However, it is not easy to extrapolate the results of these studies due to the heterogeneity of the post-transplantation follow-up times and the instruments that were used to evaluate the different spheres comprising the quality of life[10-17]. In general, studies reveal a significant short-term improvement in the quality of life with no differences observed between ALD and non-alcoholic liver disease[10-18]. Notably, although ALD patients seem less likely to be involved in structured social activities during the post-LT phase than the patients who were transplanted as a result of other etiologies, the ALD patients return to society to lead active and productive lives[19]. Few studies analyzed the quality of life long-term. As a representative study, the study by Ruppert *et al*[17]. that included a 12-year follow-up after LT does not show a progressive loss of quality of life in these patients after the first year of LT[17].

Regarding job reinsertion, the age at the time of the LT, the duration of the pre-transplant disability and the physical and general health status of the patient are the factors that correlate more with employment[14,20-22]. Globally, approximately half of the LT patients return to work[20-22], with no differences between the ALD patients and those with the remaining etiologies[15,23].

ALCOHOLIC RELAPSE/RECIDIVISM AFTER LT

We must recall that, although LT effectively restores the physiological function of the liver and reverses the complications of portal hypertension, LT does not treat the underlying alcoholism. Alcoholism is a life-long disease that is often characterized by episodes of a relapsing-remitting pattern of alcohol use despite the physical, psychological and social consequences, wherein the probability of long-term sobriety becomes robust only after 5 years of sustained abstinence[24-26].

Dimension of the problem

Addiction specialists define relapse as the prolonged resumption of heavy alcohol intake and distinguish this harmful drinking behavior from so-called slips, which are defined as sporadic drinking episodes followed by the reestablishment of abstinence[27]. This definition of alcoholic relapse is in contrast to that by most transplant centers that consider any alcohol consumption after LT to be unacceptable and define recidivism as any use of alcohol after LT[28]. Most of these episodes of alcohol abuse are effectively diagnosed with interviews and validated self-reporting questionnaires[29,30].

Reviews summarizing the post-transplantation alcoholic relapse rates note differences across studies ranging from 10%-95%, likely due to several factors, including variations in the study methodology, the definition and assessment of relapse and the duration of the follow-up (Table 2)[28,31-60]. In general, the risk that alcoholic

Table 2 Alcohol relapse (any use) after liver transplantation for alcohol liver disease

Ref.	Study design	Patients (*n*)	Year	Follow-up median or mean (mo)	Relapse rate
Bird *et al*[34]	Retrospective	18	1990	84	17%
Kumar *et al*[48]	Retrospective	52	1990	25	12%
Gish *et al*[42]	Prospective	29	1993	24	24%
Knechtle *et al*[38]	Retrospective	32	1993	Not stated	13%
Berlakovich *et al*[22]	Retrospective	44	1994	78	32%
Howard *et al*[45]	Retrospective	20	1994	43	95%
Krom *et al*[48]	Retrospective	30	1994	Not stated	13%
Osorio *et al*[51]	Retrospective	43	1994	21	19%
Gerhardt *et al*[41]	Retrospective	41	1996	47	49%
Tringali *et al*[56]	Retrospective	58	1996	27	21%
Zibari *et al*[58]	Retrospective	29	1996	Not stated	7%
Coffman *et al*[84]	Prospective	91	1997	Not stated	20%
Anand *et al*[31]	Retrospective	39	1997	25	13%
Everson *et al*[38]	Retrospective	42	1997	Not stated	17%
Foster *et al*[40]	Retrospective	63	1997	49	21%
Lucey *et al*[50]	Retrospective	50	1997	63	34%
Stefanini *et al*[53]	Retrospective	18	1997	Not stated	27%
Fabrega *et al*[39]	Prospective	44	1998	40	18%
Tang *et al*[54]	Retrospective	56	1998	24	50%
Yates *et al*[57]	Retrospective	43	1998	21	19%
Gledhill *et al*[44]	Retrospective	24	1999	14	25%
Pageaux *et al*[75]	Retrospective	53	1999	32	42%
Pereira *et al*[13]	Retrospective	56	2000	30	50%
Burra *et al*[73]	Prospective	34	2000	40	33%
Jain *et al*[79]	Retrospective	185	2000	94	20%
Dimartini *et al*[37]	Prospective	36	2001	12	38%
Gish *et al*[43]	Prospective	61	2001	83	20%
Mackie *et al*[28]	Retrospective	46	2001	25	53%
Bellamy *et al*[32]	Retrospective	123	2001	84	13%
Karman *et al*[57]	Retrospective	49	2001	36	21%
Bravata *et al*[93]	Retrospective	313	2001	Not stated	32%
Pageaux *et al*[52]	Retrospective	128	2003	54	31%
Jauhar *et al*[86]	Retrospective	111	2004	44	15%
Cuadrado *et al*[36]	Retrospective	54	2005	99	26%
Bjornsson *et al*[35]	Retrospective	103	2005	31	33%
Kelly *et al*[85]	Retrospective	90	2006	67	31%
Pfitzman *et al*[83]	Retrospective	300	2007	89	19%
Karim *et al*[91]	Retrospective	80	2010	Not stated	10%
Schmeding *et al*[60]	Retrospective	300	2011	84	27%
Rice *et al*[74]	Retrospective	300	2013	78	16%

recipients return to any alcohol use after LT is between 10%-50% with 8-year follow-ups[28,31-60]. More specifically, between 20 and 50% of the patients who received a liver transplant for end-stage ALD acknowledge some alcohol use in the first 5 years after LT, and 10%-15% will resume heavy drinking[28,55,59]. This finding compares favorably to post-treatment relapse rates as high as 80%-95% in treatment studies of alcoholics without ALD[24].

In a meta-analysis performed in 2008 on the risk of recurrence of substance use after solid organ transplantation that included 54 studies, 50 of which were on LT, it was concluded that the relapse rate of alcohol consumption after LT was 5.6 cases per 100 patients/year, and the relapse rate of excessive consumption was 2.5 cases per 100 patients/year[27]. Additionally, the authors concluded that it was possible that these cumulative incidence rates would become stable at some point that could not be established because few of the studies had a post-transplant follow-up over 7-8 years[27].

Being able to determine the threshold of initiation of alcohol consumption after a liver transplant would be of great clinical and therapeutic utility because this knowledge would allow us to plan specific interventions more accurately. DiMartini *et al*[61] have described four different patterns of alcohol consumption depending on the starting date, quantity and duration as follows: (1) Minimum consumption over a long period; (2) Early consumption that progresses rapidly to moderate consumption; (3) Early consumption that progresses continuously to a harmful consumption; and (4) Moderate consumption with a late start. These results indicate that we should maintain surveillance after the first year post-LT, despite the fact that the rates for the initiation of consumption generally attenuate over time post-transplantation, probably due to the increase in the stability of sobriety over time[30].

Consequences

The impact of alcohol use on the patient is not entirely clear. The available literature suggests that abusive drinking leads to a decrease in both graft and patient survival and may also lead to the lack of therapeutic compliance.

Adherence to immunosuppressant medication: In LT, adherence to the immunosuppressant treatment and any other drugs medically prescribed is crucial for positive short- and long-term results in the transplanted patients because non-adherence to these measures might lead to graft rejection and failure[62]. Reviews summarizing the nonadherence rates post-transplantation note differences across studies ranging from 3%-47%, probably due to several factors, including variations in study methodology, definitions and the small number of patients included in the studies[63-65].

Therefore, we question whether the consumption or abuse of substances pre-LT increases the risk of non-adherence to immunosuppressant treatment[63,66] and whether an alcoholic relapse is associated with pre-LT alcohol use[36,62,63]. Berlakovich *et al*[63] studied the effect of alcohol consumption on adherence and found that the patients who relapsed (15 of the 118 transplanted ALD patients) had a non-adherence rate that was no different from that of the patients who did not relapse. This finding was also demonstrated in a study performed in our hospital, where there was no association between the adherence to drug treatment and the presence or absence of alcoholic relapse in a series of transplanted ALD patients[36]. We believe that this concept is endorsed by the meta-analysis of Dew *et al*[27], which showed a lack of association. Specifically, these authors observed that European studies presented a lower non-adherence rate to immunosuppressant treatment compared to the North American studies, despite presenting significantly higher relapse rates of harmful alcohol consumption[27].

Thus, the lack of adherence seems to be linked to the personality of the patient, the acknowledgement of their

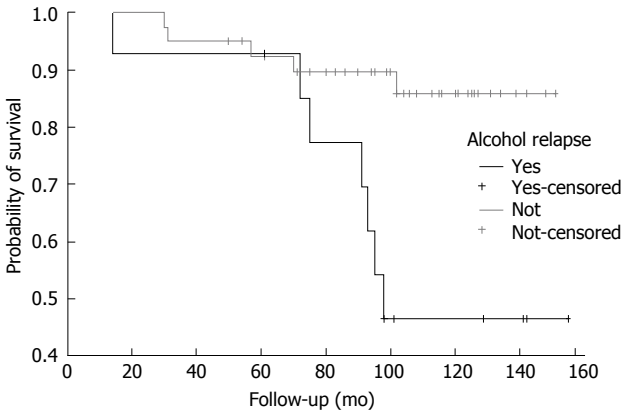

Figure 1 Kaplan-Meier survival curves from patients with alcoholic liver diseases, with or without alcohol recidivism[36].

disease, the complexity of the medical prescriptions, the presence of family support and the doctor-patient relationship, more so more than to alcohol consumption[67,68].

Liver graft: Resuming alcohol consumption after LT may damage the graft because of poor compliance with immunosuppressive drug treatment and alcohol-related liver injury. Graft loss from recurrent disease related to alcohol use is rare[69,70]. Globally, graft dysfunction related to relapse ranges from 0%-17%, although deaths related to relapse range from 0%-5%[52,71]. There are few studies on the severity of the liver lesions associated with alcohol consumption after LT on ALD patients[60,72-74]. Rice *et al*[74] found that alcohol relapse is associated with advanced fibrosis on biopsy. In contrast, our histologic study revealed only mild hepatic changes directly attributable to alcohol[36]. As reported by Pageaux *et al*[52,75], fatty changes and pericellular fibrosis represented the most relevant histological findings in patients who resumed heavy alcohol intake.

In contrast, several studies have shown that ALD LT patients have a lower rejection risk compared to other LT indications, suggesting an inhibitory effect of alcohol over some components of the immune response[2,76-78]. We have corroborated this finding and observed a lower incidence of acute rejection among patients who relapse to alcohol consumption compared to abstemious patients[36].

Long-term survival: Jain *et al*[79] observed that the 5-year post-transplantation survival rate was significantly lower for transplanted ALD patients compared to transplanted non-alcoholic liver disease patients, mainly due to cardiovascular events and *de novo* neoplasms, especially of the aerodigestive tract, which suggests that immunosuppression by itself is not an initiation factor for malignant changes[80,81]. We reached a similar conclusion after we evaluated the alcoholic relapse risk in a series of transplanted ALD patients and the influence of a relapse on survival[35]. In our case, the 5-year survival rate was similar between relapsers and non-relapsers (92.9% *vs* 92.4%, respectively), but after 10 years, the survival rate decreased significantly in the relapse patients (45.1% *vs* 85.5%), with malignant tumors and cardiovascular events the main cause of death in these patients (Figure 1)[36]. In addition, tobacco consumption was observed in all the patients with an alcoholic relapse and in only one quarter of the abstemious patients, which might explain the higher mortality rate due to cardiovascular events and neoplasms in these patients; this finding has been observed in other studies, as will be discussed later. However, the transplanted ALD patients are potentially affected not only by alcohol consumption but also by liver diseases with other etiologies. This finding has been shown in a recent study in which excessive alcohol consumption had a negative impact on long-term survival after LT regardless of the indication[82].

Despite these results, it is important to distinguish from among the relapsers those who are "slip" drinkers (mild alcohol consumption that is usually isolated or self-limited) and those who are "heavy" drinkers (a long period of alcohol consumption with a loss of control) because the former have a better survival rate compared to the latter[83].

IDENTIFICATION OF THESE PATIENTS

Because of the above findings, it is important to identify relapse patients, but it is more important to prevent this relapse by identifying the patients at risk.

In order to predict the post-LT alcoholic relapse risk with a high degree of accuracy, it is necessary to acknowledge the risk factors that have a strong correlation, which has not yet been achieved. In this regard, numerous studies have identified factors related to the risk of post-LT alcoholic relapse, such as alcohol dependence, an age less than 40 years at the time of the transplantation, a lack of family and social support, a family history of alcoholism, personality or psychiatric disorders, previous abstinence or substance abuse failures, younger age at LT, and the refusal of further rehabilitation before the LT[30,37,40,42,43,58,78,83-87]. However, this association has not yet been corroborated in other studies[26,30,37,54,75,84-86]. For this reason, Kotlyar *et al*[88] decided to perform a critical review of the literature on LT in ALD candidates and concluded that patients with a lack of social support, active smoking, psychotic or personality disorders or a pattern of nonadherence should be listed only with reservation, and those who have a diagnosis of alcohol abuse as opposed to alcohol dependence may make better transplant candidates. Finally, the most controversial among these risk factors is the 6-mo pre-LT period of abstinence, about which many studies have reported a high predictive power regarding relapse[27,30,34,89-91], while others have not found such a correlation[40,79,85,86,88,92].

Most LT centers in Europe and the United States require a minimum of 6 mo of abstinence before being included in the waiting list. This common practice is based on two points: first, the possibility of improving liver function and possibly avoiding the LT, and second,

the higher alcoholic relapse rate reported in patients with a period of abstinence less than 6 mo[51,93]. Both points have been discussed. Veldt *et al*[94] demonstrated that those with irreversible ALD were identified with 3 mo of abstinence; out of 74 patients with a Child-Pugh C liver function, the percentage of patients with improvement after 1, 2 and 3 mo of abstinence was 23%, 40% and 66%, respectively, and the remaining 33% did not show improvement at a 1-year follow-up. Furthermore, it has not been proven that this 6-mo abstinence period improves survival after LT[95]. Considering all of this, and although improved post-LT abstinence rates have been documented with a longer pre-LT abstinence period, a cut-off point has not yet been established[30,40]. Therefore, the pre-LT alcohol abstinence period could be shortened for some patients because this factor by itself is a poor indicator of post-LT relapse. In addition, some patients, especially those with a high Model for End-Stage Liver Disease score, have a considerable risk of mortality during the 6 mo abstinence period[96,97].

In conclusion, a thorough assessment by a trained alcoholism and addiction professional, rather that defined sobriety periods, should be the tool used to assess the future risk of alcoholic relapse in the alcoholic patient.

ALCOHOL AND TOBACCO

Patients who undergo LT have an unexpectedly high rate of *de novo* extrahepatic cancer[98,99], including lung and upper aerodigestive tract cancer[98,100,101], and studies have reported that patients with ALD are particularly affected[79,99,102]. Indeed, these tumors are known to be associated with alcohol intake and smoking because the carcinogenic or co-carcinogenic effects of smoking and drinking might be enhanced by the post-LT immunosuppressive therapy[103]. The purported mechanisms of alcohol-mediated oncogenesis are poorly understood, but these pathways may involve the carcinogenic properties of acetaldehyde and/or the inhibition of DNA methylation *via* the alteration of retinoid processing[104,105]. Saigal *et al*[106] found that patients who underwent LT for ALD appeared to have an increased risk of developing post-transplantation malignancies compared with those who underwent LT for other liver diseases. These authors hypothesized that a tumorigenic action mediated by the immunosuppressive effect of alcohol on natural killer cells could explain this observation. In fact, in the non-immunosuppressed population, alcoholism is associated with an increased risk for several malignancies, including liver and alimentary tract tumors[107,108]. Jain *et al*[79] observed a higher rate of *de novo* oropharyngeal and pulmonary neoplasms in transplanted ALD patients than in those with a non-alcoholic disease, similar to Duvoux *et al*[102], suggesting the presence of other initiators of malignant changes in addition to immunosuppression. Among the identified risk factors, alcohol and tobacco consumption were highlighted[98,99,109], data also obtained in our study[36].

Regarding tobacco, nearly 90% of alcoholics smoke[110], compared to 26.7% of the general population of the United States[111]. Regarding the candidates for LT, approximately 60% are smokers[112,113] and 15%-40% continue to smoke after the LT[112,114]. DiMartini *et al*[114] found that nearly 40% of ALD recipients resume smoking and resume it early post-LT, increase their consumption over time and quickly become tobacco dependent. In a recent meta-analysis, active smoking was revealed as one of the major risk cofactors, independent of alcoholic relapse, of long-term morbidity and mortality in transplant recipients, either from cardiovascular complications or from *de novo* neoplasms[88]. This data was confirmed in numerous studies[36,109,114-121]. In an interesting study from a conceptual point of view, Herrero *et al*[122] showed that smoking withdrawal after LT may have a protective effect against the development of neoplasia. In particular, these researchers observed that patients with a smoking history who continued smoking after the LT, presented a hazard ratio of approximately 20 for the development of neoplasms associated with tobacco (head and neck, lung, esophagus, kidney and urinary tract carcinomas), while the risk of developing these neoplasms was reduced significantly in ex-smokers[122]. Furthermore, as noted earlier, smoking is also a risk for cardiovascular disease, and this is one of the most frequent causes of late mortality after LT[36,123]. Pungpapong *et al*[113] found a higher rate of vascular complications in LT recipients who had a history of smoking. Those who quit smoking 2 years prior to the transplantation reduced the incidence of vascular complications by 58%. Therefore, our pre- and post-LT follow-up efforts regarding ALD should be focused not only on alcoholic relapse but also on treating and avoiding other modifiable risk factors such as tobacco, not simply because of what was discussed earlier, but because we now acknowledge that tobacco is a risk factor for alcohol abuse. In a recent study on mice, it was observed that the rodents exposed to nicotine tended to ingest alcohol more frequently than those that were not administered such a substance due to a reduction in the dopamine response of the reward-response system in the brain, which thus decreased the pleasurable response to alcohol[124]. Given all the above-mentioned observations, the establishment of control programs and post-LT interventions could perhaps reduce the mortality in these patients, as will be discussed below.

POST-LT FOLLOW-UP IN ALD

Several approaches have been evaluated to reduce alcohol recidivism in alcoholic patients after LT, but there is no standardized approach, and the available data are few and often controversial. In some liver transplant centers, alcoholic patients are encouraged to attend support groups, even if the data demonstrating the efficacy of such treatment in this cluster of patients are currently lacking. In a pilot study, Georgiou *et al*[125] reported that psychological interventions could be a valid approach to enhance motivation in these patients. However, this study was con-

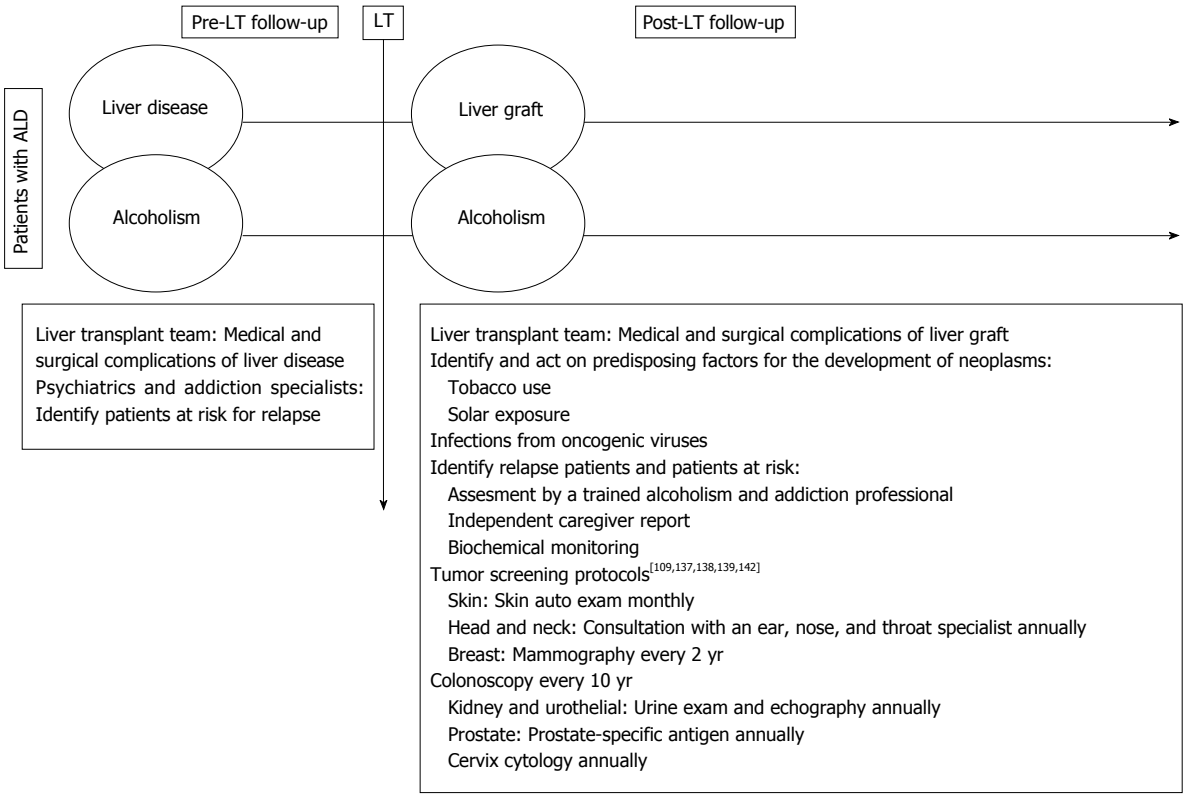

Figure 2 Proposed pre- and post-liver transplantation follow-up in alcohol liver disease. LT: Liver transplantation; ALD: Alcohol liver disease.

ducted on a limited number of patients, and the efficacy of this intervention on alcohol recidivism after LT was not evaluated. Björnsson *et al*[35] evaluated the impact of the management of alcoholic patients by addiction psychiatrists, social workers and tutors in the period before LT and reported a 22% prevalence of alcohol recidivism in the treated group *vs* 48% in the untreated group. The presence of an alcohol addiction unit within a liver transplant center is not usual, but the study of Addolorato *et al*[126] suggests that it could represent a useful approach to reducing alcohol recidivism after LT. However, objective and accurate indicators of abstinence are required[127]. Direct detection in the blood or breath only assesses alcohol intake within the preceding 10-12 h[39,128]. Carbohydrate-deficient transferrin (CDT) is an indirect marker that reflects alcohol intake in the previous 1-4 wk[129,130]; however, the daily consumption of 60-89 g of ethanol for a period of at least 7-10 d is required for a positive result. Therefore, CDT is inappropriate for the detection of low-to-moderate alcohol intake. Furthermore, wide ranges in sensitivity and specificity of 46%-73% and 70%-100%, respectively, have been reported[131]. Nevertheless, a high rate of false-positives with the CDT test has been reported, particularly in patients with severe liver damage[131].

Currently, the determination of ethyl glucuronide (EtG), a metabolic product of alcohol, either in the urine or in the hair of patients offers a new, reliable possibility for the detection of alcohol intake[132-135]. Urinary EtG (uEtG) remains positive for up to 80 h after alcohol consumption and allows for the detection of very small amounts of ethanol (uptake of < 5 g)[133,135] with a sensitivity and specificity of 89% and 99%, respectively[131]. However, positive uEtG tests may occur after the accidental consumption of foods containing alcohol, such as chocolate, cake and others. To reduce this problem in the transplant setting, a higher cut-off level for uEtG than what is routinely used (> 0.5 mg/L instead of > 0.1 mg/L) is recommended[132].

Furthermore, the detection of EtG in the scalp hair of patients is a powerful tool for monitoring abstinence over a retrospective period of up to 6 mo. Each hair segment of 1 cm in length reflects alcohol consumption over a period of approximately 1 mo. The test has been validated for a maximal hair length of 6 cm[135].

Thus, based on the above, we can infer that regular monitoring after LT is critical for determining the ongoing abstinence from tobacco and alcohol and for providing treatment assistance when tobacco or alcohol use are identified. Using a combination of methods (patient interviews by a trained alcoholism and addiction professional connected to the transplant team, independent caregiver reports and biochemical monitoring) provides the greatest yield because every method can add to the number of identified cases[27].

As we have discussed previously, patients who undergo LT have an unexpectedly high rate of *de novo* extrahepatic cancer[98,99], including lung and upper aerodigestive tract cancer[100,102], and studies have reported that patients with ALD are particularly affected[79,99,102]. Therefore,

apart from identifying and acting on predisposing factors for the development of neoplasms (such as tobacco use, solar exposure and infections from oncogenic viruses), intensive tumor screening protocols have been suggested for these patients. Herrero *et al*[109] concluded that ALD transplant patients, smokers or ex-smokers, should have a further follow-up, including a low-radiation-dose thorax computed tomography (CT) scan, a consultation with an ear, nose and throat specialist and a urine exam, as suggested by Benlloch *et al*[136], who recommend an annual head and neck cancer screening due to the high risk of this type of cancer. However, only two studies have shown that intensive screening protocols increase survival[137,138]. Therefore, at the present time, patient education, mainly to avoid smoking and sun exposure, and periodic clinical follow-ups continue to be the standard of care regarding treatment[98].

CONCLUSION

In the last 10 years, ALD is the LT indication that has seen the greatest increase in prevalence[3] as well as in post-LT survival rate compared with other causes of liver disease, although concerns over alcoholic relapse remain. Even though less than 5% of grafts are rejected at 5 years post-LT due to a direct or indirect consequence of alcohol consumption[139], transplanted ALD patients who relapse have an increased long-term mortality due to cardiovascular pathologies and the onset of *de novo* neoplasms.

Much has been discussed regarding the risk factors for relapse, and the most controversial has been, and continues to be, the pre-LT abstinence period. In view of the foregoing, we can say that this period by itself should not be a determining factor to include a patient on the list because many other factors exist; therefore, a good psychiatric and psychosocial evaluation that identifies and addresses such factors before and after the LT is important[140,141].

The major incidence of *de novo* neoplasms in this type of patient could be remedied with the detection of and action on the predisposing factors for the development of neoplasm in addition to the development of more intensive programs for the detection of neoplasm; however, the efficacy of this approach must be demonstrated. What we conclude is that the pre-LT evaluation and the post-LT follow-up in ALD patients should be a multidisciplinary task that includes transplant specialists, psychiatrists and addiction treatment specialists (Figure 2).

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TOPIC HIGHLIGHT

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Liver transplantation for hilar cholangiocarcinoma

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Core tip: The most appropriate treatment for Klatskin tumor (KT) with a curative intention is multimodal therapy based on achieving R0 resection combined with other types of neoadjuvant or adjuvant treatment. In irresectable non-disseminated KT patients, using liver transplantation without neoadjuvant treatment, the 5-year survival rate increase to 38%, reaching 50% survival in early stage. In selected cases, with liver transplantation and neoadjuvant treatment (chemotherapy and radiotherapy), the actuarial survival rate is 65% at 5 years and 59% at 10 years. In conclusion, correct staging, neoadjuvant treatment, living donor and priority on the LT waiting list may lead to improved results.

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Abstract

The most appropriate treatment for Klatskin tumor (KT) with a curative intention is multimodal therapy based on achieving resection with tumour-free margins (R0 resections) combined with other types of neoadjuvant or adjuvant treatment (the most important factor affecting KT survival is the possibility of R0 resections, achieving 5-year survival rate of 40%-50%). Thirty to forty percent of patients with KT are inoperable and present a 5-year survival rate of 0%. In irresectable non-disseminated KT patients, using liver transplantation without neoadjuvant treatment, the 5-year survival rate increase to 38%, reaching 50% survival in early stage. In selected cases, with liver transplantation and neoadjuvant treatment (chemotherapy and radiotherapy), the actuarial survival rate is 65% at 5 years and 59% at 10 years. In conclusion, correct staging, neoadjuvant treatment, living donor and priority on the liver transplant waiting list may lead to improved results.

INTRODUCTION

The most appropriate treatment for cholangiocarcinoma (CC) with a curative intention is multimodal therapy based on achieving R0 resection combined with other types of neoadjuvant or adjuvant treatment such as external radiation therapy or brachytherapy, systemic or arterial chemotherapy, chemoradiation therapy and photodynamic therapy[1-10].

Thirty to forty per cent of patients with Klatskin tumour (KT) are inoperable and present a 5-year survival rate of 0%. The other 60%-70% of patients may be eligible for surgery, although some 15%-50% are non-resectable and have an identical prognosis to inoperable

patients. In these non-resectable cases (due to spread to the second-generation intrahepatic radicals), and providing there is no lymph node dissemination, liver metastases or extrahepatic spread, some authors classically have proposed liver transplant (LT)[9]. The most important factor affecting KT survival is the possibility of tumour resection with tumour-free margins (R0 resections)[9,11-15]. The rate of resectability has increased over the last 20 years[16-22] as a result of several factors: (1) extending tumour resection to the hepatic parenchyma, especially caudate lobe resection[16-18], since bile drainage occurs at the bifurcation of the hepatic ducts, performing extended right-sided resections also increases resectability[9] and it is often necessary to increase the residual liver volume with portal vein[23,24] or arterial[16] embolization; (2) extending tumour resection to the pancreatic head, associating a cephalic duodenopancreatectomy (CDP) to the liver resection[16,25-30]. This technique has been used when performing both liver resection and LT[16,25-30]. Most authors generally only consider CDP for KT when there is invasion of the lower biliary resection margin; (3) performing vascular resections has led to higher rates of morbidity and mortality, although it increases the possibility of resection and also of performing R0 resections[16]. Portal vein resection does seem to increase the 5-year survival rate, whereas hepatic artery resection does not appear to increase survival but does increase postoperative morbidity and mortality; and (4) performing a lymphadenectomy appears to be fundamental for achieving an R0 resection by removing the lymphatic pathways of dissemination and eliminating the frequent perineural dissemination through the periduodenal and peripancreatic elements of the hepatic hilum[31].

RESULTS OF LIVER TRANSPLANTATION WITHOUT NEOADJUVANCY

The indications for LT in KT patients were not well established due to the poor results reported in the literature, and each case needs to be analysed separately. When the tumour was non-resectable and not disseminated, palliative treatment obtains a zero 5-year survival rate but LT may achieve complete R0 resection of the tumour[16]. Some authors associated a CDP to the LT[16,25-30] and Starzl *et al*[32] and Alessiani *et al*[33] even extended the resection to neighbouring organs (cluster transplantation). The drawback with LT is immunosuppression, which favours the dissemination of tumour remains that might have gone unnoticed, which is why the fundamental cause of death following LT is usually abdominal tumour recurrence (occurring in 56%-96% of cases)[34-39]. The 5-year survival rate in LT series for KT is 0%-38%[34-39] and did not exceed 38% when it was more aggressive (cluster transplantation)[32]. An example of these poor results was published by the Cincinnati Transplant Tumour Registry in 2000[36] in an analysis of 207 cases of LT for CC, with a 23% 5-year survival rate, a 51% rate of early tumour recurrence and a 10% rate of postoperative mortality. The survival rate was lower than with LT for other indications, and because of the scarcity of organs many centres considered LT contraindicated for KT. These poor results have been related to three factors: (1) poor patient selection, LT being indicated in patients with non-resectable tumours with biliary, portal and arterial invasion; (2) not performing a preoperative exploratory laparotomy and therefore many patients undergoing transplantation with disseminated peritoneal disease (16% of the cases) and affected regional lymph nodes; and (3) none of the patients receiving neoadjuvant therapy.

The Spanish series[40] reported 36 LTs for KT over a period of 18 years [3 of them associated with primary sclerosing cholangitis (PSC)], with 52.7% recurrence and 8.3% postoperative mortality and 30% and 18% survival at 5 and 10 years, respectively. In the early stages (stages Ⅰ-Ⅱ) the survival rate was suitable for indicating LT (47% at 5 years), whereas in very advanced stages (Ⅲ-Ⅳ) it was only 15%. Despite the bad results (30% survival at 5 years) we showed that a small group of patients undergoing LT with negative lymph nodes had prolonged survival rates and that LT might therefore be an option in carefully selected cases. Subsequently Kaiser *et al*[41], in a similar study, presented their experience in Germany and reported 47 patients undergoing transplantation for KT, with a higher postoperative mortality rate (20%) and a 5-year survival rate of 22%. As with the Spanish series, when the selection criteria were strict (from 1998 onwards) the 5-year survival rate of the 15 transplant patients was 48% ($P < 0.014$). Friman *et al*[42] have reported the Scandinavian experience with LT for CC and 20 of the 53 patients in the series were KT. The same results were reported as for the Spanish and German series, with a 48% 5-year survival rate in patients with tumor node metastasis (TNM) stages ⩽ 2 who received transplantation from 1995 onwards (the rate of PSC in this series was 64%, compared to 8% in the Spanish series). As Friman *et al*[42] state, this survival rate was similar to that obtained in some series with LT for hepatic cirrhosis secondary to C virus.

Some authors have compared resection with LT without neoadjuvant. Hidalgo *et al*[43] have reported 106 patients with KT, managing resection in 44 cases and performing LT in 12. There were no differences between resection and LT for sex, early stages, tumour size, lymph node invasion (7 from the LT group were N1), differentiation grade, perineural invasion and vascular invasion. There were also no differences for 5-year survival (28% with resection *vs* 20% with LT). As in our series, the patients undergoing LT were much younger than those with resection ($P < 0.012$). Factors of poor prognosis were stages Ⅲ-Ⅳ, R1-2 resections, presence of lymph node invasion and liver metastases, undifferentiated tumours and vascular invasion. The Mayo Clinic in Rochester, in a study published in 2005[44], compared 38 LTs selected among 71 patients with the neoadjuvant protocol and 26 patients with liver resection from a total of 54 patients in whom it was attempted (48% resectability). The authors

concluded that transplantation may be the ideal treatment for KT due to a better 5-year survival rate (82% *vs* 21%) and lower rates of recurrence (13% *vs* 27% with resection), but the drawback with the study was that the two series were not homogeneous. The age of the LT group was 48 years, compared to 63 years in the resection group, and they were all stages I - II whereas 14% of the resection group had hepatic metastases, 39% vascular invasion, 25% positive lymph nodes in the hepatic hilum and 18% peritoneal metastases. They also performed just 38% of caudate lobe resections in the resection group, a technique which all authors currently claimed to be fundamental for preventing KT recurrence. They also selected PSC patients with an early diagnosis for the transplant group, as 58% of the transplanted patients had a Klatskin tumour besides PSC, compared to 8% of the resected patients. These results were in contrast to those published by Iwatsuki *et al*[45], who found no differences between LT and resection, in this case without neoadjuvant. When we have compared[46] 11 LTs and 29 KT resections without neoadjuvant, we also found no differences for 5-year survival (38% with LT and 36% with resection), and in no case was PSC associated.

RESULTS OF LIVER TRANSPLANTATION WITH NEOADJUVANCY

In 1987 the University of Nebraska initiated a protocol of brachytherapy and chemotherapy with fluorouracil (5-FU) up until transplantation and in 2002[47] published a series of 11 patients showing prolonged survival rates for a select group of patients with non-resectable KT who received neoadjuvant internal radiation therapy alone or with chemotherapy with 5-FU (external radiation therapy was associated in 2 patients). Of these, 45% were alive and disease-free between 2.8 and 15.5 years after the transplant. The authors reported a high postoperative mortality rate of 27% and a low recurrence rate of 18%.

Subsequently in 1993 the Mayo Clinic initiated a protocol[26,44,48-53] of neoadjuvant treatment with external radiation therapy, chemotherapy with 5-FU for three days and internal radiation therapy, followed by capecitabine up until transplantation. All the patients were considered non-resectable by an experienced group of hepatobiliary surgeons and all the patients had to belong to stages I and II of the TNM classification[54]. For this they established an exhaustive selection process, performing exploratory laparotomy 2 mo after the end of radiation therapy and excluding the patient from the study if the tumour was disseminated. In 2008[50] they reported 148 patients (90 having completed neoadjuvant and LT), of whom 71 were alive, 19 died (8 due to tumour recurrence), 19 were awaiting LT and 39 failed to complete neoadjuvant due to progression of the disease. The 5-year survival rate in the group was 55%, and 71% among the transplant patients. The good results with this protocol were related to several factors: external and internal radiation therapy (useful for controlling wall and perineural invasion); strict patient selection, as all were stages I - II, unlike other series in which stages III-IV exceed 40%, and most were young patients; and lastly the significant rate of PSC (65%). The neoadjuvant treatment was so effective that no tumour was found in the explanted liver (even though cytology prior to LT had been positive). Factors of poor prognosis in their series were age > 45 years, carbohydrate antigen [carbohidrate antigen (CA) 19-9] < 100, previous cholecystectomy, residual tumour of > 2 cm, perineural invasion, and waiting time > 100 d, hence the importance of living donor LT and application of a scoring system besides the Model for End-Stage Liver Disease (MELD) system. The drawback of this protocol were a higher rate of late vascular complications and a greater need for the use of grafts[55], especially when living donor LT was used. This greater difficulty was related to the significant fibrosis encountered during the transplant as a result of radiation therapy, although it does not affect patient or organ survival.

Results after acknowledgement of LT for KT by UNOS

The good results reported by the Mayo Clinic in Rochester lead to United Network Organ Sharing (UNOS) adopting this protocol on 17 November 2009 and beginning to allow priority MELD exception scores for CC patients who have completed the neoadjuvant chemoradiation protocol and for whom staging laparotomy was negative[26,30]. Darwish Murad *et al*[26] re-published the results of the Mayo Clinic in Rochester, including 199 patients in the protocol, both intrahepatic CCs and KTs. Twenty patients did not reach the staging laparotomy, due in 15 cases to progression of the disease and to 4 dying from causes unrelated to the disease and 1 from intolerance to the treatment. An exploratory laparotomy was performed in 179 at the end of the protocol and 42 patients were excluded: 36 for metastases, 40 for progression of the disease and 2 who died without progression prior to transplantation. One hundred and thirty-seven patients underwent transplantation: 131 in their hospital (66%) and 6 in other hospitals. Thirty-six patients died (27%): 24 due to recurrence and 12 from other causes. The actuarial 5-year survival rate was 71%, with tumour recurrence in 26 patients, of whom 24 died as a result.

Darwish Murad *et al*[26] analysed pre-LT dropout factors and found that 62 of the 199 patients (31%) abandon the waiting list, their mean survival being just 3.6 mo. Statistically significant factors of poor prognosis in the univariate analysis were presentation with painless jaundice, weight loss, visible tumour mass of ⩾ 3 cm, positive or suspicious intraluminal brushing or biopsy, high CA 19-9 (> 500) and higher MELD score. Statistically significant in the multivariate analysis were mass size of ⩾ 3 cm, positive or suspicious intraluminal brushing or biopsy, high CA 19-9 and higher MELD score.

Darwish Murad *et al*[26] also analysed the prognostic factors related to tumour recurrence following liver transplantation. Statistically significant in the univariate analysis were age over 50 years, size ⩾ 3 cm, CA 19-9 over

500 and vascular encasement. Statistically significant in the multivariate analysis were high CA 19-9 and complete portal vein encasement, perineural invasion and tumour persistence in the explant. In the multivariate study tumour persistence in the explanted liver was exclusively significant. It is worth noting in this series that the patients undergoing transplantation for KT associated with PSC had a lower risk of recurrence than the patients with the novo KT.

In 2012, after approving the neoadjuvant protocol in 2009 in the United States, Darwish Murad *et al*[30] send a survey to 50 American centres to collected their experience in LT for cholangiocarcinoma between 1993 and July 2010 and received 30 responses (8 of the 20 non-respondents were because they did not applied the neoadjuvant protocol). Selection for transplantation from the waiting list was done with a MELD score of 22 points and with the same criteria as with hepatocarcinoma: every 3 mo 10% drop off the waiting list. The objectives have been: (1) to assess the efficacy of neoadjuvant for KT; (2) to analyse the intercentre impact of neoadjuvant; and (3) to evaluate whether the MELD system applied is appropriate. They included 287 patients, of whom 22 were excluded (16 due to progression, 3 who died of causes unrelated to cancer, and 3 who did not tolerate the treatment). Staging laparotomy was performed in 229 patients and extrahepatic disease detected in 40, who were also excluded. Nine patients were excluded after the laparotomy (7 for tumour progression and 2 who died of non-tumour-related causes). Transplantation was done in 184 cases following staging and in another 30 who underwent transplantation without staging after neoadjuvant (214 liver transplants in total).

Of the 287 patients included in the study 193 belonged to the Mayo Clinic in Rochester and 94 to other centres (between 2 and 12 transplants). A CDP was associated in 22 cases. One hundred and twenty-two patients died: 60 prior to liver transplantation and 62 after transplantation (22%). There was a post-transplant recurrence in 43 patients (20%), of whom 40 died. The actuarial survival rate was 65% at 5 years and 59% at 10 years. They analysed the prognostic factors and found no differences between living and deceased donor transplantation or between KT associated with PSC and the novo KT; there were also no differences between patients with and without exploratory laparotomy: 36/184 recurrences (20%) *vs* 7/30 recurrences (23%), respectively. A poorer prognosis was shown by patients who do not fulfil UNOS criteria for MELD exception: existence of a mass of > 3 cm (21 patients), with a 5-year survival rate of 32%, *vs* 69% for < 3 cm masses; those with liver metastases (4 patients); and when a percutaneous biopsy was done for tumour diagnosis (16 cases). As in previous series there was a group of patients with no preoperative biopsy for diagnosis, no tumour in the explanted specimen and whose deaths were not tumour-related. Of 87 patients with no reliable preoperative tumour diagnosis 55 did have a tumour in the explant and another 17 presented with tumour recurrence during evolution. The remaining 15 cases (5%) had no tumour in the preoperative period or in the explant and the authors claim that even if they had been excluded the 5-year survival rate was 50% in the other 272. They concluded that neoadjuvant was effective, there were no inter-centre differences and that the MELD system was valid for waiting list selection.

Validation of the results of other centres

These results show that LT is a valid therapeutic option for both hilar and intrahepatic cholangiocarcinoma, especially when neoadjuvant treatment is used. However, there are doubts in the literature, as not all centres reproduce these results, something which, as also claimed by Friman *et al*[56], may be related to the high % of patients with PSC, strict criteria for a preoperative diagnosis of malignancy and especially[55] strict criteria for selection (performing a staging laparotomy after performing neoadjuvant). Other American centres have recently reported their results for LT for cholangiocarcinoma. Panjala *et al*[27], from the Mayo Clinic in Florida, reported 22 patients in whom the protocol of the Mayo Clinic in Rochester was applied between 2001 and 2008. Seventeen cases (77%) were associated with PSC and 5 were the novo KTs. They did not perform an exploratory laparotomy and staging was done during LT with 2 recipients. The preoperative diagnosis was certainty in 12 cases and suspicion in 10. During transplantation 3 of the 12 patients with a preoperative diagnosis of certainty presented with liver metastases in 2 cases and an intestinal implant in 1 case. Overall survival was 63%, similar to that reported by the Rochester group. As with the Rochester group there were patients with no tumour in the explant, which influenced the survival rate: there was still tumour in 77% (17 cases) but no tumour remains could be identified in 5 cases (23%), a factor with which survival was related, such that patients with tumour in the explant had a survival rate of 52% at 3 years and those with no tumour in the explant had a survival rate of 100%. Nine patients (41%) died as a result of recurrence and 3 for other non-tumour-related causes, the recurrence rate being 27% at one year, 4.5% the second year and 4.5% the third year. When the association with PSC was analysed, these patients made up 93% of the group of patients with no tumour recurrence, whereas it constituted 50% of the patients who did have tumour recurrence, which implies that patients with *de novo* KT carry a higher risk of recurrence than those associated with PSC: of the 17 with PSC there were 4 recurrences and 1 with visceral metastases, whereas in the 5 *de novo* KTs there were 4 recurrences and 2 visceral metastases.

Of a total of 132 cholangiocarcinomas, the UCLA University[57,58] selected 57 for surgery and perform LT in 38 of them[57]. In a subsequent publication they reported 40 liver transplants for CC, of which 14 were for KT[58]. Only 13 patients received neoadjuvant treatment with chemotherapy plus radiation therapy[58] and the 5-year survival rate was 47% when neoadjuvant was applied, compared to 20% without neoadjuvant treatment and

33% when adjuvant treatment was administered. These results were lower than those reported by the Rochester group and similar to those reported for European groups without neoadjuvant on selected early cases[40-42].

In 2012 the Anderson Cancer Center reported the efficacy of neoadjuvant treatment in patients with resection of tumours of the bile duct. Of 157 patients 94 were cholangiocarcinomas[59]. Forty-eight point seven per cent received adjuvant chemotherapy, 17.8% had neoadjuvant chemotherapy and 15.8% had chemotherapy plus neoadjuvant radiation therapy (the latter treatment delayed surgery by 6.8 mo). The 5-year survival rate was 30.4%, and when immediate tumour resection was achieved without neoadjuvant with a margin of at least 1 cm the survival rate was 52.4%. Thus, immediate tumour resection increased survival from 42.3 to 53.5 mo. This protocol was applied to patients considered initially resectable, and not as occurs in CC patients considered non-resectable to whom the Rochester protocol was applied before transplantation.

The results for other non-American groups[60-62] has been contradictory, with favourable[60] and unfavourable[61] cases, very small series and an absence of multicentric prospective studies. Wu *et al*[62] achieved good results with the protocol. They reported 6 patients with PSC and cholangiocarcinoma, with a similar early detection protocol to that of the Mayo Clinic in Rochester; the patients only received neoadjuvant radiation therapy before LT with CDP and only 1 died from a non-tumour-related cause, the other 5 having survived more than 5 years.

In conclusion, R0 resection is the most accepted treatment of KT. In non-disseminated unresectable tumours, liver transplantation in early stages have an acceptable survival (50% at 5 years). In these same patients (KT and early stages), treatment with neoadjuvant chemoradiotherapy and very strict selection criteria achieves a 5-year survival rate of over 65%. The series with neoadjuvant treatment are not homogeneous and most tumours are associated with PSC as compared to other series where most are the novo KT. Therefore, some authors consider it necessary prospective randomized studies, comparing KT associated to PSC and the novo KT, to discover the proper role of neoadjuvant chemoradiation[63]. Staging correct, the priority in the waiting list LT (MELD) and living donor LT may lead to better results.

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TOPIC HIGHLIGHT

A M El-Tawil, MSc, MRCS, PhD, *Series Editor*

Current management of fecal incontinence: Choosing amongst treatment options to optimize outcomes

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Abstract

The severity of fecal incontinence widely varies and can have dramatic devastating impacts on a person's life. Fecal incontinence is common, though it is often under-reported by patients. In addition to standard treatment options, new treatments have been developed during the past decade to attempt to effectively treat fecal incontinence with minimal morbidity. Non-operative treatments include dietary modifications, medications, and biofeedback therapy. Currently used surgical treatments include repair (sphincteroplasty), stimulation (sacral nerve stimulation or posterior tibial nerve stimulation), replacement (artificial bowel sphincter or muscle transposition) and diversion (stoma formation). Newer augmentation treatments such as radiofrequency energy delivery and injectable materials, are minimally invasive tools that may be good options before proceeding to surgery in some patients with mild fecal incontinence. In general, more invasive surgical treatments are now reserved for moderate to severe fecal incontinence. Functional and quality of life related outcomes, as well as potential complications of the treatment must be considered and the treatment of fecal incontinence must be individualized to the patient. General indications, techniques, and outcomes profiles for the various treatments of fecal incontinence are discussed in detail. Choosing the most effective treatment for the individual patient is essential to achieve optimal outcomes in the treatment of fecal incontinence.

Key words: Fecal incontinence; Treatment; Sacral nerve stimulation; Sphincteroplasty; Artificial bowel Sphincter; Biofeedback

Core tip: An increasing number of treatment options for the management of fecal incontinence have been developed. In addition to traditional options such as sphincteroplasty and colostomy, non-surgical options such as biofeedback and dietary modification may be considered for mild incontinence. Injectable materials and radiofrequency energy delivery are two newer treatments for mild incontinence. Surgical options for moderate to severe incontinence include sacral nerve stimulation, artificial bowel sphincter implantation, muscle transposition, antegrade continence enemas, sphincteroplasty, and colostomy formation. Treatment for fecal incontinence (repair, stimulation, replacement, augmentation, or diversion) must be individualized to the patient, considering the underlying cause and impact on quality of life of the fecal incontinence.

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INTRODUCTION

Fecal incontinence is a common problem; one that is likely underreported in the general population. The prevalence of fecal incontinence varies in the literature, with one study of over 4000 surveyed American adults finding a prevalence of 8.3%[1]. The much larger and more recent Mature Women's Health Study of over 5800 American women found an even higher incidence of accidental bowel leakage of almost 20%[2]. Incontinence to liquid or solid stool, mucous, or flatus occurs with varying frequency and can have a range of impact on daily function[1]. The Mature Women's Health Study found that nearly 40% of women with accidental bowel leakage have severe symptoms impacting their quality of life, even though less than one third of women sought medical care for their bowel leakage[3,4]. While there can be many etiologic factors contributing to its development, there are some common risk factors. Age, diarrhea or frequent bowel movements, nocturnal bowel movements, other bowel disorders, and the presence of urinary incontinence are commonly associated with fecal incontinence[1,4,5]. In women, internal sphincter injury and reduced perineal descent related to obstetrical trauma independently predict the development of fecal incontinence[6]. Other risk factors include neurological disorders, congenital anorectal malformations, trauma, iatrogenic injury during anorectal procedures, and chronic diseases such as diabetes[6-9].

It is necessary to complete a physiological and anatomical assessment of the pelvis and colon in order to choose the most appropriate treatment option for a patient's fecal incontinence. This caveat is especially important since many women with fecal incontinence have associated genital and urinary anatomical or functional problems[10]. A rectal examination may identify a sphincter defect or decreased rectal tone. This finding may be helpful to identify potential etiologies and treatments for a patient's fecal incontinence. Though not all investigations are required for every patient, options include anal or pelvic ultrasound, anal manometry, defecography, magnetic resonance imaging, and electromyography with pudendal nerve terminal motor latency testing. Anatomical imaging can help identify sphincter defects and associated pelvic floor disorders such as rectocele or prolapse, which may be contributing to the severity of incontinence[11,12]. A physiology lab is helpful for the assessment of incontinence and other pelvic floor disorders.

The impact of fecal incontinence varies and can greatly alter a person's ability to perform daily activities. One may alter timing of meals or eating habits, and possibly avoid all social occasions for fear of embarrassment[8]. While fecal incontinence is not a normal part of aging it may be perceived as such, and older people may not seek treatment until symptoms are severe. Treatment options for fecal incontinence range from dietary modification and physical therapy to major surgery, such as colostomy formation. In recent decades, many new treatments for fecal incontinence have been developed with good success, adding to traditional options of sphincteroplasty and ostomy formation. These alternatives include biofeedback, radiofrequency, injectable materials, and surgical approaches such as sacral nerve stimulation, the artificial bowel sphincter, and muscle transposition. A recent Cochrane review concluded that there is insufficient evidence to allow for quality comparisons to be made among the various surgical approaches to fecal incontinence[13]. The decision among these options is multifactorial and the severity of the incontinence, patient anatomy, and patient wishes must all be carefully considered. The aim of this article is to review current options for the management of fecal incontinence, their indications, and reported outcomes. The treatments most commonly offered by the authors, from the five available categories of repair, stimulation, replacement, augmentation, and diversion, are discussed.

DIETARY MODIFICATION AND MEDICATION

Modifiable diet and lifestyle factors may be identified which can provide simple interventions to try to improve symptoms. Smoking and sedentary lifestyle are associated with fecal incontinence[14]. Weight loss has been shown to improve fecal incontinence in obese women[15]. Medications should be reviewed with the help of a pharmacist to identify potentially incriminating medications. Low fiber and high fat diets may be contributory to loose stools. Loose stools and diarrhea often precipitate symptoms of fecal incontinence and may be improved with dietary and medication alterations. Other factors may be identified that may suggest the need for further testing or anatomical causes of fecal incontinence. For example, cholecystectomy may lead to persistent diarrhea and flatulence which may amplify symptoms of fecal incontinence; cholestyramine may help relieve these symptoms[16,17].

The addition of a daily fiber supplement should be advocated in fecal incontinence. It acts as a bulking agent to allow for more solid stool and adds little to no morbidity to the patient. A randomized, blinded, placebo controlled study found that fiber improved fecal incontinence and stool consistency within 1 mo in the community living population[18]. In addition to fiber, medications with a constipating effect may be useful for patients with fecal incontinence with loose stools. These pharmacologic agents include loperamide, diphenoxylate and atropine, and codeine. Loperamide is most commonly used and may also have beneficial effects on anal sphincter resting tone[19]. Unfortunately, studies comparing various medications are lacking and trials of medications for the treatment of fecal incontinence include very heterogeneous populations and treatments[20]. A Cochrane review conducted in 2013 concluded that there is insufficient evidence to guide the decision between medications for the treatment of incontinence in various clinical situations[20]. Clearly, no medication will cure moderate to severe fecal incontinence, but it should certainly be utilized in mildly

Table 1 Success of biofeedback for fecal incontinence

Ref.	Year	Patients (*n*)	Significant reduction in incontinence (percentage of patients)	Improvement in quality of life (percentage of patients)	Adjuncts to traditional biofeedback
Keck *et al*[33]	1994	15	73%	NR	None
Solomon *et al*[28]	2003	102	70%	69%	Anal manometry, transanal ultrasound
Terra *et al*[34]	2006	239	60%	NR	EMG, electrostimulation
Naimy *et al*[30]	2007	49	None	None	Electrostimulation
Byrne *et al*[32]	2007	385	70%	87%	None
Heymen *et al*[27]	2009	45	76%	NR	None
Schwandner *et al*[29]	2010	158	50%	NR	EMG, electrostimulation
Bartlett *et al*[26]	2011	72	86%	100%	None
Jodorkovsky *et al*[31]	2013	12	80%	NR	None

NR: Not reported; EMG: Electromyography.

symptomatic patients where indicated.

BIOFEEDBACK

Biofeedback is a form of physical therapy and muscle re-training offered to patients refractory to medical treatment of fecal incontinence. There are numerous regimens, most of which involve many weeks of treatment lead by a physical therapist. Numerous studies have attempted to define the most effective regimen and most responsive patient population, but overall there are few high quality studies showing a definitive impact of biofeedback on fecal incontinence[21]. It has been suggested by some authors that biofeedback should be offered to all patients who have not responded to medical interventions of fecal incontinence because it is safe, inexpensive, and effective long term[22]. Older patients with normal defecation physiology appear to respond well[23]. Advanced anorectal physiology tests such as manometry, defecography, pelvic magnetic resonance imaging, and pudendal nerve terminal motor latency testing do not seem to predict who will respond best to biofeedback[24]. Patients with mild or moderate fecal incontinence who have not responded well to medical treatments are likely the best candidates for biofeedback[25].

The technique of biofeedback may include monitored or home sessions, pelvic floor exercises, digital feedback, electrical stimulation, balloons, and manometric or ultrasound monitoring of response. Pelvic floor exercises alone have been shown to improve fecal incontinence scores and quality of life[26]. In one study of pelvic floor exercises, no differences in treatment effect were found between the different regimens, but symptoms improved in both groups[26]. The addition of biofeedback using manometry is more effective than pelvic floor exercises alone to improve fecal incontinence scores and achieve more physiologically normal defecation[27]. Biofeedback with digital feedback alone may be just as effective as manometry and ultrasound guided treatment, providing enough feedback to guide re-training, as found in a randomized controlled trial of different methods of biofeedback[28]. Some literature suggests that electrical stimulation leads to more effective results over biofeedback alone, while others have found that biofeedback alone is adequate to improve patient symptoms[29,30]. A multicenter randomized and blinded trial found that the combination of electrical stimulation with extended treatment duration (longer than 3 mo) achieved the best results[29]. Such treatment regimens may not be available in many centers, but access to a trained biofeedback therapist who is aware of the various treatment modalities may be invaluable to the population with fecal incontinence.

Biofeedback requires the patient and therapist to commit to treatment for a number of weeks to months. One study found that only 44% of patients with fecal incontinence who were recommended to undergo biofeedback therapy completed the treatment[31]. This finding was largely due to lack of insurance coverage and distance to treatment centers[31]. It is important to note that in this study those patients who did undergo biofeedback reported an 80% positive response to the treatment[31]. Other studies have confirmed improvement in over 70% of patients when fecal incontinence scores and quality of life scores were assessed[32,33]. Table 1 summarizes the success of biofeedback. Physiologic parameters such as squeeze pressure and maximum tolerated volume have also been reported to improve with biofeedback[34]. Improvements in fecal incontinence scores are durable over at least 1 year, but some patients may require additional sessions to boost the effect[35]. Pelvic floor training with biofeedback is likely beneficial to many patients with fecal incontinence long term, but patients and therapist must be willing to devote the time to a complete set of sessions to see maximum benefit. In those able to do so, biofeedback may achieve improvement in symptoms without invasive procedures.

REPAIR

Sphincteroplasty

Sphincteroplasty has long been the standard of care of the management of fecal incontinence related to anal sphincter injury[36]. The vast majority of patients who undergo sphincteroplasty have a history of vaginal delivery[37]. However, only about one third of women who have had a known sphincter injury related to vaginal delivery develop fecal incontinence over time[36]. Pudendal nerve injury, failed prior sphincteroplasty, multiple

Table 2 Success of overlapping sphincteroplasty

Ref.	Year	No. of patients with follow-up	Mean follow-up (mo)	Success[1] (percentage of patients)
Karoui *et al*[52]	2000	74	40	28%
Halverson *et al*[40]	2002	49	69	46%
Bravo Gutierrez *et al*[39]	2004	130	120	41%
Barisic *et al*[49]	2006	65	80	48%
Maslekar *et al*[55]	2007	64	84	80%
Oom *et al*[50]	2009	120	111	60%
Mevik *et al*[51]	2009	25	84	53%
Zutshi *et al*[53]	2009	31	129	0%

[1]Success variably defined in studies. Good, excellent or complete continence included as success.

vaginal deliveries, history of third of fourth degree tear, and instrument-assisted vaginal deliveries are all factors which may predispose to fecal incontinence associated with sphincter defect and impact the success of sphincteroplasty[38]. It is important to note that the majority of recent studies indicate that pudendal nerve injury as demonstrated by prolonged pudendal nerve terminal motor latency does not independently predict the success of sphincteroplasty[39-41]. Many women who undergo sphincteroplasty have associated pelvic floor injuries, which do not seem to impact the success of sphincteroplasty[42]. In addition, the combination of internal and external anal sphincter defect repair can lead to successful and equivalent outcomes when compared to external anal sphincter defect repair, alone[43].

While various techniques for sphincteroplasty have been described, the most commonly performed procedure is the anterior overlapping sphincteroplasty. A curvilinear incision is made on the perineum and dissection proceeds until the edges of the external anal sphincter are identified and isolated. Care is taken to not dissect too far laterally to avoid nerve injury. The ends are overlapped and sutured together, providing new bulk to the sphincter complex and an intact circumferential ring of sphincter. Separate attention to the imbrication of the internal anal sphincter does not seem to add to the overall durability of the sphincteroplasty if the internal sphincter is not injured[44]. Post-operative manometry shows significant increases in the length of the high pressure zone and resting and squeeze pressures[37]. A diverting stoma is not required to achieve optimal outcomes in early repair of third and fourth degree tears during vaginal delivery[45]. Delayed repair is associated with higher overall cost in this situation, but may still achieve good long term outcomes and may be the safer option depending on the clinical scenario[45,46].

Posterior sphincter repair is rarely needed, given that most sphincter injuries are associated with traumatic vaginal delivery. However, posterior repair may be occasionally utilized for neurogenic fecal incontinence, multifocal sphincter defects, or after failed anterior sphincteroplasty in order to avoid any significant scar tissue in the area. A similar technique is used as in the anterior technique, with a curvilinear posterior incision being used for access to the external anal sphincter. Some surgeons may proceed with a combined anterior and postanal approach, though this combination is not common. The success rate of the postanal approach is likely equivalent or less durable compared to anterior sphincteroplasty[47,48]. In the absence of a specific iatrogenic posterior sphincter injury or excessive anterior scar tissue, the anterior sphincteroplasty should be considered the preferred approach.

The long term functional outcomes following anal sphincteroplasty are not ideal. The Wexner fecal incontinence score is commonly used to assess for incontinence following sphincteroplasty. In the short term, good results are achieved in over 70% of patients and excellent results in over half of patients[49]. However, the long term outcomes which have been reported in numerous retrospective studies reveal a consistent decrease to 15% to 60% good long term continence[39-40,50-56]. Interestingly, there is poor correlation between long term quality of life scores and fecal incontinence scores, with one study reporting that 95% of patients were satisfied with their operation a mean of 7 years following sphincteroplasty[39,53]. A summary of long term outcomes is found in Table 2. Age has long been felt to be a predictor of success of sphincteroplasty, with many studies reporting that older patients do not have as durable long term outcomes compared to younger patients[39,53,56]. However, a recent large review of 321 women who underwent sphincteroplasty showed that age is not a predictor of long term incontinence scores[57]. A review of both sphincteroplasty and sacral nerve stimulation concluded that sphincteroplasty remains a good option for the management of incontinence due to sphincter defect, despite new technologies[58]. Patients must be chosen after appropriate preoperative evaluation to achieve optimal outcomes.

STIMULATION

Sacral nerve stimulation

For many patients and practitioners, sacral nerve stimulation has revolutionized the treatment of moderate to severe fecal incontinence. Adapted from its use in urinary incontinence, it may provide effective relief from fecal incontinence without any direct intervention on the anal sphincter complex. Interestingly, one study found that the only positive predictors of successful treatment with sacral nerve stimulation were loose stools and low stimulation intensity during the test phase of the procedure[59]. Conversely, age, gender, etiology of fecal incontinence, and physiology study results did not impact the efficacy of sacral nerve stimulation[59]. Though sacral nerve stimulation and sphincteroplasty have not been directly compared in the literature, numerous studies have shown that patients with sphincter defects can have excellent results with sacral nerve stimulation[60-64]. The success of sacral nerve stimulation in these patients also does not appear to be correlated to the degree of sphincter defect[63]. Pa-

Table 3 Studies of outcomes of sacral nerve stimulation

Ref.	Year	Patients (*n*)	Significant reduction in incontinence scores and incontinent episodes	Significant increase in quality of life
Leroi *et al*[73]	2005	27	Y	Y
Boyle *et al*[63]	2009	15	Y	NR
Brouwer *et al*[64]	2010	55	Y	Y
Wexner *et al*[79]	2010	120	Y	Y
Hollingshead *et al*[76]	2011	18	Y	NR
Lim *et al*[78]	2011	41	Y	Y
Mellgren *et al*[81]	2011	83	Y	Y
George *et al*[77]	2012	23	Y	Y
Devroede *et al*[83]	2012	78	Y	Y
Hull *et al*[75]	2013	76	Y	Y
Damon *et al*[82]	2013	92	Y	Y

Y: Yes; NR: Not reported.

tients known to have pudendal nerve injuries or previous sphincteroplasty can have good responses to sacral nerve stimulation[64].

The mechanism by which sacral nerve stimulation improves fecal incontinence is not well defined, as it is multifactorial. A systematic review found that sacral nerve stimulation likely works in 3 ways: stimulation of a somato-visceral reflex, direct effect on the anal sphincter complex, and afferent nerve modulation[65]. It is postulated that sacral nerve stimulation may induce a change in anal sphincter muscle type from fast to slow twitch, thus reducing muscle fatigue, though this has not been definitively demonstrated in the sacral nerve stimulation population[66]. Sensory changes include the sensation of rectal filling and urge to defecate at higher rectal volume[67]. Sacral nerve stimulation alters colonic transit by inducing retrograde colonic propagating sequences, activity which may slow transit in the setting of fecal incontinence[68]. In an animal model, sacral nerve stimulation was found to increase activity in the central cerebral cortex[69]. The effects of sacral nerve stimulation are well beyond local effect on the anal sphincter complex.

There are two approaches to the implantation of the sacral nerve stimulator. Some surgeons introduce a peripheral nerve stimulator wire in the office, guided by anatomical landmarks. The patient is tested for response for a period of 1-2 wk and if good response is achieved, the permanent tined lead and stimulator device are implanted in the same setting in the operating room. The authors' preferred approach is a two-stage operative technique. The first stage is the insertion of the tined lead into the S3 foramen in the operating room with careful fluoroscopic and patient-directed guidance. Local anesthetic injections and light sedation allow the patient to signal when stimulation is felt in the perianal, perineal, or saddle regions during lead electrostimulation. In addition, sphincter bellows and plantar flexion of the great toe on the side of lead placement are used to further indication stimulation of the sacral nerve. Once a good response is achieved the lead is tunneled into position. A temporary device is used during a 2 wk test phase. If a good response is achieved during the test phase, the patient undergoes a second procedure to implant the permanent device which is attached to the tined lead. This approach is associated with very little lead migration during the test phase but does require two operations. A test phase is important in both approaches, as not all patients will have a good response to lead placement[70]. Each permanent device is programmed to the individual's response pattern. Successful strategies to prolong the durability of the device battery beyond the average of six years include cyclical stimulation and subsensory stimulation[71,72].

Results of the first randomized multi-center study of sacral nerve stimulation were reported in 2005, showing that fecal incontinence was improved when the sacral nerve stimulator was activated[73]. Longer term results are now available. Compared to medical treatment of fecal incontinence, sacral nerve stimulation is significantly more effective[74]. A recent report from the SNS Study Group showed that in patients followed for at least 5 years, 89% have significant continued reduction in fecal incontinence and 36% had a complete response to sacral nerve stimulation[75]. Numerous other studies from around the world have demonstrated significant long term reduction in fecal incontinence scores[75-79]. Table 3 summarizes the results of studies of outcomes of sacral nerve stimulation. Furthermore, in women who have undergone sacral nerve stimulation for fecal incontinence; urinary, sexual, and vaginal symptoms also improve with a global benefit on pelvic floor health[80]. Quality of life scores are also improved in the short and long term after sacral nerve stimulation[79,81-84].

There are potential morbidities with sacral nerve stimulation including a 5% risk of lead displacement associated with the percutaneous lead testing technique[85]. Pain at the surgical site and paresthesias are the most commonly reported complaints[81]. Infection of the permanent device or surgical site occurs in 10%, with about half of those infections requiring surgical management[81,85]. Overall, about one third of patients required surgical manipulation of the device in a study of long term outcomes[75]. Despite potential morbidity associated with the device, sacral nerve stimulation has been shown to be cost-effective in the treatment of fecal incontinence[86,87]. When balancing the effectiveness, morbidity profile, and cost-effectiveness of the technique, sacral nerve stimulation is a very valuable tool for the treatment of fecal incontinence, especially in its more severe forms.

REPLACEMENT

Artificial bowel sphincter

The artificial bowel sphincter is considered only for patients with severe fecal incontinence. It is an effective device, but requires long term follow up and a motivated patient. The use of an artificial bowel sphincter requires both manual dexterity and mental capacity to operate the device[88]. Due to the high incidence of adverse events,

Table 4 Outcomes of artificial bowel sphincter

Ref.	Year	Patients (*n*)	Explanted devices (*n*)	Success (percentage of patients), intention to treat	Complications
Lehur *et al*[93]	2000	24	7	83%	Obstructed defecation
Altomare *et al*[94]	2001	28	3	75%	Obstructed defecation, infection, device erosion
Devesa *et al*[95]	2002	53	10	65%	Perforation, infection, sepsis, device erosion, pain, impaction
Wong *et al*[97]	2002	112	41	53%	Infection, pain
Lehur *et al*[101]	2002	16	4	69%	Erosion
Parker *et al*[96]	2003	45	18	49%	Infection, pain
O'Brien *et al*[98]	2004	14	1	NR as percentage	Obstructed defecation, non-healing of wound
Melenhorst *et al*[103]	2008	33	7	NR as percentage	Pain, perforation, infection, obstructed defecation
Ruiz Carmona *et al*[99]	2009	17	11	53%	Infection, erosion
Wexner *et al*[90]	2009	51	31	NR as percentage	Infection, malfunction, erosion, pain
Wong *et al*[100]	2011	52	14	67%	Perforation, cuff leak

NR: Not reported.

other treatment options should be considered and attempted before proceeding to artificial bowel sphincter[89]. Contraindications include Crohn's disease, local sepsis, prior radiation, poor quality of the perineal tissues, severe constipation, and incontinence associated irritable bowel syndrome[89]. Disruption of the anal sphincter complex due to trauma, severe obstetrical injury, and imperforate anus are common indications[89,90]. Sacral nerve stimulation and the artificial bowel sphincter have largely replaced muscle transposition and dynamic graciloplasty for the treatment of severe fecal incontinence, with better functional outcomes and quality of life parameters[91,92]. Patients must be carefully selected and extensively counselled on the risks and benefits of the artificial bowel sphincter, as discussed below.

Meticulous sterile technique and thorough bowel preparation are essential to reduce the risk of infection associated with the artificial bowel sphincter. The 3 components of the artificial bowel sphincter are connected *via* tubing and compose the sphincter cuff, the reservoir balloon, and control pump. These components are inserted *via* perineal, Pfannenstiel, and labial or scrotal incisions, respectively. The cuff itself is chosen for size based on circumferential length around the rectum and width. It is inserted first and great care is taken to ensure there is adequate tissue bulk distal to the cuff, in an attempt to avoid device erosion and infection. The balloon holds approximately 40 mL of liquid and is left filled with the device deflated at the end of the procedure after testing the control pump. The device is not activated for four to six weeks to allow for complete healing. The patient is taught how to fill and empty the cuff by using the implanted control pump.

Patients who retain the artificial bowel sphincter long term have reported very good functional and qualitative results. Manometry results show that the artificial bowel sphincter achieves normal resting tone when the cuff is filled[93]. Improved continence is achieved in over 75% of patients, with one series reporting normal continence in two-thirds of patients[94,95]. Though adverse events are significant, patients who retain the device have excellent responses to artificial bowel sphincter implantation based on incontinence scores[93-100]. Quality of life scores are also markedly improved after successful treatment of fecal incontinence with the artificial bowel sphincter[96,98,99,101]. A systematic review of the safety of the artificial bowel sphincter noted that functional outcomes and quality of life scores for those patients who do not retain a functioning device are not reported in the literature[102].

Complications following artificial bowel sphincter implantation unfortunately remain high and often lead to device explantation, mitigating the overall population benefit in fecal incontinence. Unfortunately, these complications continue to accrue long term[90]. The rate of revision of the device has been reported to be up to 50%, with infection and device failure the most common reasons[100]. About 25%-40% of artificial bowel sphincters become infected over time[90,100,103]. Erosion of the cuff or control pump and post-operative constipation may also occur[92,104,105]. The outcomes and complications associated with the artificial bowel sphincter are included in Table 4. In summary, a balanced consideration of potential benefits and adverse events is important and artificial bowel sphincter may still be the optimal treatment consideration for select patients with severe fecal incontinence.

Muscle transposition

Muscle transposition is a technique used to physically replace the sphincter with *in vivo* muscle bulk. It is most often used in the setting of a traumatic or iatrogenic disruption of the anal sphincters to recreate a wrap of muscle around the anus. A substantial congenital or post-traumatic defect is indicated to consider muscle transposition. The two muscles widely described in the literature for transposition are the gluteus maximus and gracilis muscles. These are useful because of their proximity to the anus, sizeable muscle bulk, and nerve locations which are amenable to preservation upon transposition. In addition, the gluteus maximus was thought to be a good

Table 5 Outcomes of graciloplasty

Ref.	Year	Type of graciloplasty	Patients (*n*)	Success (percentage of patients)
Kumar *et al*[114]	1995	Unstimulated	9	100%
Eccersley *et al*[113]	1999	Unstimulated	8	100%
Madoff *et al*[109]	1999	Stimulated	128	66%
Wexner *et al*[110]	2002	Stimulated	115	62%
Bresler *et al*[112]	2002	Stimulated	24	79%
Rongen *et al*[111]	2003	Stimulated	200	72%
Thornton *et al*[117]	2004	Stimulated	38	73%
Hassan *et al*[107]	2010	Stimulated	31	71%

choice for transposition given that involuntary gluteal contraction occurs with the strong urge to avoid involuntary defecation[106].

The surgical technique of muscle transposition is complex and requires significant experience to gain expertise. Three main options exist: gluteoplasty, graciloplasty, and dynamic (or stimulated) graciloplasty. Gluteoplasty is performed with the patient in the prone position with the table flexed at the hips. Bilateral incisions over the gluteus are made and two tongues (one from each side) of the lower 10% of the muscle are raised with care taken to preserve the neurovascular bundles[106]. The mobilized muscle is then tunnelled and delivered through separate bilateral curvilinear incisions around the anus. The contralateral mobilized segments are sutured together to create a ring of muscle.

In a graciloplasty procedure, the patient is placed in the modified lithotomy position. Two or three incisions are made along the longitudinal access of the gracilis muscle on the chosen side to harvest the entire length of the gracilis. The neurovascular bundle is preserved through its identification during medial dissection. The muscle is released distally and tunneled medially. A perineal incision is made and the gracilis is wrapped circumferentially around the anus. In the dynamic graciloplasty technique, an electrode is placed in the gracilis muscle and an implantable device similar to that used for sacral nerve stimulation is implanted in the abdominal wall. Modified approaches to dynamic graciloplasty include temporary stimulation with an external stimulator for muscle retraining, similar to biofeedback[107]. It must be noted that the stimulator and leads for dynamic graciloplasty are not currently approved for use in North America.

Much like the artificial bowel sphincter, muscle transposition has fairly good functional outcomes but high rates of complications and re-operation; graciloplasty has largely replaced gluteoplasty. The largest and most recent study of gluteoplasty reported a good functional outcome in 59% of patients[108]. Successful functional outcomes for gracilplasty, dynamic and unstimulated, is consistently reported to be about 60%-75%, with earlier success of unstimulated graciloplasty being even higher[107-114]. Table 5 lists the published success rates of graciloplasty. If a patient has a stoma at the time of the graciloplasty, eventual outcomes are equivalent to those who do not have a stoma, but are delayed in achieving them[110]. Complications of the procedure are common, and include surgical site infections, pain, rectal injury, and erosion of the device in the case of dynamic graciloplasty[112,115,116]. In addition, constipation due to obstructed defecation is commonly reported in as many as 50% of patients[115-117]. There are no studies directly comparing muscle transfer to other surgical treatments of fecal incontinence. Graciloplasty followed by artificial bowel sphincter implantation may be the best combination option for adult patients with fecal incontinence attributable to congenital imperforate anus[118].

DIVERSION

Antegrade continence enema

The antegrade continence enema was first described by Malone *et al*[119] in 1990. It is used to control fecal soiling in both adults and children, but is most commonly used and reported in the pediatric population. Neurogenic conditions, such as spina bifida, resulting in neurogenic bowel and urinary symptoms are the most common indications in children. While the antegrade continence enema may be helpful in pure fecal incontinence, most often patients who undergo this procedure have the combination of constipation or colonic dysmotility with associated overflow fecal incontinence. Patients also commonly undergo urological procedures at the same time to control neurogenic bladder symptoms, with good results for these combined indications[120]. In adults, good functional outcomes are better in this setting, when compared to those patients who undergo the procedure for constipation alone[121]. While an antegrade continence enema does not alter anorectal physiology or anatomy, it provides a mechanism to empty the colon in a controlled fashion, allowing the patient to perform their daily activities with little worry of fecal soiling or incontinent episodes.

Since Malone's original description, various techniques have been described for the creation of an antegrade continence enema. The appendix, ileum, cecum, and left colon may be used successfully as the access point for irrigation[122-124]. The appendix is most commonly used, where it is inverted and fixated to the skin at the umbilicus or right lower quadrant. This can be performed open or laparoscopically with good results[124]. The access point is left intubated with a catheter for about 3 wk after the operation before intermittent intubations begin. Patients or their caregivers then intubate the bowel daily to every few days and perform colonic irrigation with tap water or an electrolyte or bowel cleansing solution. Both tap water and commercial products have good irrigation results, with solution irrigants achieving slightly better continence rates[125]. The volume of irrigation is gradually increased over time after the procedure and the timing and frequency of irrigation through the site may be largely patient directed. In the pediatric patient population, the operation is performed around the age of 10 years.

Few studies report on outcomes of antegrade con-

Table 6 Outcomes of antegrade continence enema in incontinent adults

Ref.	Year	Patients using antegrade continence enema on follow-up	Percentage of patients achieving continence	Complication rate
Gerharz *et al*[121]	1997	8	100%	44%
Teichman *et al*[120]	1998	7	86%	71%
Teichman *et al*[128]	2003	4	75%	67%
Lefevre *et al*[127]	2006	18	94%	33%
Poirier *et al*[126]	2007	14	78%	67%

tinence enemas in adults. Overall, functional results are very good, with about 75% of adults achieving continence with the procedure[126-128]. Quality of life improves in adult patients with antegrade continence enemas, although not all patients continue to use their antegrade continence enema in the long term[127,128]. See Table 6 for a summary of antegrade continence enema study results. In children, full continence is achieved in 65%-100% of patients[122,125,129-133]. Even though the amount of time devoted to bowel care may not significantly change, satisfaction and quality of life scores improve for most children and parents[131,134-137]. Persistent leakage, stoma stenosis, and surgical site infections are common complications, with one study quoting a 13% chance of requiring stoma revision due to stoma complications[130,131,138]. While the antegrade continence enema is not commonly performed in adults, the patients who have grown to adulthood require long term follow up and attention to these possible complications.

Fecal diversion

The creation of a colostomy or ileostomy provides definitive control of fecal incontinence. An ileostomy may be considered in patients with colonic transit abnormalities but the colostomy is the standard ostomy utilized in the treatment of fecal incontinence. In many patients the ostomy can be created using a laparoscopic approach to improve recovery time. While a colostomy is not without short and long term risks, such as bleeding, anesthesia related cardiac or respiratory morbidities, and parastomal hernia, it is a safe and effective treatment of severe fecal incontinence. It is generally only offered if other treatment modalities have failed. Patients are usually understandably very resistant to the idea of a permanent colostomy, fearing it will be difficult to manage and have great impact on self-image and social interactions.

When patients who had undergone colostomy creation for fecal incontinence were surveyed, general quality of life and fecal incontinence quality of life scores were actually higher in the colostomy group when compared to other patients with fecal incontinence[139]. Another study found that patients generally reported high satisfaction levels with their stomas for fecal incontinence, with over 80% of patients stating that they would likely or definitely choose to undergo the procedure again[140]. Compared to other surgical treatments of sever incontinence (dynamic graciloplasty and artificial bowel sphincters), a British study found colostomy to be most cost effective in terms of quality adjusted life years[92]. While fecal diversion is not required in the majority of patients presenting for treatment of fecal incontinence, it is a viable, definitive, and well-tolerated treatment which offers good quality of life.

Table 7 Outcomes of radiofrequency energy treatments

Ref.	Year	Patients (*n*)	Significant improvement in incontinence scores after treatment	Significant improvement in quality of life
Efron *et al*[143]	2003	50	Y	Y
Felt-Bersma *et al*[147]	2007	11	Y	NR
Takahashi-Monroy *et al*[142]	2008	19	Y	Y
Lefebure *et al*[144]	2008	15	Y	N
Kim *et al*[148]	2009	8	N	N
Ruiz *et al*[145]	2010	24	Y	Y
Abbas *et al*[146]	2012	27	Y	NR

Y: Yes; N: No; NR: Not reported.

AUGMENTATION

Radiofrequency energy

There is a gap between medical and surgical treatment options in fecal incontinence[141]. Radiofrequency energy delivery and injectable materials are becoming increasingly popular as minimally invasive procedural treatments that may bridge this gap. The delivery of radiofrequency energy to the internal anal sphincter, known as the SECCA® procedure, is proposed to induce local restructuring of collagen, leading to a more robust internal anal sphincter and better continence. It can be used for patients with mild or moderate fecal incontinence who are unwilling or not candidates to undergo surgical treatment after failing medical management. It may also be applied to patients with idiopathic or sphincter defect-associated fecal incontinence.

The technique of radiofrequency energy delivery is simple. It is done with conscious sedation and local anesthesia on an outpatient basis in endoscopy or the operating room. A commercial device is utilized and the procedure takes about 30 min. The device resembles a clear plastic anoscope with four retractable needles. The needles are electrodes which are deployed into the anorectal mucosa to deliver radiofrequency energy to the internal anal sphincter, starting just distal to the dentate line and moving proximally. The device delivers radiofrequency while simultaneously monitoring the temperature and impedance of the tissues to avoid burning. The device is activated four or five times per quadrant of the anorectum, moving 5 mm more proximal before each activation in a quadrant. The machine provides constant feedback on the contact with the tissues, temperature and impedance during the device activation, and the timing of each activation, giving visual and sound cues to the surgeon

Table 8 Outcomes of dextranomer in hyaluronic acid gel for fecal incontinence

Ref.	Year	Patients (*n*)	> 50% reduction in fecal incontinence episodes (percentage of patients)	Significant quality of life improvement
Dodi *et al*[150]	2010	115	64%	Yes
Graf *et al*[161]	2011	136	52%	Yes
Schwandner *et al*[159]	2011	21	56%	Yes
Danielson *et al*[160]	2012	34	76%	Yes
La Torre *et al*[156]	2013	83	63%	Yes

throughout the procedure.

Reports of the success of radiofrequency energy treatment are generally, though not universally, positive and are summarized in Table 7. Numerous studies have reported long term improvement in fecal incontinence scores[142-145]. The cohort with the longest reported follow-up showed a durable reduction in mean Wexner fecal incontinence scores from 14 to 8 and found that most participants had a greater than 50% improvement in symptoms after 5 years[142]. Similarly, patient satisfaction and quality of life scores show improvement after radiofrequency energy treatment[142-145]. Another study with a higher average baseline fecal incontinence score compared to other trials found that only 22% of patients had sustained treatment benefits at an average follow up of 40 mo[146]. Despite the overall favorable outcomes of radiofrequency energy delivery, anal manometry testing does not show any significant change in physiologic parameters[143,147,148]. No major adverse events have been reported following radiofrequency energy delivery, though there have been reports of infection, hematoma, minor bleeding, and anal pain[145,147,148].

Injectable materials

Various injectable materials have included trialed for local injection of the sphincter complex to treat fecal incontinence. Benefits of this approach are that it is an outpatient procedure with little discomfort that has low morbidity. The materials used have included collagen, silicone, autologous fat, glutaraldehyde, carbon-coated beads, dextranomer in hyaluronic acid gel, and others[149]. Dextranomer in hyaluronic acid gel (NASA/Dx) has received the most extensive recent investigation and attention in the literature. Injectables may be used in patients who have failed medical treatment and have fecal leakage or mild to moderate fecal incontinence[149]. The bulking effect may not be permanent and may require repeat injections at subsequent office visits.

The technique of injection is relatively simple. The open-label multicenter trial of NASHA/Dx involved four quadrant injections of 1 mL of NASA/Dx into the deep submucosa of the anal canal[148]. This was performed through an anoscope and done with the patient in the prone jack-knife or lithotomy positions. The injections were placed at a 30 degree angle 5-10 mm proximal to the dentate line[150]. The needle was kept in place for up to 30 s so that the gel would not leak from the site[150]. There are very few comparative trials amongst injectable materials. A small study of 40 patients found that silicone was more effective than carbon-coated beads to reduce incontinence[151]. No published studies have compared NASHA/Dx with other injectables. One randomized controlled trials comparing NASHA/Dx to biofeedback and found no significant difference in functional outcomes[152]. Biofeedback, however, certainly requires more dedication and long term commitment from the patient. The effect of injectables on manometry parameters are an increase in the length of the high pressure zone and asymmetry index[153]. The impact on resting pressure is variable in the literature, ranging from improvements in resting pressure to no effect[153,154].

There are no long term outcomes reported yet for NASHA/Dx, the most popular injectable. The longest reported outcomes are at 2 years[155,156]. A Cochrane review published in 2013 noted the absence of long term studies, making definitive conclusions about the utility of injectables difficult[157]. See Table 8 for a summary of cohort studies investigating the utility of NASHA/Dx gel. A good response is considered a 50% reduction in the number of reported incontinence episodes, which is reported to occur in over 50% of patients who have been treated with injectables[150,154,156,158-161]. In addition, the majority of patients have good quality of life improvement, as reported on both global quality of life and fecal incontinence quality of life scores[150,155,156,158]. Morbidity from the use of injectables is low, with fever and proctalgia being the two most common adverse events and bleeding, abscess, and pain being other rare reported events[150,156,160,161]. Though many patients with fecal incontinence may be candidates for the use of injectables, the ideal candidate is one who has seepage or mild to moderate incontinence who has failed medical management but is not yet ready to pursue surgical treatment. Prior use of an injectable such as NASHA/Dx does not preclude future surgical treatments such as sacral nerve stimulation, sphincteroplasty or artificial bowel sphincter.

CONCLUSION

Successful treatment of fecal incontinence requires careful consideration of the individual patient's severity of incontinence. Treatments range from inexpensive medications and physical therapy to complex surgical procedures such as artificial bowel sphincter implantation and muscle transposition. In general, more invasive treatments are required for more severe incontinence or after less invasive treatments have failed. A careful history including obtaining an incontinence score, physical examination, bowel diary, and adjunctive anal physiology tests should be utilized to define the nature of the fecal incontinence. Minimally invasive approaches including biofeedback, radiofrequency energy, and injectables have moderate long term success. Sphincteroplasty remains an acceptable option for patients with documented sphincter defects. Be-

cause initially adequate functional outcomes decline over time, quality of life improvement after sphincteroplasty is not robust long-term. Sacral nerve stimulation is very effective in managing moderate to severe fecal incontinence and has had a great impact on the treatment of fecal incontinence. In the very long-term, patients will require additional procedures to change the battery of the sacral nerve stimulator but the procedure has excellent reproducible long term functional and quality of life outcomes. The artificial bowel sphincter has similar outcomes in those patients who retain the device, but further studies aimed at reducing infection, erosion, and device failure must be undertaken. Fecal diversion remains a good option for severe fecal incontinence and actually provides the patient with satisfying quality of life. Knowledge of these currently used treatments is essential to honest and thorough counseling of the patient with fecal incontinence to improve treatment success. Together with the patient, the surgeon can then best select treatment from the five available categories of repair, replacement, augmentation, stimulation, and diversion.

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REVIEW

Sleep, immunity and inflammation in gastrointestinal disorders

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Abstract

Sleep disorders have become a global issue, and discovering their causes and consequences are the focus of many research endeavors. An estimated 70 million Americans suffer from some form of sleep disorder. Certain sleep disorders have been shown to cause neurocognitive impairment such as decreased cognitive ability, slower response times and performance detriments. Recent research suggests that individuals with sleep abnormalities are also at greater risk of serious adverse health, economic consequences, and most importantly increased all-cause mortality. Several research studies support the associations among sleep, immune function and inflammation. Here, we review the current research linking sleep, immune function, and gastrointestinal diseases and discuss the interdependent relationship between sleep and these gastrointestinal disorders. Different physiologic processes including immune system and inflammatory cytokines help regulate the sleep. The inflammatory cytokines such as tumor necrosis factor, interleukin-1 (IL-1), and IL-6 have been shown to be a significant contributor of sleep disturbances. On the other hand, sleep disturbances such as sleep deprivation have been shown to up regulate these inflammatory cytokines. Alterations in these cytokine levels have been demonstrated in certain gastrointestinal diseases such as inflammatory bowel disease, gastro-esophageal reflux, liver disorders and colorectal cancer. In turn, abnormal sleep brought on by these diseases is shown to contribute to the severity of these same gastrointestinal diseases. Knowledge of these relationships will allow gastroenterologists a great opportunity to enhance the care of their patients.

Key words: Sleep; Immune function; Immunity; Irritable bowel syndrome; Inflammatory bowel disease; Gastro-esophageal reflux disease; Liver disorders; Colon cancer; Circadian rhythm

Core tip: Sleep disorders have become a global issue, and discovering their causes and consequences are the focus of many research endeavors. Recent research suggests that individuals with sleep abnormalities are at greater risk of all-cause mortality and serious adverse health and economic consequences. Several studies support the associations among sleep, immune function and inflammation. We review the current research linking sleep, immune function, and gastrointestinal diseases and discuss the interdependent relationship between sleep, overall immune function with emphasis on inflammatory bowel disease, irritable bowel syndrome, gastro-esophageal reflux and colorectal cancer.

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INTRODUCTION

Research into sleep and its associated health abnormalities has had a relatively recent surge, and sleep quality has been shown in many investigations to be an important, if not essential element of good health[1-3]. Sleep disorders can be primary, secondary or behavioral. Primary disorders are related to neurologic defects like narcolepsy and restless leg syndrome, breathing problems like obstructive sleep apnea and central sleep apnea, or circadian rhythm abnormalities like jet lag and delayed sleep phase syndrome. Secondary sleep disorders are secondary to primary diseases such as depression, chronic illness *etc*. Behavioral sleep problems such as insomnia or insufficient sleep are caused or perpetuated by poor sleep hygiene.

Sleep disorders have become a global issue. Sleep abnormalities occur in 17%-22% Japanese[4,5], while sleep disorders are estimated to range from 7% to 50% in people living in Portugal and Finland[6-8]. In the United States, more than 70 million people suffer from a sleep disorder, and modern lifestyles have led to Americans sleeping approximately 2 h less per night than 100 years ago[4,7,9]. Abnormalities in the sleep cycle are linked with neurocognitive consequences ranging from performance decrements, slower response times, and decreased cognitive ability[10].

Receiving fewer hours of sleep may also impact metabolism in a manner that contributes to obesity[10]. A strong association has been found between disruption in sleep and gastrointestinal disease. We will review the interdependent relationship of sleep dysfunction and gastrointestinal issues including inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), gastro-esophageal reflux disease (GERD), liver disorders and colon cancer. Sleep abnormalities have been shown to worsen symptoms of IBS, IBD and GERD which, in turn, can worsen sleep abnormalities. Sleep disorders and circadian dysfunction have also been shown to increase the risk of colon cancer.

HUMAN SLEEP

Sleep is classified based on polysomnographic data into two main categories known as rapid eye movement (REM) sleep and non-REM (NREM) sleep. NREM sleep is further divided into three stages based on increasing depths of sleep and increasing arousal thresholds. These sleep stages cycle through REM and NREM approximately every 90 min[11]. More time is spent in slow-wave delta sleep each cycle during the first half of the night, with increasing time in REM sleep in the later portions of the night. Humans spend around 25% of total sleep time in REM sleep[12]. The exact biological purpose of sleep is unknown. However, slow-wave sleep is thought to be restorative, restful sleep, and REM sleep is associated with dream recall and memory consolidation[13]. Although the ideal quantity of sleep is different among individuals, most studies recommend seven to eight hours a night for adults as an optimal amount of sleep[14]. Alterations in normal sleep patterns are thought to be a significant contributor to a vast array of illness including depression, metabolic syndrome, inflammation, gastrointestinal diseases, and also cancer[15,16].

REGULATION OF SLEEP

Sleep regulation is often described by a two process model[17]. Process S, or the sleep homeostatic drive, linearly increases the longer an individual stays awake[18]. Process C, or the circadian alerting drive, oscillates with body temperature on an approximate 24-h cycle[15,18]. During the later hours of the day, Process C enters its decline in the circadian pattern, and Process S has accumulated approximately 16 h of continuous wakefulness. The combination of declining alertness and a sufficient amount of prior wakefulness facilitates the onset of sleep[18]. Biological clocks have evolved based on a 24-h cycle that allow organisms to anticipate and physiologically adjust to daily environmental changes and this circadian system provides a temporal organization of waking and sleep[15,19]. The circadian clock is entrained or synchronized to the specific day-night cycle (phase) of the environment through signals such as light, meals, and social interaction. These affect neuro hormonal pathways which influence the circadian clock. Light is the most important factor affecting the circadian rhythm. Light travels from the retina *via* the retinohypothalamic pathway to the suprachiasmatic nucleus (SCN), and then *via* a multisynaptic pathway to the pineal gland where it suppresses melatonin production. Melatonin is a neurohormone that serves to synchronize circadian rhythms both with the environment and the human body as melatonin receptors are found in nearly all human tissue. Furthermore, the 24-h circadian rhythm is governed by a main circadian clock and a system of peripheral clocks located in multiple tissues including the pancreas, liver, and adipose tissue[20]. The SCN also serves as a "standard time" which synchronizes peripheral tissue clocks[21]. A series of "clock genes" help regulate the timing through both positive and negative feedback loops. CLOCK and Brain and Muscle Arnt-like protein (BMAL-1) form heterodimers that accumulate throughout the day. These heterodimers then bind to the promoter regions of the genes Period (PER) and Cryptochrome (CRY) to activate their transcription. PER and CRY proteins then accumulate and form heterodimers that inhibit transcription of CLOCK and BMAL-1 proteins[22]. Point mutations in these clock genes have been linked to altered circadian function and sleep abnormalities in mammals including familiar ad-

vanced sleep phase syndrome and delayed sleep phase syndrome[23-25].

Research has also focused on determining whether similar feedback-loop clock genes are present within the gastrointestinal tract. PER2 expression has been identified in the myenteric plexus and affects the rhythmic releases of acetylcholine and nitric oxide, ultimately regulating peristalsis[26,27]. Hypotheses on circadian rhythms affecting nutrient transport in the small intestine, gastric acid secretion, gut motility, and production of digestive enzymes have also been proposed[27].

IMMUNE ACTIVATION AND CYTOKINE EFFECTS ON SLEEP

Many immune and endocrine pathways exhibit a diurnal profile including cortisol and growth hormone. The onset of sleep corresponds with an increase in the serum levels of some cytokines, peaking at 2.5 h after sleep onset[28]. This surge of cytokines and their pro-inflammatory effects are suggested to be linked with nocturnal exacerbations of diseases like asthma and rheumatoid arthritis[11]. Increasing evidence supports a reciprocal relationship between sleep and the immune system. An activated immune system alters sleep and sleep abnormalities affect immune function[29,30]. Studies have also shown that an immune response elicits a pro-inflammatory cytokine response that helps to modulate sleep[22]. This was first illustrated in the 1970s[31] after the identification of a sleep-inducing muramyl peptide known as factor S was found to have both immune and sleep regulatory properties[18,32]. Although the diverse range of cytokines released in early inflammation limits our ability to isolate individual contributions[33], tumor necrosis factor (TNF)-α, interleukin-1 (IL-1), and IL-6 have shown the strongest potential[30]. However, numerous other cytokines with at least partial sleep regulatory properties have been identified. In animal models, IL-1 and TNF-α elevations have correlated with increased time in NREM sleep. Furthermore, an inhibitory effect on both spontaneous sleep and sleep rebound (increased REM sleep after sleep deprivation) was produced when IL-1 was inhibited by anti-IL-1 specific antibodies[34]. In addition, high serum levels of TNF-α has been linked to sleepiness in patients with obstructive sleep apnea and rheumatoid arthritis[35,36]. IL-6 also plays a role in sleep modulation. Sleep deprivation can increase IL-6 levels leading to daytime fatigue[37]. In a human study, subjects received an injection of IL-6 that simulated the levels found in infection, and they experienced marked subjective fatigue, inhibition of REM sleep, and elevated CRP in 6.5 h[33]. The inhibition of REM and the promotion of NREM sleep appear to play key roles in the immune response. IL-1, IL-6 and TNF-α are at high levels at time of infection and correlated with increased duration of NREM, changes in core body temperatures, more shivering, and an overall greater capacity to fight off illness[32]. This was confirmed in several studies evaluating the effect of infection with human immunodeficiency virus (HIV) on sleep. In early stages of HIV infection, polysomnographic data showed larger percentage of time spent in NREM than in REM and prolonged REM sleep latency[18,38]. Serotonin also is an integral component to IL-1 activity. Depletion of serotonin or inhibition of the serotonin receptor led to a reduction in the IL-1-induced increase in the amount of NREM sleep[39,40]. Thus, there appears to be an interaction of IL-1 and its ability to modulate sleep based on baseline levels of serotonin. Infection caused by viral, bacterial, fungal or even parasites was evidenced to increase the amount of time spent in NREMS and decrease the amount of time spent in REMS[41] based on severity of infection[12].

SLEEP EFFECTS ON THE IMMUNE RESPONSE

Both human and animal studies have shown that sleep has an overall protective role and that sleep deprivation is associated with an increased susceptibility to infection[18,22]. A study on infected rabbits showed that animals who had longer periods of sleep had less morbidity and mortality[42]. In humans, long-term sleep deprivation was shown to increase risk of septicemia[43,44]. Furthermore, decreased sleep has been linked to impaired antibody response to hepatitis A vaccine[29], influenza[45], and increased risk of getting a upper respiratory infection[46]. The timing of sleep is also important because most immune cells have their highest response to immune challenges during the night[12,18] and their lowest response in the morning[45]. This antibody impairment is very similar to the decrease in the immune response seen with human aging as both have a lowered T-cell response to antigens and impaired response to vaccinations[47].

GASTROESOPHAGEAL REFLUX DISEASE AND SLEEP

It is well established that gastroesophageal reflux and its most common symptoms, heartburn and regurgitation, is among the most frequently dealt with conditions encountered by gastroenterologists[48].

Approximately, 10%-20% of the people in the United States have GERD[49]. One study found that approximately 74% of patients with GERD had nocturnal symptoms[50]. A Gallup survey revealed that approximately 63% of the people with nocturnal GERD felt it impaired their ability to sleep and 40% felt it impaired their ability the following day[51]. Several factors likely contribute to nocturnal GERD. Numerous studies now have documented that reflux during sleep presents physiologic issues not encountered during the waking state. For example there is a notable prolongation of acid clearance due to the suppression of swallowing and salivation during sleep. This results in enhanced back diffusion of hydrogen ions and subsequent mucosal damage.

These issues are discussed in detail in a review by Orr *et al*[51] in which he presents an argument for considering nighttime reflux and its clinical manifestations as a distinct clinical entity[52]. However, sleep and GERD have been shown to have a more interdependent relationship. A study by Dickman *et al*[52] noted that poor quality of sleep led to exacerbations of reflux the following day. They also found that longer durations of reflux events correlated with reduced sleep quality. This was supported by the Gallup survey, a higher frequency of reflux was associated with higher frequency of sleep difficulties[51]. A likely contributing factor is the hyperalgesia due to sleep disturbances[54,55]. This was first reported by Onen *et al*[53] who found that sleep deprivation led to a somatic hyperalgesia. This hyperalgesia was evidenced after loss of REM sleep or cumulative 2 d loss of non-REM sleep[54]. Recently, Schey *et al*[54] have documented a visceral hyperalgesia and increased sensitivity to reflux in GERD patients with documented poor sleep prior to undergoing an acid perfusion test[55]. Further research in this area is needed, but current studies indicate that discussion and treatment of sleep abnormalities in patients with GERD may lead to improved management.

PEPTIC ULCER DISEASE

Patients with sleep apnea sustain cessation of breath during sleep, leading to intermittent hypoxia, systemic inflammation and sympathetic activation. These insults are not only be a threat to cardiovascular system but can also contribute to damage to the gastrointestinal mucosa and hence initiation or progression of peptic ulcers[56]. In a very large study of nearly 35000 patients from Taiwan, patients with sleep apnea experienced 2.4 fold higher risk for peptic ulcer bleeding[56]. This may warrant surveying for sleep apnea as a potential predisposing factor in patients with peptic ulcer bleeding and without any apparent risk factors.

INFLAMMATORY BOWEL DISEASE AND SLEEP

IBD is characterized by a chronic immune mediated inflammation of the gastrointestinal tract. It is estimated that approximately 400/100000 Americans suffer from IBD[57]. The relationship between sleep and IBD has been a topic of more recent consideration. Ranjbaran *et al*[57] used the Pittsburgh Sleep Quality Index (PSQI) to show a relationship with sleep abnormalities and the quality of life in patients with IBD. They noted several sleep-related issues: more sleep latency, less day time energy, and increased sleeping pill use[57].

Abnormal sleeping habits may also play a role on disease severity. One study noted both worsened severity of UC and higher mortality in phase-shifted mice than in unaltered circadian-phase mice[59]. They noted that chronic circadian phase shifts led to worsening mucosal inflammation and colitis likely secondary to altered inflammatory cascade regulation[59]. Another study found that occupations that have artificial working conditions (such as light) and irregular hours had higher odds ratio (1.6-1.7) for development for IBD[60,61].

Patients with Crohn's disease (CD) and sleep loss may also have a greater risk for disease relapse. These patients had twice the risk of active disease in 6 mo than patients who did not have sleep abnormalities[62]. In fact, Tang *et al*[62] performed a study examining sleep deprivation on mice with colitis and noted both acute and chronic sleep deprivation led to worsening colitis likely secondary to heightened sensitivity to pro-inflammatory cytokines such as IL-6 and TNF-α[9,30,61,63]. A large survey study looking at sleep disturbances in over 3100 participants found that CD patients in clinical remission and subjective sleep disturbances had a 2-fold increased risk of active disease at 6 mo. They discovered approximately 75% of patients with active disease have subjective sleep complaints compared to 48% inactive disease[62].

Recently, we performed a prospective observational cohort study looking at the sleep disturbances of IBD patients. We discovered that 100% of patients with active disease had poor sleep while only 72% of patients with clinically inactive disease had poor sleep. The difference between sleep disturbances became even higher when histology was used to define the disease activity. We found 100% of those in histologically active group had poor sleep while only 54% in the histologically inactive group had poor sleep (OR = 6.0, 95%CI: 2.9-12.5, $P < 0.0001$). An abnormal PSQI had a positive predictive value for histologic inflammatory activity of 83%[64]. These patients were prospectively followed for 6 mo, and the relapse rate in clinically inactive patients with poor sleep was found to be 67%. No patients with normal sleep patterns relapsed (RR = 3, 95%CI: 1.5-6.1, $P = 0.03$). We detected a significant correlation between the baseline PSQI and disease activity at the 6-mo follow up (CD: $r = 0.56$, $P = 0.0046$; UC: $r = 0.54$, $P = 0.024$)[65]. Although the study was limited by the small number of patients, the results are intriguing and hold very important therapeutic implication in the management of immune-mediated inflammatory diseases.

Melatonin has recently been investigated as a possible method of improving outcomes for patients with UC Data from several animal models indicate that melatonin administration increased serum levels of IL-10 (an anti-inflammatory cytokine) and decreased serum levels of pro-inflammatory cytokines such as IL-6 and TNF-α[66-69]. Patients with UC had abnormally high levels of pro-inflammatory cytokines, and melatonin may play a role in reducing the severity of UC by reducing these specific cytokines[69-73].

IRRITABLE BOWEL SYNDROME AND SLEEP

IBS is a chronic gastrointestinal syndrome that is associated with abdominal pain and distorted bowel behavior.

IBS is commonly diagnosed and there is an estimated 10%-15% of the North American population suffering from this syndrome[74]. IBS appears to have a significant association with anxiety, stress, and overall environment. Interestingly, sleep dysfunction also has similar associations. The study conducted by Kim *et al*[74] examined IBS occurrence among irregular-shift workers and traditional day-shift workers. They found that the prevalence of IBS in irregular-shift workers was significantly higher (32.7%) than in the day-shift workers (16.7%). They also found that many of the individuals that worked irregular shifts experienced less sleep quality, higher rates of daytime sleepiness, and higher levels of stress[75]. Chen *et al*[75] compared sleep patterns and rectal sensitivity using anorectal manometry among patients with IBS and healthy subjects. They noted that IBS patients with lower amounts of quality sleep were prone to lower thresholds for rectal sensitivity and altered anal sphincter function[76]. This rectal hyperalgesia in patients with sleep abnormalities and IBS is consistent with the visceral hyperalgesia noted in patients with sleep abnormalities and GERD[55].

COLON CANCER AND SLEEP

Colorectal cancer is the second most commonly diagnosed cancer in the world in women and the third most common in men[77]. Surgery is often the primary method of intervention while adjuvant chemotherapy and radiation therapy are often employed to improve survival or quality of life[78-80]. Several surveys noted that fatigue was one of the highest concerns for people with cancer[78,81,82].

Animal studies indicate that both circadian disruption by nocturnal light exposure or sleep deprivation accelerated tumor formation[83-85]. A recent study by Thompson and colleagues evaluated sleep and colon cancer and noted that shorter duration of sleep (< 6 h) led to an almost 50% increase in the risk for colorectal adenomas[86]. Shift work, abnormal clock gene expression, and other causes of disruption of circadian rhythms are emerging as cancer risk factors[83,87]. A study by Schernhammer *et al*[87] found an increased risk for colon cancer in women who worked night shifts[88]. Several theories have been proposed to explain the relationship between sleep and colon cancer. Increased obesity is a known risk factor for cancer[89]. Sleep disorders are also known to alter metabolism and contribute to obesity[10]. Sleep disturbance may play an indirect role in increasing the risk for cancer by increasing adiposity[90]. Another theory suggests melatonin and its anti-carcinogenic properties are a key factor. Nocturnal light exposure suppresses melatonin production, and the lack of melatonin and its anti-proliferative effects may contribute to intestinal cancer formation[88,91]. Open discussion, evaluation, and treatment of lower-than-normal duration of sleep may be an under-appreciated method of colorectal cancer risk modification.

SLEEP DYSFUNCTION AND THE LIVER

Sleep disturbances are seen in numerous types of liver diseases. One study found 47.7% of cirrhotic patients had unsatisfactory sleep when compared to 4.5% seen in controls[92]. Elevated levels of ammonia seen in hepatic encephalopathy is also evidenced to induce sleep wake cycle reversal and progressive electroencephalography changes with triphasic wave changes in Stage I hepatic encephalopathy and eventually delta waves and comatose state in Stage IV[93]. Another study found that women with primary biliary cirrhosis slept nearly twice as much during the day when compared to controls[94]. Although the exact mechanism behind this is known, it is thought that elevated IL-6 plays a role[95]. Patients with hepatitis C also are at higher risk for sleep abnormalities with 60%-65% reporting abnormal sleep complaints[96]. In addition, patients undergoing treatment with interferon-a are also at increased risk for sleep abnormalities as 22%-24% of patients experience sleep disturbance as a side effect[97].

Summa *et al*[97] study on mice found that circadian disorganization *via* Clock$^{\Delta 19/\Delta 19}$ mutation led to elevated liver/body weight ratios and advanced alcohol induced steatohepatitis[98]. The etiology behind this connection is thought to rely on abnormal intestinal epithelial permeability. Ideally, the intestinal epithelial barrier serves to protect the body from unwanted luminal contents while also allowing a fraction of permeability to allow immune surveillance and regulation[99]. Summa *et al*[97] followed the absorption of sugars in the gastrointestinal tract in phase shifted mice and found increased permeability in the colon when compared to control. This evidence indicates that circadian dysfunction may be a separate risk factor for alcohol induced liver damage[98].

Patients with sleep apnea sustain cessation of breath during sleep, leading to intermittent hypoxia, systemic inflammation, and sympathetic activation. These insults may contribute to initiation or progression of peptic ulcers[56]. In a very large study of nearly 35000 patients from Taiwan, patients with sleep apnea experienced 2.4 fold higher risk for peptic ulcer bleeding[56].

TREATMENT IMPLICATIONS

As the complexities regarding the association between sleep and gastrointestinal disorders continue to become better understood, it begs the question as to how the medical and psychiatric community should address comorbid sleep and gastrointestinal disorders. Though current clinical trials have not directly addressed this population, several small preliminary trials have investigated the efficacy of cognitive behavioral therapy for insomnia in patients with comorbid chronic pain[100-103]. Collectively, these studies suggest that insomnia can be effectively treated among patients with chronic pain and that improvement in sleep confers some clinical improvement in pain. Therefore, given the state of the current science, it seems prudent that medical providers would recommend the evaluation and treatment of sleep disorders in patients with gastrointestinal disorders. Treating both disorders in parallel may not only result in a better outcome

for the patient, but also allow the medical provider to use less invasive and expensive means to improve the patient's overall quality of life.

CONCLUSION

Sleep abnormalities are a global issue and its effects on well-known pathologies is both an interesting and relevant field of research. Sleep abnormalities contribute to many gastrointestinal diseases and conversely, gastrointestinal diseases often lead to sleep abnormalities. This interdependent relationship represents a novel approach to treating GERD, IBS, IBD, liver disorders and colon cancer. The evaluation, discussion, and treatment of sleep abnormalities may play a key role in further preventing and improving many gastrointestinal disorders.

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REVIEW

Pathophysiology of cerebral oedema in acute liver failure

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Abstract

Cerebral oedema is a devastating consequence of acute liver failure (ALF) and may be associated with the development of intracranial hypertension and death. In ALF, some patients may develop cerebral oedema and increased intracranial pressure but progression to life-threatening intracranial hypertension is less frequent than previously described, complicating less than one third of cases who have proceeded to coma since the advent of improved clinical care. The rapid onset of encephalopathy may be dramatic with the development of asterixis, delirium, seizures and coma. Cytotoxic and vasogenic oedema mechanisms have been implicated with a preponderance of experimental data favouring a cytotoxic mechanism. Astrocyte swelling is the most consistent neuropathological finding in humans with ALF and ammonia plays a definitive role in the development of cytotoxic brain oedema. The mechanism(s) by which ammonia induces astrocyte swelling remains unclear but glutamine accumulation within astrocytes has led to the osmolyte hypothesis. Current evidence also supports an alternate 'Trojan horse' hypothesis, with glutamine as a carrier of ammonia into mitochondria, where its accumulation results in oxidative stress, energy failure and ultimately astrocyte swelling. Although a complete breakdown of the blood-brain barrier is not evident in human ALF, increased permeation to water and other small molecules such as ammonia has been demonstrated resulting from subtle alterations in the protein composition of paracellular tight junctions. At present, there is no fully efficacious therapy for cerebral oedema other than liver transplantation and this reflects our incomplete knowledge of the precise mechanisms underlying this process which remain largely unknown.

Key words: Cerebral oedema; Acute liver failure; Ammonia; Hepatic encephalopathy; Intracranial pressure; Intracranial hypertension; Cerebral blood flow

Core tip: Cytotoxic and vasogenic cerebral oedema have been implicated in acute liver failure (ALF) with a preponderance of experimental data favouring cytotoxic mechanisms. Astrocyte swelling is a consistent neuropathological finding in human ALF and ammonia plays a definitive role. The mechanism(s) by which ammonia induces astrocyte swelling remains unclear but glutamine plays a central role inducing oxidative stress, energy failure and ultimately astrocyte swelling. Although complete breakdown of the blood-brain barrier is not evident in human ALF, increased permeation to water and ammonia has been demonstrated. There is no efficacious therapy other than liver transplantation reflecting the incomplete knowledge base.

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INTRODUCTION

Acute liver failure (ALF) is a complex clinical syndrome that results from a sudden and severe loss in hepatocyte function in a patient without pre-existing liver disease[1]. This rapid loss of function is the result of massive hepatocyte necrosis and is typically associated with hepatic encephalopathy (HE) and coagulopathy, the hallmark features of ALF. In many cases progressive multi-organ failure ensues. Although ALF is rare, with an incidence of one to six cases per million people every year in the United States and Western Europe, the mortality rate and the cost of treatment is high[2]. The majority of those affected are young adults.

ALF is sometimes referred to as fulminant hepatic failure (FHF), a term first used in 1970 by Trey and Davidson[3] who described a potentially reversible disorder resulting from severe hepatic injury, with an onset of encephalopathy within 8 wk of symptom appearance in the absence of chronic liver disease. Whilst the main features of this definition remain relevant today, O'Grady *et al*[4] proposed a new classification for adults with ALF, dividing them into three groups based on the time between the onset of jaundice to the development of encephalopathy: hyperacute (within 7 d), acute (8-28 d) and subacute (5-12 wk). This classification recognises that ALF complications and prognosis depend on the rate of evolution of the disorder. It has now gained wide acceptance in clinical and research studies. Those with a hyperacute presentation, such as following an acetaminophen overdose, are at highest risk of developing cerebral oedema.

The most reliable clinical signs of severe ALF include coagulopathy [international normalised ratio (INR) $\geqslant$ 1.5], which may become severe enough to cause spontaneous bleeding, and HE (any degree of altered mentation). HE presents with a rapid onset of initially subtle mental alterations such as minor confusion, disorientation and agitation, progressing to delirium, seizures and coma. When severe, HE is typically associated with the development of cerebral oedema[5].

Historically, cerebral oedema was thought to occur in up to 80% of patients with ALF and be the most common cause of death[6]. However, recent data following a review of 3300 patients presenting to a single tertiary liver centre has shown that the proportion of patients with intracranial hypertension (ICH) fell from 76% in 1984-1988 to 20% in 2004-2008 ($P < 0.0001$). In those who developed ICH, mortality fell from 95% to 55% ($P < 0.0001$). This mirrored a fall in the admission markers of disease severity and most likely reflects earlier illness recognition, improved intensive care, and use of salvage liver transplantation[7]. A further study from Bernal and colleagues from King's College Hospital on 165 patients presenting with ALF and grade 3/4 HE found that only 29% showed evidence of ICH. However, only one third had intracranial bolts inserted which raises the possibility that some of this cohort may have developed ICH without showing clinical sequelae. Whether the development of cerebral oedema is similar or higher in patients with ALF in developing countries remains to be determined. Nevertheless, along with sepsis and multi-organ failure, it is one the leading causes of death in these patients[1,8].

Patients with ALF are acutely ill and are best managed in intensive care units within tertiary liver transplant centres. The armamentarium of treatments available to alleviate cerebral oedema include mannitol, hyperventilation, hypertonic sodium chloride, induced hypothermia and barbiturates which aim to decrease the total fluid volume within the brain either by reducing the interstitial fluid and/or by reducing cerebral blood flow[8]. However, at present, no fully efficacious medical therapy for ALF is available and the only effective treatment is an emergency liver transplantation[1]. Nevertheless, liver transplantation is not always an option, with co-morbidities, sepsis, multi-organ failure and graft availability posing a major obstacle to a patient qualifying for a life-saving liver transplant.

The pathophysiological mechanisms underpinning the development of cerebral oedema are complex and remain to be fully unravelled. The central role of ammonia in the pathogenesis of cerebral oedema in ALF however, remains undisputed. Indeed, arterial ammonia concentrations greater than 100 μmol/L have been shown to predict the onset of severe HE with 70% accuracy with ICH developing in 55% of patients with ALF with an arterial ammonia concentration > 200 μmol/L[9]. Furthermore, Clemmesen and colleagues have shown that blood ammonia levels in excess of 150 μmol/L predicted a greater likelihood of dying from brain herniation[10].

CEREBRAL OEDEMA AND ACUTE LIVER FAILURE: AN OVERVIEW

Cerebral oedema is a net increase in total brain water content. The rigid skull bone protecting the brain limits the compliance of the brain and as a consequence a small increase in fluid can cause a significant rise in intracranial pressure. ICH can lead to a decrease in cerebral perfusion pressure and capillary blood flow, culminating in ischaemia[11].

Cerebral oedema as a complication of massive hepatic necrosis was first described by Ware *et al*[12] in 1971 and was found to be present in 80% of comatose ALF patients on post-mortem examinations. Increased water content of brain tissue has been considered to be a cardinal feature of cerebral oedema in ALF. However, ICH caused by an increase in cerebral blood flow has also been demonstrated in experimental models of ALF[13] in addition to patients with ALF[14]. Impaired autoregulation of CBF is well documented in patients with ALF and can be explained by the presence of vasodilatation of cerebral arterioles resulting in increased intracranial blood volume (cerebral hyperemia or the so-called luxury perfu-

sion)[15-17].

More recent studies have suggested that neuroinflammatory mediators, particularly pro-inflammatory cytokines such as the interleukins (IL)-1β and IL-6 and tumour necrosis factor-alpha (TNF-α), play an important role in the development of ICH[18] and progression of HE[19,20]. The presence of an infection or systemic inflammation (also known as 'systemic inflammatory response syndrome' or 'SIRS') is common in ALF and has been shown to be a major prognosticator of both the progression of HE and mortality in patients with ALF[21,22]. Moreover, evidence suggests that this inflammatory response may not only be peripheral but may arise within the brain itself[18,23,24]. Neuroinflammation is now widely considered to result from a direct interaction between microglia and ammonia[25,26]. The released pro-inflammatory cytokines from activated microglial cells and ammonia appear to act synergistically to induce cerebral oedema[27].

The neuropathological aspects of cerebral oedema were first described by Klatzo[28] in a presidential address classifying the underlying mechanisms of cerebral oedema into cytotoxic or vasogenic. This was further explored within the context of ALF by Ede *et al*[29]. In cytotoxic oedema the BBB is intact and there is intracellular swelling[30], whereas in vasogenic oedema there is breakdown of the BBB and water and plasma constituents accumulate in the extracellular space[31].

CYTOTOXIC OEDEMA AND ASTROCYTE SWELLING

The most prominent neuropathological finding from studies of brain autopsies of patients with ALF[32] and from animal models of cerebral oedema due to ALF is astrocyte swelling[30,33-35]. Astrocytes found within the gray matter are mainly affected and swelling of astrocytic foot processes rather than cell bodies is more commonly seen[32].

Magnetic resonance imaging (MRI) studies using diffusion tensor imaging (DTI) in humans support the view that astrocyte swelling, *i.e.*, cytotoxic oedema, represents the major component of cerebral oedema in ALF[36]. A reduction in the apparent diffusion coefficient (ADC) has been demonstrated in patients with ALF, indicative of a reduction in the size of the extracellular space. This implies that the development of cerebral oedema in ALF results from the accumulation of intracellular fluid.

Approximately one third of the brain volume is made up of astrocytes. They have an important function supporting neurones and have many biochemical, neurochemical and regulatory roles. Swelling of astrocytes therefore impacts upon their function. Abnormal membrane depolarisation has been demonstrated which could affect the ability of astrocytes to maintain ionic gradients and regulate neurotransmitter uptake and processing[37,38]. Impairment of astrocytic function can have deleterious effects on the rest of the central nervous system (CNS) leading to impairment of neuronal excitability and function.

The precise mechanism by which astrocytes swell remains to be determined, although many factors have been implicated. The evidence is most compelling for a role for ammonia in the development of astrocyte swelling in ALF[35]. Whilst other factors, including cerebral blood flow, vaso paralysis, hyperthermia, hyponatremia, substances derived from the necrotic liver, infection, inflammatory cytokines, lactic acid and glutamate have all been implicated in astrocyte swelling, the data is insufficient and often conflicting[34,39]. These factors may all act synergistically to induce cytotoxic swelling with ammonia playing a central role[20].

AMMONIA-GLUTAMINE HYPOTHESIS

Ammonia is mainly produced in the small bowel by the enzyme glutaminase, which breaks down glutamine into ammonia and glutamate. Ammonia is metabolised to urea primarily by the liver and to a lesser extent by the kidneys. In ALF this detoxification pathway, known as the urea cycle, is impaired from the loss of hepatocytes and the concentration of ammonia in the blood rises. Arterial concentrations of ammonia have been shown to correlate with the development of intracranial hypertension[9] and cerebral herniation[10]. Numerous experimental models of ALF have unequivocally associated ammonia exposure with the induction of astrocyte swelling. Treatment of cultured astrocytes with ammonia has consistently caused astrocytes to swell[40]. *In vivo* animal models of hyperammonemia have also demonstrated the presence of astrocyte swelling[30,41]. Rose *et al*[42] treated rats in ALF with *L*-ornithine-*L*-aspartate, an ammonia-lowering agent which acts by stimulating the urea cycle, and found a reduction in plasma ammonia concentrations and, more importantly, a reduction in cerebral oedema. Lastly, in the absence of liver pathology, patients with genetic disorders of urea cycle enzymes culminating in hyperammonemia develop cerebral oedema, suggesting that elevated levels of ammonia alone are sufficient to cause brain swelling[43]. The precise mechanisms underlying ammonia-induced astrocyte swelling are still poorly understood. Ammonia is able to enter the brain by diffusion[44,45] and its increased uptake from the circulation[46,47] leads to disturbances in astrocyte function[48-50].

The exclusive localisation within astrocytes of glutamine synthetase[51], a cytosolic enzyme which converts ammonia to glutamine, has led to the 'osmolyte' or 'ammonia-glutamine' hypothesis. Ammonia is detoxified to glutamine within the astrocyte, a precursor for the neurotransmitter glutamate. In addition to causing astrocyte swelling, ammonia has been shown to increase cerebral glutamine levels in the ALF setting[41]. Elevated glutamine levels have been found in brain tissue from animal models of HE[52] and in cerebrospinal fluid (CSF) and brain from patients with HE due to ALF[53,54]. These findings collectively suggest a potential role for glutamine in the development of astrocyte swelling, with hyperammone-

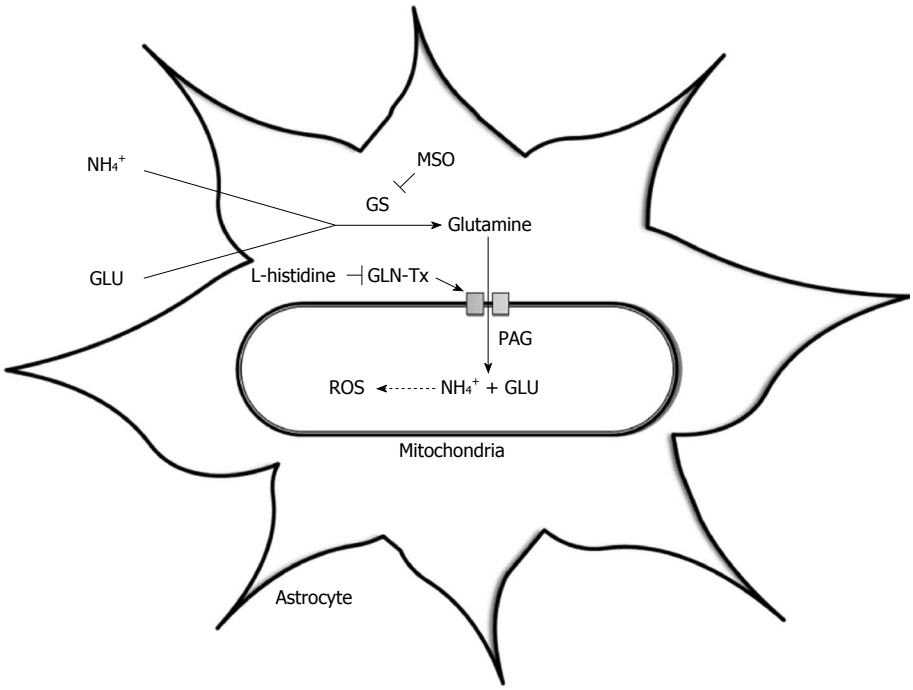

Figure 1 The 'Trojan Horse' hypothesis. This illustrates the synthesis of glutamine *via* the enzyme glutamine synthetase; its transport into mitochondria *via* the glutamine transporter (GLN-Tx); its hydrolysis by phosphate-activated glutaminase (PAG) resulting in glutamate (GLU) and ammonia (NH_4^+) production and the subsequent generation of reactive oxygen species (ROS). MSO: *L*-methionine *S*-sulfoximinel; GS: Glutamine synthetase.

mia causing increased synthesis and accumulation of glutamine in astrocytes, resulting in astrocyte swelling[55,56].

Originally, it was thought that glutamine acted as an organic osmolyte increasing the intracellular osmolarity, resulting in an influx of water into the cell and culminating in astrocyte swelling and dysfunction. In order to verify whether glutamine accumulation induces astrocyte swelling in hyperammonemic states, studies utilising *L*-methionine *S*-sulfoximine (MSO), an irreversible inhibitor of glutamine synthetase, have been performed[57]. Firstly, MSO lowers glutamine in normal brains[58] and prevents cerebral oedema in ammonia-infused healthy rats[59]. Subsequently, it was found to significantly diminish astrocyte swelling both *in vivo*[41] and in cell culture[60]. Therefore, inhibition of glutamine synthesis may have a protective effect, preventing glutamine accumulation, astrocyte swelling and thus cerebral oedema.

Although these findings suggest that glutamine accumulation within astrocytes plays an important role in cerebral oedema, more recent studies have questioned the glutamine-osmolyte hypothesis. In rats with ALF, glutamine concentrations do not correlate well with the degree of encephalopathy and associated cerebral oedema[61]. In two experimental models of ALF, rats were cooled to reduce brain swelling. Although cerebral oedema was ameliorated by mild hypothermia, it was not accompanied by a similar decrease in glutamine level[62,63]. Jayakumar *et al*[64] further tested the hypothesis using cultured astrocytes exposed to ammonia. They found no direct correlation between astrocyte swelling and glutamine levels. More importantly, astrocyte swelling was absent when glutamine levels peaked and cell swelling was maximal when glutamine levels were low. Furthermore, the duration and persistence of hyperammonemia, rather than its absolute level is most likely to determine brain glutamine levels and correlate with the development of cerebral oedema and raised intracranial pressure[65]. This delay in astrocyte swelling in relation to an increase in cellular glutamine content is not consistent with the concept of glutamine acting as an osmolyte in ALF and suggests that astrocyte swelling may not be the result of a direct osmotic effect of glutamine.

The 'Trojan horse' hypothesis has recently been proposed as an alternative theory by Albrecht *et al*[66] to explain the development of astrocyte swelling and brain oedema and suggests an important role for both ammonia and glutamine. The excess glutamine synthesised within astrocytes is transported into mitochondria where it is metabolised by phosphate-activated glutaminase (PAG) to ammonia and glutamate[67]. Glutamine, the "Trojan horse", thereby acts as a carrier of ammonia into mitochondria, where its accumulation can lead to oxidative stress and ultimately astrocyte swelling (Figure 1).

OXIDATIVE STRESS, MITOCHONDRIAL PERMEABILITY TRANSITION AND ENERGY FAILURE

Oxidative stress has been implicated as an important factor in the pathophysiology of ammonia-induced neurotoxicity[68]. O'Connor *et al*[69] first suggested oxidative stress might play a role in the pathogenesis of HE when they found evidence of lipid peroxidation in hyperam-

monemic mice. Norenberg *et al*[70] subsequently described the concept that protein peroxidation as well as lipid peroxidation may occur in astrocytes treated with ammonia. Further studies revealed that ammonia was able to generate free radicals such as superoxide in cultured astrocytes[71] and *in vivo*[72]. Ammonia also increases mRNA levels of heme-oxygenase-1 (HO-1), which is considered to be one of the best markers of oxidative stress, in a portacaval shunt rat model of HE[73]. Finally, decreased activity of the antioxidant enzymes glutathione peroxidase, superoxide dismutase and catalase were described in rats exposed to ammonia toxicity adding to the burden of oxidative stress[72].

Oxidative stress has been shown to be a key component of the cerebral oedema which develops in rats following hepatic devascularization[74]. Moreover, administration of antioxidants such as superoxide dismutase, catalase and vitamin E have been shown to inhibit the ammonia-induced astrocyte swelling[64]. Although most evidence supporting the development of oxidative stress in ALF comes from animal and cell culture studies, clinically, the antioxidant and anti-inflammatory agent *N*-acetylcysteine has proven to be beneficial in the management of patients with ALF[75-77], and agents such as mannitol and sodium benzoate, which are occasionally used in the treatment of ALF, have also been shown to have antioxidant effects[78].

Nitrosative stress is also considered to play an important role in ammonia-induced neurotoxicity. Data from experimental models of HE revealed increased nitric oxide synthase (NOS) gene expression and activity in the brain[79,80]. Inhibition of NOS by nitroarginine significantly reduced deaths in mice exposed to ammonia neurotoxicity[81]. In line with these findings, nitric oxide (NO), was shown to have increased in brains of portacaval-shunted rats given continuous ammonia infusions[82]. This animal model is a well-standardised paradigm of cerebral oedema which occurs in the absence of ALF.

Free radicals such as NO and superoxide can be categorised into reactive nitrogen and oxygen species (RNOS), respectively. In cultured astrocytes and in rat brain *in vivo*, ammonia triggers their formation through *N*-methyl-*D*-aspartate (NMDA)-receptor and calcium (Ca^{2+})-dependent mechanisms[71,83-86]. Activation of the NMDA receptor is thought to result from the depolarisation-induced removal of the magnesium blockade, which can be induced by ammonia and swelling of the cell itself. Ammonia induces glutamate release from cultured astrocytes[87] and NMDA receptor activity can be further amplified by subsequent Ca^{2+}-dependent astroglial glutamate release and autocrine NMDA receptor stimulation[88]. There is a close relationship between oxidative stress and astrocyte swelling which makes it difficult to separate them temporally as both events are causally interlinked[85,89,90]. This suggests a self-amplifying cycle[91] whereby on the one hand, astrocyte swelling induces oxidative/nitrosative stress through NMDA receptor and Ca^{2+}-dependent mechanisms, and on the other, NMDA receptor activation and oxidative stress trigger astrocyte swelling.

Exactly how ammonia-induced free radicals lead to cell swelling and cerebral oedema is not known. One possibility is that they cause direct damage to proteins and lipids in the membranes of cells and organelles such as mitochondria, thereby altering membrane permeability by affecting ion transport systems. In mitochondria, oxidative injury could lead to altered bioenergetics. Controlled ion transport systems and energy production are essential in maintaining normal cell volume, and alterations in their activity could lead to disturbed volume regulation.

One critical consequence of oxidative and nitrosative stress is induction of the mitochondrial permeability transition[92]. The MPT usually develops in response to an increase in mitochondrial Ca^{2+} levels and results in a sudden opening of the permeability transition pore (PTP), a large non-selective permeability pore in the inner mitochondrial membrane. This leads to increased permeability of the inner mitochondrial membrane to protons, ions and other small solutes. As a result, the inner mitochondrial membrane potential dissipates causing mitochondrial dysfunction. The MPT is therefore associated with movement of metabolites across the inner mitochondrial membrane, swelling of the mitochondrial matrix, defective oxidative phosphorylation and adenosine triphosphate (ATP) production, and generation of free radicals[93]. Production of free radicals through MPT induction further aggravates the MPT, resulting in a vicious cycle. Induction of the MPT was described in cultured astrocytes exposed to ammonia[60]. The mechanism underlying MPT induction most likely involves oxidative stress, as antioxidants including superoxide dismutase, catalase and vitamin E were able to inhibit the development of the MPT by ammonia[94].

Cyclosporine A (CsA) blocks ammonia-induced astrocyte swelling in culture during the evolution of swelling[95]. Nevertheless, the mechanism(s) by which the MPT mediates astrocyte swelling in hyperammonemia remains unclear. Interestingly, glutamine is capable of inducing the MPT in cultured astrocytes[55] as well as causing mitochondrial swelling in isolated rat cerebral mitochondria[96]. It is notable that, like ammonia, glutamine has been shown to induce oxidative stress by forming free radicals[97]. How glutamine acts to induce oxidative stress, the MPT and consequent astrocyte swelling, is less clear however although it has been suggested that glutamine mediates its deleterious effects through ammonia. Glutamine is hydrolysed in the mitochondria by PAG to yield high levels of ammonia which leads to oxidative stress and the MPT. In support of this concept is the finding that inhibition of PAG by 6-diazo-5-oxo-*L*-norleucine (DON) blocks free radical production[97], MPT formation[98] as well as ammonia-induced astrocyte swelling[64]. In a further study, *L*-histidine, an inhibitor of mitochondrial glutamine transport, was further used to study the role of mitochondrial glutamine in a rat model of ALF[99]. *L*-histidine was found to inhibit HO-1 overexpression, the MPT and brain oedema, supporting the involvement

of glutamine in the development of oxidative stress. Taken together, the above data supports the key role of glutamine transport into mitochondria and subsequent metabolism to ammonia in the pathogenesis of cerebral oedema in ALF. Furthermore, these findings support the "Trojan horse" theory, which suggests that glutamine acts as a "stealth" carrier of ammonia in ammonia-induced neurotoxicity.

In terms of a timeline, it was shown that exposure of cultured rat astrocytes and mice brain slices to ammonia results in rapid ROS formation and astrocyte swelling[89,90], whereas MPT-induction and glutamine accumulation occurs later[60,64] implying astrocyte swelling occurs primarily through oxidative/nitrosative stress and is then further aggravated by glutamine accumulation in astrocytes[100].

Cell volume regulation is an energy-dependent process and involves ion homeostasis through ionic transporters and exchangers and extrusion of osmotically active amino acids[101]. In particular, the Na/K/Cl cotransporter-1 (NKCC1) was found to be implicated in astrocyte swelling. NKCC1 expression and activity was increased in cultured astrocytes exposed to ammonia and its activation appears to be mediated by oxidative/nitrosative stress[102]. Energy failure following MPT induction is another possible mechanism underlying cell swelling. Ammonia is thought to interfere with mitochondrial energy metabolism and several studies have reported depletion of ATP *in vitro* and *in vivo* models of ammonia neurotoxicity[103]. The implications of energy failure in ALF have largely been ignored despite the presence of higher lactate levels in patients with ALF, which is a consequence of energy failure[104,105]. Indeed, Zwingmann *et al*[104] in an experimental ALF rodent model showed that in the early (pre-coma) stages of encephalopathy there was a significant 2 to 4.5-fold increase in total brain glutamine and lactate but in the severe (coma) stages of encephalopathy and brain oedema there was a further significant increase in brain lactate but no such increase in glutamine suggesting that impaired glucose oxidative pathways rather than intracellular glutamine accumulation *per se* may play a more dominant role[101]. This is supported by data by Bernal *et al*[105] that unequivocally shows lactate to be an important prognostic marker in ALF[102] and data from Rose *et al*[106] in a pig model of ALF which demonstrated using cerebral microdialysis that ALF animals had increased levels of lactate dehydrogenase activity and mitochondrial complex IV activity.

Mitogen-activated protein kinases (MAPKs) are activated by oxidative/nitrosative stress in cultured astrocytes exposed to ammonia and inhibition of MAPK phosphorylation abrogates astrocyte swelling[107]. Activation of MAPKs may therefore play an important protective role in cell volume regulation through phosphorylation of key proteins.

Water flow across cell membranes in astrocytes is largely dependent on aquaporin 4 (AQP4)[108]. Upregulation of AQP4 has been found to precede cell swelling in cultured astrocytes treated with ammonia and CsA can inhibit this upregulation, indicating that MPT induction is a key step in AQP4 upregulation in ammonia-induced astrocyte swelling[109]. Although it has been suggested that AQP4 is important in initiating signalling events associated with cerebral oedema[110], Wright *et al*[111] in a rat model of ALF could not find any association of the expression of AQP4 with the development of brain oedema, hyperammonemia or sepsis. The exact role of AQP4 in ALF therefore remains hotly debated.

In recent years, ammonia-induced and swelling-induced oxidative/nitrosative stress has been shown to result in multiple functional consequences. In addition to protein phosphorylation, oxidative/nitrosative stress can trigger protein tyrosine nitration, RNA oxidation and altered zinc metabolism, which can lead to changes in gene expression, intracellular signalling and synaptic plasticity[112]. Furthermore, nitration of glutamine synthetase inactivates the enzyme[113], which suggests this regulatory mechanism leads to reduced glutamine production and therefore astrocyte swelling (Figure 2).

VASOGENIC OEDEMA AND BLOOD-BRAIN BARRIER DYSFUNCTION

The BBB plays a critical role in establishing and maintaining homeostasis of the brain. It exerts tight control over any exchange of metabolites between the circulating blood and the central nervous system. The BBB consists of brain capillary endothelial cells, pericytes and the enveloping end foot processes of astrocytes. Together they form a neurovascular unit capable of regulating the special composition of the CNS fluid[114]. The main structural constituent of the BBB, and the first to come into direct contact with potentially toxic substances, is the endothelial cell. By spreading itself to cover the entire luminal surface of the capillary and sealing its two surface edges with junctional complexes known as tight junctions (TJ), the endothelial cell forms a physical barrier. These tight junctions consist of transmembrane proteins, including junctional adhesion molecules (JAM), occludin, claudins and intracellular proteins [zona occludin (ZO)-1, -2, and -3] linked to the cytoskeleton which control the stability and functioning of the TJ. Together with adherens junctions located in the basal region below the TJ, they prevent circulating compounds from freely entering the brain parenchyma and limit paracellular diffusion of small molecules. Transport of larger molecules into the brain occurs in a transcellular fashion utilising specific transport systems within endothelial cells. Water is able to diffuse through the bilayer of endothelial cell plasma membranes but can also enter the brain through water channels known as aquaporins, the predominant one in the brain being AQP4[108].

Recent MRI studies of patients with ALF demonstrate evidence of interstitial brain oedema as well as cytotoxic oedema, implying there may be a vasogenic component to the cerebral oedema in ALF[115,116]. In an animal model of ALF, astrocyte swelling, extravascular

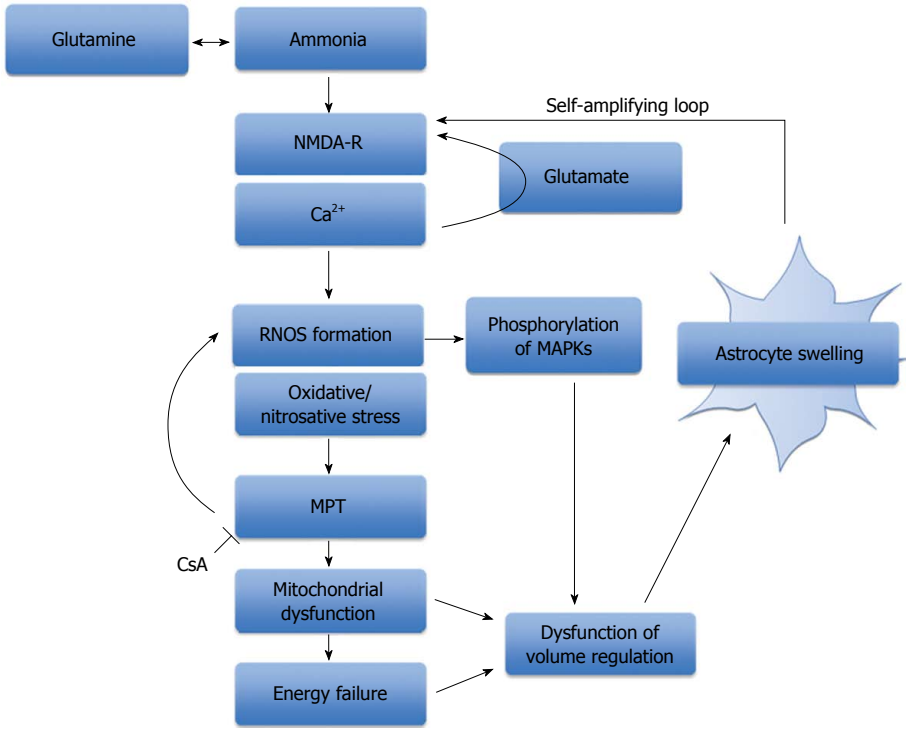

Figure 2 The role of oxidative stress, mitochondrial permeability transition and energy failure in ammonia-induced neurotoxicity. A schematic representation of the central role that ammonia plays in the production of oxidative/nitrosative stress and astrocyte swelling. Ammonia-induced astrocyte swelling is mediated by oxidative and nitrosative stress resulting in the induction of the MPT, activation of intracellular signaling kinases and alterations in gene expression. Mitochondrial dysfunction and energy failure culminates in astrocytes failing to regulate their cell volume, thereby resulting in astrocyte swelling. NMDA-R: *N*-methyl-*D*-aspartate-receptor; RNOS: Reactive nitrogen and oxygen species; MPT: Mitochondrial permeability transition; MAPKs: Mitogen-activated protein kinases; CsA: Cyclosporine A.

and interstitial oedema have been described. However, brain capillary endothelial cells and their tight junctions appeared intact[30,117]. Similar findings were also reported in patients who died of ALF[32]. Apart from an increase in cytoplasmic vesicles, suggesting altered transcellular transport across the BBB, no gross structural damage was found in capillary endothelial cells. Similarly, Nguyen[118] has described physically intact tight junctions in ALF, but these were lengthened and tortuous in shape. Thus, electron microscopic examination of the BBB reveals only minimal ultrastructural changes in the brain capillaries of animals and humans with ALF.

Nevertheless, subtle increases in BBB transport of amino acids and energy metabolites have been widely described in the context of hyperammonemia[119]. Changes in BBB penetration of ammonia itself have also been reported in hyperammonemic states. However, the results of these reports, which used PET with ^{13}N-labeled ammonia to study BBB passage of ammonia, are inconsistent[47,120,121]. Nevertheless, in animal models of ALF, ammonia uptake into the brain is thought to increase[122]. Investigating possible changes in BBB permeability to ammonia has been hampered by the recent discovery that ammonia may be able to cross the BBB *via* two possible routes, and it is not known which of the two may be affected in hyperammoneamic states. Circulating ammonia is largely present as a cation (NH_4^+) and transport across the BBB was originally considered to occur *via* diffusion in its gaseous form (NH_3), the amount of which is rather small at physiological pH levels[45]. In ALF, due to the acidosis caused by lactic acid, the amount of NH_3 and hence its diffusion across the BBB would be expected to be reduced still further. The electric charge of the ionic form was thought to prevent ammonia transport across the BBB, but now an alternative, transcellular route, through potassium channels and transporters, has been suggested[123]. This transcellular transport of ammonia may be affected in ALF, resulting in increased ammonia concentrations within the CNS. Pathological increases in BBB permeability could also result in gaseous ammonia entering the brain *via* a paracellular route.

Although there has been little evidence for a complete BBB breakdown, findings from more recent studies suggest vasogenic oedema may still contribute to the development of cerebral oedema in ALF. Nguyen and colleagues used Evans blue dye, which binds to albumin and is normally unable to penetrate the BBB, and injected it into the circulation of mice with azoxymethane-induced ALF to assess brain extravasation. They found that BBB permeability to Evans blue dye and water was significantly increased in mice with experimentally-induced ALF. Under electron microscopy, they noted that the leakage of Evans blue dye, *i.e.*, extravasation, occurred mostly in the surrounding region of the brain capillaries. Consistent with previous findings, the BBB and tight junctions were found to be structurally intact[124]. Furthermore, they were able to demonstrate that BBB permeability and brain water was reduced in ALF mice given monoclonal

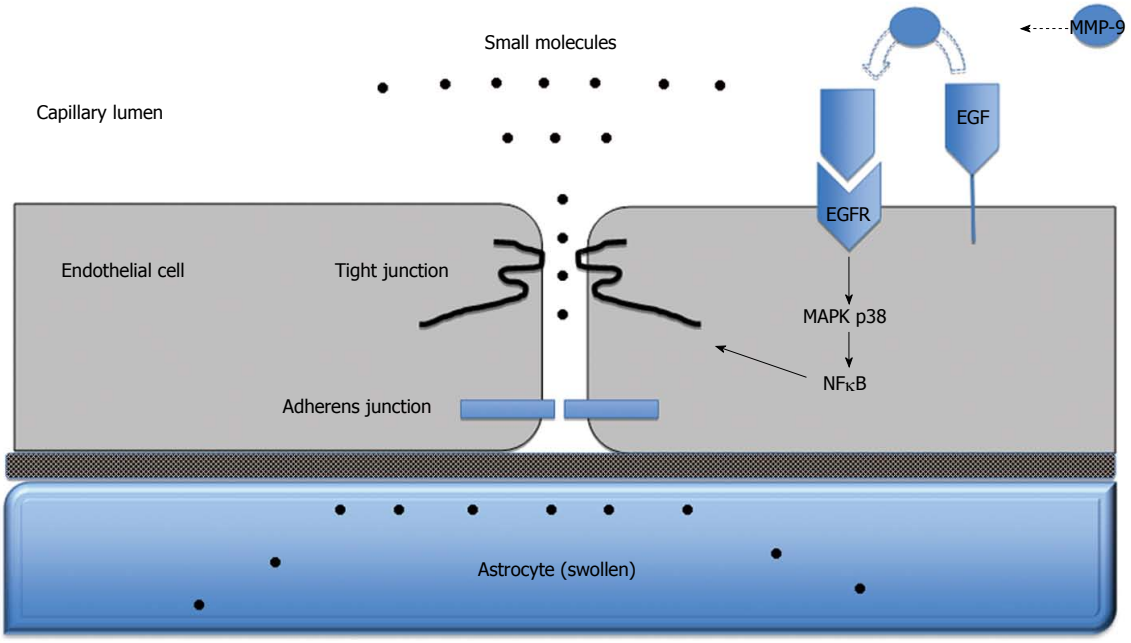

Figure 3 Blood-brain barrier dysfunction in acute liver failure. Anatomy of the blood-brain barrier (BBB) created by the brain capillary endothelial cell and its paracellular tight junction and adherens junction. In acute liver failure, activation of epidermal growth factor receptor (EGFR) and other signaling pathways results in a loss of BBB tight junction integrity. Tight junctional proteins are altered, resulting in increased permeability to small molecules, leading to astrocyte swelling. MMP-9: Matrix metalloproteinase-9; MAPK p38: Mitogen activated protein kinase p38; NFκB: Nuclear factor-κB.

antibodies specific for active matrix metalloproteinase-9 (MMP-9), a member of the matrix metalloproteinase (MMP) family of endopeptidase enzymes that degrade the extracellular matrix in normal and disease states. MMP-9 in particular, causes protein degradation of tight junctions and is upregulated in the liver of ALF mice. Increased blood concentrations of MMP-9 can also be found. These findings collectively show increased BBB permeation to water and plasma constituents in experimental ALF mice and suggest that BBB dysfunction is associated with protein deregulation in tight junctions but not necessarily with a structural breakdown. Circulating MMP-9 derived from the necrotic liver contributes to fine perturbation in BBB integrity and increased brain extravasation in mice with azoxymethane-induced ALF and inhibition of MMP-9 may be useful in preventing the development of brain oedema. Chen *et al*[125] further demonstrated that MMP-9 induces significant degradation of the TJ proteins occludin and claudin-5 in brain endothelial cells *in vitro* and in mice with azoxymethane-induced ALF; these alterations in TJ proteins correlated with increased BBB permeability and were reversed by inhibiting MMP-9. Chen *et al*[126] went on to demonstrate that MMP-9 induces activation of the epidermal growth factor receptor (EGFR) and p38 mitogen activated protein kinase/nuclear factor-κB (MAPK/NFκB) in brain endothelial cells. Activation of this pathway in turn leads to degradation of the TJ protein occludin and deregulation of the TJ. Taken together, these findings suggest that substances derived from the injured liver, such as MMP-9, reach the BBB and induce increased permeability through subtle changes in TJ composition (Figure 3). An important role for a vasogenic mechanism in the development of cerebral oedema in ALF is thus supported by these studies.

Interestingly, activation of the p38 MAPK pathway as a result of oxidative/nitrosative stress is also thought to mediate ammonia-induced astrocyte swelling[107]. The p38 MAPK pathway and subsequent phosphorylation of key proteins appears to play an important role in the pathophysiology of cell swelling[127,128] and thus cerebral oedema, and therefore, this pathway may be a potential therapeutic target.

In recent years, there has been some controversy as to whether ALF *per se* causes the changes seen within the BBB integrity or whether these changes are due to secondary complications associated with ALF such as infection and sepsis[129]. Consistent with this viewpoint is the evidence that neurosteroid biosynthesis is increased in the brains of rats with ALF[130] and that these neurosteroids protect against BBB breakdown induced by ammonia[131]. Jayakumar *et al*[132] have also reported neuroprotective effects of neurosteroids in some models of ALF but not in all suggesting that there may be differences in outcomes depending on which hepatotoxin-induced ALF model is used and that this may explain the inconsistent reports on BBB breakdown in ALF.

TREATMENT OF CEREBRAL OEDEMA IN ACUTE LIVER FAILURE

Management principles

In the absence of overt HE patients in the early stages of ALF may be observed and managed conservatively.

However such patients are susceptible to extrahepatic manifestations including the development of multiorgan dysfunction, acute kidney injury and infections[21] both of which can accelerate the development of advanced HE and brain oedema[8]. Frequent clinical and neurological examinations, concentrating on pupil size, coma grade, evidence of delirium and reflexes, are imperative to detect features which may herald the development of brain oedema. The development of grade 3/4 coma, indicative of impending raised intracranial pressure (ICP), typically necessitates intubation and ventilation[133]. ICH should be suspected in patients with sudden onset systemic hypertension, changes in pupillary reactivity, abnormal oculovestibular reflexes or decerebrate posturing. ICH becomes problematic when the ICP is above 20 mmHg due to the risk of compromising cerebral perfusion pressure. Ultimately severe ICH can result in brain stem compression causing ischaemia, haemorrhage and death[134].

Transcranial doppler ultrasonography is a non-invasive device which can continuously measure middle cerebral artery blood flow velocity, producing a velocity-time waveform that indirectly monitors changes in cerebral hemodynamics, including ICP avoiding the complications associated with more invasive monitoring devices which include haemorrhage and infection. In a small retrospective study of 16 patients with ALF four features in the waveform were found to capture the cerebral hemodynamic state and potentially can be used to predict dynamic changes in ICP or CPP. This included the slope of the Windkessel upstroke, the slope of the Windkessel downstroke, the slope of the diastolic downstroke, and the angle between the end systolic downstroke and start diastolic upstroke[135]. ICP monitoring, involving intracranial bolt insertion, is used in patients who are at high risk for the development of ICH. ICP monitoring is indicated in a subset of patients with grade 3/4 coma[136] (Glasgow Coma Scale < 8) who also display a combination of the following features; fever and tachycardia, arterial ammonia > 150 μmol/L, hyponatraemia, seizures or pupillary abnormalities, acute/hyperacute liver failure, vasopressor requirement, are less than age 40 or have jugular venous oxygen saturations or have middle cerebral artery doppler monitoring indicative of a very high or very low cerebral blood flow[8]. Additionally reverse jugular vein oxygen saturation should also be monitored, which gives an indication of cerebral oxygenation and metabolism which is often reduced as a result of the loss of CBF autoregulation in patients with ALF[15]. In terms of imaging the brain for evidence of cerebral oedema, computed tomography is only of benefit if cerebral herniation or intracranial bleeding is suspected and has no role in the routine surveillance. Electroencephalography is very useful for the detection of subclinical seizures and to measure brain activity in comatose patients, but due to its lack of specificity it is not employed routinely to diagnose encephalopathy or cerebral oedema[8].

Metabolic changes contributing to the development of raised ICP in ALF can be monitored utilizing *in vivo* cerebral microdialysis and have been documented in research settings in human ALF but this technique is currently only reserved for experimental studies and is not used in routine clinical settings[137].

Specific therapies

The treatment of cerebral oedema is aimed at preventing infection, reducing or controlling inflammation, ensuring sufficient sedation and correcting hypo-osmolality. The objective of ICH management is to maintain the ICP at less than 20 mmHg and to keep the cerebral perfusion pressure over 70 mmHg although this can be very difficult to practically achieve and the evidence base in human ALF to support such strategies is very limited. Patients are nursed in the 20°-30° head-up position favouring venous drainage to reduce ICP whilst maintaining cerebral perfusion pressure. Hypoxaemia should be avoided with target arterial oxygenation of above 95%. Patients with grade 3 encephalopathy and above should be intubated and ventilated. Propofol and other short acting sedatives are commonly utilised to ease mechanical ventilation and reduce seizure risk. Opiates, such as fentanyl, are often used for analgesia[133]. Most patients are normoventilated but hyperventilation is employed in those displaying signs indicative of imminent cerebral herniation, such as pupillary dilatation and extensor posturing. Hyperventilation results in reduced ICP by inducing hypocapnia which causes precapillary vasoconstriction decreasing CBF[17].

Patients should be adequately fluid resuscitated. Plasma volume expansion results in a significant reduction in plasma ammonia concentration by increasing urinary ammonia excretion[138]. Hypertension can reduce cerebral perfusion pressure by increasing intracranial blood volume and is best avoided; sedation can help to combat this. Arterial hypotension, especially in the presence of reduced cerebral blood flow autoregulation, will also compromise cerebral perfusion pressure. Theoretically, diastolic blood pressure should be kept > 40 mmHg higher than the ICP in patients with severe cerebral oedema and ICH who have ICP bolt monitoring in situ to guarantee adequate CBF but again this is often hard to achieve in practice[139]. Vasopressors, commonly noradrenaline, may be necessary to maintain this.

Hyponatraemia should be corrected. Background hypertonic saline (30%) infusions are used to induce and maintain serum sodium levels between 145 and 150 mmol/L thus maintaining the BBB osmotic pressure gradient[140]. Hypertonic saline acts as a dehydrating agent reducing brain water content and subsequently lowers ICP. Mannitol may also be used for the same purpose but it may be more rational to use hypertonic saline instead of mannitol as the BBB has a reflection coefficient of 1 for sodium chloride *vs* 0.9 for mannitol making it more efficient to exclude saline from the brain. It is also recommended that serum osmolarity be maintained at < 320 mOsm/L. Boluses of hypertonic saline or mannitol are used for sustained increases in ICP (> 25 mmHg) but resistant rises in ICP may be treated with indomethacin[141]

or hypothermia[142]. Indomethacin (a non-selective cyclooxygenase inhibitor) induces cerebral vasoconstriction by inhibiting the endothelial cyclooxygenase pathway, reducing cerebral temperature and modifying extracellular pH. However, it has a number of adverse effects, including nephrotoxicity, platelet dysfunction and gastrointestinal bleeding, and therefore its use in ALF patients is limited to when all other management options to reduce ICP have been exhausted[8]. Moderate hypothermia (32-34 ℃) may be useful in patients with resistant ICH awaiting liver transplantation by decreasing brain ammonia uptake and also through its role in reducing brain cytokine production, OS and CBF[143]. Barbiturates are postulated to reduce brain metabolism and consequently lead to a decrease in cerebral blood volume. Thiopental infusion has been shown to be efficacious in 14 patients with ALF as measured by extradural transducers with minimal side effects although additional data in the context of human ALF is scarce[144]. However, due to their hepatic metabolism and negative inotropic effects they are only used to reduce ICP surges as a last resort[8].

ALF has many similarities to septic shock[145] and there is evidence that patients exhibiting a systemic inflammatory response progress more rapidly to severe encephalopathy[21]. Broad spectrum intravenous antibiotics and antifungals are therefore used empirically to reduce the risk of sepsis and development of severe encephalopathy.

Intravenous *N*-acetylcysteine (NAC) is now considered as standard of care in the treatment of acetaminophen-induced and non-acetaminophen induced ALF as it acts as both as an antioxidant and anti-inflammatory agent. Early administration of intravenous NAC after an overdose of acetaminophen replenishes glutathione stores and helps to alleviate hepatic necrosis[146]. NAC also has beneficial hemodynamic effects and has been shown to improve cerebral perfusion pressure[147] mediated by enhanced activity of the nitric oxide soluble cyclic GMP system[76].

Ultimately emergency liver transplantation reverses cerebral oedema, although a variety of neurological manifestations including intracerebral haemorrhage and seizures, precipitated by cerebral hypoperfusion, coagulopathy and transfusion may still occur post-operatively. If the graft is functioning well ICH is expected to resolve 48 h post-transplant[148,149].

Bernal *et al*[7] reviewed 3305 patients with acute liver dysfunction from 1973-2008 and found a significant reduction in the proportion of patients with ICH (from 76% in 1984-1988 to 20% in 2004-2008 ($P < 0.0001$)). Furthermore, mortality of patients with ICH decreased from 95% to 55% ($P < 0.0001$). The cause for this improvement is likely to be multifactorial. Patients now present and are diagnosed earlier and the prompt use of *N*-acetylcysteine, fluid resuscitation, empirical antibiotics and renal replacement therapy may have reduced the incidence of cerebral oedema and ICH by modulating principal contributory factors. Such approaches may also limit hepatotoxicity, reduce plasma ammonia levels and prevent sepsis. The more timely use of emergency liver transplantation for those at greater risk may also have contributed to the reduction in ICH.

NOVEL THERAPIES IN DEVELOPMENT

Minocycline

Jiang *et al*[74] studied the use of minocycline, a broad-spectrum tetracycline antibiotic which has been shown to attenuate lipopolysaccharide-induced neuroinflammation[150], in an experimental model of ALF[24]. They were able to demonstrate that it delayed the progression of HE and brain oedema by exerting a potent inhibitory action on microglial activation independently of its antimicrobial properties.

NMDA receptor antagonists

NMDA receptor antagonists have been shown to prevent the oxidative stress induced by acute ammonia intoxication[83]. This is likely to be the case as the production of ROS is mediated by NMDA-receptor activation in hyperammonemic states[151]. Memantine is a non-competitive NMDA-receptor antagonist and has been shown to improve EEG activity, clinical grading, ICP and brain water content in portocaval shunted rats infused with ammonia, and in rats with ALF induced by ischaemia which was independent of ammonia concentration[152].

Endotoxin removal

An albumin replacement system with a novel endotoxin ligation (ARSeNEL) function has been developed at University College London and tested in an ALF pig model. Early data have reported an improvement in survival, endotoxemia and ICP index which warrant further studies in clinical settings[153].

Novel anti-inflammatory agents

It make sense that if agents were used to reduce the systemic inflammation that is frequently seen in ALF and which sensitises the brain to the effects of ammonia, then we might be able to prevent the development of cerebral oedema. Unfortunately however, the proinflammatory response which develops in the wake of acute liver injury is also key in initiating liver repair and regeneration. One would postulate therefore that it may be detrimental to use agents and antibodies which target prominent pro-inflammatory mediators. Neutrophil malfunction, akin to that seen in septic shock is a consistent finding in patients with ALF and recent data support an intimate relationship with hyperammonemia[127,145]. Strategies that target innate and adaptive immune dysfunction in ALF including TLR expression and production of ROS would certainly be of therapeutic interest and warrant further study. Granulocyte colony stimulating factor (GCSF) has been shown in 2 small studies to improve neutrophil phagocytic capability in patients with ALF[154,155] and as such may have utility in the prevention of advanced HE.

Plasmapheresis

Larsen *et al*[156] have previously shown that high volume plasmapheresis can alleviate brain oedema in some patients with ALF with favourable changes in systemic hemodynamics despite increasing cerebral blood flow. Plasmapheresis may also have a positive impact on alleviating the systemic immune dysfunction and endothelial dysfunction that commonly develops. This was the stimulus for performing a randomised clinical trial of high volume plasmapheresis of which the preliminary analysed data suggests that it may improve survival in patients unsuitable for liver transplantation (verbal communication-Dr Finn Stolze Larsen).

CONCLUSION

This detailed review has unequivocally presented the evidence to support the critical role of the neurotoxin ammonia in the development of astrocyte swelling and cytotoxic oedema in ALF. Although a generalised breakdown of the BBB cannot be demonstrated in patients with ALF, more recent studies have described a "leaky" BBB resulting from subtle changes in the integrity of the tight junctions, supporting a role of a vasogenic component in the pathophysiology of cerebral oedema in ALF.

Exactly how both cytotoxic and vasogenic mechanisms interact to bring about cerebral oedema in ALF, and the extent of their involvement, remains unknown. Moreover, the sequence of events is unclear. Is BBB dysfunction the result of a cytotoxic insult or is cytotoxic oedema a consequence of increased BBB permeability? It has been postulated that increased BBB permeability to small molecules such as water and ammonia arises as a result of BBB dysfunction as an initial event in the pathophysiology of brain oedema in ALF. Increased BBB permeability may then invoke vasogenic oedema. The subsequent development of ammonia neurotoxicity and cytotoxic oedema may then occur as a downstream manifestation. This sequence of events is supported by the observation that in rats with ALF an early increase in BBB permeability correlates with increased ICP and results in vasogenic oedema followed by a progressive increase in brain ammonia and glutamine levels[157]. However, it is difficult to determine to what extent these data definitively support a vasogenic mechanism in ALF. For example, the potency of mannitol in treating ICH in the context of cerebral oedema in patients with ALF supports the BBB being intact and the predominant mechanism being a cytotoxic one. Another possibility however, is that certain brain areas may behave respond differently to others. For example, Cauli *et al*[157] were able to show in a rodent experimental ALF model that the mechanism and time course of the appearance of brain oedema differed between 12 different brain regions with the cerebellum showing predominantly vasogenic oedema whilst the frontal cortex exhibited cytotoxic oedema.

The syndrome of ALF arises in the context of various aetiological toxic insults to the liver and is frequently associated with the development of multiple organ dysfunction and sepsis. It is not known how these manifestations independently impact on BBB integrity and function. Furthermore, the liver toxins utilised in the various animal ALF models could directly affect the BBB independently of ALF. Changes to the BBB in ALF are very different in nature to that seen in brain ischemia or traumatic brain injury, where complete BBB breakdown is commonly observed. The mechanisms underpinning cerebral oedema in ALF are therefore also different and therapeutic interventions that are beneficial in other types of brain injury may not be useful in the treatment of ALF.

It is clear that the development of more effective therapies in ALF will require further knowledge of the pathophysiology of cerebral oedema, which is a devastating and frequently fatal feature of ALF. Greater knowledge of the sequence of events and key mediators involved in the development of brain oedema will allow for specific targets to be identified.

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REVIEW

Therapeutic potential of curcumin in digestive diseases

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Abstract

Curcumin is a low-molecular-weight hydrophobic polyphenol that is extracted from turmeric, which possesses a wide range of biological properties including anti-inflammatory, anti-oxidant, anti-proliferative and anti-microbial activities. Despite its diverse targets and substantial safety, clinical applications of this molecule for digestive disorders have been largely limited to case series or small clinical trials. The poor bioavailability of curcumin is likely the major hurdle for its more widespread use in humans. However, complexation of curcumin into phytosomes has recently helped to bypass this problem, as it has been demonstrated that this new lecithin formulation enables increased absorption to a level 29-fold higher than that of traditional curcuminoid products. This allows us to achieve much greater tissue substance delivery using significantly lower doses of curcumin than have been used in past clinical studies. As curcumin has already been shown to provide good therapeutic results in some small studies of both inflammatory and neoplastic bowel disorders, it is reasonable to anticipate an even greater efficacy with the advent of this new technology, which remarkably improves its bioavailability. These features are very promising and may represent a novel and effective therapeutic approach to both functional and organic digestive diseases.

Key words: Curcumin; Curcumin-phythosome; Curcumin bioavailability; Digestive disorders

Core tip: Curcumin is a well-established molecule with multiple pharmacological activities, mainly anti-inflammatory and anti-proliferative. The major hurdle for a widespread clinical use has been represented by its poor bioavailability, which has been recently overcome by the development of a new formulation combining curcumin with phospholipids (curcumin-phytosome). This compound permits to improve markedly intestinal absorption of curcumin and guarantees a greater tissue delivery than the traditional curcuminoid mixtures. So, curcumin-phythosome has the potential to be exploited in many gastrointestinal diseases, both functional and organic.

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INTRODUCTION

In recent years, we have witnessed a shortage of certain types of drugs synthesized from chemical laboratories and a growing interest in therapeutic substances derived from natural plants. Curcumin represents one of these compounds, and this nutraceutical has already undergone many experimental and clinical studies to assess its use in the treatment of various human diseases.

This polyphenol has been shown to possess anti-inflammatory, anti-oxidant, immuno-modulatory, wound-healing, anti-proliferative and antimicrobial activities. These diverse properties, together with the fact that curcumin is innocuous, inexpensive and easily available,

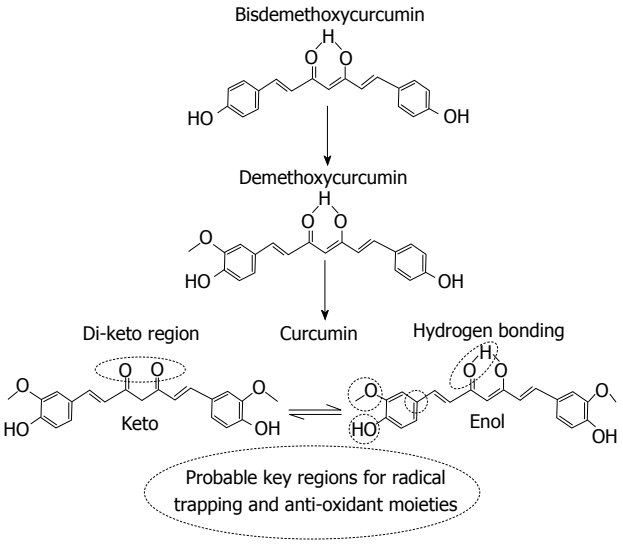

Figure 1 Proposed molecular pathway for the conversion of bisdemethoxycurcumin to demethoxycurcumin and finally to curcumin and the co-existence of keto and enol isomers of curcumin.

have sparked interest in its therapeutic application for several digestive disorders. Moreover, recent progress in the formulation of curcumin complexes with other substances, in particular with phospholipids, has remarkably increased the bioavailability of this compound, leading to greater absorption and a higher concentration in human tissues. This allows us to use lower dosages of curcumin than have been used in the past, which greatly reduces the number of tablets taken during the day while maintaining no adverse side effects.

Finally, distribution studies of curcumin in human tissues have shown that it preferentially accumulates in the intestine, colon and liver. This finding might be one major reason for the anticipation and observation of its most promising *in vivo* effects in gastrointestinal diseases when compared with other organ systems.

This review presents current knowledge of the physical and molecular properties of curcumin, its pharmacokinetics and metabolism, its mechanism of action and results of the few published clinical trials, as well as the potential therapeutic perspectives in patients with various digestive disorders.

Literature searches were performed in PubMed, Ovid, EMBASE and the Cochrane Library databases in accordance with published recommendations. We critically analyzed all full-text papers and reviews written in the English language and searched them using the terms curcumin, turmeric, colorectal cancer (CRC), inflammatory bowel diseases (IBD), functional digestive disorders, irritable bowel syndrome and liver diseases. Both animal and human studies were reviewed.

PHYSICAL AND MOLECULAR PROPERTIES OF CURCUMIN

Turmeric (the common name for *Curcuma longa*) is an Indian spice derived from the rhizomes of the plant and has a long history of use in Ayurvedic medicine as a treatment for inflammatory conditions[1].

The primary active constituent of turmeric, which is responsible for its vibrant yellow color, is curcumin, which was first identified in 1910 by Lampe and Milobedzka[2]. Curcumin exists as a bright yellow powder that provides the pigmentation of turmeric, which is used in the dye industry. Turmeric is composed of volatile oils (tumerone, atlantone, and zingiberone), sugars, proteins, resins and a group of the following three curcuminoids: about 75% curcumin (diferuloylmethane), about 16% demethoxycurcumin (DMC), about 8% bisdemethoxycurcumin (bDMC). DMC and bDMC possess similar molecular and biological properties. It is proposed that within natural pathways (Figure 1), bDMC is converted to DMC, which is then converted to curcumin[3].

Curcumin (or diferuloylmethane) is a poly-phenolic molecule that exhibits keto-enol tautomerism and has a predominant keto form in acidic and neutral solutions and a stable enol form in alkaline medium[4]. The molecule is lipophilic and consists of two aromatic rings connected by two unsaturated carbonyl groups; therefore, it has poor solubility in water. The molecule is stabilized by hydrogen-bonding associated with the central OH group. This may be one of the important functional sites that is responsible for the array of molecular biological activities[5]. Curcumin is photosensitive, and precautions should be taken to avoid exposure and subsequent degradation.

PHARMACOKINETICS AND METABOLISM OF CURCUMIN

Absorption and systemic bioavailability

Over the past three decades, animal studies have shown that curcumin is hydrolytically unstable at intestinal pH, rapidly metabolized, conjugated in the liver, and excreted in the feces. Therefore, it has limited systemic bioavailability. The effects of reduced bioavailability of any agent within the body are low intrinsic activity, poor absorption, high rate of metabolism, inactivity of metabolic products and/or rapid elimination and clearance from the body. In this section, problems of limited curcumin bioavailability such as low serum levels, limited tissue distribution, apparent rapid metabolism and short half-life are described in detail.

Serum concentration

One of the major observations from curcumin studies is very low serum levels. The first reported study to examine the uptake, distribution, and excretion of curcumin was by Wahlstrom and Blennow[6] in 1978 using Sprague-Dawley rats. Negligible amounts of curcumin in the blood plasma of rats after oral administration of 1 g/kg of curcumin showed that this molecule was poorly absorbed from the gut.

In 1980, Ravindranath *et al*[7] showed that after oral administration of 400 mg of curcumin in rats, no curcumin

was found in the heart blood, whereas a trace amount (less than 5 μg/mL) was found in the portal blood from 15 min to 24 h after curcumin administration.

When curcumin was given orally at a dose of 2 g/kg in rats, a maximum serum concentration of 1.35 ± 0.23 μg/mL was observed after 0.83 h, whereas in humans, the same dose of curcumin resulted in either undetectable or extremely low (0.006 ± 0.005 μg/mL at 1 h) serum levels[8].

A phase I clinical trial[9] conducted among 25 patients with various precancerous lesions demonstrated that oral doses of 4, 6 and 8 g of curcumin administered daily for three months yielded serum curcumin concentrations of only 0.51 ± 0.11, 0.63 ± 0.06, and 1.77 ± 1.87 μm, respectively. This finding indicates that curcumin is poorly absorbed and may have limited systemic bioavailability. Serum levels peaked between one and two hours after administration and declined rapidly thereafter. This study did not identify curcumin metabolites, and urinary excretion of curcumin was undetectable.

Another phase I trial[10] involving 15 patients with advanced colorectal cancer administered curcumin at doses between 0.45 and 3.6 g daily for four months. In three of six patients who were given the 3.6 g dose, the mean plasma curcumin measured after one hour on day 1 was 11.1 ± 0.6 nmol/L. This measurement remained relatively consistent at all-time points measured during the first month of curcumin therapy. The molecule was not detected in the plasma of patients taking lower doses.

A very recent study by Yang *et al*[11] showed that 10 mg/kg of curcumin given *iv* in rats yielded a maximum serum curcumin level of 0.36 ± 0.05 μg/mL, whereas a 50-fold higher curcumin dose administered orally yielded a maximum serum level of only 0.06 ± 0.01 μg/mL.

These studies clearly suggest that the route of administration affects achievable serum levels of curcumin, and they further indicate that the serum levels of this compound in rats and in humans are not directly comparable.

Tissue distribution

The uptake and distribution of curcumin in body tissues are obviously important factors determining its biological activity, yet a limited number of studies have addressed this issue.

Ravindranath *et al*[7] showed that after oral administration of 400 mg of curcumin in rats, only traces of the unchanged molecule were found in the liver and kidney. At 30 min, 90% of the curcumin was found in the stomach and small intestine, but only 1% was present at 24 h.

Another study of the same group evaluated the tissue distribution of curcumin using a tritium-labeled molecule[12]. They found that radioactivity was detectable in the blood, liver, and kidney following doses of 40080, or 10 mg of (3H) curcumin. With 400 mg, considerable amounts of the radio-labeled products were present in tissues 12 d after dosing. The percentage of curcumin absorbed (60%-66% of the given dose) remained constant regardless of the dose, indicating that increased administration of the drug does not result in greater absorption.

Similarly, the concentrations of curcumin in normal and malignant colorectal tissue of patients receiving 3600 mg of the compound were 12.7 ± 5.7 and 7.7 ± 1.8 nmol/g, respectively, and these doses had pharmacological activity in the colorectum as measured by their effects on levels of M(1)G and cyclooxygenase-2 (COX-2) protein[13]. Another study by the same authors showed no curcumin in the liver tissue of patients with hepatic metastases from colorectal cancer who received 450-3600 mg of curcumin daily for 1 wk prior to surgery[14].

Metabolites

Various studies have evaluated the metabolism of curcumin in rodents and in humans. Once absorbed, curcumin is subjected to conjugations such as sulfation and glucuronidation at various tissue sites. The very first biodistribution study reported the metabolism of the major part of curcumin orally administered in rats[6]. The liver was indicated as the major organ responsible for metabolism of this drug[15].

Holder *et al*[16] reported that the major biliary metabolites of curcumin in rats are glucuronides of tetrahydrocurcumin (THC) and hexahydrocurcumin. A minor biliary metabolite was dihydroferulic acid together with traces of ferulic acid. In addition to glucuronides, sulfate conjugates were found in the urine of curcumin-treated mice[13].

Asai *et al*[17] evaluated the absorption and metabolism of orally administered curcumin in rats. The enzymatic hydrolysis of plasma samples showed that the predominant metabolites in plasma following oral administration were glucuronides/sulfates of curcumin. The plasma concentrations of conjugated curcuminoids reached a maximum at 1 h after administration. The presence of conjugative enzyme activities for glucuronidation and sulfation of curcumin in the liver, kidney and intestinal mucosa suggests that orally administered curcumin is absorbed from the alimentary tract and is present in the general blood circulation after largely being metabolized to form glucuronide/sulfate conjugates.

Whether curcumin metabolites are as active as curcumin itself is not clear[18-20]. While most studies indicate that curcumin glucuronides and THC are less active than curcumin itself, other studies suggest that they may actually be more active than curcumin[19-21].

Half-life

Systemic elimination or clearance of curcumin from the body is another important factor that determines its relative biological activity. Wahlstrom and Blennow[6] reported that when 1 g/kg curcumin was given orally to rats, 75% of it was excreted in the feces, and negligible amounts were found in the urine. Intravenous (*iv*) and intraperitoneal (*ip*) administration of curcumin resulted in biliary excretion of the molecule from cannulated rats.

A clinical study of 15 patients receiving oral curcumin in doses between 36 and 180 mg daily for up to 4 mo found neither curcumin nor its metabolites in urine, but the drug was recovered from feces[22]. The absorption and

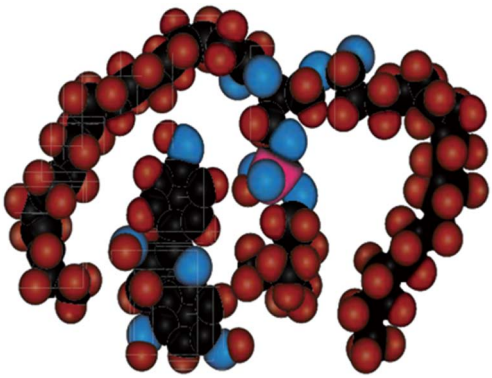

Figure 2 Phytosome molecular complex.

elimination half-lives of orally administered curcumin (2 g/kg) in rats were reported to be 0.31 ± 0.07 and 1.7 ± 0.5 h, respectively. However, in humans, the same dose of curcumin did not allow the calculation of these half-life values because the serum curcumin levels were below the detection limit at the majority of time points in most of the experimental subjects.

The existing evidence in the literature is not sufficient to make conclusions about the factors controlling the *in vivo* elimination half-life of curcumin, and future studies are warranted to address this issue.

METHODS TO OVERCOME THE TRADITIONAL LOW BIOAVAILABILITY OF CURCUMIN

Because of the above-mentioned poor bioavailability, which limits the therapeutic usefulness of curcumin, many attempts have been made to improve oral absorption of the compound[23]. Among them, the complexation of curcumin with phospholipids using so-called phytosome technology has emerged as one of the most documented approaches from a preclinical and clinical standpoint.

Phytosome technology was developed in 1989 (Figure 2). Water-soluble phytosomes can be converted into a lipid-compatible molecular complex. Phytosomes are more available than uncomplexed products due to their enhanced capacity to cross the lipid biomembranes and to reach the systemic circulation[24].

It is inferred that, at the intestinal level, the water-miscible phosphatidylcholine (PC) molecules enhance the dispersion of the poorly water-soluble polyphenol molecules into the water-soluble environment of the gastrointestinal lumen. PC further enhances transfer from the lumen into the lipid-soluble environment of the outer cell membrane of the epithelial absorptive cells (enterocytes). The enterocyte outer membrane has a lipid molecular bilayer that consists largely of PC. It is feasible that the PC in the phytosome merges into this PC domain of the enterocyte membrane, and by carrying the polyphenol with it, the PC "ushers" the polyphenol into the cell.

The bioavailability of the curcumin phytosome (CP)

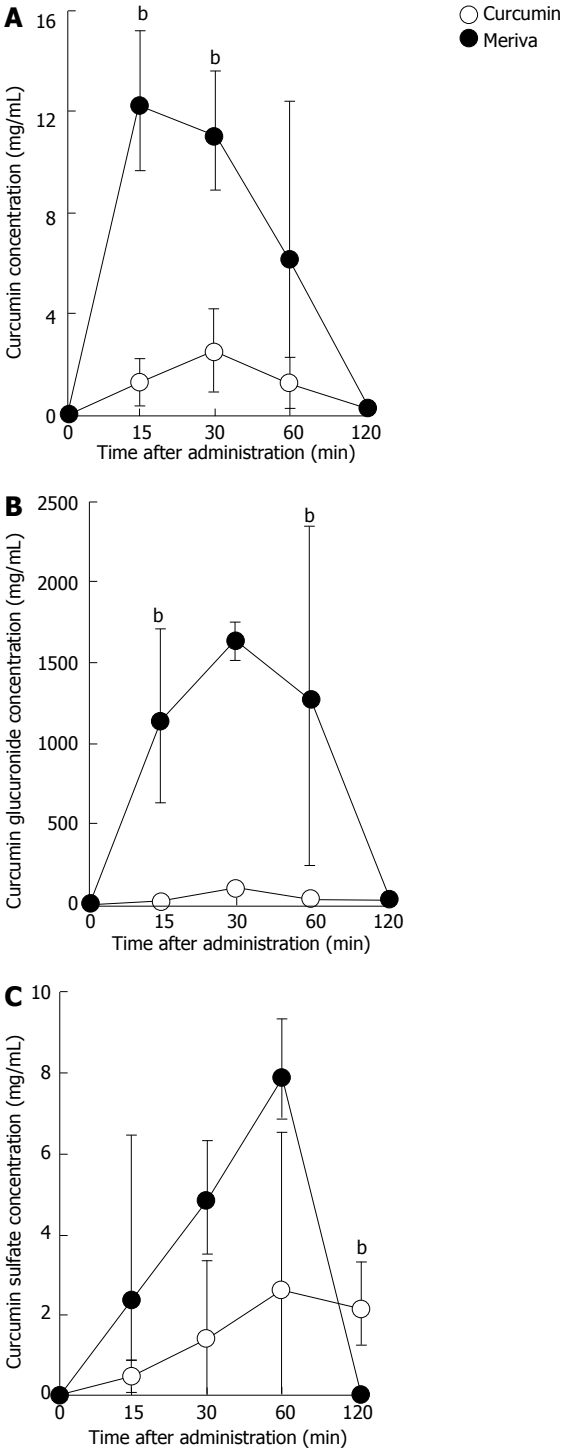

Figure 3 Plasma curcumin I in rats from curcumin phytosome or non-complexed curcumin. A: Curcumin concentration; B: Curcumin glucuronide concentration; C: Curcumin sulfate concentration. $^{b}P < 0.01$ *vs* curcumin phytosome.

preparation (Meriva®, Indena Spa, Milan, Italy) has been tested against an equivalent non-phytosome curcumin extract by Marczylo *et al*[25]. These authors administered equivalent dosages (340 mg of curcumin) of curcumin or curcumin phytosome preparation to rats and reported a dramatic increase in the bioavailability among the animals that received the curcumin phytosome preparation (Figure 3). Peak plasma levels of curcumin were approximately

Table 1 Pharmacokinetics parameters in healthy volunteers after administration of curcuminphytosomes or unformulated curcumin

Curcuminoid	Formulation	AUC (ng/mL)	C_{max} (ng/mL)	t_{max} (h)	Relative absorption[2]
Curcumin (1a)	Curcuminphytosome high	538.0 ± 130.7	50.3 ± 12.7	3.8 ± 0.6	19.2[1]
	Curcuminphytosome low	272.6 ± 68.52	24.2 ± 5.9	4.2 ± 0.8	17.5[3]
	Reference	122.5 ± 29.3	9.0 ± 2.8	6.9 ± 2.2	1
Demethoxycurcumin (1b)	Curcuminphytosome high	655.0 ± 195.7	134.6 ± 40.6	2.4 ± 0.3	68.3[4]
	Curcuminphytosome low	297.4 ± 107.3	39.1 ± 11.4	3.1 ± 0.4	55.5[4]
	Reference	55.8 ± 15.5	4.2 ± 1.1	4.4 ± 1.0	1
Bisdemethoxycurcumin (1c)	Curcuminphytosome high	142.2 ± 58.2	24.9 ± 8.1	2.2 ± 0.4	56.8[5]
	Curcuminphytosome low	70.1 ± 34.3	8.8 ± 3.1	2.4 ± 0.6	53.1[5]
	Reference	24.6 ± 10.3	2.1 ± 0.8	3.4 ± 1.2	1
Total curcuminoids	Curcuminphytosome high	1336.0 ± 357.1	206.9 ± 54.9	2.7 ± 0.3	31.5[6]
	Curcuminphytosome low	640.2 ± 197.7	68.9 ± 16.9	3.3 ± 0.3	27.2[6]
	Reference	202.8 ± 53.8	14.4 ± 4.2	6.9 ± 2.2	1

[1]Actual results not baseline subtracted, and errors are standard error of the mean ± SE; [2]Area under the curve (AUC) normalized; [3]Average: 18.3; [4]Average: 61.9; [5]Average: 54.1; [6]Average: 29.14.

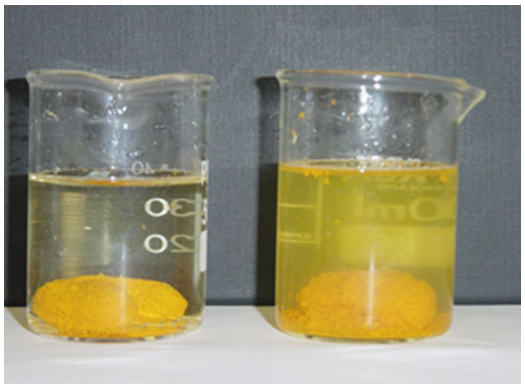

Figure 4 Image of Norflo® tablet in water after few seconds.

5-fold higher for CP than for traditional curcumin. Plasma levels of curcumin sulfate and curcumin glucuronide observed after the administration of CP were 3- to 20-fold higher, respectively, than those observed after the administration of uncomplexed curcumin. In the same study, significant amounts of curcumin were also measured at the tissue level and were found to have particular relevance for the liver and intestine.

More recently, Cuomo *et al*[26] reported the results of a comparative pharmacokinetic study of healthy volunteers. In this randomized, double-blind, cross-over study, subjects received curcumin and the CP formulation at 2 dosage levels (209 and 376 total curcuminoids). The average dose-related absorption of curcumin following the 2 doses of CP was approximately 18-fold higher than the absorption of the reference curcumin. Moreover, the absorption of total curcuminoids was approximately 29-fold higher for CP in comparison with the unformulated reference, as the plasma concentration of demethoxycurcumin and bis-demethoxycurcumin from the former compound was approximately 50- to 60-fold higher than the concentration from the unformulated curcumin (Table 1).

CP is a powder that contains 20% curcumin, 40% microcrystalline cellulose and 40% phospholipids. It is utilized as an active ingredient in several food supplements in different markets. The product is available in various formulations including hard gel capsules and tablets. In Italy, for example, the product has been developed as 500-mg tablets that combine CP with some dissolving substances (Curcusol) under the name Norflo® (Eyepharm, Genoa, Italy). These tablets dissolve very rapidly in the first part of the intestine, favoring the formation of an emulsion with bile acids (Figure 4), which permits almost complete absorption of phospholipids (unpublished data). The use of this formulation overcomes the risk that undissolved tablets may pass through the entire intestine and be eliminated in the feces either intact or only partially dissolved.

CP has been widely documented in several health settings, but few studies have focused on gastrointestinal disorders, which, nevertheless, seem to be a very promising therapeutic area. From this perspective, a colon-targeted delivery preparation could further optimize the clinical effects.

TOXICITY AND TOLERABILITY OF CURCUMIN

Curcumin has been reported to be safe in many human studies, and only minimal toxicity has been associated with this polyphenol[27]. In a dose escalation study among 34 healthy volunteers, in whom the doses of curcumin ranged from 500 to 12000 mg, safety was assessed after 72 h. Only 7 subjects complained of disturbances, which were mild and included headache, skin rash, diarrhea and yellow stool[9]. In another investigation lasting for 1-4 mo, escalating doses of curcumin from 0.45 to 3.6 g/d found rare instances of nausea and diarrhea, as well as an increase in alkaline phosphatase and LDH[10]. Some patients treated with doses as high as 8 g/d for 2 wk reported abdominal pain and complained about the bulky volume of the tablets[28]. As curcumin is particularly concentrated in the human liver, the risk of hepatotoxicity has been closely evaluated, but liver function tests have been shown to be unaffected with doses as high as 2-4 g/d[29]. As one of the most documented bioavailable curcumin formulations, the CP formulation has been widely employed in the clinical setting with a daily dosage rang-

ing between 1 and 2 g, and this preparation has shown good tolerability and compliance, even in medium-term trials. However, we must stress that studies of more than 6 mo of treatment are lacking, and it is not possible to draw any firm conclusions regarding the long-term safety profile of this compound.

MECHANISMS OF ACTION AGAINST INFLAMMATORY AND NEOPLASTIC CONDITIONS

Anti-inflammatory mechanisms

Curcumin is a highly pleiotropic molecule capable of interacting with numerous molecular targets involved in inflammation. It has been proposed that this compound modulates the inflammatory response by the following mechanisms[30,31]: (1) Down-regulation of COX-2, lipoxygenase, and inducible nitric oxide synthase (iNOS) enzymes; (2) Inhibition of the inflammatory cytokines, tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1, -2, -6, -8, and -12, monocyte chemoattractant protein, and migration inhibitory protein; and (3) Down-regulation of mitogen-activated and Janus kinases.

COX-2 inhibition and iNOS inhibition are likely achieved *via* curcumin suppression of nuclear factor kappa B (NF-κB) activation. NF-κB is a ubiquitous eukaryotic transcription factor involved in the regulation of inflammation, cellular proliferation, transformation, and tumorigenesis[32]. NF-κB is not a single gene but rather a family of interrelated transcription factors that include the following five genes: NF-κB1 (p50/p105), NF-κB2 (p52/p100), RelA (p65), c-Rel, and RelB[33]. The member proteins form homo- or heterodimers, of which the p50/p65 heterodimer is the most abundant and is responsible for the majority of NF-κB canonical transcriptional activity. Generally, NF-κB dimers associate with an inhibitory-κB (IκB-α) protein that keeps the dimer in the cytoplasm in an inactive state.

NF-κB activation begins with the activation of an IκB kinase (IKK) complex that consists of catalytic subunits IKK-α and IKK-β and the scaffolding subunit IKK-γ (the NF-κB essential modifier)[34]. Several mitogen-activated protein (MAP) kinases that also include NF-κB-inducing kinase (NIK) activate IKK through the phosphorylation of IKK-α and IKK-β. IKK-β has higher activity than IKK-α for IκB-α and is considered important in the canonical pathway.

In the canonical pathway, as shown in Figure 4, phosphorylation of I-kappa B kinase (IκB) kinase α/β by mitogen-activated protein kinase (MAPK) is followed by phosphorylation of IκB-α, which occurs in an inactive complex with p50/p65. Phosphorylated IκB-α is released and degraded in the cytoplasm. The active heterodimer of p50/p65 enters the nucleus to regulate expression of multiple genes[35].

Curcumin is thought to suppress NF-κB activation and proinflammatory gene expression by blocking phosphorylation of inhibitory factor IκB. Suppression of NF-κB activation subsequently down-regulates COX-2 and iNOS expression, thus inhibiting the inflammatory process and tumorigenesis[33]. In an animal model of inflammation, curcumin also inhibited arachidonic acid metabolism and inflammation in mouse skin epidermis *via* down-regulation of the cyclooxygenase and lipoxygenase pathways[36].

In vitro studies indicate that curcumin inhibition of inflammatory cytokines is achieved through suppression of cytokine gene expression and down-regulation of intercellular signaling proteins, such as protein kinase C[36].

Curcumin anticancer effects

There has been some promising research concerning curcumin as a safe therapeutic agent for many cancers, including colorectal cancer. This has been shown through various studies in cell cultures, animal models, and humans[2,37].

Carcinogenesis is a complex process mainly consisting of the following three phases: initiation, promotion, and progression[38]. There is suggestive evidence that inflammation may play a role in the three phases of carcinogenesis[39]. Cancer initiation is produced by oxidative stress and chronic inflammation[2]. Inflammation acts as a key regulator in the promotion of these initiated cells, possibly by providing them with proliferating signals and by preventing apoptosis[40]. The role of inflammation in tumor induction and subsequent malignant progression has also been investigated[41]. An inflammatory response produces cytokines, which act as growth and/or angiogenic factors, leading transformed cells to proliferate and undergo promotion. Leukocytes produce cytokines and angiogenic factors as well as matrix-degrading proteases that allow the tumor cells to proliferate, invade, and metastasize. Tumor-infiltrating lymphocytes secrete matrix-degrading proteinases such as matrix metallo-peptidase 9 (MMP-9) and thus promote neoplastic proliferation, angiogenesis, and invasion[42].

These details demonstrate the role of inflammation in all three stages of carcinogenesis. Substantial evidence for the role of inflammation in cancer is provided by the frequent up-regulation of inflammatory mediators such as NF-κB. The pathways activated by NF-κB up-regulators are implicated not only in tumor growth and progression but also in the development of cancer cell resistance to anti-cancer drugs, radiation and death cytokines. NF-κB is an excellent target for anti-cancer therapy[43].

Effects on tumor initiation by curcumin

Curcumin has demonstrated a significant reduction in the levels of iNOS, which produces oxidative stress, which is itself one of the main causes of tumor initiation. Curcumin inhibits the induction of nitric oxide synthase and is a potent scavenger of free radicals such as nitric oxide[44].

NF-κB has been implicated in the induction of iNOS. Curcumin prevents phosphorylation and degradation

of inhibitor κB-α and thereby blocks NF-κB activation, which down-regulates iNOS gene transcription[45]. Curcumin was found to inhibit cell proliferation and cytokine production by inhibiting NF-κB target genes involved in this mitogen induction of T-cell proliferation, interleukin IL-2 production and nitric oxide generation. The overexpression of cytokines, such as IL-10, IL-6, and IL-18, is accompanied by NF-κB induction that is controlled and inhibited by curcumin[46]. Curcumin has been shown to increase expression of conjugation enzymes (phase Ⅱ), which suppress ROS-mediated NF-κB, activator protein 1 (AP-1) and MAPK activation[47].

Tumor proliferation and progression suppression by curcumin

We have already mentioned that NF-κB has an important role in cancer initiation, promotion and progression. In addition to suppressing various cell survival and cell proliferative genes, including Bcl-2, cyclin D1, IL-6, COX-2, and MMP-9, curcumin induces apoptosis, as shown by caspase activation and poly (ADP-ribose) polymerase-cleavage[48-50].

Curcumin is also able to block NF-κB signaling and inhibit IKK activation. The suppression of cell survival and cell proliferation genes, including Bcl-2, cyclin D1, IL-6, COX-2 and MMP, has also been noted[48,49]. It has been suggested that COX-2 induction is mediated by the NF-κB intracellular signaling pathway, and overexpression of COX-2 leads to malignant cell proliferation and invasion[51,52]. Curcumin inhibits COX-2 expression by repressing degradation of the inhibitory unit inhibitor κB-α and hindering the nuclear translocation of the functionally active subunit of NF-κB, thereby blocking improper NF-κB activation[34].

Curcumin has been found to reduce the invasion and subsequent metastasis of cancer cells. It suppresses MMP expression, which is believed to play a major role in mediating neovascularization and is increased during tumor progression[53].

Curcumin down-regulates MMP-9 expression by inhibiting NF-κB and AP-1 binding to the DNA promoter region. MMP-9 is one of the two determinants of neovascularization that help to form new capillaries from preexisting blood vessels[54].

Curcumin has been noted to cause significant inhibition of tumor necrosis factor α-induced VCAM-1 expression, which is related to the activation of the MAPK NF-κB pathway[55,56]. Curcumin has been shown to reduce cell migration and invasion induced by osteopontin, an extracellular matrix protein, through the NF-κB pathway[57].

Curcumin may inhibit cancer cell growth through down-regulation of IL-1- and IL-8-induced receptor internalization. It controls cancer progression by either blocking tumor growth or inhibiting its invasive and aggressive potential. In both cases, most of the effects are exerted by curcumin-induced NF-κB inhibition[57].

However, curcumin has been found to arrest the cell cycle and to induce apoptotic cell death through inhibition of the Janus family of kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway[58].

The JAK and STAT comprise an important signaling pathway involved in dysregulation of cell growth, invasion, angiogenesis, metastasis and resistance to apoptosis[59,60]. The JAK-STAT system consists of the following three main components: (1) A receptor; (2) JAK; and (3) STAT.

The receptor is activated by a signal from interferon, IL-6, growth factors, or other chemical messengers[61]. This activates the kinase function of the JAKs (JAK1, JAK2, and JAK3), which autophosphorylation (phosphate groups act as "on" and "off" switches on proteins). The STAT protein then binds to the phosphorylated receptor, where STAT is phosphorylated by JAK. The phosphorylated STAT protein binds to another phosphorylated STAT protein (dimerizes) and translocates into the cell nucleus. In the nucleus, it binds to DNA and promotes transcription of genes responsive to STAT[62-63]. Studies have evaluated the regulators of cytokine signaling including protein tyrosine phosphatases (PTPases) such as Src homology 2 (SH2) domain-containing PTPases (SHP)-1 and SHP-2. Potential roles for SHP-1 and SHP-2 have been investigated for their use in the control of cytokine signaling through the dephosphorylation of JAKs and their receptors[64].

Of the seven STAT proteins identified thus far, only activated STAT3 and STAT5 have been implicated in multiple myeloma, lymphomas, leukemias and several solid tumors[65]. Aberrant STAT3 signaling is an important process in the development and progression of cancer; thus, agents that block its activation have therapeutic potential. Rajasingh *et al*[66] have demonstrated that *in vitro*, treatment with curcumin induced a dose-dependent decrease in JAK and STAT phosphorylation, resulting in the induction of growth-arrest and apoptosis in T cell leukemia. Curcumin reversibly inhibits STAT3 activation in human multiple myeloma cells and, by this mechanism, suppresses IL-6-induced cell proliferation[67-68]. It also inhibits STAT3 activation in five different human Hodgkin and Reed-Sternberg lymphoma cell lines[69].

It has been shown that curcumin inhibits lysophosphatidic acid-induced IL-6 and IL-8 secretion and STAT3 phosphorylation in ovarian cancer cells[70], and curcumin has also been shown to have a significant effect upon CRC by blocking STAT3-driven cancer cell growth[69]. In summary, the anti-inflammatory and anticancer effects of curcumin are listed in Table 2.

CLINICAL TRIALS EXPLORING THE THERAPEUTIC POTENTIAL OF CURCUMIN IN GASTROINTESTINAL DISEASES

Because of its higher bioavailability in the gastrointestinal

Table 2 Curcumin's anti-inflammatory and anticancer effects

Anti-inflammatory effects	Anticancer effects
Downregulation of NF-κB,	Inhibition of carcinogen activation
Inhibition, *via* NF-κB, of COX-2, lipoxygenase, and iNOS enzymes	Stimulation of carcinogen detoxification
Inhibition of the inflammatory cytokines, such as TNF-α, interleukin (IL)-1, -2, -6, -8, and -12, MCP, and migration inhibitory protein	Suppression of pro-inflammatory signaling
Inhibition of PPAR-g	Inhibition of STAT
	Induction of cancer cell apoptosis cell cycle arrest
	Inhibition of angiogenesis and metastasis
	Modulation of oncogenes and tumor suppressor genes

TNF-α: Tumor necrosis factor-α; MCP: Monocyte chemoattractant protein; PPAR-g: Peroxisome proliferator-activated receptor-g; iNOS: Inducible nitric oxide synthase; STAT: Signal transducer and activator of transcription; NF-κB: Nuclear factor kappa B; COX-2: Cyclooxygenase-2.

tract than in other organs, the therapeutic potential of curcumin has been investigated in several studies of digestive diseases including IBD, CRC and hepatic fibrosis.

Inflammatory bowel disease

Idiopathic IBD comprises the following two types of chronic intestinal disorders: Crohn's disease (CD) and ulcerative colitis (UC)[71-73]. Accumulating evidence suggests that IBD results from an inappropriate inflammatory response to intestinal microbes in a genetically susceptible host[74]. Pathogen recognition by innate immune cells is coupled to the secretion of cytokines that inform the adaptive immune system about the nature of the pathogen and instruct naïve T cells to differentiate into the appropriate T cell subtypes required to clear the infection[75]. Thus, naïve T cells are induced to differentiate into Th1, Th2, Th17 and/or regulatory T cells (Treg) depending on the pathogen eliciting the response[76]. Recent studies reveal that IL-6/IL-12 family cytokines (IL-6, IL-12, IL-23, IL-27 and IL-35) play pivotal roles in these lymphocyte cell-fate decisions, and their influence on the T cell developmental program is mediated primarily through activation of an evolutionarily conserved family of latent cytoplasmic transcription factors called STATs[77,78].

The progressive damage to the gut is characterized by an aberrant inflammatory response to components of the bacterial microflora, and Th17 cells are thought to contribute to the destruction of gut tissues by inducing secretion of the extracellular matrix-degrading enzymes MIP-3α and IL-21. Autocrine secretion of IL-21, which perpetuates a cycle of elevated IL-21 secretion, and sustained STAT3 activation in the gut play important roles in exacerbating the disease[79]. In addition, pSTAT3 enhances survival of the pathogenic Th17 cells by up-regulating *Bcl-2*, *Bcl-xL*, and *Mcl-1* genes[80] and may thereby contribute to maintaining the chronic inflammatory process.

Very recently, the role of NF-κB in IBD has been elucidated[73]. Colon biopsies in IBD patients with active disease showed increased levels of NF-κB p65 protein, a member of the NF-κB family of proteins. The amount of NF-κB p65 in the tissue samples correlated with the severity of intestinal inflammation. This increased expression of NF-κB results in an increased ability to secrete inflammatory cytokines, such as TNF-α, IL-1, IL-6, IL-12, and IL-23, the latter of which are directly responsible for mucosal damage in IBD. TNF-α is also able to up-regulate the production of NF-κB, which results in a cyclical feedback loop of inflammation[81]. Additionally, the findings that the degree of gut tissue inflammation correlates with the level of pSTAT3 in histological sections of IBD patients support a role of STAT3 and Th17 cells in IBD[82].

Anti-inflammatory drugs, immunosuppressants, and TNF blockers are used to manage IBD. However, the high cost and adverse effects associated with these drugs encourage the use of alternative management options[83].

Because curcumin plays a key role in the inhibition of both the activation of NF-κB pro-inflammatory cytokines and the IL-6/STAT3 signaling pathway, it could be proposed as a novel therapeutic agent in several inflammatory diseases, such as IBD[84]. However, to date, there have been only two human studies of curcumin in patients with IBD that have achieved encouraging results. Holt *et al*[85] conducted a small, open-label, pilot study of curcumin in five patients with ulcerative colitis/proctitis and five patients with Crohn's disease. Patients with ulcerative proctitis, who were currently using 5-aminosalicylic acid (5-ASA) compounds and corticosteroids (four of five patients were on corticosteroids + 5-ASA compounds), were given 550 mg curcumin twice daily for one month and then 550 mg three times daily for the second month. Patients with CD were treated with 360 mg curcumin three times daily for 1 mo followed by 360 mg four times daily for another 2 mo. All patients were assessed at baseline and after two months of curcumin administration *via* hematological, biochemical, and inflammatory analysis (C-reactive protein and erythrocyte sedimentation rate) and by sigmoidoscopy and biopsy. Subjective analysis was performed *via* a self-reported symptom diary. In the ulcerative proctitis group, all five patients had significant improvement with reductions in concomitant medications in 4 patients. Although only four of five CD patients completed the study, they also improved, as evidenced by a lowered Crohn's Disease Activity Index. There was a mean reduction of 55 points and a mean reduction in the sedimentation rate of 10 mm/h. Based on the symptom diary ($P < 0.02$), all patients improved from baseline after two months of ther-

apy, and the inflammatory markers decreased to normal limits.

Subsequently, Hanai *et al*[86] evaluated the use of curcumin in 89 patients with quiescent UC in a randomized, double-blind, multicenter trial. After a four-week washout period, subjects were randomly assigned to a six-month regimen of either placebo (n = 44) or curcumin. The treatments consisted of 1000 mg after breakfast and 1000 mg after dinner (n = 45) in combination with sulfasalazine (SZ) (1-3 g/ d; median 2 g/d) or mesalamine (1.5-3 g/d; median 2.25 g/d).

Patients were followed during treatment and for six months after the treatment ended; they received only SZ or mesalamine during the six-month follow-up period. Of 43 patients (2 patients violated the protocol) who received curcumin, 2 relapsed during the 6 mo of therapy (4.65%), compared to 8 of 39 patients (20.51%) in the placebo group (P = 0.040).

Recurrence rates evaluated on the basis of intention to treat showed a significant difference between curcumin and placebo (P = 0.049). Furthermore, curcumin improved both the clinical activity index (CAI) (P = 0.038) and the endoscopic index (EI) (P = 0.0001), measures that are used to evaluate the morbidity associated with UC. The authors drew the following three major conclusions: (1) Curcumin had better clinical efficacy over placebo in the prevention of relapse; (2) Curcumin significantly improved the CAI and EI; and (3) Curcumin was well-tolerated.

Based on these two studies, curcumin seems to be a promising and safe therapy for maintaining remission in patients with quiescent UC as well as for improving symptoms in patients with proctitis and CD. It is evident that further rigorous randomized controlled trials in larger samples of IBD patients are needed to validate the results of the above clinical studies. Considering its effect on multiple inflammatory pathways, curcumin also has the potential to be used as a steroid-sparing induction agent in mild to moderate colitis or as an adjunct to maintain remission in patients who are losing response to immunomodulators.

Colorectal cancer

Currently, it appears that the anti-carcinogenic properties of curcumin are most likely due to its effects on multiple molecular targets, such as NF-κB factor and AP-1. These are both major transcription factors that regulate inflammation and thus affect cell proliferation, differentiation and even apoptosis.

We have already mentioned that curcumin has been shown to affect a variety of other key players involved in carcinogenesis, such as cyclooxygenase-2, matrix metallopeptidases 2 and 9 and tumor necrosis factor α-induced vascular cell adhesion molecule.

Sharma *et al*[10] conducted two separate clinical trials exploring the effect of curcumin on malignancies and tumor marker levels. In the first pilot study, the pharmacokinetics and pharmacodynamics of a standardized Curcuma extract in capsule form (Phytopharm, United Kingdom) at doses ranging from 440 to 2200 mg/d, corresponding to 36-180 mg of curcumin, were evaluated. Fifteen patients with advanced CRC refractory to standard chemotherapies received Curcuma extract daily for up to 4 mo. In one patient, measurement of a serum tumor marker revealed a decrease in carcinoembryonic antigen levels from 310 ± 15 to 175 ± 9 μg/L after two months of treatment with 440 mg Curcuma extract. Stable disease *via* computed tomography scan was observed in five of 15 patients. Oral Curcuma extract was well-tolerated, and dose-limiting toxicity was not observed.

In the second dose-escalation study[10], 15 patients with advanced CRC refractory to standard chemotherapies consumed capsules compatible with curcumin doses of between 0.45 and 3.6 g/d for up to 4 mo. Levels of curcumin and its metabolites in plasma, urine, and feces were analyzed. Blood and imaging tests were performed at baseline and at various points throughout the trial. A daily dose of 3.6 g of curcumin caused decreases of 62% and 57% in inducible prostaglandin E2 (PGE2) production in blood samples taken 1 h after the dose was administered on days 1 and 29, respectively. PGE2 is an end product of cyclooxygenase that has been shown to stimulate the growth of human colorectal cancer cells.

Garcea *et al*[14] studied curcumin levels in the colorectum and the pharmacodynamics of curcumin in 12 patients with confirmed CRC. The staging of patients was noted; 2 patients were Duke A, 3 patients were Duke B, and 7 patients were Duke C. Patients were assigned to 450, 1800 or 3600 mg of curcumin per day for 7 d prior to surgery. The recoveries of curcumin in normal and malignant colorectal tissues of patients receiving 3.6 g of curcumin were 12.7 ± 5.7 and 7.7 ± 1.8 nmol/g, respectively. Curcumin levels were highest in the normal tissue of the cecum and the ascending colon as opposed to the transverse colon, the splenic flexure and the descending colon, which suggests a local effect. The levels of M1G were also decreased by curcumin treatment in malignant colorectal tissue. COX-2 levels were undetectable in normal tissue but were detectable in malignant colorectal tissue. Curcumin was not found to modulate the expression of Cox-2 in malignant tissues. The study concluded that a daily dose of 3.6 g of curcumin is pharmacologically efficacious in CRC patients.

Curcumin has also demonstrated potential for the prevention and treatment of CRC in combination with other agents. Familial adenomatous polyposis (FAP) is an autosomal-dominant disorder characterized by hundreds of colorectal adenomas that eventually develop into CRC. One study[87] evaluated whether the combination of curcumin and quercetin could suppress adenomas in patients with FAP. Five patients with FAP received combinations of curcumin (480 mg) and quercetin (20 mg) orally three times a day, and the number and size of polyps were assessed at baseline and after therapy. Four patients had a retained rectum, and one had an ileoanal anastomosis. After 6 mo of combination treatment, all five patients had a

decrease in the number and size of polyps from baseline. Polyp number decreased by a mean of 60.4% ($P < 0.05$), and polyp size decreased by a mean of 50.9% ($P < 0.05$). This is the first human demonstration of the reduction in size and number of ileal and rectal polyps in patients with FAP by a curcumin-containing agent. Although the combinations seemed to reduce the adenomas, randomized controlled trials are needed to further validate these findings.

In a non-randomized, open-label clinical trial, Carroll *et al*[28] assessed the effects of oral curcumin (2 or 4 g per day for 30 d) on PGE2 within abnormal crypt foci (ACF) as the primary endpoint using 5-hydroxyeicosatetraenoic acid (5-HETE), ACF number, and proliferation in 44 eligible smokers with eight or more ACF on screening colonoscopy. They assessed pre- and post-treatment concentrations of PGE2 and 5-HETE by liquid chromatography tandem mass spectroscopy in ACF and normal-tissue biopsies; ACF number *via* rectal endoscopy; proliferation by Ki-67 immunohistochemistry; and curcumin concentrations by high-performance liquid chromatography in serum and rectal mucosal samples. Forty-one subjects completed the study. A significant 40% reduction in ACF number occurred with the 4-g dose ($P < 0.005$), whereas ACF were not reduced in the 2-g group. The ACF reduction in the 4-g group was associated with a significant, five-fold increase in post-treatment plasma curcumin/conjugate levels (*vs* pretreatment, $P = 0.009$).

In summary, the above studies suggest that curcumin is safe and has bright prospects for the treatment of patients with CRC. In fact, curcumin has been shown to be beneficial in all 3 stages of carcinogenesis and in all multifactorial illnesses such as cancer. An agent that acts at a number of different cellular levels offers the potential for effective prophylaxis and treatment. It is hoped that larger and methodologically sound clinical trials in patients with CRC will lead to the consideration of curcumin as an anticancer agent.

Liver disease

We are still remote from having available and effective drug therapies in hepatic diseases, with the exception of those with viral etiology. Especially in emerging liver diseases, such as non-alcoholic fatty liver disease (NAFLD), the only currently available therapies that have proven to be effective are those with nutritional agents such as vitamin E or those that are associated with antidiabetic drugs[88,89]. The only effective therapy for NAFLD/NASH remains non-pharmacological and involves a multidisciplinary treatment based not only on diet but also on frequent aerobic physical activity. In this scenario, curcumin appears to provide an opportunity to cure or improve liver pathologies. Curcumin has the following 4 basic effects on the hepatobiliary system[90]: (1) Choleretic-cholagogue; (2) Antifibrotic; (3) Hepatoprotective; and (4) Antioxidant.

Choleretic-cholagogue effect

Experimental studies have shown large hepatoprotective effects for curcumin against a variety of hepatotoxic endogenous (from cholestasis to fatty infiltration) and exogenous insults (alcohol to xenobiotics), a significant percentage of which may progress to cirrhosis or hepatocellular carcinoma[91,92]. In pharmacological terms, curcumin is a complete choleretic-cholagogue. The cleavage products of curcumin (feluric and hydrofeluric acids) have cholecistokinetic properties because they squeeze the gallbladder, while another principle product, paratolilmethilcarbinol, has strong choleretic activity[15].

The choleretic effect of curcumin increases bile production by approximately 62%. Its effect is not limited to the stimulation of contraction and is also expressed in the bile composition. Indeed, it has been reported that sodium curcuminate increases the excretion of bile salts, cholesterol, and conjugate bilirubin, which increases the solubility and prevents the formation of stones in the gallbladder[93].

Antifibrotic effect

Curcumin may attenuate hepatic fibrosis induced experimentally by various pathogenetic mechanisms due to its protective effect on the inhibition of tissue growth factor TGF-β[94]. In the development of liver fibrosis, this profibrinogenic cytokine plays a key role by promoting the activation of stellate cells to myofibroblasts and through the production of extracellular matrix.

TGF-β is one of the main targets of curcumin, which likely occurs through NF-κB[86]. A second target of the antifibrotic effect of curcumin is its effect on metalloproteinases, which are involved in remodeling the extracellular matrix. In an experimental model of cirrhosis, curcumin normalized some parameters (ALT, glutathione, glycogen), thus signifying a resumption of hepatic metabolism. Other parameters, such as fibrosis, were only attenuated, which is likely due to the activation of metalloproteinases by curcumin itself [95,96].

Hepatoprotective effect

Table 3 shows some examples of the hepatoprotective effects of curcumin against many hepatotoxic insults such as paracetamol, *Aspergillus aflatoxins*, or nitrosamines. The hepatoprotective effect derives mainly from its antioxidant activities, as well as its ability to reduce the formation of pro-inflammatory cytokines[97].

Given its hepatoprotective effects, curcumin can be used in cases of insult by exogenous toxins derived from both the environment and lifestyle. It should be recalled that curcumin is able to induce the synthesis of phase Ⅱ enzymes that protect cells from oxidative stress, such as glutathione transferase, heme-oxygenase and the NAPH-quinone reductase, which results in detoxification and reduced stress[98].

Antioxidant effect

Curcumin is characterized by its high antioxidant activity, which is comparable to, if not higher than, that of vitamin C and is more than ten times higher than the activity of the scavenger vitamin E[99].

Table 3 Substances and hepatic intoxication mechanisms contrasted by curcumin

Intoxications	Pathogenetic mechanisms	Curcumin effects
Iron (alcoholic liver disease; steatosis, viral hepatitis; anemia)	Fibrosis induced by oxidation	Anti-oxidant enzymatic activity
Alcohol (chronic or acute intoxication)	Phospholipase A2 activation	Phospholipase A2 inhibition by NF-κB
High-fat diet (lipid storage)	Focal degeneration, micronecrosis	Acil-CoA, cholesterol biliary acids; LDL peroxidation
Xenobiotics induced acute damage	ROS, lipid peroxidation, inflammation	Scavenger activity on NF-κB; anti-oxidant enzymatic activity
Xenobiotics induced chronic damage	Inflammation and hepatocellular necrosis	Hepatic fibrosis inhibition by NF-κB
Poisons (carbon tetrachloride)	Inflammatory self-maintenance	Hepatic inflammation inhibition by NF-κB

ROS: Reactive oxygen species; LDL: Low density lipoprotein; NF-κB: Nuclear factor kappa B.

Overall, the antioxidant action, especially towards cells subjected to increased oxidative stress such as hepatocytes, results in an increase of cellular resistance to oxidative damage for at least 18 h[99]. The antioxidant properties of curcumin reside in the same chemical structure. Numerous natural antioxidants can be classified into the following two types of compounds: phenolic (sesame extract) and β-diketonics (extracts of eucalyptus).

Curcumin is one of the few antioxidants that possess both a phenolic group and one diketonic in the same molecule. This explains why curcumin possesses the ability to interrupt the chain that transmits the oxidation of biological structures until the oxidant energy is sufficient[99].

In summary, the multiple positive effects of curcumin on both the biliary system and on liver structure and function encourage its clinical use, which needs to be validated in future controlled clinical trials.

Functional digestive disorders

The mechanisms of symptom generation in patients with functional digestive disorders are poorly understood due to the lack of a mucosal injury that enables us to explain their troublesome disturbances[100]. Recent studies have shown that transient receptor potential vanilloid type 1 (TRPV1) receptors play a critical role in somatic and visceral nociceptive neural detection and transmission[101], and they have been implicated in the induction of symptoms in these diseases. TRPV1 is a polymodal sensory transducer that can be activated by multiple noxious stimuli such as heat, low pH, and endogenous lipid derivatives such as anandamide as well as by exogenous substances that possess a vanilloid moiety such as capsaicin[102]. Of remarkable importance, the curcumin molecule has the same vanilloid ring moiety as capsaicin, making TRPV1 its likely target, and it has been shown in animals that curcumin blocks TRPV1 activation by capsaicin in a competitive manner[103]. It has been suggested that up-regulation of TRPV1 signaling may contribute to visceral hypersensitivity in functional gastrointestinal diseases, including esophageal hypersensitivity[104]. This condition can be found in more than 50% of patients with non-erosive reflux disease, which represents the most frequent form of gastro-esophageal reflux disease[105]. Recent epidemiological studies have shown that the rate of reflux patients with negative endoscopy can be as high as 75%[106]. This relevant population contains subgroups of patients with hypersensitive esophagus to both acid and non-acid reflux or patients with functional heartburn, who are difficult to treat with antisecretory therapies and who therefore may benefit from drugs that are able to act on TPRV1 receptors. In fact, curcumin has been shown to antagonize the vanilloid receptors even at low dosages and thus has the potential to modulate the response of TPRV1 to various stimulants and to prevent the generation of symptoms in patients with hypersensitive esophagus and functional heartburn[103].

Moreover, the TPRV1 receptors are widely expressed in the entire gastrointestinal tract and enteric nervous system, and there is evidence that curcumin can inhibit GI nociception and reverse gut hypersensitivity by acting on peripheral terminals. Taking into account this mechanism of action, it cannot be excluded that this molecule may be beneficial in treating patients with functional dyspepsia and irritable bowel syndrome, which are disorders that remain clinically challenging in the setting of current drugs and whose patients may benefit from the pharmacological properties of curcumin on TRPV1 as a novel pain modulator.

Finally, as it has been shown that low-grade inflammation of the intestinal mucosa is responsible for symptoms of irritable bowel syndrome[107], we cannot exclude that the well-known anti-inflammatory effects of curcumin may also improve the quality of life of patients with this disease.

CONCLUSION

In summary, curcumin is a well-known molecule with multiple pharmacological activities that have the potential to be used to treat many gastrointestinal diseases, both functional and organic. It appears to be a very promising therapeutic compound on the basis of thousands of pre-clinical studies, but its poor bioavailability has greatly hampered more widespread clinical use. However, the new formulation of curcumin with phospholipids has allowed us to overcome this problem by markedly improving intestinal absorption compared with the traditional unformulated curcuminoid mixtures. If curcumin is truly beneficial, as has been suggested by prior clinical trials using curcumin with limited bioavailability, we can expect to see greater therapeutic effectiveness from phospholipid-complexed curcumin, which enables increased absorption

and appropriate tissue delivery. These improved pharmacokinetic and pharmacodynamic properties are also able to significantly reduce the required dosages of curcumin and to increase the compliance of the product. Overall, these features make curcumin a very promising new therapeutic option for the treatment of gastrointestinal and hepatic diseases for which present therapies are largely unsatisfactory.

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Early respiratory complications after liver transplantation

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Abstract

The poor clinical conditions associated with end-stage cirrhosis, pre-existing pulmonary abnormalities, and high comorbidity rates in patients with high Model for End-Stage Liver Disease scores are all well-recognized factors that increase the risk of pulmonary complications after orthotopic liver transplantation (OLT) surgery. Many intraoperative and postoperative events, such as fluid overload, massive transfusion of blood products, hemodynamic instability, unexpected coagulation abnormalities, renal dysfunction, and serious adverse effects of reperfusion syndrome, are other factors that predispose an individual to postoperative respiratory disorders. Despite advances in surgical techniques and anesthesiological management, the lung may still suffer throughout the perioperative period from various types of injury and ventilatory impairment, with different clinical outcomes. Pulmonary complications after OLT can be classified as infectious or non-infectious. Pleural effusion, atelectasis, pulmonary edema, respiratory distress syndrome, and pneumonia may contribute considerably to early morbidity and mortality in liver transplant patients. It is of paramount importance to accurately identify lung disorders because infectious pulmonary complications warrant speedy and aggressive treatment to prevent diffuse lung injury and the risk of evolution into multisystem organ failure. This review discusses the most common perioperative factors that predispose an individual to postoperative pulmonary complications and these complications' early clinical manifestations after OLT and influence on patient outcome.

Key words: Respiratory complications; Postoperative respiratory failure; Liver transplantation; Postoperative edema; Post-transplant pneumonia

Core tip: This "minieview" underlines the most important perioperative factors that predispose to early post-liver transplant respiratory complications. Despite advances in surgical techniques and anesthesiological management the lung may still suffer throughout the perioperative period from various types of injury, with different ensuing ventilatory impairments, and different clinical outcomes. The incidence, etiology, pathophysiological features, clinical manifestations, preventing measures, and outcomes of post-operative respiratory disorders in this setting are also reported.

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INTRODUCTION

Orthotopic liver transplantation (OLT) is currently the only definitive treatment for patients with acute liver failure and end-stage liver cirrhosis. Due to recipients' generally poor preoperative clinical conditions, the extensive surgical field, and lengthy operating times, postop-

erative respiratory disorders are very common after OLT and significantly contribute to the related morbidity and mortality, both in the acute postoperative stage and in the long term.

Several factors are involved in the onset of postoperative pulmonary complications (PPCs), and many preoperative and intraoperative variables have been associated with different degrees of severity of respiratory impairment after OLT.

Although refinements in surgical techniques, antimicrobial prophylaxis, immunosuppression, anesthesia, and intensive care management have most likely altered the frequency and overall spectrum of post-OLT respiratory disorders, it is still common for pulmonary infiltrates, atelectasis, pleural exudates, and other radiological abnormalities to be documented on chest X-ray at any time during a patient's stay at an intensive care unit (ICU).

All of these respiratory disorders can affect lung compliance and alveolar gas exchange and, when severe, may necessitate tracheal intubation and mechanical ventilation. In the early stages after transplantation, pulmonary complications may prolong intubation time and increase the risk of systemic infectious complications. Prolonged mechanical ventilation due to refractory respiratory failure is an extremely morbid event, as this event is a marker of poor recipient recovery, predisposes a recipient to long-term ventilator dependency, and predicts further complications.

This review focuses on the most common perioperative factors that predispose an individual to PPCs occurring early after OLT, along with these complications' clinical manifestations and contribution to outcome. The main strategies for preventing the development of post-OLT respiratory disorders are also mentioned.

PREOPERATIVE RISK FACTORS FOR POST-OLT RESPIRATORY COMPLICATIONS

The most commonly identified risk factors for PPCs are detailed in Table 1 and relate to a recipient's age, the severity of liver dysfunction, cirrhotic encephalopathy, acute renal failure, smoking history, emphysema, high systolic pulmonary artery pressure, hypoxia, and hepatopulmonary syndrome. Pre-existing pulmonary abnormalities *per se* may also make a liver transplant recipient more vulnerable to pulmonary complications. Patients with chronic liver disease have pulmonary regional hemodynamic disturbances, with greater differences in alveolar-arterial oxygen tension, a weaker pulmonary vascular tone, and a poor hypoxic pulmonary vasoconstrictive response[1].

Levesque *et al*[2] reported that evidence of a preoperative restrictive pulmonary syndrome is one of the main risk factors for PPCs. An association between abnormal preoperative spirometry findings and a higher rate of PPCs was also mentioned by Bozbas *et al*[3].

The relationship between patients' Model for End-Stage Liver Disease (MELD) scores and the incidence of PPCs has yet to be clearly elucidated, but liver transplant recipients with high MELD scores often have a higher incidence of pleural effusion, a need for more perioperative blood transfusions, a greater risk of fluid retention, severe restrictive pulmonary patterns, and muscle atrophy related to poor nutritional status. Given this higher rate of comorbidities in patients with higher MELD scores, cases of postoperative respiratory impairment or failure may be more common as well[4,5].

Table 1 Common preoperative risk factors for post-orthotopic liver transplantation pulmonary complications

Recipient's age[2,8]
Female sex[5]
Smoking history[3]
Severity of liver dysfunction[2] (Child-Pugh class[5], MELD score[12,43])
Cirrhotic encephalopathy
Cerebral dysfunction[5]
Acute renal failure
Emphysema[3]
High systolic pulmonary artery pressure[3]
Hypoxia, orthodeoxia[3]
Hepatopulmonary syndrome
Pre-existing pulmonary abnormalities[1]:
Intrinsic cardiopulmonary disease: chronic obstructive pulmonary disease, congestive heart failure, pneumonia, asthma
Specific to liver disease: association with specific liver diseases (alpha-1 antitrypsin deficiency, primary biliary cirrhosis), fluid retention com plicating portal hypertension (ascites, hepatic hydrothorax), pulmonary vascular abnormalities (hepatopulmonary syndrome, portopul monary hypertension)
Evidence of a restrictive pulmonary syndrome[2]
Abnormal spirometry findings[3]
Preoperative ventilator support[6]
Severe preoperative respiratory failure requiring mechanical ventilation[8,9]
Higher value of INR[2]
Preexisting diabetes mellitus[6,7]
Impaired renal function[6]
Preoperative MARS use[6]
Deceased donor source of organ transplantation[6]

MELD: Model End Stage Liver Disease; INR: International normalised ratio; MARS: Molecular adsorbent re-circulating system.

In a retrospective study, Huang *et al*[6] found that preoperative ventilator support, diabetes mellitus, impaired renal function, and OLT with grafts from deceased donors were the most significant preoperative predictors of the risk of postoperative respiratory failure (PRF). John *et al*[7] demonstrated that patients suffering from diabetes mellitus prior to liver transplantation had a higher incidence of pulmonary complications afterward than did non-diabetic patients.

The main reasons why liver recipients given a graft from a deceased donor are at a higher risk of postoperative respiratory complications relate to the higher MELD scores of such recipients compared with patients receiving grafts from living donors, the "urgent" nature of the transplantation surgery, and the greater "marginality" of cadaveric grafts.

Severe preoperative respiratory failure requiring me-

Table 2 Major intraoperative and common postoperative risk factors for post-orthotopic liver transplantation pulmonary complications

Major intraoperative risk factors
Surgical procedure[2] (wide incision[19])
Intraoperative fluid transfusion volume[2,8,12]
Intraoperative blood transfusion volume[6,12]
Perioperative fluid balance[12]
Intraoperative fluid retention[14]
Intraoperative bleeding volumes[14]
Common postoperative risk factors
Excessive perioperative fluid administration[2]
Postoperative duration of mechanical ventilation[2] (delayed removal of endotracheal tube[13,19])
Acute rejection during the hospital stay[2]
Postoperative acute renal failure[5]
Postoperative hypoproteinemia
Onset of renal insufficiency
Poor postoperative myocardial function
Right hemidiaphragm paralysis[24]
Greater exposure to nosocomial agents[34]
Significant decline in the recipient's immune function[34]
Surgical complications[34]
Re-interventions or need for retransplantation[34]

chanical ventilation prior to OLT is one of the most serious events leading to the onset of PPC[8,9], as the presence of an endotracheal tube is a well-recognized factor that predisposes an individual to lower respiratory tract infectious complications[10].

INTRAOPERATIVE RISK FACTORS FOR POST-OLT RESPIRATORY COMPLICATIONS

OLT is a lengthy procedure that may cause numerous physiological changes, such as mechanical derangement of the chest wall and diaphragm, hydrostatic and oncotic pressure abnormalities, increases in pulmonary vascular resistance and pulmonary artery pressure, abnormal pulmonary vascular permeability, and variable coagulopathies (Table 2).

Although the administration of fluids and blood products is adjusted in an effort to ensure hemodynamic stability and to correct unanticipated coagulation abnormalities and bleeding, a significant loss of blood and fluids during OLTx may be associated with excess fluid administration and a positive fluid balance.

At the end of the transplantation procedure, significantly lower respiratory compliance than before the operation is highly suggestive of increased extravascular pulmonary water content, as demonstrated by Tallegren *et al*[11].

In a report by Lin *et al*[12], a MELD score $\geqslant$ 25 points, an intraoperative fluid transfusion volume >10 L, and an intraoperative blood transfusion volume > 4 L were all independent predictors of the risk of PPCs, whereas a fluid balance of $\leqslant$ -300 mL on the first two postoperative days appeared to be a protective factor.

Huang *et al*[6] found that OLT recipients who developed PRF had significantly different intraoperative blood loss, *i.e.*, more patients in the non-PRF group completed the surgical procedure without needing any blood transfusions. This difference greatly influenced outcome, with patients who developed PRF staying longer in the ICU and exhibiting significantly higher morbidity and mortality rates.

Other clinical studies have demonstrated that intraoperative fluid overload is the strongest risk factor for PPCs[8-13]. Jiang *et al*[14] investigated the link between intraoperative and postoperative fluid therapy and early PPCs, showing that patients with net intraoperative fluid retention volumes < 5000 mL and intraoperative bleeding volumes < 800 mL had fewer PPCs than did patients needing more fluid therapy. The group that was administered less fluid intraoperatively experienced a faster postoperative recovery, with shorter times to extubation and ICU stays.

Severe reperfusion syndrome is mainly characterized by prolonged hypotension, bradycardia, hyperkalemia, vasodilation, and pulmonary hypertension and may also trigger generalized endothelial injury, resulting in acute pulmonary edema and/or acute respiratory distress syndrome (ARDS)[15]. Liver ischemia-reperfusion may lead to an increase in the levels of multiple inflammatory mediators that become active in the lungs. Inflammatory lung-liver interactions, and the activation of nuclear factor $\varkappa$B in particular, may be implicated in the pathogenesis of permeability-type pulmonary edema[16,17].

A greater susceptibility to interstitial lung edema can seriously impair patients' postoperative oxygenation, worsen oxygen delivery to the newly transplanted organ, and increase the need for ventilation.

Preservation-related or graft-related factors, potentially contaminated preservation fluids, the amount of intraoperative blood transfusion, longer ischemia times, and poor initial graft function are other important factors that predispose an individual to postoperative infections that may also involve the respiratory tract[18,19].

POSTOPERATIVE RISK FACTORS FOR POST-OLT RESPIRATORY COMPLICATIONS

The most important factors involved in the development of PPCs following the transplantation procedure are reported in Table 2. After admission to the ICU, the residual effect of anesthetics, an excessive need for opioids for analgesia, and a high fluid input may all interfere with a patient's weaning from a ventilator in various ways. Inadequate deep inspiration due to a wide incision and the inhibitory effect of wound pain on coughing and mucus removal also predispose patients to various respiratory complications[20].

As in other patients undergoing upper abdominal surgery, changes in respiratory pressures and chest wall

Table 3 Major post-orthotopic liver transplantation pulmonary complications

Complication	Frequency
Pleural effusion[4,8,12,30]	32%-47%
Atelectasis[8,12,28,30]	5%-29%
Pulmonary edema[8,12-14,28,30]	4%-47%
Acute respiratory distress syndrome[4,8,9,12,14,28,30,55]	0.8%-42%
Pneumonia[2,4,8,12,14,28,30,36-39]	5%-38%

movement anomalies due to transection of the abdominal oblique muscles and rectus muscles and prolonged retraction of the right hemidiaphragm, which is associated with diaphragmatic dysfunction, may result in a 50%-60% reduction in vital capacity and a 30% reduction in functional residual capacity[21].

Early weaning from mechanical ventilation is a primary goal for a favorable outcome, but primary graft dysfunction, the need for re-laparotomy, respiratory distress syndrome, the persistence of severe encephalopathy, or surgery-related emboligenic problems may delay removal of the endotracheal tube and correspondingly increase the risk of respiratory infections[14].

One of the most severe, although rare, adverse effects of massive intraoperative transfusion is transfusion-related acute lung injury (TRALI), which has the potential to cause lung edema and severe postoperative respiratory distress. TRALI is particularly relevant in post-OLT patient care because this injury can lead to pulmonary infiltrates, hypoxia, and respiratory failure during or within 6 h after a blood transfusion, with no other apparent cause. According to the "two-hit" theory about the pathogenic mechanism of TRALI, a first event (*e.g.*, sepsis or trauma) could induce pulmonary endothelial activation, cytokine release, and "neutrophil priming". Subsequent exposure to lipids, cytokines, or antibodies associated with massive transfusion would then prompt the activation of adherent neutrophils and a release of inflammatory mediators, thus leading to lung injury[22,23]. TRALI and acute lung injury (ALI) share the same pathophysiological pathway and clinical definition, except that TRALI is temporally and mechanistically related to the transfusion of blood or blood components. In both conditions, capillary permeability results in plasma moving into the alveolar space and causing pulmonary edema[24].

Postoperative hypoproteinemia, the onset of renal insufficiency, and poor postoperative myocardial function can also set the stage for interstitial edema, reduce pulmonary compliance, increase the effort of breathing, and prolong the need for invasive ventilation.

Right hemidiaphragm paralysis after OLT is another complication responsible for the development of right lower lobe atelectasis. In an old study, McAlister *et al*[25] found that 79% of liver recipients had right phrenic nerve injury, and approximately half of these patients also had hemidiaphragm paralysis. Phrenic nerve conduction generally tends to recover within a few months, and most patients with phrenic nerve injury and right hemidiaphragm elevation rarely develop substantial respiratory dysfunction or need more prolonged mechanical ventilator support[25,26].

A considerable risk of acute rejection invariably persists early after OLT, which is associated with a need for higher levels of immunosuppression. Acute allograft rejection demanding high-dose corticosteroids or cytolytic agents is known to raise the risk of systemic infection, which may also involve the respiratory tract.

INCIDENCE AND PATHOLOGICAL FEATURES OF PPCS IN LIVER TRANSPLANT PATIENTS

Pulmonary complications after OLT can be classified as infectious and non-infectious (Table 3). Although uncommon in the first few days, the former complications later become an important cause of overall morbidity, whereas non-infectious complications account for most early problems but have less impact on patient outcome[27,28].

In an old study conducted by the Pittsburgh group[29], pulmonary infiltrates characterized as pulmonary edema occurred in 40% of patients; pneumonia, in 38%; atelectasis, in 10%; and ARDS, in 8%. Of the cases of infiltrates, 48% occurred within 30 d of transplantation. In total, 78% of the cases of pulmonary infiltrates and 87% of the cases of pneumonia diagnosed at the ICU involved mechanically ventilated patients.

Glanemann *et al*[30] reported that 11% of liver transplant patients required ventilatory support due to pulmonary complications, and 36.1% had to be reintubated. Among the patients who developed pulmonary complications and needed reintubation, 44.6% were intubated within 24 h after OLT.

Hong *et al*[31] reported that early post-OLT pulmonary infiltrates were detected in 68 of 131 liver recipients (42.7%), with pleural effusion in 50 patients (73.5%), pneumonia in 6 (8.8%), atelectasis in 6 (8.8%), pulmonary edema in 5 (7.4%), and ARDS in 1 (1.5%). Jiang *et al*[14] found that 29 of 62 patients (46.77%) had pulmonary complications after OLT, including pulmonary edema (4 cases, 13.79%), acute lung injury (7 cases, 24.14%), pneumonia (14 cases, 48.28%), and ARDS (4 cases, 13.79%).

In a series described by Bozbas *et al*[4], pulmonary complications were detected in 42.1% of liver recipients; pneumonia, in 21.1%; and pleural effusion on early postoperative chest radiographs, in 32.5%. Right hemidiaphragm elevation was the most common disorder (25.4%).

PLEURAL EFFUSIONS

Many patients undergoing OLT develop pleural effusions that usually mainly involve the right side, with variable amounts of fluid accumulation. The effusions are transudative and unrelated to primary cardiovascular disease. Pleural effusion after OLT is generally not a serious com-

plication, but if the effusion continues to expand beyond the first week or remains isolated to the left side, the fluid should be sampled to rule out other causes. Patients with large effusions may experience shortness of breath or a nonproductive cough.

Disruption of the diaphragmatic lymphatics during hepatectomy, along with diaphragmatic defects that allow the transfer of ascites developing in the abdominal cavity directly into the pleural space, are postulated to be the principal mechanisms behind fluid accumulation[26,32]. The negative intrathoracic pressure draws ascitic fluid into the pleural space, and analysis shows that this fluid has many of the same characteristics as abdominal ascites.

Pleural effusions may expand during the first postoperative week but frequently disappear in the following weeks. These effusions are usually asymptomatic and self-limiting, and thoracentesis or chest tube placement is rarely necessary.

Persistent pleural effusions may lead to respiratory dysfunction by causing atelectasis or may predispose patients to pneumonia and prolong recovery. Effusions may also recur at any time and are occasionally a sign heralding allograft rejection.

Postoperative atelectasis can also be the result of bronchial obstruction due to changes in bronchial secretions, a defective expulsion mechanism, or a reduced bronchial caliber.

A limited intraoperative production of surfactants reduces alveolar surface tension and thus prevents the lung from stabilizing at low volumes, predisposing the lung to collapse. The residual effects of anesthetics and postoperative narcotics can cause hypoventilation, ineffectual respiration, depression of the cough reflex, immobilization, and splinting.

POST-LT PNEUMONIA

Early nosocomial pneumonia after OLT is nearly exclusively a perioperative complication and is characterized by the presence of pulmonary infiltrate, fever, leukocytosis, and new-onset respiratory symptoms (cough, sputum, and dyspnea). When the typical radiological picture of pneumonia is identified, it is important to isolate the responsible microorganism from deep tracheal aspirate or sputum cultures or bronchoalveolar lavage cultures to prescribe the appropriate, specific treatment.

The breakdown of the mucocutaneous defensive barriers that occurs after prolonged orotracheal intubation is a major risk factor for post-OLT pneumonia. Massive intraoperative bleeding during the transplantation procedure, the persistence of severe encephalopathy, diffuse pleural exudates, postoperative ALI/ARDS, and severe renal impairment are frequently associated with delayed weaning from mechanical ventilation and contribute to the development of infectious diseases[19,33].

Other important risk factors for early pneumonia include a greater exposure to nosocomial agents, a significant decline in recipients' immune function, surgical complications, re-interventions, and the need for retransplantation[34].

Hospital-acquired pneumonia (HAP) and ventilator-associated pneumonia are usually diagnosed in cases of early- or late-onset pneumonia, depending on whether the pneumonia occurs within or after the first 4-6 d of hospitalization, respectively[35].

The incidence of post-LT pneumonia has been shown to vary from 5%-38%[8,36-39]. Pirat *et al*[8] reported an incidence of 22.7% and a mortality rate of 40%. These authors found that individuals who developed pneumonia had longer times to extubation and higher mortality. In a study by Xia *et al*[38], the overall incidence of severe pneumonia was 18.2%, with an associated mortality rate of 37.5%. Bozbas *et al*[4] reported a higher rate of bacterial pneumonia (> 70%), with a 26% rate of fungal pneumonia; lung infections were noted in 21% of patients in their study and were responsible for 45.8% of deaths.

In a report by Weiss *et al*[39], early HAP (within 6 d after OLTx) occurred in 15.5% of liver recipients. As in the above-mentioned reports, these cases of pneumonia were associated with prolonged postoperative mechanical ventilation, a long ICU stay, and a trend toward higher short- and long-term mortality rates.

Levesque *et al*[2] recently reported a 22% incidence of postoperative pneumonia, and 43% of their liver recipients who had pneumonia developed respiratory failure that required mechanical ventilation. Based on a univariate analysis, the researchers found that several preoperative factors and the number of intraoperative transfusions (units of blood and fresh frozen plasma) were associated with pneumonia However, in a multivariate analysis, only a preoperative restrictive pulmonary pattern and the international normalized ratio measured prior to OLT were independent predictors of pneumonia after surgery. Ikegami *et al*[40] reported the prevalence and characteristics of bacterial pneumonia after living-donor liver transplantation (LDLT), stating that 50 of 346 patients (14.5%) experienced bacterial pneumonia after LDLT. The incidence of bacterial pneumonia was highest on postoperative day 6, whereas the incidence declined on postoperative days 8 and 9. Pneumonia was associated with a prolonged use of mechanical ventilation, a prolonged stay in the ICU, the creation of a tracheostomy, primary graft dysfunction, and a need for renal replacement therapy. The mortality rate of patients with early-onset pneumonia was 25.7%. Delayed-onset pneumonia (at least 10 d after liver transplantation) was significantly associated with graft dysfunction and resulted in a higher mortality rate (73.3%) than did early-onset pneumonia.

A wide variety of community-acquired and hospital-acquired microorganisms may be responsible for post-OLTx pneumonia, but Gram-negative pathogens dominate in the early post-transplant stages, as in the population undergoing general surgery. The Gram-negative bacteria that frequently colonize the oropharyngeal cavity are most often responsible for lower respiratory

tract infections. In liver recipients on prolonged mechanical ventilation, nosocomial pathogens, including *Pseudomonas aeruginosa*, *Escherichia coli*, *Klebsiella* species, *Acinetobacter* species, and *Staphylococcus aureus* (including MRSA), are usually detected in bronchoalveolar lavage samples[41,42]. It is worth emphasizing, however, that the microbiological ecology may vary considerably from one ICU to another, and previous antimicrobial consumption may have a major influence on microbial ecology.

In a report by Weiss *et al*[39], in a subgroup of patients with HAP occurring within the first 4 d after ICU admission, 61.5% of the causative pathogens were Gram-negative bacilli, and 38.5% were Gram-positive cocci. Of these microorganisms, 73% were classified as community acquired. More than 30% of liver recipients had a history of hospital stays and antibiotic treatments.

Given patients' obligatory immunosuppression, prompt isolation of the microorganisms causing post-OLT pneumonia and appropriate treatment are mandatory for a favorable outcome. An early diagnosis may not be achievable with "conventional" diagnostic techniques in certain patients, however, making it necessary to resort to more "invasive" methods. If pulmonary infiltrates persist or become worse, a histopathological diagnosis by bronchial brushing, telescope catheter culture, fiberoptic bronchoscopy with transbronchial biopsy, or even surgical pulmonary biopsy may be needed to rule out opportunistic infectious agents.

POST-LT PULMONARY EDEMA

Severe pulmonary edema is unusual in the early postoperative period, unless the liver recipient experiences acute-onset, severe left ventricular dysfunction or acute fluid overload in the case of renal impairment. Despite a high incidence of postoperative radiological findings suggestive of acute pulmonary edema, most episodes are clinically easily overlooked, with only a mild deterioration in gaseous exchange.

In patients with fulminant hepatic failure, pulmonary edema is an ominous sign because it may predict evolving acute lung injury[43].

Subclinical forms of interstitial/alveolar edema may prompt findings of a transient increase in pulmonary capillary hydrostatic pressure or hypoalbuminemia or nonspecific signs of mild or moderate lung endothelial injury. Additional causes of acute pulmonary edema include excessive amounts of fresh frozen plasma and total fluids being administered intraoperatively, postoperative changes in renal function (urine volume and serum creatinine), massive transfusions, large-volume thoracentesis, and reduced lymph flow. It has been speculated that the greater pulmonary vessel permeability associated with end-stage liver disease may be exacerbated by the systemic inflammatory reaction induced by liver transplantation[14].

In a study by Aduen *et al*[44], a worse preoperative MELD score could predict the risk of pulmonary edema developing soon after the transplantation procedure. Preoperative right ventricular systolic pressure, as estimated by echocardiography, was also higher in patients who developed postoperative pulmonary edema, suggesting that elevated pulmonary pressures are associated with increased interstitial lung loading.

Chen *et al*[45] postulated that nitric oxide (NO) flow-mediated vasodilation is the pathogenic mechanism behind the high incidence of pulmonary edema after LDLT. In this study, the total volume of intraoperative fluid administered was higher in patients who developed pulmonary edema, but their net fluid retention did not significantly differ from that of the patients who did not experience this complication. Pulmonary edema did not prolong the hospital stay or increase the risk of infection and was overcome by administering diuretics.

In cirrhotic patients undergoing OLT, increased blood flow in the lung may increase shear stress on the endothelium, and this phenomenon is associated with an increased release of vasodilators, including NO, prostaglandins, and endothelium-derived hyperpolarizing factors[46,47].

Pulmonary edema is diagnosed based on the strength of radiographic criteria, clinical symptoms, the PaO2/FIO2 (PF) ratio (< 300), and hemodynamic data. According to the American-European Consensus Conference (AECC) on ARDS, permeability edema may be characterized by a pulmonary artery wedge pressure < 18 mmHg, whereas the hydrostatic type is usually associated with a wedge pressure > 18 mmHg[48]. Patients with persistent permeability-type edema may also have a higher mean pulmonary arterial pressure and a higher pulmonary vascular resistance, consistent with a resistance-dependent mechanism.

In a study by Snowden *et al*[13], patients with pulmonary edema stayed longer in the ICU and were on mechanical ventilation for longer. Aduen *et al*[44] also found that the time on mechanical ventilation and in the ICU and hospital stays were longer in patients with persistent permeability-type edema. In contrast to the situation observed for the hydrostatic type, permeability-type pulmonary edema was associated with an increase in both mean pulmonary arterial pressure and pulmonary vessel resistance. In the series, 29% of patients with persistent permeability-type pulmonary edema died, as opposed to 7% of patients who never developed pulmonary edema and 0% of patients who developed hydrostatic-type pulmonary edema.

POST-OLT ARDS

Post-OLT acute lung injury and even severe ARDS may develop within 24 h or the first few days after the procedure. Frequent causes of ARDS include crystalloid infusion overload, massive transfusion of blood or blood products, prolonged operating times, severe bleeding during liver removal, and severe ischemia-reperfusion syndrome. In the early postoperative course, serious systemic infections, gastric aspirations, disseminated intravascular

Table 4 Major strategies to prevent postoperative pulmonary complications after orthotopic liver transplantation

Preoperative strategies	Intraoperative strategies
Pulmonary rehabilitation prior to OLT	Reduction in the degree of surgical insult
	Reduction in the level of aggressiveness
	Reduction in the duration of procedure
	Reduction in the amount of blood lost
Postoperative ventilation[60,61,64,65]	Postoperative care[68]
Early extubation	Adequate postoperative pain relief
Lung expansion maneuvers	Optimal hemodynamic and fluid management
Deep breathing exercises	Improvement of general health and nutrition
Timely execution of bronchial toilette	
NIV	
Chest percussion and vibration	
Invasive mechanical ventilation: assisted modes with minimal sedation	

OLT: Orthotopic liver transplantation; NIV: Non-invasive ventilation.

coagulation, and other nonspecific generalized insults may also be involved.

The intraoperative transfusion of blood products, and platelets in particular, has been identified as a risk factor for a poor outcome after OLT. The negative impact cannot be explained simply by the activation of the coagulation system and platelet aggregation at the endothelium; the poor outcome most likely has to do with ischemia-related endothelial cell injury[49]. Platelets contain many cytokines and vasoactive and inflammatory mediators that are rapidly released and activated by various stimuli after reperfusion and that may affect the lung. Several other factors, such as the potential for viral transmission and bacterial contamination, the risk of alloimmunization, nonspecific immunosuppressive effects, and graft-versus-host disease, may also contribute to a worse outcome[50,51].

Pereboom *et al*[52] demonstrated that platelet transfusion during OLT is associated with higher postoperative mortality due to severe lung edema causing heaviness of the lungs, as described in the clinical diagnosis of TRALI or ARDS.

ARDS after OLT is a serious multifactorial complication associated with diffuse, bilateral pulmonary infiltrates of acute onset (and non-cardiogenic etiology), with a PF ratio of < 200. Based on an "old" concept, ALI was once defined as a milder form of ARDS and was distinguished by a PF ratio of between 200 and 300[48]. Currently, according to the Berlin definition, the term ALI is avoided and replaced by mutually exclusive subcategories of ARDS based on the degree of hypoxemia. ALI is now be called "mild ARDS" and applies to cases with a PF ratio of up to 201-300 mmHg, the upper limit for ALI according to the AECC definition[53].

A poorly controlled systemic inflammatory response induced by severe reperfusion syndrome, along with transfusion related-adverse events, can substantially increase the risk of postoperative pulmonary injury. Inflammatory mediators cause damage to both the alveolar and the microvascular endothelia, and this damage alters the alveolar-capillary barrier, causing extravascular fluid accumulation. This pulmonary damage results in an increase in extravascular lung water, which is one of the hallmarks of mild ARDS and ARDS[54,55].

Major clinical findings in ARDS include severely impaired pulmonary oxygen diffusion, with pulmonary edema developing in the presence of normal pulmonary capillary-filling pressures and in the absence of a marked reduction in oncotic pressure.

ARDS is an important cause of PRF after OLT. In an old study, the reported incidence of ARDS was in the range of 4.5%-15.7%, with a mortality rate nearing 80%[9].

More than 10 years ago, Golfieri *et al*[56] also reported that 4%-16% of patients who developed post-OLT lung injury deteriorated to severe ARDS, and the mortality rate of these patients was as high as 80%-100%.

Treatment for ARDS is primarily supportive, with fluid restriction, lung-protective mechanical ventilation, mild hypercapnia, and optimal PEEP[57]. The use of high PEEP has raised certain concerns, however[58], because of the potentially reduced venous return in a newly engrafted liver. Given the still limited data available, the literature affords no definitive answers on the use of "permissive" PEEP in this setting.

When critical hypoxemia ensues in patients with severe ARDS, additional rescue therapies may be administered, such as inhaled NO and prostaglandins[59].

PREVENTING PPCS AFTER OLT

The period following transplantation surgery is marked by variable changes in the structure and function of the respiratory system, which can particularly affect severely debilitated patients. The normal activity of liver recipients is usually reduced due to a low physical performance status both before and after liver transplantation.

Similar to what is advisable after upper abdominal surgery, important strategies for PPC reduction may include early extubation associated with lung expansion maneuvers, which comprise incentive spirometry, deep breathing exercises, intermittent positive-pressure breathing, and continuous positive airway pressure (CPAP)[60]. Manual techniques, including chest percussion and vibration, are alternative treatment approaches if airway clearance is not sufficient (Table 4).

Early extubation is the key element to reduce PPCs and ICU stay and to speed patients' recovery. There is a substantial body of evidence proving that patients who undergo OLT can be extubated immediately after surgery, with few pulmonary complications, a lower risk of postoperative infection, and no effect on 1- or 3-year graft survival[61].

The specific benefit of each chest physical therapy technique has not been fully evaluated, and even combining various methods does not seem to provide additional

risk reduction. However, CPAP is particularly useful for patients who cannot perform deep breathing or incentive spirometry exercises after extubation[62].

In the case of reduced postoperative lung volumes, the elevation of both hemidiaphragms, and lower-lobe atelectasis, the work of breathing can be consistently augmented, making it difficult to achieve and maintain postoperative ventilatory autonomy. In liver recipients who remain under invasive mechanical ventilation, ventilator use may significantly influence the disuse of muscle dysfunction. Assisted modes of ventilation with minimal sedation should be favored over "controlled" modes, as complete diaphragm rest will rapidly lead to atrophy[63].

Due to the important restrictive respiratory pattern of cirrhotic patients and abdominal hypertension, weaning from a ventilator after OLT can take longer because of unsatisfactory gas exchange during various T-piece trials. Rapid extubation followed by immediate noninvasive ventilation (NIV) application should be considered in this setting to shorten and accelerate the weaning process in those recipients who do not completely fulfill the criteria for safe extubation[64]. By resting and unloading the inspiratory muscles, NIV with pressure support enables both hypercapnic and hypoxic patients to improve faster and may prevent basal atelectasis induced by abdominal distension. Chest physical therapy associated with CPAP or NIV stimulates lung expansion and improves lung ventilation, thereby preventing or reducing the build-up of liquid in the pleural space.

The early and "prophylactic" use of NIV may also reduce the risk of reintubation[65]. Because NIV leaves the upper airways intact, this method can reduce not only bacterial colonization and nosocomially acquired infections but also hemorrhagic complications in cases of underlying coagulopathy.

Many respiratory disorders following OLT respond to specific treatments, such as hemofiltration, pleural drainage, bronchial toilette, and abdominal drainage, with expected improvements over a period of hours or days. Supporting the failing recipient with early NIV may reduce the work of breathing and maintain gas exchange while awaiting an improvement in spontaneous ventilation.

Adequate postoperative pain relief, optimal hemodynamic and fluid management, the timely execution of bronchial toilette, airway clearance maneuvers (assisted cough and expiratory airflow techniques), and trials of NIV in the case of respiratory fatigue are extremely useful to facilitate the rehabilitation process.

Postoperative pain is a major cause of shallow breathing and impaired coughing, resulting in retention of secretions, atelectasis, hypoxemia, hypercapnia, and respiratory failure, especially in patients with pre-existing lung disease. Adequate treatment of pain will prevent hypoventilation and reduce the respiratory rate. Paracetamol at reduced doses, along with rescue doses of tramadol, should be offered as a valid analgesic regimen[66].

The improvement of sedation management, simple interventions aiming at actively mobilizing the recipient, and increasing the amount of time out of bed are further advantageous for lung function.

Although the level of evidence for implementing multimodal preventive measures is relatively low[67], and although many of the procedures believed to reduce the risk of PPCs are supported by a conventional "traditional" consensus, early respiratory disorders associated with cirrhosis and transplantation surgery undoubtedly benefit from an optimized patient care program with a multidisciplinary approach.

Promising new interventions may rely on more accurate preoperative respiratory assessment, *e.g.*, with maximal inspiratory pressure and maximal expiratory pressure measurements, quantification of the degree of respiratory muscle weakness, optimization of chronic inflammatory pulmonary disease, preoperative lung expansion maneuvers, inspiratory muscle training performed in a chest physical therapy outpatient setting or a pulmonary rehabilitation clinic in the hospital, and improvement of general health and nutritional status[68].

It should be noted, however, that pulmonary rehabilitation prior to OLT may be of unpredictable value, as the variable waiting times before surgery, along with malnutrition and end-stage cirrhosis-related muscle weakness, may insufficiently affect exercise capacity and are thus unlikely to reduce postoperative risk.

Additional interventions of more expected benefit include an effort to reduce the degree of surgical insult, the level of aggressiveness, the duration of the procedure, and the amount of blood lost.

Better identification of patient- and procedure-related risks of pulmonary complications, the recognition of independent predictors of PRF, and the application of a local treatment protocol in high-risk patients, may favorably influence both the incidence and the outcome of PPCs.

CONCLUSION

Many reports underscore that infectious and other PPCs are important contributors to early morbidity and mortality in liver transplant patients[4,8,55]. Despite advances in surgical techniques and anesthesiological management, the lung may still suffer throughout the perioperative period from various types of injury, with different ensuing ventilatory impairments and different clinical outcomes.

Postoperative respiratory complications are not always related to preoperative respiratory disorders, but rather may be the result of systemic inflammatory responses induced by surgical trauma, hemodynamic impairment, reperfusion syndrome, "distant" organ dysfunction, or early graft dysfunction. The severity of any PPC is also believed to depend on a recipient's clinical condition at the time when the complication occurs[31]. The stress response to the surgery is maximal soon after OLT and is expressed by disrupted circulating hormone concentrations, with increased antidiuretic activity. Electrolyte abnormalities and water retention are also common at this

time due to the nephrotoxic effects of the immunosuppressants administered.

Pleural effusion, atelectasis, pneumonia, and ARDS may be severe enough to demand or prolong the need for tracheal intubation, resulting in a higher risk of nosocomial infections, longer stays at the ICU and/or in the hospital, and a worse clinical outcome[69]. The lungs are particularly vulnerable to infectious diseases after OLTx and represent the second most common site (after the abdominal cavity) of colonization by nosocomial pathogens. Infectious complications involving the respiratory tract are acknowledged to be an important cause of death in liver transplant recipients[18]. In certain old studies by Plevak *et al*[70] and Shieh *et al*[71], patients who developed pneumonia in the early postoperative period and required prolonged mechanical ventilation had a mortality rate of 43%. Singh *et al*[29] also reported an overall mortality rate of 28% in transplant recipients with pulmonary infiltrates in the ICU and a mortality rate of 47% after 14 d among patients with pneumonia. Bozbas *et al*[4] found significantly lower survival rates for patients yielding microorganisms by deep tracheal aspirate culture. The early mortality rate was higher for patients whose thoracentesis cultures were positive.

Currently, the overall 1- and 5-year survival rates after OLT are approximately 85% and 68%, respectively, with a 10-year survival rate approaching 50%[72]. Judging from the multicenter-based prospective data collected by Watt *et al*[73], post-transplant respiratory diseases now account for only 2.4% of all deaths. Among the deaths after OLT, those of an infectious nature (> 19%) occur earlier, and pneumonia is among the most important contributors to the overall morbidity and mortality rates.

In conclusion, numerous perioperative factors may be responsible for impaired respiratory function after OLT. It is of paramount importance to accurately identify any lung disorders because pulmonary infectious complications need to be treated rapidly and aggressively to prevent diffuse lung lesions and potential evolution into multisystem organ failure.

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ORIGINAL ARTICLE

Refining pathological evaluation of neoadjuvant therapy for adenocarcinoma of the esophagus

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Abstract

AIM: To assess tumour regression grade (TRG) and lymph node downstaging to help define patients who benefit from neoadjuvant chemotherapy.

METHODS: Two hundred and eighteen consecutive patients with adenocarcinoma of the esophagus or gastro-esophageal junction treated with surgery alone or neoadjuvant chemotherapy and surgery between 2005 and 2011 at a single institution were reviewed. Triplet neoadjuvant chemotherapy consisting of platinum, fluoropyrimidine and anthracycline was considered for operable patients (World Health Organization performance status $\leqslant$ 2) with clinical stage T2-4 N0-1. Response to neoadjuvant chemotherapy (NAC) was assessed using TRG, as described by Mandard *et al*. In addition lymph node downstaging was also assessed. Lymph node downstaging was defined by cN1 at diagnosis: assessed radiologically (computed tomography, positron emission tomography, endoscopic ultrasonography), then pathologically recorded as N0 after surgery; ypN0 if NAC given prior to surgery, or pN_0 if surgery alone. Patients were followed up for 5 years post surgery. Recurrence was defined radiologically, with or without pathological confirmation. An association was examined between t TRG and lymph node downstaging with disease free survival (DFS) and a comprehensive range of clinicopathological characteristics.

RESULTS: Two hundred and eighteen patients underwent esophageal resection during the study interval with a mean follow up of 3 years (median follow up: 2.552, 95%CI: 2.022-3.081). There was a 1.8% (n = 4) inpatient mortality rate. One hundred and thirty-six (62.4%) patients received NAC, with 74.3% (n = 101) of patients demonstrating some signs of pathological tumour regression (TRG 1-4) and 5.9% (n = 8) having a complete pathological response. Forty four point one percent (n = 60) had downstaging of their nodal disease (cN1 to ypN0), compared to only 15.9% (n = 13) that underwent surgery alone (pre-operatively overstaged: cN1 to pN_0), (P < 0.0001). Response to NAC was associated with significantly increased DFS (mean DFS; TRG 1-2: 5.1 years, 95%CI: 4.6-5.6 *vs* TRG 3-5: 2.8 years, 95%CI: 2.2-3.3, P < 0.0001). Nodal down-staging conferred a significant DFS advantage for those patients with a poor primary tumour response to NAC (median DFS; TRG 3-5 and nodal down-staging: 5.533 years, 95%CI:

3.558-7.531 *vs* TRG 3-5 and no nodal down-staging: 1.114 years, 95%CI: 0.961-1.267, $P < 0.0001$).

CONCLUSION: Response to NAC in the primary tumour and in the lymph nodes are both independently associated with improved DFS.

Key words: Esophageal cancer; Gastro-esophageal cancer; Neoadjuvant; Regression

Core tip: Predictive markers of benefit from neoadjuvant chemotherapy (NAC) in esophageal adenocarcinoma are urgently required to provide a "personalised medicine" approach: directing treatment to those most likely to benefit. Before prospective studies can be initiated, retrospective series need to be interrogated to identify likely candidate markers of a positive response. In defining a positive response attention needs to be given to both response in the primary tumour and in the lymph nodes, as a previously unidentified group of patients who appear to have a poor tumoural response to NAC (tumour regression grade 3-5) do benefit from combination therapy by nodal downstaging.

Noble F, Nolan L, Bateman AC, Byrne JP, Kelly JJ, Bailey IS, Sharland DM, Rees CN, Iveson TJ, Underwood TJ, Bateman AR. Refining pathological evaluation of neoadjuvant therapy for adenocarcinoma of the esophagus. *World J Gastroenterol* 2013; 19(48): 9282-9293 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i48/9282.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i48.9282

INTRODUCTION

Neoadjuvant therapy followed by surgery is established as the gold standard in the management of patients with locally advanced adenocarcinoma of the esophagus/esophagogastric junction. In the United Kingdom neoadjuvant chemotherapy (NAC) in conjunction with transthoracic esophagogastrectomy is the current standard of care for these patients[1]. The potential benefits of neoadjuvant therapy include: downstaging of the primary tumour[2] and lymph nodes[3], an increase in the resectability of the tumour[4], elimination of micrometastases[5] and improved survival[6]. A recently suggested advantage of neoadjuvant therapy and early assessment of response is the potential for assessing *in vivo* the chemosensitivity of the tumour and so providing information to tailor multimodal therapy[7]. Both NAC and surgery are associated with considerable morbidity and mortality[8] and evidence remains inconsistent for the survival benefit for patients who undergo NAC[4,8,9]. The most recent meta-analysis to compare NAC *vs* surgery alone in 2062 patients suggests a 5.1% survival advantage at 2 years for patients treated with NAC for adenocarcinoma[6]. Patients who have a significant pathological response to neoadjuvant therapy have consistently been shown to have improved survival when compared to patients who have not had a significant response[10-13]. For those patients who do not have a significant pathological response, the consequences of delay to surgery and the benefits of neoadjuvant chemotherapy are not known. Furthermore, it is unclear which patients should be considered for tailored adjuvant systemic therapy or alternative neoadjuvant therapy.

The pathological response to chemotherapy is most widely assessed using Tumour Regression Grading (TRG)[1] as described by Mandard *et al*[14] although this has not gained universal acceptance[15]. This system is based on the amount of residual tumour and the degree of fibrosis at the primary tumour[14]. Other proposed pathological systems for measuring neoadjuvant treatment response include complete pathological response[16], size of residual tumour[17], number of residual tumour cells[15,18], response classification system[19], size based pathological response[17] and downstaging of cT and cN stage[10]. These grading systems have predominately been developed following chemoradiotherapy with heterogeneous histology with few studies assessing their utility following chemotherapy in patients with esophageal adenocarcinoma[2,20-23]. A number of clinically important questions could be addressed by a robust and universally accepted measure of response to neoadjuvant treatment including: the ability to accurately predict an individual patient's tumour response to preoperative therapy leading to non-responders proceeding directly to surgery or being considered for alternative neoadjuvant regimes; assessment of new neoadjuvant regimes, and identification of patients who are likely to benefit from adjuvant therapy.

We have therefore assessed pathological response to neoadjuvant chemotherapy by assessing the tumour response as well as the response in the lymph nodes in a large contemporary cohort of patients with esophagogastric adenocarcinoma managed with neoadjuvant platinum based triplet chemotherapy, and describe their associations with short- and long-term outcomes. In addition we suggest combining both local tumour and nodal responses to NAC.

MATERIALS AND METHODS

Patients

For this retrospective study, a prospectively collected database of consecutive patients undergoing esophagogastric resection treated at University Hospital Southampton National Health Service Foundation Trust (UHSFT) between January 2005 and December 2011 was reviewed. All patients were discussed at a specialist multidisciplinary team meeting (MDT). Standard staging investigations included endoscopic ultrasonography, high-resolution computed tomography, integrated fluorodeoxyglucose positron emission tomography/computed tomography (PET-CT) and staging laparoscopy, where indicated and were uniformly applied during the study interval. Patients considered suitable for potential surgical resection with tumours staged as T2N0M0 or above were considered for neoadjuvant chemotherapy.

Neoadjuvant chemotherapy consisted of three 21 d

Table 1 Tumour regression scoring according to Mandard *et al*[14]

Grade	Definition
TRG 1	No residual cancer
TRG 2	Rare residual cancer cells
TRG 3	Fibrosis outgrowing residual cancer
TRG 4	Residual cancer outgrowing fibrosis
TRG 5	Absence of regressive changes

TRG: Tumour Regression Grade.

cycles of anthracycline, platinum and fluoropyrimidine: ECF (epirubicin 50 mg/m^2, cisplatin 60 mg/m^2, both intravenously on 1 d and protracted venous infusion 5-FU 200 mg/m^2 per day) or ECX (epirubicin 50 mg/m^2, cisplatin 60 mg/m^2, both intravenously on 1 d and capecitabine 625 mg/m^2 orally twice daily for 21 d) or EOX (epirubicin 50 mg/m^2 *iv* bolus and oxaliplatin 130 mg/m^2 *iv* infusion over 2 h on 1 d, capecitabine 625 mg/m^2 orally twice daily for 21 d).

Surgery was performed at UHSFT after initial staging or 4-6 wk following neoadjuvant chemotherapy. A repeat CT scan was performed, prior to surgery, for those who received chemotherapy to assess their response to chemotherapy and disease operability. Types of esophagogastrectomies included Ivor Lewis, left thoracoabdominal with or without cervical anastomosis and transhiatal esophagogastrectomy or minimally invasive esophagogastrectomy (MIO) either 2 stage (MIO-2) or 3 stage (MIO-3) in accordance with recommendations arising from the consensus statement from the Association of Upper Gastrointestinal Surgeons and the Association of Laparoscopic Surgeons for introduction of MIO[24].

Data recorded included demographics, tumour characteristics, resection type, estimated blood loss (calculated from suction bottles and weighed swabs) and histopathological analysis of the surgical specimen. TNM-7 (International Union Against Cancer TNM Classification 7th Edition) was used to report tumour stage after analysis of pathology reports[25]. Pathological tumour clearance ("R"-status) was determined according the Royal College of Pathologists' guidance.

Postoperative complications were graded according to the Clavien-Dindo (CD) classification[26]. An AL was defined as a leak sufficient to cause symptoms and confirmed by radiology (contrast enhanced multi-detector CT scan with on-table oral contrast or water soluble contrast studies), endoscopy or during surgical exploration.

All patients were cared for by a specialist esophagogastric team who applied a similar perioperative regime to all patients. Patients were routinely followed-up for 5 years post surgery according to the following protocol: 2-4 wk post-discharge, 3 monthly for 1 year, 6 monthly for 2 years and yearly thereafter. Patients were also seen on an "as required" basis if symptomatic. Recurrence of disease during follow-up was defined as the first site or sites of recurrence with radiological or pathological confirmation. For assessment of disease free survival (DFS), recurrence was defined as time from operation to development of local, nodal (regional) and distant metastasis (whichever occurred first).

Factors analysed

Pathological response to chemotherapy was assessed using the TRG system developed by Mandard *et al*[14] who scored regression based on the degree of fibrosis and residual cancer cells (TRG 1-5)[14,27], see Table 1. All dissected lymph nodes were stained with hematoxylin and eosin and microscopically analysed for metastatic disease. TRG was scored by specialist gastrointestinal pathologists; initially by one pathologist (Bateman AC) prior to its introduction by all pathologists as part of routine pathological reporting.

Statistical analysis

Descriptive data are represented as median and range unless indicated with Kruskal-Wallis, Mann Whitney *U*, *P* and χ^2 test, which were used as appropriate for comparison. Kaplan-Meier, univariate and multivariate cox logistic regression modelling were used to assess the relationship between pathological response grading systems with DFS. All factors that showed statistical significance on univariate analysis were entered to derive the final model. DFS curves of the patients were plotted by using the Kaplan-Meier method and analysed using the Log-rank test. Stratified analyses were performed based on receipt of neoadjuvant chemotherapy, nodal stage and response to chemotherapy. A $P < 0.05$ was considered statistically significant for all tests. Statistical analysis was performed with SPSS® version 19 (SPSS, Chicago, Illinois, United States).

RESULTS

Study patients

A total of 218 patients underwent esophageal resection during the study interval with a mean follow up of 3 years (median follow up: 2.552, 95%CI: 2.022-3.081). There was a 1.8% ($n = 4$) inpatient mortality rate. Detailed patient characteristics and clinical and pathological outcomes are summarised in Table 2, grouped by treatment.

Patients who underwent surgery alone ($n = 82$; 37.6%) were significantly older ($P < 0.0001$), had worse physiological status (ASA $P = 0.005$; performance status $P = 0.001$; O-POSSUM $P < 0.0001$) and lower preoperative staged disease (cT stage $P < 0.0001$; cN stage $P < 0.0001$) compared to patients that underwent multimodal therapy.

One hundred thirty-six (62.4%) patients received multimodal therapy, neoadjuvant chemotherapy and surgery, with 74.3% ($n = 101$) of patients demonstrating some signs of pathological tumour regression (TRG 1-4) with 5.9% ($n = 8$) having a complete pathological response. Forty four point one percent ($n = 60$) had downstaging of their nodal stage compared to only 15.9% ($n = 13$) whose lymph node status was cN1 on preoperative staging and pN$_0$ following surgery alone ($P < 0.0001$).

There were no statistically significant differences in postoperative pathological tumour stage (yp or pT, $P = 0.692$); yp or pN $P = 0.758$), postoperative complications (CD maximum grade, $P = 0.590$) or completeness of resection ($P = 0.772$) in patients that underwent multimodal therapy *vs* surgery alone.

Table 2 Clinical and pathological characteristics of the 218 patients operated on for esophageal and gastro-esophageal adenocarcinoma, according to treatment *n* (%)

Characteristic		Surgery only 82 (37.6)	Neoadjuvant chemotherapy and surgery 136 (62.4)	*P* value
Preoperative status				
Age (range)[1] yr		74.32 (42.08-85.41)	63.76 (32.77-81.28)	< 0.0001
Sex ratio (M:F)[1]		68 (82.9):14 (17.1)	118 (86.8):18 (13.2)	0.439
cT stage	1	17 (20.7)	0 (0.0)	< 0.0001
	2	30 (36.6)	16 (16.0)	
	3	34 (41.5)	114 (84.0)	
	4	1 (1.2)	6 (4.4)	
cN stage	0	36 (43.9)	19 (14.0)	< 0.0001
	1	46 (56.1)	117 (86.0)	
cM stage	0	80 (97.6)	134 (98.5)	0.613
	1	1 (2.4)	2 (1.4)	
Performance status	0	8 (11.6)	35 (25.7)	0.001
	1	51 (73.9)	96 (70.6)	
	2	10 (14.5)	5 (3.7)	
ASA	1	3 (3.7)	11 (8.1)	0.005
	2	56 (68.3)	106 (78.5)	
	3	23 (28)	18 (13.3)	
O-POSSUM		18 (12-30)	16 (12-26)	< 0.0001
Tumour site	Middle 1/3	1 (1.2)	1 (0.7)	0.418
	Lower 1/3	32 (39)	57 (41.9)	
	GEJ-S1	19 (23.2)	23 (16.9)	
	GEJ-S2	18 (22.0)	34 (25.0)	
	GEJ-S3	12 (14.6)	20 (14.7)	
Operative outcomes				
Length of operation (min)[1]		255 (120-480)	261 (120-471)	0.409
Blood loss (mL)[1]		300 (0-2200)	318 (0-3000)	0.429
Clavien Dindo Max	0	26 (31.7)	53 (39.3)	0.59
	1	5 (6.1)	8 (5.9)	
	2	35 (42.7)	40 (29.6)	
	3	6 (7.3)	17 (12.6)	
	4	6 (7.3)	17 (12.6)	
	5	4 (4.9)	0 (0)	
Anastomotic leaks		8 (9.8)	9 (6.7)	0.413
Pathological outcomes				
pT or ypT	0	3 (3.6)	8 (5.9)	0.692
	1	23 (28)	23 (16.9)	
	2	17 (20.7)	34 (25)	
	3	34 (41.5)	66 (48.5)	
	4	5 (6.1)	5 (3.7)	
pN or ypN	0	40 (48.8)	73 (53.7)	0.758
	1	20 (24.4)	21 (15.4)	
	2	11 (13.4)	25 (18.4)	
	3	11 (13.4)	17 (12.5)	
pM or ypM	0	82 (100)	136 (100)	1.00
Tumour regression grade	1	-	8 (5.8)	n/a
	2	-	28 (20.6)	
	3	-	20 (14.7)	
	4	-	45 (33.1)	
	5	-	35 (25.7)	
Nodal downstaged (cN1 to p or ypN1)		13 (15.9)	60 (44.1)	< 0.0001
Positive nodes[1]		1 (0-21)	0 (0-24)	0.789
Nodal yield[1]		18 (4-49)	18 (3-53)	0.242
Resection clearance	R0	65 (79.3)	110 (80.9)	0.772
Vascular invasion		24 (29.3)	41 (30.1)	0.891
Lymphatic invasion		9 (11)	22 (16.2)	0.28
Perineural invasion		8 (9.8)	20 (14.7)	0.291
Maximum tumour diameter (mm)[1]		25 (0-90)	25 (0-155)	0.998
Morphology	Ulcer	48 (60)	96 (74.4)	0.029
	Polypoid	22 (27.5)	23 (17.8)	
	Fungating	2 (2.5)	3 (2.3)	
	Diffuse infiltrating	8 (10)	7 (5.4)	
Grade	G1	6 (7.3)	16 (11.8)	0.669
	G2	30 (36.6)	37 (27.2)	
	G3	46 (56.1)	82 (60.3)	
	G4	0 (0)	1 (0.7)	
Sites of recurrence	Local	3 (3.7)	8 (5.9)	0.461

	Nodal	5 (6.1)	14 (10.4)	0.281
	Distant	18 (22.0)	44 (32.6)	0.093

Values in parentheses are percentages unless indicated. [1]Values in parentheses are range. ASA: American society of anesthesiologists classification; O-POSSUM: Oesophagogastric surgery-physiological and operative severity score for the enumeration of mortality and morbidity; GEJ-S1-3: Gastro-esophageal junction-Siewert type 1-3.

The relationship of tumour regression grade and clinicopathological characteristics

The relationship between patient and tumour characteristics and response to neoadjuvant chemotherapy, as defined by tumour regression grade, are presented in Table 3.

Of the 136 patients that underwent NAC, 36 (26.5%) patients had a significant pathological response (TRG 1-2; responders) compared to 100 (73.5%) patients with no significant pathological response (TRG 3-5; non-responders). Responders and non-responders had similar preoperative clinical features (age, sex and physiological status) and clinical stage of disease (cT stage, $P = 0.396$; cN stage, $P = 0.987$; cM stage, $P = 0.456$), yet responders had markedly reduced ypT stage ($P < 0.0001$), maximal pathological tumour diameter ($P < 0.0001$), and ypN stage ($P < 0.0001$) and were more likely to have their nodal stage downstaged ($P < 0.0001$) compared to non-responders (Table 3). In addition, responders had tumours that were more likely to be ulcers ($P = 0.003$), showing less vascular ($P = 0.004$), and perineural invasion ($P = 0.072$) compared to non-responders. Complete resection (R0) was achieved in 97.2% ($n = 35$) of responders compared with 75% ($n = 75$) of non-responders ($P = 0.04$). There was no significant difference in postoperative complications as classified by the Clavien Dindo system, nodal yield, blood loss or operative time between groups.

The relationship of TRG and lymph node downstaging with DFS

There was a significant difference in survival between responders compared to non-responders, shown in Figure 1A [mean DFS; TRG 1-2: 5.064 years, 95%CI: 4.560-5.569 (median DFS: not reached) *vs* TRG 3-5: 2.759 years, 95%CI: 2.193-3.325 (median DFS: 1.613, 95%CI: 0.834-2.39), $P < 0.0001$].

There was no statistically significant difference in survival between patients graded as TRG 1 compared to TRG 2 [mean DFS; TRG-1: 5.021, 95%CI: 4.069-5.973 *vs* TRG-2: 4.983, 95%CI: 4.069-5.973, $P < 0.0001$ (median DFS's: not reached)].

Patients with lymph node downstaging following NAC had improved DFS *vs* patients without downstaging, Figure 1B [median DFS; lymph node (LN) downstaged: 5.316 years, 95%CI: 4.504-6.127 (median DFS: 5.544) *vs* LN not downstaged: 2.118 years, 95%CI: 1.594-2.643 (median DFS: 1.210, 95%CI: 1.026-1.394), $P < 0.0001$].

Univariate and multivariate analysis for predicting DFS following neoadjuvant chemotherapy

Univariate and multivariate analysis confirmed known predictors of DFS in esophageal adenocarcinoma (OAC) that are detailed in Table 4. Factors that retained significance for the prediction of worse DFS on multivariate analysis were: vascular invasion (HR = 1.929, 95%CI: 1.034-3.6, $P = 0.039$), perineural invasion (HR = 2.766, 95%CI: 1.444-5.3, $P = 0.002$), no significant response to NAC (HR = 6.315, 95%CI: 1.261-31.616, $P = 0.025$) and the absence of lymph node downstaging (HR = 6.161, 95%CI: 1.683-22.554, $P = 0.006$).

The relationship of lymph node downstaging and status with clinicopathological characteristics and DFS

Patients with no pathological lymph node involvement were compared (pN_0 *vs* ypN_0), grouped as those who had surgery alone (pN_0) *vs* multimodal therapy (ypN_0), with detailed clinical and pathological characteristics presented in Table 5 and DFS shown in Figure 1C.

For patients with no evidence of pathological lymph node involvement increased pre-operative clinical stage (cT stage, $P < 0.0001$; cN stage, $P < 0.0001$) of disease and increased nodal downstaging (NAC 83.6% *vs* surgery alone 37.5%, $P < 0.0001$) was observed in patients who received multimodal therapy *vs* surgery alone despite pathological stage being similar (yp or pT stage, $P = 0.224$; yp or pN stage, $P = 1.00$).

Patients who underwent surgery alone (pN_0) had increased DFS compared to patients who underwent NAC and surgery (ypN_0) (mean DFS; pN_0: 6.285 years, 95%CI: 5.647-6.923 *vs* ypN0: 5.102 years, 95%CI: 4.314-5.891 (median DFS's: not reached, $P = 0.042$).

Evaluation of combined local tumour response grade and lymph node downstaging

Eighty-three point three percent of responders' additionally demonstrated downstaging of their regional lymph nodes compared to only 30% of non-responders, spread across TRG 3-5, Figure 2.

The presence of lymph node downstaging in apparent non-responders was associated with significantly improved DFS (median DFS; TRG 3-5 and nodal down-staging: 5.544, 95%CI: 3.558-7.531 *vs* TRG 3-5 and LN not downstaged: 1.114, 95%CI: 0.961-1.267, $P < 0.0001$), Figure 3.

DISCUSSION

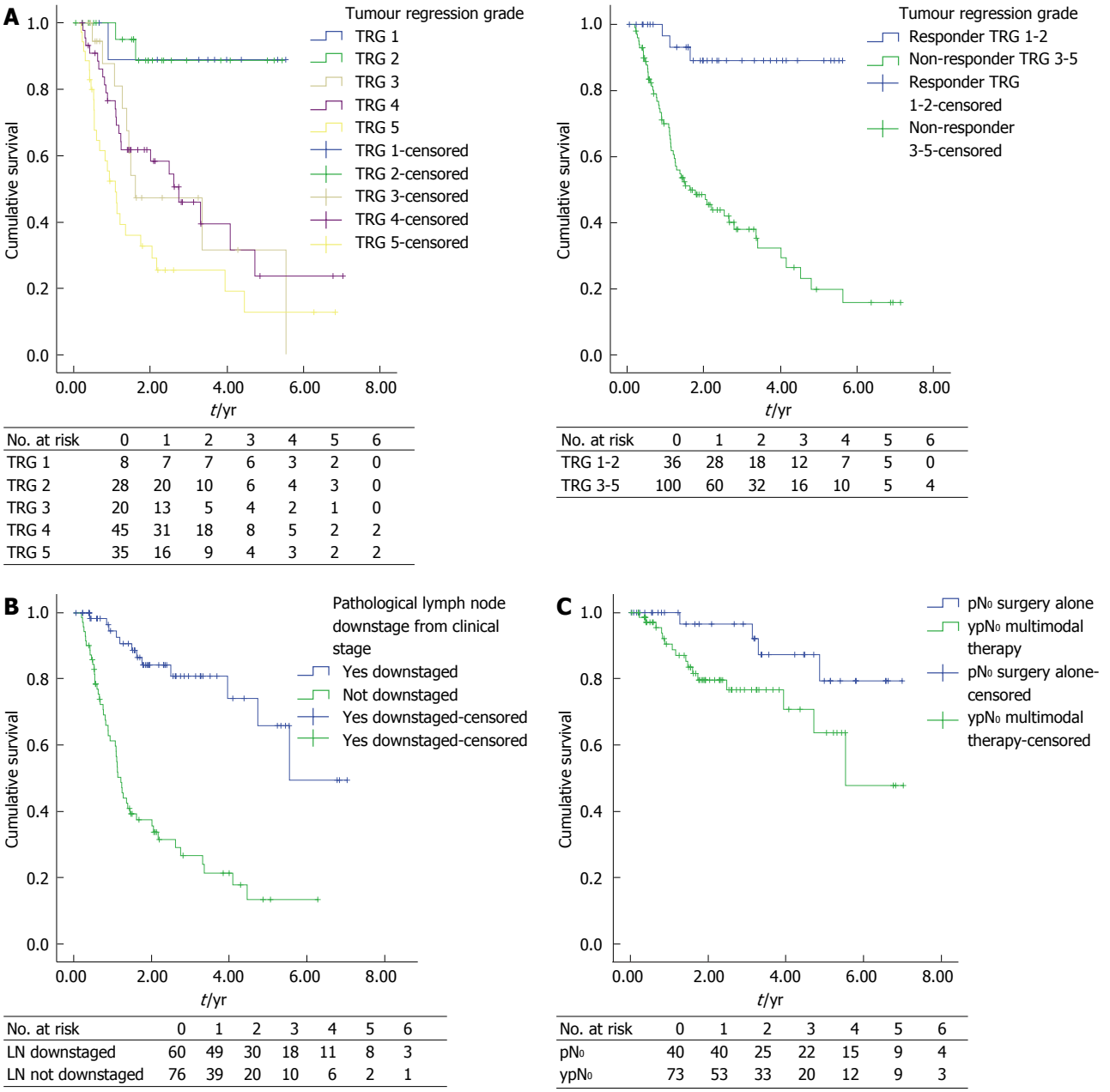
Neoadjuvant treatment for esophageal cancer is associated with increased survival. However, it is clear that not all patients (and their tumours) respond to neoadjuvant therapy in the same way. It is likely that improved outcomes will be observed by the tailoring of neoadjuvant and adjuvant therapy based on patient stratification according to tumour response.

In this study we have analysed a consecutive cohort of patients with esophageal adenocarcinoma (OAC) undergoing treatment with curative intent to assess the primary tumour and regional lymph node response to NAC. We have described three main findings: firstly, we

Table 3 Clinical and pathological characteristics of the 136 patients treated with neoadjuvant chemotherapy for esophageal and gastro-esophageal adenocarcinoma, classified as responders Tumour regression grade 1-2 or non-reponders tumour regression grade 3-5 *n* (%)

		TRG 1-2 36 (26.5)	TRG 3-5 100 (73.5)	*P* value
Preoperative status				
Age (range) yr[1]		65.27 (26.99-76.04)	63.51 (32.77-81.28)	0.410
Sex ratio (M:F)[1]		32 (88.9):4 (11.1)	86 (86):14 (14)	0.662
cT stage	1	0 (0)	0 (0)	0.396
	2	2 (5.6)	14 (14)	
	3	33 (91.7)	81 (81)	
	4	1 (2.8)	5 (5)	
cN stage	0	5 (13.9)	14 (14)	0.987
	1	31 (86.1)	86 (86)	
cM stage	0	35 (97.1)	99 (99)	0.456
	1	1 (2.8)	1 (1)	
Performance status	0	12 (33.3)	23 (23)	0.225
	1	23 (63.9)	73 (73)	
	2	1 (2.8)	4 (4.0)	
ASA	1	2 (5.6)	9 (9.1)	0.408
	2	32 (88.9)	74 (74.7)	
	3	2 (5.6)	16 (16.2)	
O-POSSUM		15 (12-23)	16 (12-26)	0.476
Tumour site	Middle 1/3	1 (2.8)	0 (0)	0.738
	Lower 1/3	15 (41.7)	42 (42)	
	GEJ-S1	7 (19.4)	16 (16)	
	GEJ-S2	9 (25)	25 (25)	
	GEJ-S3	4 (11.1)	16 (16)	
Operative outcomes				
Length of operation (min)[1]		262 (163-427)	260 (120-471)	0.513
Blood loss (mL)[1]		300 (0-3000)	325 (0-1700)	0.673
Clavien Dindo Max	0	14 (38.9)	39 (39.4)	0.531
	1	2 (5.6)	6 (6.1)	
	2	14 (38.9)	26 (26.3)	
	3	4 (11.1)	13 (13.1)	
	4	2 (5.6)	15 (15.2)	
	5	0 (0)	0 (0)	
Anastomotic leaks		1 (2.8)	8 (8.1)	0.276
Pathological outcomes				
yPT	0	8 (22.2)	0 (0)	< 0.0001
	1	11 (30.6)	12 (12)	
	2	9 (25)	25 (25)	
	3	8 (22.2)	58 (58)	
	4	0 (0)	5 (5)	
yPN	0	34 (94.4)	39 (39)	< 0.0001
	1	0 (0)	21 (21)	
	2	2 (5.6)	23 (23)	
	3	0 (0)	17 (17)	
yPM	0	36 (100)	100 (100)	0.579
Nodal downstaged (cN1 to ypN0)		30 (83.3)	30 (30)	< 0.0001
Positive nodes[1]		0 (0-5)	1 (0-24)	< 0.0001
Nodal yield[1]		18 (4-25)	18 (3-53)	0.984
Resection clearance	R0	35 (97.2)	75 (75)	0.004
Vascular invasion		4 (11.1)	37 (37)	0.004
Lymphatic invasion		4 (11.1)	18 (18)	0.338
Perineural invasion		2 (5.6)	18 (18)	0.072
Maximum tumour diameter[1]	(mm)	15 (0-110)	30 (0-155)	< 0.0001
Morphology	Ulcer	30 (93.8)	66 (68)	0.003
	Polypoid	2 (6.3)	21 (21.6)	
	Fungating	0 (0)	3 (3.1)	
	Diffuse infiltrating	0 (0)	7 (7.2)	
Grade	G1	8 (22.2)	8 (8)	0.104
	G2	9 (25)	28 (28)	
	G3	19 (52.8)	63 (63)	
	G4	0 (0)	1 (1)	
Sites of recurrence	Local	0 (0)	8 (8.1)	0.080
	Nodal	1 (2.8)	13 (13.1)	0.082
	Distant	2 (5.6)	42 (42.4)	< 0.0001

Values in parentheses are percentages unless indicated. [1]Values in parentheses are range. TRG: Tumour Regression Grade; ASA:American Society of Anesthesiologists classification; O-POSSUM: Oesophagogastric surgery-physiological and operative severity score for the enumeration of mortality and morbidity; GEJ-S1-3: Gastro-esophageal junction-Siewert type 1-3.



No. at risk	0	1	2	3	4	5	6
TRG 1	8	7	7	6	3	2	0
TRG 2	28	20	10	6	4	3	0
TRG 3	20	13	5	4	2	1	0
TRG 4	45	31	18	8	5	2	2
TRG 5	35	16	9	4	3	2	2

No. at risk	0	1	2	3	4	5	6
TRG 1-2	36	28	18	12	7	5	0
TRG 3-5	100	60	32	16	10	5	4

No. at risk	0	1	2	3	4	5	6
LN downstaged	60	49	30	18	11	8	3
LN not downstaged	76	39	20	10	6	2	1

No. at risk	0	1	2	3	4	5	6
pN0	40	40	25	22	15	9	4
ypN0	73	53	33	20	12	9	3

Figure 1 Kaplan-Meier curve of patients. A: Patients (n = 136) received neoadjuvant chemotherapy grouped by tumour regression grade. Left: Tumour Regression Grade (TRG) 1-5 (P < 0.0001); Right: TRG 1-2 *vs* TRG 3-5 (P < 0.0001); B: Patients (n = 136) received neoadjuvant chemotherapy grouped by presence or absence of lymph node downstaging (P < 0.0001); C: Patients (n = 113) with no pathological lymph node metastasis grouped by treatment (P = 0.042).

have confirmed that a significant pathological response as described by Mandard *et al*[14] is associated with improved DFS; Secondly we have confirmed that lymph node downstaging leads to improved DFS[10]; Thirdly, and most importantly, we describe that when tumour and nodal response are combined, a group of patients who previously would have been classified as non-responders to NAC actually have significantly increased DFS.

There is considerable debate regarding the role of tumour regression in OAC. Conflicting opinions are evident, for what represents a significant tumour response, even within the TRG grading system. In our study TRG-3 tumours, despite representing tumours whose fibrosis outgrows the residual tumour, clearly grouped with TRG-4 and TRG-5 and not TRG-1 and TRG-2 tumours in terms of DFS. This is in keeping with previous studies that have observed a significant increase in survival and/or metabolic response on serial PET imaging for TRG groups 1 and 2 compared to TRG groups 3 to 5[14,18,19,22,28,29]. In addition, we found there to be no significant difference in DFS between complete pathological responders (TRG-1) *vs* major responders (TRG-2) consistent with other studies[14,22]. As has been previously suggested this may reflect a type II error due to insufficient sample size or the intensity of pathological sampling[22]. The observed increase in DFS in patients with a significant tumour response to NAC in this study may also reflect the significantly increased resectability (R0 rate) of the primary tumour. It may also reflect the selection of tumours that are biologically more favourable as suggested by reduced vascular invasion (P = 0.004), tu-

Table 4 Univariate and multivariate Cox regression analyses of patient and tumour factors with disease free survival for patients undergoing neoadjuvant chemotherapy (n = 136)

		Univariate			Multivariate		
		HR	95%CI	*P* value	HR	95%CI	*P* value
Patient factors							
Age		0.972	(0.944-1.00)	0.054			
Sex	Female	1.000	Ref				
	Male	0.953	(0.453-2.005)	0.899			
ASA	1	1.000	Ref				
	2	0.696	(0.313-1.548)	0.374			
	3	0.947	(0.352-2.546)	0.914			
Performance status	0	1.000	Ref				
	1	1.016	(0.578-1.789)	0.955			
	2	0.950	(0.218-4.129)	0.945			
O-POSSUM							
Tumour response							
TRG	1	1.000	Ref				
	2	1.099	(0.099-12.148)	0.939			
	3	8.404	(1.071-65.929)	0.043			
	4	7.829	(1.054-58.163)	0.044			
	5	15.422	(2.083-114.189)	0.007			
TRG grouped	1-2	1.000	Ref		1.000	Ref	
	3-5	9.504	(2.973-30.380)	< 0.0001	6.315	(1.261-31.616)	0.025
Lymph node response							
Lymph nodes downstaged	Yes	1.000	Ref		1.000	Ref	
	No	5.784	(3.064-10.919)	< 0.0001	6.161	(1.683-22.554)	0.006
Tumour factors							
ypT stage	0	1.000	Ref		1.000	Ref	
	1	2.085	(0.232-18.711)	0.512	0.281	(0.020-3.928)	0.345
	2	5.214	(0.687-39.549)	0.110	0.286	(0.022-3.705)	0.338
	3	9.490	(1.293-69.635)	0.027	0.469	(0.034-6.460)	0.571
	4	52.907	(6.008-465.873)	< 0.0001	1.519	(0.087-26.389)	0.774
ypN stage	0	1.000	Ref		1.000	Ref	
	1	4.791	(2.434-9.431)	< 0.0001	0.476	(0.133-1.700)	0.253
	2	4.102	(2.005-8.392)	< 0.0001	0.254	0.070-0.927)	0.038
	3	7.449	(3.522-15.756)	< 0.0001	0.476	(0.129-1.755)	0.265
ypM stage	0	1.000	Ref		1.000	Ref	
	1	3.172	(1.253-8.031)	0.015	2.693	(0.924-7.847)	0.069
Vascular invasion	No	1.000	Ref		1.000	Ref	
	Yes	3.444	(2.080-5.702)	< 0.0001	1.929	(1.034-3.600)	0.039
Lymphatic invasion	No	1.000	Ref		1.000	Ref	
	Yes	2.201	(1.268-3.821)	0.005	1.253	(0.637-2.462)	0.514
Perineural invasion	No	1.000	Ref		1.000	Ref	
	Yes	5.073	(2.896-8.886)	< 0.0001	2.766	(1.444-5.300)	0.002
Resection clearance	R0	1.000	Ref		1.000	Ref	
	R1	3.869	(2.272-6.588)	< 0.0001	1.805	(0.940-3.468)	0.076

TRG: Tumour Regression Grade; ASA: American Society of Anesthesiologists classification; O-POSSUM: Oesophagogastric surgery-physiological and operative severity score for the enumeration of mortality and morbidity.

mour morphology (P = 0.003) and increased lymph node downstaging (P < 0.0001).

In this study we confirmed the association between lymph node downstaging after NAC and improved DFS[10]. Bollschweiler *et al*[3] showed regression in lymph nodes, such as central fibrosis, to predict improved survival and response to chemoradiotherapy[3]. This would require additional pathological time and expertise whereas downstaging can be more simply assessed from the data available to the multidisciplinary team (MDT) after surgery, to assess a patient's prognosis and potential for adjuvant therapies. The number of positive lymph nodes is consistently the most important prognostic factor associated with survival[30]. However, the clinical significance of downstaging is controversial due to the difficulties in evaluating preoperative status. This study has the advantage of using contemporary and uniformly implemented clinical staging based on current United Kingdom practice. The comparison of nodal stage based on pre-operative staging assessment (cN) and post-operative pathology (pN) is open to the criticism that any downstaging simply reflects overdiagnosis of lymph node metastases on preoperative staging. To address this point we assessed the survival of patients with no positive lymph nodes in the pathological specimen, comparing NAC with surgery alone (ypN$_0$ *vs* pN$_0$). We found that patients receiving NAC with ypN$_0$ disease had reduced DFS across all sites of recurrence compared to patients treated by surgery alone with pN$_0$ disease. This reached statistical significance when overall DFS was assessed (P = 0.042). Whilst the patients that underwent multimodal or surgery only had comparable pathological

Table 5 Clinical and pathological characteristics of the 113 patients with pathological N0 stage, according to treatment *n* (%)

		pN0 Surgery alone 40 (35.4)	ypN0 Neoadjuvant chemotherapy and surgery 73 (64.6)	*P* value
Preoperative status				
Age (range) yr[1]		73.62 (56.73-85.41)	65.59 (32.77-78.43)	< 0.0001
Sex ratio (M:F)[1]		31 (77.5):9 (22.5)	66 (90.4):7 (9.8)	0.061
cT stage	1	13 (32.5)	0 (0)	< 0.0001
	2	17 (42.5)	9 (12.3)	
	3	10 (25)	61 (83.6)	
	4	0 (0)	3 (4.1)	
cN stage	0	27 (67.5)	13 (17.8)	< 0.0001
	1	13 (32.5)	60 (82.2)	
cM stage	0	40 (100)	71 (97.3)	0.293
	1	0 (0)	2 (2.8)	
Performance status	0	3 (9.4)	16 (21.9)	0.045
	1	25 (78.1)	54 (74)	
	2	4 (12.5)	3 (4.1)	
ASA	1	2 (5)	6 (8.2)	0.268
	2	31 (77.5)	59 (80.8)	
	3	7 (17.5)	8 (11)	
O-POSSUM[1]		17 (14-29)	16 (12-26)	0.015
Tumour site	Middle 1/3	0 (0)	1 (1.4)	0.190
	Lower 1/3	15 (37.5)	35 (47.9)	
	OGJ-S1	11 (27.5)	12 (16.4)	
	OGJ-S2	8 (20)	16 (21.9)	
	OGJ-S3	6 (15)	9 (12.3)	
Operative outcomes				
Length of operation (min)[1]		240 (120-360)	278 (120-471)	0.082
Blood loss (mL)[1]		200 (0-2200)	350 (0-3000)	0.167
Clavien Dindo Max	0	14 (35)	24 (32.9)	0.709
	1	1 (2.5)	3 (4.1)	
	2	17 (42.5)	27 (37)	
	3	4 (10)	10 (13.7)	
	4	2 (5)	9 (12.3)	
	5	2 (5)	0 (0)	
Anastomotic leaks		4 (10)	7 (9.6)	0.944
Pathological outcomes				
TRG 1-2		-	34 (46.6)	NA
TRG 3-5		-	39 (53.4)	
pT or ypT	0	2 (5)	11 (15.1)	0.224
	1	22 (55)	20 (27.4)	
	2	5 (12.5)	20 (27.4)	
	3	10 (25)	24 (32.9)	
	4	0 (0)	1 (1.4)	
Nodal Downstaged (cN1 to p or ypN0)		15 (37.5)	61 (83.6)	< 0.0001
Nodal yield[1]		16 (4-49)	18 (3-52)	0.150
Resection clearance	R0	35 (87.5)	69 (94.5)	0.189
Vascular invasion		7 (17.5)	10 (13.7)	0.590
Lymphatic invasion		2 (5)	6 (8.2)	0.525
Perineural invasion		2 (5)	5 (6.8)	0.698
Maximum tumour diameter (mm)[1]		24 (0-50)	24 (0-110)	0.324
Morphology	Ulcer	25 (65.8)	53 (79.1)	0.135
	Polypoid	10 (26.3)	11 (16.4)	
	Fungating	1 (2.6)	1 (1.5)	
	Diffuse infiltrating	2 (5.3)	2 (3)	
Grade	G1	4 (10)	13 (17.8)	0.811
	G2	17 (42.5)	20 (27.4)	
	G3	19 (47.5)	40 (54.8)	
	G4	0 (0)	0 (0)	
Site of recurrence	Local	0 (0)	2 (2.7)	0.293
	Nodal	1 (2.5)	4 (5.5)	0.463
	Distant	3 (7.5)	12 (16.4)	0.182

Values in parentheses are percentages unless indicated. [1]Values in parentheses are range. ASA: American society of anesthesiologists classification; O-POSSUM: Oesophagogastric surgery-physiological and operative severity score for the enumeration of mortality and morbidity; NA: Not available.

staged disease they are different based on their clinical stage and survival. It is therefore unlikely that our clinical staging was inadequate and suggests that the majority of patients with ypN0 disease in fact had lymph node metastases prior to treatment.

The increased survival observed with lymph node

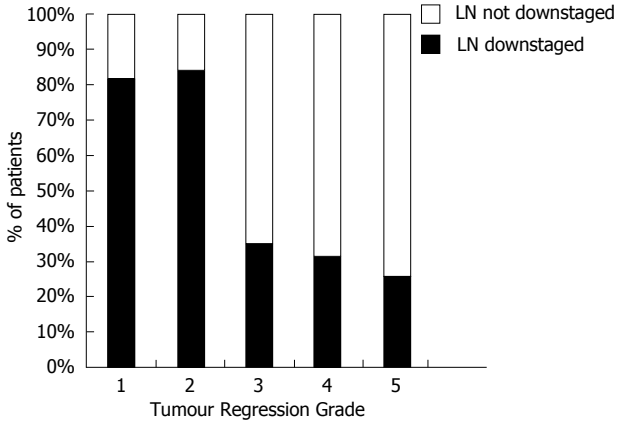
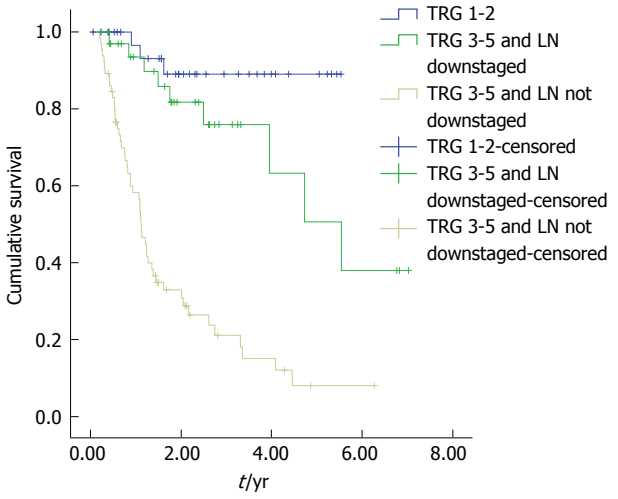


Figure 2 Percentage of patients who received neoadjuvant chemotherapy (*n* = 136) having lymph node downstaging grouped by Tumour Regression Grade. LN: Lymph node.



No. at risk	0	1	2	3	4	5	6
TRG 1-2	36	28	18	12	7	5	0
TRG 3-5 and LN downstaged	30	25	16	9	5	4	3
TRG 3-5 and LN not downstaged	70	35	16	7	5	1	1

Figure 3 Kaplan-Meier curves of patients undergoing multimodal therapy (*n* = 136) grouped based on a combination of tumour regression grade and lymph node downstaging (*P* < 0.0001). TRG: Tumour Regression Grade; LN: Lymph node.

downstaging has important implications for the staging of OAC as neoadjuvant therapy is increasingly used. Although the final pathological stage of disease may be similar between patients treated with either multimodal therapy or surgery alone we have demonstrated that the long-term DFS of these patients are different. This would suggest revisions for the staging system for OAC to take into account the differences in outcomes for patients who have similar pathologically staged disease after multimodal therapy compared to those treated by surgery alone. This hypothesis is further supported by the results of our multivariate analysis of factors independently related to outcome in neoadjuvant chemotherapy for OAC. This showed that nodal downstaging and TRG were independent predictors of DFS but that the classical markers of disease burden, PT stage and PN stage, were only statistically significant on univariate analysis. Similar observations and suggestions have been made for patients who have undergone neoadjuvant chemoradiotherapy followed by surgery when compared to patients who underwent surgery alone[31].

There are several advantages of our study compared to other published series. This study consists of a large number of consecutive patients (*n* = 218) of uniform histological type, with consistent clinical and pathological staging and treatment provided over a contemporary time period. The retrospective nature of this study and the use of multiple pathologists assessing TRG on an individual basis are potential limitations. However, the data was vigorously collected prospectively and the use of multiple pathologists reflects the usefulness of TRG in clinical practice and is pragmatic. A debate also remains as to what system to use to assess a local tumour response to neoadjuvant therapy[10,11,14,15,17-19]. The use of TRG is not without controversy as significant tumour regression has been reported in patients who underwent surgery alone, in up to 13.7% of cases. It has been suggested that this reflects tumour growth within abundant stroma and/or lymphocytic infiltration leading to partial tumour regression[21]. While the association of lymphocytic infiltration and stromal features with survival in cancer is not new their association with survival in OAC is yet to be fully understood and the clinical impact is unknown[32].

Although a good pathological response of the primary tumour might be expected to represent a prognostic predictor after NAC, the low response rate observed following NAC remains problematic. In this study we observed a significant response rate of 26.5% (*n* = 36) as assessed by TRG. However when lymph node downstaging is also considered this proportion increases to 48.5% (*n* = 66). It can be hypothesised that patients who have a partial response to NAC reflected by downstaging of lymph nodes with modest or no response in the primary tumour (TRG 3-5) may be the most appropriate to be considered for trials of adjuvant treatment; as there is limited data from other disease sites to suggest only patients responding to neoadjuvant treatment benefit from further treatment[33]. This is relevant as the role of adjuvant therapy in esophageal cancer is controversial due to concerns over the additional benefit of post operative treatment over neoadjuvant alone[8] and toxicity[34], and has resulted in the lack of adoption in the United Kingdom[1]. What is clear is that the group of patients with no significant downstaging and ypN+ post neoadjuvant treatment have a particularly poor outlook. This group urgently requires identification at diagnosis and new trial treatments. This requires the ongoing studies of prognostic and predictive biomarkers from this cohort and others to yield meaningful and validated results.

One can now begin to consider an evolving algorithm for perioperative treatment of OAC that may involve induction chemotherapy followed by an early assessment of response and the curtailment of, or a change of, neoadjuvant therapy for non-responders. Further analysis of the primary tumour and lymph nodes after surgery would

direct patients with modest or no tumour response (TRG 3-5) to NAC, but with nodal downstaging, to adjuvant therapy. This kind of stratified therapy will be supported by ongoing studies of biomarkers and molecular imaging. The contribution of the tumour microenvironment is also likely to offer new targets for therapy and may be the place to look to explain the different responses to therapy observed between otherwise similar tumours.

In summary, this study has shown that a response to NAC in the primary tumour and in the lymph nodes is associated with improved outcomes after surgery for adenocarcinoma of the esophageal and gastro-esophageal. A previously unidentified group of patients who appear to have a poor tumoural response to NAC (TRG 3-5) do benefit from NAC with nodal downstaging and increased DFS.

We propose that methods to assess the pathological response to NAC are refined so that both the response in the primary tumour and the regional lymph nodes is used to guide selection of tailored post operative treatment strategies, identify biomarkers of response to chemotherapy, provide prognostic information and assess multimodal therapies.

COMMENTS

Background

Adenocarcinoma of the esophagus and esophageal adenocarcinoma (OAC) is a significant and increasing health problem in many countries; linked to rates of obesity, smoking, gastro-esophageal reflux disease and Barrett's oesophagus. At presentation, even in operable cases, tumours are often locally advanced (T3N1) with multi-institutional randomised studies of surgery alone giving 5 years survival rates in the order of 15%-24%. So as well as a focus on earlier detection and screening of at risk groups, clinical research has focused on adjuvant and specifically neo-adjuvant treatments prior to resection.

Research frontiers

Neoadjuvant chemotherapy can be considered one standard of care, with a modest improvement in outcome over surgery alone; detailed in a recent meta-analysis as HR = 0.83 (95%CI: 0.71-0.95), or an absolute benefit of 5%-10% at 2 years. A key focus now is on identifying optimum neoadjuvant approaches (which chemotherapy regimens, chemoradiotherapy, small molecule inhibitors, biologic agents *etc*) and which patients should receive them *e.g.*, patients with human epidermal growth factor receptor (HER)-2 over expressing tumours receiving a Trastuzumab containing regimen.

Innovations and breakthroughs

To date prognostic information for OAC has been from standard clinicopathological data, and bar HER-2 expression predictive markers of response to treatment are lacking. The authors cannot predict at diagnosis who is going to gain from neoadjuvant treatment. Globally collaborative groups have been set up to generate large clinical datasets to link patient outcomes to molecular features: groups such as the oesophageal cancer clinical and molecular stratification study group in the United Kingdom, which are beginning to highlight important molecular determinants of OAC behaviour and identify attractive targets for therapy. The expectation is this will lead to valuable prognostic information and also identify who should, and should not proceed to a particular neoadjuvant strategy.

Applications

The identification here that both T and N downstaging post neoadjuvant treatment need to be accounted for will help refine clinical datasets and provide prognostic information, as well as inform decisions concerning adjuvant treatment.

Terminology

When reporting the anatomical extent of cancer after preoperative treatment has been given pathologists include the prefix "y" to the PTNM.

Peer review

This study is an excellent clinical research as it confirms the association between regression grade and prognosis in a large and histologically homogenous group of patients treated with platinum based triplet chemotherapy and staged uniformly. It contains novel findings that are clinically relevant to physicians treating oesophageal cancer and assessment of both T and N responses to neoadjuvant therapy may be of relevance and interest to specialists treating other solid tumours.

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ORIGINAL ARTICLE

Hepatitis B virus subgenotype A1 predominates in liver disease patients from Kerala, India

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Abstract

AIM: To molecularly characterize hepatitis B virus (HBV) isolates from Kerala and to relate them to the clinical manifestation of infection.

METHODS: Sera and clinical data were collected from 91 patients diagnosed with chronic HBV infection and HBV-related hepatocellular carcinoma (HCC). HBV from 44 HCC, 22 cirrhotic and 25 chronic hepatitis patients were genotyped by sequencing of the complete S region or by restriction fragment length polymorphism assays. The basic core promoter/precore region was sequenced. The complete surface DNA sequences were assembled and aligned manually, and then compared with the sequences of HBV of genotypes (A-J) from GenBank. The evolutionary history was inferred using the Neighbor-Joining method and the evolutionary distances computed using the Kimura 2-parameter method. Bootstrapping was performed using 1000 replicates. The TaqMan BS-1 probe was used to quantify HBV DNA at a lower detection limit of approximately 20 IU/mL. Continuous variables were compared using an independent Student's t test. The χ^2 test or Fisher's exact test was used to compare categorical variables. The differences were considered statistically significant at $P < 0.05$.

RESULTS: Irrespective of disease status, the predominant genotype was A (72%); 95% belonging to subgenotype A1, followed by genotypes D (27%) and C (1%). HCC patients infected with subgenotype A1 were significantly younger than those infected with D. Mutation A1762T/G1764A was significantly associated with HCC in both genotypes A and D. Mutation G1862T was more frequent in subgenotype A1 ($P < 0.0001$), and in combination with A1762T/G1764A, it was significantly associated with HBV from HCC patients. Mutation C1766T/T1768A was significantly associated with

genotype A (P = 0.05) and HCC (P = 0.03). The preS2 start codon M1T/I mutation was unique to genotype A strains (15.6%) from all disease groups and occurred at a higher frequency in isolates from HCC patients (P = 0.076). A higher frequency of preS deletion mutants (33.3%) was observed in genotype A from HCC compared with non-HCC patients, but did not reach statistical significance. The preS2:F22L mutation was found in genotypes A and D.

CONCLUSION: Kerala is the first Indian state in which subgenotype A1 has been found to predominate in liver disease patients who developed HCC at a relatively young age.

Key words: Hepatocellular carcinoma; Cirrhosis; Chronic hepatitis; Phylogenetic analysis; Genotype; India

Core tip: This study shows the predominance of subgenotype A1 in liver disease patients in Kerala, and its high prevalence in hepatocellular carcinoma (HCC) patients. Subgenotype A1 could be more hepatocarcinogenic and HCC could develop at an earlier age, regardless of host ethnicity. The S open reading frame of subgenotype A1 isolates from Kerala clustered separately within the "Asian" cluster and encoded distinct subgenotype A1 amino acids. A higher frequency of G1862T was detected compared with subgenotype A1 isolates from other geographical regions. This is the first time that preS deletion mutants have been described in Indian HCC patients.

Gopalakrishnan D, Keyter M, Shenoy KT, Leena KB, Thayumanavan L, Thomas V, Vinayakumar KR, Panackel C, Korah AT, Nair R, Kramvis A. Hepatitis B virus subgenotype A1 predominates in liver disease patients from Kerala, India. *World J Gastroenterol* 2013; 19(48): 9294-9306 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i48/9294.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i48.9294

INTRODUCTION

Hepatitis B virus (HBV) is the prototype member of the family *Hepadnaviridae*. HBV replicates by reverse transcription using a polymerase that lacks proof reading ability, and sequence heterogeneity is a feature of this virus. Phylogenetic analysis of HBV full-length genomes has led to the classification of HBV into nine genotypes (A-I), defined by an intergroup divergence in the complete HBV genome sequence of 7.5% or more. A tenth genotype J, which was found in a single individual, has been proposed[1]. Genotypes A, B, C, D, F and I are further classified into subgenotypes. Most genotypes, and some subgenotypes, display distinct geographical distributions. Moreover, HBV genotypes and, in some cases, subgenotypes, have been shown to play an important role in the clinical consequences of the infection, as well as in the response to antiviral treatment.

HBV infection remains a significant global health problem, with an estimated two billion people infected and more than 240 million chronic carriers of the virus, leading to 600000 deaths from the clinical consequences of infection, including cirrhosis, liver failure and hepatocellular carcinoma (HCC). With a population of more than 1.2 billion people, India has the second largest global pool of chronic HBV infection and HBV is the major cause of liver disease in India[2].

Most studies have estimated the hepatitis B surface antigen (HBsAg) carrier rate to be between 2% and 8%, placing India within the zone of intermediate endemicity. An HBsAg prevalence rate of 2.97% was found among the rural population[3], and a meta-analysis has reported the mean prevalence in the general population of India as 3.3%[4]. However, these estimates have been questioned because, according to Phadke and Kale[5], the often quoted estimate for India of 4.7% was obtained by incorrectly pooling results of a set of studies including unrepresentative high risk groups and also equating the single test HBsAg positivity rate with the carrier rate. By correcting for these errors, they estimated a carrier rate of 1.4%.

The known HBV genotype distribution in India is summarized in Figure 1. Overall, at approximately 65%, genotype D predominates, being the dominant genotype in Delhi in the north, Pune in the west and the Nicobar Islands in the south. Genotype A has been found in approximately 30%, with the highest frequency found in northern India. At approximately 5%, genotype C is found in the minority, with the highest frequency in eastern and southern India. The subgenotypes that have been described in India include A1, A2, C1, C2, D1, D2, D3, D5 and D9[6-9].

Kerala is the most densely populated state of India, with a population of 33 million. HBsAg prevalence of 0.5% in the normal population has been reported in northern Kerala[10] and an HBsAg prevalence of 1.5% was detected among voluntary blood donors from Trivandrum, South Kerala[11]. There is a paucity of information on the prevalence of HBV genotypes and the respective subgenotypes in Kerala, as well as their association, if any, with different clinical manifestations following infection with HBV. We investigated the distribution of HBV genotypes/subgenotypes among patients with different clinical manifestations of HBV infection and characterized the viral isolates molecularly.

MATERIALS AND METHODS

Patients

The cross-sectional study was conducted from January 2005 to December 2009 during which sera and clinical data were collected from 91 patients diagnosed with chronic HBV infection and HBV-related HCC from

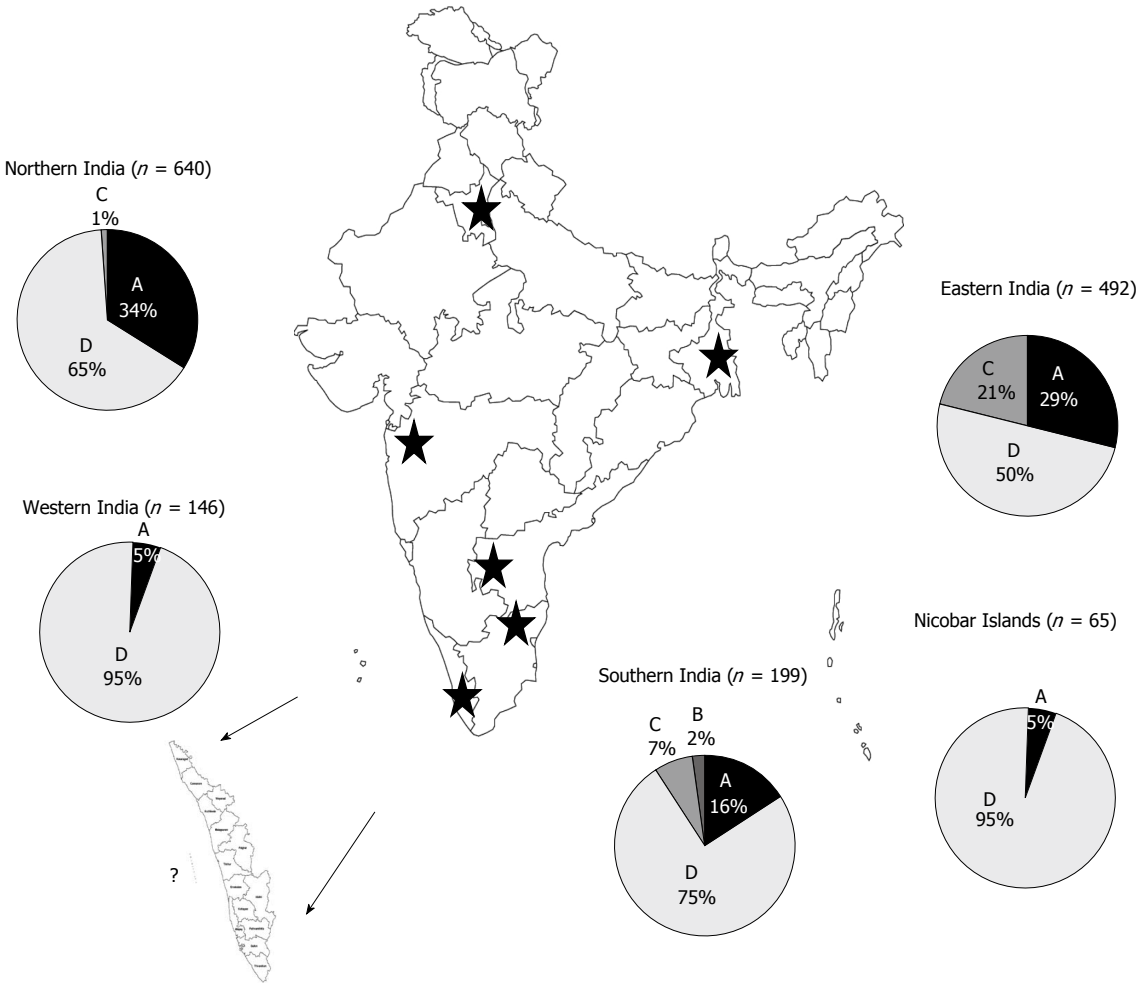


Figure 1 Prevalence of genotypes in different geographical areas of India compiled from previous reports. Northern[22,23,28,41,42]; Western[18,43]; Eastern[8,24,32,43-46]; Southern[6,47]; Nicobar Islands[48]. No hepatitis B virus genotyping data was available for Kerala before this study.

Medical College Trivandrum, Kerala, India. The serum samples were stored at -80 ℃ until use. A serum alanine transaminase (ALT) level of < 10 times the upper limit of normal (ULN), a serum bilirubin level of less than 2.5 times the ULN and detectable HBsAg for ≥ 6 mo were used as inclusion criteria. The presence of the hepatitis B e antigen (HBeAg) was examined at the time of screening. All patients were negative for antibodies to hepatitis C virus, hepatitis D virus and human immunodeficiency virus. The study protocol conformed to the 1975 Declaration of Helsinki. The ethics committees of the Medical College Trivandrum, India and the University of the Witwatersrand, South Africa approved the study.

The diagnosis of HBV-related liver disease was based on clinical data, laboratory tests, liver biopsy and imaging studies. The patients were classified into three groups: group- Ⅰ (HCC): the 44 patients with HCC were diagnosed by ultrasound scan and elevated serum α-fetoprotein levels (≥ 400 ng/mL) and the presence of a lesion of ≥ 5 cm; group- Ⅱ (CR-Cirrhosis): 22 patients, with necro-inflammatory damage, fibrosis with nodule formation confirmed by liver biopsy, and with ultrasonographical evidence of portal hypertension; group- Ⅲ (CH-Chronic Hepatitis): 25 patients, with HBsAg positive status for ≥ 6 mo with normal or intermittently elevated ALT (1.5 times the ULN). Patients in this group were considered for liver biopsy on the basis of elevated ALT levels and HBeAg-status, and diagnosed with cirrhosis using histological activity index (HAI) and Fibrosis scores.

Serological assays

All serum samples were screened for HBsAg and HBeAg using enzyme linked immunsorbent assay kits (DiaSorin S.*P*.A, Italy), according to the manufacturer's instructions. Laboratory evaluation included routine liver biochemistry (ALT and aspartate transaminase levels), total bilirubin, albumin, alkaline phosphatase, total protein and prothrombin time. Liver function tests were performed to find necro-inflammatory activity using a Hitachi 902 Fully Automated Chemistry Analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan). The ULN of ALT (40 IU/L) was used for diagnosis.

Real-time polymerase chain reaction quantification of HBV DNA

Polymerase chain reaction (PCR) primers, HBV-Taq1

and HBV-Taq2, covering a region of the S gene (321 to 401 from the *Eco*RI site) with a FAM/TAMRA labeled TaqMan BS-1 probe were used to quantify HBV DNA in an ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, United States). The second WHO International Standard for HBV Nucleic Acid Amplification Techniques (product code 97/750 National Institute for Biological Standards and Control; Hertfordshire, United Kingdom), which has a final concentration of 10^6 IU/mL, was used as the internal standard. The lower detection limit of our assay was approximately 20 IU/mL. The conversion formula of IU = copies/4.7 was used[12].

PCR and restriction fragment length polymorphism assay for genotyping and molecular characterization

Total HBV DNA was extracted from serum using a QIAamp DNA Blood Mini kit (QIAGEN GmbH, Hilden, Germany), according to the manufacturer's instructions. The complete S open reading frame (ORF) was amplified using nested PCR.

Primers S1F 5'-CAATCGCCGCGTCGCAGAAGATCTCAATC-3' (2410-2439 from the *Eco*RI site) and S1R 5'-TCCAGACCXGCTGCGAGCAAAACA-3' (1314-1291 from the *Eco*RI site) were used for the first round and S2F 5'-AATGTTAGTATTCCTTGGACTCATAAGGTGGG-3' (2451-2482 from the *Eco*RI site) and S2R 5'-AGTTCCGCAGTATGGATCGGCAGAGGA-3' (1280-1254 from the EcoRI site) were used for the second round PCR using previously reported reaction conditions[13]. The samples that did not amplify in the full S region were genotyped using restriction fragment length polymorphism (RFLP)[14]. Subgenotypes of A were also determined using a previously described RFLP assay, which uses the StuI recognition site, 5' AGG↓CCT3' at position 967-972 from the *Eco*RI site, found only in subgenotype A2 and genotype D, but not in subgenotype A1[15]. Thus, subgenotypes A1 and A2 could be differentiated. The basal core promoter (BCP)/Pre C region of HBV isolates was amplified using nested PCR.

Primers BCP1F 5'-GCATGGAGACCACCGTGAAC-3' (1606-1625 from the *Eco*RI site) and BCP1R 5'-GGAAAGAAGTCCGAGGGCAA-3' (1974-1955 from the *Eco*RI site), were used for the first round and BCP2F 5'-CATAAGAGGACTCTTGGACT-3' (1653-1672 from the *Eco*RI site) and BCP2R 5'-GGCAAAAAACAGAGTAACTC-3' (1959-1940 from the *Eco*RI site) were used for the second round, using previously reported reaction conditions.

Sequencing

The amplicons were prepared for direct sequencing using the BigDye Terminator v3.0 Cycle Sequencing Ready Reaction Kit and sequencing was performed with the ABI 3130XL Genetic analyzer (Applied Biosystems). The complete S ORF was analyzed as three overlapping fragments[13].

Phylogenetic analysis

The complete surface DNA sequences were assembled and aligned manually using MEGA 5 (http://www.megasoftware.net/mega.php). The sequences were compared with the sequences of HBV of genotypes (A-J) from GenBank. The evolutionary history was inferred using the neighbor-joining method and the evolutionary distances computed using the Kimura 2-parameter method. Bootstrapping was performed using 1000 replicates to determine the support for the specific nodes. The accession numbers of HBV isolates sequenced in this study have been deposited in GenBank as KC752137-KC752206.

Statistical analysis

Data were represented as mean ± SD. Continuous variables were compared using an independent Student's *t* test. The χ^2 test or Fisher's exact test was used to compare categorical variables. Odds ratio was calculated to assess the risk of HCC. All *P* values were two sided, and the difference was considered statistically significant for $P < 0.05$. The analysis was performed using Statistical package for Social Sciences (SPSS 15) program (SPSS Inc., Chicago, IL, United States).

RESULTS

Genotyping and phylogenetic analyses of HBV isolated from liver disease patients

Of the 91 HBsAg-positive sera, 86 were successfully genotyped using either RFLP or phylogenetic analysis of the S region (Table 1). Using the Lindh RFLP assay[14] for 36 HBV isolates, 30 belonged to genotype A and six to genotype D. Of the 30 genotype A isolates, 28 were subgenotype A1 and two were subgenotype A2, as determined by an alternative RFLP[15].

Following phylogenetic analysis of the complete S ORF of 50 isolates, 32 belonged to genotype A (subgenotype A1:A2, 31:1) (Figure 2A), 17 to genotype D (subgenotypes D1:D2:D3, 4:12:1) (Figure 2B) and one to genotype C (subgenotype C1) (Figure 2A). The genotype A strains belonged to serotype *adw*2 (84.4%) and *ayw*1 (15.6%). The genotype D strains were of serotype *ayw*3 (58.8%), *ayw*2 (35.3%) and *adw*3 (5.9%). The single subgenotype C1 strain was *adr*.

The subgenotype A1 isolates split into an "African" and an "Asian" cluster[16](Figure 2A). The 31 subgenotype A1 isolates from Kerala clustered within the Asian clade as a separate monophyletic clade and encoded the distinct subgenotype A1 amino acids, preS1:Q54, preS1:V74, preS1:A86, and preS1:V91 in the preS1 region and preS2:L32 in the preS2 region[17]. The majority of the isolates in the "Asian" cluster, including the Kerala isolates, had preS1:S5, preS1:S6, preS1:F25. The isolates in the African cluster displayed greater variation, with preS1:S5, preS1:S6; preS1:S5, preS1:A6 or preS1:5L, preS1:6P. There were, however, a number of amino acids in the preS1 and preS2 regions that differentiated the Kerala clade

Table 1 Demographic, clinical and virological characteristics of hepatitis B surface antigen-positive patients with different disease profiles

	Characteristic	Group Ⅰ HCC (*n* = 44)	Group Ⅱ CR (*n* = 22)	Group Ⅲ CH (*n* = 25)	Total (*n* = 91)
Demographic and clinical data	Gender (M/F)	33/11	18/4	18/7	69/22
	Age (yr) (mean ± SD)	48.70 ± 10.94[a]	40.68 ± 11.52	26.28 ± 11.10	40.87 ± 14.66
	ALT, IU/L	73.50 ± 11.65	70.50 ± 41.09	56.32 ± 30.01	67.45 ± 37.74
	HBeAg positive[1]	7 (24.14)	4 (14.3)	9 (37.5)	20 (26.67)
Virological characteristics	HBV DNA $\log_{10}$ copies/mL[1]	4.79 ± 1.41[a]	3.38 ± 1.69	3.27 ± 2.12	4.03 ± 1.83
	Number genotyped by direct sequencing/RFLP	21/19	9/12	19/6	86
	Genotype A	33 (82.5)[a]	14 (66.6)	15 (60)	62 (72.1)
	Subgenotype A1	30	14	15	59 (95)
	Subgenotype A2	3	-	-	3 (5)
	Genotype C	-	1 (4.8)	-	1 (1.2)
	Genotype D	7 (17.5)	6 (28.6)	10 (40)	23 (26.7)
	Subgenotype D1[2]	-	1	3	
	Subgenotype D2	4	2	6	
	Subgenotype D3	1	-	-	

[a]$P < 0.05$ *vs* chronic hepatitis. [1]Depletion of serum allowed the viral loads to be determined for only 28, and hepatitis B e antigen for 29 HCC sera; [2]Subgenotyping of genotype D was performed by direct sequencing. CH: Chronic hepatitis; HCC: Hepatocellular carcinoma; CR: Cirrhosis; SD: Standard deviation; ALT: Alanine aminotransferase; RFLP: Restriction fragment length polymorphism; HBeAg: Hepatitis B e antigen.

from other Asian strains. The majority of Kerala strains (22/31; 71%), had preS1:V48 in the preS1, whereas V/I/N/T was found in the other clades. In the preS2, 28/31 (90%), had preS2:T7, whereas the other Asian strains had either preS2:T7 or preS2:A7. In contrast to the other Asian strains that had preS2:T37, the Kerala strains had preS2:N37, as did the strains in the African clade. PreS2:P54 in the preS2 was found in 90% of the Kerala strains, whereas the other clades had a higher diversity of amino acids at this position (Figure 2). A cut off of 60% amino acid sequence identity was used to define the consensus sequence within the clades.

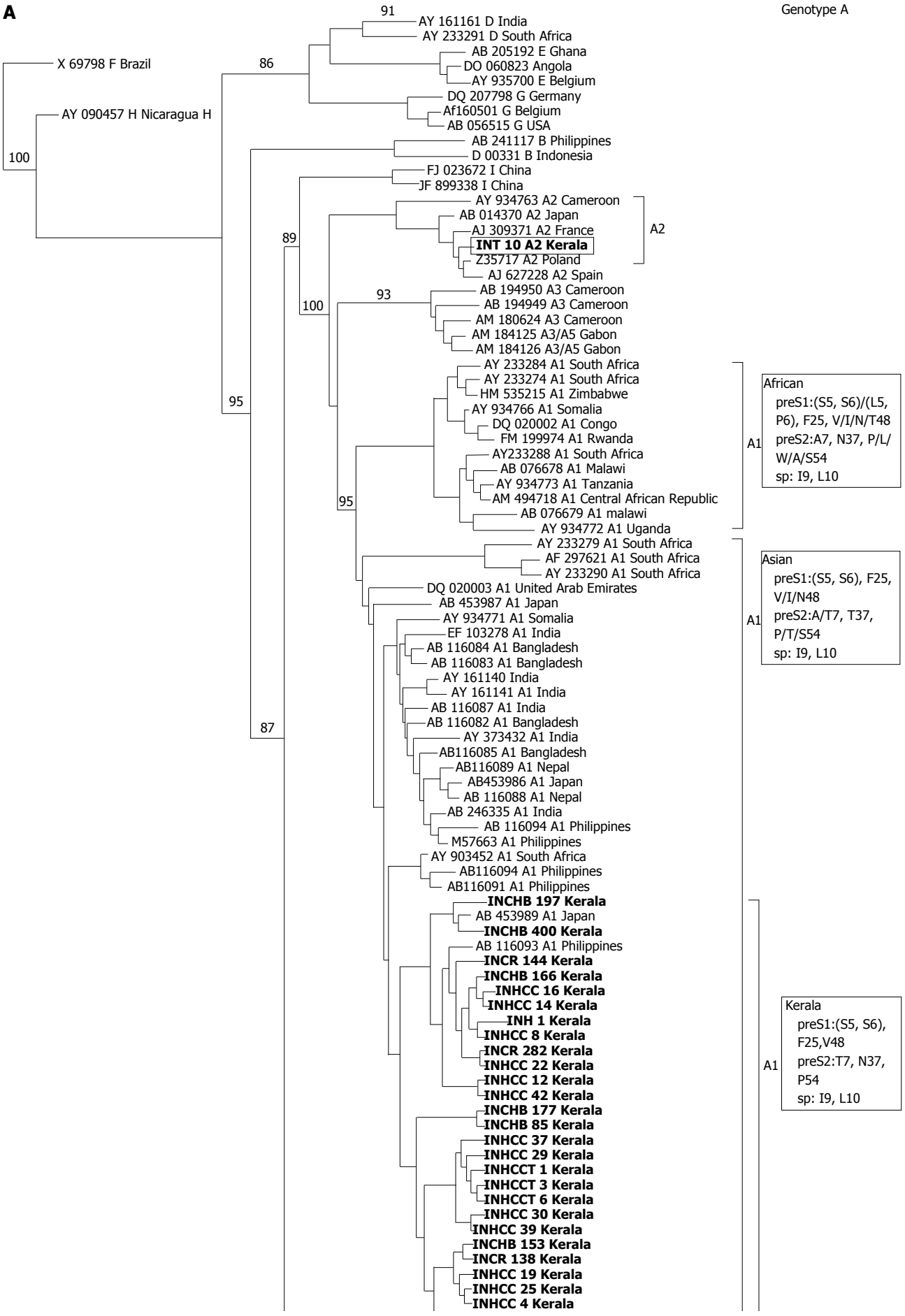
The Keralite genotype D isolates had the characteristic 33-nucleotide deletion in the preS1 region and had a relatively well-conserved polymerase overlapping preS1/preS2/S region when compared to subgenotype A1 sequences. The preS2 signature amino acids, preS2:I42, preS2:S43 and preS2:L54, were found in the Keralite strains belonging to subgenotype D1[7]; however, they differed by having preS2:39V instead of preS2:39A.

Demographic, clinical and virological characteristics

In all three disease groups, the frequency of males was significantly higher (Table 1). Patients with HCC were significantly older than CH patients ($P = 0.0001$). Twenty seven percent of the whole cohort was HBeAg-positive, with no significant difference in the frequency between the three disease groups. HBeAg-positive individuals were significantly younger than HBeAg-negative (32.1 ± 17.9 years *vs* 39.6 ± 11.1 years, $P = 0.032$) and had higher viral loads (5.4 ± 1.8 $\log_{10}$ IU/mL *vs* 3.6 ± 1.6 $\log_{10}$ IU/mL, $P = 0.016$). The ALT levels differed significantly between HBeAg-positive and negative patients (52.3 IU/L *vs* 37.4 IU/L, $P = 0.012$, equal variances not assumed). HCC patients had higher viral load defined by HBV DNA level ≥ 4.7×10^4 IU/mL compared with the non-HCC patients (18/29(62.1%) *vs* 10/33 (30.3%), respectively, $P = 0.012$, OR = 3.76, 95%CI: 1.16-12.52). The mean ALT values did not vary significantly between the three disease groups.

The majority of patients (72%) were infected with HBV genotype A, 27% with genotype D and one patient was infected with genotype C. The majority of genotype A strains (95%) belonged to subgenotype A1, and three to A2. Compared with the other disease groups, HCC patients were predominantly infected with subgenotype A1 ($P < 0.05$). Subgenotypes D1, D2 and D3 were found, with D2 (70.6%) predominating followed by D1 (23.5%) and a single strain of D3. Age, HBV viral load, the frequency of HBeAg-positivity and ALT levels, did not differ between those patients infected with genotype A and D in all the three groups. However, HBeAg-negative HCC patients, infected with genotype A, were significantly younger (44.1 ± 8.0 years) than those infected with genotype D (53.0 ± 8.8 years) ($P = 0.02$). There was no significant difference in the mean HAI (5.8 ± 2.8 *vs* 4.6 ± 3.1) and fibrosis scores (1.0 ± 1.8 *vs* 2.0 ± 2.0) between those with genotypes A and D. Genotype A was seen in 70% of the patients with HAI ≥ 4.

Detection of mutations in the BCP/Pre C region was performed for 63 HBV isolates (Table 2). BCP and/or Pre C mutants were detected in 63% of the isolates, with different mutational patterns found in genotypes A and D (Figure 3). Mutation 1773T was characteristic of genotype A and 1773C of genotype D, with no significant difference in the frequency of the mutation in the disease groups. Mutation G1896A occurred only in genotype D, whereas C1766T and G1862T occurred more frequently in genotype A (Figure 3). The double mutation A1762T/G1764A was found in 26 (41.2%) isolates, with the single mutation, G1764A, occurring in 12 (19%) isolates. Both the single and double BCP mutations were significantly


A
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X 69798 F Brazil
AY 090457 H Nicaragua H
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AY 233291 D South Africa
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DO 060823 Angola
AY 935700 E Belgium
DQ 207798 G Germany
Af160501 G Belgium
AB 056515 G USA
AB 241117 B Philippines
D 00331 B Indonesia
FJ 023672 I China
JF 899338 I China
AY 934763 A2 Cameroon
AB 014370 A2 Japan
AJ 309371 A2 France
INT 10 A2 Kerala
Z35717 A2 Poland
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A2
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AM 180624 A3 Cameroon
AM 184125 A3/A5 Gabon
AM 184126 A3/A5 Gabon
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AY 233274 A1 South Africa
HM 535215 A1 Zimbabwe
AY 934766 A1 Somalia
DQ 020002 A1 Congo
FM 199974 A1 Rwanda
AY233288 A1 South Africa
AB 076678 A1 Malawi
AY 934773 A1 Tanzania
AM 494718 A1 Central African Republic
AB 076679 A1 malawi
AY 934772 A1 Uganda
A1
African
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preS2:A7, N37, P/L/W/A/S54
sp: I9, L10
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AF 297621 A1 South Africa
AY 233290 A1 South Africa
DQ 020003 A1 United Arab Emirates
AB 453987 A1 Japan
AY 934771 A1 Somalia
EF 103278 A1 India
AB 116084 A1 Bangladesh
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AY 161140 India
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M57663 A1 Philippines
AY 903452 A1 South Africa
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A1
Asian
preS1:(S5, S6), F25, V/I/N48
preS2:A/T7, T37, P/T/S54
sp: I9, L10
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AB 453989 A1 Japan
INCHB 400 Kerala
AB 116093 A1 Philippines
INCR 144 Kerala
INCHB 166 Kerala
INHCC 16 Kerala
INHCC 14 Kerala
INH 1 Kerala
INHCC 8 Kerala
INCR 282 Kerala
INHCC 22 Kerala
INHCC 12 Kerala
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INHCCT 1 Kerala
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INHCC 30 Kerala
INHCC 39 Kerala
INCHB 153 Kerala
INCR 138 Kerala
INHCC 19 Kerala
INHCC 25 Kerala
INHCC 4 Kerala
A1
Kerala
preS1:(S5, S6), F25,V48
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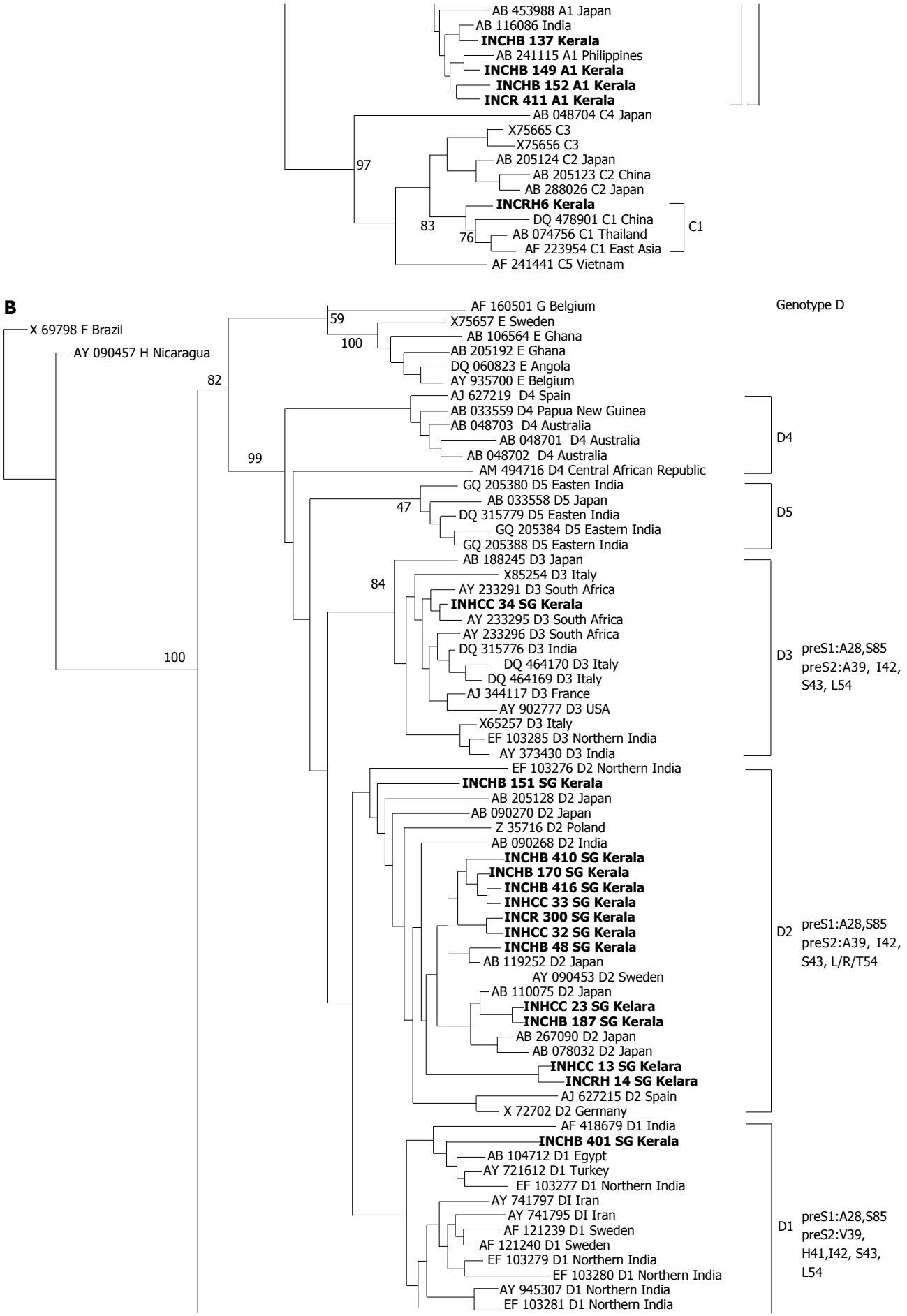
95
87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X75665 C3
X75656 C3
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C1
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83
76
AF 241441 C5 Vietnam
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AF 160501 G Belgium
X75657 E Sweden
AB 106564 E Ghana
AB 205192 E Ghana
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AY 935700 E Belgium
AJ 627219 D4 Spain
AB 033559 D4 Papua New Guinea
AB 048703 D4 Australia
AB 048701 D4 Australia
AB 048702 D4 Australia
AM 494716 D4 Central African Republic
D4
GQ 205380 D5 Easten India
AB 033558 D5 Japan
DQ 315779 D5 Easten India
GQ 205384 D5 Eastern India
GQ 205388 D5 Eastern India
D5
AB 188245 D3 Japan
X85254 D3 Italy
AY 233291 D3 South Africa
INHCC 34 SG Kerala
AY 233295 D3 South Africa
AY 233296 D3 South Africa
DQ 315776 D3 India
DQ 464170 D3 Italy
DQ 464169 D3 Italy
AJ 344117 D3 France
AY 902777 D3 USA
X65257 D3 Italy
EF 103285 D3 Northern India
AY 373430 D3 India
D3
preS1:A28,S85
preS2:A39, I42,
S43, L54
EF 103276 D2 Northern India
INCHB 151 SG Kerala
AB 205128 D2 Japan
AB 090270 D2 Japan
Z 35716 D2 Poland
AB 090268 D2 India
INCHB 410 SG Kerala
INCHB 170 SG Kerala
INCHB 416 SG Kerala
INHCC 33 SG Kerala
INCR 300 SG Kerala
INHCC 32 SG Kerala
INCHB 48 SG Kerala
AB 119252 D2 Japan
AY 090453 D2 Sweden
AB 110075 D2 Japan
INHCC 23 SG Kelara
INCHB 187 SG Kerala
AB 267090 D2 Japan
AB 078032 D2 Japan
INHCC 13 SG Kelara
INCRH 14 SG Kelara
AJ 627215 D2 Spain
X 72702 D2 Germany
D2
preS1:A28,S85
preS2:A39, I42,
S43, L/R/T54
AF 418679 D1 India
INCHB 401 SG Kerala
AB 104712 D1 Egypt
AY 721612 D1 Turkey
EF 103277 D1 Northern India
AY 741797 DI Iran
AY 741795 DI Iran
AF 121239 D1 Sweden
AF 121240 D1 Sweden
EF 103279 D1 Northern India
EF 103280 D1 Northern India
AY 945307 D1 Northern India
EF 103281 D1 Northern India
D1
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preS2:V39,
H41,I42, S43,
L54
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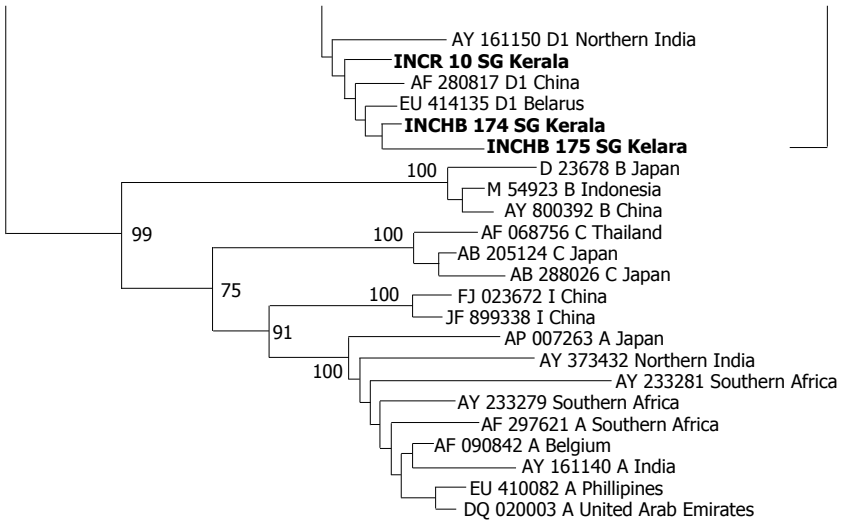

Figure 2 Phylogenetic relationships among complete preS1/pre S2/S sequences (nt 2854-835 numbering according to GenBank accession AY233274). A: Subgenotype A1 from hepatitis B virus (HBV) positive patients from Kerala (marked in bold) compared with sequences obtained from GenBank established using the neighbor joining method; B: Genotype D isolates from HBV positive patients from Kerala (marked in bold) compared with HBV isolates obtained from GenBank established using the neighbor joining method. Bootstrap statistical analysis was performed using 1000 replicates. Each sequence obtained from GenBank is designated by its accession number and its country of origin. The characteristic amino acids in the preS1 and polymerase spacer regions are indicated next to the sequences or relevant clades.

Table 2 Multiple logistic regression analysis of basal core promoter/precore region in different disease groups

		HCC		Non HCC (CR/CH)		OR (95%CI)	*P* value
		Gen A (*n* = 20)	Gen D (*n* = 6)	Gen A (*n* = 26)	Gen D (*n* = 11)		
Basic core promoter/ pre core region	A1762T/G1764A + G1764A only	16 (80)	5 (83)	14 (54)	3 (27)	20.2 (6.3-65)[a]	0.008[a] NS[d]
	C1766T/T1768A	9 (45)	1 (5)	5 (25)	0	25 (7.3-86)[a] 14.3 (1.7–119)[d]	0.03[a] 0.05[d]
	1773T (genotype A)	19 (95)	-	25	-		
	1773C (genotype D)	-	4	-	7	-	NS[a]
	G1862T	18 (90)	2 (33)	21 (81)	1(9)	30.33 (5.62-192.6)[d]	NS[a] 0.0001[d]
	G1896A	0	4 (67)	0	2 (18)	-	NS[a] 0.0002[c]
	A1762T/G1764A + G1862T	13 (65)	1 (2)	13 (50)	0	4.81 (0.6-39.4)[b]	NS[a] 0.0004[b]
Complete S region	Pre-S deletions	5 (33.3)[e]	0	3 (17.6)	1 (9)	1.89 (0.54-6.60)[b]	NS[b] NS[d]

[a]Comparison between hepatocellular carcinoma (HCC) and non-HCC, all genotypes; [b]Comparison between HCC and non-HCC, restricted to genotype A isolates; [c]Comparison between HCC and non-HCC, restricted to genotype D isolates; [d]Comparison between genotype A and D isolates; [e]Percentage out of 15 genotype A HCC isolates that were sequenced in the S region. CR: Cirrhosis; CH: Chronic hepatitis.

associated with HCC in both genotypes A and D (P = 0.03). Mutation C1766T/T1768A was significantly associated with HCC and found predominantly in subgenotype A1 (P = 0.05) (Table 2). Although G1862T was significantly associated with subgenotype A1, occurring in 85%, there was no significant difference between its presence in HCC and non-HCC patients (Table 2) and between isolates from HBeAg-positive and -negative patients (38.5% *vs* 61.5%, respectively; P = 0.08). However, in combination with A1762T/G1764A, G1862T was significantly associated with HCC in patients infected with subgenotype A1 (P = 0.0004). There was no correlation between the presence of BCP/Pre C mutations with either the age, gender, or the viral loads.

PreS deletion mutants, whose patterns are depicted in Table 3, were detected in nine isolates from five HCC and four CH, but in none of the CR patients. Overall, seven different types of preS mutations were detected (Table 3). The mean age of the patients, with and without preS deletions, did not differ significantly (35.4 ± 11.5 years *vs* 37.0 ± 15.8 years, P = 0.78), nor did the HBV DNA (3.8 ± 2.0 *vs* 4.2 ± 1.7, P = 0.62) and mean ALT levels (58.2 ± 20.8 *vs* 60.0 ± 28.2, P = 0.88). A higher frequency of preS deletion mutants was observed in HCC patients infected with genotype A, although this did not reach statistical significance (Table 2). The preS2 start codon M1T/I mutation, was unique to genotype A strains, occurring in 5/32 (15.6%) isolates from all disease groups

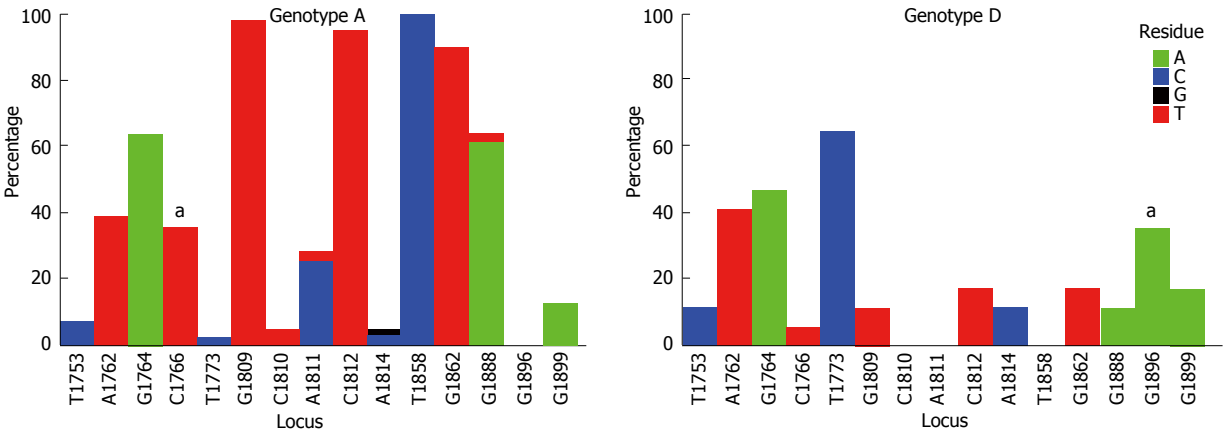

Figure 3 Comparison of the distribution of mutations in the basic core promoter/precore region (1742-1901 from the *Eco*R1 site) in genotypes A (62 isolates) and D (23 isolates). Graphs showing the percentage of mutant residues relative to the reference motif found at the 15 loci of interest (1753, 1762, 1764, 1766, 1773, 1809-1812, 1814, 1858, 1862, 1888, 1896 and 1899). The study sequence files were submitted to the Mutation Reporter Tool[49] to produce the graphs. The reference motifs used were TAGCTGCACACGGGG (genotype A) and TAGCTGCACATGGGG (genotype D) for comparison. This is also shown by the letter preceding each locus on the X-axis. To facilitate direct comparisons between the graphs, conserved loci were not suppressed and the Y-axis was scaled to 100% by selecting the appropriate controls on the input page of the Mutation Reporter Tool. Nucleotides: A (green), C (dark blue), G (black), T (red). [a]Significantly associated with the respective genotype.

Table 3 Summary of the pre-S mutations prevalent among the three clinical groups and genotypes

Isolate	Age/sex	Clinical status	Subgenotype	PreS1	PreS2					Functions affected
				Start codon	Nucleotide from the *Eco*RI start			Amino acids from preS2 start codon		
					Start codon	Deletion size	Position	Deletion size	Position	
CHB42	40/M	CH	A1	ATG	ACG	-	-	-	-	A
HCC12	60/M	HCC	A1	ATG	ACG	-	-	-	-	A
HCC25	50/M	HCC	A1	ATG	ACG	-	-	-	-	A
CHB137	16/F	CH	A1	Deletion	ATG	18	2854-2871	6	1-6	I
CHB170	29/F	CH	D2	ATG	ATG	21	35-55	7	16-22	T, B, P
CHB202	33/M	CH	A1	ATG	ATG	24	28-51	8	13-21	T, B, P
CHB413	35/M	CH	A1	ATG	ATG	6	49-54	2	21-22	B, P
HCC29	50/F	HCC	A1	ATG	ATA	33	22-54	11	11-22	A, T, B, P
HCC30	32/M	HCC	A1	ATG	ATG	24	30-53	8	13-21	T, B, P
HCC37	30/F	HCC	A1	ATG	ATG	33	24-56	11	11-22	T, B, P
HCC39	55/M	HCC	A1	ATG	ATG	33	24-56	11	11-22	T, B, P
HCCT3	38/M	HCC	A1	ATG	ATA	54	1-54	18	4-22	A, T, B, P, M

A: PreS2 initiation codon abolished; M1T, M1I; I: PreS1 initiation start codon abolished; M: Morphogenesis domain ps1:103-119 and ps2: 1-4; T: T cell epitope ps1: 109-119/ps2: 1-13; B: B cell epitope, amino acids 14-26; P: Putative neutralizing anti-preS2 antibody, amino acids 1-26; HCC: Hepatocellular carcinoma; CHB: Chronic hepatitis B.

and occurred at a higher frequency in isolates from HCC patients (P = 0.076). The preS2: F22L/I mutation was detected in 13 isolates (genotype A-9/32, 28%; genotype D4/17, 23%). The F22 mutation was significantly associated with HCC (10/13, 77%) compared with CR (2/13, 15%) and CH (1/13, 8%), respectively (P = 0.0065). This significance remained when comparing genotype D isolates only, but not genotype A isolates alone.

DISCUSSION

The present study demonstrated that genotype A was the most prevalent HBV genotype infecting liver disease patients in Kerala. This prevalence differed from other geographical regions of India, where genotype D predominates or occurs at an equal prevalence with genotype A (Figure 1). Ninety five percent of the genotype A isolates belonged to subgenotype A1, which has also been found to be the predominant subgenotype of A in India[18].

The Keralite subgenotype A1 strains clustered with the Asian subgenotype strains but differed from them in some molecular characteristics in the preS2 region, which they shared with African strains. A minority of genotype A strains belonged to subgenotype A2, which was previously described to be restricted to the peripheral blood lymphocytes of eastern Indian blood donors[19]. Subgenotype A1 of HBV was the first subgenotype to be recognized and is the dominant genotype A strain in Africa, with unique molecular characteristics that differentiate it from A2, the genotype A strain prevailing outside Africa. Subgenotype A1 has its origin in Africa and its

global dispersal coincides with historical events, including the slave trade and colonization[20]. Calicut and Cochin on the west coast of Kerala were major sea ports frequented by both the Dutch East India Company and Portuguese colonists[21].

The patients infected with genotype A and D did not differ from each other in terms of age, HBV viral loads, the frequency of HBeAg-positivity and ALT levels (Table 1). However, compared to cirrhotic and chronic hepatitis patients, a significantly higher proportion of HCC patients were infected with subgenotype A1, and HBeAg-negative HCC patients infected with subgenotype A1 were significantly younger than HCC patients infected with genotype D. Although the number of HCC patients in the present study is relatively low, the results concur with a South African study that showed that patients infected with subgenotype A1 had a 4.5 fold increased risk of developing HCC compared with those infected with non-A genotypes and they developed the cancer 6.5 years earlier[15], thus intimating a higher hepatocarcinogenic potential of subgenotype A1, regardless of host ethnicity. However, studies with a larger number of HCC patients would be required to confirm this. These findings differ from a New Delhi study, which found a comparable distribution of genotype A and D between disease groups and that genotype D, and not genotype A, was associated with HCC and were of younger age[22]. Other studies from New Delhi[23] and Western India[24] found no association between genotype A, D and disease progression. The subgenotypes of A were not differentiated in any of these three studies.

Irrespective of the genotype, the frequency of the *A1762T/G1764A* and *1764A* mutations was significantly higher in HCC patients compared with non-HCC patients. The majority of HCC patients were infected with subgenotype A1. Similarly, the BCP double mutation occurred at a higher frequency in HCC patients compared with asymptomatic carriers in southern Africa, where subgenotype A1 predominates[25]. This was not the case in Western India where genotype D is prevalent[26]. Although the BCP mutants have been reported to contribute to the HBeAg-negative phenotype by downregulating precore mRNA transcription[27], in the present study, and in agreement with others[28], there was no correlation between the presence of the *A1762T/G1764A* mutation and HBeAg-negativity.

The double mutation C1766T/T1768A was significantly associated with HCC and subgenotype A1 (Table 2). The T1768A mutation results in F132Y in HBx and may play a synergetic role with K130M and V131I, introduced by A1762T/G1764A, leading to carcinogenesis[29]. Moreover, mutation C1766T/T1768A has been reported as an independent predictor of cirrhosis in HBeAg-negative patients and is associated with higher viral replication by increasing the encapsidation of pg RNA[30].

Mutation G1862T was found in 85% of subgenotype A1 isolates (Table 2). Previously, G1862T was detected in 79% of global subgenotype A1 isolates, but in none of the subgenotype A2 isolates, and has been shown to be a characteristic of subgenotype A1[31]. This high frequency of G1862T in the Keralite strains is much higher than that reported in either Eastern Indian and Southern African studies (60%[32] and 25%[33], respectively). In the present study, the combination of A1762T/G1764A and G1862T was significantly associated with HCC in patients infected with subgenotype A1. A previous study showed that G1862T was significantly associated with HBeAg-negativity in South African HCC patients[34], but not in asymptomatic carriers[33]. Moreover, mutation *A1762T/G1764A* is found frequently in South African HCC patients, but not in asymptomatic carriers[25], and the majority of South African HCC patients are infected with subgenotype A1[15].

The present study showed a significant association of the preS2:F22L mutation with the development of HCC, particularly in genotype D. Recent studies identified the F22L mutation in the preS2 region as a risk factor for HCC among patients infected with genotype C[35], and showed significant association of this mutation with liver cirrhosis in Eastern India[36]. Our study supports the possibility that F22L may be associated with severe liver disease progression.

The preS deletion and initiation codon mutations were prevalent in strains isolated from all clinical groups (Table 3). This is the first study to describe the preS mutants from Indian HCC patients infected with subgenotype A1. A strong correlation between preS mutants and the development of HCC has been shown in patients infected with genotypes B or C[37,38]. In studies carried out in isolates belonging to genotypes B and C, mutated envelope proteins were shown to accumulate within the hepatocyte endoplasmic reticulum (ER) and result in a characteristic histopathological hallmark of HCC, known as ground glass hepatocytes[39]. HBV induced ER stress has been shown to dysregulated several cell cycle regulatory pathways, which may contribute to hepatocarcinogenesis[40].

In conclusion, genotypes A and D were isolated from liver disease patients in Kerala, Southern India, with subgenotype A1 predominating. The relatively high prevalence of subgenotype A1 in HCC patients supports previous studies in Africa, which showed an association of subgenotype A1 with HCC and its development at a younger age[15]. This association appears not to depend on host ethnicity. The combination of BCP and/or preC mutations, as well as the described preS mutations, could lead to the accumulation of replicative intermediates and viral proteins, contributing to viral integration and cellular stress or damage. These combined characteristics could induce severe liver disease, including HCC, and should be explored further.

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COMMENTS

Background

Genotype D, followed by genotype A, has been reported to predominate in India. Hepatitis B virus (HBV) isolates with the A1762T/G1764A variations are prevalent in hepatocellular carcinoma (HCC) patients. In addition, variation G1862T has been reported to be a characteristic of subgenotype A1. Subgenotype A1 has been reported to be more hepatocarcinogenic in southern Africans, who develop HCC at a younger age than those infected with other genotypes.

Research frontiers

The hepatocarcinogenic potential of subgenotype A1 of HBV has been linked to disease progression in regions outside India. Subgenotype A1 has its origin in Africa and its global dispersal coincides with historical events, including the slave trade and colonization.

Innovations and breakthroughs

This is the first study to report the predominance of subgenotype A1 in liver disease patients in India and its high prevalence in HCC patients. The relatively high prevalence of subgenotype A1 in HCC patients supports previous studies in Africa that showed an association of subgenotype A1 with HCC and its development at a younger age. The S open reading frame of subgenotype A1 isolates from Kerala clustered within the Asian clade as a separate clade and encoded distinct subgenotype A1 amino acids. The subgenotype A1 isolates from Kerala had a higher frequency of G1862T compared to subgenotype A1 isolates from other geographical regions. This is the first time that preS deletion mutants have been described in Indian HCC patients. Pre-S2: F22L was found in genotypes A and D.

Applications

The prevalence of different mutations in the various genotypes of HBV may serve as biomarkers for disease risk and development of HCC. The differences in the geographical distribution of genotypes and subgenotypes of HBV may require different treatment algorithms. Knowledge of HBV genotypes and/or mutations may facilitate personalized treatment.

Terminology

A genotype is generally defined as the genetic constitution of an organism. In the case of viruses, the term genotype applies to the forms into which the genomic sequence has stabilized after a prolonged period of time and that are replication competent. The genotypes of HBV are defined by an intergroup divergence of more than 7.5%-8% in the complete genome sequence and by more than 4% at the level of the S gene. The term subgenotype is used to identify subgroups of HBV genotypes with an intergroup nucleotide difference between 4% and 8% across the complete genome.

Peer review

The study contributes to the understanding of the relationship between HBV variability and clinical outcomes in different populations worldwide. The relationship between the preS deletion and HCC in this study is a critical and hot point.

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ORIGINAL ARTICLE

Anti-miRNA-221 sensitizes human colorectal carcinoma cells to radiation by upregulating PTEN

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Abstract

AIM: To investigate the regulative effect of miRNA (miR)-221 on colorectal carcinoma (CRC) cell radiosensitivity and the underlying mechanisms.

METHODS: A human CRC-derived cell line was cultured conventionally and exposed to different doses of X-rays (0, 2, 4, 6 and 8 Gy). The total RNA and protein of the cells were extracted 24 h after irradiation, and the alteration of miR-221 and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) gene mRNA expression was detected by real-time reverse transcriptase polymerase chain reaction (PCR). The protein alteration of PTEN in the cells was detected by Western blotting. Caco2 cells were pretreated with or without anti-PTEN-siRNA prior to the addition of pre-miR-221 or anti-miR-221 using Lipofectamine 2000. Colony formation assay and flow cytometry analysis were used to measure the surviving cell fraction and the sensitizing enhancement ratio after irradiation. Additionally, PTEN 3′-untranslated region fragment was PCR amplified and inserted into a luciferase reporter plasmid. The luciferase reporter plasmid construct was then transfected into CRC cells together with pre-miR-221 or anti-miR-221, and the luciferase activity in the transfected cells was detected.

RESULTS: The X-ray radiation dose had a significant effect on the expression of miR-221 and PTEN protein in human Caco2 cells in a dose-dependent manner. The miR-221 expression level improved gradually with the increase in irradiation dose, while the PTEN protein expression level reduced gradually. miR-221 expression was significantly reduced in the anti-miR-221 group compared with the pre-miR-221 and negative control groups ($P < 0.01$). Anti-miR-221 upregulated expression of PTEN protein and enhanced the radiosensitivity of Caco2 cells ($P < 0.01$). Moreover, the inhibitory effect was dramatically abolished by pretreatment with anti-PTEN-siRNA, suggesting that the enhancement of radiosensitivity was indeed mediated by PTEN. A significant increase of luciferase activity was detected in CRC cells that were cotransfected with the luciferase reporter plasmid construct and anti-miR-221 ($P < 0.01$).

CONCLUSION: Anti-miR-221 can enhance the radiosensitivity of CRC cells by upregulating PTEN.

Key words: Colorectal carcinoma; miR-221; Phosphatase and tensin homolog deleted on chromosome 10; Radiosensitivity

Core tip: Previous studies have shown that miRNA (miR)-221 expression is elevated in radioresistant colorectal carcinoma (CRC) cells; however, it is unknown whether and how miR-221 controls cellular response to irradiation. We demonstrated that knockdown of miR-221 upregulated phosphatase and tensin

homolog deleted on chromosome 10 (PTEN) expression, and PTEN was identified as a direct target of miR-221 in CRC. Upregulated PTEN expression suppressed AKT activity and increased radiation-induced cell death, enhancing radiosensitivity in CRC cells. This study provides evidence for antioncogenic activity of anti-miR-221 in the irradiation of CRC and may be a useful biomarker or therapeutic target in CRC.

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INTRODUCTION

Colorectal carcinoma (CRC) is one of the most frequent cancers and a common cause of cancer-related death worldwide, with an increasing incidence expected in the next few decades[1]. The overall incidence of CRC is 5% in the general population and the 5-year survival rate ranges from 40% to 60%[2]. The national comprehensive cancer network guidelines on CRC treatment include radiotherapy as standard for patients with a high risk of recurrence (http://www.nccn.org/index.asp). However, the radiotherapeutic efficiency is often limited by the occurrence of radioresistance, reflected as a diminished susceptibility of the irradiated cells to undergo apoptosis. As a result, this therapeutic strategy cannot substantially improve the survival rate[3]. With the understanding of the molecular biology of CRC, it has been recognized that development of radioresistance is related to changes of tumor environment and the dysregulation of certain genes, including some genes involved in a variety of cell signaling pathways as well as oncogenes and tumor suppressor genes[4].

miRNAs are a new class of small noncoding RNAs that regulate the expression of target genes through translational repression or mRNA cleavage/decay[5]. Genome-wide studies have demonstrated that miRNA genes are frequently located at cancer-associated genomic regions, indicating the potential roles of miRNAs in tumorigenesis[6]. miRNAs play an important role in the multistep processes of carcinogenesis, either by oncogenic or tumor suppressor function in CRC[7]. However, only a few studies have determined the roles of miRNAs in radiation response in CRC[8]. miRNA (miR)-221, encoded in tandem from a gene cluster located on chromosome X, is a recently discovered miRNA and is involved in tumor development by regulating cell proliferation cycle[9]. In our previous study, we have demonstrated that miR-221 promotes CRC occurrence and progression, which makes it a potential antitumor candidate for treatment and prevention of CRC[10]. However, the miR-221 response for CRC to survive radiation-induced injury remains largely unknown.

The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) gene, located at 10q23.3, encodes a central domain with homology to the catalytic region of protein tyrosine phosphatases[11]. This gene is an important regulator of protein phosphatases and 3'-phosphoinositol phosphatases. PTEN dephosphorylates phosphatidylinositol-3,4,5-triphosphate (PIP3), the second messenger produced by phosphoinositide 3-kinase, to regulate negatively the activity of the serine/threonine protein kinase, AKT[12]. PTEN is inactivated in some malignant tumors, resulting in AKT hyperactivation, thereby promoting cell proliferation, inhibition of apoptosis, and enhanced cell invasion and radioresistance[13]. miRNAs, specifically miR-221, have been established as regulators of PTEN expression[14]. However, how miR-221 affects PTEN in CRC radiation has not been elucidated. Therefore, in this study, we observed the effect of miR-221 on the radiosensitivity of CRC cells and its underlying mechanisms.

MATERIALS AND METHODS

Cell culture and transfection

Human CRC-derived cell lines, including HT-29, Lovo, SW-480, Caco2, and control human umbilical vein endothelial cells (HUVECs), provided by Shanghai Institutes For Biological Science, CAS, were resuscitated routinely, resuspended with RPMI-1640 supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT, United States), 100 kU/mL penicillin G and 100 g/L streptomycin, and then planted in a 25-cm^2 culture bottle and incubated in a 5% CO_2 humidified atmosphere at 37 ℃. The media were changed every 3 d and the cells were trypsinized using trypsin/edetic acid when they reached 80%-90% confluence. Cells aged at passages 4-8 were used for the experiments. The day before transfection, cells were seeded in antibiotic-free medium. Cells (1×10^4/well) were seeded in a 96-well plate, incubated for 24 h to allow them attach to the bottom of the well, and then transfected with 50 nmol/L negative control, pre-miR-221 or anti-miR-221 oligonucleotides, respectively (Shanghai GenePharma, Shanghai, China). Transfection of miRNAs was carried out using Lipofectamine 2000 in accordance with the manufacturer's procedure (Invitrogen, Carlsbad, CA, United States). The above experiment was repeated at least three times.

Cell proliferation analysis of transfected cells by MTT assay

The status of cell proliferation was determined by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl trozolium bromide (MTT; Amresco, Solon, OH, United States) assay. In short, exponentially growing CRC cells were adjusted to 1.5×10^4 cells/mL with RPMI-1640, planted in 96-well plates (Corning, Corning, NY, United States) at 200 μL/well, and incubated for 12 h. After transfection with 50 nmol/L pre-miR-221 or anti-miR-221 and incubation for

48 h (five duplicate wells for each sample), 20 μL/well MTT (5 g/L) was added to each well. The medium was removed after 4 h incubation and 100 μL/well dimethylsulfoxide was added to dissolve the reduced formazan product. Finally, the plate was read in an enzyme-linked immunity implement (Bio-Rad 2550, Hercules, CA, United States) at 490 nm. Cellular proliferation inhibition rate (CPIR) was calculated using the following formula: CPIR = (1 - average *A* value of experimental group/average *A* value of control group) × 100%. The above experiment was repeated at least three times.

miRNA target prediction

The analysis of miR-221-predicted targets was performed using the algorithms TargetScan (http://targetscan.org/), PicTar (http://pictar.mdc-berlin.de/) and MiRanda (http://www.microrna.org/microrna/home.do).

Luciferase activity assay

The human 3'-untranslated region (UTR) of the PTEN gene was amplified by PCR using the primers 5'-CGATTCTAGAAATCATGTTCTGGTGG-3' for PTEN-3'-UTR-Forward and 5'-GCATTCTAGAATTCTGCACAGTAAGCATA-3' for PTEN-3'-UTR-Reverse and cloned into the *Xba*I site of the pGL3-control vector (Promega, Madison, WI, United States), downstream of the luciferase gene, to generate the vector pGL3-PTEN. For luciferase assay, the CRC cells were cultured in 24-well plates and transfected with 500 ng of either pGL3-PTEN or pGL3-control vector and 50 pmol pre-miR-221, anti-miR-221 or negative control. Transfection was performed using Lipofectamine 2000 (Invitrogen) as described by the manufacturer. At 24 h after transfection, firefly luciferase activity was measured using the Dual Luciferase Reporter Assay (Promega, Madison, WI, United States). The above experiment was repeated at least three times.

Radiation exposure

Irradiation was performed at room temperature in a linear accelerator (Varian 600; Palo Alto, CA, United States) at a dose rate of 3.2 Gy/min. Monolayer cells were plated into six-well plates, placed 100 cm from the source, and exposed to the specified dose (0, 2, 4, 6 and 8 Gy) of X-rays[14].

Detection of miR-221 and PTEN mRNA expression by real-time reverse transcriptase polymerase chain reaction

Total RNA was extracted with routine Trizol reagent (Invitrogen). The precipitation was dissolved in diethylpyrocarbonate-treated water. Nucleic acid protein analyzer (Beckman Coulter, Fullerton, CA, United States) was used to determine RNA concentration. The purity and integrity of RNA were identified by two aspects: $A_{260nm}/A_{280nm} \geqslant 1.8$, and a band ratio of 28S to 18S RNA ⩾ 1.5 in formaldehyde denaturing gel electrophoresis. miR-221 and PTEN mRNA was quantified as described previously[10]. The comparative $2^{-\Delta\Delta CT}$ method was used for relative quantification and statistical analysis. The above experiment was repeated at least three times.

Detection of target protein expression by Western blotting

The cells were rinsed twice with cold phosphate buffered solution (PBS) buffer, and were then lysed in an ice-cold lysis buffer containing 150 mmol/L NaCl, 50 mmol/L Tris-HCl (pH 7.6), 0.1% SDS, 1% Nonidet P-40, and protease inhibitor cocktail (Boehringer Mannheim, Lewes, Sussex, United Kingdom). The samples were cleared by centrifugation at 13000 *g* for 10 min. Fifty micrograms of protein from the tissue was subjected to SDS-PAGE and electrotransferred to polyvinylidine fluoride membranes (Immobilon, Bedford, MA). After blocking in 20 mmol/L Tris-HCl, pH 7.6 (containing 150 mmol/L NaCl, 0.1% Tween-20, and 5% nonfat dry milk), membranes were incubated with primary antibodies against target protein or β-actin (used as a sample loading control) overnight at 4 ℃ and then incubated with horseradish-peroxidase-conjugated secondary antibody. The blot was developed using the ECL detection kit (Amersham Pharmacia Biotech, Piscataway, NJ, United States) according to the manufacturer's instructions and the protein imprinting band was obtained. The above experiment was repeated at least three times.

Colony formation assay

At 24 h after irradiation, all cells were trypsinized and counted. Corresponding numbers of cells were seeded into 10-cm dishes containing RPMI-1640 supplemented with 10% FBS, in triplicate, incubated for 14 d to allow colony growth, and colonies were stained with crystal violet. Colonies containing ⩾ 50 cells were counted. The plating efficiency was calculated by dividing the average number of colonies per dish by the number of cells plated. Survival fractions were calculated by normalization to the plating efficiency of appropriate control groups. The above experiment was repeated at least three times.

Flow cytometry assay

The effects of miR-221 and irradiation on CRC cell death were examined by flow cytometry. Pretreated CRC cells were harvested and washed twice with PBS, fixed with 70% ethanol at -20 ℃ for 30 min, and stored at 4 ℃ overnight, then washed with PBS again, treated with 100 mL 100 mg/L RNase at 37 ℃ for 30 min, and stained with 100 mL 50 mg/L propidium iodide at 4 ℃ for 30 min in the dark. The multiplication cycle and apoptotic rate were assayed using flow cytometry, and the data were analyzed using CellQuest software. The percentages of necrotic and apoptotic cells were measured by calculating the ratio of the number of corresponding cells to the number of total cells. For each sample, 10000 cells were measured.

Statistical analysis

All data in the experiment are presented as mean ± SD.

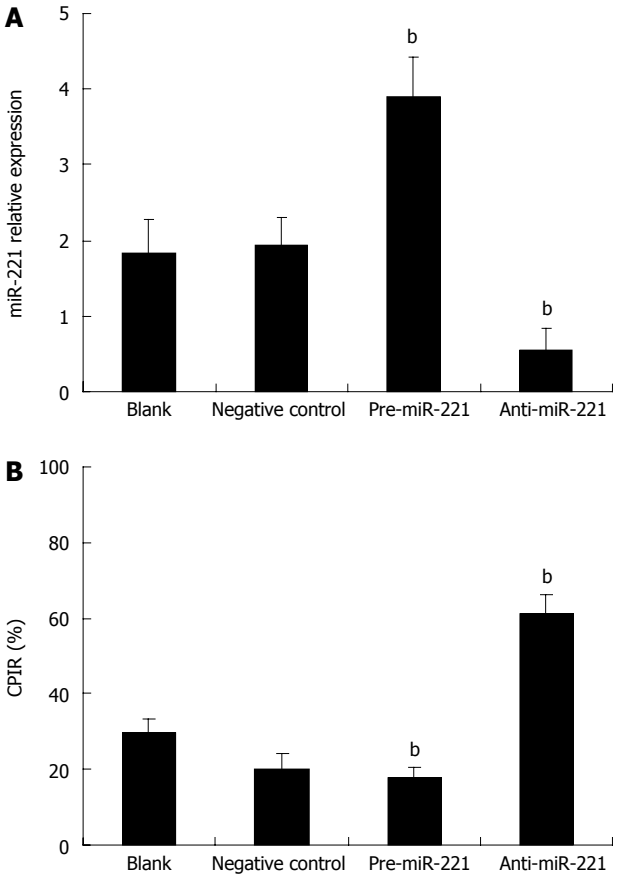

Figure 1 Modulation of miR-221 expression and cell proliferation in Caco2 cells. A: Expression of miR-221 was detected by real-time RT-PCR. Expression of U6 snRNA was used as internal control; B: The status of cell proliferation was determined by MTT assay. CPIR in the presence of pre-miR-221 or anti-miR-221 was compared with those of controls. $n = 6$, mean ± SD; $^{b}P < 0.01$ *vs* control group.

Comparisons between groups were analyzed with one-way ANOVA and Student-Newman-Keuls Q test using SPSS version 15.0 (SPSS, Chicago, IL, United States). $P < 0.05$ was considered to be statistically significant.

RESULTS

Modulation of miR-221 expression in CRC cell lines

To study the expression pattern of miR-221 in CRC cells, we performed real-time RT-PCR to detect miR-221 expression in four CRC-derived cell lines. The expression values of miR-221 in HT-29, Lovo, SW-480 and Caco2 were 4.094 ± 0.208, 1.122 ± 0.138, 3.927 ± 0.232 and 1.831 ± 0.149, respectively. A significant overexpression of miR-221 was observed in all four CRC cell lines relative to HUVEC (0.223 ± 0.047, $P < 0.01$). The Caco2 cell line was chosen for both pre-miR-221 and anti-miR-221 transfection in the successive experiment because it exhibits, among the four cell lines tested, an intermediate miR-221 expression level.

To determine the biological impact of miR-221 in the CRC-derived cell line, Caco2 cells were transfected with pre-miR-221 or anti-miR-221 to increase or reduce miR-221 level, respectively. Real-time RT-PCR analysis revealed that introduction of pre-miR-221 caused a significant increase of miR-221 value; conversely, anti-miR-221 caused a significant decrease of miR-221 value (Figure 1A, $P < 0.01$). These strategies were then used as the basis of the remaining experiments.

We further tested whether the cell proliferation potential of the transfected CRC cells was modified and the status of cell proliferation was determined by MTT assay. We observed a significant increase in proliferation after transfection of pre-miR-221. In contrast, anti-miR-221 significantly decreased cell proliferation (Figure 1B, $P < 0.01$). These data indicate that cell proliferation can be significantly enhanced by increase of miR-221 expression, a result which strongly supports the potential oncogenic activity of miR-221 in CRC.

PTEN is a target of miR-221 in CRC

Most miRs are thought to control gene expression by base-pairing with the miR-recognizing elements found in their messenger target. We utilized all three currently available major prediction programs, TargetScan, Miranda and PicTar, to analyze the potential interactions between miR-221 and PTEN. All these algorithms reveal a potential miR-221 target site in the PTEN mRNA 3'-UTR region (Figure 2A). To demonstrate the direct interaction between miR-221 and PTEN mRNA, we cloned PTEN-3'-UTR segment, which includes a potential target site for miR-221, downstream of the pGL3 luciferase reporter gene, to generate the pGL3-PTEN vector. This vector was cotransfected into Caco2 cells together with pre-miR-221 or anti-miR-221. Luciferase activity in Caco2 cells cotransfected with pGL3-PTEN vector and miR-221 was decreased markedly compared with negative controls. On the contrary, luciferase activity in Caco2 cells transfected with anti-miR-221 was increased significantly compared with negative controls (Figure 2B). These results support the bioinformatic prediction indicating the 3'-UTR of PTEN mRNA as a target for miR-221.

To check whether miR-221 actually affects PTEN expression in CRC cells, we analyzed the consequence of the ectopic expression of miR-221. We transfected the pre-miR-221 or anti-miR-221 into Caco2 cells, and we searched for changes in PTEN protein levels by Western blotting. Introduction of miR-221 caused a significant increase of miR-221 value and decreased PTEN protein levels. Conversely, anti-miR-221 caused a significant decrease of miR-221 value and increased PTEN protein amounts (Figure 2C). No significant changes in the PTEN mRNA levels were observed in the cells either transfected with miR-221 or anti-miR-221 (Figure 2D). This result strongly validates a post-transcriptional regulation of PTEN protein by miR-221, and also excludes its role in PTEN mRNA degradation.

miR-221 modulates Caco2 cell radiosensitivity

To determine whether miR-221 and PTEN were involved in the cellular response to radiotherapy in CRC,

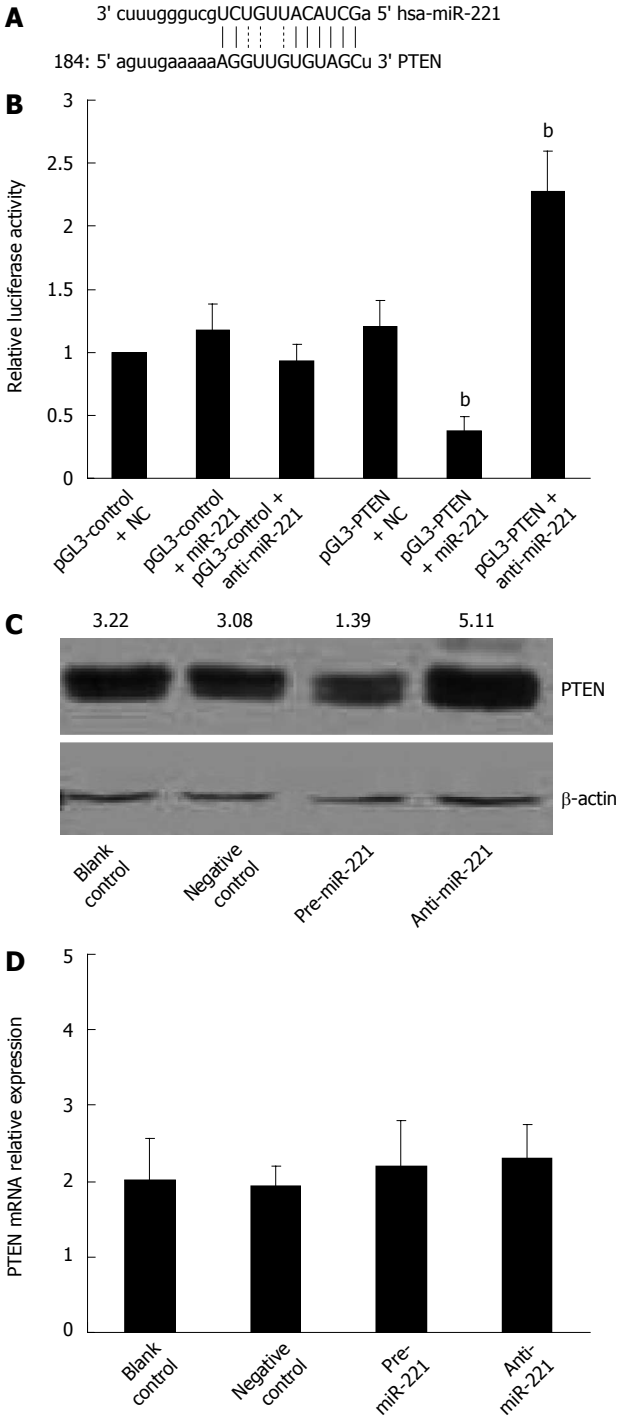

Figure 2 miR-221 regulated expression of phosphatase and tensin homolog deleted on chromosome 10 in colorectal carcinoma-derived cells. A: Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) 3'-UTR site potentially targeted by miR-221 as predicted by Miranda; B: Luciferase activity assay showing direct interaction between miR-221 and PTEN 3'-UTR site. Firefly luciferase reporter activity in the presence of both pGL3-PTEN vector and pre-miR-221 or anti-miR-221 was compared with that of the controls. Luciferase activity in Caco2 cells cotransfected with pGL3-PTEN vector and pre-miR-221 was decreased markedly compared with the negative control. Luciferase activity in Caco2 cells transfected with anti-miR-221 was increased significantly compared with the negative control, $^{b}P < 0.01$ *vs* control group; C: Western blotting showing PTEN protein expression in Caco2 cells transfected with pre-miR-221 or anti-miR-221. β-actin was used as a housekeeping gene to normalize PTEN protein expression. The relative PTEN protein levels normalized against β-actin are shown at the top of each panel; D: Real-time RT-PCR analysis showing PTEN mRNA expression in Caco2 cells transfected with pre-miR-221 or anti-miR-221. All the results are representative of three independent experiments.

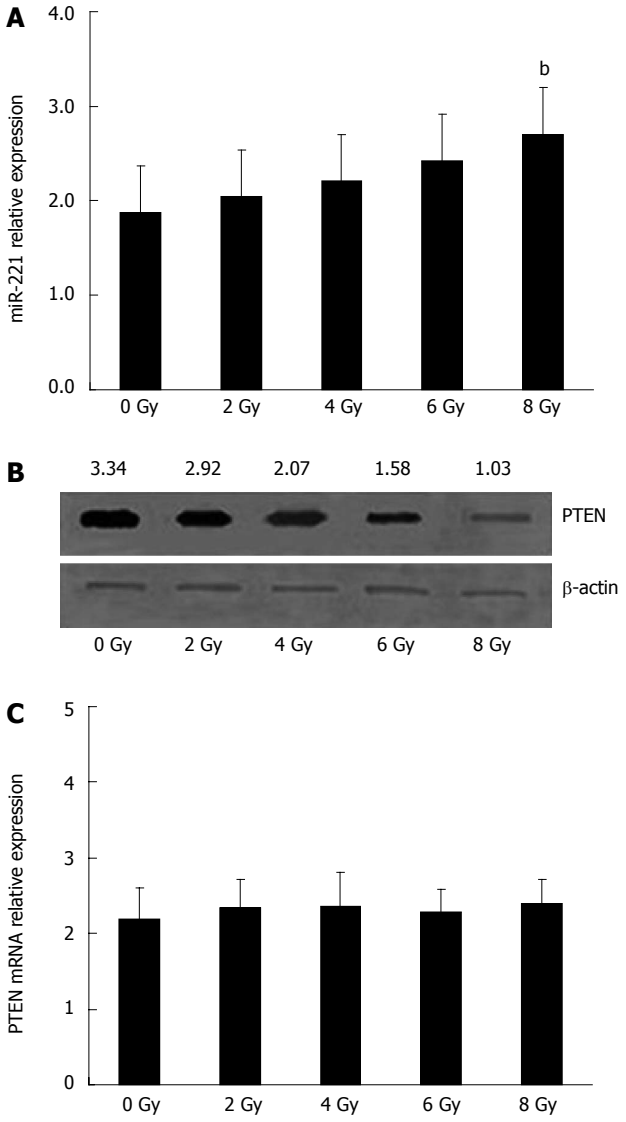

Figure 3 Effects of X-ray dose on miR-221 and phosphatase and tensin homolog deleted on chromosome 10 expression in colorectal carcinoma-derived cells. A: Expression of miR-221 was detected by real-time RT-PCR. The miR-221 expression level improved in a dose-dependent manner with the increase in radiation dose, $^{b}P < 0.01$ *vs* control group; B: Expression of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) protein was detected by Western blotting, β-actin was used as a housekeeping gene to normalize PTEN protein expression. The relative PTEN protein levels normalized against β-actin are shown at the top of each panel. The expression of PTEN protein was decreased in a dose-dependent manner with the increase in radiation dose; C: Expression of PTEN mRNA was detected by real-time RT-PCR. No significant changes in the PTEN mRNA levels were observed in the cells exposed to irradiation. The results are representative of three independent experiments.

Caco2 cells were exposed to different doses of X-rays to observe the regular pattern of miR-221 and PTEN. The radiation dose had a significant effect on the expression of miR-221 and PTEN protein in human Caco2 cells in a dose-dependent manner. The miR-221 expression level improved gradually with the increase in radiation dose, while the PTEN protein expression level reduced gradually (Figure 3A and B). However, no significant changes in the PTEN mRNA levels were observed in the cells exposed to irradiation (Figure 3C). All these results gave us a first hint that the expression of miR-221 might be

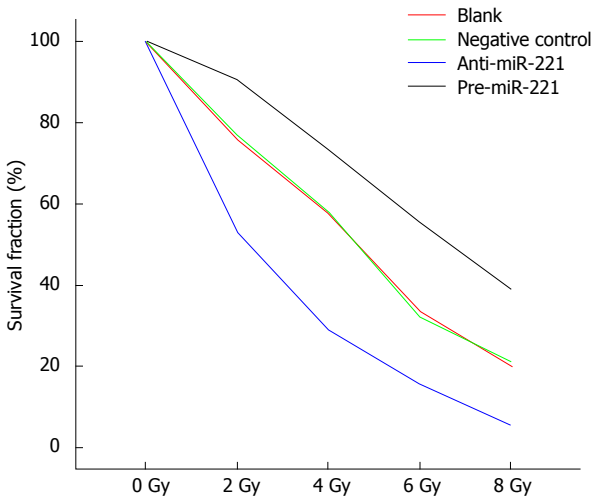

Figure 4 miR-221 modulates colorectal carcinoma cell radiosensitivity. Caco2 cells transfected with negative control, pre-miR-221 or anti-miR-221 oligonucleotides were exposed to 0-8 Gy radiation and incubated for 14 d prior to fixation, staining and assessment of colony formation. The colony formation assays were performed in triplicate.

one of the mechanisms acting to regulate radiosensitivity in CRC cells negatively.

To determine whether miR-221 affected Caco2 cell radiosensitivity, cells were transfected with pre-miR-221 or anti-miR-221 and colony formation was assessed following 0-8 Gy radiation. Transfection of Caco2 cells with pre-miR-221 significantly increased survival following 0-8 Gy radiation compared to blank and negative controls. Conversely, transfection of Caco2 cells with anti-miR-221 significantly decreased survival following radiation exposure (Figure 4). The D_0 value, the radiation dose required to reduce the level of cell survival from 100% to 37%, which is considered a measure of the intrinsic radiosensitivity of the cell, was calculated following genetic manipulation of miR-221. The quasi-threshold dose (D_q) value represents the sublethal damage repair capacity of the cells, which is also a sensitive indicator for the evaluation of cell radiosensitivity. Blank control cells, cells transfected with negative control or pre-miR-221 or anti-miR-221 oligonucleotides exhibited D_0 values of 1.681, 1.666, 2.208 and 1.068 Gy, respectively. The sensitization enhancement ratio (SER), calculated by determining the ratio of the D_0 of the control group *vs* treated cells, was 1.009, 0.761 and 1.574 for negative control-, pre-miR-221-, or anti-miR-221-treated cells, respectively (Table 1), indicating a radiosensitization potential for targeting miR-221.

To study the mechanism of miR-221 knockdown-induced radiosensitization, we measured irradiation-induced cell death in cells transfected with negative control or anti-miR-221 by flow cytometry. We found that in unirradiated cells transfected with anti-miR-221, there was an increase in necrosis and apoptosis compared to that in the controls. This is consistent with our previous observations that miR-221 knockdown leads to inhibition of cell growth[10]. More interestingly, in irradiated cells, anti-miR-221 transfection enhanced cell death (Figure 5), demonstrating a synergistic effect of miR-221 knockdown with irradiation. Collectively, these results provide strong evidence that miR-221 regulates the radiosensitivity of Caco2 cells.

Table 1 Impact of miR-221 expression on Caco2 cell radiosensitivity

Group	D_0	D_q	SF_4	SER
Blank control + irradiation	1.681	5.630	0.5966	
Negative control + irradiation	1.666	5.813	0.5858	1.009
Pre-miR-221 + irradiation	2.208	7.828	0.7453	0.761
Anti-miR-221 + irradiation	1.068	4.655	0.2984	1.574

Caco2 cells were transfected with negative control, pre-miR-221 or anti-miR-221 oligonucleotides. D_0 and D_q were determined by standardized software, and the sensitization enhancement ratio (SER) was calculated by determining the ratio of the D_0 of the control group *vs* treated cells. SF_4: Surviving fraction at 4 Gy.

Enhancement of anti-miR-221 on CRC cell radiosensitivity is mediated by PTEN

PTEN is a tumor suppressor protein, thus, we hypothesized that anti-miR-221 might sensitize Caco2 cells to radiotherapy by upregulating PTEN protein expression. To study further downstream pathways of miR-221, we conducted Western blotting to look at phosphorylation of AKT, a downstream target of PTEN. In Caco2 cells transfected with anti-miR-221, there was a significant increase in PTEN expression accompanied by downregulation of AKT phosphorylation (Figure 6A).

Additionally, if anti-miR-221 enhancement of CRC cell radiosensitivity was indeed mediated by PTEN, we would expect that the PTEN-specific and irreversible antagonist, anti-PTEN-siRNA, would abolish this effect. To test this hypothesis, we measured the cell radiosensitivity variations induced by pre-miR-221 or anti-miR-221 in CRC cells previously transfected with anti-PTEN-siRNA. The aim of this experiment was to study if and how the PTEN-depleted cellular environment responds to pre-miR-221 or anti-miR-221 addition and irradiation. Caco2 cells were pretreated with or without anti-PTEN-siRNA (80 nmol/L) for 24 h prior to the addition of pre-miR-221 (50 nmol/L) or anti-miR-221 (50 nmol/L) and the status of cell radiosensitivity was determined by colony formation assay following different doses of X-ray exposure. The data showed that a reduction of PTEN dosage by means different from miR-221 overexpression led to analogous outcomes: when we transfected Caco2 cells with anti-PTEN-siRNA, which was able to reduce both PTEN mRNA and protein by about 80% (Figure 6B and C), we observed a sharp increase in cell survival following radiation as compared with the negative controls (Figure 6D). Thus, reducing PTEN levels in CRC cells, either by miR-221 overexpression or by anti-PTEN-siRNA transduction, is sufficient to induce a comparable cell survival increase.

When pre-miR-221 was transfected into Caco2 cells previously treated with anti-PTEN-siRNA, we observed

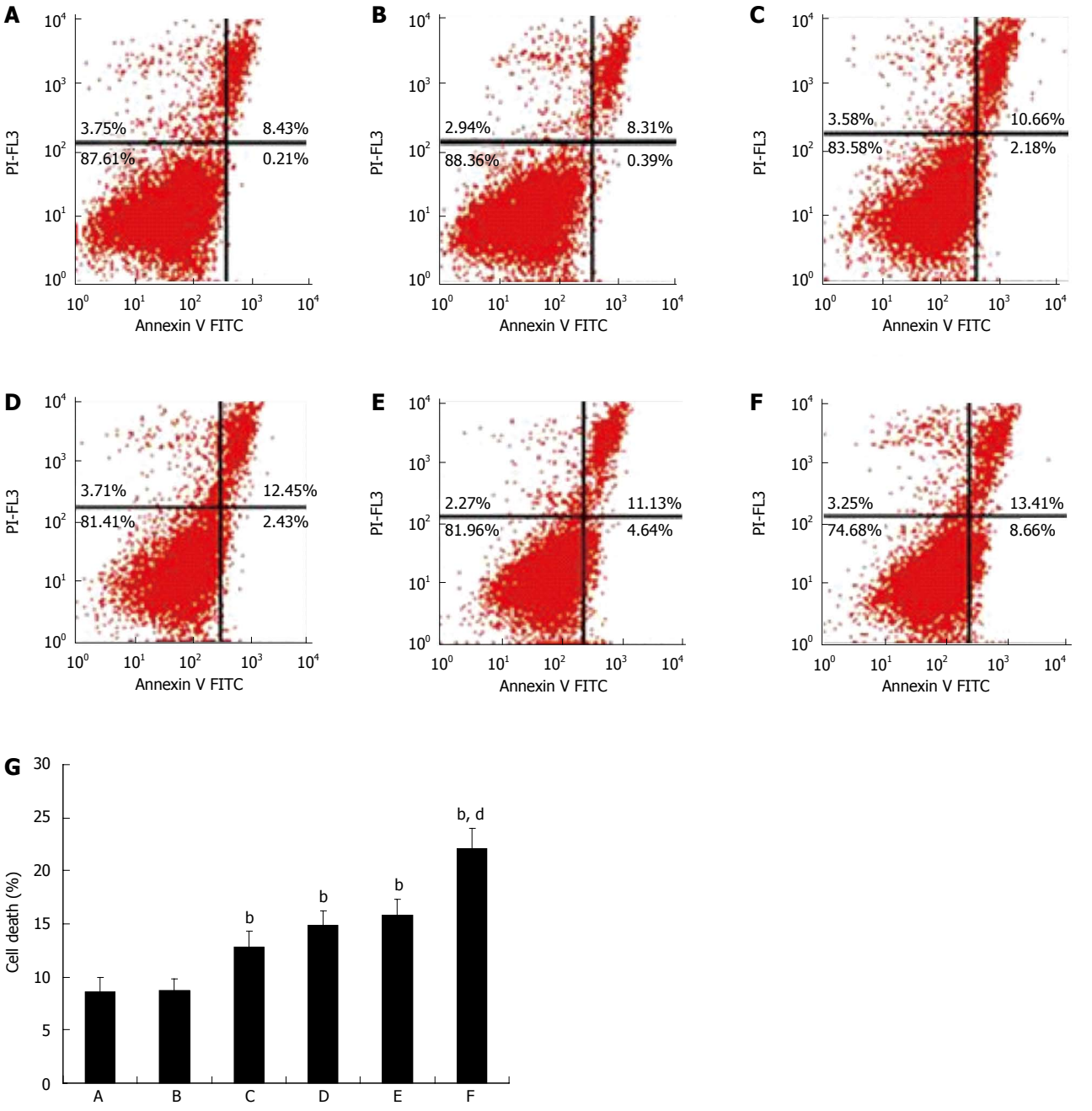

Figure 5 Effects of anti-miR-221 on irradiation-induced death in Caco2 cells. The percentages of necrotic and apoptotic cells were measured by calculating the ratio of the number of corresponding cells to the number of total cells. A: Blank control; B: Caco2 cells transfected with negative control; C: Caco2 cells transfected with anti-miR-221; D: Caco2 cells under irradiation; E: Caco2 cells transfected with negative control under irradiation; F: Caco2 cells transfected with anti-miR-221 under irradiation. The right upper quadrant ($FITC^+/PI^+$) shown as necrotic cells. The right lower quadrant ($FITC^+/PI^-$) shown as apoptotic cells; G: The status of cell death was determined by flow cytometry. Groups A-F are described as above. $n = 3$, mean ± SD; $^bP < 0.01$ *vs* blank or negative control group. $^dP < 0.01$ *vs* sole anti-miR-221 or irradiation group.

that anti-PTEN-siRNA and miR-221 seemed to cooperate to increase the survival rate (Figure 6D). However, when anti-miR-221 was transfected into Caco2 cells previously treated with anti-PTEN-siRNA, we observed that the suppression of cell survival by anti-miR-221 was partially abrogated by anti-PTEN-siRNA (Figure 6D, Table 2). These results indicated that the inhibitory effect of anti-miR-221 on CRC cell survival following irradiation was partially, but not completely, mediated by PTEN, suggesting that anti-miR-221 could also activate some PTEN-independent signaling pathways to repress CRC cell growth in addition to the up-regulation of PTEN.

DISCUSSION

Radiotherapy is one of the most important treatment methods for CRC. However, only one-third of CRC patients are highly responsive to radiation. The other patients are relatively resistant to radiation, and they tend to progress even after high-dose treatment[15-17]. Factors

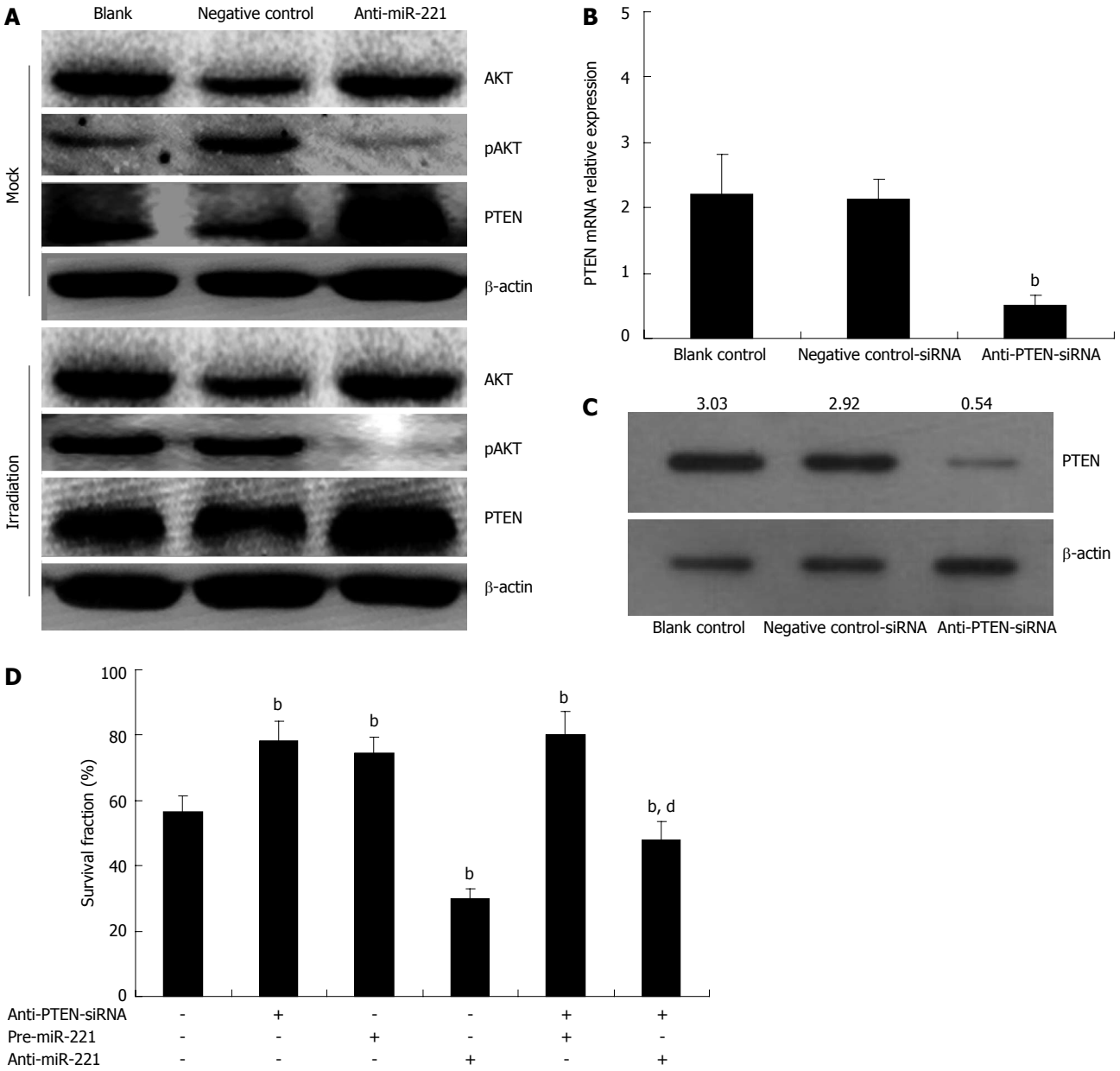

Figure 6 Ectopic expression of miR-221 affects the radiosensitivity of colorectal carcinoma cells by targeting phosphatase and tensin homolog deleted on chromosome 10. A: miR-221 regulates the phosphatase and tensin homolog deleted on chromosome 10 (PTEN)/AKT pathway. Caco2 cells transfected with negative control or anti-miR-221 were mock treated or irradiated (4 Gy). Total cell lysates were obtained for Western blotting using the indicated antibodies. Caco2 cells were pretreated with or without anti-PTEN-siRNA (80 nmol/L) for 24 h prior to the addition of pre-miR-221 (50 nmol/L) or anti-miR-221 (50 nmol/L); B, C: Real-time RT-PCR and Western blotting showing PTEN mRNA and protein reduced markedly after transfection with anti-PTEN-siRNA ($^bP < 0.01$ *vs* blank control and negative control group); D: The status of cell radiosensitivity was determined by colony formation assay. The suppression of Caco2 cell survival fraction at 4 Gy by anti-miR-221 was partially, but not completely, abrogated by anti-PTEN-siRNA (SF_4 from 29.77% to 47.88%). $n = 3$, mean ± SD; $^bP < 0.01$ *vs* negative control group; $^dP < 0.01$ *vs* sole anti-miR-221 group.

leading to CRC radioresistance include location, size, and microenvironment such as inadequate vascular supply[18,19]. More importantly, cellular and genetic factors that are related to radiation responses may explain radiation-resistant cellular phenotypes[20,21].

Activation of oncogenes and inactivation of tumor suppressor genes lead to aberrant activity of signal transduction pathways, and radioresistance can be a result of abnormal functioning of these signaling pathways[22-24]. PTEN functions as a tumor suppressor gene, specifically by negatively regulating the AKT/PKB signaling pathway[25]. Previous studies have shown that PTEN is a dual-specificity phosphatase possessing both lipid and protein phosphatase activities. Activated PTEN affects a dephosphorylation of PIP3, generates PIP2, and decreases the phosphorylation level of AKT, which result in cell growth arrest and apoptosis[26]. Genetic inactivation of PTEN is a hallmark of many cancers, including CRC, and reduced expression occurs in many other tumor types. Deficiency of PTEN in the intestine has been reported to induce precancerous polyps, *via* the induction of formation and fission of crypts, structures located

Table 2 Impact of PTEN on miR-221-mediated Caco2 cell radiosensitivity

Group	D_0	D_q	SF_4	SER
Blank control + irradiation	1.677	5.590	0.5667	
Anti-PTEN-siRNA + irradiation	2.327	7.788	0.7569	0.718
Anti-miR-221 + irradiation	1.082	4.702	0.2977	1.550
Anti-miR-221 and anti-PTEN-siRNA + irradiation	1.511	5.010	0.4788	1.110

Caco2 cells were pretreated with anti-PTEN-siRNA for 24 h prior to the addition of anti-miR-221. D_0 and D_q were determined by standardized software, and the sensitization enhancement ratio (SER) was calculated by determining the ratio of the D_0 of the control group *vs* treated cells. SF_4: Surviving fraction at 4 Gy.

at the base of the intestine containing a rapidly dividing pool of intestinal stem cells[27,28]. Moreover, restoring PTEN expression in PTEN-deficient tumor cells has been shown to enhance radiosensitivity; however, little is known regarding the impact of miRNAs on PTEN expression in CRC[29,30].

Zhang *et al*[31] studied the miR-221 expression in a gastric cancer-derived cell line and demonstrated that PTEN was the target of miR-221. miR-221 is a newly discovered miRNA which is upregulated in multiple malignant tumors such as hepatocellular carcinoma, bladder cancer, and pancreatic cancer, and facilitates tumors entering S phase from G_0/G_1 phase by inhibiting the expression of cyclin dependent kinase inhibitor (CDKI)[32]. miR-221 therefore represents an attractive candidate for selective treatment with miR-221-specific inhibitor. miR-221 expression is abnormally increased in CRC and can promote CRC development, which has been confirmed by our previous studies[10]. However, the mechanism by which miR-221 modulates the malignant phenotype, including radioresistance, within CRC remains unknown. Here, we observed miR-221 upregulation in several human CRC-derived lines compared with HUVEC, corroborating the findings of our previous studies[10]. In this study, we predicted that PTEN would be a target gene of the miR-221 by computer-aided algorithm. Moreover, we found binding sites for human miR-221 in the PTEN 3'-UTR by using luciferase activity assay, suggesting that miR-221 might affect PTEN expression. Indeed, we demonstrated that introduction of miR-221 caused a significant increase of miR-221 value and decreased PTEN protein levels. Conversely, anti-miR-221 caused a significant decrease in miR-221 value and increased PTEN protein. Based upon these findings, we hypothesized PTEN as a target of miR-221 in CRC to regulate cell radiosensitivity.

As PTEN is a target of miR-221, and has been described previously as an important regulator of radiation sensitivity, these results suggest that increasing PTEN expression by silencing miR-221 could enhance the radiosensitivity of CRC cells. In this study, transfection of Caco2 cells with pre-miR-221 significantly increased survival following X-ray exposure compared to blank and negative controls. Conversely, transfection of Caco2 cells with anti-miR-221 significantly decreased survival following irradiation. Indeed, we proved that the Caco2 cells were sensitized to radiation by knockdown of miR-221; however, whether PTEN was the sole or main target for miR-221 regulation of radiosensitivity remains unknown. Thus, by using the PTEN-specific antagonist, anti-PTEN-siRNA, we demonstrated that the regulatory effect of anti-miR-221 on CRC cell radiosensitivity was partially, but not completely, mediated by PTEN, suggesting that miR-221 could regulate other PTEN-independent signaling pathways to enhance CRC radiosensitivity. It has been previously shown that CDKN1B/p27 and CDKN1C/p57 are also the target of miR-221 and consistently, CDKN1B/p27 and CDKN1C/p57 expressions inversely correlate in most cancers with miR-221 overexpression, which suggests that the inhibitory effect of anti-miR-221 on CRC cell radiosensitivity is only partially abrogated by anti-PTEN-siRNA[33]. We think that our results, which identify PTEN as a target for miR-221 in the context of CRC cell lines, fit well within a dynamic view of the miRNA-mediated regulation of gene expression: it is well known and widely predicted that the relationship between miRNAs and target mRNAs is not a "one to one" connection, because the same mRNA can be regulated by more than one miRNA, and that the choice of how many and which miRNAs target one 3'-UTR is strongly determined by the specific cellular environment[34-36]. An miRNA that regulates targets playing opposite roles in the control of cell proliferation may act as a tumor suppressor in some cancers and as an oncogene in others, depending on which targets are driving tumorigenesis in that specific cellular milieu.

In summary, we demonstrated that miR-221 could regulate CRC cell radiosensitivity by targeting PTEN. Our data suggest that upregulation of PTEN expression by transfection of anti-miR-221 has important biological effects on the radiosensitivity of CRC cells. These results identify anti-miR-221 as a potential therapeutic approach for CRC *via* upregulation of PTEN. However, it is noteworthy that the results in this study are based on only one cultured CRC cell line that might not necessarily comprehensively reflect other lines and the *in vivo* situation. Therefore, further experiments are required to elucidate the antitumor mechanisms of anti-miR-221 in *in vivo* systems.

COMMENTS

Background

miRNAs regulate gene expression by mainly binding to the 3'-untranslated region (UTR) of target mRNAs, leading to mRNA degradation or translation inhibition. miRNAs are aberrantly expressed in various cancers, suggesting that they play a vital role as a novel class of oncogenes or tumor suppressor genes, depending on the targets they regulate.

Research frontiers

Colorectal carcinoma (CRC) is one of the most dangerous malignancies in China. Previous studies have shown that miR-221 expression is elevated in radioresistant CRC cell lines; however, it is not known whether and how miR-221 controls the cellular response to irradiation. In this study, the authors investigated the alterations of miR-221 and phosphatase and tensin homolog

deleted on chromosome 10 (PTEN) gene expression in CRC cells after X-ray irradiation, and the mechanisms underlying the enhancement of radiosensitivity to irradiation in CRC cells transfected with anti-miR-221.

Innovations and breakthroughs

Some human miRNAs are consistently deregulated in human cancer, suggesting a role for these genes in tumorigenesis. This study showed that knocking down miR-221 by antisense oligonucleotides upregulated PTEN expression and PTEN was identified as a direct target of miR-221 in CRC. Moreover, upregulated PTEN expression suppressed AKT activity and increased radiation-induced cell death, resulting in enhancement of radiosensitivity in CRC cells.

Applications

This study indicated that anti-miR-221 enhanced the radiosensitivity of CRC cells by upregulating PTEN, and miR-221 might be a novel potential strategy for CRC treatment.

Peer review

Anti-miR-221 could enhance the radiosensitivity of CRC cells by upregulating PTEN. This study provides evidence for the antioncogenic activity of anti-miR-221 in the irradiation of CRC and this may be a useful biomarker or therapeutic target in CRC.

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ORIGINAL ARTICLE

Effectiveness of a hydroxynaphthoquinone fraction from *Arnebia euchroma* in rats with experimental colitis

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Abstract

AIM: To evaluate the potential effectiveness of hydroxynaphthoquinone mixture (HM) in rats with 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis.

METHODS: Colitis was induced by intracolonic administration of TNBS (80 mg/kg, dissolved in 50% ethanol). Rats were treated daily for 7 d with HM (2.5, 5, 10 mg/kg) and mesalazine 100 mg/kg 24 h after TNBS instillation. Disease progression was monitored daily by observation of clinical signs and body weight change. At the end of the experiment, macroscopic and histopathologic lesions of rats were scored, and myeloperoxidase (MPO) activity was determined. We also determined inflammatory cytokine tumor necrosis factor (TNF)-α level by ELISA, Western blotting and immunochemistry to explore the potential mechanisms of HM.

RESULTS: After intracolonic instillation of TNBS, animals developed colitis associated with soft stool, diarrhea and marked colonic destruction. Administration of HM significantly attenuated clinical and histopathologic severity of TNBS-induced colitis in a dose-dependent manner. It abrogated body weight loss, diarrhea and inflammation, decreased macroscopic damage score, and improved histological signs, with a significant reduction of inflammatory infiltration, ulcer size and the severity of goblet cell depletion (all $P < 0.05$ *vs* TNBS alone group). HM could reduce MPO activity. In addition, it also decreased serum TNF-α level and down-regulated TNF-α expression in colonic tissue. This reduction was statistically significant when the dose of HM was 10 mg/kg ($P < 0.05$ *vs* TNBS alone group), and the effect was comparable to that of mesalazine and showed no apparent adverse effect. The underlying mechanism may be associated with TNF-α inhibition.

CONCLUSION: These findings suggest that HM possesses favourable therapeutic action in TNBS-induced colitis, which provides direct pharmacological evidence for its clinical application.

Key words: *Arnebia euchroma* (Royle) Johnst; Hydroxynaphthoquinones; Inflammatory bowel disease; 2,4,6-trinitrobenzene sulfonic acid-induced colitis; Tumor necrosis factor

Core tip: Current therapies for inflammatory bowel disease are limited by lack of effectiveness, drug refractoriness or severe adverse effects. Therefore, there is

an urgent need for more effective and safe therapeutic approaches. Due to their favourable effect and less side effects, looking for novel agents from herbal and natural products has been a research focus for a long time. Previous studies demonstrate that hydroxynaphthoquinones exert therapeutic action on chronic inflammatory disease. In this study, hydroxynaphthoquinones showed beneficial effect on 2,4,6-trinitrobenzene sulfonic acid-induced colitis *via* tumor necrosis factor-α inhibition, and the effect was comparable to that of mesalazine. This provides pharmacological evidence for its clinical application.

Fan HY, Zhang ZL, Liu K, Yang MY, Lv WH, Che X, Xu H, Song WW. Effectiveness of a hydroxynaphthoquinone fraction from *Arnebia euchroma* in rats with experimental colitis. *World J Gastroenterol* 2013; 19(48): 9318-9327 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i48/9318.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i48.9318

INTRODUCTION

Inflammatory bowel disease (IBD), comprising Crohn's disease (CD) and ulcerative colitis (UC), is a chronic, relapsing inflammatory intestinal disorder[1]. The incidence and prevalence of IBD are now increasing worldwide. The highest incidences have been reported in North American and Northern Europe. In recent years, the incidence rates appear to be increasing in developing countries in Europe and Asia with westernisation of lifestyle and industrialization, including China, South Korea and India[2]. The exact pathogenesis remains elusive, but thus far, IBD is thought to be the results of interaction between genetic alterations and environment factors that induce an aberrant mucosal immune response, in which inflammatory cytokines play a critical role in the induction of colonic tissue damage[3,4]. Available therapies for IBD include conventional anti-inflammatory agents (such as 5-aminosalicylates and corticosteroids), immune modulators and biological therapy. Biological therapy aims at antagonizing pro-inflammatory molecules. Thus, inflammatory cytokines are the most logical targets for IBD treatment. Among various cytokines, tumor necrosis factor (TNF)-α is the cytokine that has been widely studied. Currently, the use of TNF-α blockers is the only licensed biological therapy for IBD and several TNF-α blockers (infliximab, adalimumab and certolizumab) have been applied in clinical practice. Although these available agents have shown clinical benefits to some degree, they are not entirely effective and have multiple adverse effects. Furthermore, IBD management requires long-term treatment that often leads to drug refractoriness or intolerance[3]. Patients who are unresponsive to the current therapy still suffer from this common disease. Therefore, it is necessary to develop novel therapeutic approaches.

Zicao, the dried root of *Arnebia euchroma* (Royle) Johnst, is a traditional Chinese herbal medicine. It has been used in China for thousands of years for the treatment of various diseases[5,6]. Naphthoquinones (also termed as hydroxynaphthoquinones in Pharmacopoeia of China) have been identified as the main representative active ingredients of *Zicao*. Hydroxynaphthoquinones mainly consist of alkannin and shikonin as well as their derivatives. Modern pharmacological studies have demonstrated that hydroxynaphthoquinones possess multiple biological activities such as anti-inflammation, wound healing, antibacterial and antifungal[5-9]. In particular, shikonin exerts significant anti-arthritic and immunomodulatory effects. It could inhibit the expression and transcriptional activation of TNF-α and reduce the production of inflammatory mediators[10-12]. Furthermore, the ointment containing alkannin derivatives has been applied successfully to the treatment of traumatic ulcers and acute anal fissures[6]. Based on these characteristics, we hypothesized that hydroxynaphthoquinones may prevent and even cure IBD.

In previous research, our group isolated a mixture of hydroxynaphthoquinone alkannin derivatives and validated its protective effect against experimental arthritis and its analgesic effect, as well as its modulatory effect on the serum TNF-α level[13,14]. Chemical analysis identified seven constituents: alkannin, acetylalkannin, β-acetoxyisovalerylalkannin, deoxyalkannin, β,β'-dimethylacrylalkannin, α-methybutyrylalkannin, and isovalerylalkannin[14]. The objective of this study was to investigate the potential therapeutic action of this hydroxynaphthoquinone mixture (HM) in the murine model of 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley (SD) rats (weight, 200-220 g) were purchased from the Animal Department of the College of Medicine, Beijing University [certificate No. SCXK (Jing) 2006-0008)]. All animals were allowed to acclimate for at least 1 wk at a temperature of 24 ± 1 ℃ and humidity of 55% ± 5%. All rats were housed in cages with food and tap water ad libitum. The experiment procedures were approved by Office of Experimental Animal Management Committee of Shandong Province, China.

Materials and regents

HM was provided by Shandong Target Drug Research Co. Ltd. Mesalazine slow-release granule was the product of Ethypharm Industries (France). TNBS was supplied by Sigma Chemical Co. (United States). Polyclonal anti-TNF-α antibody was purchased from Santa Cruz Biotechnology (CA, United States). Anti-β-actin antibody was obtained from Beyotime Institute of Biotechnology (Jiangsu Province, China). Rabbit anti-goat IgG was purchased from Boster Biotechnology (Wuhan, Hubei Province, China). Myeloperoxidase (MPO) detection kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). TNF-α ELISA Kit was the

product of R and D System (United States), which was obtained from Shanghai Chuanxiang Biotechnology Co. Ltd (Shanghai, China).

Induction of colitis and study design

After a 24 h fasting, rats were anaesthetized with pentobarbital, and then TNBS (80 mg/kg, dissolved in 50% ethanol) was instilled into the colon[15]. Control rats received saline instead. Animals were orally administered with mesalazine 100 mg/kg and HM (2.5, 5, 10 mg/kg) 24 h after TNBS instillation, daily for 7 d. Body weight and diarrhea of rats were recorded daily. On day 8, rats were anaesthetized with pentobarbital. Blood was collected from the abdominal aorta of rats. The serum was prepared by centrifuging the blood at 3000 *g* for 15 min for TNF-α assay. The colon was removed and placed on an ice-cold plate, cleared of fat and mesentery, and blotted on filter paper. Then, the colon was longitudinally opened, washed gently with ice-cold saline and blotted dry with filter paper. Finally, colon tissue was weighted. Its length was measured and the extent of macroscopic damage was evaluated. Afterwards, the colon was divided into several segments. One segment of the colon (approximately 2 cm) was used for histological examination. The rest of tissue segments were snap-frozen in liquid nitrogen and stored at -80 ℃ for MPO activity measurement and Western blotting analysis.

Macroscopic damage evaluation

Macroscopic colonic damage was assessed using a magnifying glass by an independent observer and was scored. The scale for macroscopic damage ranged from 0-10 and was based on the appearance of ulceration, thickening of the bowel wall, sites of ulceration and sites of inflammation[16].

Histopathological assessment

The tissue was fixed in 10% neutral buffered formalin, embedded in paraffin and stained with hematoxylin and eosin (H and E). Colonic damage and inflammation were scored blindly according to the criteria described previously[17].

Determination of myeloperoxidase activity

The assessment of MPO activity is a well established biochemical assay for quantifying intestinal inflammation[18]. The colonic samples (100 mg) were thawed and homogenized on ice in PBS buffer containing hexadecyl trimethyl ammonium bromide to prepare a 5% homogenate. The subsequent assay was performed according to the manufacturers' instructions. The absorbance was read at 460 nm using visible spectrophotometer. MPO activity was determined using the *O*-dianisidine method and the final results were expressed as units per gram of wet tissue.

TNF-α assay

Serum was obtained from all groups of rats on the final experimental day and then stored at -20 ℃ until analysis. The supernatant was used to measure the level of TNF-α with a rat TNF-α ELISA kit. The procedure was performed according to the manufacturer's instructions.

Immunohistochemical analysis

Paraffin-embedded colonic tissue sections (5 μmol/L) were fixed in 4% paraformaldehyde, deparaffinized and dehydrated through graded ethanol. The sections were washed three times with PBS for 5 min each, blotted drying, and then treated with 3% hydrogen peroxide for 30 min at room temperature to block the endogenous peroxidase activity. Afterwards, the sections were immersed in antigen retrieval solution (citrate buffer, pH 6.0) for 10 min. This was followed by rinsing with PBS. After blocking with normal goat serum for 30 min at 37 ℃, sections were co-incubated with a primary anti-TNF-α antibody (1:150 dilution in PBS) overnight and then with a peroxidase-conjugated anti-rabbit IgG secondary antibody for 1 h at room temperature. Thereafter, the sections were incubated with 3,3-diaminobenzidine (DAB) regents for 10 min, counterstained with hematoxylin, dehydrated and mounted for microscopy analysis. The intensity of immunoreactivity was examined with a pathological image analyzer and IMAGE-PRO PLUS analyzing program. The results were represented as mean integrated optical density (*A*) value for each sample.

Western blotting analysis

Frozen colonic tissues (60 mg) were thawed and later mechanically homogenized on ice. Homogenates were centrifuged at 10000 *g* for 20 min and total proteins were collected from the supernatant. The protein concentration was measured with a BCA protein assay kit. The protein was boiled in SDS sample loading buffer (Tris-HCl pH 6.8; 10% SDS; 0.5% bromophenol blue; 50% glycerine; 5% β-mercaptoethanol) for 5 min. Then equal amounts of protein (120 μg) were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis for 60 min, the protein was transferred onto polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 3% skim milk and saturated in Tris buffered saline with 1% Tween 20 for 1 h at room temperature. Subsequently, the membrane was incubated with the primary anti-TNF-α antibody (diluted 1:100 in TBST) overnight at 4 ℃. After that, the membrane was washed three times for 5 min each and probed with a horseradish peroxidase-conjugated anti-rabbit IgG antibody (diluted 1:10000 in TBST). Protein was detected using an enhanced chemiluminescence (ECL) detection kit (Beyotime Institute of Biotechnology), and bands were visualized by exposure to photographic film. Densitometric analysis of protein bands was performed using Quantity One v4.4.0.36 analyzer software (BIO-RAD).

Statistical analysis

Statistical analysis was performed using SPSS software 11.5 for windows. All results are expressed as mean ±

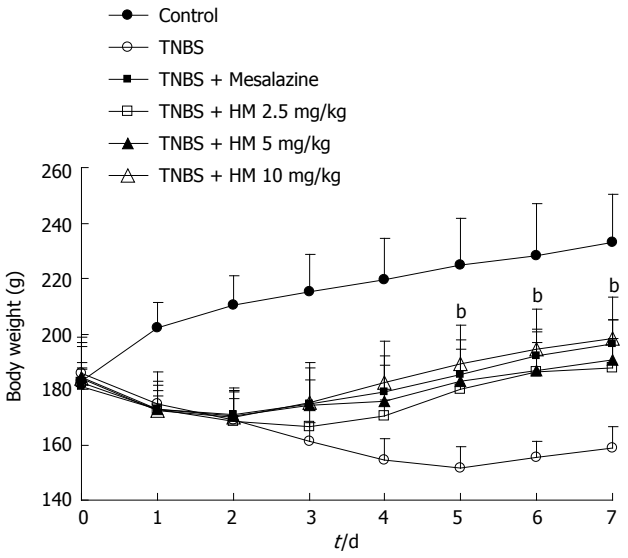

Figure 1 Treatment with hydroxynaphthoquinone mixture ameliorates body weight loss of 2,4,6-trinitrobenzene sulfonic acid-induced rats. Colitis was induced by intracolonic administration of TNBS (80 mg/kg, dissolved in 50% ethanol). Rats were treated daily for 7 d with HM (2.5, 5, 10 mg/kg) and mesalazine 100 mg/kg 24 h after TNBS instillation. Disease progression was monitored by observation of clinical signs and body weight change. Data are represented as mean ± SD of 8 animals of each group. $^{b}P < 0.01$ *vs* TNBS alone. HM: Hydroxynaphthoquinone mixture; TNBS: 2,4,6-trinitrobenzene sulfonic acid.

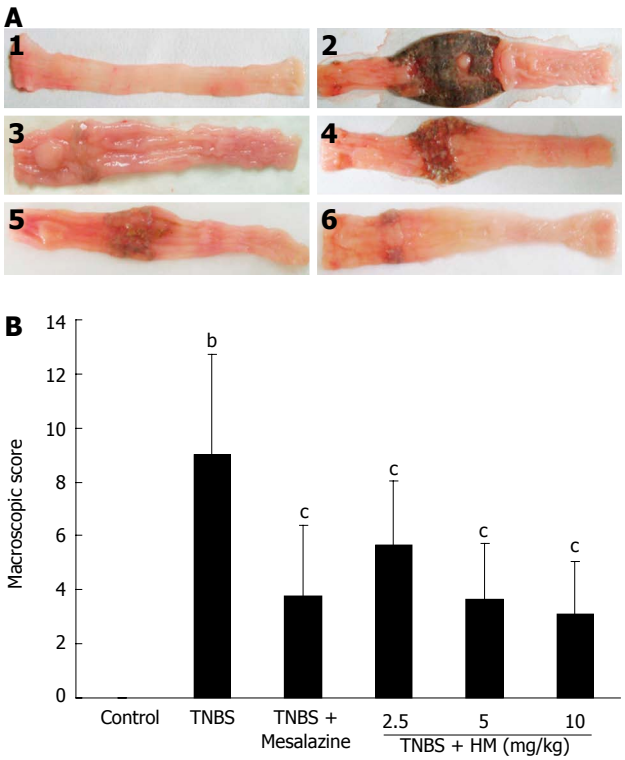

Figure 2 Hydroxynaphthoquinone mixture reduces colonic macroscopic damage in rats with 2,4,6-trinitrobenzene sulfonic acid-induced colitis. A: Intestinal macroscopic changes; B: Macroscopic pathological scores. Colitis was induced by intracolonic administration of TNBS (80 mg/kg, dissolved in 50% ethanol). Rats were treated daily for 7 d with HM (2.5, 5, 10 mg/kg) and mesalazine 100 mg/kg 24 h after TNBS instillation. 1: Control; 2: Rat treated with TNBS alone; 3: Rat treated with TNBS and mesalazine; 4-6: Rats treated with TNBS and HM (2.5, 5, 10 mg/kg). Data are represented as mean ± SD of 8 animals of each group. $^{b}P < 0.01$ *vs* control; $^{c}P < 0.05$ *vs* TNBS alone. HM: Hydroxynaphthoquinone mixture; TNBS: 2,4,6-trinitrobenzene sulfonic acid.

SD. Comparisons between groups of nonparametric data were made with the Kruskal-Wallis test followed by the Mann-Whitney *U* test. For evaluation of the body weight and TNF-α level, one-way ANOVA followed by Tukey's *post hoc* test was used. $P < 0.05$ was considered significant.

RESULTS

HM improves clinical symptoms in rats with TNBS-induced colitis

After intracolonic instillation of TNBS, animals developed colitis associated with soft stool and diarrhea. All TNBS-treated rats had profound weight loss compared with the weight gain seen in controls (all $P < 0.01$). Rats treated with HM and mesalazine gradually recovered the lost body weight beginning on day 3, accompanied by improving symptoms (Figure 1).

HM reduces colonic macroscopic damage in rats with TNBS-induced colitis

Control animals showed no colonic damage and the colonic damage score was zero. Rats in the TNBS group displayed hyperemia, thickening of the bowel, necrosis, inflammation, and a large area of ulceration. Moreover, moderate to severe adhesion of the colon to the surrounding organs was also observed. The macroscopic colon damage score was significantly increased. The severity of colonic destruction was markedly ameliorated after oral administration of HM and mesalazine, with reduced area of inflammation and ulcer (Figure 2).

HM prevents TNBS-induced histopathological change

There was no histopathological change in the colons of control rats. Histologic evaluation of the colon of TNBS-treated rats showed transmural inflammation involving all layers of the bowel. The inflammatory process was associated with patchy ulceration, epithelial cell loss, pronounced depletion of goblet cells, distortion of the tubular glands, numerous inflammatory cell infiltrations, and dilated crypts. When rats were administered with HM, these histologic signs were much improved, with significant reduction of inflammatory infiltration and ulcer size. The transmural involvement of the lesions was reduced, and the goblet cell depletion was less severe (Figure 3).

HM reduces MPO activity in rats with TNBS-induced colitis

Myeloperoxidase in the intestine is a well-known enzyme that is directly correlated with the degree of neutrophil infiltration[18]. As shown in Figure 4A, MPO activity was markedly increased in colonic tissue following TNBS instillation compared to controls ($P < 0.01$, 2.29 ± 0.78 U/g *vs* 0.65 ± 0.11 U/g tissue). After treatment with HM,

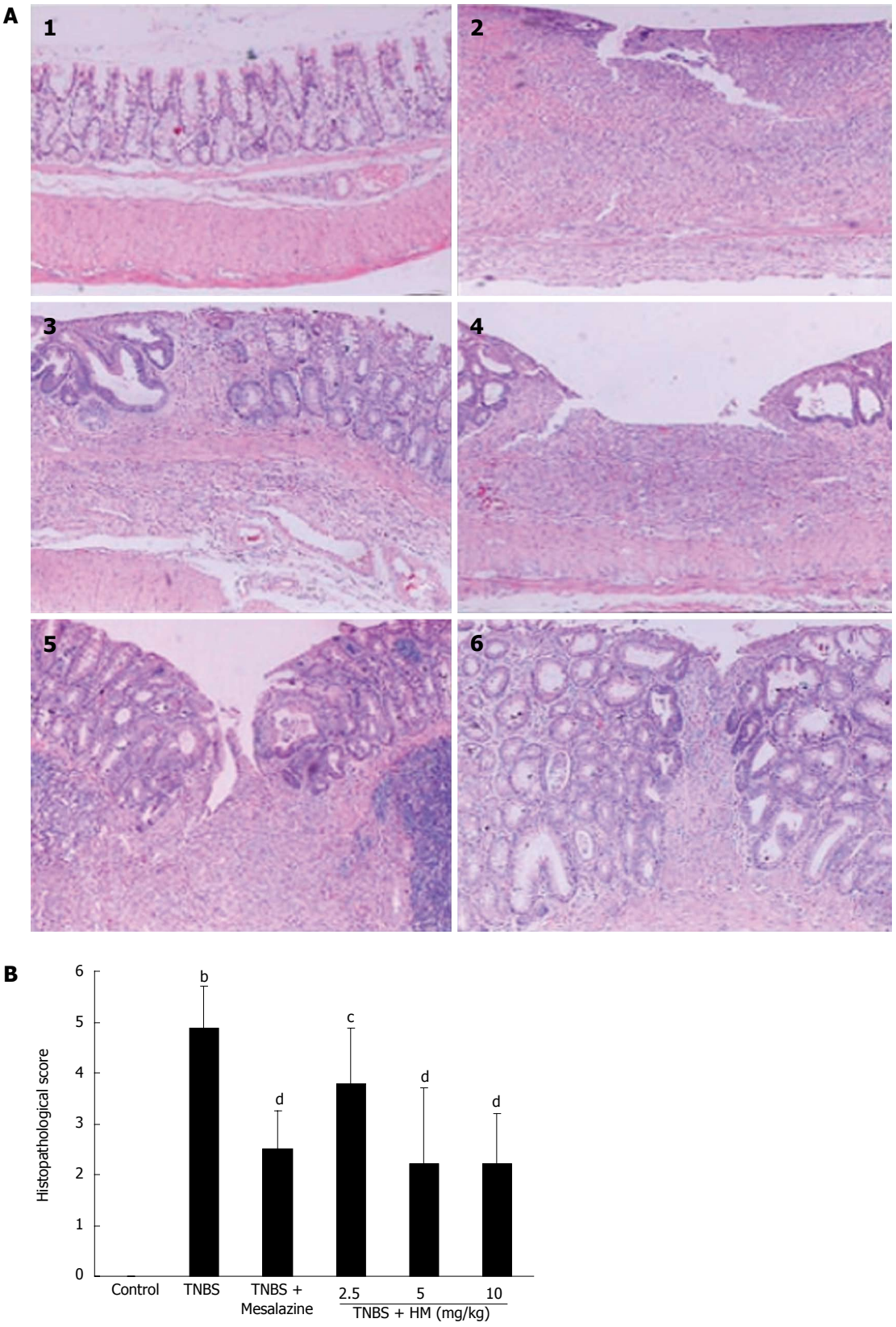

Figure 3 Hydroxynaphthoquinone mixture prevents 2,4,6-trinitrobenzene sulfonic acid-induced histopathological changes. A: Intestinal histopathological features; B: Pathological scores of representative colonic samples of each group. Colitis was induced by intracolonic administration of TNBS (80 mg/kg, dissolved in 50% ethanol). Rats were treated daily for 7 d with HM (2.5, 5, 10 mg/kg) and mesalazine 100 mg/kg 24 h after TNBS instillation. Histopathological analysis was performed in HE-stained sections of colons. 1: Control; 2: Rat treated with TNBS alone; 3: Rat treated with TNBS and mesalazine; 4-6: Rats treated with TNBS and HM (2.5, 5, 10 mg/kg). Data are represented as mean ± SD of 5 animals of each group. $^{b}P < 0.01$ *vs* control; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* TNBS alone. HM: Hydroxynaphthoquinone mixture (original magnification, × 100); TNBS: 2,4,6-trinitrobenzene sulfonic acid.

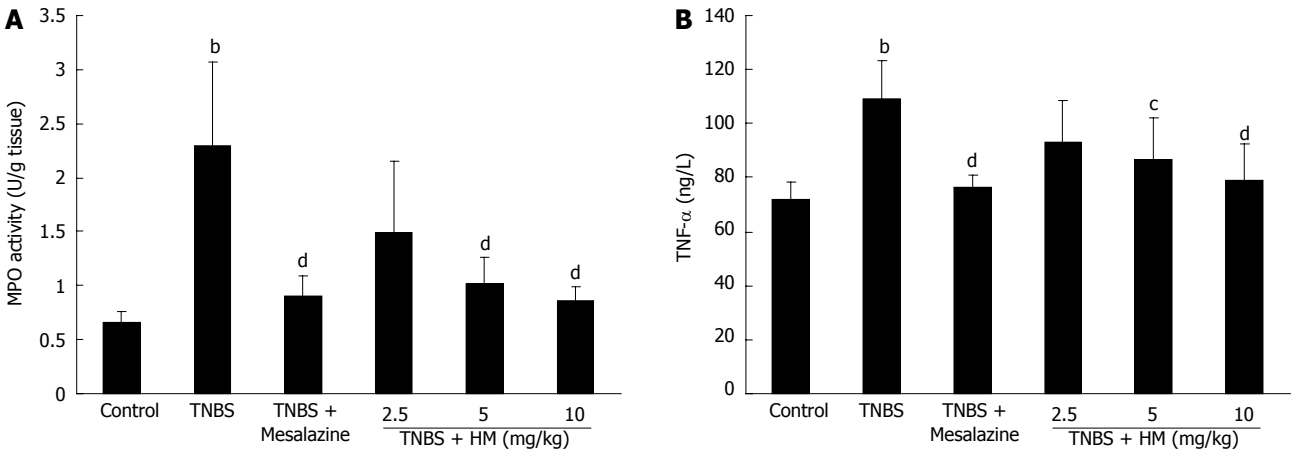

Figure 4 Hydroxynaphthoquinone mixture decreases myeloperoxidase activity (A) and serum tumor necrosis factor-α level (B) in rats with 2,4,6-trinitrobenzene sulfonic acid-induced colitis. Colitis was induced by intracolonic administration of TNBS (80 mg/kg, dissolved in 50% ethanol). Rats were treated daily for 7 d with HM (2.5, 5, 10 mg/kg) and mesalazine 100 mg/kg 24 h after TNBS instillation. TNF-α level was determined by ELISA. Data are represented as mean ± SD of 8 animals of each group. $^{b}P < 0.01$ *vs* control; $^{c}P < 0.05$, $^{d}P < 0.01$ *vs* TNBS alone. HM: Hydroxynaphthoquinone mixture; TNBS: 2,4,6-trinitrobenzene sulfonic acid; MPO: Myeloperoxidase; TNF-α: Tumor necrosis factor-α.

MPO activity were reduced to be 1.49 ± 0.66, 1.02 ± 0.24 and 0.86 ± 0.13 U/g tissue for HM 2.5, 5 and 10 mg/kg, respectively. The effect was significant at doses of 5 and 10 mg/kg. This indicated that treatment with HM attenuated the degree of inflammation response. The similar efficacy for mesalazine was also observed.

HM decreases serum TNF-α level in rats with TNBS-induced colitis

As depicted in Figure 4B, serum TNF-α level was obviously higher in TNBS-treated rats compared with that in controls. In contrast, pretreatment with HM and mesalazine prevented the increase in TNF-α level. The mean serum TNF-α levels were determined to be 71.90 ± 6.33 ng/L for the control group, 109.05 ± 14.30 ng/L for the TNBS group, 76.16 ± 4.64 ng/L for the mesalazine group, 93.00 ± 15.24 ng/L for the HM 2.5 mg/kg group, 86.96 ± 15.26 ng/L for the HM 5 mg/kg group and 78.72 ± 13.94 ng/L for the HM 10 mg/kg group.

HM attenuates immunostaining for TNF-α in rats with TNBS-induced colitis

Representative samples of each group were immunohistochemically stained for the expression of TNF-α. As shown in Figure 5A1, colonic section obtained from the control group showed negative staining. TNBS-treated rats showed strongly positive staining for TNF-α. Positively stained cells appeared in the mucosal and submucosal inflammatory cells (Figure 5A2 and B). Administration of HM attenuated the degree of TNF-α staining in the colon tissue. Significantly less positive cells were observed in tissues from HM 5 mg/kg- and 10 mg/kg-treated rats than in those from TNBS-treated rats (Figure 5A4, A5 and Figure 5B).

HM decreases TNF-α expression in colonic tissue of rats with TNBS-induced colitis

As shown in Figure 6, higher TNF-α expression was observed after TNBS instillation as compared with controls. HM treatment reduced the expression of TNF-α in a dose-dependent manner.

DISCUSSION

Many plant-derived extracts or chemicals have pharmacological effects and clinical benefits, which provide a great potential in improving the symptoms of IBD. The published literature has revealed that several natural products exhibit encouraging anti-IBD activity by inhibition of cytokine production, such as flavonoids or polyphenolic compounds[19]. TNBS-induced colitis is a Th1 cell-mediated inflammatory disease, associated with excessive secretion of cytokines as a consequence of exaggerated macrophage and neutrophil infiltration and activation, giving rise to transmurally inflamed intestinal mucosa, which displays clinical, biochemical, and pathological similarities to human CD. It is a commonly used experimental model system to test potential therapeutic agents[20]. Thus, the present study was performed to investigate the potential effect of HM on CD using TNBS-induced colitis.

The results obtained from the study demonstrate for the first time the efficacy of HM in intestinal inflammation, confirming the hypothesis mentioned in the introduction section that hydroxynaphthoquinones possess the ability to attenuate symptoms of IBD. Administration of HM at the onset of the disease ameliorated the clinical severity of the wasting disease, abrogating body weight loss, diarrhea, inflammation and the area of ulceration. This beneficial effect was further evidenced by histological evaluation, with a marked reduction in the extent and severity of inflamed tissue damage and infiltration of inflammatory cells. The beneficial effect was also established by a decrease in MPO activity. As mentioned above, MPO is a marker of neutrophil infiltration. It has been reported that MPO activity is increased in several

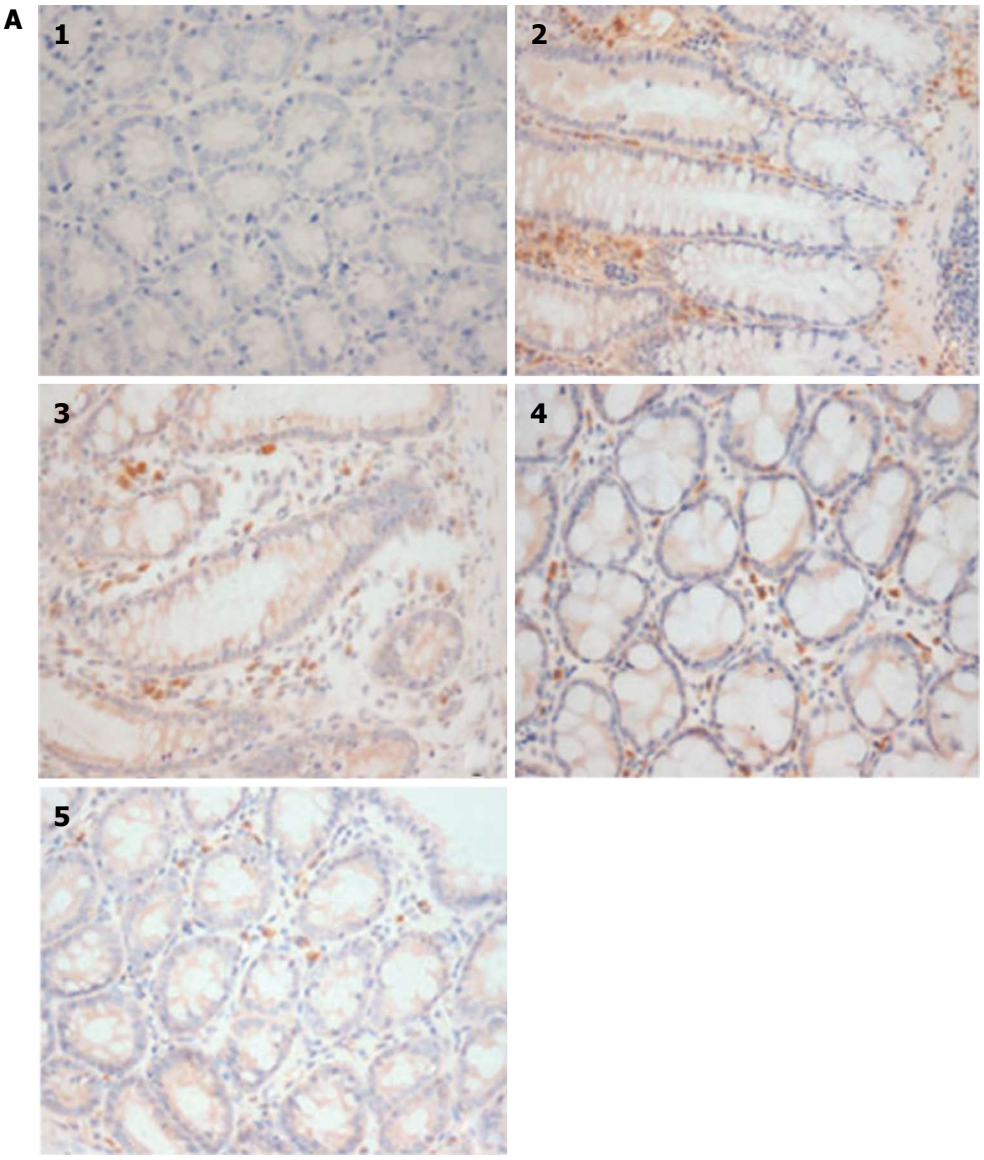

Figure 5 Immunostaining for tumor necrosis factor-α. A: Immunostaining sections; B: Integrated optical density (*A*) values of representative colonic samples of each group. Colitis was induced by intracolonic administration of TNBS (80 mg/kg, dissolved in 50% ethanol). Rats were treated daily for 7 d with HM (2.5, 5, 10 mg/kg) 24 h after TNBS instillation. Colons were excised and stained with anti-TNF-α antibody. 1: Control; 2: Rat treated with TNBS alone; 3-5: Rats treated with TNBS plus HM (2.5, 5, 10 mg/kg). Data are represented as mean ± SD of 4 animals of each group. $^{a}P < 0.05$ *vs* control; $^{d}P < 0.01$ *vs* TNBS alone. HM: Hydroxynaphthoquinone mixture (original magnification, × 400); TNBS: 2,4,6-trinitrobenzene sulfonic acid; TNF-α: Tumor necrosis factor-α.

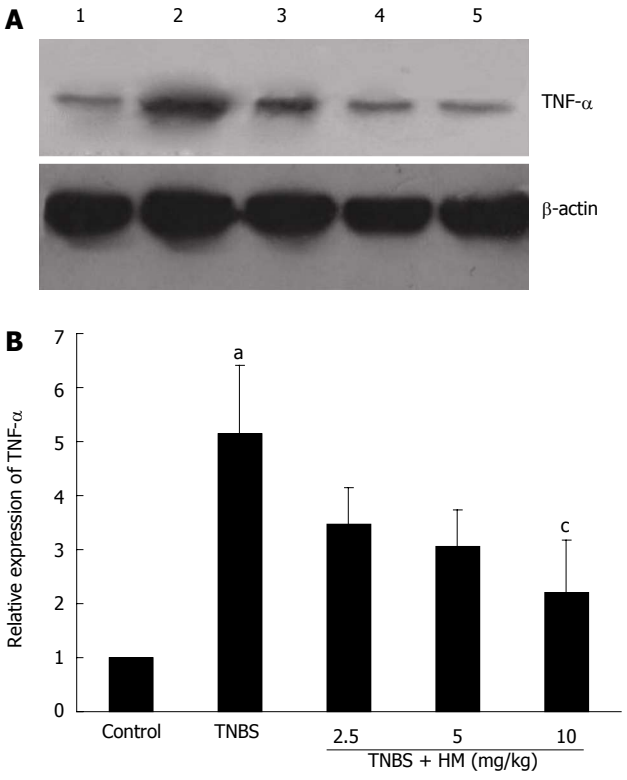

Figure 6 Hydroxynaphthoquinone mixture decreases tumor necrosis factor-α expression in colonic tissue of rats with 2,4,6-trinitrobenzene sulfonic acid-induced colitis. A: Tumor necrosis factor (TNF)-α protein bands; B: Integrated optical density (*A*) values of protein bands. Colitis was induced by intracolonic administration of TNBS (80 mg/kg, dissolved in 50% ethanol). Rats were treated daily for 7 d with HM (2.5, 5, 10 mg/kg) 24 h after TNBS instillation. Protein extracts were obtained from colons and TNF-α expression level was detected by Western blotting analysis. Lane 1: Controls; lane 2: Rats treated with TNBS alone; lane 3-5: Rats treated with TNBS plus HM (2.5, 5, 10 mg/kg). Data are represented as mean ± SD of 4 animals of each group. $^{a}P < 0.05$ *vs* control; $^{c}P < 0.05$ *vs* TNBS alone. HM: Hydroxynaphthoquinone mixture; TNBS: 2,4,6-trinitrobenzene sulfonic acid.

experimental colitis models, including TNBS-induced colitis[18], and it is widely used to quantify intestinal inflammation and assess the degree of inflammation. Thus, a reduction of MPO activity can be interpreted as a manifestation of the anti-inflammatory effect of a given compound[18,21,22]. Inhibition of MPO activity by HM was consistent with the results observed in the histological examination, in which the level of inflammatory cell infiltration in colonic tissues was lower in HM-treated animals than in TNBS-treated rats.

Mesalazine was used as a reference in this study. Several reports suggest that mesalazine acts locally in the colon. After intragastric administration of mesalazine, it is absorbed from intestinal lumen and concentrates in the mucosa. The effectiveness of the drug directly depends on its mucosal concentration[23,24]. In the present experiment, oral administration with mesalazine 100 mg/kg resulted in a significant improvement in intestinal lesions, which was consistent with previous findings[24,25]. The effect of HM 10 mg/kg was comparable to that of mesalazine.

The anti-inflammatory effect and protection against tissue injury exerted by HM was further confirmed by the down-regulation of TNF-α in serum and colonic tissue. TNF-α is an important inflammatory mediator and plays a key role in the colonic damage. Among the various cytokines involved in the pathogenesis of TNBS colitis, TNF-α appears to be a key regulator since it has been reported that TNBS-induced colitis could not be induced in TNF-α-deficient mice and is far more severe in mice that over-express this inflammatory cytokine[26]. Different agents interfering with TNF-α signaling have been successful in the treatment of subsets of CD patients, and display favourable therapeutical effect, particularly TNF-α blockers[27]. Moreover, the majority of previous preclinical studies and current therapeutic approaches[27-29] have confirmed the idea that therapy that can address an essential element of a final common pathologic pathway participating in IBD could potentially treat this disease[30]. Therefore, we assessed the impact of HM on TNF-α signaling and explored the underlying mechanism. The results obtained from the present study showed that HM significantly lowered TNF-α level in serum and reduced TNF-α expression in colonic tissue. This indicates that the preventive effect of HM against TNBS-induced colonic injury is directly or indirectly related to its TNF-α inhibition. However, given that many other signaling pathways and transcription factors are involved in the regulation of TNF-α activity, additional research is needed to examine the exact mechanisms of action of HM.

In summary, the present study demonstrates that treatment with HM attenuates the clinical symptoms of TNBS-induced colitis, resulting in significant histological improvement, reduced MPO activity and TNF-α level in serum, and down-regulation of TNF-α expression in colonic tissue. The underlying mechanism of action of HM may be associated with TNF-α inhibition. These results provide supporting evidence for the clinical application of HM.

COMMENTS

Background

The incidence and prevalence of inflammatory bowel disease (IBD) are increasing at a disturbing rate worldwide, and now it has become a global disease. Current therapies for IBD are limited by lack of effectiveness, drug refractoriness or severe adverse effects. Therefore, there is an urgent need for more effective and safe therapeutic approaches.

Research frontiers

Looking for novel agents from herbal or natural products has been a research focus for a long time. Many plant-derived extracts or chemicals have pharmacological effects and clinical benefits. The published literature has revealed that several natural products exhibit encouraging anti-IBD activity by inhibition of cytokine production, such as flavonoids or polyphenolic compounds. Hydroxynaphthoquinones have been identified as the main active ingredients of *Zicao*, a traditional Chinese herbal medicine, and possess potent anti-inflammatory, wound healing and antibacterial activities. Yet no studies have evaluated the therapeutical effect of hydroxynaphthoquinones on treating inflammatory intestinal diseases so far. In this study, the authors isolate a hydroxynaphthoquinone mixture (HM) from *Zicao*, which mainly contains seven alkannin derivatives, and demonstrate that HM showed beneficial effect on TNBS-induced colitis.

Innovations and breakthroughs

This is the first study to report that hydroxynaphthoquinones exert therapeutical

effect on mucosal inflammation by modulating tumor necrosis factor-α, and the effect of hydroxynaphthoquinones is comparable to that of mesalazine, a classical anti-IBD drug.

Applications

Elucidation of the effect and mechanism of action of hydroxynaphthoquinone mixture (HM) in the treatment of inflammatory intestinal disease provides pharmacological evidence for its further development as a candidate for treating IBD, and for its clinical application.

Peer review

This study describes the beneficial effects of HM from *Arnebia euchroma* in an experimental model of colitis in rats. It revealed that HM significantly attenuated the severity of mucosal lesions. The authors have made a complex and interesting study.

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BRIEF ARTICLE

Latent hepatitis B is a risk factor for hepatocellular carcinoma in patients with chronic hepatitis C

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Abstract

AIM: To study the potential association between hepatocellular carcinoma (HCC) in patients with chronic hepatitis C (CHC), cirrhosis and latent hepatitis B (LHB) infection, defined as the absence of detectable serum hepatitis B surface antigen (HBsAg) and the presence of hepatitis B core antibody (HBcAb).

METHODS: This retrospective analysis is comprised of 185 cirrhotic patients with HCC who were hepatitis C virus antibody (HCV Ab) (+) and HBsAg(-) at Wayne State University between 1999 and 2008. From these, 108 patients had HCV polymerase chain reaction confirmation of viremia while the remaining (77) were considered to have CHC on the basis of a positive HCV Ab and the absence of any other cause of liver disease. Controls were drawn from our institutional database from the same time period and consisted of 356 HBsAg(-) age, race and gender matched patients with HCV RNA-confirmed CHC and without evidence of HCC. A subgroup of controls included 118 matched patients with liver cirrhosis. χ^2 test and *t* test were used for data analysis.

RESULTS: Seventy-seven percent of patients in all 3 groups were African Americans. Patients with HCC had a significantly higher body mass index ($P = 0.03$), a higher rate of co-infection with human immunodeficiency virus (HIV) ($P = 0.05$) and a higher prevalence of alcohol abuse ($P = 0.03$) than the controls. More patients with HCC had LHB than controls (78% *vs* 39%, $P = 0.01$). Sixty three percent of patients with HCC were both hepatitis B surface antigen (HBsAb)(-) and HBcAb(+) compared to 23% of controls ($P < 0.01$). When compared to cirrhotic controls, the frequency of HBcAb(+) remained higher in patients with HCC (78% *vs* 45%, $P = 0.02$). Patients with HCC were more likely to be both HBsAb(-) and HBcAb(+) than the cirrhotic controls (63% *vs* 28%, $P = 0.01$). Although not statistically significant, 100% of CHC and HIV co-infected patients with HCC ($n = 11$) were HBcAb(+) when compared to controls (44%; $n = 9$).

CONCLUSION: These data suggest that LHB occurs at a significantly increased frequency in patients with CHC and HCC than in patients with CHC without HCC.

Key words: Hepatocellular carcinoma; Chronic hepatitis C; Latent hepatitis B; Hepatitis C virus

Core tip: Latent hepatitis B (LHB) has recently received significant attention among researchers and clinicians managing chronic liver disease. It is defined as a combination of hepatitis B surface antigen negative and hepatitis B core antibody positive. The potential association of LHB with hepatocellular carcinoma among patients with chronic hepatitis C infection has been studied and reported in this manuscript.

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INTRODUCTION

Emerging data suggest that the mortality rate in cirrhotic patients with hepatocellular carcinoma (HCC) is rising whereas the mortality rate from other complications of cirrhosis is either stable or declining[1]. In the United States, chronic hepatitis C (CHC) accounts for the majority of cases of HCC. Among patients with CHC, factors such as older age, male gender, severity of liver disease, metabolic syndrome and poor response to interferon therapy are established risk factors for hepatocarcinogenesis[1]. "Latent hepatitis B (LHB)", defined as the presence of detectable hepatitis B core antibody (HBcAb) with undetectable hepatitis B surface antigen (HBsAg)(-) in serum and usually with detectable HBV DNA in hepatocytes, has not been studied as a risk factor for HCC in the United States[2]. Patients with previous exposure to hepatitis B virus but with no evidence of chronic infection are HBsAg(-) and HBcAb(+). This finding alone is now considered as unrecognized LHB[3]. In a large study, the majority of patients with LHB had detectable hepatitis B DNA (HBV DNA) in serum as well as in liver tissue[4]. Various other studies have also confirmed the same findings[5,6]. This led to the identification of a unique group of patients who are HBcAb(+) and at risk for latent hepatitis B.

Early studies from the 1990s suggested that patients with HCC in the absence of chronic hepatitis B and C had detectable covalent closed circular hepatitis B DNA (ccc DNA) in liver parenchyma although they were HBsAg(-) in serum. These patients were considered to have "occult hepatitis B"[7]. A single prospective study by Squadrito *et al*[8] revealed that among HBsAg(-) patients with CHC, patients with occult hepatitis B with ccc DNA in liver biopsy specimens were at a higher risk for the development of HCC. With the availability of highly sensitive real-time polymerase chain reaction (PCR) assays for the measurement of HBV DNA, tissue analysis for HBV DNA is largely unnecessary to make a diagnosis of latent hepatitis B[2]. Patients with cirrhosis from alcoholic and non-alcoholic fatty liver disease are also at a significantly higher risk for developing HCC when associated with LHB particularly in those who were HBcAb(+) but HBsAg(-)[9]. Injection drug users, patients on hemodialysis, patients with CHC and human immunodeficiency virus (HIV)-infected patients are at increased risk for LHB[10]. In patients with CHC, occult hepatitis B seems to be associated with rapid progression of liver disease[11]. Studies from areas with high prevalence of chronic hepatitis B have associated occult hepatitis B with HCC among patients with CHC[12,13]. This association is much stronger among CHC patients who are non-responders to currently available therapy[14]. Another study reported that although occult hepatitis B may not have a significant impact on response of CHC to interferon, it does increase the risk for HCC among non-responders but not among responders[15].

A large multicenter Japanese study concluded that CHC patients with LHB are at a significantly higher risk for developing HCC[16]. In the same study, interferon was less effective in preventing HCC in patients with LHB when compared to those without evidence of previous HBV exposure. This association was independent of the presence of HBV DNA in serum and therefore, LHB is clinically and prognostically more relevant than serum DNA status.

The above referenced studies establishing LHB as a risk factor for development of HCC are from countries with high endemicity for chronic hepatitis B infection. We studied this potential association among predominantly African American patients with CHC and cirrhosis in an area with low endemicity for chronic hepatitis B.

MATERIALS AND METHODS

This retrospective study, included patients with CHC who were diagnosed with HCC between January 1999 and December 2008 at the Detroit Medical Center, Detroit, Michigan. The primary sites were the Wayne State University Gastroenterology clinic and the Department of Pathology at Harper University Hospital in Detroit. Patients with a diagnosis of HCC who were > 18 years old, hepatitis C Ab(+) and HBsAg(-) were included in our study. The diagnosis of HCC was made either by histopathology or by non-invasive criteria *i.e.*, alpha fetoprotein (AFP) > 200 ng/mL and a mass lesion in the liver with radiological features typical for HCC observed on two or more imaging modalities (European Association for the Study of the Liver, EASL criteria)[17]. The study group consisted of both inpatients and outpatients although the majority were outpatients.

A control comparison group consisted of patients with CHC who were HBsAg(-) without evidence of HCC. These patients were drawn from our institutional database of CHC patients. They were age, race and gender matched to the cases and were from the same time period. Patients with HIV and CHC coinfection were included in the study and were part of a subset analysis. The study was approved by the Institutional Review Board at Wayne State University and the Detroit Medical Center.

A total of 185 patients fulfilled the selection criteria and were evaluated for inclusion in the study. Of these, 108 had serum RNA confirmation of CHC by PCR, while CHC viremia was presumed in the remaining 77. A total of 356 matched (1:2) non-HCC controls with CHC were selected from our database. All controls had PCR confirmation of CHC, were HBsAg(-) and were selected from the same time period. Non HCC controls included 118 matched patients with cirrhosis diagnosed by histopathology or by clinical criteria. Since the majority of patients with CHC who develop HCC have underlying advanced liver disease[10], a selected sub-group of matched

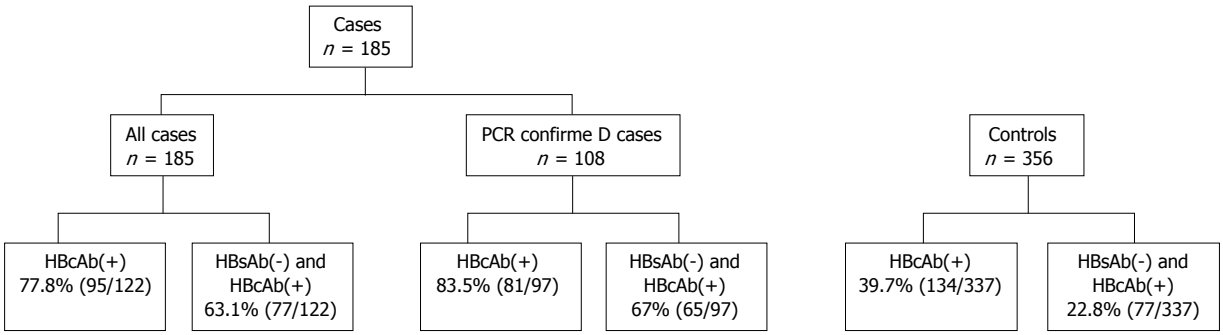

Figure 1 Hepatitis B serology in cases and controls. HBcAb: Hepatitis B core antibody; HBsAb: Hepatitis B surface antigen; PCR: Polymerase chain reaction.

Table 1 Baseline characteristics of hepatocellular carcinoma cases

	HCV Ab(+), HCV RNA(+) (*n* = 108)	HCV Ab(+) (*n* = 77)	*P* value
Age (yr), mean ± SD	60.88 ± 8.6	59.42 ± 7.4	0.88
Males	73.10%	67.53%	0.32
African American	75.90%	72.70%	0.22
	n = 70	*n* = 52	0.20
BMI (kg/m^2)	29.56 ± 6.11	27.88 ± 5.2	
HIV coinfection	10.20%	7.60%	0.16
Heavy alcohol use	37.03%	48.05%	0.04

HCV Ab: Hepatitis C virus antibody; HCV DNA: Hepatitis C DNA; BMI: Body mass index; HIV: Human immunodeficiency virus.

controls with cirrhosis and CHC was utilized in the analysis (Figure 1). In addition to demographic data, alcohol intake was assessed by chart review. Patients were classified into three categories based on alcohol consumption. Those who consumed 1 to 2 servings of liquor or wine a week or ⩽ 6 beers (12 oz) a week were categorized as "mild drinkers". Patients with alcohol consumption that exceeded this amount were considered "heavy drinkers". The third group was designated as "non drinkers". Lab values closest to the date of the diagnosis of HCC were collected. In controls, lab values at the time of their initial evaluation were recorded.

Statistical analysis

Data was analyzed using SPSS version 12. χ^2 test was used to analyze nominal data while *t* test was used to compare means among groups. Univariate analysis was then performed after controlling for covariates in the final analysis. Among cases, baseline characteristics were compared in patients with PCR confirmation of CHC and in those without PCR confirmation (Table 1). Since these groups were identical in baseline characteristics, they were combined for subsequent analysis. Additionally, subset analysis of African-American patients, patients with PCR confirmation of CHC, and cirrhotic patients was performed.

RESULTS

The mean age of patients with HCC was 60 years, and 71% were male (Table 2). More than seventy five percent of patients in each group were African-American. HCC was diagnosed by biopsy in 129 patients and by non-invasive (EASL) criteria in the remainder. Patients with HCC had a significantly higher body mass index (BMI), AFP, aspartate aminotransferase, alanine aminotransferase and a more prolonged prothrombin time (PT), but they had a lower albumin and platelet count. HIV-HCV coinfection was seen more commonly in patients with HCC (8.1%) than in controls (2.5%, $P = 0.05$). While mild alcohol consumption was not different in both groups, patients with HCC were more likely to be heavy drinkers (42% *vs* 27%; Table 2). Furthermore, HCV patients without HCC were more likely to be non-drinkers (32%) compared to patients with HCC (11%, $P < 0.01$; Table 2).

HBcAb was positive in 78% of patients with HCC but in only 40% of controls ($P = 0.01$). When hepatitis B surface antibody (HBsAb) status was determined, 63% of HCC cases were both HBsAb(-) and HBcAb(+) as compared to only 23% of controls ($P < 0.01$). When analysis was restricted to patients with cirrhosis, the prevalence of HBcAb was higher in cirrhotic controls at 42%, and the combination of HBsAb(-) and HBcAb(+) was also more prevalent when compared to total controls (27.6% *vs* 63.1%, $P < 0.01$). Despite this difference in prevalence of HBsAb and HBcAb among control groups, overall prevalence remained significantly higher in patients with HCC (63.1% *vs* 22.8%). Although statistical significance was not achieved, 100% of HIV-HCV coinfected patients with HCC (44.4%) were HBcAb(+) when compared to < 50% among coinfected controls.

Univariate analysis predicting HCC showed that HBcAb(+) status had an odds ratio of 1.9 (95%CI: 1.28-3.04, $P = 0.02$) where as a combination of HBsAb(-) and HBcAb(+) had a higher odds ratio of 3.24 (95%CI: 2.28-4.62, $P < 0.01$). In this analysis, BMI, albumin, PT, alcohol consumption and HIV coinfection were identified covariates. When regression analysis was performed after controlling for these covariates, these odds ratios were 1.84 (95%CI: 1.22-3.08, $P = 0.01$) and 2.98 (95%CI: 2.12-5.08, $P < 0.01$) respectively. Analysis of cirrhotic patients when controlled for covariates showed that HBcAb(+) had an odds ratio of 1.66 and a combination of HBsAb(-) and HBcAb(+) was at 2.10 (95%CI: 2.12-4.04, $P < 0.01$).

Table 2 Comparison of variables in cases and controls

	CHC with HCC (n = 185)	CHC without HCC (n = 356)	P value
Age (yr), mean ± SD	60.3 ± 9.71	59.72 ± 9.2	
Males	70.80%	71.10%	
Race AA	74.60%	78.70%	
CAU	21.60%	18.50%	
Other	3.80%	2.80%	
BMI (kg/m^2)	n = 122	n = 320	
	28.8 ± 6.01	27.26 ± 5.9	
Albumin	n = 178	n = 289	
	2.67 ± 0.7	3.88 ± 0.6	< 0.01
PT (s)	n = 171	n = 242	
	16.69 ± 8.6	11.57 ± 2.5	< 0.01
AFP (ng/mL)	n = 163	n = 284	
	99035.1 ± 263605.5	21.12 ± 90.4	
Platelets	n = 172	n = 337	
	176.5 ± 127	202.98 ± 83.4	0.04
ALT (IU/L)	n = 166	n = 345	
	263.8 ± 518.2	78.19 ± 58.3	< 0.01
AST (IU/L)	n = 160	n = 324	
	283.8 ± 63.5	75.2 ± 55.9	< 0.01
HIV co-infection	8.10%	2.50%	0.05
Alcohol			
Mild	29.7%	27.50%	NS
Heavy	41.60%	27%	0.03
Non drinkers	10.80%	31.70%	< 0.01

AST: Aspartate aminotransferase; ALT: Alanine aminotransferase; HIV: Human immunodeficiency virus; BMI: Body mass index; PT: Prothrombin time; AFP: Alpha feto-protein; NS: Not significant.

Subset analysis of African-American patients with cirrhosis when controlled for covariates resulted in an odds ratio of 2.08 (95%CI: 1.42-3.60, $P < 0.01$) when HBcAb was positive and 2.58 (95%CI: 1.82-4.44, $P < 0.01$) when HBsAb was negative in addition to a positive HBcAb.

Table 3 Univariate analysis predicting the following

	OR (95%CI)	P value
HCC in patients with CHC		
HBcAb(+)	1.90 (1.28-3.04)	0.02
HBsAb(-) and HBcAb(+)	3.24 (2.28-4.62)	< 0.01
HCC in cirrhotic patients with CHC		
HBcAb(+)	1.54 (1.18-2.54)	0.02
HBsAb(-) and HBcAb(+)	2.14 (1.68-3.82)	0.01
HCC in patients with CHC when controlled for covariates[1]		
HBcAb(+)	1.84 (1.22-3.08)	0.01
HBsAb(-) and HBcAb(+)	2.98 (2.12-5.08)	< 0.01
HCC in cirrhotic patients with CHC when controlled for covariates[1]		
HBcAb(+)	1.66 (1.22-3.24)	0.01
HBsAb(-) and HBcAb(+)	2.10 (1.72-4.04)	< 0.01
HCC in cirrhotic African American patients with CHC when controlled for covariates[1]		
HBcAb(+)	2.08 (1.42-3.6)	< 0.01
HBsAb(-) and HBcAb(+)	2.58 (1.82-4.44)	< 0.01

[1]Body mass index, albumin, prothrombin time, human immunodeficiency virus co-infection and alcohol consumption. HCC: Hepatocellular carcinoma; CHC: Chronic hepatitis C; HBcAb: Hepatitis B surface antibody; HBcAb: Hepatitis B core antibody.

DISCUSSION

In this study, HCC and advanced liver disease, shown by higher transaminases, lower albumin and platelet count and prolonged prothrombin time were associated with increased frequency of HBcAb(+) among patients with CHC. Previous studies have also noted an increased association of HBcAb(+) with advanced liver disease and HCC[1,11,12,14,16,18]. However, these data, as well as a prospective study[16], originated from regions of relatively high prevalence for both chronic hepatitis B and HCC. Our study presents findings from an area of relatively low endemicity in a population comprised predominantly of urban African-Americans, yet the prevalence of HBcAb(+) was even higher in our study (74%) than previously reported[11,14]. Multivariate analysis restricted to this group of patients revealed a much stronger association of LHB with HCC (Table 3). Our study included a total of 418 African-American patients and is by far the largest analysis studying this association in a select group.

Certain potential limitations to our study need further discussion. A majority of patients with HCC (66%) were diagnosed by histopathology. This is largely due to inclusion of patients before the EASL non-invasive criteria for diagnosis of HCC were proposed[17]. One could argue that lack of RNA confirmation of CHC viremia in 40% of our cases detracts from our conclusions. However, patients with or without RNA confirmation of HCV viremia had similar baseline characteristics and no other etiology for their liver disease, and were therefore appropriately grouped together. Alcohol consumption is a well established risk factor for HCC among patients with chronic liver disease. In the present study, details pertaining to alcohol consumption were not available in some patients despite extensive review of both inpatient and outpatient medical records. Every effort was made to classify patients based on alcohol consumption when information was available. Controls, who were drawn from a prospectively maintained institutional database were more likely to have reliable information regarding alcohol intake. Nevertheless, in concurrence with previous literature, our results suggest that BMI and alcohol consumption play an important role in the progression to HCC in cirrhotics with CHC.

Although the majority of patients with HCC were drawn from an outpatient setting, some were hospitalized and likely sicker. Controls were selected from a predominantly outpatient database. This may account for the significantly higher AFP and transaminases in the CHC patients with HCC. Although PCR measurement of serum HBV DNA assessment was not accomplished in our patients, we do not consider this as a significant limitation. In previous studies, HBcAb had a stronger association with HCC than serum HBV DNA among patients with CHC[5,6]. Latent hepatitis B, defined as a previous exposure to hepatitis B [HBcAb(+) and HBsAg(-)] is a clinically more relevant tool for predicting risk for HCC than is the assessment of HBV DNA in serum or liver tissue.

Another limitation to the study is that the frequency

of smoking, injection drug use and diabetes was not analyzed. Patients with HIV-HCV co-infection have rapid progression of liver disease and development of HCC. Liver disease is the leading cause of mortality in these patients[19-21]. Of 20 patients with HIV-HCV coinfection, 11 patients had HCC and all 11 were HBcAb(+) compared to 40% among coinfected controls. Although this difference did not achieve statistical significance, due to low numbers, this raises the intriguing observation that HIV-HCV coinfected patients with HCC may have an increased frequency of LHB.

Our study is consistent with previous studies from areas with high prevalence of hepatitis B that suggests that LHB increases the risk for HCC. Furthermore, patients sero-negative for HBsAb are at even higher risk, which may suggest longer time since acquisition of HBV. Our data also suggest that African-Americans have a greater risk of HCC when associated with LHB. There is additional data from our institution (preliminary) to support the observation that patients with CHC and LHB are more likely to have advanced liver disease and respond poorly to Interferon-based therapies. The current study takes this concept further by associating LHB with HCC.

In conclusion, LHB occurs at a significantly increased frequency in patients with CHC and HCC than in patients with CHC without HCC. This association is even stronger in African Americans. It is important to recognize this risk during surveillance for HCC in these patients.

COMMENTS

Background

Patients with chronic hepatitis C (CHC) and cirrhosis have an increased risk of developing hepatocellular carcinoma (HCC). Several risk factors for this progression have so far been identified. The authors studied the potential association between HCC in patients with CHC, cirrhosis and latent hepatitis B (LHB) infection, defined as the absence of detectable serum hepatitis B surface antigen (HBsAg) and the presence of hepatitis B core antibody (HBcAb).

Research frontiers

LHB has recently received significant attention among researchers and clinicians managing chronic liver disease. It is defined as a combination of HBsAg(-) and HBcAb(+). The potential association of LHB with HCC among patients with chronic CHC has been studied and reported in this manuscript.

Innovations and breakthroughs

Interestingly, subset analysis among human immunodeficiency virus-CHC co-infected patients showed a 100% association of HCC with LHB suggesting much higher association in this group. This identifies a unique group of CHC patients at much higher risk for development of HCC.

Peer review

Risk factors for HCC in patients with chronic hepatitis C are only partially understood. Here the authors showed that there is a clear association between latent hepatitis B infection and HCC development in an area with low endemicity for chronic hepatitis B.

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BRIEF ARTICLE

Inhibitor of differentiation proteins do not influence prognosis of biliary tract cancer

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Abstract

AIM: To investigate the expression and clinical relevance of inhibitor of differentiation (ID) proteins in biliary tract cancer.

METHODS: ID protein expression was analyzed in 129 samples from patients with advanced biliary tract cancer (BTC) (45 extrahepatic, 50 intrahepatic, and 34 gallbladder cancers), compared to normal controls and correlated with clinical an pathological parameters.

RESULTS: ID1-3 proteins are frequently overexpressed in all BTC subtypes analyzed. No correlation between increased ID protein expression and tumor grading, tumor subtype or treatment response was detected. Survival was influenced primary tumor localization (extrahepatic *vs* intrahepatic and gall bladder cancer, OS 1.5 years *vs* 0.9 years *vs* 0.7 years, $P = 0.002$), by stage at diagnosis (OS 2.7 years in stage Ⅰ *vs* 0.6 years in stage Ⅳ, $P < 0.001$), resection status and response to systemic chemotherapy. In a multivariate model, ID protein expression did not correlate with clinical prognosis. Nevertheless, there was a trend of shorter OS in patients with loss of cytoplasmic ID4 protein expression ($P = 0.076$).

CONCLUSION: ID protein expression is frequently deregulated in BTC but does not influence clinical prognosis. Their usefulness as prognostic biomarkers in BTC is very limited.

Key words: Biliary tract cancer; Cholangiocarcinoma; Inhibitor of differentiation; Prognostic factors

Core tip: Cholangiocarcinoma present as heterogeneous tumors with generally poor prognosis. Molecular changes that drive tumor development are poorly understood, and no valid prognostic markers other than stage and performance status have been identified. Here we analyzed the protein expression of the four inhibitor of differentiation (ID)-proteins by immunohistochemistry in 129 patients with advanced biliary tract cancer, which showed a deregulated ID protein expression in cancer cells and this protein expression partly correlated with the overall survival of patients. Therefore the ID-proteins maybe useful prognostic markers.

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INTRODUCTION

Cholangiocarcinomas/biliary tract cancers (BTC) form a heterogeneous group of tumors consisting of intrahepatic mass forming type biliary tract cancer (IHC), perihilar Klatskin tumors, extrahepatic BTC (EHC), and gallbladder cancer (GBC)[1,2]. More than 50% of tumors are diagnosed at an advanced stage. Prognosis is dismal with a mean overall survival of 7 to 8 mo. Although expression of oncogenes such as *K-ras*, *c-myc*, *c-neu*, *c-met*, and *bcl-2*, or inactivation of tumor suppressor genes like p53 in BTC has been described[3,4], the pathogenesis and molecular biology of this tumor entity is poorly understood. A recent analysis of liver-fluke associated biliary tract cancer on eight tumors and matched normal tissue identified mutations in three signaling pathways, namely histone modification, G protein activation, and genomic instability[5]. Validation of 15 of the identified 187 mutated genes in another 46 cases of BTC confirmed the role of p53, K-ras, and SMAD-4, and identified additional mutations in genes not prior associated to BTC[5].

The inhibitor of DNA-binding (ID) proteins, ID1-4, are members of the larger family of basic Helix-Loop-Helix (bHLH) transcription factors, which share a basic domain necessary for DNA-binding[6]. ID proteins lack this DNA-binding domain and inhibit transcription of target genes such as $p21^{CIP1/WAF1}$, $p16^{INK4B}$, and pRb by forming DNA-binding incompetent heterodimers with other bHLH factors. Various cellular processes are regulated by individual ID-proteins: Inhibition of cellular differentiation by interference with differentiation-specific bHLH and non-bHLH transcription factors[7], extension of cellular life span[8-10], regulation of angiogenesis[11] and maintenance of embryonic and adult stem cells[12], and chromosomal instability[13,14]. ID expression is deregulated in many tumors including pancreatic cancer[6] a malignancy somehow related to BTC. In some cases ID-expression is associated with poor clinical prognosis[15]. Until now, no data on ID protein expression in biliary tract cancer is available, although methylation of the ID4 promoter has been reported in some instances[16].

To investigate the role of ID proteins in BTC we analyzed the expression of ID proteins 1-4 in tumor specimen from 129 patients with advanced BTC and in 9 normal controls by immunohistochemistry (IHC).

MATERIALS AND METHODS

Archival tumor samples and controls

The institutional database of the University Medical Center Freiburg, Germany, was retrospectively searched for patients presenting with advanced BTC between 1996 and 2007. Archival hematoxylin and eosin (HE) stained slides from routinely processed paraffin-embedded samples collected at time of the initial diagnosis were reviewed to verify the diagnosis and to choose representative blocks for further evaluation. Control tissue samples included normal liver with normal biliary structures obtained from 9 male autopsy cases that had died without any evidence of liver or cardiac disease. Samples from tonsils, lymph nodes and colon mucosa were used as positive controls. The study was in accordance with the ethical standards set by the institutional ethics committee and was conducted according to the declaration of Helsinki.

Staging

Staging was performed according to UICC/AJCC recommendations.

Immunohistochemistry

Fresh serial sections were cut at 2 μm from the original diagnostic paraffin-embedded tissue blocks, mounted on Superfrost Plus slides, dried overnight at 37 ℃, deparaffinized in xylene and rehydrated through a graded series of alcohol solutions. Antigen retrieval was performed in Target Retrieval Solution (ID1, ID2, ID4: pH6; ID3: pH8; Dako, Glostrup, Denmark) at 95 ℃ for 60 min. Immunostaining was performed using a semi-automatically autostainer (Dako). The sections were incubated for 60 min at room temperature with primary polyclonal rabbit antibodies against relevant ID proteins (Santa Cruz Biotechnology, Santa Cruz, CA, United States) at different dilutions (ID1, C-20: sc-488 at 1:150; ID2: C-20: sc-489 at 1:1000; ID3, C-20: sc-490 at 1:100; ID4: H-70: sc-13047 at 1:100). After washing with PBS, the samples were incubated with biotinylated goat anti-polyvalent antibodies for 15 min and subsequently by streptavidin alkaline phosphatase for 15 min according to the labeled streptavidin-biotin (LSAB) method. The ID protein binding sections was visualized by K 5005, Fast-Red-Chromogen (Dako) detection for 10 min. Nuclei were counterstained with Mayer´s hemalaun solution. Vascular smooth muscle cells served as internal positive controls for the ID proteins. As a negative control, the primary antibody was omitted, with all other experimental conditions kept constant. The staining specificity was further confirmed by blocking the antibody binding to the antigen with the corresponding blocking peptides for ID1, ID2 and ID3 (Santa Cruz). No blocking peptide was commercially available for ID4.

Scoring of the immunohistochemical staining

Two blinded observers independently evaluated all cases. For cases with discordant scoring results, a consensus was obtained by reevaluating the slides. When the tumor samples showed a heterogeneous staining pattern, the "hot spots" showing the highest staining intensity were selected for further quantification. Cytoplasmic and nuclear immunolabeling were evaluated. Cytoplasmic ex-

Table 1 Patient characteristics, survival and best response to treatment *n* (%)

Age (yr)	Median (62.2)	Range (32-84)		
Sex	Male	Female		
	67 (51.9)	62 (48.1)		
BTC subtype	EHC	IHC	GBC	Total
	45 (34.9)	50 (38.8)	34 (26.4)	129
Stage	I	II	III	IV
	10 (7.8)	20 (15.5)	40 (31.0)	65 (50.4)
Grade	G1	G2	G3	
	7 (5.4)	81 (62.8)	41 (31.8)	
Treatment	BSC	Cx	Sx	Total
	56 (43.4)	64 (49.6)	9 (7.0)	129
Response to Cx	PR	SD	PD	
	11 (17.2)	27 (42.2)	26 (40.6)	
OS	BSC	Cx	Sx	All
(mo)	15.1	17.7	41.8	18.3

BSC: Best supportive care; Sx: Surgery; Cx: Chemotherapy; PR: Partial remission; SD: Stable disease; PD: Progressive disease; EHC: Extrahepatic BTC; IHC: Intrahepatic BTC; GBC: Gallbladder cancer.

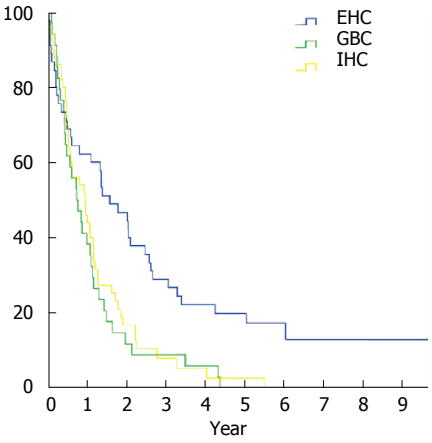


Figure 1 Survival of patients with biliary tract cancer by primary tumor localization. Kaplan-Meier estimates of overall survival in patients with extrahepatic biliary tract cancer (BTC) (n = 45, blue line), intrahepatic BTC (n = 50, yellow line), and gall bladder cancer (n = 34, green line).

pression was scored according to the staining intensity (0, negative; 1, low; 2, moderate to 3, strong). Nuclear staining was scored according to the percentage of positive nuclei. At least 100 nuclei were assessed in the hot spots at high magnification (× 400). The percentage of positive cells was rated as follows: 0: 0%-10%, 1: 11%-50%, 2: 51%-80%, and 3: 81%-100% of tumor cell nuclei positive.

Statistical analysis

Statistical analysis was performed using SPSS 15.0 statistical software (SPSS, Inc, Chicago, Illinois). Both the analysis of the association of ID1-4 expression levels in BTC with the overall survival (OS) and the correlation of tumor subtypes with OS were performed by the chi-square test with $P < 0.05$ considered as statistically significant. Overall survival was defined as the interval from date of diagnosis (histopathology) until death from any cause. Additionally to the analysis with the whole study cohort (n = 129), the patients were divided into the two following subgroups: Patients treated by chemotherapy (n = 64) and patients not treated by chemotherapy (n = 56). Survival estimates were calculated using the Kaplan-Meier method. To test for independent relevance of the candidate prognostic factors a multivariate Cox proportional hazards regression model was fit for each of the ID-proteins. All baseline characteristics plus cytoplasmic and nuclear expression of the respective ID-protein were included in the model. Tumor stage and grading were dichotomized (1/2 *vs* 3/4).

RESULTS

We identified 129 cases of advanced BTC (unresectable at time of presentation in our institution) that had been treated at our institution between June 1998 and June 2005. Thirty-five percent (n = 45) were of extrahepatic origin (EHC), 39% (n = 50) were of intrahepatic origin (IHC), and 26% (n = 34) were gallbladder cancers (GBC). Median age at diagnosis was 62.2 years (range 32-84 years) with even gender distribution (48% female, 52% male). The majority of patients had developed metastatic disease; most tumors were G2 tumors (63%). Patients were treated with best supportive care (n = 56) and various chemotherapeutic regimens (n = 64). Nine patients had become secondary resectable (R0). Chemotherapy regimens were 5-FU or gemcitabine based, often in combination with cisplatin or oxaliplatin. Response to treatment was monitored by CT, MRI or ultrasound according to the standard WHO criteria (WHO, 1979). Eleven patients (17%) showed a partial remission, 27 (42%) had stable disease, and 26 patients (41%) had progressive disease despite chemotherapy. Follow-up data were available from all 129 patients with a median follow-up of 16.5 mo. The median overall survival for all patients was 18.3 mo (Table 1).

To identify prognostic subgroups standard clinical prognostic variables were correlated with clinical outcome. As expected from clinical experience, site of the primary tumor clearly influenced prognosis. Patients with EHC had a significantly longer OS compared to patients with IHC and GBC (Median OS 1.5 years *vs* 0.9 years *vs* 0.7 years, P = 0.002) (Figure 1). Overall survival from time of diagnosis was also influenced by stage at diagnosis (Stage I : 2.7 years *vs* stage II : 2.0 years *vs* stage III 0.9 years *vs* stage IV 0.6 years, $P < 0.001$). Resection status also influenced survival, which was longer in patients with completely resected (R0) tumors compared to incompletely resected (R1/R2) and not surgically treated tumors (OS 2.0 years *vs* 1.3 years *vs* 0.6 years, $P < 0.001$). Likewise, OS was better in patients whose tumors responded to chemotherapy (PR 2.0 years *vs* SD 1.3 years *vs* PD 0.6 years, P = 0.003). To exclude an influence of curative resection (n = 9) on the results, a sensitivity analysis excluding these cases was performed that confirmed the results (P = 0.004). Because of the small sample size no additional subgroup analyses were performed.

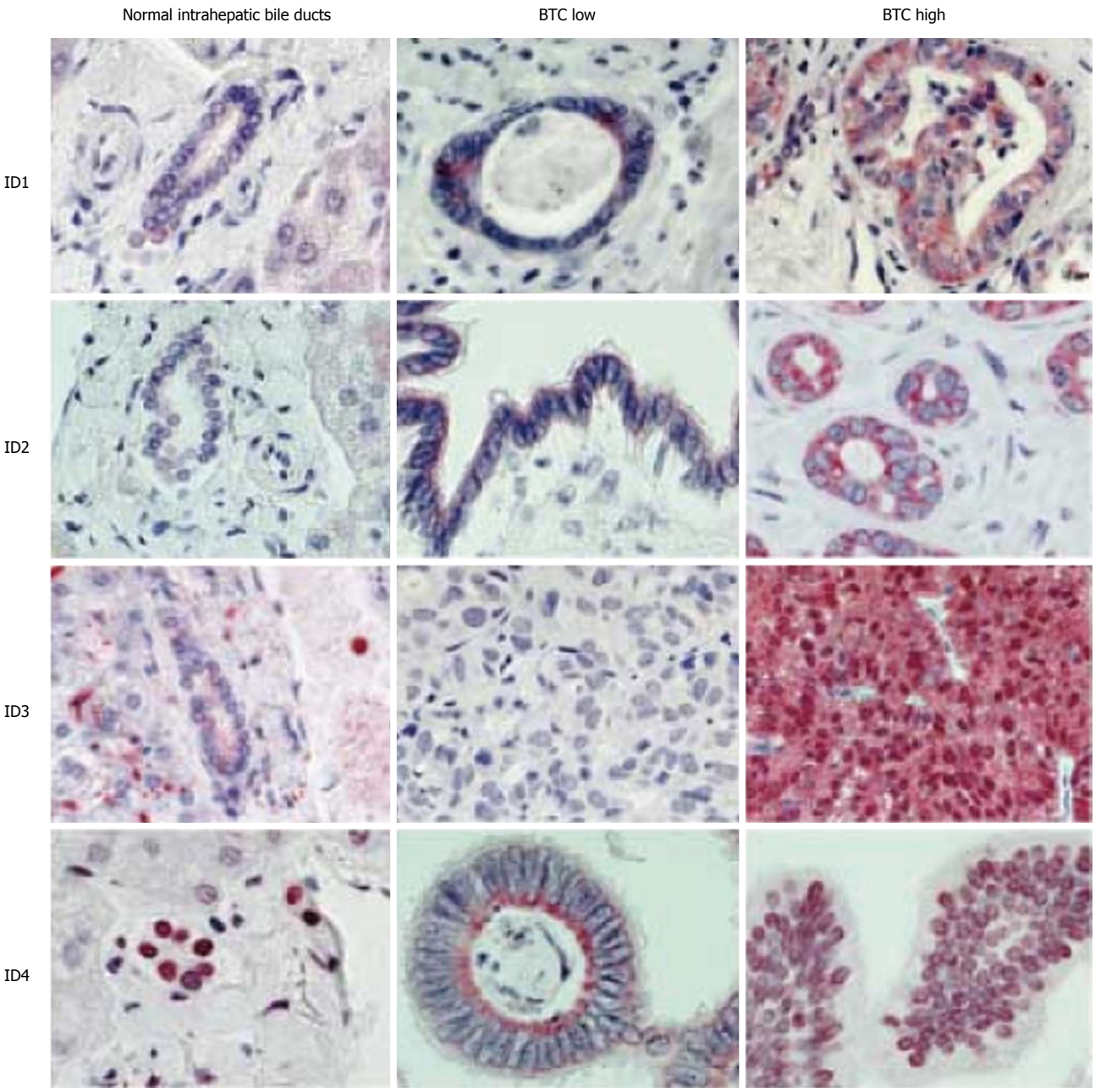


Figure 2 Inhibitor of differentiation protein expression in normal intrahepatic bile ducts and biliary tract cancer. Representative photographs. Depicted are representative stains of normal intrahepatic bile ducts, low and high expressing biliary tract cancer cases for each inhibitor of differentiation (ID) protein.

ID proteins are frequently expressed in BTC

To determine the role of the ID proteins in BTC, we analyzed ID protein expression and subcellular localization of ID proteins in archival samples from all 129 patients collected at initial diagnosis. Normal bile ducts expressed only low amounts of ID proteins with the exception of ID4, where strong nuclear expression of ID4 was detected in all cases analyzed. In contrast to normal controls, high levels of ID1, ID2 and ID3 were detected in the majority of BTC cases (Figure 2). Table 2 summarizes the results.

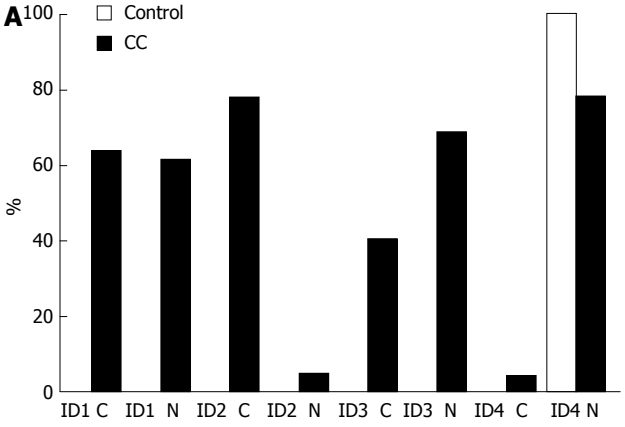
Detailed analysis of expression levels and subcellular localization of the ID proteins showed clear differences between normal bile ducts and BTC. While expression of ID1, ID2, and ID3 was undetectable or low in normal tissue, similarly high cytoplasmic and nuclear ID1 expression was detected in 63.6%, and 61.3% of BTC cases (Figure 3). Likewise, high ID2 expression was detected, which was mainly cytoplasmic (77.5% cytoplasmic *vs* 4.7% nuclear). ID3 was highly expressed in the cytoplasm (40.3%) and even more pronounced in the nucleus (68.3%). In contrast, all normal controls analyzed expressed high levels of nuclear ID4, but only low levels of cytoplasmic ID4. In BTC, this predominant nuclear ID4 staining was reduced to 77.8%.

To investigate the clinical relevance of the above findings, ID protein expression was correlated with overall survival (OS), tumor grade, tumor stage, prior

Table 2 Inhibitor of differentiation protein expression and tumor characteristics *n* (%)

	Total (*n*)	IHC	EHC	GBC	Stage I	Stage II	Stage III	Stage IV	G1	G2	G3
ID1 neg	17	6 (5)	6 (5)	5 (4)	1 (1)	2 (2)	4 (3)	10 (8)	1 (1)	9 (7)	7 (5)
ID1 pos	112	44 (34)	39 (30)	29 (22)	9 (7)	18 (14)	30 (23)	55 (43)	6 (5)	72 (56)	34 (26)
ID2 neg	29	13 (10)	9 (7)	7 (5)	1 (1)	3 (2)	9 (7)	16 (12)	2 (2)	19 (15)	8 (6)
ID2 pos	100	37 (29)	36 (28)	27 (21)	9 (7)	17 (13)	25 (19)	49 (38)	5 (4)	62 (48)	33 (26)
ID3 neg	25	9 (7)	9 (7)	7 (5)	1 (1)	7 (5)	3 (2)	14 (11)	2 (2)	15 (12)	8 (6)
ID3 pos	104	41 (32)	36 (28)	27 (21)	9 (7)	13 (10)	31 (24)	51 (40)	5 (4)	66 (51)	33 (36)
ID4 neg	26	11 (9)	9 (7)	6 (5)	2 (2)	7 (6)	9 (7)	8 (7)	1 (1)	15 (12)	10 (8)
ID4 pos	96	35 (29)	35 (29)	26 (21)	8 (7)	13 (11)	23 (19)	52 (43)	6 (5)	61 (50)	29 (24)

EHC: Extrahepatic biliary tract cancer (BTC); IHC: Intrahepatic BTC; GBC: Gallbladder cancer; G: Grade.



B

	ID-expression		Low	High%	*n*
ID1	CC	Cyto/nuclear	36.4%/38.7%	63.6%/61.3%	129
	Control	Cyto/nuclear	100%/100%	0%/0%	9
ID2	CC	Cyto/nuclear	22.5%/95.3%	77.5%/4.7%	129
	Control	Cyto/nuclear	100%/100%	0%/0%	9
ID3	CC	Cyto/nuclear	59.7%/31.8%	40.3%/68.3%	129
	Control	Cyto/nuclear	100%/100%	0%/0%	9
ID4	CC	Cyto/nuclear	95.9%/22.1%	4.1%/77.8%	122
	Control	Cyto/nuclear	100%/0%	0%/100%	9

Figure 3 Proportion of inhibitor of differentiation protein expression in biliary tract cancer and normal intrahepatic bile ducts. A: Percentage of biliary tract cancer (BTC) with high expression of the respective inhibitor of differentiation (ID) protein in BTC (black bars) and normal intrahepatic bile ducts (control; grey bar); B: Summary of ID protein expression. Overall ID protein expression ID protein expression was scored in low (0+1) and high (2+3) and assessed for nuclear and cytoplasmic (cyto) staining pattern. Cytoplasmic staining intensity was scored 0 (negative) to 3 (strong), nuclear expression was scored based on the percentage of positive nuclei (0: 0%-10%; 1: 11%-50%; 2: 51%-80%; and 3: 81%-100%). For ID4, only 122 samples could be analyzed. C: Cytoplasmic expression; N: Nuclear expression.

chemotherapy, and response to chemotherapy. Correlation of ID expression with OS was calculated for the whole study cohort (n = 129; n = 122 for ID4) and for the subgroups of patients treated with chemotherapy (n = 64) and patients not treated by chemotherapy (n = 56). While neither cytoplasmic nor nuclear ID1 expression was correlated with OS, a clear trend for shorter OS was observed for nuclear negative (n = 27) *vs* nuclear positive (n = 102) cases (0.9 years *vs* 1.2 years, P = 0.058) (Figure 4A). Further subgroup analyses identified a strong correlation of ID1 expression and overall survival in chemotherapy naïve patients (n = 56). Here, patients without nuclear (n = 10) ID1 expression had an OS of 2.1 years compared to 0.5 years in patients with nuclear (n = 46) ID1 expression (P = 0.001) (Figure 4B). ID2 did not have a prognostic value for the overall study population (P = 0.79 for cytoplasmic expression, P = 0.28 for nuclear expression). Nevertheless, in the subgroup of patients who had received chemotherapy (n = 64) patients without nuclear ID2 expression (n = 51) had a significantly better prognosis than patients with nuclear ID2 expression (n = 13), with OS of 1.3 years and 0.5 years, respectively (P = 0.001) (Figure 4C). As for ID1 and ID2, ID3 expression in the overall study population did not correlate with OS (P = 0.28 for cytoplasmic, and P = 0.44 for nuclear ID3 expression). The subgroup of patients who had received chemotherapy (n = 64) patients without cytoplasmatic ID3 expression (n = 36) had a slightly better prognosis than patients with cytoplasmatic ID3 expression (n = 28), with OS of 1.0 years and 1.1 years, respectively (P = 0.037) (Figure 4D). Using the same subgroup analyses as for the other IDs, no relevant subgroup was identified. Quite strikingly, while nuclear ID4 expression did not correlate with OS (P = 0.27), cytoplasmic ID4 expression seemed to correlate with prognosis (Figure 4E). Overall survival in patients with cytoplasmic ID4 expression (n = 60) was 1.2 years compared with 0.6 years in patients without cytoplasmic ID4 expression (n = 62, P = 0.001) in univariate analysis. This was reproduced in the subgroup of patients who had not received prior chemotherapy (n = 56). Here, OS was 1.1 years *vs* 0.5 years in patients with cytoplasmic (n = 23) *vs* no cytoplasmic (n = 33) ID4 expression (P = 0.025) (Figure 4F). Analyses of ID1-4 expression and other clinical-pathological variables failed to show any significant correlation (data not shown). Specifically, ID expression levels were similar in EHC, IHC, and GBC.

To test the relevance of the above findings a multivariate Cox proportional hazards regression model was used. Multivariate testing confirmed the importance of tumor localization, surgical treatment, and response to chemotherapy as factors influencing survival (Table 3). Patients with extrahepatic BTC have the best prognosis (HR = 0.32, 95%CI: 0.18-0.6, P < 0.0005), as have patients who had been treated surgically (HR = 0.3, 95%CI: 0.17-0.55, P < 0.0001). Patients responding to chemotherapy with

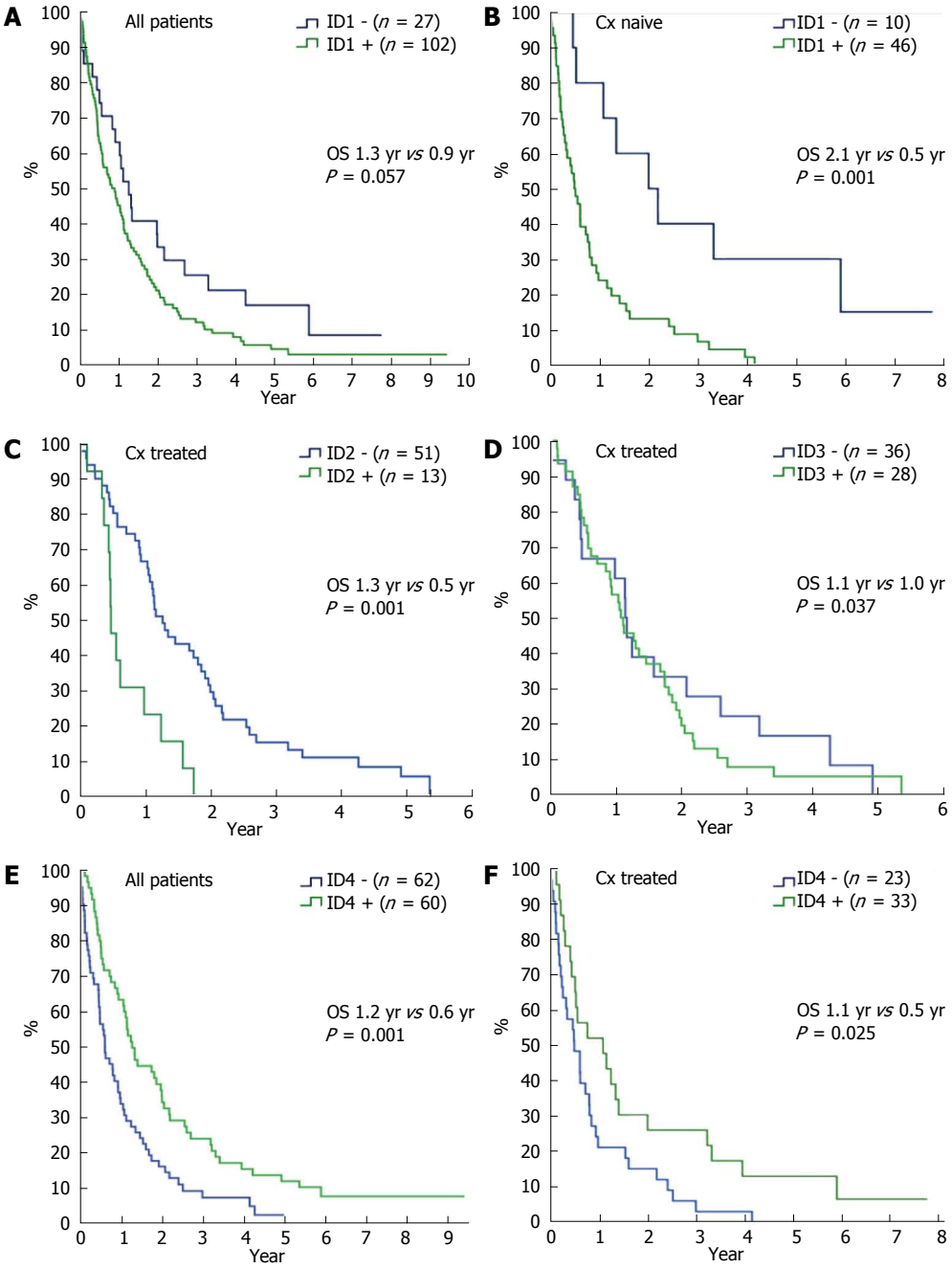


Figure 4 Kaplan-Meier survival estimates for overall survival from time of diagnosis for patients with biliary tract cancer expressing ID1-ID4 with or without systemic chemotherapy. A: Survival in patients (n = 129) with (green line) and without nuclear ID1 expression (blue line); B: Survival in patients who have not received systemic chemotherapy (n = 56) with (green line) or without nuclear ID1 expression (blue line); C: Survival in patients who have received systemic chemotherapy (n = 64) with (green line) or without nuclear ID2 expression (blue line); D: Survival in patients who have not received systemic chemotherapy (n = 64) with (green line) or without cytoplasmic ID3 expression (blue line); E: Survival in patients (n = 122) with (green line) and without cytoplasmic ID4 expression (blue line); F: Survival in patients who have not received systemic chemotherapy (n = 56) with (green line) or without cytoplasmic ID4 expression (blue line). Cx: Chemotherapy.

a partial remission (PR) or disease stabilization (SD) likewise seem to have a better clinical prognosis (HR = 0.43, 95%CI: 0.20-0.91, P = 0.267; and HR 0.4, 95%CI: 0.22-0.71, P = 0.002, respectively). The effects of the ID proteins observed in univariate testing did not reach statistical significance in this multivariate model. Only tumors with loss of cytoplasmatic ID4 expression showed a trend for shorter survival (HR = 0.68, 95%CI: 0.45-1.04, P = 0.0736).

DISCUSSION

Our analysis of 129 cases of biliary tract cancer confirmed the importance of primary tumor localization, tumor stage, resection status and response the treatment as strong predictors for patients' prognosis. This difference in survival was independent of other confounding factors such as stage, resection, or response to chemotherapy. We believe that our findings are valid, as also patients who

Table 3 Multivariate analysis of clinical characteristics and inhibitor of differentiation protein expression

	ID1			ID2			ID3			ID4		
	HR	95%CI	*P* value	HR	95%CI	*P* value	HR	95%CI	*P* value	HR	95%CI	*P* value
Gender (male)	1.17	0.76-1.80	0.4717	1.07	0.71-1.64	0.7363	1.03	0.68-1.56	0.8877	1.29	0.82-2.02	0.267
IHC	0.57	0.33-0.97	0.0399	0.66	0.39-1.11	0.1193	0.72	0.42-1.23	0.2312	0.75	0.44-1.28	0.295
EHC	0.32	0.18-0.60	0.0003	0.36	0.20-0.64	0.0005	0.36	0.20-0.63	0.0003	0.32	0.18-0.57	0.0001
Stage Ⅲ-Ⅳ	1.51	0.80-2.83	0.2037	1.5	0.80-2.80	0.2051	1.72	0.91-3.25	0.0928	1.24	0.65-2.35	0.5129
Grade 3	1.57	0.99-2.50	0.0532	1.58	1.01-2.47	0.0471	1.43	0.90-2.27	0.1326	1.53	0.96-2.45	0.0732
Surgery	0.3	0.17-0.55	< 0.0001	0.35	0.20-0.61	0.0003	0.33	0.18-0.61	0.0004	0.31	0.17-0.55	< 0.0001
Age	0.99	0.97-1.01	0.2327	0.99	0.97-1.01	0.3835	0.99	0.97-1.01	0.3118	0,99	0.97-1.02	0.6452
Response to Cx PD	0.81	0.44-1.47	0.4836	0.83	0.45-1.53	0.5442	0.65	0.35-1.20	0.1669	0.7	0.37-1.31	0.261
Response to Cx SD	0.4	0.22-0.71	0.002	0.4	0.22-0.72	0.0021	0.33	0.19-0.60	0.0002	0.37	0.20-0.67	0.0012
Response to Cx PR	0.43	0.20-0.91	0.0267	0.48	0.23-0.98	0.0426	0.46	0.22-0.94	0.0329	0.5	0.25-1.01	0.0531
Nuclear low	0.93	0.46-1.88	0.8447	1.41	0.80-2.50	0.2333	1.32	0.62-2.79	0.4721	0.94	0.38-2.33	0.8979
Nuclear high	1.2	0.65-2.20	0.5563	1.51	0.19-11.84	0.696	0.97	0.47-2.00	0.9275	0.63	0.27-1.47	0.2813
Cytoplasmic low	0.81	0.34-1.91	0.6276	0.59	0.07-4.80	0.6195	1.0	0.60-1.68	0.9948	0.68	0.45-1.04	0.0736
Cytoplasmic high	0.81	0.34-1.90	0.6261	0.64	0.08-5.04	0.6703	0.71	0.33-1.54	0.3866	0.31	0.03-2.85	0.3028

n = 129, number of events = 119, r^2 = 0.386 for ID1-3, r^2 = 0.392 for ID4; EHC: Extrahepatic biliary tract cancer (BTC); IHC: Intrahepatic BTC; Cx: Chemotherapy; PR: Partial remission; SD: Stable disease; PD: Progressive disease; ID: Inhibitor of differentiation.

had not been curatively resected had a significantly longer OS (P = 0.004). Hence, information of primary BTC localization should be prospectively evaluated and used for stratification in clinical trials in advanced BTC to verify its prognostic relevance. It is hard to speculate on factors influencing this different prognosis. One report could hint to a role of the multidrug resistance proteins (MRP) showing lower levels of MRP3 in EHC compared to gallbladder carcinomas[17]. Alternatively, differential gene expression as of matrix metalloproteinases and growth factors might contribute to the different biological behavior of EHC[3,18].

While data on ID protein expression in BTC was scarce, more comprehensive data is available for ID expression in hepatocellular carcinoma (HCC). In HCC, ID1 is frequently expressed and higher ID1 expression was reported to correlate with decreased $p16^{INK4A}$ expression[19]. ID protein expression decreased with loss of differentiation, suggesting a role of ID proteins in early carcinogenesis[20]. Contrasting these results are data that imply a role of ID1 in progression, metastasis, and tumor vessel formation[19,21-23]. Immunohistochemical analysis of liver tissue from 112 patients with liver cirrhosis showed elevated ID1 expression in 38% (n = 42) of patients. These patients were at higher risk of developing HCC[23]. In an analysis of 80 matched pair biopsies of HCC and normal liver, and cirrhotic and chronic hepatitis samples no ID1-protein expression was observed in normal liver, whereas moderate to strong ID1 expression was detected in 50% of HCCs. In 60 matched pairs of primary tumor and metastasis, expression in the metastases was higher (90%) than in the primary tumors (42%), correlating with increased VEGF expression[22]. In a nude mouse model ID1 induced VEGF by stabilizing HIF alpha, and antisense-inhibition of ID1 resulted in decreased tumor growth due to decreased VEGF expression and decreased tumor vascularization[22].

Analysis of ID protein expression in our cohort of cholangiocarcinomas showed expression of all four ID proteins. ID4 was the only ID protein expressed in normal bile ducts. Neither overall ID protein expression levels nor subcellular localization correlated with clinical prognosis. While loss of ID4 protein expression was frequently detected and patients with ID4 negative tumors had a trend for shorter OS this needs to be confirmed in a larger sample. The role of ID4 on tumor development is still not fully understood. Recent studies revealed that the ID4 gene can be silenced through promoter hypermethylation in various tumors, including BTC[16,24-30], suggesting a tumor suppressive role. On the other hand ID4 was identified as an upstream regulator of BRCA1 in breast and ovarian cancer[31]. Also supporting an oncogenic role of ID4 are reports describing activating translocations of ID4 in some patients with bladder cancer and a subset of patients with acute lymphoblastic leukemia[32-34]. Our data suggest a tumor suppressive role of ID4 in BTC, as patients without or with low cytoplasmic levels of ID4 had shorter overall survival. While hypothesis generating, this must be confirmed and validated in a larger patient population. In summary, we have shown that ID protein expression is deregulated in biliary tract cancer but that their use as prognostic biomarkers is very limited.

ACKNOWLEDGMENTS

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COMMENTS

Background

Cholangiocarcinoma present as heterogeneous tumors with generally poor prognosis. Molecular changes that drive tumor development are poorly understood, and no valid prognostic markers other than stage and performance status have been identified.

Innovations and breakthroughs

To investigate the role of inhibitor of differentiation (ID) proteins in biliary tract cancer (BTC) we analyzed the expression of ID proteins 1-4 in tumor specimen

from 129 patients with advanced BTC and in 9 normal controls by immunohistochemistry.

Applications

Authors have shown that ID protein expression is deregulated in biliary tract cancer but that their use as prognostic biomarkers is very limited.

Peer review

The authors described the expressions of ID1-4 proteins in specimens of cholangiocarcinoma and compared to the normal control. An interesting paper looking for new histological parameters in cholangiocarcinoma.

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BRIEF ARTICLE

Smoothelin, a new marker to determine the origin of liver fibrogenic cells

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Abstract

AIM: To explore this hypothesis that smooth muscle cells may be capable of acquiring a myofibroblastic phenotype, we have studied the expression of smoothelin in fibrotic conditions.

METHODS: Normal liver tissue (n = 3) was obtained from macroscopically normal parts of hepatectomy, taken at a distance from hemangiomas. Pathological specimens included post-burn cutaneous hypertrophic scars (n = 3), fibrotic liver tissue (n = 5), cirrhotic tissue (viral and alcoholic hepatitis) (n = 5), and hepatocellular carcinomas (n = 5). Tissue samples were fixed in 10% formalin and embedded in paraffin for immunohistochemistry or were immediately frozen in liquid nitrogen-cooled isopentane for confocal microscopy analysis. Sections were stained with antibodies against smoothelin, which is expressed exclusively by smooth muscle cells, and α-smooth muscle actin, which is expressed by both smooth muscle cells and myofibroblasts.

RESULTS: In hypertrophic scars, α-smooth muscle actin was detected in vascular smooth muscle cells and in numerous myofibroblasts present in and around nodules, whereas smoothelin was exclusively expressed in vascular smooth muscle cells. In the normal liver, vascular smooth muscle cells were the only cells that express α-smooth muscle actin and smoothelin. In fibrotic areas of the liver, myofibroblasts expressing α-smooth muscle actin were detected. Myofibroblasts co-expressing α-smooth muscle actin and smoothelin were observed, and their number was slightly increased in parallel with the degree of fibrosis (absent in liver with mild or moderate fibrosis; 5% to 10% positive in liver showing severe fibrosis). In cirrhotic septa, numerous myofibroblasts co-expressed α-smooth muscle actin and smoothelin (more than 50%). In hepatocellular carcinomas, the same pattern of expression for α-smooth muscle actin and smoothelin was observed in the stroma reaction surrounding the tumor and around tumoral cell plates. In all pathological liver samples, α-smooth muscle actin and smoothelin were co-expressed in vascular smooth muscle cells.

CONCLUSION: During development of advanced liver fibrosis, a subpopulation of myofibroblasts expressing smoothelin may be derived from vascular smooth muscle cells, illustrating the different cellular origins of myofibroblasts.

Key words: Smooth muscle cells; Myofibroblasts; α-smooth muscle actin; Smoothelin; Fibrosis/cirrhosis; Hepatocellular carcinoma

Core tip: In fibrotic conditions, it has been suggested that smooth muscle cells can acquire a myofibroblastic phenotype. To explore this hypothesis, we studied the expression of smoothelin, a specific marker of end-stage differentiation of smooth muscle cells, in cutaneous and hepatic fibrotic conditions, using immunohistochemistry and confocal microscopy. We showed that during advanced liver fibrosis, a subpopulation of α-smooth muscle actin-expressing myofibroblasts also express smoothelin and thus may be derived from vascular smooth muscle cells. This finding, which illustrates the different potential cellular origins of myofibroblasts involved in liver fibrogenesis, may represent an interesting tool to distinguish advanced stages of cirrhosis.

Lepreux S, Guyot C, Billet F, Combe C, Balabaud C, Bioulac-Sage P, Desmoulière A. Smoothelin, a new marker to determine the origin of liver fibrogenic cells. *World J Gastroenterol* 2013; 19(48): 9343-9350 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i48/9343.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i48.9343

INTRODUCTION

Smoothelin, a constituent of the smooth muscle cell cytoskeleton, has been described as a marker of end-stage differentiation of smooth muscle cells because it has been found only in contractile smooth muscle cells[1-3]. Smoothelin has two major isoforms in adults, which are expressed in a tissue specific manner: a 59 kDa isoform, smoothelin-A, which is expressed in visceral and urogenital tissues, such as the digestive tract, bladder, and prostate; and a 110 kDa isoform, smoothelin-B, which is expressed in blood vessel walls[4]. Transient synthesis of a third smoothelin isoform has been detected in embryonic striated muscle cells in chicken[5]. In cultured smooth muscle cells, smoothelin colocalizes with α-smooth muscle actin stress fibers[3] and smoothelin can bind to α-smooth muscle actin, which suggested a direct role of smoothelin in contraction[6]. In contrast, cells with smooth muscle cell-like features, such as myofibroblasts, do not express smoothelin[3]. Myofibroblasts are contractile cells that express α-smooth muscle actin[7]. These cells are involved in tissue repair processes and, particularly, in extracellular matrix deposition and remodeling[8]. In normal connective tissues, myofibroblasts are rare[9]. After tissue injury, myofibroblasts appear, and it is commonly accepted that most are derived from locally recruited connective tissue fibroblasts[10]. In the liver, the most important cells involved in fibrogenesis are hepatic stellate cells and portal fibroblasts, which are able to differentiate into myofibroblasts and are responsible for matrix deposition[11]. However, the involvement of different cell types with various origins, such as smooth muscle cells, circulating cells or bone marrow-derived cells has also been suggested in the establishment of liver fibrosis/cirrhosis[12-16]. However, the degree to which this process contributes to fibrosis remains a matter of intense debate and is likely to be context-dependent. To determine the possible contribution of smooth muscle cells to the appearance of myofibroblasts during fibrotic processes, we studied the expression of smoothelin in different diseases that show myofibroblast involvement; *i.e.*, post-burn cutaneous hypertrophic scars, liver fibrosis and cirrhosis, and hepatocellular carcinomas.

MATERIALS AND METHODS

Human liver samples and tissue processing

Human tissue samples used in this study were selected from the files of the tissue bank of the Department of Pathology (CHU Bordeaux, Pellegrin Hospital, Bordeaux, France). Normal liver tissues (n = 3) were obtained from macroscopically normal parts of livers after hepatectomy, taken at a distance from hemangiomas. Pathological specimens included post-burn cutaneous hypertrophic scars (n = 3); fibrotic liver tissue (n = 5), ranging from F1 to F3 stage according to the Metavir score[17]; cirrhotic tissues (viral and alcoholic hepatitis) (n = 5); and hepatocellular carcinomas (n = 5). Tissue samples were fixed in 10% buffered formalin, embedded in paraffin and processed for diagnostic purposes and immunohistochemistry, or were immediately frozen in liquid nitrogen-cooled isopentane for confocal microscopy analysis. The procedures were carried out in accordance with the European Guidelines for the use of human tissues.

Immunohistochemistry and confocal microscopy

Mouse monoclonal antibodies against smoothelin (immunoglobulin G, IgG1 clone R4A, which reacts with smoothelin A and B, MUbio Products, Maastricht, The Netherlands), and α-smooth muscle actin (IgG2a clone 1A4, Dako SA, Trappes, France) were used. These two antibodies have been extensively used and their specificity has been clearly documented[1,18]. Immunohistochemistry was essentially performed as previously described[19]. Briefly, after incubation with the first antibody, the epitopes were detected using the Vectastain® ABC system (Vector Laboratories, Peterborough, United Kingdom) or the Envision™ system (DakoCytomation, Trappes, France), with diaminobenzidine as the color substrate. Slides were then counterstained with hematoxylin.

For double immunofluorescence, cryostat sections were first incubated with the antibodies against smoothelin and α-smooth muscle actin, then with a TRITC-conjugated goat anti-mouse IgG1 (Southern Biotech, Birmingham, AL, United States) and an Alexa Fluor® 488 goat anti-mouse IgG2a (Molecular Probes, Eugene, OR, United States). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI).

The specificity of staining was confirmed by incuba-

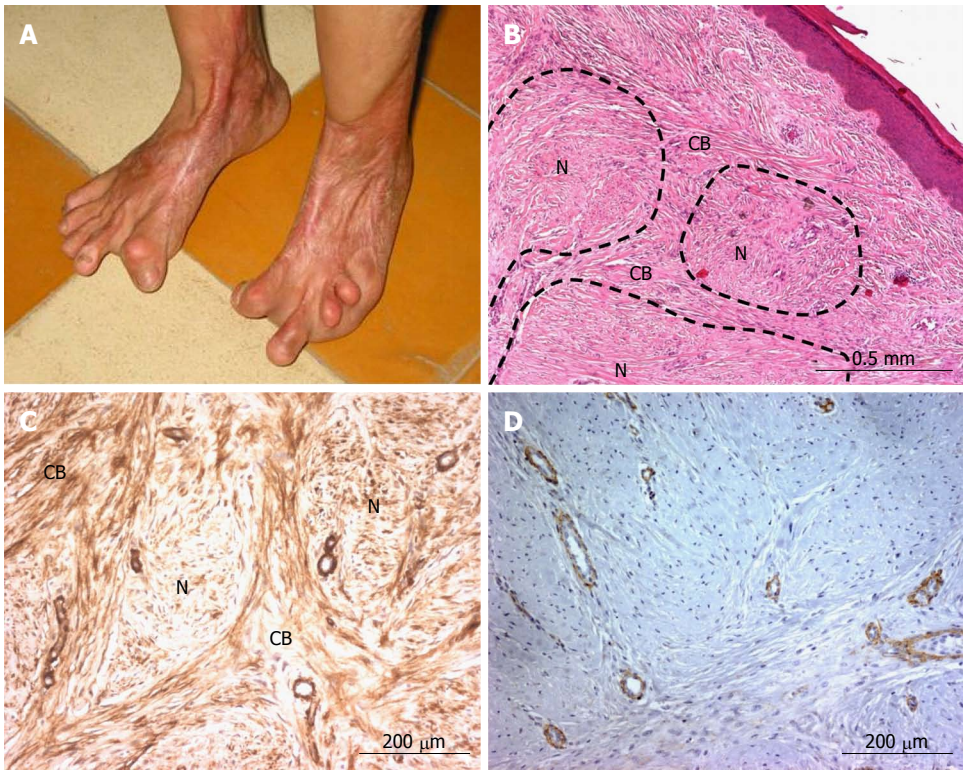

Figure 1 α-smooth muscle actin and smoothelin expression in a cutaneous hypertrophic scar. A: Following a burn injury, a retractile hypertrophic scar was observed (from Vincent Casoli, Plastic Surgery and Burns Unit, University Hospital of Bordeaux, France); B: Hematoxylin and eosin staining shows typical hypertrophic scar architecture with nodules (N) surrounded by cell bundles (CB); C: α-smooth muscle actin is expressed in vascular smooth muscle cells and myofibroblasts both in and around the nodules; D: Smoothelin is only expressed by vascular tunica media smooth muscle cells.

tion in non-immune serum and in the absence of the primary antibody. For immunohistochemistry, sections were examined with a Zeiss Axioplan 2 microscope (Carl Zeiss Microscopy, Jena, Germany). Images were acquired with an AxioCam camera (Carl Zeiss Vision, Hallbergmoos, Germany) by means of the AxioVision image processing and analysis system (Carl Zeiss Vision). For double immunofluorescence, sections were analyzed with a confocal microscopy (LSM Meta 510, Carl Zeiss Microscopy).

For quantitative evaluation of staining, cell counting was performed in the septa of fibrotic and cirrhotic livers, and in the stroma reaction of hepatocellular carcinoma. Vessels were not included in this evaluation. For each field, the ratio of the number of smoothelin-positive and α-smooth muscle actin-positive cells over the number of cells only expressing α-smooth muscle actin was calculated. The analysis was performed on an average of 10 fields/zone using the × 40 objective. Only positive cells containing a nucleus were counted.

RESULTS

α-smooth muscle actin and smoothelin expression in hypertrophic scars

In many situations, hypertrophic scars develop significant contractile activity (because of the presence of myofibroblasts) (Figure 1A), and are usually organized in a nodular pattern (Figure 1B). By immunohistochemistry, α-smooth muscle actin was detected in blood vessel walls and in numerous myofibroblasts present in and around the nodules (Figure 1C), whereas smoothelin was exclusively shown in the vessel walls (Figure 1D). Our data showed that myofibroblasts present in hypertrophic scars do not express smoothelin.

α-smooth muscle actin and smoothelin expression in normal and pathological livers

In the normal liver, α-smooth muscle actin was exclusively expressed by the smooth muscle cells within the tunica media of portal arteries and veins, and of the centrilobular veins (Figure 2A). The expression of smoothelin in the normal liver was also detected in the smooth muscle cells of these vessels (Figure 2B). Smoothelin and α-smooth muscle actin showed a similar distribution.

In livers with mild or moderate fibrosis (F1-F2), myofibroblasts present in fibrotic areas clearly expressed α-smooth muscle actin, but did not express smoothelin (data not shown). In livers showing severe fibrosis (F3), strong staining for α-smooth muscle actin was detected in myofibroblasts present in fibrotic areas (Figure 2C). Weak expression of smoothelin was present in a few myofibroblasts (Figure 2D). In cirrhotic livers (F4), myofibroblasts present in cirrhotic septa expressed high amounts of α-smooth muscle actin (Figure 2E). Numerous myofibroblasts also expressed smoothelin (Figure 2F). In all stages of liver fibrosis, α-smooth muscle actin

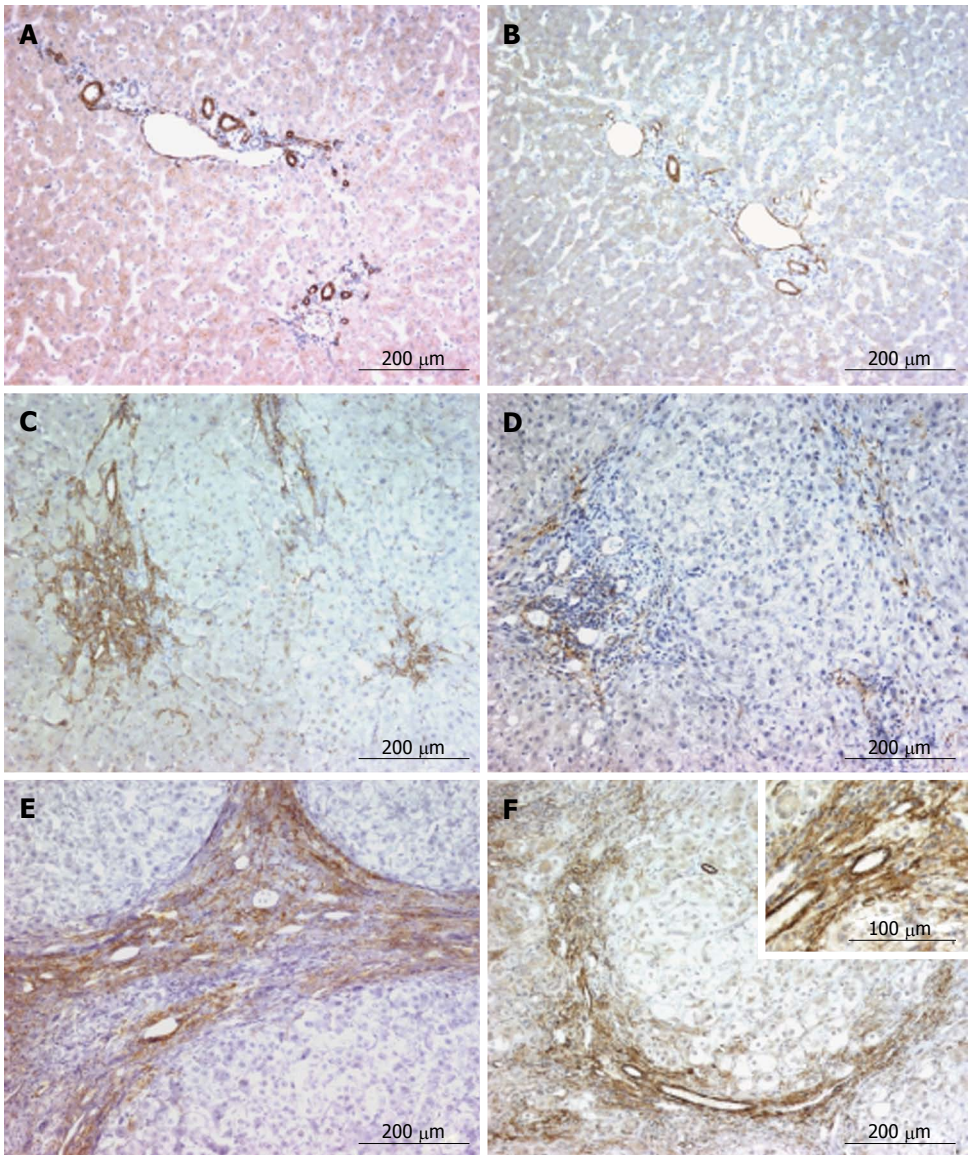

Figure 2 α-smooth muscle actin and smoothelin expression in normal, fibrotic and cirrhotic livers. A and B: In the normal liver, α-smooth muscle actin (A) and smoothelin (B) show similar expression in tunica media smooth muscle cells of the portal arteries and veins and in the centrilobular veins; C and D: In severe fibrosis, myofibroblasts express high amounts of α-smooth muscle actin (C), while smoothelin is only expressed at low levels (D) (α-smooth muscle actin and smoothelin are coexpressed in vascular smooth muscle cells); E and F: In the cirrhotic liver, myofibroblasts express α-smooth muscle actin in fibrotic septae (E) and numerous myofibroblasts also express smoothelin (F); however, smoothelin expression is lower compared with α-smooth muscle actin expression (insert). α-smooth muscle actin and smoothelin are co-expressed in vascular tunica media smooth muscle cells (F).

and smoothelin were co-expressed in vascular smooth muscle cells (Figure 2C-F).

The co-localization of α-smooth muscle actin and of smoothelin in the same cells was confirmed by double immunofluorescence (Figure 3). All cells expressing smoothelin also expressed α-smooth muscle actin, including vascular smooth muscle cells and myofibroblasts. Moreover, a quantitative evaluation revealed that in livers showing severe fibrosis (F3), 5% to 10% of myofibroblasts co-expressed α-smooth muscle actin and smoothelin. In cirrhotic septa, more than 50% of myofibroblasts co-expressed α-smooth muscle actin and smoothelin.

In hepatocellular carcinomas, we observed the same expression pattern of α-smooth muscle actin and smoothelin in the stroma reaction surrounding the tumor (Figure 4A and B). These two proteins were also expressed by fusiform pericyte-like cells between endothelial cells of the sinusoidal capillaries and tumoral cell plates (Figure 4C and D).

DISCUSSION

In normal conditions, several cell types that have contractile properties express α-smooth muscle actin, such as smooth muscle cells and, to a lesser extent, pericytes. Smooth muscle cells display a large variation in phenotype among, and even within, tissues and organs. Different patterns of marker expression reflect, in part, the heterogeneity of smooth muscle cell subpopulations and their phenotypic modulation[20]. Smoothelin, a recently

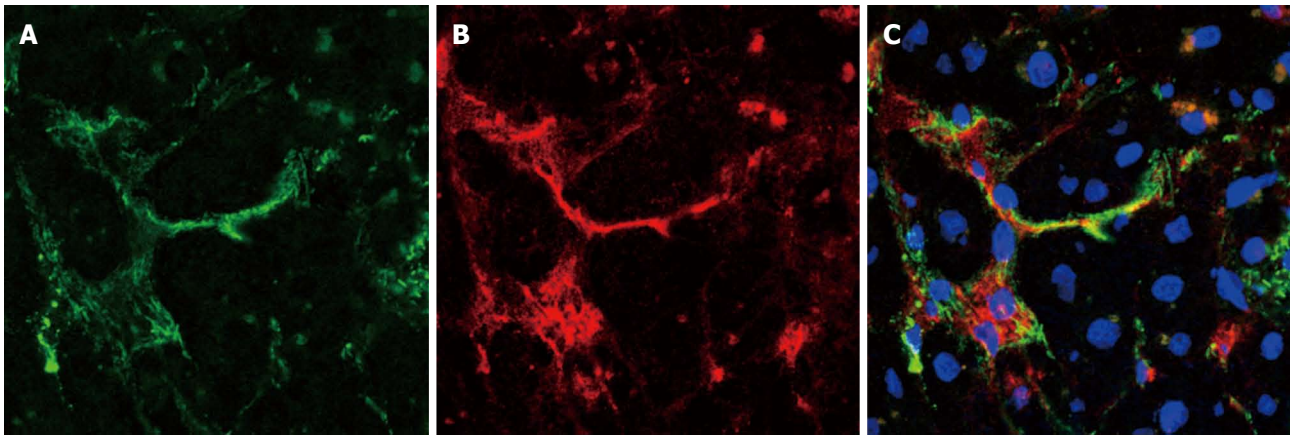

Figure 3 Cellular co-localization of α-smooth muscle actin and smoothelin in a fibrotic liver. A: α-smooth muscle actin expression (green); B: Smoothelin expression (red); C: Merged (nuclei are stained with DAPI). Smoothelin-expressing cells also express α-smooth muscle actin.

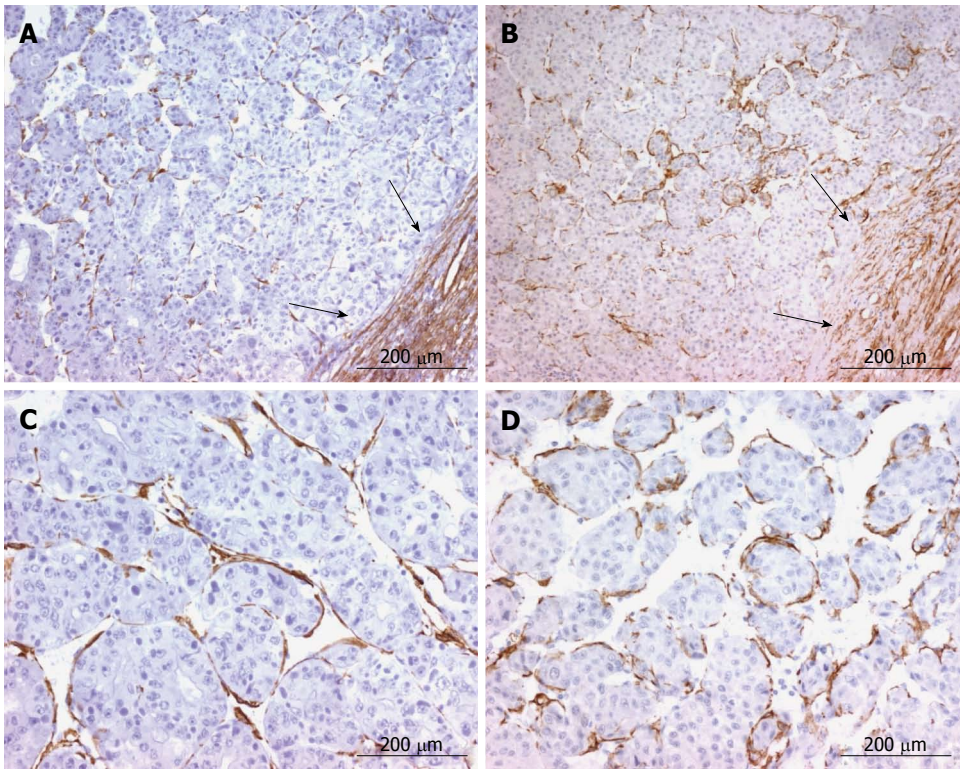

Figure 4 α-Smooth muscle actin and smoothelin expression in hepatocellular carcinoma. A and B: α-smooth muscle actin (A) and smoothelin (B) are expressed similarly in the stroma reaction surrounding the tumor (arrows) and around tumoral hepatocytes; C and D: α-smooth muscle actin (C) and smoothelin (D) are expressed by pericyte-like cells underlying capillaries between tumoral hepatocytes.

described smooth muscle cell marker, illustrates these phenotypic features. Firstly, smoothelin expression varies, with isoform A being expressed in visceral smooth muscle cells, and isoform B being expressed in vascular smooth muscle cells[4]. Secondly, smoothelin is expressed only by fully differentiated smooth muscle cells and not by proliferative or non-contractile smooth muscle cells[1]. Thirdly, visceral smoothelin expression is different according to the location of the smooth muscle cells within the organ. For example, in the digestive tract and urinary tract, most of the smooth muscle cells of the muscularis propia express smoothelin, but the smooth muscle cells of the muscularis mucosae do not[21,22]. During fibrotic processes, it is assumed that it is mainly fibroblasts that are recruited and acquire a myofibroblastic phenotype, *i.e.*, a smooth muscle cell-like phenotype. However, it has been suggested that smooth muscle cells can also contribute to the appearance of myofibroblasts. Similarly to smooth muscle cells, fully differentiated myofibroblasts express α-smooth muscle actin and are contractile cells; in addition, myofibroblasts are responsible for extracellular matrix deposition. Like myofibroblasts observed within bladder carcinoma[21], blood vessel adventitia[23] or the airway wall of patients with asthma[24], our study shows

that myofibroblasts in cutaneous hypertrophic scars do not express smoothelin. In the skin, myofibroblasts are mostly derived from locally recruited connective tissue fibroblasts, which, prior to injury, do not express α-smooth muscle actin or smoothelin[8]. These fibroblasts, when transformed into myofibroblasts, express α-smooth muscle actin but not smoothelin. Additionally, it has been shown that, in hypertrophic scars, a population of fibrogenic circulating cells, called fibrocytes, is recruited and participates in scar formation[25]. Here, we showed that, in hypertrophic scars, myofibroblasts do not express smoothelin, which suggests that these myofibroblasts probably do not derive from local smooth muscle cells. However, our study showed that, in fibrotic liver diseases, myofibroblasts can express smoothelin. Double immunofluorescence and confocal analysis confirmed the co-expression of α-smooth muscle actin and smoothelin in the same cells. This expression is stronger in parallel with the progression and the chronicity of the fibrotic lesion: i.e., there are more smoothelin-expressing myofibroblasts within advanced stages of fibrosis or cirrhosis than in the first stages of fibrosis. Smoothelin expression may thus appear gradually during fibrosis development; therefore, smoothelin could represent a good marker for evaluating the prognosis of liver fibrosis. In the liver, numerous cell types can be recruited and acquire a myofibroblastic phenotype; generally these are fibroblasts within the portal tract and hepatic stellate cells in the space of Disse[11]. As shown here, these cells do not express smoothelin. However, myofibroblasts can also originate from the smooth muscle cells present in portal vessels and centrilobular veins[11], which, as described here, do express smoothelin. In chronic hepatic schistosomiasis, it has been suggested that smooth muscle cells from the artery and vein tunica media are involved in the portal fibrogenesis that occurs in this pathology[12]. In the experimental model of porcine serum-induced fibrosis in rats, mesenchymal cells, located around the centrilobular vein, called second layer cells, participate in the formation of fibrotic septa[26]. The expression of smoothelin within smooth muscle cells can also vary. In early atherosclerotic lesions, smoothelin expression decreases in the vicinity of the affected area[27,28]; however, when the plaques become "quiescent", smoothelin expression can be detected again[2]. Finally, we cannot exclude the possibility that the expression of smoothelin within septal myofibroblastic cells is a residual smooth muscle feature, underlining the possible smooth muscle cell origin of a subpopulation of myofibroblasts. Lastly, the tumoral stroma is the connective tissue surrounding tumoral cells[29]. No smoothelin-expressing stromal cells were described in published studies of bladder carcinoma[21,30]. In hepatocellular carcinoma, we show that numerous α-smooth muscle actin expressing myofibroblasts in the stroma reaction also express smoothelin, underlining the possible smooth muscle origin of these myofibroblasts. Surprisingly, we also showed that, in hepatocellular carcinomas, cells located between endothelial cells and tumoral hepatocytes express α-smooth muscle actin and smoothelin. Pericytes and hepatic stellate cells, also termed liver-specific pericytes[31,32], can express α-smooth muscle actin, but not smoothelin[2]. This pattern of smoothelin expression, which was not found in the fibrotic liver, illustrates the complete reorganization of the capillarized liver sinusoids during tumor development, with the appearance of specific cells, and may represent an additional feature for diagnosing hepatocellular carcinomas. In summary, we hypothesize that during liver fibrosis, a subpopulation of myofibroblasts express smoothelin. These myofibroblasts may be derived from smooth muscle cells coming from vascular walls, which are recruited to participate in the fibrotic process. These cells expressing smoothelin are recruited in advanced stages of cirrhosis, when a significant vascular reorganization occurs, including portovenous and arteriovenous shunting; therefore, smoothelin could be a useful marker to distinguish advanced stages of cirrhosis. In addition, in hepatocellular carcinomas, smoothelin expression shows a very specific pattern. These data again illustrate the different cellular origins of the so-called myofibroblastic cells involved in liver fibrogenesis.

COMMENTS

Background

Myofibroblasts are fibrogenic and contractile cells involved in remodeling and healing processes. In most organs, myofibroblasts are rare in normal physiological conditions. After tissue injury, myofibroblasts appear, which are derived from local stromal cells, such as resident fibroblasts or organ specific stromal cells, such as hepatic stellate cells in the liver.

Research frontiers

To explore the origin of myofibroblasts, the authors studied the expression of smoothelin, a constituent of the smooth muscle cell cytoskeleton, in normal and fibrotic livers, in hepatocellular carcinoma and in a cutaneous hypertrophic scar. Immunohistochemistry was used to identify smoothelin-expressing cells.

Innovations and breakthroughs

This is the first study exploring smoothelin expression in the normal liver, fibrotic liver and in hepatocellular carcinoma. The results show an increase in expression that correlated with the degree of fibrosis, suggesting the progressive involvement of resident smooth muscle cells in myofibroblast recruitment. In hepatocellular carcinoma, smoothelin is expressed by pericyte-like cells between the endothelial cells of the capillaries and the tumoral cell plates.

Applications

This preliminary study underlines that tracing the origin of myofibroblasts may be useful to evaluate the degree of fibrosis in the liver and the level of reorganization in hepatocellular carcinomas.

Terminology

Smoothelin is a constituent of the smooth muscle cell cytoskeleton. It has been described as a marker of end-stage differentiation of smooth muscle cells because its expression has been found only in contractile smooth muscle cells.

Peer review

This is a good descriptive study in which the authors explore the expression pattern of a smooth muscle marker, smoothelin, within normal, fibrotic and tumoral livers, as well as in a cutaneous hypertrophic scar. The results are interesting and suggest that smoothelin expression increases with the degree of fibrosis, allowing the evaluation of the transition between fibrosis and cirrhosis. Moreover, in hepatocellular carcinoma, smoothelin is expressed by some stromal cells present beneath endothelial cells of the capillary surrounding the tumoral cell plates and this distinctive feature could be useful for hepatocellular carcinoma diagnosis.

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BRIEF ARTICLE

Epidemiology and clinical features of cystic hydatidosis in Western Sicily: A ten-year review

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Abstract

AIM: To assess retrospectively the epidemiological and clinical aspects of cystic echinococcosis (CE) and to evaluate follow-up and response to treatment in patients affected by CE.

METHODS: From January 2000 to December 2010, all patients affected by CE at the Infectious Diseases Units of the University of Catania and of Basilotta Hospital in Nicosia-Enna, were enrolled as participants in the study. Epidemiological, clinical and laboratory data were collected for each patient. Diagnosis of CE was performed using clinical imaging and laboratory parameters. Response to treatment was categorized as follows: "cure" as the disappearance or complete calcification of cyst/s; "improvement" as a reduction in the diameter and/or number of existing cysts; and "impairment" as an increase in the diameter and/or number of existing cyst/s and the onset of relapses (*i.e.*, the onset of new cyst/s and an increase in the diameter of previously existing cyst/s and/or complications. Immunoglobulin E (IgE) titers and eosinophil percentages were evaluated at diagnosis, at six months after the initiation of treatment and again in the case of relapse. Hyper-eosinophilia was defined as an eosinophil percentage of $\geqslant$ 6%.

RESULTS: Thirty-two patients were diagnosed with CE in our Unit during the research period, with a male-female ratio of 2:1. At the time of diagnosis, 40% of patients presented a single CE cyst. Sixty percent showed multi-organ involvement. The liver-lung localization ratio was 2:1. Patients below the age of 50 at diagnosis were more likely to have multiple cysts (73.7% *vs* 35.5%, $P < 0.05$). Regarding treatment, 30 patients were treated medically and 16 surgically. Fourteen patients were treated both medically and surgically. Relapses were seen to be less frequent in patients treated with albendazole before and after surgery. Complete cure or an improvement was achieved in 23 patients. Impairment was observed in one patient. Two patients showed no improvement. Relapses were more frequent in those patients treated before 2005. At diagnosis, 71% of patients were positive for specific CE IgE, and 56.3% showed an eosinophil percentage of $\geqslant$ 6%. Patients who were diagnosed with hyper-eosinophilia developed complications more frequently than the other patients, but did not suffer relapses.

CONCLUSION: On the basis of our results, we propose cystic echinococcosis screening for family members of patients, appropriate pre- and post-surgery treatment and the assessment of anti-echinococcus IgE titer or eosinophil percentage as a therapy response marker in settings with limited resources.

Key words: Cystic echinococcosis; Hydatid disease; Cestode infections; Epidemiology; Diagnosis

Core tip: On the basis of the data presented, we suggest the use of specific immunoglobulin E detection and eosinophil percentage counts as therapeutic response markers, particularly in settings with limited resources. We also recommend: (1) routine screening for cystic echinococcosis in relatives (and/or close associates) of patients to facilitate the diagnosis of asymptomatic infection; (2) extension of the follow-up period after surgical and/or medical treatment for the early diagnosis of relapses; and (3) appropriate pre- and post-surgery therapy.

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INTRODUCTION

Human cystic echinococcosis (CE), or hydatid disease, is a parasitic zoonosis caused by the larval stages of the cestode *Echinococcus granulosus*. The definitive hosts of this parasite are usually members of the canid family, such as dogs, which develop the adult worm in the gut following ingestion of the larvae that are present in the tissues of the intermediate host. Following the ingestion of eggs that are expelled in the feces of the definitive host, larval cysts then go on to develop in the visceral tissue of the intermediate hosts (typically sheep and goats and occasionally, humans), particularly in the liver and lungs.

CE is found worldwide, especially where livestock breeding and farming are widespread and in areas where human, definitive and natural intermediate hosts are found in close proximity. In the human host, a hydatid cyst can lead to life-threatening complications, such as cyst rupture, with possible anaphylactic shock, the spread of new cysts, and bacterial infection. In Italy, Sicily is one of the most endemic areas for CE because of the high levels of farming and livestock breeding, with an average annual incidence of 3.2/100000 inhabitants[1]. CE is often underdiagnosed because it is frequently a silent condition that develops over several years and whose symptoms are only apparent when compression of internal organs occurs.

The aim of this study was to assess retrospectively the epidemiological and clinical characteristics of CE, and evaluate follow-up and response to treatment in patients affected by this disease.

MATERIALS AND METHODS

From January 2000 to December 2010 all CE patients admitted to the Infectious Diseases Units of the University of Catania and of Basilotta Hospital in Nicosia-Enna were enrolled as participants in the study. Epidemiological, clinical and laboratory data were collected from the clinical records of each patient. Diagnosis of CE was made using clinical, imaging and laboratory parameters.

Epidemiological data included sex, age, race, job, place of residence, possible contact with stray dogs or hunting dogs, countryside activities, hunting and presence of other people in the family affected by CE. Patients were classified as being "exposed" or "not exposed" to CE on the basis of occupation and recreational activities potentially at risk of acquiring CE. Chest X-ray and abdominal ultrasound provided the parameters for an initial diagnosis of CE. None of the patients enrolled in this study were assessed using high-sensitivity tests, such as specific anti-Echinococcus immunoglobulin E (IgE)-ELISA ImmunoCAP or on the basis of micro/macroscopic morphology alone. Serum alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (gamma-GT) and alkaline phosphatase (ALP) values were also collected.

Where appropriate, treatment consisted of albendazole at a dosage of 10-15 mg per kg per day, administered in two separate doses, each individual course of treatment lasting up to 3 mo. Ultrasound and X-ray assessed the response to treatment after 1 year. A complete "cure" was defined as the disappearance or the complete calcification of the cyst/s, thereby rendering them non-viable. "Improvement" was defined as a reduction in the diameter and/or number of cysts and/or partial calcification, and "impairment" as an increase in the diameter and/or number of cyst/s and the onset of relapses and/or complications. A "relapse" was defined as the onset of new cyst/s (regardless of location) and/or an increase in diameter of previously existing cyst/s.

Response to treatment was recorded together with details of any adverse events, the severity of such events and any further complications. Correlations between side effects and albendazole treatment were assessed using the Naranjo algorithm[2]. Side effect severity was measured using the FDA drug reaction severity scale[3].

As the average time before relapse was observed to be 30 ± 6.4 mo, for the purposes of calculating the relapse rate, the decision was made to include only those patients diagnosed before 2008. IgE titers were evaluated at diagnosis, at six months after beginning treatment and again in the case of relapse. Eosinophil percentages were evaluated at diagnosis and six mo after starting treatment. Hypereosinophilia was defined as an eosinophil percentage of $\geqslant 6\%$.

Ethics statement

This study was conducted in accordance with the principles expressed in the Declaration of Helsinki. The Ethics Committee of the University of Catania approved the study. All patients provided written informed consent for their data to be analyzed.

Statistical analysis

Anti-echinococcus IgE was performed using ELISA Immu-

Table 1 Main characteristics of the 32 patients affected by cystic echinococcosis *n* (%)

Characteristics	Value
Age (yr)	
Median (range)	46 ± 16.5
Sex	
Male	21 (65.7)
Female	11 (34.3)
Risk factors	
Exposed	14 (43.7)
Not exposed	18 (56.3)
Comorbidities	
Affected	17 (53.1)
Not affected	15 (46.9)
Diagnosis	
Abdominal ultrasound	16 (50)
Chest X-ray	9 (28.1)
Unknown	7 (21.9)
Hydatid cyst	
Single	13 (40)
Multiple	18 (60)
Treatment	
Medical	30 (93.5)
Surgical	16 (50)
Medical/surgical	14 (46.8)

no-CAP (Phadia AB, Uppsala). Inferential statistical analysis was conducted using the χ^2 test with Yates' correction, Fishers' exact test, Mann-Whitney's *U* test and Student's *t*-test.

RESULTS

Gender and age

Thirty-two patients were diagnosed with CE in the course of this study, of whom 21 were male and 11 were female, with a male-female ratio of 2:1. Their median age was 46 ± 16.5 years.

Risk factors

Fourteen patients, (43.7%) were classified as having been exposed to CE. In 12 of these cases (85.7%), exposure was a result of occupation (dog or cattle breeders, farmers, butchers, veterinarians), and in two cases (14.2%) exposure occurred during recreational activities (hunting). A strong correlation was observed between exposure to risk factors and the presence of other subjects affected by CE in the patients' extended families (Figure 1A). Comorbidities were observed in 17 (53.1%) patients, hypertension in nine, diabetes in four, chronic pulmonary diseases in two, hepatitis B in one and hepatitis C in one.

Diagnosis and localization

CE was detected in 16 patients (50%) by abdominal ultrasound, and in nine (28.1%) by chest X-ray, though in some cases diagnostic examinations were not carried out directly in the two units involved in the study. When diagnosed, 13 patients (40%) were observed as having a single hydatid cyst either in the liver (nine patients) or in the lung (four patients). Nineteen patients (60%) presented multiple cysts with multi-organ involvement. Multiple cysts were observed more frequently (73.7% *vs* 35.5%, $P < 0.05$) in patients below 50 years of age at the time of diagnosis (Figure 1B). When diagnosed, 24 patients (75%) were symptomatic, the most frequent symptom being a dry cough. The liver-lung cyst localization ratio was 1.8:1 and although pulmonary cysts tended to be more frequently symptomatic than hepatic cysts, this difference was not significant (Figure 1C).

Medical and surgical treatment

Thirty patients were treated medically, receiving an average of two cycles of albendazole (6 mo treatment; interquartile range of two to four cycles). Sixteen patients were treated surgically, while 14 patients were treated both medically and surgically. Table 1 summarizes the main characteristics of the 32 patients affected by CE. Of the 30 medically treated patients, 10 developed severe adverse events: leukopenia was observed in four patients, pancytopenia in one patient, and an increase in ALT levels in five patients (for three of whom it resulted in the discontinuation of treatment). Gamma-GT and ALP levels were monitored during albendazole treatment and were found to be normal. The onset of adverse events was not related to ALT levels observed before treatment with albendazole. Of the 30 patients treated with albendazole alone or in combination with surgery, 23 had either a complete cure of CE (disappearance or calcification of cysts) or an improvement (reduction in either the number or size of cysts). An increase in cyst diameter (impairment) was observed in one patient. Four patients could not be evaluated for response to treatment because the cysts were surgically removed immediately after therapy. Two patients showed no improvement.

Relapses

Of the 21 patients diagnosed before 2005, five patients (23.8%) relapsed. Relapses tended to be more frequent in patients that were treated with albendazole before surgical treatment alone than in those treated both before and after surgery, although this difference was not statistically significant (Figure 1D).

Complications

Complications were observed in six patients (18.8%), these being cyst rupture, mostly traumatic, in five cases and bacterial infection in one case.

Specific IgE and eosinophil cell count

At diagnosis, 71% of patients were positive for specific CE IgE. The median value of anti-echinococcus IgE titer was 16 kU$_A$/L (25th percentile = 2; 75th percentile = 24.3; min = 0; max = 34). Six months after beginning treatment with albendazole, 60% of patients were positive for specific IgE. A significant reduction in anti-echinococcus IgE titer values was also observed (IgE titer median = 1.2; 25th percentile = 0.75; 75th percentile = 9.5;

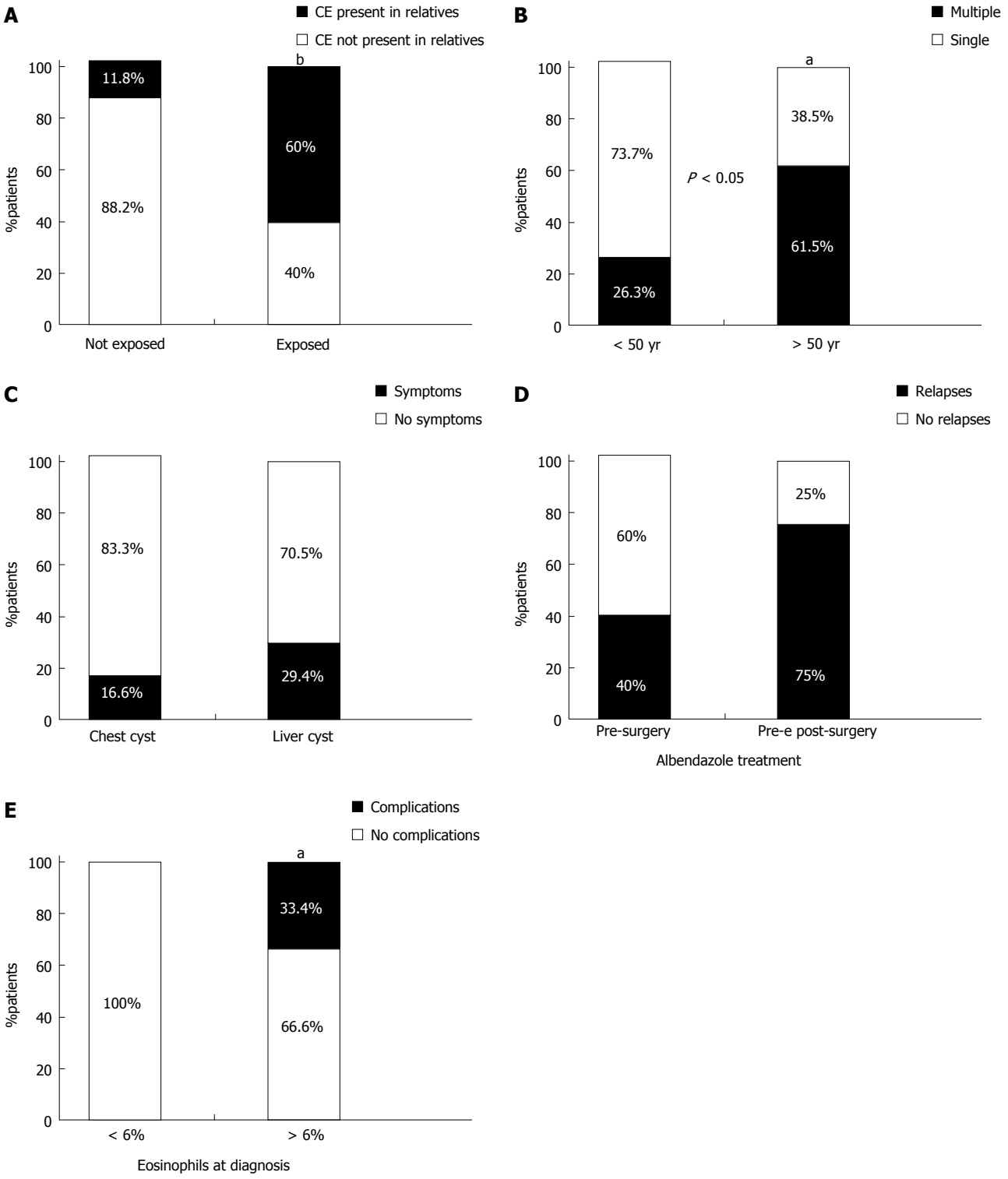

Figure 1 Correlation between different groups. A: Correlation between exposure of patients to risk factors and presence of relatives affected by cystic echinococcosis (CE); B: Correlation between age at diagnosis and presence of single or multiple CE; C: Correlation between cysts location and presence of symptomatic CE; D: Correlation between treatment modality and development of relapses; E: Correlation between rate of eosinophils at diagnosis and development of complications. [a]$P < 0.05$, [b]$P < 0.01$ *vs* control group.

min = 0; max = 369, $P < 0.05$). In cases of recurrence (five patients), the median value of anti-echinococcus IgE titer was 22 kUA/L (25th percentile = 4.93; 75th percentile = 38.5; min = 1.24; max = 42).

An eosinophil percentage of ⩾ 6% was observed in 18 patients (56.3%) at diagnosis. After 6 mo of albendazole treatment, a significant reduction in eosinophil count was observed, with only three patients (12%) showing more than 6%. The median percentage value at diagnosis was 5% (25th percentile = 3; 75th percentile = 9; min = 0; max = 35.2), while 6 mo after beginning treatment with albendazole this has fallen to 2% (25th percentile = 1; 75th percentile

= 3; min = 0.75; max = 12), with a highly significant difference in titres registered at these two times ($P < 0.001$).

Patients with hyper-eosinophilia at diagnosis developed complications, but not relapses, more frequently than other patients (33.4% *vs* 0%, $P < 0.05$) (Figure 1E). Patients with positive specific IgE results at diagnosis had a higher median eosinophil count than those with negative specific IgE.

DISCUSSION

As hydatid cysts usually develop slowly, CE is often an under-diagnosed (and consequently under-treated) disease. It is often difficult to assess its prevalence in a given area. The aim of this study was to assess the epidemiological and clinical characteristics of CE and to evaluate responses to therapy and follow-up by analyzing patterns in patients affected by the disease admitted to two Infectious Diseases Departments from 2000 to 2010.

Gender and age

During the ten-year observation period, 32 patients were diagnosed with CE, with a male/female ratio of 1.8/1. This ratio is comparable with that reported in other endemic areas and perhaps reflects the more frequent occupational exposure of males to the risk of infection[4-7]. The slow development of hydatid cysts, the nature of the host's immune response, and the structure of the most typically affected tissues, may all contribute to late diagnosis[6,7].

Risk factors

Of the total number of patients, 43% were classified as having been exposed to CE through specific risk factors. This is probably caused by the sharing of occupational and/or recreational risk factors among patients and their relatives. It is, therefore, crucial to evaluate patient exposure in family contexts and suggest CE screening for relatives and co-workers.

Diagnosis and localization

Abdominal ultrasound was used as the primary diagnostic tool for 50% of patients. Ultrasound is widely considered one of the best hepatic diagnostic tools for CE, as it allows diagnosis even before the evidence of an increase in specific IgE antibodies[8,9]. In addition, abdominal ultrasound is fundamental for the staging of hepatic CE purposes and for establishing appropriate treatment options[10,11]. At the time of diagnosis, 60% of patients had multiple cysts. Those below the age of 50 were more likely to have multiple cysts ($P < 0.05$) than those diagnosed later. This probably reflected the fact that multiple cysts tend to become more symptomatic than single cysts.

In other studies, the majority of CE patients diagnosed during non-hospital based screening programs in endemic areas did not present any symptoms of CE[12]. By contrast, our patients presented CE-related symptoms in 75% of cases. This may reflect the higher probability that an already symptomatic patient is more likely to be referred to a hospital for investigation and/or treatment. In addition, a higher percentage of symptomatic patients was observed among patients affected by pulmonary-CE compared with hepatic-CE. This may be because hepatic hydatid cysts can grow for many years before symptom onset, while the symptoms of pulmonary CE tend to be more evident. Screening programs in endemic areas show a prevalence of hepatic-CE that is higher than pulmonary-CE, with a liver:chest localization ratio of 6-12:1[12]. In the patients of this study, the ratio was around 2.5:1, thus confirming the greater probability of finding cases of pulmonary-CE rather than hepatic CE in a hospital setting[12].

Medical and surgical treatment

The patients in this study were given albendazole in 93.8% of cases, with an average treatment duration of 6 mo and a maximum of 120 mo in one patient affected by non-surgical multi-organ CE. Studies have shown that Albendazole is more effective than mebendazole in the treatment of CE[13]. Some studies suggest treating CE patients with albendazole for no less than 3 mo. but no longer than 6 mo, longer treatment being offered only to patients with non-abdominal and non-pulmonary CE[14,15].

Response to medical treatment was evaluated by means of ultrasound and X-ray, performed 1 year after beginning of therapy with albendazole. A complete cure (disappearance or calcification of cysts) or improvement (reduction in number or size of cysts) was achieved in 76% of patients treated with albendazole alone or in combination with surgery. This confirmed the results from other studies conducted on around 2000 CE patients treated with benzimidazoles, which showed a response to therapy of 50%-70% after a 12-mo follow-up[16-18]. Albendazole-related adverse events are usually self-limiting and rarely severe. However, in CE, factors such as the longer period of treatment needed, the frequently older age of patients and drug-cyst interaction, may lead to higher occurrence and severity[17-20]. One third of the patients in the current study developed moderate to severe adverse events. In three of the five hypertransaminasemia cases, this led to the discontinuation of treatment with this drug. Similarly, in a study carried out by Gil-Grande *et al*[15], 10%-20% of CE patients were seen to develop hypertransaminasemia, which was always reversible after drug discontinuation or during the pauses between albendazole cycles[13-19].

The surgical treatment of CE can potentially lead to a complete cure of the disease and is indicated in the following cases: for the removal of large cysts with multiple daughter vesicles; for cysts exerting pressure on adjacent vital organs; in the case of cysts communicating with the biliary tree (as an alternative to percutaneous treatments or PTs); or where there are single superficial liver cysts and infected cysts (when PTs are not possible). In non-surgical cases, alternative techniques such as Puncture Aspiration Injection Respiration (PAIR) can be considered[6]. In our study, 50% of the patients were surgically treated, but only half of these cases were given albendazole both pre- and

post-surgery. The WHO recommendations stipulate that albendazole should be given at least 4 d before surgery and for 3 mo following surgery[20]. Other studies suggest that a 3 mo albendazole cycle before surgery is more effective in reducing cyst vitality, rather than treatment for only 4 d or even one mo[15]. Appropriate post-surgical albendazole therapy has also been observed to prevent CE relapses because of surgical dissemination[13-24].

Relapses

Among the patients of this study, 60% of those treated with albendazole before surgery alone suffered a relapse, compared to only 25% of those treated both before and after surgery. This suggests that appropriate medical therapy offered to the patient both pre- and post-surgery is necessary.

The literature reports a relapse incidence in the treatment of CE of between 0% and 30%, with an average time for the onset of relapse symptoms of 3-4 years[21]. The patients in this study developed symptomatic relapses, hepatic in 80% of cases and pulmonary in 20% of cases, in an average time of 20.7 mo after the cessation of treatment or following surgery, with a relapse rate of 23.8%.

Complications

Six of the patients in this study (18.8%) developed complications, these being bacterial super-infection of a cyst in one case and cyst rupture (mostly traumatic and easily managed using a surgical approach) in five cases. The most frequent complication reported in CE endemic areas is cyst rupture into the biliary tree (5%-17%), followed by super-infection (5.1%)[25,26].

Specific IgE and eosinophil cell count

ELISA for specific anti-echinococcus IgG and IgE titer detection is frequently used in CE serological diagnosis and screening, as it requires a short preparation time, has a relatively limited cost, and shows a sensitivity and specificity of 95%[27-30]. In our study, at the time of diagnosis, 71% of patients were positive for specific CE IgE, with a median titer of 16 kU$_A$/ which dropped significantly to 1.2 kU$_A$/ (with 60% of positive specific IgE patients) 6 mo after beginning treatment with albendazole. This confirmed that monitoring IgE titer variation over time is likely to be more helpful in evaluating CE activity than considering single absolute values[31]. However, it should be noted that fluctuations in specific IgE titer that are unrelated to the clinical stage of the disease are possible. Extremely prolonged positivity (*e.g.*, 3-7 years) is possible even after surgical cyst treatment[32-34]. Persistence of high specific IgE titer beyond 3-7 years usually indicates the onset of a relapse. This appeared to be confirmed by the patients of this study that developed relapses: their median specific IgE value was 22 kU$_A$/ at the time of relapse diagnosis[32-34]. On this basis, it is possible to claim that in CE-affected patients, specific IgE titer detection can be considered as a useful tool in post-therapeutic follow-up to predict cure and eventual relapse.

In the literature, CE diagnosis and follow-up eosinophil cell counts have usually been considered of limited value because it is significantly high in no more than half of CE affected patients, as confirmed in our own study[4]. In this study, although 56.3% of patients presented a percentage value of eosinophils $\geqslant$ 6% (defined as hyper-eosinophilia) at diagnosis, six months after beginning treatment with albendazole, only 12% of patients continued to display hyper-eosinophilia. At diagnosis, the median percentage value of eosinophils was 5%; however, 6 mo after beginning treatment with albendazole, this value had dropped to 2%. This significant difference in titers registered at the two different times ($P < 0.001$) may be a result of the positive response to therapy obtained in the majority of patients. Finally, we observed that patients with positive specific IgE at diagnosis had a higher median eosinophil percentage than those with negative specific IgE ($P < 0.05$). This suggested that, in settings with limited resources, or where specific IgE detection is not feasible, eosinophil percentage value at diagnosis could be used as an effective tool for post-therapeutic follow-up, to assess the response to therapy and as an aid in patient prognosis.

In conclusion, on the basis of our data, three main proposals can be made. Firstly, we advocate the routine screening for CE in relatives (and/or close associates) of patients classified as being exposed to CE in settings where this is feasible. We also recommend the use of anti-echinococcus IgE titer as a response marker of albendazole therapy (in low resource settings this could be replaced by eosinophil percentage value). Our results also highlight the need for an extended and well managed period of follow-up after surgical and/or medical therapy, as indicated by the average time for the onset of relapse, which in our patients was about 2 years. Finally, while the surgical approach remains the first-line treatment in most CE cases, appropriate medical therapy before and after surgery should also be considered a useful tool to reduce the incidence of relapse[35].

COMMENTS

Background

Endemic in sheep raising areas, cystic echinococcosis (CE) is a neglected disease. This neglect is also reflected in the clinical management of CE, which has evolved over the last few decades with little or no comparative evaluation of the efficacy, effectiveness, rate of adverse events and relapse rates. In addition, CE is often under-diagnosed as it is frequently undetected, developing over the course of several years.

Research frontiers

On the basis of their data, some proposal can be made: (1) routine screening for CE in relatives (and/or close associates) of patients exposed, according to occupation and recreational activities, who are potentially at risk for acquiring CE, to speed up CE diagnosis; (2) extended and manage the period of follow-up after surgical and/or medical therapy to avoid late CE relapse diagnosis; and (3) administer appropriate medical therapy before and after surgery to reduce the incidence of relapse.

Innovations and breakthroughs

These findings suggest that anti-echinococcus immunoglobulin E (IgE) titer may be successfully replaced by eosinophil percentage count in low resource settings as a marker of response to therapy.

Applications

Cystic echinococcosis is a helminthic zoonosis that can affect humans, most frequently involving the liver or the lungs. Symptoms typically show themselves only when the compression of internal organs occurs. In humans, the natural history of CE can lead to life-threatening complications, such as cyst rupture (with possible anaphylactic shock), the spread of new cysts, and bacterial infection.

Terminology

Cystic echinococcosis is a helminthic zoonosis that can affect human beings, most frequently involving the liver or the lungs, showing symptoms only when the compression of internal organs occurs. In humans, the natural history of CE can lead to life-threatening complications, such as cyst rupture, with possible anaphylactic shock, the spread of new cysts, and bacterial infection.

Peer review

Cystic echinococcosis is a helminthic zoonosis that can affect humans, most frequently involving the liver or the lungs. Symptoms typically show themselves only when the compression of internal organs occurs. In humans, the natural history of CE can lead to life-threatening complications, such as cyst rupture (with possible anaphylactic shock), the spread of new cysts, and bacterial infection.

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BRIEF ARTICLE

Risk of cancer, with special reference to extra-intestinal malignancies, in patients with inflammatory bowel disease

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Abstract

AIM: To determine the incidence and characteristics of intestinal and extra-intestinal cancers among patients with inflammatory bowel disease in a Spanish hospital and to compare them with those of the local population.

METHODS: This was a prospective, observational, 7-year follow-up, cohort study. Cumulative incidence, incidence rates based on person-years of follow-up and relative risk were calculated for patients with inflammatory bowel disease and compared with the background population. The incidence of cancer was determined using a hospital-based data registry from Hospital Universitario de Fuenlabrada. Demographic data and details about time from diagnosis of inflammatory bowel disease to occurrence of cancer, disease extent, inflammatory bowel disease treatment, cancer therapy and cancer evolution were also collected in the inflammatory bowel disease cohort.

RESULTS: Eighteen of 590 patients with inflammatory bowel disease developed cancer [cumulative incidence = 3% (95%CI: 1.58-4.52) *vs* 2% (95%CI: 1.99-2.11) in the background population; RR = 1.5; 95%CI: 0.97-2.29]. The cancer incidence among inflammatory bowel disease patients was 0.53% (95%CI: 0.32-0.84) per patient-year of follow-up. Patients with inflammatory bowel disease had a significantly increased relative risk of urothelial carcinoma (RR = 5.23, 95%CI: 1.95-13.87), appendiceal mucinous cystadenoma (RR = 36.6, 95%CI: 7.92-138.4), neuroendocrine carcinoma (RR = 13.1, 95%CI: 1.82-29.7) and rectal carcinoid (RR = 8.94, 95%CI: 1.18-59.7). Colorectal cancer cases were not found.

CONCLUSION: The overall risk of cancer did not significantly increase in our inflammatory bowel disease patients. However, there was an increased risk of urinary bladder cancer and, with less statistical power, an increased risk of appendiceal mucinous cystadenoma and of neuroendocrine tumors. Colorectal cancer risk was low in our series.

Key words: Extra-intestinal cancer; Inflammatory bowel disease; Cancer risk; Background population; Urothelial carcinoma; Appendiceal mucinous cystadenoma; Neuroendocrine carcinoma; Rectal carcinoid

Core tip: Several studies have reported increased rates of colorectal cancer in patients with inflammatory bowel diseases but limited data are available regarding incidence of extraintestinal malignancies in these patients. The present study demonstrates a higher risk of urinary bladder cancer, mucinous cystadenoma of the appendix and of neuroendocrine tumors, and a low colorectal cancer risk, in patients with inflammatory bowel dis-

ease in our environment. We raised the question of whether current cancer screening strategies need to be reviewed and adapted to the characteristics of each patient.

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INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic condition that involves several portions of the gastrointestinal tract and includes periods of activity of variable severity. This pathology can be associated with involvement of other organs in 35% of patients with IBD, with rheumatologic, ophthalmologic and dermatologic disorders being the most common extra-intestinal manifestations[1,2].

To date, a number of studies have reported increased rates of colorectal cancer (CRC) in patients with IBD[3-8]. The reported risk varies widely between studies due to the different methodologies used. The current trend published in the most recent studies suggests a lower rate of CRC than previously described[9]. The location and duration of IBD[6-12], as well as previous family history of CRC[13,14] and primary sclerosing cholangitis[15,16], have been described as determinant factors associated with CRC. Additionally, the severity of chronic inflammation of the colon is another important risk factor for this kind of cancer in patients with both ulcerative colitis (UC) and Crohn's disease (CD)[17,18]. IBD-specific and non-IBD specific medications have also been associated with an increased or decreased risk of CRC[19,20].

On the other hand, limited and disparate data are available for incidences of extra-intestinal malignancy in these patients[21-24]. In addition, several of these studies are not population based, or have retrospective designs. Other studies only contain information on cumulative cancer risk or represent select populations.

We present a prospective, cohort study designed to determine the incidence and characteristics of intestinal and extra-intestinal cancers among patients with IBD in our environment and to compare these incidences with those of the local population.

MATERIALS AND METHODS

Patients and design

This was a prospective, observational, 7-year follow-up, cohort study. We identified all cases of cancer observed between January 2005 to the end of December 2011 in a cohort of patients diagnosed with IBD in our hospital (n = 590). Diagnosis of IBD was confirmed by routine clinical, radiological, endoscopic and histological criteria[25]. The incidence and characteristics of intestinal and extra-intestinal cancers were obtained in the IBD group and were compared to those of the background population (n = 222219). The incidence of cancer in our population was determined using a hospital-based data registry from Hospital Universitario de Fuenlabrada. This registry contains all cases of cancer diagnosed and/or treated in a 7-year follow-up period in the patients of our area. In each case, cancer was confirmed by a pathologist who classified the lesions according to the International Classification of Diseases for Oncology (ICD-O-3 histology codes).

Demographic data and details of time from diagnosis of IBD to occurrence of cancer, disease extent according to the Montreal Classification[26], IBD treatment, cancer therapy and cancer evolution were also collected in the IBD cohort. The study was approved by the Research Ethics Committee at Hospital Universitario de Fuenlabrada.

Statistical analysis

The descriptive analysis of quantitative variables calculated the mean and standard deviation or median and interquartile range (IQR) depending on whether or not the data were normally distributed. Qualitative variables were expressed as percentages with 95%CI. Cumulative incidences for each cancer were calculated for patients with IBD and compared with the background local population. The incidence rates based on person-years of follow-up and relative risk (RR) with 95%CI were also analysed. Analysis was performed assuming that the IBD cohort had the same risk of developing malignancies as the general population.

RESULTS

Eighteen of 590 patients with IBD were diagnosed with cancer between 2005 and 2011 in our hospital. The clinical and demographic characteristics of all patients with IBD are shown in Table 1. The cumulative incidence of cancer was 3% (95%CI: 1.58-4.52) *vs* 2% (95%CI: 1.99-2.11) in the local population; RR = 1.5; 95%CI: 0.97-2.29. The mean age in IBD patients with a diagnosis of cancer was 49.9 ± 11.9 years; 61.1% were males. In the cohort of patients with IBD, 9 had CD (50%) and 9 UC (50%). The clinical characteristics of these patients are shown in Table 2. By type of IBD, the cumulative incidence was 2.8% (95%CI: 0.86-4.84) for CD *vs* 3.5% (95%CI: 1.06-5.92) for UC patients. At the time of cancer diagnosis, 33% of patients were being treated with thiopurines [median duration of treatment was 6 mo (IQR 2.4-27)], and one patient (6%) was on anti-tumour necrosis factor-alpha therapy (39 mo of treatment with adalimumab). The median time from IBD diagnosis to cancer development was 54 mo (IQR 21-111). The cancer incidence among IBD patients was 0.53% (95%CI: 0.32-0.84) per patient-year of follow-up.

Ten different kinds of cancers were identified. The

Table 1 Clinical features of the total studied inflammatory bowel disease patients *n* (%)

Clinical features	Value
Age (yr, mean ± SD)	43.4 ± 13.8
Gender	
Female	305 (51.70)
Male	285 (48.3)
Type of IBD	
CD	313 (53.05)
UC	256 (43.39)
IBDU	21 (3.56)
Disease extension (UC)[1]	
Proctitis	61 (23.83)
Left-sided colitis	124 (48.44)
Pancolitis	71 (27.82)
Age at diagnosis (CD)[1]	
A1 < 17	24 (7.67)
A2 17-40	208 (66.45)
A3 > 40	81 (25.88)
Disease location (CD)[1]	
L1 ileal	112 (35.78)
L2 colic	76 (24.28)
L3 ileocolic	111 (35.46)
L4 upper gastrointestinal tract	6 (1.92)
L1 + L4	5 (1.60)
L3 + L4	3 (0.96)
Behaviour (CD)[1]	
B1 non-stricturing non-penetrating	179 (57.19)
B2 stricturing	30 (9.58)
B3 penetrating	41 (13.10)
B1 + perianal disease	51 (16.29)
B2 + perianal disease	1 (0.32)
B3 + perianal disease	11 (3.52)
Immunosuppressive or biological treatment	
Thiopurines	259 (43.90)
Anti-TNF-α drugs	84 (14.23)

[1]In accordance with the Montreal classification. IBD: Inflammatory bowel disease; IBDU: Inflammatory bowel disease type unclassified; CD: Crohn's disease; UC: Ulcerative colitis.

Table 2 Clinical features of the cohort of patients with inflammatory bowel disease and cancer diagnosis *n* (%)

Features	Value
Disease extension (UC)[1]	
Proctitis	1 (11.1)
Left-sided colitis	4 (44.45)
Pancolitis	4 (44.45)
Age at diagnosis (CD)[1]	
A2 17-40	8 (88.89)
A3 > 40	1 (11.11)
Disease location (CD)[1]	
L1 ileal	4 (44.45)
L2 colic	2 (22.22)
L3 ileocolic	2 (22.22)
L3 + L4 upper gastrointestinal tract	1 (11.11)
Behaviour (CD)[1]	
B1 non-stricturing non-penetrating	7 (77.78)
B2 stricturing	1 (11.11)
B1 + perianal disease	1 (11.11)
Treatment at time of cancer diagnosis	
Aminosalicylates	10 (55.56)
Thiopurines	6 (33.34)
Anti-TNF-α drugs	1 (5.55)
Nonspecific inflammatory bowel disease treatment	1 (5.55)
Immunosuppressive or biological treatment previous cancer diagnosis	
Thiopurines	1 (5.55)
Anti-TNF-α drugs	1 (5.55)

[1]In accordance with the Montreal classification. CD: Crohn's disease; UC: Ulcerative colitis; TNF-α: Tumor necrosis factor α.

Table 3 Specific cancers diagnosed in the cohort of patients with inflammatory bowel disease between 2005 and 2011

Location	*n*	Histological type
Urinary bladder	4	Urothelial carcinoma
Breast	3	Carcinoma
Skin	1	Melanoma
	2	Basal cell carcinoma
Appendix	2	Mucinous cystadenoma
Stomach	1	Adenocarcinoma
Pancreas	1	Adenocarcinoma
Lung	1	Adenocarcinoma
Liver	1	Hepatocellular carcinoma
Small intestine	1	Neuroendocrine carcinoma
Rectum	1	Carcinoid

specific types of cancer observed in the cohort of IBD patients are shown in Table 3. Compared with the local population, patients with IBD had a significantly increased RR of urothelial carcinoma, mucinous cystadenoma of the appendix, neuroendocrine carcinoma and rectal carcinoid (Table 4). The RR of breast, skin, stomach, pancreas, lung and liver cancers were not significantly different with respect to the background local population (Table 4). CRC diagnoses were not found, and only two patients had biopsies with low grade dysplasia despite dysplasia screening by colonoscopy being performed following standard recommendations[27].

All patients with a diagnosis of urinary bladder cancer were men: 2 UC and 2 CD, 1 smoker and 3 former smokers. All patients with breast cancer had a previous family history (none of the remaining patients had family history for other types of tumours).

Regarding the evolution of cancer, 16 of the 18 patients diagnosed with cancer (88.9%) needed oncological surgery and 27.7% (n = 7) were treated with chemotherapy or radiation therapy. Treatment was maintained in 50% of patients on thiopurines at the time of their cancer diagnosis. In the remaining patients, immunosuppressive therapy was withdrawn and in only one of them was thiopurine therapy reintroduced three years later in agreement with the oncologist's recommendations. An association between thiopurine treatment and malignancy risk was not found. Adalimumab treatment was also withdrawn after the cancer diagnosis. Patients were followed up for an average of 3.5 ± 2.3 years from tumour diagnosis. During this period, two patients died (11.1%) due to cancer (patients with lung and liver cancer diagnoses) and 3 (16.7%) had tumour recurrence.

DISCUSSION

The present cohort study of patients with IBD revealed

Table 4 Cumulative incidences in both the inflammatory bowel disease cohort and the non-inflammatory bowel disease cohort and relative risk for different types of cancer

Cancer site	Cumulative incidence in IBD cohort	Cumulative incidence in local population	RR	95%CI
Urinary bladder	0.68	0.13	5.23	1.95-13.87[a]
Breast	0.51	0.26	1.95	0.63-5.87
Melanoma	0.17	0.06	2.56	0.34-17.63
Basal cell carcinoma	0.33	1.26	0.26	0.06-1.00
Appendix	0.33	0.009	36.6	7.92-138.4[a]
Stomach	0.17	0.06	2.83	0.41-20.9
Pancreas	0.17	0.028	6.07	0.84-40.4
Lung	0.17	0.067	2.54	0.34-17.6
Liver	0.17	0.037	4.59	0.64-32.5
Small intestine	0.17	0.013	13.1	1.82-29.7[a]
Rectum	0.17	0.019	8.94	1.18-59.7[a]

[a]$P < 0.05$. IBD: Inflammatory bowel disease.

that the overall risk of cancer did not significantly increase in our IBD patients compared to the background population. However, the study found patients with IBD to have an increased risk of developing urothelial carcinoma and, with less statistical power, an increased risk of mucinous cystadenoma and neuroendocrine tumours.

The results obtained in our population regarding overall risk of cancer and risk of urinary bladder cancer are in accordance with those reported previously by Pedersen *et al*[23]. The association between CD and urinary bladder cancer described in other populations by other authors[23,28] could be related to the high prevalence of smokers. Pedersen *et al*[23] explain in their meta-analysis that tobacco smoking could have a causal role in the development of a number of cancers including urinary bladder cancer. They did not find this association in UC patients. In our study, out of the two patients diagnosed with urothelial carcinoma with UC and two patients with CD, only one patient was a smoker at time of cancer diagnosis, while the remaining three patients were former tobacco users. Although different factors may be involved in the development of bladder cancer, the results obtained in several studies, including our work, indicate that tobacco could be a key factor and it would be recommended to encourage CD patients to quit smoking. A recent publication finds that the risk of bladder cancer in former smokers remains elevated more than 32 years after quitting, even among those with moderate smoking histories[29]. Nonsteroidal anti-inflammatory drugs seem to have a chemo-preventive role in urothelial carcinoma of the bladder in subjects who have quit for long periods[30]. Dietary factors may also affect the risk of these carcinomas[31-33]. Ros *et al*[32] suggest that high consumption of certain types of vegetables and fruits may reduce the risk of aggressive or non-aggressive urothelial cell carcinoma of the bladder.

On the other hand, the increased risk of appendiceal mucinous cystadenoma and neuroendocrine tumours observed in the present study were not described before in the meta-analysis carried out by Pedersen *et al*[23].

Mucocele of the appendix is a rare group of lesions that includes four histological types: retention cyst, mucosal hyperplasia, cystadenoma and cystadenocarcinoma. In our study, IBD patients were at an increased risk of mucinous cystadenoma of the appendix. Although a causal relationship between IBD and this type of mucocele cancer is still being elucidated, some authors have suggested that obstruction of the appendiceal orifice might play a role in the development of appendiceal mucocele. This obstruction could be due to inflammation by IBD or associated with colorectal neoplasm[34-36].

Several publications suggest that appendectomy appears to reduce the extent and recurrence of UC and is associated with a less severe course of this pathology[37,38]. These findings could support the hypothesis that an appendicectomy related to mucinous cystadenoma diagnosis may have a secondary beneficial effect on the severity and the course of UC. In contrast, data regarding CD patients are controversial[39,40]. In our series, the two patients with mucocele of the appendix had CD and ileocolic involvement.

The present study also identified one case of neuroendocrine carcinoma of the small intestine and one carcinoid of the rectum. Carcinoids and, in particular, neuroendocrine neoplasms other than carcinoids are uncommon tumours and are infrequently described in UC and CD. Some authors suggest that a permanent inflammation at the level of the colon could increase the number of neuroendocrine cells and help the development of this kind of neoplasm[41]. Greenstein *et al*[42] included eleven patients with IBD-associated carcinoid tumours (11 in the appendix, 2 in the ileum). They found that all carcinoids were diagnosed incidentally after surgery for IBD. These findings are consistent with our results, in which the patient was diagnosed with carcinoid of the rectum during a colectomy. Sigel *et al*[43] evaluated 14 cases of neuroendocrine neoplasms. All of the tumours arose in areas with IBD involvement. Tumour sites were the rectum (6 cases), appendix (4 cases), small bowel (2 cases) and sigmoid colon (2 cases). Conversely, in our case, the patient with neuroendocrine carcinoma of the small intestine had UC (extensive colitis) without ileum involvement.

Concerning the IBD-specific medications, it has been reported that patients with IBD who receive thiopurines are at increased risk of non-melanoma skin cancer or lymphoproliferative disorders[44,45]. However, data about other malignancies are less clear. Moreover, studies in patients taking azathioprine for a long time are scarce. In the present study, a possible effect of thiopurines on the risk of extra-colonic cancer was evaluated but a clear association between variables was not found; this probably could be due to the small size of our series and the short period of treatment.

A higher RR of CRC has been described in patients with IBD[5,7,46]. However, in the present study, CRC was not found despite dysplasia screening by colonoscopy. These data are consistent with current publications

that show a lower incidence of CRC than previously reported[9,47-49]. Some authors have found that only IBD patients with a longer disease duration, extensive disease and an IBD diagnosis at a young age have a significantly higher risk of CRC. IBD patients without these characteristics would have a similar risk for colorectal cancer as the general population[9,47,48,50]. IBD-specific and non-IBD specific medications have also been associated with an increased or decreased risk of CRC[19,20,51]. The anti-inflammatory action of IBD drugs such as 5-ASA seems to have a protective effect on the occurrence of CRC[52,53]. Jess *et al*[48] suggest that changes in IBD-specific treatment may reduce the risk of CRC among UC patients. Currently, it has also been suggested that agents that control chronic inflammation, such as thiopurines and tumour necrosis factor-alpha antagonists, could have a protective role against the development of CRC[48,50,54,55].

Study design could also have an influence on the CRC risk obtained. Patients with IBD from population-based cohorts have a lower risk for CRC than reference centre cohorts[47]. Regarding this fact, we present a cohort study that included all patients with IBD followed in our hospital. We believe that this cohort of patients is a good representative for the entire IBD population in our area. Our hospital is a reference centre with a specific unit for patients with IBD. There are no other hospitals or reference centres in our area. In our opinion, only an insignificant number of patients with IBD are treated by general practitioners at primary care centres. In addition, the incidence of IBD is not lower than that expected for our population: 590 cases in a population of 222219 people. The present manuscript has the value of its prospective design and its well-defined population using a data registry that contains all cases of cancer diagnosed and/or treated in a 7-year follow-up period. In this regard, the results obtained in this survey probably will serve as a base for future studies aimed at elucidating the real risk of extra-intestinal malignancies and the utility of current cancer screening strategies used in these patients.

However, the observations obtained in the present study should be considered with caution due to the potential limitations of this study, which included a small number of cases over a limited period of time.

In conclusion, our prospective study revealed that patients with IBD in our area have a similar overall risk of cancer as the local population. However, they are at a higher risk of developing specific types of extra-intestinal cancers such as bladder, appendiceal cystadenoma or neuroendocrine tumours. Smoking and specific IBD characteristics could be risk factors associated with the development of cancer, so it is recommended that patients be encouraged to quit smoking. On the other hand, the present study did not show a higher risk of CRC, in line with recent publications that suggest a lower incidence of CRC than previously reported. Further evaluations are required to know if the current CRC screening strategies need to be reviewed and adapted to patients according to the characteristics of their IBD.

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COMMENTS

Background

To date, many studies have analyzed the rates of colorectal cancer in patients with inflammatory bowel disease. The reported risk varies widely between studies due to the different methodologies used. However, limited and disparate data are available for incidences of extra-intestinal malignancy in these patients.

Research frontiers

Inflammatory bowel disease is a chronic condition that involves several portions of the gastrointestinal tract but also can be associated with involvement of other organs. Accordingly with the special characteristics of this pathology, the risk of development of different kind of cancers in these patients could be different from the general population.

Innovations and breakthroughs

The present study demonstrates a higher risk of urinary bladder cancer, appendiceal mucinous cystadenoma and of neuroendocrine tumors and a low colorectal cancer risk in patients with inflammatory bowel disease in a Spanish hospital setting. These results could suggest the revision and adaptation of current cancer screening strategies according to characteristics of each patient.

Applications

The results obtained in this survey could serve as the basis of future studies aimed at elucidating the real risk of extra-intestinal malignancies and the utility of current cancer screening strategies used in these patients.

Terminology

Appendiceal mucinous cystadenoma is a rare tumour of the appendix characterized by a cystic dilatation of the appendiceal lumen with stasis of mucus inside it.

Peer review

The authors present a prospective, cohort study designed to determine the incidence and characteristics of intestinal and extra-intestinal cancers among patients with inflammatory bowel disease and to compare these incidences with those of the local population.

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BRIEF ARTICLE

Transforming growth factor-β and toll-like receptor-4 polymorphisms are not associated with fibrosis in haemochromatosis

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Abstract

AIM: To investigate the role of genetic polymorphisms in the progression of hepatic fibrosis in hereditary haemochromatosis.

METHODS: A cohort of 245 well-characterised C282Y homozygous patients with haemochromatosis was studied, with all subjects having liver biopsy data and DNA available for testing. This study assessed the association of eight single nucleotide polymorphisms (SNPs) in a total of six genes including toll-like receptor 4 (TLR4), transforming growth factor-beta (TGF-β), oxoguanine DNA glycosylase, monocyte chemoattractant protein 1, chemokine C-C motif receptor 2 and interleukin-10 with liver disease severity. Genotyping was performed using high resolution melt analysis and sequencing. The results were analysed in relation to the stage of hepatic fibrosis in multivariate analysis incorporating other cofactors including alcohol consumption and hepatic iron concentration.

RESULTS: There were significant associations between the cofactors of male gender (P = 0.0001), increasing age (P = 0.006), alcohol consumption (P = 0.0001), steatosis (P = 0.03), hepatic iron concentration (P < 0.0001) and the presence of hepatic fibrosis. Of the candidate gene polymorphisms studied, none showed a significant association with hepatic fibrosis in univariate or multivariate analysis incorporating cofactors. We also specifically studied patients with hepatic iron loading above threshold levels for cirrhosis and compared the genetic polymorphisms between those with no fibrosis *vs* cirrhosis however there was no significant effect from any of the candidate genes studied. Importantly, in this large, well characterised cohort of patients there was no association between SNPs for TGF-β or TLR4 and the presence of fibrosis, cirrhosis or increasing fibrosis stage in multivariate analysis.

CONCLUSION: In our large, well characterised group

of haemochromatosis subjects we did not demonstrate any relationship between candidate gene polymorphisms and hepatic fibrosis or cirrhosis.

Key words: Haemochromatosis; Genetic polymorphism; Liver fibrosis; Toll-like receptor 4; Interleukin 10; Monocyte chemoattractant protein 1; Chemokine (C-C motif) ligand 2; Transforming growth factor beta; 8-oxoguanine DNA glycosylase

Core tip: This study does not support the previously proposed role of mutations in both toll-like receptor 4, transforming growth factor-beta in the progression of hepatic fibrosis associated with hereditary haemochromatosis.

Wood MJ, Powell LW, Dixon JL, Subramaniam VN, Ramm GA. Transforming growth factor-β and toll-like receptor-4 polymorphisms are not associated with fibrosis in haemochromatosis. *World J Gastroenterol* 2013; 19(48): 9366-9376 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i48/9366.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i48.9366

INTRODUCTION

It is generally believed that genetic factors may influence the progression of hepatic fibrosis in chronic liver disease of differing aetiologies. Many case-control studies have been performed in an attempt to elucidate these genetic influences, however, results have been inconsistent. Possible explanations for this include relatively small sample sizes and the difficulties in controlling for factors such as the duration of hepatic insult (*e.g.*, in chronic hepatitis C virus infection and disease co-morbidities (*e.g.*, alcohol)[1]. In hereditary haemochromatosis iron accumulation begins in early adulthood in males and despite similarity in the age of onset, there is a highly variable disease progression both in iron loading and in hepatic fibrosis progression[2-6]. It is likely that genetic factors play a role in influencing both iron accumulation and the development of cirrhosis[7]. The aim of this study was to explore potential genetic polymorphisms involved in hepatic disease progression in haemochromatosis with particular attention to candidate molecules associated with the processes of hepatic fibrogenesis. This study was conducted using a well-characterised cohort of patients with HFE-associated hereditary haemochromatosis with known fibrosis stage and quantitative hepatic iron loading.

Candidate genes for analysis were chosen based either on their existing association between gene mutations and fibrogenesis in other disease aetiologies, or their demonstrated role in hepatic stellate cell biology and hepatic injury/fibrosis. Candidate genes included: (1) molecules associated with hepatic inflammation including monocyte chemoattractant protein 1 (MCP-1), the MCP-1 receptor, chemokine C-C motif receptor 2 and interleukin-10 (IL10); and (2) mediators of hepatic injury/inflammation/fibrosis including transforming growth factor-beta (TGF-β), toll-like receptor 4 (TLR4) and human 8-oxoguanine DNA glycosylase (hOGG1).

MCP-1, also known as chemokine (C-C motif) ligand 2, is a cytokine belonging to the CC chemokine family which acts as a potent inducer of monocyte, macrophage and hepatic stellate cell migration[8-11] and is involved in the early stages of hepatic inflammation and fibrogenesis[9]. Several clinical studies have shown an association between single nucleotide polymorphisms (SNPs) in the *MCP-1* gene and fibrosis in various organs including liver, kidney, and skin[12-14] although others have shown conflicting results. The MCP-1 receptor, CCR2, mediates much of chemokine response of MCP-1[15]. While the precise role of CCR2 in human liver disease is relatively unknown, studies investigating CCR2 variants in alcoholic liver disease or liver carcinoma have produced varying results[16,17]. IL10 acts as an anti-inflammatory cytokine[18] in different forms of human chronic liver disease, regulating inflammatory and fibrogenic responses (reviewed in[19]). In haemochromatosis, hepatic IL10 mRNA expression is decreased[20].

TGF-β has been described as a "master switch" in hepatic fibrosis due to its central role in the activation of hepatic stellate cells and the production of fibrillar collagen *via* Smad[21]. SNPs in the *TGF-β* gene have previously been studied in a relatively small cohort of subjects with haemochromatosis where results suggested that the presence of the proline substitution (C) may accelerate hepatic fibrosis[22]. TLRs are a group of receptors involved in both the recognition of pathogens and in mediating non-infectious and ischaemic causes of liver injury[23,24]. TLR4 has been shown to be associated with signalling leading to hepatic fibrosis, with hepatic stellate cells being the main direct mediator promoting fibrogenesis *via* TGF-β signalling[25]. Studies designed to assess the role of *TLR4* polymorphisms in the susceptibility to inflammatory or infectious diseases have provided conflicting results[26,27]. In haemochromatosis, however, one study has shown that a *TLR4* polymorphism was associated with clinical disease without any notable effect on iron loading[28], although again this was conducted in a relatively small cohort of patients. hOGG1 is an enzyme responsible for repairing the 8-oxo-7,8-dihydroguanine 8 lesion of DNA subjected to oxidative stress. Although oxidative stress is one of the common mechanisms for hepatocyte injury and an inflammatory cascade, particularly in iron loading, to our knowledge no studies have considered the role of *OGG1* gene polymorphisms in the progression of liver damage.

Few studies have assessed the contribution of polymorphisms in genes associated with hepatic injury and fibrosis in the phenotypic disease expression in haemochromatosis. Those that have were limited by both

cohort size and the lack of well characterised patients with liver biopsy-proven fibrosis staging and quantitative hepatic iron loading. This study represents one of the largest cohorts of patients with haemochromatosis to be assessed for the role of SNPs associated with hepatic disease expression.

MATERIALS AND METHODS

Ethics statement

All subjects in this study provided written informed consent and the study was approved by the Human Research Ethics Committees of the Royal Brisbane and Women's Hospital (RBWH) and the Queensland Institute of Medical Research (QIMR), Brisbane, Australia. Written informed consent was witnessed and documented in each patient hospital file, a procedure approved by both ethics committees.

Study subjects

The subjects in this study were derived from the Haemochromatosis Database at the QIMR. This is a cohort recruited over approximately 30 years from clinical review at the RBWH. The database lists more than 2400 patients of whom 722 are C282Y homozygous. The database includes clinical and laboratory data, with DNA available from a subset of these patients.

Inclusion criteria for selection for this study were: (1) genetic testing confirming C282Y homozygosity; (2) patients had previously undergone a liver biopsy for clinical indications and information was available with respect to iron loading and fibrosis stage; and (3) patients had previously provided blood for the extraction of DNA from peripheral white cells or were available to do so prospectively.

Patients were excluded from this study if aged less than 16 years at the time of liver biopsy as iron loading at this age may indicate the presence of other mutations in iron homeostatic genes. Patients with viral hepatitis were excluded. Excessive alcohol consumption was not an exclusion criterion however this information was included in the data collection.

Control subjects were obtained from the QIMR DNA bank and represented a selection of healthy subjects who had previously provided peripheral blood for the extraction of DNA. Those with European ethnicity were preferentially utilised for this study in order to provide a genetically comparable group for the study subjects. Allele frequencies were also compared to subjects in the International HapMap project selected from United States residents with Northern and Western European ancestry[29].

Rationale for candidate gene analysis

While this cohort of haemochromatosis patients is one of the largest and most well characterised studied to date, investigation using genome wide association in a cohort of this size was not considered viable, thus we used a candidate gene approach.

MCP-1 and CCR2: Human MCP-1 production is regulated in part by a region 1.8 to 2.7 kb upstream of the transcriptional start site and a polymorphism at position -2518 (G/A rs1024611) affects the transcriptional activity[30]. Individuals with a G allele at this site (G/A or G/G) produce more MCP-1 from monocytes in response to stimulation with interleukin-1β[30]. A variant in the *CCR2* gene Val64Ile (A/G rs1799864) has been shown to be associated with a delay in progression in human immunodeficiency virus[31] and other studies have shown a possible role for this polymorphism in inflammatory conditions such as sarcoidosis and atherosclerosis[32,33]. Thus, the MCP1-2518 and CCR2-190 SNPs were chosen for study.

IL10: The promoter region of the *IL10* gene has several polymorphisms at positions -1082 (G/A: rs1800896), -819 (C/T: rs1800871) and -592(C/A: rs1800872) with only three haplotypes found in Caucasian populations: GCC, ACC and ATA[34]. Heritability factors are thought to account for some of the variability in IL10 production although environmental influences are also important[35,36]. The role of specific SNPs in differential IL10 production is controversial[37-40]. The GCC promoter haplotype may have greater transcriptional activity compared to the ATA and ACC haplotypes[35,41] and this would be consistent with other studies showing decreased IL10 production in those with the -1082A genotype[34,39,42]. Most studies considering the relationship between *IL10* promoter polymorphisms and liver fibrosis in HCV infected patients have failed to show statistically meaningful effects although many of these have suffered from small sample sizes (reviewed in[43]). Therefore, although it seems attractive to consider IL10 as a mediator of hepatic inflammation and fibrosis, promoter polymorphisms in this gene have not been unambiguously linked with either cytokine production or hepatic fibrosis. For this study, IL10-1082 and IL10-592 SNPs were selected for investigation.

TGF-β: A single nucleotide polymorphism in the *TGF-β* gene at position -915, codon 25(G/C: rs1800471) results in an amino acid substitution of proline for arginine. Leukocytes from those homozygous for the arginine (G) molecule at this site appear to produce more TGF-β in response to stimuli suggesting this is the "high producing" genotype[44]. This report has been challenged by other groups who have suggested that there may be important differences between total TGF-β secretion and bioavailable forms[45,46]. The TGFβ-915 SNP was used in this evaluation of disease expression susceptibility in haemochromatosis.

TLR4: Two common missense mutations in the *TLR4* gene have been suggested to have functional significance with defective signalling resulting. Aspartic acid substi-

Table 1 Gene specific primers for polymerase chain reaction amplification and dissociation analysis

Gene	Forward primer	Reverse primer
MCP1-2518	TTTCTTGACAGAGCAGAAGTGGGAG	TTGCTGGCTGAGTGTTCACATAGG
CCR2 190	ATACCAACGAGAGCGGTGAAGAAG	AAAGCAGATCAGAGATGGCCAGG
IL10-592	AAAGGAGCCTGGAACACATCCTGT	AAAGTTCCCAAGCAGCCCTTCCAT
IL10-1082	TCCAAGACAACACTACTAAGGCTTC	GCTGGATAGGAGGTCCCTTACTTT
TGF-β	CTACCGCTGCTGTGGCTACTGGT	TCACCAGCTCCATGTCGATAGTCT
TLR4-299	CCGATTAGCATACTTAGACTACTACCTC	CCTTTCAATAGTCACACTCACCAGG
TLR4-399	GCTTGAGTTTCAAAGGTTGCTGTTCTC	GCCCAAGAAGTTTGAACTCATGGTAA
hOGG1	ACCCTCCTACAGGTGCTGTTCAGT	CCTTTGGAACCCTTTCTGCGCTTT

MCP-1: Monocyte chemoattractant protein 1; CCR-2: Chemokine C-C motif receptor; IL10: Interleukin-10; TGF-β: Transforming growth factor-beta; TLR4: Toll-like receptor 4; hOGG1: Human 8-oxoguanine DNA glycosylase.

Table 2 Polymerase chain reaction profiles for candidate gene high resolution melt analysis

Gene	Annealing temperature	Extension temperature	Melt analysis range
MCP-1	58 ℃	72 ℃	74 to 84 ℃
CCR-2	58 ℃	72 ℃	74 to 84 ℃
IL10-592	57 ℃	72 ℃	75 to 85 ℃
IL10-1082	59 ℃	72 ℃	70 to 81 ℃
TGF-β	65 ℃ (20 s)	72 ℃ (20 s)	79 to 89 ℃
TLR4 299	57 ℃	68 ℃	66 to 78 ℃
TLR4 399	58 ℃	70 ℃	69 to 81 ℃
hOGG1	57 ℃	68 ℃	78 to 88 ℃

MCP-1: Monocyte chemoattractant protein 1; *CCR-2*: Chemokine C-C motif receptor; *IL10*: Interleukin-10; *TGF-β*: Transforming growth factor-beta; *TLR4*: Toll-like receptor 4; *hOGG1*: Human 8-oxoguanine DNA glycosylase.

tuted for glycine at amino acid position 299 (Asp299Gly) (A/G: rs4986790) and 399 (Thr399Ile) (C/T: rs4986791) are said to induce hypo-responsiveness to lipopolysaccharide although this has not been supported by all investigations[47-49]. The CC variant of the Thr399Ile polymorphism was one SNP included in a panel of tests demonstrating discrimination of patients with advanced fibrosis in chronic hepatitis C[50]. Functional studies have shown that the polymorphisms in TLR4 which confer protection from hepatic fibrosis are associated with a reduced threshold for HSC apoptosis and attenuation of fibrogenic responses stimulated by MCP-1, BAMBI and IL6[51]. Both TLR4-299 and TLR4-399 SNPs were assessed in the present study.

OGG1: It can be shown on paraffin sections of diseased liver that there is increased staining of 8-oxodG consistent with oxidative DNA damage to hepatocytes and this does not appear to be specific to any particular type of liver disease[52]. A polymorphism exists in the human *OGG1* gene leading to Ser326Cys conversion and affecting the function of this glycosylase due to changes in localization and phosphorylation (C/G: rs1052133)[53]. There is some epidemiological evidence to suggest that certain tumours may have an increased prevalence in those with the variant of OGG1[54]. Functional studies have shown that individuals with the GG genotype in Ser326Cys have a reduced capacity for repair of DNA damage compared to wild types or heterozygous subjects[55]. Thus OGG1-326 was included for evaluation in this study.

SNP mutation analysis, real-time PCR and sequencing

Patients provided blood samples, often at the time of therapeutic venesection to allow DNA extraction from buffy coats using a high salt extraction method. DNA was utilised in all PCR experiments at a concentration of 25 ng/μL. Primers were designed using Genbank to obtain genetic sequences adjacent to the area of interest and using specific software (Primer Quest) to optimise the temperature difference for denaturing and minimise secondary structures (Table 1). Specificity was tested using specific software (Blast). Primers were purchased from Sigma-Aldrich Pty Ltd (NSW, Australia).

High resolution melt (HRM) analysis (Corbett Life Science Rotor-Gene™ 6000 HRM) was used to evaluate nucleotide sequences based on the dissociation profile of the fragment of DNA containing the polymorphism when amplification of the template occurs in the presence of specific dye. Sensimix HRM™ (Quantace, London) was used in PCR reactions with the following volumes for 1 reaction to give a total reaction volume of 25 μL. Each gene was amplified and analysed under optimised conditions (Table 2).

From initial HRM analysis, subjects with variable dissociation characteristics were selected for sequencing in order to confirm the presence of the suspected gene polymorphism. Once identified, a sample from these subjects was used in each experiment to provide a positive control. Any sample that could not be genotyped with HRM analysis was subjected to sequencing. Each patient sample was tested in duplicate. All experiments contained negative control samples (H_2O) to confirm the absence of contamination by PCR product. All patient and control sample identities were number coded for inclusion in experiments.

Sequencing was performed with ABI BigDye Version 3.1 according to specific instructions and using primers designed to incorporate the area of interest.

Table 3 Patient characteristics grouped according to the presence or absence of hepatic fibrosis (univariate analysis) *n* (%)

Parameter	No fibrosis (*n* = 136)	Fibrosis present (*n* = 109)	*P* value
Male gender	72 (53)	89 (82)	0.0001
Age at biopsy (yr) (mean ± SD)	39.7 (14.9)	44.3 (12.4)	0.006
Alcohol (g/d) (median: range)	5 (0-120)	20 (0-200)	0.0001
Steatosis present[1]	36 (37)	42 (53)	0.03
Serum ferritin (μg/L) (median: range)	531 (33-3000)	2134 (155-6000)	0.0001
HIC (μmol/g *dw*) (median: range)	124 (20-537)	218 (43-847)	< 0.0001
Iron grade			
0	1 (1)	0	
1	5 (4)	0	
2	27 (20)	7 (6)	
3	63 (46)	25 (23)	
4	40 (29)	77 (71)	< 0.0001

[1]Steatosis data available for 176 subjects (72% of total cohort).

Table 4 Allele frequency in candidate genes assessed in patient and control groups by high resolution melt analysis: Compared to published data for Caucasian ethnicity populations

Gene	Patient group	Control group	Published results[1]
MCP1	A 0.729	A 0.736	A 0.695
	G 0.271	G 0.264	G0.305
CCR2	G 0.892	G 0.976	G 0.892
	A 0.108	A 0.024	A 0.108
TGFβ	G 0.918	G 0.892	G 0.887
	C 0.082	C 0.108	C 0.113
hOGG1	C 0.695	C 0.694	C 0.776
	G 0.305	G 0.306	G 0.224
IL10-1082	G 0.539	G 0.500	G0.531
	A 0.461	A 0.500	A 0.469
IL10-592	C 0.794	C 0.794	C 0.792
	A 0.206	A 0.206	A 0.208
TLR4 299	A 0.934	A 0.930	A 0.967
	G 0.066	G 0.070	G 0.033
TLR4 399	C 0.934	C 0.931	C 0.955
	T 0.066	T 0.069	T 0.045

[1]NCBI dbSNP (HapMap CEU-Utah residents with Northern and Western European ancestry from the CEPH collection). *MCP-1*: Monocyte chemoattractant protein 1; *CCR-2*: Chemokine C-C motif receptor; *IL10*: Interleukin-10; *TGF-β*: Transforming growth factor-beta; *TLR4*: Toll-like receptor 4; *hOGG1*: Human 8-oxoguanine DNA glycosylase.

Statistical analysis

Normally distributed data were summarized by mean and standard deviation and differences tested by Student's *t* test. Non-parametric data were summarized by median and range and tested by Mann-Whitney or Kruskal-Wallis test. Categorical variables were summarized by frequencies and tested by Pearson χ^2 or Fisher's Exact test. Ordinal multivariate logistic regression analysis was performed using increasing hepatic fibrosis grade as the outcome variable incorporating predictor variables including age, gender, alcohol consumption and the genetic polymorphism in question. Results are presented as OR with 95%CI. *P* values of < 0.05 or less were considered significant. Polymorphisms were grouped in both possible ways for testing but reporting has been limited to that commonly described in the literature. Grouping for the outcome variable of hepatic fibrosis was done in several ways including "no fibrosis (F0) *vs* any hepatic fibrosis (F1-4)", "minimal fibrosis (F0-2) *vs* advanced fibrosis (F3-4)" and as an ordered logistic regression analysis with increasing hepatic fibrosis grade as the outcome variable. In order to consider those patients with the greatest difference in clinical outcome the analysis was repeated incorporating only those patients with heaviest iron loading (iron grade 3 and 4) and comparing those with no fibrosis (F0) against those with advanced fibrosis (F3/F4). Stata/IC software (version 10.1; StataCorp LP, College Station, TX) was used for statistical analysis. Deviation from Hardy-Weinberg equilibrium was tested for each gene using an on-line calculation tool (http://www.oege.org/software/hwe-mr-calc.shtml).

RESULTS

Of the 245 C282Y homozygous patients included in this study, 161 (66%) were male. The majority of biopsies performed in these patients (82%) were done so prior to 1996 when the *HFE* gene was cloned and therefore many patients are likely to have been biopsied for diagnostic purposes. Fibrosis stages were as follows; F0: 136 (56%), F1: 26 (11%), F2: 23 (9%), F3: 13 (5%), F4: 47 (19%). The demographic, laboratory and histological characteristics of the patients with and without hepatic fibrosis are summarized in Table 3. Of those with the grade of steatosis reported, 56% had no steatosis present and 12% had grade 2 or 3 steatosis. Male gender, age, alcohol consumption, steatosis and iron indices from serum and liver sections all showed significant associations with the presence of hepatic fibrosis, as has been previously demonstrated[56,57].

Allele frequencies for the genes of interest were assessed in patient and control populations and these results compared to published data for Caucasian groups (Table 4). There was a significant difference in allele frequencies for the *CCR2* gene polymorphism when comparing the haemochromatosis and control populations ($P = 0.001$). This appeared to be due to an unexpectedly low number of heterozygous and A allele homozygous control subjects. When the patient population was compared to data published from the International HapMap Project there was no significant difference ($P = 0.985$). For all other genes there were no significant differences in allele frequencies between patient and control populations.

No patient or control subject was identified to have the uncommon homozygous polymorphism (Pro/Pro or C/C) in the *TGF*-β gene but as this is present in low frequency in the population this is not an unexpected finding. For the analyses of this polymorphism, Arg/Arg (G allele homozygosity) was compared to Arg/Pro (G/C).

Table 5 Genetic polymorphisms in Hereditary Haemochromatosis patients grouped according the presence or absence of advanced fibrosis and subjected to univariate analysis *n* (%)

Gene	No/minimal fibrosis (F0-2)	Advanced fibrosis (F3-4)	*P* value
MCP1			
AA	99 (53.5)	28 (46.7)	
AG	76 (41.1)	27 (45.0)	
GG	10 (5.4)	5 (8.3)	0.546
CCR2			
GG	142 (78.0)	50 (84.8)	
AG	37 (20.3)	9 (15.3)	
AA	3 (1.7)	0 (0)	0.401
TGFβ			
GG	154 (83.2)	51 (85)	
GC	31 (16.8)	9 (15)	0.749
hOGG1			
CC	99 (53.8)	26 (43.3)	
CG	64 (34.8)	25 (41.7)	
GG	21 (11.4)	9 (15)	0.352
IL10-1082			
GG	50 (27.1)	17 (28.3)	
GA	97 (52.4)	33 (55.0)	
AA	38 (20.5)	10 (16.7)	0.806
IL10-592			
CC	120 (64.9)	37 (61.7)	
AC	58 (31.3)	17 (28.3)	
AA	7 (3.8)	6 (10.0)	0.173
TLR4 299			
AA	159 (86.4)	54 (91.5)	
AG	24 (13.0)	4 (6.8)	
GG	1 (0.6)	1 (1.7)	0.305
TLR4 399			
CC	161 (87.0)	53 (89.8)	
CT	23 (12.4)	5 (8.5)	
TT	1 (0.5)	1 (1.7)	0.502

MCP-1: Monocyte chemoattractant protein 1; *CCR-2*: Chemokine C-C motif receptor; *IL10*: Interleukin-10; *TGF-β*: Transforming growth factor-beta; *TLR4*: Toll-like receptor 4; *hOGG1*: Human 8-oxoguanine DNA glycosylase.

All genes showed no deviation from Hardy-Weinberg equilibrium in the haemochromatosis patient population except the *OGG1* polymorphism ($P = 0.03$). This may relate to selective pressure in this group. The control population showed no deviation from Hardy-Weinberg equilibrium.

No significant associations were present between the polymorphisms of any of the candidate genes and fibrosis stage when the patients were grouped into those with minimal or no fibrosis (F0-2) and compared with those having severe fibrosis (F3-4) (Table 5). Each candidate gene was assessed in ordered logistic regression analysis incorporating increasing fibrosis stage as outcome before and after adjustment for age, gender, iron loading and alcohol consumption and in each analysis, there was no statistically significant effect from the SNP of interest on fibrosis stage (Table 6). Analyses were repeated incorporating data on steatosis grade however this produced no statistically significant effect. Iron loading, alcohol consumption, male gender and age remained important in multivariate analyses as previously reported[2,4-6,58-62].

Table 6 Multivariate ordered logistic regression analysis determining role of genetic polymorphisms in increasing hepatic fibrosis stage

Gene	OR	95%CI	*P* value	Adjusted *P* value[1]
MCP1	1.11	0.69-1.80	0.660	0.681
CCR2	0.71	0.36-1.32	0.284	0.432
TGFβ	1.03	0.54-1.95	0.936	0.740
hOGG1	0.94	0.44-1.98	0.864	0.588
IL10-1082	1.10	0.65-1.88	0.720	0.897
IL10-592	0.82	0.50-1.35	0.441	0.639
TLR4 299	0.73	0.35-1.53	0.403	0.745
TLR4 399	0.87	0.42-1.81	0.706	0.990

[1]Adjusted for age at biopsy: gender: iron grade and alcohol consumption. *MCP-1*: Monocyte chemoattractant protein 1; *CCR-2*: Chemokine C-C motif receptor; *IL10*: Interleukin-10; *TGF-β*: Transforming growth factor-beta; *TLR4*: Toll-like receptor 4; *hOGG1*: Human 8-oxoguanine DNA glycosylase.

After considering only those patients with heaviest grades of iron loading (iron grade 3 and 4) there was no significant association with hepatic fibrosis (F0 *vs* F3/F4) and any of the genetic polymorphisms studied when assessed in univariate or multivariate analysis (after adjustment for age, gender and alcohol consumption) (Table 7). A threshold hepatic iron concentration for cirrhosis of 236 μmol/g dry weight has previously been identified in this haemochromatotic patient cohort[59]; this cut off was used to isolate those with the greatest risk of hepatic fibrosis and subjects with no fibrosis (F0) were again compared to those with advanced disease (F3/F4) (Table 8). Age and alcohol consumption were important in disease progression in this group but no difference was seen for any of the genetic polymorphisms studied. Gender was not a significant risk factor in this cohort with very heavy iron stores.

DISCUSSION

Studies investigating the clinical penetrance of haemochromatosis have consistently identified the severity of iron loading, male gender and alcohol consumption as being crucial factors in determining the risk of liver fibrosis (reviewed in[7]). It is clear that steatosis accelerates the hepatic injury[57] and that diabetes is a risk factor for advanced fibrosis[56]. Despite these known risk factors, family studies have suggested a clustering of phenotypes that may indicate a role for genetic disease modifiers quite separate to those influencing iron loading. Relatively few studies have explored this area in haemochromatosis subjects, particularly when compared to a large body of literature that exists with respect to viral hepatitis.

Many candidate gene studies have suffered from methodological flaws which increase the risk of misleading results. Perhaps the most common scenario is a small subject group which allows the finding of a false positive result due to chance. Although haemochromatosis is relatively common in terms of genetic diseases, its expression remains uncommon in the general popula-

Table 7 Genetic polymorphism frequencies in patients with heavy iron loading (Grade 3 and 4) grouped according to no fibrosis (F0) *vs* advanced fibrosis (F3/F4)

Gene	No fibrosis (F0) (n = 103)		Advanced fibrosis (F3/4) (n = 60)		*P* value	Adjusted *P* value[1]
MCP1	AA 53/103 51.50%	AG/GG 50/103 48.5%	AA 28/60 46.70%	AG/GG 32/60 53.3%	0.555	0.433
CCR2	GG 81/100 81%	AG/AA 19/100 19%	GG 50/59 84.70%	AG/AA 9/59 15.3%	0.549	0.277
TGF β	GG 86/103 83.50%	CG/CC 17/103 16.5%	GG 51/60 85%	CG/CC 9/60 15%	0.800	0.968
hOGG1	CC 54/102 52.90%	CG/GG 48/102 47.1%	CC 26/60 43.30%	CG/GG 34/60 56.7%	0.238	0.790
IL10-1082	AA/AG 75/103 72.8%	GG 28/103 27.20%	AA/AG 43/60 71.80%	GG 17/60 28.30%	0.874	0.998
IL10-592	AA/AC 34/103 33.0%	CC 66/103 67.0%	AA/AC 23/60 38.3%	CC 37/60 61.7%	0.492	0.659
TLR4 299	AA 87/102 85.3%	AG/GG 15/102 14.7%	AA 54/59 91.5%	AG/GG 5/59 8.5%	0.248	0.848
TLR4 399	CC 89/103 86.4%	CT/TT 14/103 13.6%	CC 53/59 89.8%	CT/TT 6/59 10.2%	0.524	0.985

[1]Adjusted for age:gender:alcohol consumption. *MCP-1*: Monocyte chemoattractant protein 1; *CCR-2*: Chemokine C-C motif receptor; *IL10*: Interleukin-10; *TGF-β*: Transforming growth factor-beta; *TLR4*: Toll-like receptor 4; *hOGG1*: Human 8-oxoguanine DNA glycosylase.

Table 8 Logistic regression analysis performed in subjects with HIC > 236 μmol/g dw and comparing outcome of F0 *vs* F3/4 (univariate analysis) (n = 47)

Factor	OR	95%CI	*P* value
Age	1.07	1.00-1.15	0.034
Alcohol	1.03	1.01-1.05	0.011
Female gender	0.27	0.05-1.55	0.140
MCP1	1.20	0.37-3.87	0.76
CCR2	0.74	0.13-4.12	0.73
TGFβ	0.58	0.09-3.52	0.553
hOGG1	1.50	0.47-4.77	0.492
IL10-1082	1.89	0.48-7.44	0.363
IL10-592	0.98	0.30-3.21	0.980
TLR4 299	1.17	0.77-7.79	0.868
TLR4 399	1.24	0.19-8.19	0.824

MCP-1: Monocyte chemoattractant protein 1; CCR-2: Chemokine C-C motif receptor; IL10: interleukin-10; TGF-β: Transforming growth factor-beta; TLR4: Toll-like receptor 4; hOGG1: Human 8-oxoguanine DNA glycosylase.

tion. Added to this is the fact that many patients have no indication, or wish to undergo a liver biopsy, it is clear that establishing a sizable cohort of subjects for study is difficult. Our group of 245 C282Y homozygous patients not only had liver biopsy data available but had also provided blood samples for extraction and storage of DNA. This is likely to represent one of the largest groups in the international literature and represents a recruitment period spanning decades, including the pre-*HFE* era when liver biopsies were used for diagnosis of the disease. This allowed inclusion of patients with early and late stage disease thus avoiding a recruitment bias. In order to allow conclusions regarding the role of genetic polymorphisms, data are also needed about other factors. We have included information from this group relating to iron grade, age, alcohol and gender and used this information to perform multivariate analyses considering gene-environment interactions.

We selected biologically plausible genes for analysis of functionally significant polymorphisms based on known mechanisms of hepatic injury and liver fibrosis and considering the results of previous studies. This approach has been used to describe the role of SNPs in many polygenic diseases although it does risk returning a null result. Genome wide association studies have since become an alternative approach which removes any need for a mechanistic approach to gene selection however these studies require very large patient cohorts in order to describe associations with a small effect on disease.

We did not find a role for the single nucleotide polymorphisms studied in genes coding *MCP-1*, *TGF*-β, *IL10*, *OGG1*, *TLR4* or *CCR2* when considered in univariate analysis with fibrosis stage or when incorporated in multivariate analysis including gender, iron loading, alcohol consumption and age. Genotyping was performed using high resolution melt analysis with confirmation of grouping controls with sequencing. Data were considered in several different analyses with the outcome variables of liver fibrosis grouped as being either present or absent, minimal or advanced and finally as an ordered logistic regression approach (F0-F4). Likewise, the predictor variables (genetic polymorphisms) were considered in all possible combinations but none proved to have a statistically significant association with the outcome variable. We particularly examined those patients where there is greatest variability in liver fibrosis despite significant iron loading. We used the previously published HIC threshold for cirrhosis in this cohort, *i.e.*, HIC > 236 μmol/g dry weight[59], and compared the groups with no fibrosis (F0) against those with advanced fibrosis (F3/F4) who had this significant level of hepatic iron deposition. We hypothesized that these patients are most likely to have other factors accounting for disease progression; however, it was still evident that there was no significant

effect seen in any of the genes tested. In this subgroup analysis of patients with significant iron loading, it is apparent that alcohol consumption and age remain the important determinants of liver disease and this is consistent with previous studies. Although our overall study size is large, it must be noted that this subgroup with very heavy iron loading is much smaller and it is possible that this accounts for the lack of association.

Our results are not consistent with previous studies which have been performed in smaller cohorts. A European study reported that the TLR4 Asp299Gly gene polymorphism modulated phenotypic expression of haemochromatosis and described the effect on both liver histology and on an amalgamated clinical expression including liver disease, arthropathy, joint disease, cardiomyopathy and endocrine disease[28]. The grouping of such diverse types of clinical expression is unusual when considering candidate gene testing and one may wonder whether this is biologically plausible. This study included 99 patients but of these, only 52 had histology available and 29 of these had liver iron quantification. Although allele frequencies were similar to our cohort, we did not replicate the SNP association with liver disease in a group almost five times larger and suspect that the European results may represent a type 1 error.

Similarly, a previous investigation into the role of TGF-β mutations in 149 biopsied haemochromatosis patients concluded that those with the proline substitution at codon 25 were more likely to be grouped into an outcome variable of cirrhosis (F4) *vs* all other stages (F0-3). This grouping could be considered somewhat arbitrary and it would be interesting to know the genotype frequencies across other fibrosis stages. A recent meta-analysis considering the role of *TGF-β* polymorphisms in liver disease (mainly viral hepatitis) concluded a lack of effect upon fibrosis progression which is in keeping with our results[63].

There are substantial difficulties in studying disorders with polygenic and environmental interactions. Future research directions may involve non-targeted analysis of either whole genomes or exomes but this is likely to require greater numbers of subjects who have been well characterised in terms of liver disease and co-factors. Collaborations between research institutions would allow more patients to be involved in such studies but newer non-invasive tests of liver fibrosis such as transient elastography will allow assessment for liver disease in almost all patients and capture a much greater proportion of C282Y homozygous subjects in such studies.

In conclusion, the role of chemokines, chemokine receptors, oxidative stress and inflammatory mediators in fibrogenesis in haemochromatosis is established; however, the influence of genetic polymorphisms in the molecules studied here is less clear. In contrast to other published associations, in our large, well-characterised group of C282Y homozygous subjects we did not demonstrate any relationship between *MCP1*, *CCR2*, *TGFβ*, *IL10*, *OGG1* and *TLR4* single nucleotide polymorphisms and hepatic fibrosis or cirrhosis. Future studies utilising techniques such as exome sequencing may provide a better approach to identify genetic polymorphisms associated with hepatic fibrosis progression in haemochromatosis.

COMMENTS

Background

Hereditary haemochromatosis can lead to liver fibrosis and cirrhosis however not all patients with iron loading develop this complication. It is thought that genetic polymorphisms influence this process however previously reported studies may have had methodological flaws.

Research frontiers

Factors associated with an increased risk of hepatic fibrogenesis have been the subject of investigation and many of the clinical cofactors are now established. Genetic factors have proven more difficult to determine although international collaborations investigating this area are ongoing. This is one of the largest cohorts of C282Y homozygous patients studied in this field.

Innovations and breakthroughs

The cohort studied in this paper is large and carefully characterised which allows us to accurately test the relationship between genetic polymorphisms, cofactors for liver injury and fibrosis stage. Authors have tested polymorphisms in molecules related to inflammation and hepatic fibrogenesis and found no significant relationship which is in contrast to previous papers. Their work suggests that further well designed studies are needed to determine what genetic factors influence fibrosis in iron loading.

Applications

By understanding the molecular differences between patients who develop progressive liver disease and those who don't, they may eventually be able to develop therapeutic targets to modify disease development. Patients could also expect more personalised prognostic information and this may allow better informed treatment decisions.

Terminology

Hereditary haemochromatosis due to homozygosity in the C282Y substitution in *HFE* is a genetic disorder seen in those of Northern European ancestry. It is one of the most common genetic disorders in this population and can lead to heavy iron loading in the liver and liver scarring (cirrhosis). The molecules studied in this paper are thought to be involved in mediating inflammation, signalling to fibrosis-producing cells or repair of oxidative stress within the liver.

Peer review

The manuscript reports the lack of association between hepatic fibrosis risk and polymorphisms in the genes encoding toll-like receptor 4, transforming growth factor-beta, in a relatively large cohort of hemochromatosis patients. These results are not consistent with previous findings, which were obtained with error prone smaller cohorts of patients. The methodology employed here is appropriate and the paper is well written. The discussion puts the negative findings into context. The conclusions will be of interest to researchers and clinicians in the field of gastroenterology.

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BRIEF ARTICLE

Efficacy and safety of tenofovir disoproxil fumarate in pregnancy for the prevention of vertical transmission of HBV infection

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Abstract

AIM: To evaluate the effects of tenofovir disoproxil fumarate (TDF) use during late pregnancy to reduce hepatitis B virus (HBV) transmission in highly viremic mothers.

METHODS: This retrospective study included 45 pregnant patients with hepatitis B e antigen (+) chronic hepatitis B and HBV DNA levels > 10^7 copies/mL who received TDF 300 mg/d from week 18 to 27 of gestation (n = 21). Untreated pregnant patients served as controls (n = 24). All infants received 200 IU of hepatitis B immune globulin (HBIG) within 24 h postpartum and 20 μg of recombinant HBV vaccine at 4, 8, and 24 wk. Perinatal transmission rate was determined by hepatitis B surface antigen and HBV DNA results in infants at week 28.

RESULTS: At week 28, none of the infants of TDF-treated mothers had immunoprophylaxis failure, whereas 2 (8.3 %) of the infants of control mothers had immunoprophylaxis failure (P = 0.022). There were no differences between the groups in terms of adverse events in mothers or congenital deformities, gestational age, height, or weight in infants. At postpartum week 28, significantly more TDF-treated mothers had levels of HBV DNA < 250 copies/mL and normalized alanine aminotransferase compared with controls (62% *vs* none, P < 0.001; 82% *vs* 61%, P = 0.012, respectively).

CONCLUSION: TDF therapy during the second or third trimester reduced perinatal transmission rates of HBV and no adverse events were observed in mothers or infants.

Key words: Hepatitis B; Tenofovir; Reverse transcriptase inhibitors; Vertical transmission; Chronic

Core tip: Tenofovir disoproxil fumarate use during late pregnancy reduced hepatitis B virus transmission in highly viremic hepatitis B e antigen positive mothers.

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INTRODUCTION

Hepatitis B virus (HBV) infection is a important medical problem affecting approximately 2 billion people globally[1]. The vertical transmission of HBV from hepatitis B surface antigen (HBsAg)-positive mothers to their infants at birth or in early infancy has a significant role in the endemicity of HBV infection and causes an increased risk of chronic hepatitis B (CHB)[2]. The prevention of perinatal or vertical transmission is crucial in the control of hepatitis B endemicity. Without immunoprophylaxis > 90% of infants, born to mothers with hepatitis B e antigen (HBeAg), become chronically infected with HBV. In recent years, active and passive immunoprophylaxis of newborns and universal vaccination programs have reduced the transmission rates of HBV[1-4]. It was reported that passive or active immunization within 12 h of birth may lead to the prevention of perinatal transmission of HBV[5]. However, some studies showed that HBV immunoprophylaxis fails in 10%-15% of infants[6,7], mainly as a result of vertical infection[8-11]. A high level of maternal viremia is a significant factor in prophylaxis failure. A positive correlation between high maternal serum HBV DNA levels and an increased risk for vaccination breakthrough was found in these studies[8-11]. These data have introduced the idea of antiviral therapy in pregnant women with a high level of maternal viremia and high maternal serum HBV DNA levels.

Among the oral anti-HBV agents approved by United States Food and Drug Administration (FDA), Tenofovir disoproxil fumarate (TDF) is an effective agent due to its potency and resistance profile[13-15]. TDF is a nucleotide analog which inhibits reverse transcriptase and blocks HBV replication in liver cells[2,16]. In over 600 human immunodeficiency virus (HIV) mono-infected and HIV/HBV co-infected mothers, it was reported that TDF had a favorable efficacy and safety profile[16-18]. However, to our knowledge, there are limited data available in the literature on the safety and efficacy of TDF therapy during pregnancy in highly viremic mothers with chronic hepatitis B and its impact on the perinatal transmission of HBV.

In the current study, we evaluated the efficacy and safety of TDF use during late pregnancy to reduce HBV transmission in highly viremic HBeAg positive mothers.

MATERIALS AND METHODS

Patients

This was a retrospective study conducted in six hopitals in South-east Anatolia, Turkey. A total of 45 pregnant women, who were diagnosed with HBeAg-positive chronic hepatitis B before 12 wk of gestation between February 2010 and January 2012, were included in this study. Twenty-one patients were treated with TDF 300 mg orally once a day (Viread; Gilead Sciences, CA, United States) from week 18 to 27 of gestation (n = 21) and served as the treated-group. Twenty-four untreated pregnant women with active hepatitis B infection served as the control group. The treated patients received TDF until the fourth week after delivery.

Eligibility criteria for inclusion in this study were: (1) pregnant women; (2) positive for serum HBsAg and HBeAg for a period of at least 6 mo; (3) HBV DNA levels ⩾ 7 log10 copies/mL before initiation of TDF; (4) treatment-naive patients; (5) patients without lamivudine resistance; and (6) patients without gestational diabetes, vaginitis, arrhythmia, anemia or proteinuria.

Forty-five pregnant women met all inclusion criteria and were included in the study. Mothers with HIV co-infection, pregnancy complications, or an abnormal sonographic examination were excluded from TDF therapy. Baseline demographic data and virological characteristics (age, race, HBeAg, and history of prior HBV therapy) of the pregnant women were recorded. Blood and urine beta-HCG were tested in all patients).

HBsAg, HBeAg, anti-HBe, HBV DNA, alanine aminotransferase (ALT), aspartate aminotransferase levels, and creatinine level were measured at intervals of 12 wk. Both the mothers and infants were evaluated at periodic intervals during the intrauterine period.

All newborns were evaluated for congenital malformations, hypothyroidism, and phenylketonuria at birth. Infant Apgar score, anthropometry, birth defects, history of immunoprophylaxis, mode of delivery and complications were evaluated and recorded.

HBV DNA was quantified using the Roche COBAS Amplicor HBV monitor assay which has a low limit of detection (LLD) of 500 copies/mL (Roche Molecular Diagnostics, Branchburg, NJ, United States). This assay was later replaced by the Roche COBAS TaqMan HBV Test with a LLD of 50 copies/mL (Roche Molecular Diagnostics). HBV serological markers were detected by enzyme-linked immunosorbent assay kits (Abbott Labs, North Chicago, IL, United States) on an ARCHITECT 2000 full automatic chemiluminescence immunoassay instrument (Abbott Labs, North Chicago, IL, United States) according to the manufacturer's instructions. Hearing screening was tested by Echo Screen (Madsen, Germering, Germany). Heel blood was taken from the infants after 72 h of breastfeeding and then dried blood-spot specimens on filter paper were sent to the laboratory for congenital phenylketonuria and hypothyroidism screening.

According to national and international treatment guidelines, all infants received 200 IU of hepatitis B immune globulin (HBIG, HyperHEP B solvent/detergent treated; Talecris Biotherapeutic, NC, United States) within 24 h postpartum and 20 μg of recombinant HBV (Recombivax HB; Merck Sharp and Dohme, NJ, United States) vaccine (4, 8, and 24 wk). Infants were evaluated in terms of serum HbsAg and HBV DNA levels at postpartum weeks 4-28. Vertical transmission was evaluated by HBsAg testing of infant peripheral blood at 4-28 wk of age.

Table 1 Maternal characteristics of the control group and tenofovir disoproxil fumarate-treated group

Maternal characteristics	Control group (n = 24)	Treated group (n = 21)
Mean age (yr)	26.9 ± 2.9	28.2 ± 4.1
HBV DNA (IU/mL)	8.31 log	8.28 log
ALT levels (U/L)	52 (19-77)	56 (22-71)
Serum creatinine levels (mg/dL)	0.81 (0.6-1.0)	0.79 (0.6-0.98)
Compensated cirrhosis	0 (0%)	2 (10%)

HBV: Hepatitis B virus; ALT: Alanine aminotransferase.

Table 2 Maternal outcomes in the control group and tenofovir disoproxil fumarate-treated group n (%)

Maternal outcomes	Control group (n = 24)	Treated group (n = 21)
HBV DNA < 50 IU/mL	0 (0)	13 (62)
Normalized ALT (U/L)	15 (61)	17 (82)
Elevated creatinine kinase (> 165 mg/dL)	0 (0)	1 (4.7)
Spontaneous abortion	1 (4)	0 (0)
Gestational diabetes	0 (0)	1 (4.7)
Vaginitis	0 (0)	1 (4.7)
Arrhythmia	0 (0)	1 (4.7)
Anemia	0 (0)	1 (4.7)
Proteinuria	1 (4.2)	2 (10)

HBV: Hepatitis B virus; ALT: Alanine aminotransferase.

Ethics

All participants gave their written informed consent and did not receive any compensation for taking part in this study. The study conformed to the standards set by the latest revision of the Declaration of Helsinki and was approved by the Ethical Committee.

Statistical analysis

Statistical analysis was performed using Stata software version 10 (Computer Resource Center, Chicago, IL, United States). Measurement data were expressed as mean ± SD and compared with analysis of variance. Fisher's exact test was used for comparison of transmission rate. $P < 0.05$ was considered statistically significant.

RESULTS

Maternal characteristics

HBV DNA levels were > 2000000 IU/mL (10^7 copies/mL) in all patients (treated-group and control group). The median maternal age was 27.7 ± 3.7 years. Serum creatinine levels were within the normal ranges in all patients. Two patients in the treated-group had compensated cirrhosis (10%) (Table 1).

Maternal outcomes

All the mothers in the treated-group continued to receive therapy during the study period. In the treated-group, all pregnant women delivered, however, one patient in the control group had a spontaneous abortion at week 9.

In the treated-group, gestational diabetes was found in one patient, vaginitis in one patient, arrhythmia in one patient, and anemia in one patient. Three patients (14.3%) had proteinuria in the treated-group. Elevated creatinine kinase (CK) was detected in one (4.7%) patient in the treated-group at week 6, and reached the highest level (341 mg/dL) at week 8. At this time, the patient had no complaints or muscle function loss. Muscle function tests in this patient were normal, and she was diagnosed with asymptomatic CK elevation.

One patient (4.7%) in the treated-group had elevated levels of ALT (258 U/L) at week 7, however, ALT levels were normal at week 11 of treatment.

At postpartum week 28, significantly more TDF-treated mothers had levels of HBV DNA < 50 IU/mL (250 copies/mL) and normalized ALT compared with controls (62% *vs* none, $P < 0.001$; 82% *vs* 61%, $P = 0.012$, respectively). There were no differences in adverse effects in mothers between the groups (Table 2). The treated mothers had no hepatic flares until the fourth week after delivery.

Infant characteristics and outcomes

Atthe 20th gestational week evaluation, no serious complications were observed in the infants of the treated-group, although 3 (14.3%) infants had growth retardation as shown on ultrasound screening. However, these infants did not show growth retardation at week 24th following ultrasound monitoring.

Birth weight was < 2500 g in two (4.7%) newborn. Hypothyroidism, phenylketonuria and congenital hearing loss were not observed in any of the newborn. At 28 wk, none of the infants whose mothers received TDF had immunoprophylaxis failure, whereas 2 (8.3%) of the infants of control mothers had immunoprophylaxis failure (HBsAg positivity was detected) ($P = 0.022$). Anti-HBs levels were < 100 mIU/mL in one (4.7%) of the vaccinated neonates of treated mothers, while levels were > 100 mIU/mL in the remaining (95%) vaccinated neonates of treated patients. No differences in infant congenital deformities, gestational age, height, or weight between the groups were observed (Table 3).

DISCUSSION

In this retrospective study, we report the efficacy and safety of TDF in the prevention of vertical transmission (VT) in pregnant women with high viremia HBV infection[15]. Immunoprophylaxis with immediate HBIG and HBV vaccine after delivery effectively prevented VT in these cases. Failure of immunoprophylaxis is generally caused by high maternal viral load[9,11,15]. It has been clearly demonstrated that there is a correlation between intrauterine serum HBV DNA levels and perinatal transmission of HBV in pregnant women[15]. HBV DNA level was found to be an independent risk factor for failure of immunoprophylaxis in HBeAg-positive mothers with high HBV DNA levels

Table 3 Outcome of infants born to control mothers and tenofovir disoproxil fumarate-treated mothers *n* (%)

Infant characteristics and outcomes	Infants of control group mothers (*n* = 23)	Infants of treated group mothers (*n* = 21)
Birth weight < 2500 g	1 (4.3)	1 (4.7)
Immunophrophylaxis failure	2 (8.3)	0 (0)
Anti-hepatitis B surface levels > 100 mIU/mL	19 (82)	20 (95)
Hypothyroidism	0 (0)	0 (0)
Phenylketonuria	0 (0)	0 (0)
Congenital hearing loss	0 (0)	0 (0)

(≥ 6 log10 copies/mL)[19]. For these reasons we included mothers with high levels of DNA in the present study.

There are many studies on the use of TDF to prevent VT in HIV mono-infected and HIV/HBV coinfected mothers in the literature[17,18]. However, there are limited data on the use of TDF in pregnancy and in the prevention of VT in HBV mono-infected mothers. According to previous studies, early post-natal immunoprophylaxis with antiviral therapy in mothers in the third trimester was safe, well-tolerated, and effectively prevented VT of HBV[2,20]. Among five FDA approved oral anti-HBV agents, TDF and entecavir are the most effective agents due to their resistance profile and potency[13,15]. TDF and telbivudine are classified as category B (no evidence of risk to humans: either animal findings indicate risk, but human findings do not; or, if no adequate human studies have been conducted, animal findings are negative) for use in pregnancy, whereas lamivudine, entecavir, and adefovir are category C (risk cannot be ruled out: human studies are lacking, and animal studies are either positive for fetal risk, or are lacking). However, potential benefits may justify the potential risk) in the FDA drug category for pregnancy[15].

In a randomized, double-blind, placebo-controlled study with lamivudine, it was found that lamivudine therapy during late pregnancy can reduce HBV perinatal transmission in highly viremic mothers. This study demonstrated that infants in the lamivudine + vaccine + HBIG group had a significant decrease in the incidence of HBsAg seropositivity (10/56, 18% *vs* 23/59, 39%, $P = 0.014$) and in detectable HBV DNA (11/56, 20% *vs* 27/59, 46%, $P = 0.003$) compared to infants who received placebo + vaccine + HBIG. The results of this study suggested that lamivudine reduced HBV transmission from highly viremic mothers to their infants following immunization[20]. In another prospective, open-label controlled study evaluating the efficacy and safety of telbivudine use during late pregnancy, a striking decline in HBV DNA levels was seen from treatment onset to week 4, and remained at a low level from week 12. According to this study, 33% of the telbivudine-treated mothers and none of the untreated controls had DNA < 500 copies/mL at delivery and seven months after delivery, and the incidence of perinatal transmission was lower in the infants of telbivudine-treated mothers than in the controls (0% *vs* 8%; $P = 0.002$)[2]. In a case series by Pan *et al*[15], TDF therapy in the third trimester was evaluated in eleven Asian women with HBV. In their uncontrolled study, a significant reduction in serum HBV–DNA was achieved at delivery compared with baseline, and all infants were HBsAg negative 28-36 wk after birth[15]. In our controlled study, at postpartum week 28 significantly more TDF-treated mothers had low levels of HBV DNA and none of the infants of 21 treated mothers had immunoprophylaxis failure. In light of these results, we suggest that TDF use in the third trimester is safe and effectively prevents VT of HBV from high viremic HBeAg-positive mothers.

When the potential benefit of an antiviral-agent is evaluated, adverse effects of that antiviral-agent should also be taken into consideration. These adverse effects include teratogenicity, long-term effects on bone development in the infant, post-treatment ALT flares, and HBV-resistant mutations. Studies have indicated that TDF can cause renal events in HIV patients and patients with preexisting renal disease. However, nephrotoxicity was not observed during a three-year period of TDF use in chronic HBV patients with preserved baseline renal function[21,22]. According to analyzed neonatal safety data from the Antiretroviral Pregnancy Registry (APR), the birth defect prevalence of earliest exposure commencing in the first trimester was 3.1% for lamivudine and 2.4% for TDF; earliest exposure commencing in the second or third trimester was 2.7% for lamivudine and 2.0% for TDF[23]. A meta-analysis of lamivudine in late pregnancy reported that no significant increase in adverse effects or complications in pregnancy was observed[24]. In the large-scale controlled study by Han *et al*[2], no serious adverse events were noted in the telbivudine-treated mothers or their infants. In a Chinese study conducted in eight pregnant HBV women receiving TDF, HBV flares, an increase in creatinine and birth defects were not observed, and all newborn parameters were appropriate for gestational age[25]. Several clinical studies and the APR have stated that anti-viral agents for hepatitis are safe in pregnancy during the second/third trimester[15,23]. The relationship between TDF and fetal growth, particularly bone development is a matter of concern. Studies of pregnant monkeys showed that the use of TDF can cause reduced fetal growth and a reduction in fetal bone porosity within two months of starting maternal therapy[26]. TDF use in HIV-infected children has been reported to result in decreases in bone mineral density[27,28]. However, long-term safety data in infants perinatally exposed to TDF demonstrated no abnormal bone metabolism or growth impairment in these children[29,30]. In the study by Pan *et al*[15], serum creatinine

levels were stable and within the normal range during TDF treatment in all mothers, and they did not encounter any adverse pregnancy outcomes and/or birth defects. Similarly, in the current study we did not observe any differences in adverse events in mothers or infant congenital deformities, gestational age, height, or weight between the groups.

In conclusion, this controlled study revealed that the use of TDF in highly viremic chronic hepatitis B mothers during the second or third trimester of pregnancy reduced the rate of perinatal transmission. Tenofovir disoproxil fumarate produced no adverse events in infants or mothers by 28 wk and is a safe and effective agent in pregnant women with high viremia.

COMMENTS

Background

The vertical transmission of hepatitis B virus (HBV) from hepatitis B surface antigen-positive mothers to their infants at birth or in early infancy has a significant role in the endemicity of HBV infection and causes an increased risk of chronic hepatitis B (CHB).

Research frontiers

Among the oral anti-HBV agents approved by the Food and Drug Administration, tenofovir disoproxil fumarate (TDF) is an effective agent due to its potency and resistance profile. However, there are limited data available in the literature on the safety and efficacy of TDF therapy during pregnancy in highly viremic mothers with CHB and on its impact on the perinatal transmission of HBV. In this study, the authors demonstrated that TDF therapy during the second or third trimester in CHB mothers reduced perinatal transmission rates with no adverse events in mothers and their infants.

Innovations and breakthroughs

This report highlighted the importance of TDF therapy in highly viremic CHB mothers during the second or third trimester to reduce perinatal transmission rates with no adverse events in infants or mothers. This is an important study which shows that TDF can be used in highly viremic mothers. Furthermore, this study suggests that TDF may be used in highly viremic CHB mothers.

Applications

TDF therapy may represent a future strategy for CHB mothers during the second or third trimester.

Terminology

TDF is a nucleotide analog which inhibits reverse transcriptase and blocks HBV replication in liver cells. TDF is a safe and effective agent which can be used during pregnancy in highly viremic mothers with CHB and does not increase perinatal transmission of HBV.

Peer review

The authors studied the influence of TDF use on perinatal transmission of HBV infection. This is an interesting report.

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BRIEF ARTICLE

Study of risk factors for gastric cancer by populational databases analysis

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Abstract

AIM: To study the association between the incidence of gastric cancer and populational exposure to risk/protective factors through an analysis of international databases.

METHODS: Open-access global databases concerning the incidence of gastric cancer and its risk/protective factors were identified through an extensive search on the Web. As its distribution was neither normal nor symmetric, the cancer incidence of each country was categorized according to ranges of percentile distribution. The association of each risk/protective factor with exposure was measured between the extreme ranges of the incidence of gastric cancer (under the 25th percentile and above the 75th percentile) by the use of the Mann-Whitney test, considering a significance level of 0.05.

RESULTS: A variable amount of data omission was observed among all of the factors under study. A weak or nonexistent correlation between the incidence of gastric cancer and the study variables was shown by a visual analysis of scatterplot dispersion. In contrast, an analysis of categorized incidence revealed that the countries with the highest human development index (HDI) values had the highest rates of obesity in males and the highest consumption of alcohol, tobacco, fruits, vegetables and meat, which were associated with higher incidences of gastric cancer. There was no significant difference for the risk factors of obesity in females and fish consumption.

CONCLUSION: Higher HDI values, coupled with a higher prevalence of male obesity and a higher *per capita* consumption of alcohol, tobacco, fruits, vegetables and meat, are associated with a higher incidence of gastric cancer based on an analysis of populational global data.

Key words: Gastric cancer; Risk factors; Epidemiologic factors; Environment; Public health

Core tip: An ecological study on gastric cancer based on public databases proved to be feasible and promising, and this method can be used to monitor the behavior of the disease globally. The results of this study indicated a higher level of development, coupled with the highest prevalence of male obesity and a higher *per capita* consumption of alcohol, tobacco, fruits, vegetables and meat, among the countries with the highest incidences of gastric cancer. In contrast, a high consumption of vegetables was associated with a lower disease incidence in other countries.

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INTRODUCTION

Although its incidence has been declining in many countries, gastric cancer is still the second leading cause of death from malignancy worldwide, accounting for 700349 deaths in 2002[1].

The identification of risk factors associated with malignancies is of great importance because this knowledge can facilitate not only the development of policies aimed at the prevention of cancer occurrence but also the vigilance of at-risk groups regarding the early identification of new cases. Such identification is usually performed through observational studies, and especially case-control and cohort studies, which involve high-cost processes from a financial perspective and a long time to completion. However, those types of studies, although numerous, are usually conducted on limited population sizes, social profiles and geographic locations, with a consequent limitation of the generalizability of the results.

The systematic recording of the health data, social characteristics and habits of living populations and the consequent creation and maintenance of public databases have been made possible by the development of techniques for ecological study. These methods, by definition, allow a shift of the focus of analysis from the individual to the population. Ecological studies allow inferences about the effect of risk conditions on rates of diseases in populations. Despite limitations in measuring individual aspects and in the analysis of the cumulative effects of factors, such studies have great value due to their simplicity and low cost of analysis, may contribute to the development of health policies and may guide research efforts specific to large populations.

Given the notable differences in the frequency and distribution of gastric cancer in the various countries of the world and the operational difficulties of running traditional epidemiological studies on global demographic trends and risk factors, we performed a study on risk factors' association with gastric cancer through populational database analysis.

MATERIALS AND METHODS

Databases

The age-standardized incidences of stomach cancer in 184 countries were obtained from the public database maintained by the GLOBOCAN Project of the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO), considering the most recent data available (from 2008).

GLOBOCAN project

Over the past 30 years, the IARC has published regular estimates of the incidence of and mortality from major types of cancer in several regions of the world at the national level, focusing on 184 countries, using new sources of data and improved methods of estimation. The results of the data collection in 2008 can be summarized as follows: (1) National incidence data - systematically collected by IARC member countries (62 countries); (2) Data from one or multiple local records - presented by the weighted average (75 countries); (3) Data derived from known frequencies of all types of cancer - adjusted for the known relative frequency of each type (13 countries); and (4) No data collected - presents data from neighboring countries in the same geographic region (34 countries).

Estimates are presented separately for each sex and divided into 10 age groups. These values are based on the most recent data available at the IARC and on publicly available information on the Internet, including data from the cancer registries of populational databases. These databases can cover an entire national population but more often cover smaller, subnational areas, and in developing countries, only large cities.

Furthermore, the degree of delay was taken into account by computing predictions. Although historical trends may not continue in the future, predictions based on linear trend patterns have been empirically shown to be reasonably accurate, particularly in the short term.

When historical data and a sufficient number of registered cases were available, the incidence rates were projected for 2008. Otherwise, the incidence rates of the most recent period were applied.

Regional models were used when data on the incidence in specific countries or locations were absent or when the data were considered to be of insufficient quality. In the absence of data, the values of neighboring countries in the same region were used.

Risk and protective factors

For the selection of risk and protective factors described in the medical literature, an extensive review was conducted through the search engines MEDLINE and PubMed, using the following keywords and limiters: gastric cancer, risk factors, etiology, epidemiology and diet factors. After characterizing these factors, we performed a search of specific databases to determine populational exposure to the factors using the Google search engine, with emphasis on references from government agencies and nongovernmental organizations linked to each marker. The year of reference for each factor was 2008 or the closest available year.

Helicobacter pylori

We analyzed data from studies published between 1990 and 2012 in PubMed and MEDLINE using the following key words and limiters: *Helicobacter pylori* (*H. pylori*), *Helicobacter*, *Helicobacter pylori*, incidence, prevalence and epidemiology. We performed this search because there is no global database containing the values of the incidence and prevalence of colonization by the agent. The values collected showed a lack of precision and a large omission of data, as we could identify references to only 54 of the 183 studied countries. Thus, we decided to exclude this risk factor from further analysis, despite its clinical relevance.

Table 1 List of countries categorized by extreme incidences

< P25	Incidence	> P75	Incidence
Botswana	0.2	Chinese Taipei	15.6
Namibia	0.7	Poland	15.6
Malawi	0.8	Singapore	15.8
Lesotho	0.9	Austria	15.9
Sudan	1.0	Bosnia and Herzegovina	16.2
Swaziland	1.0	Azerbaijan	16.4
Tanzania	1.0	Bhutan	16.6
Central African Republic	1.1	Uruguay	16.7
Chad	1.1	Guatemala	17.0
Eritrea	1.1	Honduras	17.0
Gambia	1.1	Slovakia	17.2
Cameroon	1.2	Vietnam	17.3
Comoros	1.2	Spain	17.5
Equatorial Guinea	1.2	Peru	18.1
Gabon	1.2	Georgia	18.6
Niger	1.2	Germany	18.6
Nigeria	1.2	Kyrgyzstan	18.6
Republic of Congo	1.3	Romania	18.7
Djibouti	1.3	Moldavia	18.8
Gaza Strip	1.3	Jamaica	19.3
Maldives	1.3	Hungary	20.8
Mozambique	1.5	Costa Rica	20.9
Syria	1.5	Kazakhstan	21.4
United Arab Emirates	1.5	Armenia	21.8
Saudi Arabia	1.7	Slovenia	22.3
Egypt	1.8	Chile	22.4
Togo	1.8	Ecuador	22.4
Benin	1.9	Croatia	22.6
Burkina Faso	1.9	Mongolia	22.8
Ethiopia	1.9	Macedonia	22.9
Zambia	1.9	Bulgaria	23.5
Kuwait	2.0	Montenegro	23.6
Qatar	2.0	Italy	26.0
Sri Lanka	2.0	Ukraine	26.2
Iraq	2.1	Latvia	26.8
Yemen	2.1	Albania	26.9
Angola	2.2	Portugal	27.1
Sierra Leone	2.2	Lithuania	27.6
Republic of South Africa	2.2	Estonia	27.9
Ivory Coast	2.4	Russia	28.7
Laos	2.4	China	34.5
Oman	2.4	Belarus	36.4
Somalia	2.4	South Korea	56.3
Solomon Islands	2.5	Japan	80.2
Ghana	2.6		
Libya	2.6		
Vanuatu	2.6		

Tobacco

Data were collected from the Global Health Observatory Data Repository of the WHO. The reference variable used was the percentage of the population using any tobacco product (age-standardized rate).

Alcohol

Data were collected from the World Health Statistics database of the Global Health Observatory Data Repository of the WHO. The reference variable used was liters of pure alcohol/person/year.

Obesity

Data were collected from the World Health Statistics database of the Global Health Observatory Data Repository of the WHO. The reference variable used was the percentage of adults over 20 years that present a body mass index (BMI) > 30 kg/m^2.

Consumption of fruits, vegetables, legumes, meat and fish

Data were collected using the FAOSTAT tool of the Food and Agriculture Organization (FAO) database of the United Nations. The reference variable used was consumption in g/person/d.

Salt consumption

We could not find databases regarding average *per capita* salt intake or consumption in different countries, so this factor was excluded from our analysis.

Human development index

Data on the human development index (HDI) were collected from the database of Human Development Reports of the United Nations.

Statistical analysis

The bivariate relationship of characteristics has been primarily studied by plotting data on the incidence of gastric cancer and various numerical indicators associated with risk in scatter plots. As the incidence of gastric cancer in 183 countries did not have a normal (Kolmogorov-Smirnov $P < 0.001$) or symmetric (histogram analysis) distribution, the distribution of incidences was categorized into percentile ranges (10, 25, 50, 75 and 90). A new graphical analysis of the association between the incidence of cancer (categorized) and measures of exposure to each risk factor was performed using box plots.

The Mann-Whitney test was then used to test for differences in measures of exposure to each risk factor between the extreme ranges of incidence (under the 25th percentile and above the 75th percentile) (Table 1).

A database of collected data was created using the software MS Excel, and data analysis was performed using SPSS v. 13.0, considering a significance level of 0.05.

As the research was performed using open-access public databases, it was unnecessary to obtain express authorization from the maintainers of such data. The data' s sources are properly cited along with the disclosure of the results of this study, as recommended by the sources.

We could not identify any conflicts of interest or ethical conflicts in the implementation of the present study, so submission for analysis by the Committee of Ethics in Research was not necessary, according to its own rules.

RESULTS

In the cross-analysis of variables from different databases, which is the basis of this study, it was expected that values for each risk factor under study could not be found for all countries. Thus, the availability of data ranged from 58 countries for the prevalence of *H. pylori*

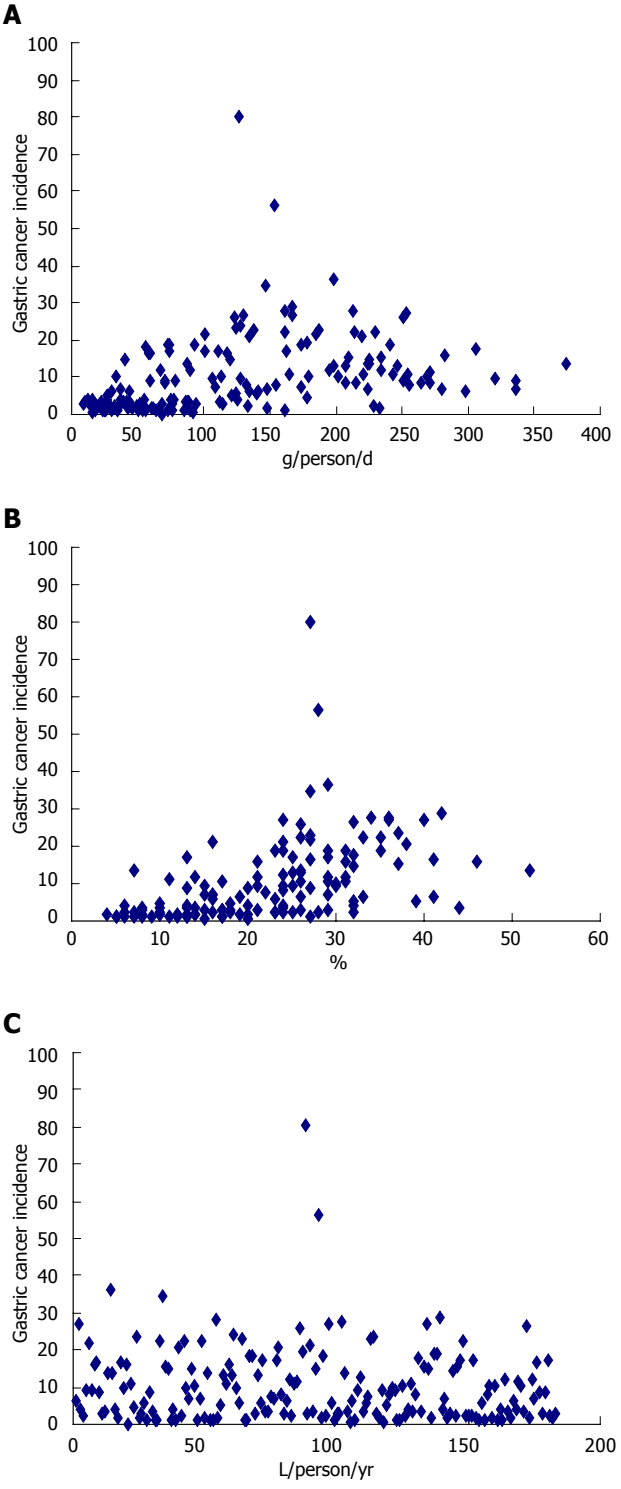


Figure 1 Several of the scatter plots. A: Meat; B: Tobacco; C: Alcohol.

to 172 countries for the consumption of alcohol and obesity, considering the 183 countries for which the incidence of gastric cancer was available in the GLOBOCAN database.

A visual analysis of the scatter plots of the incidence of gastric cancer and the study variables suggested a weak or nonexistent correlation, as exemplified by Figure 1.

By the selection of extreme incidences of gastric cancer according to percentile distribution [under the 25th percentile (< P25) and above the 75th percentile (> P75)], a different view of the association was possible. The countries categorized in the extreme incidence ranges are presented in the world map in Figure 2.

The Mann-Whitney test showed differences in measures of certain risk factors between the extreme incidence ranges, as summarized in Table 2.

Countries in the > P75 range of the incidence of gastric cancer had a significantly higher average HDI (0.76019 *vs* 0.52404) than countries in the < P25 range.

The consumption of alcohol was associated with higher incidences of gastric cancer, with an average consumption of 10.60 L/person/year, which is significantly higher than the consumption in countries with incidences below the 25th percentile (3.75 L/person/year).

The consumption of tobacco was also associated with the highest incidences of gastric cancer, with an average of 30.03% of the population being consumers of any tobacco product, compared with an average of 14.47% among countries with incidences of cancer below the 25th percentile.

A higher consumption of fruits was related to higher incidences of gastric cancer. An average consumption of 222.9 g/d was observed among countries with incidences above the 75th percentile, and consumption of 145.8 g/d was observed among countries with incidences below the 25th percentile.

The same direction of association was found for vegetable consumption, with an average consumption of 318.7 g/d among countries with the highest incidences of gastric cancer and an average of 154.4 g/d among countries below the 25th percentile of the distribution of cancer incidence.

However, a higher consumption of legumes was significantly associated with lower incidences of gastric cancer.

The highest *per capita* consumption of meat was related to higher incidences of gastric cancer, as an average consumption of 157.1 g/d/person was found in countries grouped above the 75th percentile of the distribution of the incidence of gastric cancer, in comparison with an average of 63.3 g/person/d in countries below the 25th percentile.

In contrast, no significant association was observed for between the average consumption of fish and the population rates of female obesity.

Regarding the national percentage of obese male adults, the difference was significant, as the highest rates of obesity were associated with higher incidences of gastric cancer.

These results were reinforced by the analysis of boxplot diagrams shown in Figure 3.

DISCUSSION

The prevention and treatment of stomach cancer, which is currently the fourth most common malignancy worldwide, are still major challenges[2-4].

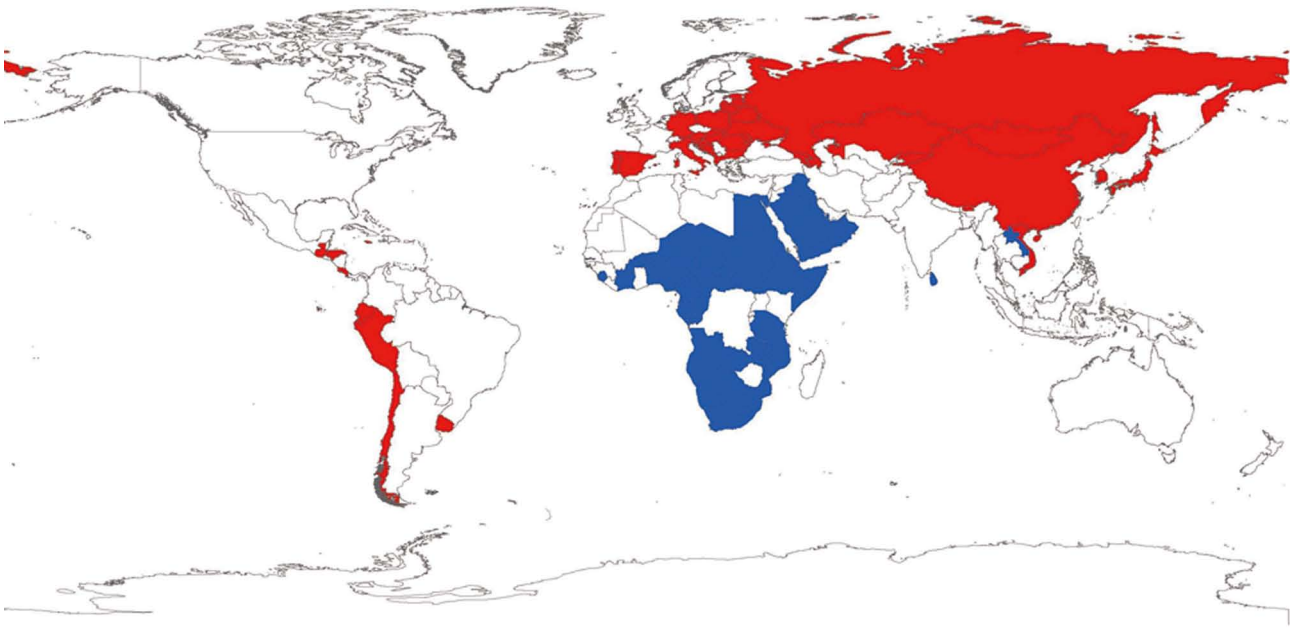


Figure 2 World map of countries with extreme incidences of gastric cancer. Blue: Countries categorized as < P25; Red: Countries categorized as > P75.

Table 2 Association between the incidence of gastric cancer and risk/protection factors based on the Mann-Whitney test

Risk/protective factors	*n*	*n* (< P25)	Average	SD	*n* (> P75)	Average	SD	*P* value
HDI	171	46	0.52	0.14	43	0.76	0.09	0.000
Alcohol, liters of alcohol/inhabitant per year	172	45	3.74	3.42	43	10.59	5.17	0.000
Tobacco, % of smoking population	132	36	14.47	7.27	35	30.03	7.11	0.000
Fruits, g/person per day	165	42	145.84	109.74	40	222.9	89.92	0.001
Vegetables, g/person per day	165	42	154.35	151.55	40	318.66	167.25	0.000
Legumes, g/person per day	165	42	23.08	19.88	41	8.42	8.91	0.000
Meat, g/person per day	165	42	63.26	51.07	42	157.05	63.16	0.000
Fish, g/person per day	162	42	42.56	74.83	38	44.93	42.14	0.260
Obesity in males, % men > 20 yr and BMI > 30 kg/m^2	172	46	9.56	102.11	42	17.03	67.73	0.000
Obesity in females, % women > 20 yr and BMI > 30 kg/m^2	172	46	18.94	150.44	42	21.66	81.28	0.100

HDI: Human development index; BMI: Body mass index.

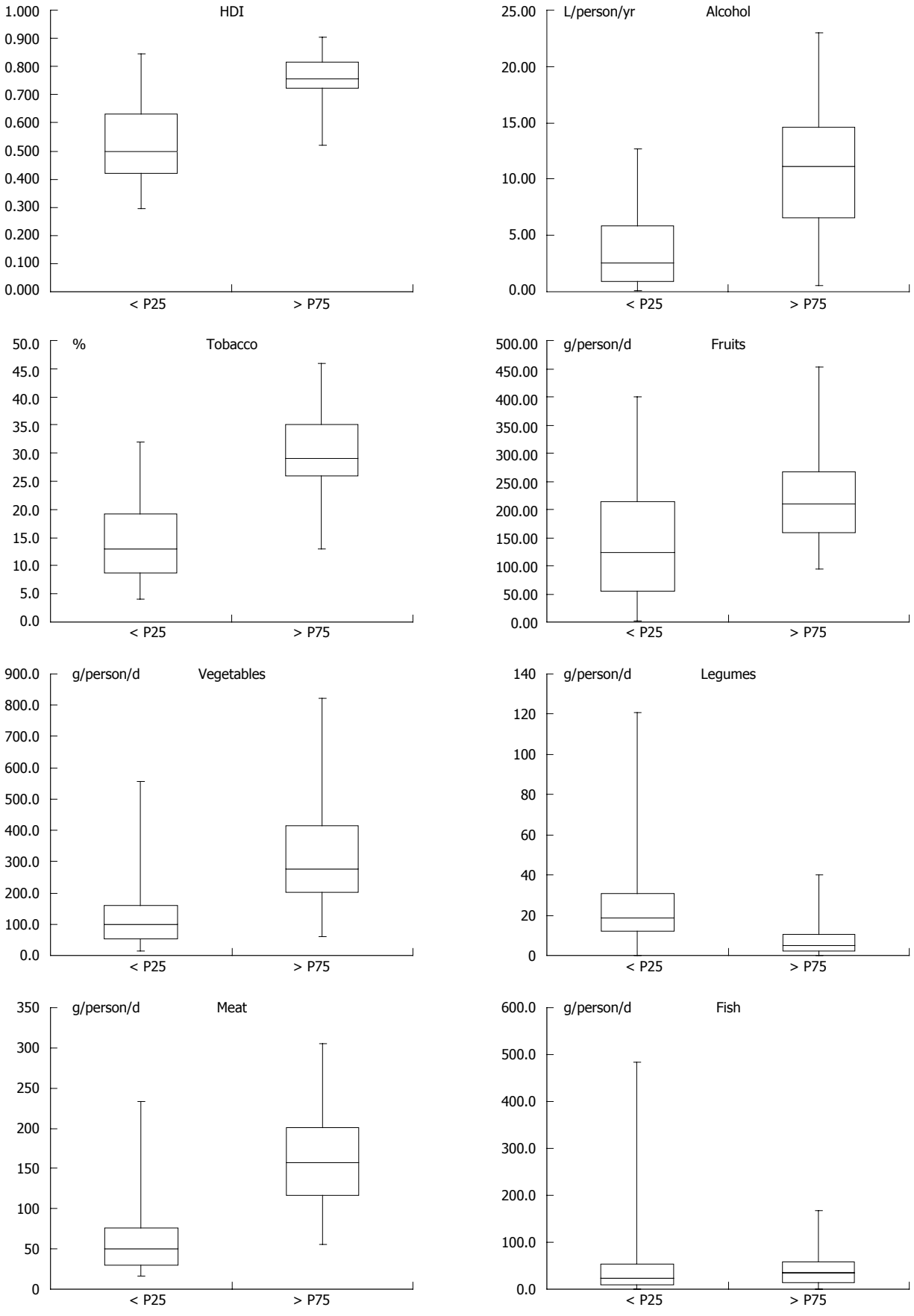
There are geographic and ethnic differences in the distribution of the incidence of gastric cancer worldwide and changing trends in each population over time, which hinder a better understanding of this cancer's etiology.

It is assumed that the incidence has been decreasing in most industrialized countries over the past three decades and that the incidence patterns observed in immigrant groups move toward the patterns in the countries of origin. These changes suggest a close association of gastric cancer with modifiable factors[5-11].

During the development of this study, when we first analyzed the crude data from all countries, we could not detect significant associations between protective/risk factors and gastric cancer due to the weak correlations observed. However, when studying the extreme incidences, we observed evidence of several of the associations already described in the literature between dietary/behavioral factors and the incidence of gastric cancer.

The IARC has presented fruits and vegetables as probable protective factors in the development of stomach cancer. Therefore, the World Cancer Research Fund recommends a daily intake of vegetables/fruits greater than 400 g for a protective effect. This association was not observed in the current study. Moreover, although gastric cancer is considered to be a multifactorial disease, we observed that several of the countries with higher incidences, such as Korea, had a *per capita* consumption of fruits and vegetables above the suggested protective intake.

Part of this result may be justified by specific dietary components and certain cooking practices that are also associated with an increased risk of gastric cancer through the formation of *N*-nitroso compounds and polycyclic aromatic hydrocarbons. These practices include grilling, baking, curing, drying in the sun, smoking, cooking, frying in open ovens and salting. Certain foods also have natural nitrate concentrations (cabbage, cauliflower, carrots, celery, radishes, beets and spinach), or these compounds may be added during preservation. In addition, the nitrate content in soil fertilizers and water


HDI
1.000
0.900
0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
< P25
> P75
L/person/yr
Alcohol
25.00
20.00
15.00
10.00
5.00
0.00
< P25
> P75
%
Tobacco
50.0
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
< P25
> P75
g/person/d
Fruits
500.00
450.00
400.00
350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
< P25
> P75
g/person/d
Vegetables
900.0
800.0
700.0
600.0
500.0
400.0
300.0
200.0
100.0
0.0
< P25
> P75
g/person/d
Legumes
140
120
100
80
60
40
20
0
< P25
> P75
g/person/d
Meat
350
300
250
200
150
100
50
0
< P25
> P75
g/person/d
Fish
600.0
500.0
400.0
300.0
200.0
100.0
0.0
< P25
> P75

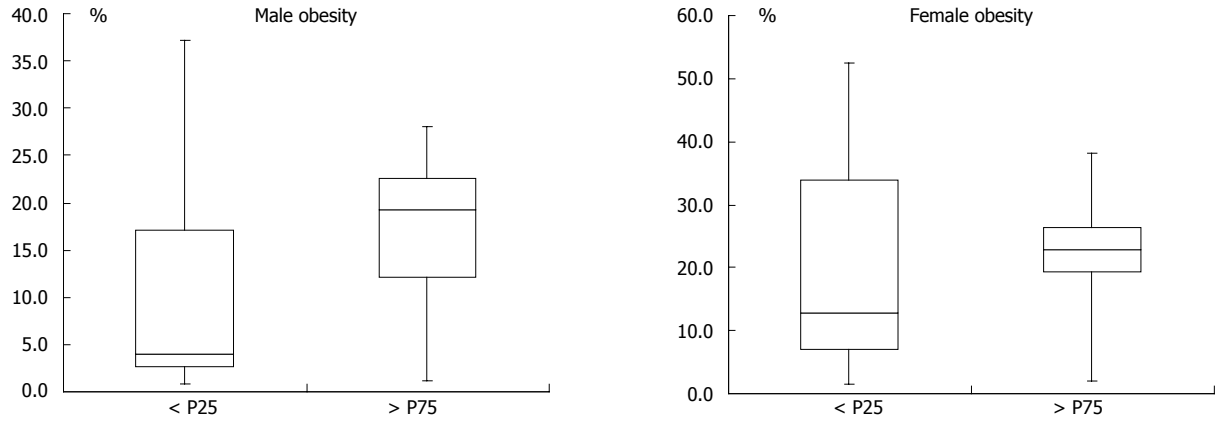


Figure 3 Boxplot diagrams of human development index values. the consumption of alcohol, tobacco, fruits, vegetables, legumes, meat and fish; and the prevalence of obesity in males and females among countries with gastric cancer incidences < P25 and > P75.

also contributes to the level of dietary nitrate[12].

When evaluating obesity in males and the consumption of meat, vegetables, tobacco and alcohol, the results were consistent with findings described in the literature, reinforcing the concept that a modification of lifestyle represents a practical strategy for preventing gastric cancer, especially in middle-aged or elderly people[12-14].

Currently, more than 80% of cases of gastric cancer may be associated with infection by *H. pylori*. In general, both cancer and infection tend to affect more individuals of lower socioeconomic classes, presumably due to poor education and sanitary conditions[15-21]. Due to the unavailability of a worldwide database representing this factor, we could not study its association with gastric cancer incidence.

It is known that the incidence rates of gastric cancer can vary up to 10 times worldwide and that nearly two-thirds of stomach cancers occur in developing countries. Even so, Japan and Korea, countries with high levels of development, have the highest rates of gastric cancer in the world[22-27].

One of the possible explanations for this finding might be the large difference not only in the etiology but also in the programs for early detection, specialized treatment and prevention in these countries. Furthermore, there is evidence that in areas of low incidence, genetic and biological characteristics seem to have a greater influence on the development of the disease. For example, the incidence of cancer in Africa is the lowest among all developing and developed countries, ranging from 2 to 5.6/100000[5,28].

It is important to remember that proximal tumors are more common in developed countries and in higher socioeconomic classes and have shown a progressive increase in incidence[29]. Distal tumors remain predominant in Japan, the country with the highest incidence, in contrast to the rest of the world[14,29].

The multifactorial etiology of gastric cancer imposes an additional challenge on the understanding and development of effective prevention and monitoring programs. The most appropriate epidemiological studies that are focused on factors associated with the disease require long and careful monitoring because these studies are based on observing individual cases, exposure to factors and cancer development, which makes these studies extremely expensive. The present study, with no claim to challenge epidemiological data or to create a method to replace epidemiological studies, proposes the implementation of a simple, low-cost methodology for the evaluation of the relationship between risk factors and outcome.

Ecological studies are valued in research on seasonal changes or geographical variations of events, especially under adverse social or territorial conditions that make it impossible to study every citizen. However, such studies have limitations. By shifting the focus from the subject to the population, one can lose sight of the direct relationship between a risk/protective factor and individual development of the disease. However, as exposure is measured in an ecological way, it is assumed that an average variation in the incidence of gastric cancer in a particular country also reflects a variation in the average exposure of each individual residing there[30-33]. This concept can be taken into consideration when evaluating individual consumption of food, tobacco products or alcohol.

The quality of a database depends on the properties of the components used in its formulation and the accuracy of the information systems used in its construction, including the database's integrity (completeness) and internal consistency (data consistent and not contradictory). The systematic application of operational definitions and standardized procedures for measuring and calculating allows inferences about and predictions of unavailable data[30,31].

Although we did not spare efforts to identify the best data needed for our research, the fact that we worked with secondary data implies a limitation of this study, especially given a lack of access to the primary measures. This lack of data disallowed the proper analysis of major risk factors, such as salt intake and exposure to *H. pylori*.

The attempt to create a database for the prevalence of infection by *H. pylori* from data from published studies

to which we had access resulted in very small number of evaluated countries, which could have affected the final outcome. Thus, we disregarded the results of this analysis in our conclusions. Due to its importance in the genesis of gastric cancer, we consider the creation of a global database for the prevalence of *H. pylori* infection in each country to be important.

The inaccuracy of cancer incidence data for certain countries due to the methodology used by GLOBOCAN, which is considered to be a relatively reliable and stable source, being widely used in technical and scientific papers, should be noted. However, the collection of data from GLOBOCAN incorporates data measured by national health agencies and data derived from approximations of incidence based on the known frequencies of all types of cancer. In addition, 34 countries do not have local data, leading to the use of data available for neighboring countries in the same geographic region[4].

The data collected from the FAO of the United Nations include global information from national statistical offices with internationally recognized definitions, concepts and classifications. Time series statistics have been compiled, processed and stored by each country since 1961, and the database contains the records of more than 245 countries and territories. The data are provided by governments through national publications and FAO questionnaires (paper or electronic). To make the data coverage as complete as possible, the official data are occasionally supplemented with data from unofficial sources.

The ecological study of gastric cancer based on public databases proved to be feasible and promising, and this method can be used to monitor the global behavior of similar diseases.

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COMMENTS

Background

The multifactorial etiology of gastric cancer imposes an additional challenge on the understanding and development of effective prevention and monitoring programs. Ecological studies are valued in research on seasonal changes or geographical variations of events, especially under adverse social or territorial conditions that make it impossible to study every citizen.

Research frontiers

The results of ecological studies can provide the opportunity for more carefully designed studies based on the initial observations. The use of databases is a simple, low-cost methodology for the evaluation of the relationship between risk factors and outcome, using data that are generally already available. The quality of a database is crucial to the analysis results.

Innovations and breakthroughs

An ecological study on gastric cancer based on public databases proved to be feasible and promising, and this method can be used to monitor the behavior of the disease globally. The results of this study indicated a higher level of development, coupled with the highest prevalence of male obesity and a higher *per capita* consumption of alcohol, tobacco, fruits, vegetables, and meat, among the countries with the highest incidences of gastric cancer. In contrast, a high consumption of vegetables was associated with a lower disease incidence in other countries.

Applications

The study's results suggest that ecological studies using populational databases can be used to monitor the global behavior of the disease. For this purpose, it is important to create a global database for the prevalence of *Helicobacter pylori* (*H. pylori*) infection in each country.

Peer review

This ecological study on gastric cancer, based on public databases, provides a useful contribution to the dimension of gastric cancer protection, and the method can be used to monitor the behavior of the disease globally, although the study implies a limitation of using secondary data. The results of this study indicated a higher level of development, coupled with the highest prevalence of male obesity and a higher *per capita* consumption of alcohol, tobacco, fruits, vegetables and meat, among the countries with the highest incidences of gastric cancer. In contrast, a high consumption of vegetables was associated with a lower disease incidence in other countries. The concern in this study is the exclusion of *H. pylori*, which is a very important risk factor for gastric cancer, from the analysis.

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BRIEF ARTICLE

Clinical significance of white gastric crypt openings observed *via* magnifying endoscopy

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Abstract

AIM: To evaluate the relationship between *Helicobacter pylori* (*H. pylori*)-induced gastritis and white gastric mucosal crypt openings (COs) in the gastric corpus.

METHODS: A total of 175 consecutive patients (including 69 patients with gastric cancer) were enrolled in this study. We used magnifying endoscopy (ME) to observe the mucosa microsurface of the lesser and greater curvature of the gastric corpus (350 areas in all). We focused on areas with a round pit microstructure (primarily observed in non-atrophied areas) and evaluated the white openings of these gastric pits. We classified the whiteness of the COs as the "white-edged dark spot" type (consisting of a dark spot bordered by white); the "white" type (pure white with no dark spot); and the "dense white pit (DWP)" type (dense white, resembling a snowball). Gastritis was also histologically evaluated according to the updated Sydney System.

RESULTS: We detected round COs using ME in 246 of the 350 areas examined. The histological examination showed significantly more mononuclear cells and neutrophil infiltration in the "white" and "DWP" types than the "white-edged dark spot" type ($P < 0.001$). Furthermore, significantly high-grade inflammation and evidence of active *H. pylori*-induced gastritis was observed in the "DWP" type ($P < 0.001$). Significant differences were observed in the whiteness of COs between *H. pylori*-positive ($n = 139$) and negative ($n = 36$) patients ($P < 0.001$). The sensitivity and specificity of the "white" and "DWP" types for predicting *H. pylori* infection were 78.5% and 81.7%, respectively. Of the patients with gastric cancer, 22.5% (18/80) had "white-edged dark spots", 51.3% (41/80) had "white" COs, and 26.3% (21/80) had "DWP"-type COs. "DWPs" were frequently observed among patients with undifferentiated gastric cancer [45.7% (16/35)].

CONCLUSION: CO whiteness detected *via* ME was associated with histological evidence of gastritis and helps to predict the severity of inflammation and *H. pylori*-induced activity.

Key words: Magnifying endoscopy; *Helicobacter pylori*; Gastritis; Gastric cancer; Inflammation

Core tip: Recent studies have reported that advances in magnifying endoscopy (ME) have led to better correlations between histopathological findings and the ME features of *Helicobacter pylori* (*H. pylori*)-induced gastritis. However, the ME findings regarding *H. pylori*-induced severe inflammation are insufficient. Therefore, we evaluated the relationship between *H. pylori*-induced gastritis and the whiteness of gastric mucosal crypt openings (COs) in the gastric corpus using ME.

Our results showed that mononuclear cell and neutrophil infiltration differed significantly among the CO subtypes. CO whiteness detected *via* ME was associated with histological evidence of gastritis and helps to predict the severity of inflammation or activity induced by *H. pylori* in the gastric corpus.

Kawamura M, Sekine H, Abe S, Shibuya D, Kato K, Masuda T. Clinical significance of white gastric crypt openings observed *via* magnifying endoscopy. *World J Gastroenterol* 2013; 19(48): 9392-9398 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i48/9392.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i48.9392

INTRODUCTION

Helicobacter pylori (*H. pylori*) infection causes acute and chronic inflammation accompanied by neutrophil or lymphocyte infiltration. This type of persistent inflammation can result in gastric mucosal changes such as glandular atrophy, intestinal metaplasia, dysplasia, and eventually carcinoma[1-5]. Previous studies using conventional standard endoscopy have reported correlations between *H. pylori*-induced gastritis and endoscopic findings. Endoscopic atrophy is correlated with the grade of glandular atrophy and intestinal metaplasia[6]. With regard to severe grades of *H. pylori*-induced inflammation, nodular gastritis in the antral area is an endoscopic marker for the early phase of *H. pylori* infection and an exaggerated immune response[7-10].

Advances in magnifying endoscopy (ME) and narrow-band imaging have enabled the real-time observation of the microsurface structure and microvascular architecture of the gastric mucosa. Recent studies have reported that these advances have led to stronger correlations between histopathological findings and the ME features of *H. pylori*-induced gastritis compared with data obtained using standard endoscopy[11-17]. Using ME, one can observe the microsurface structure of the gastric mucosa change from a round pit pattern to vertical long pits, tubular and granular patterns after the start of *H. pylori*-induced gastritis.

However, many investigators have reported that the morphological changes identified using ME are closely associated with histological glandular atrophy or intestinal metaplasia. The data regarding the characteristics of the ME findings in *H. pylori*-induced severe inflammation, such as nodular gastritis in the antral area, are insufficient. Therefore, we used high-resolution ME to investigate the characteristics of *H. pylori*-induced inflammation of the gastric corpus in *H. pylori*-negative and *H. pylori*-positive patients.

MATERIALS AND METHODS

Patients and methods

This observational study was performed in the endoscopy unit of a city hospital (JR Sendai Hospital, Sendai, Mi-, Japan). Between September 2007 and November 2010, 175 consecutive patients who had undergone ME in our hospital as part of their annual health checks to investigate digestive symptoms (or as an additional pre-treatment examination) were enrolled. Patients were excluded if they had severe systemic disease; had a history of upper digestive tract surgery; had been treated with nonsteroidal anti-inflammatory drugs, antiplatelet agents, or anticoagulants within 7 d of endoscopy; or had active bleeding, advanced gastric cancer, or other non-gastric malignancies. Patients who received *H. pylori* eradication therapy were also excluded. *H. pylori* infection was diagnosed using a rapid urease test, which examined the histology of biopsy specimens obtained from the greater curvature of the gastric antrum and body, and the urea breath test. Patients who tested positive on any of these tests were considered positive for *H. pylori* infection. Written informed consent was obtained from each patient, and the institutional review board of JR Sendai Hospital approved the study protocol.

Endoscopic procedure

Magnifying endoscopy was performed using either a CV-240 or CV-260 video system (Olympus Optical, Tokyo, Japan) and a magnifying endoscope (Model Q240Z or H260Z, Olympus). To obtain a clear view using ME, a black rubber attachment (MB-46 or MB-162, Olympus) was fitted to the tip of the videoendoscope to ensure an appropriate distance between the lens and the mucosal surface. A single experienced endoscopist (Masashi Kawamura) performed all procedures. A videoendoscope was inserted into the patient's stomach, and the diseased and uninvolved gastric mucosa were visualized using standard and magnified views. The microsurface structures of the non-cancerous areas in the greater and lesser curvatures of the upper gastric corpus were evaluated for the presence of patterns such as round pit, long pit, tubular, or granular. If areas with round pit microstructures were observed under maximum magnification, then we evaluated the whiteness of the gastric pit crypt openings (COs). Then, the specific area that had just been magnified was biopsied under magnification. The whiteness of each round CO was classified into one of the following three categories: A round dark spot bordered by white was classified as a "white-edged dark spot" CO; pure white COs without a dark spot were classified as "white" COs; and densely white COs resembling snowballs were classified as "dense white pit" ("DWP") COs (Figure 1).

Histological assessment

Biopsy specimens were fixed with buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin. An expert pathologist who was unaware of the endoscopic findings assessed the grade of histological gastritis in each biopsy sample. The degree of mononuclear cell and neutrophil infiltration, atrophy, and intestinal metaplasia was assessed and graded as normal, mild, moderate, or marked using a visual analog scale in-

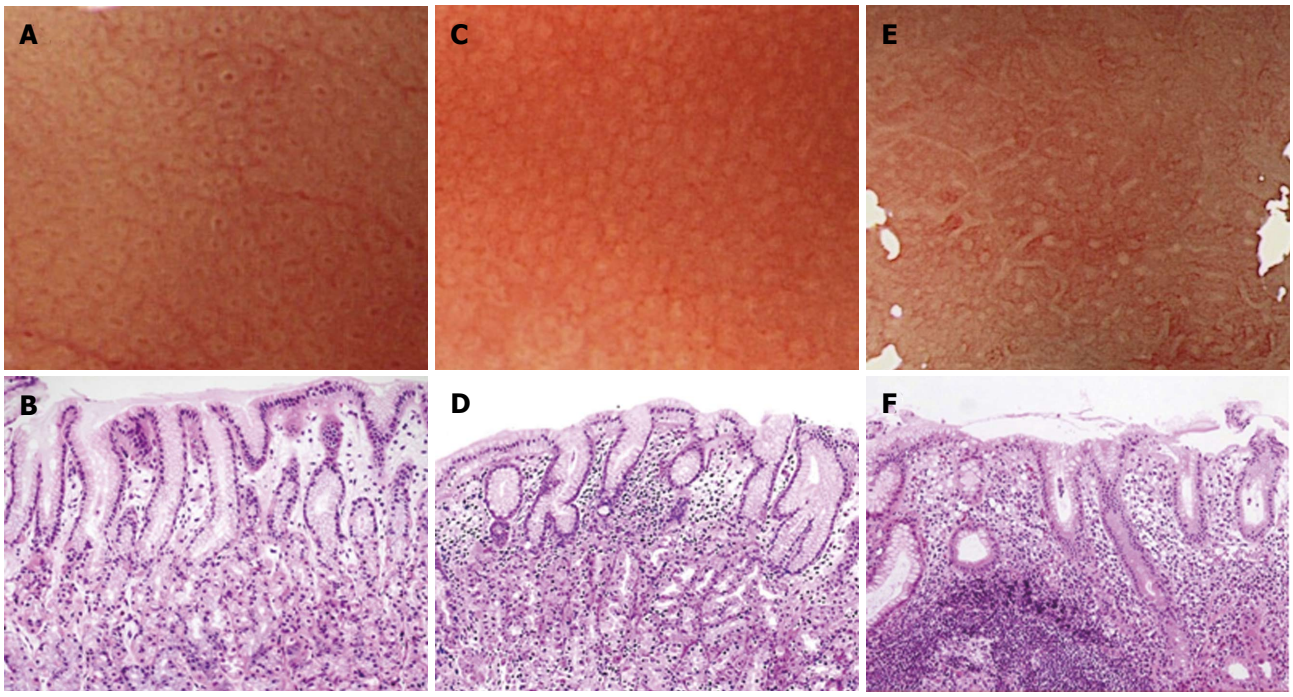

Figure 1 The whiteness of crypt openings (HE, × 100). A: "White-edged dark spot" crypt openings (COs) (a round dark spot bordered by white); B: Histological image of "white-edged dark spot" COs; C: "White" COs (pure white COs without a dark spot); D: Histological image of "white" COs; E: "Dense white pit" ("DWP") COs (densely white COs resembling snowballs); F: Histological image of "DWP" COs.

cluded in the updated Sydney System[18]. Gastric carcinomas were classified as differentiated or undifferentiated based on the degree of glandular structure formation among the tumor cells (the Japanese Classification was proposed by the Japanese Research Society for Gastric Cancer)[19]. These types match the intestinal and diffuse types of gastric carcinoma, respectively, described in the Lauren classification[20].

Statistical analysis

The results are presented as the mean ± SD. Kruskal-Wallis and Mann-Whitney *U* tests were used to evaluate the relationship between the whiteness of the COs and the histological findings. The differences between the *H. pylori*-negative and *H. pylori*-positive groups with regard to CO type were compared using the Mann-Whitney *U* test. Data analyses were performed using R Version 3.0.1 (The R Foundation for Statistical Computing, Vienna, Austria); *P* values < 0.05 were considered significant.

RESULTS

Of the 175 enrolled patients, 116 were men and 59 women; their mean age was 63.9 years. The diagnoses included 46 patients with differentiated-type gastric cancer, 23 with undifferentiated-type gastric cancer, 22 with active duodenal ulcers, and eight with active gastric ulcers. A total of 76 patients had only gastritis or normal findings. The ME observations of the lesser and greater curvatures revealed round pit patterns in 246 of the 350 areas examined (Table 1).

Regarding the whiteness of the round COs, 89 had the "white-edged dark spot", 114 were "white", and 43 were "DWP" COs. Figure 2 shows the relationship between CO whiteness and the severity of gastritis diagnosed histologically based on the updated Sydney System. In both "white" and "DWP" type COs, the histological examination tended to show moderate or marked mononuclear cell and neutrophil infiltration accompanied by normal-to-mild glandular atrophy and intestinal metaplasia. These variables were mostly classified as normal to mild among "white-edged dark spot" COs. We evaluated the histological findings according to the updated Sydney system, which uses 4 classes (none, mild, moderate and marked) for each parameter [inflammation (mononuclear cell infiltration), activity (neutrophil infiltration], atrophy (glandular atrophy), and intestinal metaplasia). Significant differences ($P < 0.001$) were found between the three CO types in the parameters of inflammation and activity; however, the degree of glandular atrophy and intestinal metaplasia did not differ significantly across CO type. The grades of inflammation and activity were higher among "DWP" COs compared with "white" COs ($P < 0.001$).

In this study, 139 patients were positive and 36 were negative for current *H. pylori* infection. Of the 186 round pit areas found in *H. pylori*-positive patients, 21.5% (40/186) were "white-edged dark spot" COs, 55.4% (103/186) were "white", and 23.1% (43/186) were "DWP" COs. Of the 60 round pit areas found among *H. pylori*-negative patients, 81.7% (49/60) were "white-edged dark spot" COs, 18.3% (11/60) were "white", and none (0/60) were "DWP" COs. Significant differences were found between the "white" and "DWP" COs in *H. pylori*-positive patients, and "white-edged dark spot" COs were found among *H. pylori*-negative patients ($P < 0.001$).

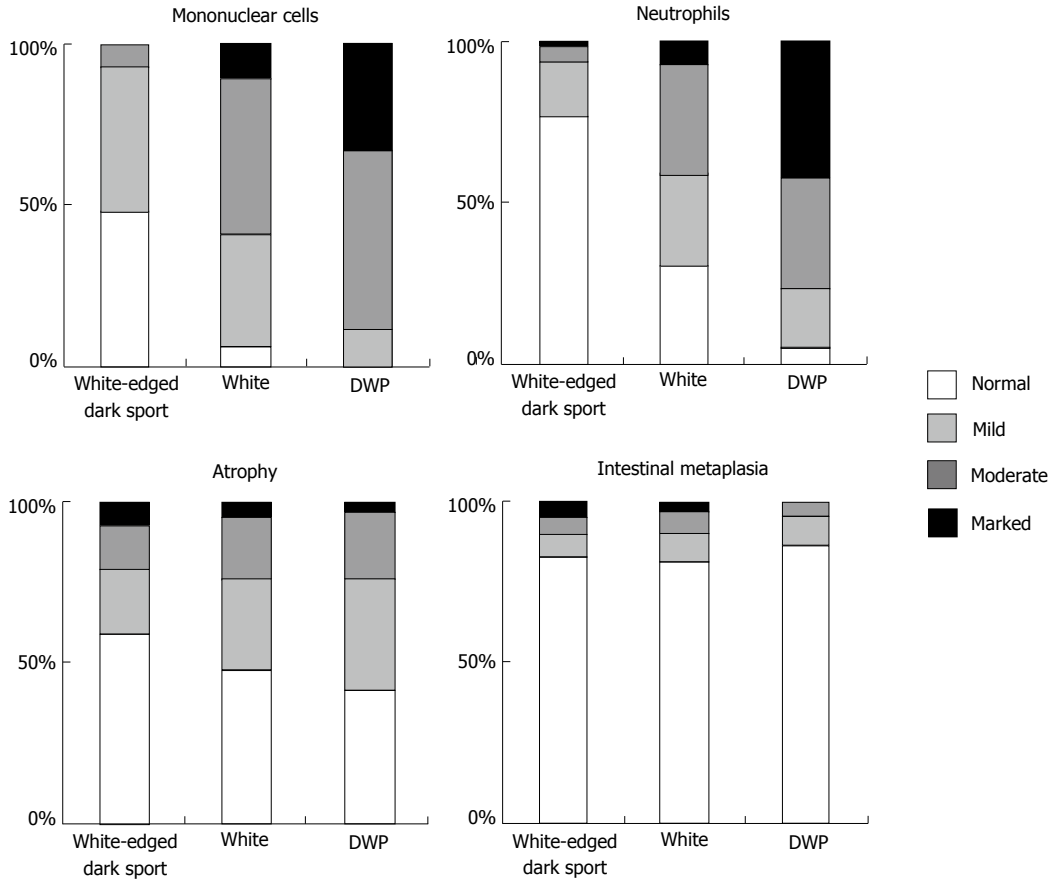

Figure 2 Whiteness of crypt opening and grade of gastritis according to the updated Sydney System. Kruskal-Wallis test showed significant correlations between grades of histological inflammation ($P < 0.001$) and activity ($P < 0.001$) and whiteness of crypt openings (COs). Glandular atrophy and intestinal metaplasia were not significantly correlated with whiteness of COs. DWP: Dense white pit.

Table 1 Characteristics of study patients *n* (%)

	Diff-GC	Undiff-GC	GU	DU	Gastritis and normal
Number of patients	46	23	8	22	76
Age (yr, mean ± SD)	70.6 ± 9.2	65.5 ± 8.2	62.6 ± 10.6	49.2 ± 12.4	63.7 ± 12.5
Sex, male/female	36/10	7/16	6/2	17/5	50/26
Current *Helicobacter pylori*-infection	40 (87.0)	21 (91.3)	8 (100)	22 (100)	48 (63.2)
Endoscopic degree of atrophy (mild/moderate/severe)	3/14/29	9/12/2	2/2/4	20/2/0	41/15/20
Round pits in LC	12 (35.3)	12 (52.2)	3 (37.5)	22 (100)	48 (63.2)
Round pits in GC	33 (71.7)	23 (100)	8 (100)	22 (100)	63 (82.9)

Diff-GC: Differentiated-type gastric cancer; DU: Duodenal ulcer; GC: Greater curvature of corpus; GU: Gastric ulcer; LC: Lesser curvature of corpus; Undiff-GC: Undifferentiated-type gastric cancer.

The sensitivity and specificity of the "white" and "DWP" COs used to predict *H. pylori* infection were 78.5% and 81.7%, respectively.

Table 2 Correlation between current disease and whiteness of crypt openings in round pit areas *n* (%)

Whiteness of COs	White-edged dark spot	White	DWP
Diff-GC	16 (35.6)	24 (53.3)	5 (11.1)
Undiff-GC	2 (5.7)	17 (48.6)	16 (45.7)
GU	2 (18.2)	6 (54.5)	3 (27.3)
DU	13 (29.5)	26 (59.1)	5 (11.4)
Gastritis and normal	56 (50.5)	41 (36.9)	14 (12.6)

COs: Crypt openings; Diff-GC: Differentiated-type gastric cancer; DU: Duodenal ulcer; DWP: Dense white pit; GU: Gastric ulcer; Undiff-GC: Undifferentiated-type gastric cancer.

In total, 22.5% (18/80) of the COs among patients with *H. pylori*-related disease and gastric cancer were "white-edged dark spot" COs, 51.3% (41/80) were "white", and 26.3% (21/80) were "DWP" COs (Table 2). The prevalence of "DWP" COs was higher [45.7% (16/35)] among patients with undifferentiated-type gastric cancer (Figure 3).

DISCUSSION

This study is the first to investigate the relationship be-

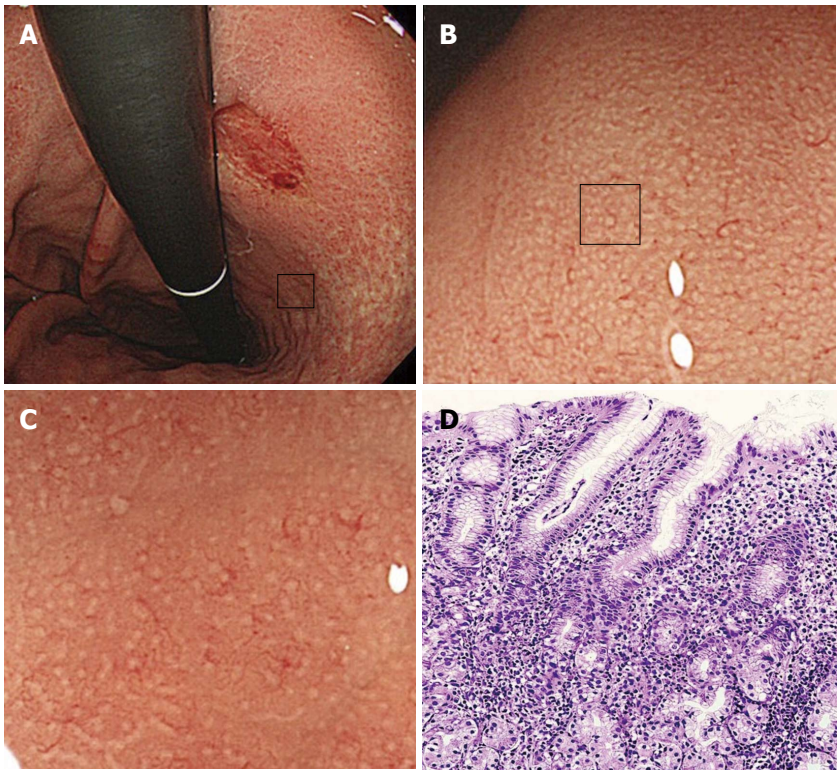

Figure 3 A case with an early undifferentiated-type gastric cancer in the middle of the corpus. A: A 67-year-old woman was found to have a depressed undifferentiated-type gastric cancer about 20 mm in diameter in an area found endoscopically to be non-atrophic; B: Magnifying endoscopy image showing minute round pits in uninvolved corpus mucosa; C: The whiteness of the crypt openings is "dense white pit" type; they resemble snowballs; D: Microscopic examination of uninvolved corpus mucosa revealed marked lymphocyte and neutrophil infiltration in the lamina propria and neutrophil infiltration within the foveolar lumens (HE stain, × 100).

tween CO whiteness type and *H. pylori*-induced gastritis. We categorized CO color in gastric corpus areas with round pit patterns, and we observed less inflammation and activity in "white-edged dark spot" COs than in other types of COs. The round dark spots observed in the ME images might correspond to tangential views of the foveolar gland COs, whereas the white portions that surround the dark spots might be tangential views of the epithelial cells that surround the ductal lumen. This CO type is the typical microsurface mucosal pattern of the gastric corpus among patients without *H. pylori* infection. A histological examination of the areas with "white" type COs revealed degenerated and hypertrophic surface epithelial cells accompanied by lymphocyte and neutrophil infiltration into the lamina propria. These histological changes might cause the dark spots to disappear and be replaced by a white color. The "DWP" COs were accompanied by a high degree of lymphocyte and neutrophil infiltration.

Several studies have evaluated the relationship between *H. pylori*-induced gastritis and the microsurface or microvascular structure of the gastric mucosa *via* ME. In 2007, Yagi *et al*[21] modified their former Z classification and created the A-B classification based on the combination of microsurface and microvascular patterns (type B-0 consists of pinhole pits, the network of true capillaries, and the regular arrangement of collecting venules; type B-1 consists of round pits and a network of capillaries; type B-2 consists of white pits and sulci; and type B-3 consists of dilated white pits with surrounding microvessels). Although this classification was considered useful for predicting the grade of *H. pylori*-induced gastritis, we often observed other combinations of microstructure and microvessel changes than those described in the A-B classification (*e.g.,* pinhole pits without capillaries). The current study classified our observations into three types of ME findings based on the color of the gastric pits and found strong correlations with histological *H. pylori*-induced inflammation and activity. Our classification is advantageous because it is simple and easy to understand and does not include variations in the combination of microstructure and microvessels.

Several reports have described that the prevalence, distribution, and grade of *H. pylori*-induced gastritis varies among individuals[22,23]. We previously reported that the ME findings of the gastric mucosa in *H. pylori*-infected patients are also heterogeneous in the stomach[15]. The present study investigated each case at two sites (the greater and lesser curvature of the upper corpus) because a multipoint evaluation of gastritis is important for assessing its status. Our results indicated that most round pit areas existed in the endoscopic non-atrophied area; furthermore, more were found in the greater curvature of the corpus. These results are in agreement with those of a previous report showing that gastric atrophy starts at the lower portion of the lesser curvature in the corpus, then extends to the upper portion and laterally involves the greater curvature[24]. The diagnosis for the CO whiteness grade seemed to be homogenous under magnified observations (see the figures). We diagnosed using maximum magnification at all sites; therefore, the CO whiteness types were recognized as a homogenous pattern using these narrow fields of view (approximately 2-mm squares).

The present study showed that the CO whiteness type had higher sensitivity and specificity than that reported

for conventional endoscopy[25,26] but lower sensitivity and specificity for detecting *H. pylori* infections than have been previously reported for ME assessment[13,14]. This discrepancy might have been caused by the differences in the *H. pylori* strain or immune responses. Our CO whiteness classification system was advantageous because it helped to predict the severity of inflammation and the *H. pylori*-induced activity in the gastric corpus.

Many reports have suggested that a relationship exists between differentiated-type gastric carcinoma and the severe atrophic gastritis caused by persistent *H. pylori* infection[2,23]. Conversely, undifferentiated-type gastric cancer is associated with *H. pylori*-induced active gastritis[23,27]. The present report showed that "DWP"-type COs that were accompanied by a histologically high grade of inflammation and activity were frequently observed in the gastric corpus of patients with undifferentiated-type gastric cancer; however, additional investigations are needed to clarify the relationship between CO whiteness and gastric cancer in a large population study.

The current study did not detect an area with round pits in 104/350 of the areas examined. As noted in earlier reports[12,21], the microsurface pattern changes a round pit pattern to vertical long pits, tubular and granular patterns with continuous *H. pylori* inflammation. Thus, in the case of severe endoscopic atrophy, tubular and granular patterns (but not pit patterns) were often observed *via* ME. Given the difficulty of assessing histological inflammation (which differs from histological glandular atrophy) under endoscopic observations, our results suggest that ME is a useful method for predicting *H. pylori* inflammation in detail. However, our ME classification might not be acceptable in cases with severe atrophy.

Another limitation of this study is its small number of patients. An analysis of patients with other *H. pylori*-related diseases is needed. In addition, assessments of the inter- and intra-observer variability with regard to the classification of CO whiteness are required to generalize the diagnostic ability of our findings. Another limitation is that we did not investigate the nature of the white substance in "DWP"-type COs. As described in the updated Sydney System[11], the marked neutrophil infiltration of foveolar lumens induced by *H. pylori*-infection might cause the formation of "pit abscesses". We speculate that the appearance of "DWP"-type COs is attributable to the severe degenerative and hypertrophic changes in surface epithelial cells that are accompanied by lymphocyte and neutrophil infiltration; additional analyses are needed to investigate this possibility.

In conclusion, we found that CO whiteness in ME images of the gastric corpus was correlated with histological findings of inflammation and activity. ME observation of CO whiteness might facilitate the histological diagnosis of the inflammation and activity induced by *H. pylori*.

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COMMENTS

Background

Magnifying endoscopy (ME) can be used for approximately x 80 magnified observation. Advances in ME have enabled the real-time observation of the microsurface structure and microvascular architecture of the gastric mucosa.

Research frontiers

Helicobacter pylori (*H. pylori*) infection causes chronic inflammation of the gastric mucosa. This persistent inflammation leads to morphological changes in gastric mucosa as observed *via* ME.

Innovations and breakthroughs

ME provides a detailed observation of *H. pylori*-induced gastritis, and the relationship between ME findings and histological gastritis was reported. Although many investigators have reported that the morphological changes identified *via* ME are closely associated with histological glandular atrophy and intestinal metaplasia, the data regarding the characteristics of these ME findings in *H. pylori*-induced gastritis are insufficient in cases of severe inflammation and activity. The current results indicate that the white gastric mucosa crypt openings observed *via* ME are useful for assessing histological inflammation and activity.

Applications

H. pylori infection causes chronic gastritis, gastric or duodenal ulcer, and carcinoma. Conventional standard endoscopy has shown a poor relationship between *H. pylori* infection and active *H. pylori* gastritis. The results enable the assessment of histological activity and inflammation without a biopsy.

Peer review

This article is well-written and well-designed for publication. It gives very new information for gastroenterologist.

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BRIEF ARTICLE

Stapled gastro/duodenojejunostomy shortens reconstruction time during pylorus-preserving pancreaticoduodenectomy

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Abstract

AIM: To investigate whether a stapled technique is superior to the conventional hand-sewn technique for gastro/duodenojejunostomy during pylorus-preserving pancreaticoduodenectomy (PpPD).

METHODS: In October 2010, we introduced a mechanical anastomotic technique of gastro- or duodenojejunostomy using staplers during PpPD. We compared clinical outcomes between 19 patients who underwent PpPD with a stapled gastro/duodenojejunostomy (stapled anastomosis group) and 19 patients who underwent PpPD with a conventional hand-sewn duodenojejunostomy (hand-sewn anastomosis group).

RESULTS: The time required for reconstruction was significantly shorter in the stapled anastomosis group than in the hand-sewn anastomosis group (186.0 ± 29.4 min *vs* 219.7 ± 50.0 min, $P = 0.02$). In addition, intraoperative blood loss was significantly less (391.0 ± 212.0 mL *vs* 647.1 ± 482.1 mL, $P = 0.03$) and the time to oral intake was significantly shorter (5.4 ± 1.7 d *vs* 11.3 ± 7.9 d, $P = 0.002$) in the stapled anastomosis group than in the hand-sewn anastomosis group. There were no differences in the incidences of delayed gastric emptying and other postoperative complications between the groups.

CONCLUSION: These results suggest that stapled gastro/duodenojejunostomy shortens reconstruction time during PpPD without affecting the incidence of delayed gastric emptying.

Key words: Pylorus-preserving pancreaticoduodenectomy; Stapled anastomosis; Gastrojejunostomy; Duodenojejunostomy; Delayed gastric emptying

Core tip: The operative procedure of pylorus-preserving pancreaticoduodenectomy (PpPD) includes reconstruction of the pancreatic, biliary, and digestive systems, thus requiring a significant amount of time. We compared clinical outcomes between 19 patients who underwent PpPD with a stapled gastro/duodenojejunostomy and 19 patients who underwent PpPD with a conventional hand-sewn duodenojejunostomy. We demonstrate that stapled gastro/duodenojejunostomy shortens reconstruction time during PpPD without affecting the incidence of delayed gastric emptying.

Sato N, Yabuki K, Kohi S, Mori Y, Minagawa N, Tamura T, Higure A, Yamaguchi K. Stapled gastro/duodenojejunostomy shortens reconstruction time during pylorus-preserving pancreatico-

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INTRODUCTION

Pancreaticoduodenectomy (PD) remains one of the major and challenging operations associated with a relatively high mortality and morbidity rate. The PD operative procedure includes reconstruction of the pancreatic, biliary, and digestive systems, thus requiring a significant amount of time. Because prolonged operative time has been demonstrated to be a risk factor for mortality and postoperative complications[1-3], efforts should be made to shorten the operative time by improving surgical skills and techniques.

The introduction of mechanical suture/stapling devices has provided surgeons with options for simple and sophisticated reconstruction methods in the field of gastrointestinal surgery. Recently, anastomotic techniques using staplers have been increasingly used for operations of the esophagus, stomach, and colorectum, particularly since the advent of laparoscopic surgery. In general, stapled anastomoses require less operative time and provide equal or better results in terms of the rate of leakage compared with hand-sewn anastomoses[4].

Although stapled anastomoses can be used for reconstruction of the alimentary tract in virtually all operations, only a few studies have described such a method in the setting of pancreatic resection[5,6]. We introduced a mechanical anastomotic technique of gastro- or duodenojejunostomy using staplers during pylorus-preserving pancreaticoduodenectomy (PpPD). In an attempt to investigate the feasibility and efficacy of stapled gastro/duodenojejunostomy, we compared the outcomes between the stapled and conventional hand-sewn anastomotic techniques in patients undergoing PpPD.

MATERIALS AND METHODS

Patients

The study included 38 patients (25 men and 13 women with a mean age of 66 years) who underwent PpPD for cancers of the pancreatic head, ampulla of vater, lower bile duct, and gallbladder; cystic neoplasms of the pancreas; neuroendocrine tumors; and others (chronic pancreatitis and duodenal submucosal tumor) at our institution between January 2009 and March 2012. Patients who underwent classical pancreaticoduodenectomy (whipple operation), subtotal stomach-preserving pancreaticoduodenectomy (SSPPD), and laparoscopy-assisted pancreaticoduodenectomy were excluded from this study. In October 2010, we altered the technique of alimentary tract reconstruction (gastro/duodenojejunostomy and Braun anastomosis) from the conventional hand-sewn technique to a mechanical anastomosis technique using staplers. The patients were divided into two groups according to the method of alimentary tract reconstruction: 19 patients who underwent hand-sewn duodenojejunostomy (hand-sewn anastomosis group) and 19 patients who underwent stapled gastro/duodenojejunostomy (stapled anastomosis group).

Operative procedure

The detailed PpPD operative procedure was previously described elsewhere[7]. We routinely use the modified Child method for reconstruction. After removal of the pancreatic head, the anal stump of the jejunum was lifted through the mesocolon right to the middle colic artery. The pancreaticojejunostomy was performed using a modified Kakita's method[8]. A mucosa-to-mucosa anastomosis of the pancreaticojejunostomy was performed with interrupted sutures using 5-0 monofilament absorbable sutures (PDS, Ethicon Inc., Tokyo, Japan). A pancreatic tube was placed from the jejunum to the main pancreatic duct. The hepaticojejunostomy was performed by interrupted sutures using 4-0 monofilament absorbable sutures (PDS II, Ethicon Inc.), and the biliary tube was placed from the jejunal lumen to the hepatic duct of the liver. The biliary and pancreatic tubes were taken from the jejunal stump to the outside of the body.

The conventional hand-sewn duodenojejunostomy (end-to-side anastomosis) was performed by a two-layer Albert-Lembert method (whole layer, running sutures of 4-0 PDS, and seromuscular layer, interrupted sutures of 4-0 silk). A Braun anastomosis (side-to-side jejunojejunostomy) was also performed using the same method.

The stapled gastro/duodenojejunostomy was performed using a circular stapler (CDH25, Ethicon Inc.). For duodenojejunostomy, the anvil was inserted into the stomach through a small gastrotomy incision, moved to the duodenum, and fixed at the duodenal stump by a purse-string suture. A circular stapler was inserted into the jejunal loop through a small opening and connected to the anvil to complete the anastomosis (Figure 1A). For gastrojejunostomy, the anvil was inserted into and fixed at the jejunum. A circular stapler was inserted into the stomach through a small opening made in the anterior wall of the antrum and connected to the anvil to complete the anastomosis at the posterior wall of the stomach (Figure 1B). The openings made in the stomach or jejunum were closed by either running or interrupted sutures. A Braun anastomosis (side-to-side jejunojejunostomy) was also performed mechanically using a linear stapler (GIA, Covidien Japan, Tokyo, Japan). The gastro/duodenojejunostomy was made *via* an antecolic route in both the hand-sewn and stapled techniques.

Drainage tubes were placed at the posterior aspect of the hepaticojejunostomy and the anterior side of the pancreaticojejunostomy. They were drained to the outside of the abdomen. Biliary and pancreatic tubes were placed from the stump of the jejunum using Witzel's method.

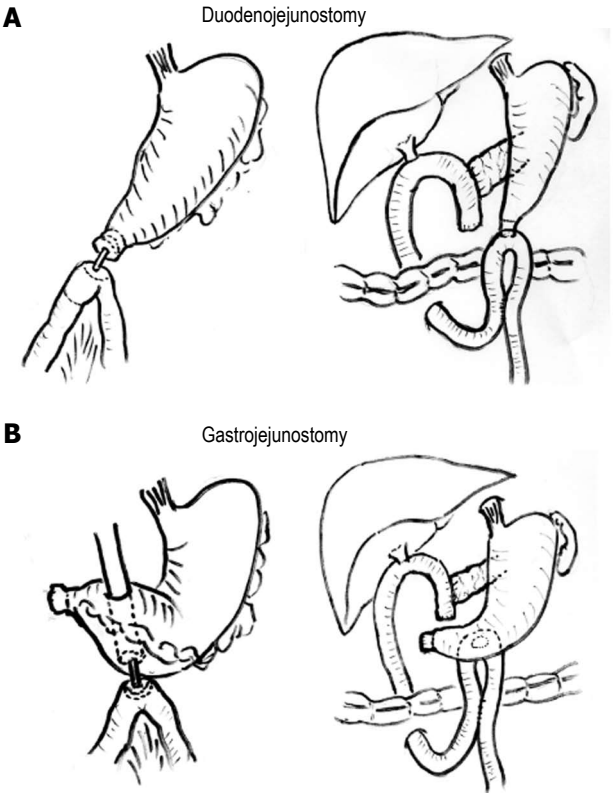

Figure 1 Schema of stapled gastro/duodenojejunostomy. A: Stapled duodenojejunostomy. The anvil was inserted into the stomach through a small gastrotomy incision, moved to the duodenum, and fixed at the duodenal stump by a purse-string suture. A circular stapler was inserted into the jejunal loop through a small opening and connected to the anvil for completion of anastomosis; B: Stapled gastrojejunostomy. The anvil was inserted into and fixed at the jejunum. A circular stapler was inserted into the stomach through a small opening made in the anterior wall of the antrum and connected to the anvil for completion of anastomosis at the posterior wall of the stomach.

All procedures were performed by one of the authors (Yamaguchi K).

Postoperative management

The nasogastric tube was removed when the amount of drainage fluid was less than 200 mL/d and the nature of the fluid was not bloody. Liquid oral intake was resumed after gas passage if there was no evidence of pancreatic fistula. Delayed gastric emptying (DGE) was defined based on the International Study Group on Pancreatic Surgery classification[9]. Grade B (unable to tolerate solid oral intake by POD 14 with/without vomiting) or C (unable to tolerate solid oral intake by POD 21 with/without vomiting) was considered clinically relevant. Requirement of nasogastric tube reinsertion after POD 7 was also considered DGE.

Statistical analysis

All statistical analyses were performed using JMP 10 software (SAS Institute Inc., Cary, NC). Categorical variables were analyzed using Fisher's exact probability test, and continuous variables were analyzed using the Mann-Whitney *U*-test. A *P* value of less than 0.05 was considered statistically significant.

Table 1 Patient characteristics in the stapled anastomosis group and hand-sewn anastomosis group

	Stapled anastomosis group	Hand-sewn anastomosis group	*P* value
Age (yr, mean ± SD)	67.2 ± 11.7	65.2 ± 11.2	0.59
Gender (M/F)	11/8	14/5	0.50
ASA			
1	4	1	
2	10	16	
3	5	2	0.11
Comorbidities *n* (%)	8 (42)	8 (42)	1.00
Diabetes *n* (%)	5 (26)	6 (32)	1.00
Previous history of upper abdominal surgery	2 (11)	2 (11)	1.00
Preoperative albumin level (g/dL, mean ± SD)	3.96 ± 0.35	3.74 ± 0.44	0.10
Disease			
Pancreatic cancer	6	4	0.08
Ampullary cancer	3	6	
Lower bile duct cancer	2	2	
Gallbladder cancer	1	0	
IPMN	3	3	
SCN	0	1	
PNET	3	0	
Duodenal GIST	0	1	
Mass-forming pancreatitis	0	1	
Others	1	1	

ASA: American Society of Anesthesiologists; IPMN: Intraductal papillary mucinous neoplasms; SCN: Solid cystic neoplasms; PNET: Pancreatic neuroendocrine tumor; GIST: Gastrointestinal stromal tumor.

RESULTS

Patient characteristics in the stapled anastomosis group and hand-sewn anastomosis group

The patient characteristics in the stapled anastomosis group and the hand-sewn group are shown in Table 1. There were no significant differences in age, gender, American Society of Anesthesiologists (ASA) score, comorbidities, history of diabetes mellitus, previous history of upper abdominal surgery, preoperative level of serum albumin (as a nutritional status), or disease distribution between the groups (Table 1).

Operative variables and postoperative outcomes in the stapled anastomosis and hand-sewn anastomosis groups

The operative variables were compared between the groups (Table 2). The mean operative time tended to be shorter in the stapled anastomosis group than in the hand-sewn group (500 min *vs* 530 min), although the difference was not statistically significant. However, the time required for reconstruction (removal of the pancreatic head to completion of surgery) was significantly shorter in the stapled anastomosis group than in the hand-sewn anastomosis group (186.0 ± 29.4 min *vs* 219.7 ± 50.0 min, $P = 0.02$). The total amount of blood loss during

Table 2 Operative and postoperative outcomes in the stapled anastomosis group and hand-sewn anastomosis group

	Stapled anastomosis group	Hand-sewn anastomosis group	*P* value
Total operative time (min)	500 ± 68.3	530 ± 88	0.33
Reconstruction time (min)	186 ± 29.4	219.7 ± 50	0.02
Intraoperative blood loss (mL)	391 ± 212.3	647.1 ± 482.1	0.03
Duration of nasogastric tube insertion (d)	1.42 ± 1.22	1.3 ± 0.58	0.67
Resuming liquid oral intake (POD)	5.4 ± 1.7	11.3 ± 7.89	0.002
Starting solid diet (POD)	13.8 ± 8.56	17.7 ± 11.2	0.26
Postoperative complications	6 (31.6)	12 (63.2)	0.10
Delayed gastric emptying	1 (5.3)	3 (15.8)	0.60
Pancreatic anastomotic leakage/pancreatic fistula	1 (5.3)	3 (15.8)	0.60
Intraabdominal abscess	1 (5.3)	4 (21.1)	0.34
Postoperative hospital stay (d)	35.8 ± 12	39.4 ± 15.4	0.67

Values shown are mean ± SD or *n* (%).

surgery was significantly less in the stapled anastomosis group than in the hand-sewn group (391.0 ± 212.0 mL *vs* 647.1 ± 482.1 mL, $P = 0.03$).

We next compared the postoperative outcomes between the groups (Table 2). Although there was no difference in the duration of nasogastric tube insertion between the groups, the time from surgery to resuming liquid oral intake was significantly shorter in the stapled anastomosis group than in the hand-sewn anastomosis group (5.4 ± 1.7 d *vs* 11.3 ± 7.9 d, $P = 0.002$). Overall, postoperative complications occurred in 18 patients, including 6 patients (31.6%) in the stapled anastomosis group and 12 patients (63.2%) in the hand-sewn group (not significant). Among the complications, pancreatic fistula/anastomotic leakage occurred in 1 patient (5.3%) in the stapled anastomosis group and in 3 patients (15.8%) in the hand-sewn anastomosis group (not significant). Intra-abdominal abscess was observed in 1 patient (5.3%) in the stapled anastomosis group and in 4 patients (21.1%) in the hand-sewn anastomosis group (not significant). No patient in either group developed leakage of the gastro/duodenojejunostomy. DGE was observed in 1 patient (5.3%; grade C) in the stapled anastomosis group and in 3 patients (15.8%; grade B in 1 patient and grade C in 2 patients) in the hand-sewn anastomosis group (not significant). There was no difference in the duration of postoperative hospital stay between the groups. No 30-d postoperative mortality was observed in either group.

DISCUSSION

In this study, we compared clinical outcomes between the stapled and conventional hand-sewn alimentary tract anastomotic techniques in a total of 38 patients undergoing PpPD. The major findings obtained were as follows: (1) the reconstruction time was significantly shorter in the stapled anastomosis group than in the hand-sewn anastomosis group; (2) intraoperative blood loss was significantly less and the time from the operation to resuming oral intake was significantly shorter in the stapled anastomosis group than in the hand-sewn anastomosis group; and (3) there were no differences in the incidences of delayed gastric emptying and other postoperative complications between the groups. These findings suggest that stapled gastro/duodenojejunostomy shortens reconstruction time during PpPD without affecting the incidence of delayed gastric emptying.

Despite the disseminated use of mechanical suture/stapling devices in the field of gastrointestinal surgery, the application of these devices to reconstruction in PD remains uncommon. To date, only one Japanese group has described the method of gastro/duodenojejunostomy using staplers during PD[5,6]. In their method of stapled reconstruction, an antecolic gastrojejunostomy or duodenojejunostomy was performed by Roux-en-Y reconstruction using a linear or circular stapler[5], which is slightly different from our technique in terms of dividing the jejunum for Roux-en-Y loop in their technique. The authors also demonstrated that the incidence of delayed gastric emptying was significantly lower in patients who underwent stapled gastro/duodenojejunostomy than in those who underwent hand-sewn reconstruction[6]. In the present study, we also found that the time to resuming oral intake was significantly shorter in the stapled anastomosis group than in the hand-sewn anastomosis group. Although the exact mechanism for improved oral intake and DGE by mechanical anastomosis is unknown, one possible explanation is that edema around the anastomotic site can be prevented by stapled anastomosis, particularly in the early postoperative period.

It has been reported that prolonged operative time is associated with an increased incidence of postoperative mortality and morbidity after PD[1-3]. According to a recent study in a total of 4817 patients undergoing PD[1], longer operative time was linearly associated with increased 30-d morbidity ($P < 0.001$) and mortality ($P < 0.01$). Therefore, it is important for surgeons to avoid prolonged operative time by improving surgical techniques. With an aim to shorten the operative time, we introduced a technique of stapled gastro/duodenojejunostomy and Braun anastomosis. Although the difference in total operative time did not reach statistical significance, the reconstruction time was significantly shorter (by approximately 30 min) in the stapled anastomosis group than in the hand-sewn group. Importantly, intraoperative blood loss was significantly less in the stapled anastomosis group than in the hand-sewn anastomosis group (a mean volume of 391 mL *vs* 647 mL). Because a variety of factors can affect the volume of intraoperative blood loss, this difference is unlikely to be attributable solely to the different reconstruction techniques used. Furthermore, because of the small number of patients in each group, the mean volume of blood loss can be affected by a small number of patients with an unexpectedly large intraoperative blood loss. However, the reduced reconstruction time observed using rapid stapling devices may

have played a role, at least in part, in the reduced blood loss observed during surgery.

One concern that might be raised against our technique of stapled gastrojejunostomy is the significance of preserving the pylorus because food may not pass the pylorus in this anastomosis. By analyzing the plasma motilin concentration and phase Ⅲ activity of the migrating motor complex of the stomach, it has been shown that preservation of the duodenum is important to maintain gastric motility and to prevent so-called "gastroparesis"[10,11]. In contrast to these findings, several lines of evidence have suggested that PD with pylorus resection (SSPPD) is comparable or even superior to that with pylorus preservation (PpPD) in terms of dietary intake and DGE[12-15]. Therefore, the clinical relevance of pylorus preservation requires further investigation.

Our study had several limitations. First, this study was a retrospective and historical cohort analysis; therefore, the possibility of bias cannot be eliminated. Second, the small number of patients in each anastomosis group may have underpowered our statistical evaluation. Third, we were unable to perform a cost comparison between the groups because of a lack of information. Therefore, to precisely determine the exact benefits of stapled gastro/duodenojejunostomy during PpPD, a prospective randomized trial, including an analysis of cost effectiveness, should be performed in the future.

In conclusion, our preliminary results suggest that stapled gastro/duodenojejunostomy is a feasible technique that could shorten the reconstruction and operative time during PpPD without increasing the incidence of postoperative complications including delayed gastric emptying. More recently, a mechanical anastomosis technique using a circular stapler has been applied to the hepaticojejunostomy during PD in selected patients with a dilated bile duct[16]. Thus, the introduction and standardization of these stapled anastomosis techniques can shorten the reconstruction time during PD and ultimately reduce the incidence of postoperative mortality and morbidity.

COMMENTS

Background

The operative procedure of pylorus-preserving pancreaticoduodenectomy (PpPD) includes reconstruction of the pancreatic, biliary, and digestive systems, thus requiring a significant amount of time.

Research frontiers

Although stapled anastomosis can be applied to the reconstruction of alimentary tract in virtually all operations, only a few studies have described the reconstruction method of alimentary tract using staplers in the setting of pancreatic resection.

Innovations and breakthroughs

The authors compared clinical outcomes between 19 patients who underwent PpPD with a stapled gastro/duodenojejunostomy and 19 patients who underwent PpPD with a conventional hand-sewn duodenojejunostomy.

Applications

This study showed that stapled gastro/duodenojejunostomy shortens reconstruction time during PpPD without affecting the incidence of delayed gastric emptying.

Peer review

This is a very novel report about the pylorus-preserving pancreaticoduodenectomy.

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BRIEF ARTICLE

Sphincterotomy by triple lumen needle knife using guide wire in patients with Billroth Ⅱ gastrectomy

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Abstract

AIM: To investigate the usefulness of a guide wire and triple lumen needle knife for removing stones in Billroth Ⅱ (B-Ⅱ) gastrectomy patients.

METHODS: Endoscopic sphincterotomy in patients with B-Ⅱ gastrectomy is challenging. We used a new guide wire technique involving sphincterotomy by triple lumen needle knife through a forward-viewing endoscopy. This technique was performed in nine patients between August 2010 and June 2012. Sphincterotomy as described above was performed. Adequate sphincterotomy, successful stone removal, and complications were investigated prospectively.

RESULTS: Sphincterotomy by triple lumen needle knife using guide wire was successful in all nine patients. Sphincterotomy started towards the 4-5 o'clock direction and continued to the upper margin of the papillary roof. Complete stone removal in one session was achieved in all patients. There were no procedure related complications, such as bleeding, pancreatitis, or perforation.

CONCLUSION: In patients with B-Ⅱ gastrectomy, guide wire using sphincterotomy by triple lumen needle knife through a forward-viewing endoscopy seems to be an effective and safe procedure for the removal of common bile duct stones.

Key words: Billroth Ⅱ gastrectomy; Endoscopic sphincterotomy; Forward-viewing endoscopy; Guide wire; Triple lumen needle knife

Core tip: Guide wire using sphincterotomy by triple lumen needle knife through a forward-viewing endoscopy seems to be a safe, easy, and effective method for removing common bile duct stones in patients with B-Ⅱ gastrectomy.

Park SB, Kim HW, Kang DH, Choi CW, Yoon KT, Cho M, Song BJ. Sphincterotomy by triple lumen needle knife using guide wire in patients with Billroth Ⅱ gastrectomy. *World J Gastroenterol* 2013; 19(48): 9405-9409 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i48/9405.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i48.9405

INTRODUCTION

Endoscopic sphincterotomy (EST) is essential for the endoscopic removal of common bile duct (CBD) stones. However, EST is more difficult in an altered anatomy, such as Billroth Ⅱ (B-Ⅱ) gastrectomy, in which the major papillae are inverted[1]. To overcome this problem, several techniques

and specialized accessories have been devised, including Soehendra sphincterotome[2], Sohma sphincterotome[3], needle knife sphincterotomy guided by a biliary endoprosthesis[4,5], S-shape sphincterotome[6], and papillary balloon dilation[7,8]. These methods may be more difficult to control and frequently produce complications[5-7].

Large balloon papillary dilation after minor EST with newly developed papillotomes, such as rotatable papillotome[9] and scissors papillotome[10], has been reported for the removal of CBD stones in B-Ⅱ gastrectomy. The triple lumen needle knife, which is capable of accepting a guidewire in one channel while simultaneously injecting and/or cutting in other lumens, is used primarily for precut sphincterotomy or fistulotomy in difficult cases of deep cannulation of the bile duct. These characteristics allow the simultaneously maintenance of the guide wire in CBD and sphincterotomy without the assistance of other accessories. Additionally, a guide wire positioned in the CBD can make sphincterotomy by triple lumen needle knife easier.

Herein, we detail our technique and present experiences of guide wire sphincterotomy using a triple lumen needle knife through forward-viewing endoscopy in patients with B-Ⅱ gastrectomy.

MATERIALS AND METHODS

Patients

From August 2010 to June 2012, endoscopic retrograde cholangiopancreatography (ERCP) for removal of CBD stones was performed on 469 patients. Of these patients, 20 with B-Ⅱ gastrectomy underwent ERCP. Eleven patients who had previous EST procedures (n = 5), failure to reach major papilla (n = 4), and needle knife fistulotomy due to difficult cannulation (n = 2) were excluded. The remaining nine patients (6 men and 3 women; mean age 66.3 years) were enrolled. The same endoscopist performed all procedures. All patients provided written informed consent for their participation and the study was approved by the Institutional Review Board of Pusan National University Yansan Hospital.

Technique

All ERCP procedures were performed under conscious sedation and coverage using prophylactic antibiotics with a model GIF-H260 cap-attached forward-viewing endoscopy apparatus (Olympus Optical, Tokyo, Japan). A transparent cap (Distal Attachments D-201-11804; Olympus) was attached to the tip of the endoscope. Selective cannulation of the CBD was achieved using a cannulation catheter with a straight tip. A 0.025-inch guide wire (Jagwire; Boston Scientific, Natick, MA) was advanced through the catheter into the CBD. The catheter was then removed, and a triple lumen needle knife (Microknife™ XL; Boston Scientific) was introduced into the major papilla over the guide wire. The needle tip was controlled with a length of 2-3 mm (Figure 1A), and sphincterotomy was performed along the guide wire as a guidance mark directed at 4-5 o'clock with sophisticated maneuver of the needle knife and endoscopy (Figure 1B). The model PSD-30 electrosurgical unit (Olympus) was used at a setting of blended one current with a power setting of 30 W/s for both the cutting and coagulation currents (cut: coagulation ratio of 3:1). During sphincterotomy, the pattern of cutting in the current method closely resembles the action of a pair of cutting scissors. Although the direction of the needle tip was toward the 1-2 o'clock direction, sphincterotomy toward the 4-5 o'clock direction was possible because the guide wire acted as a guide when the needle knife approached the bile duct pathway. At this time, the cap enabled us to perform safe and effective sphincterotomy by keeping a visual field during the procedure. After sphincterotomy to the upper margin of the papillary roof (Figure 1C), a stone retrieval basket or balloon catheter was used to extract CBD stones (Figure 1D). In cases when EST was insufficient in extracting the stone, papillary balloon dilation or mechanical lithotripsy was used in an attempt to removal the stone. To assess the efficacy and safety of this technique, we evaluated the sphincterotomy of the desired direction, use of balloon dilation or mechanical lithotripsy, successful stone removal, and complications after ERCP.

RESULTS

ERCP with B-Ⅱ gastrectomy was performed in 20 patients by the same endoscopist. Nine patients (6 men and 3 women) underwent EST using a guide wire and triple lumen needle knife sphincterotomy. The mean age of patients was 66.3 (range, 56 -85) years. The mean size and number of stones were 7.89 (range, 5-12) and 1.78 (range, 1-4) mm, respectively (Table 1).

Sphincterotomy in the 4-5 o'clock direction and to the upper margin of the papillary roof were successful in all nine patients (100%). Complete endoscopic stone removal was achieved in a single session in all patients. Papillary balloon dilatation was performed in only one patient owing to the large CBD stones (12 mm diameter) and stenosis in the distal CBD they exhibited. No mechanical lithotripsy was performed in any patient (Table 1). Serum amylase and lipase were measured before and after the procedure (4 and 24 h, respectively). Complete blood count and a liver function test were performed the morning after the procedure. There were no complications, such as bleeding, pancreatitis, or perforation.

DISCUSSION

Diagnostic and therapeutic ERCP in patients with B-Ⅱ gastrectomy can be hindered by difficulties in the identification and intubation of the afferent loop, negotiation of abrupt turns in the afferent loop, cannulation, and adequate sphincterotomy of papilla due to inverted position[11-14]. In particular, the standard pull-type sphincterotome cannot cut toward the 6 o'clock position[15,16]. To

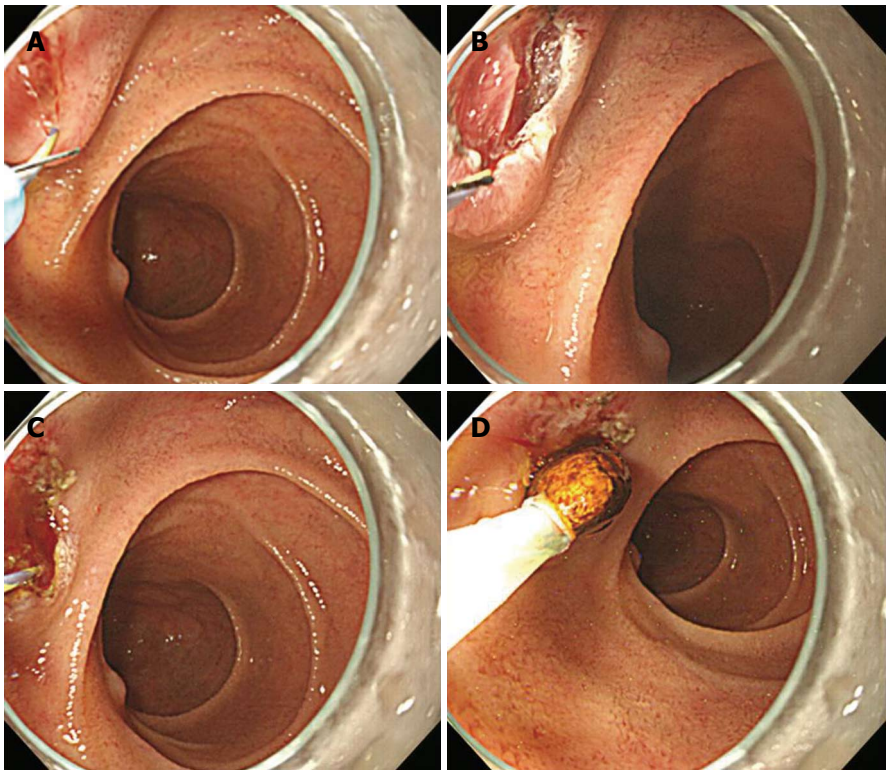

Figure 1 Guide wire using sphincterotomy by triple lumen needle knife. A: A triple lumen needle knife was introduced into the major papilla over the guidewire and the tip of needle was then controlled with 2-3 mm length; B: Sphincterotomy was performed along the guide wire as a guidance mark directed in the 4-5 o'clock direction with sophisticated maneuver of needle knife and endoscopy; C: Sphincterotomy to the upper margin of the papillary roof was performed; D: A stone was removed by basket without balloon dilation or mechanical lithotripsy.

Table 1 Baseline characteristics of patients and treatment outcomes

			Stone					
Patient No.	Age (yr)	Sex	Size, mm	No.	Procedure sessions	Balloon dilation (size, mm)	Stone removal	Complications
1	63	F	12	4	1	12	Success	None
2	69	M	5	2	1	0	Success	None
3	56	M	6	2	1	0	Success	None
4	68	F	10	2	1	0	Success	None
5	67	M	5	1	1	0	Success	None
6	65	M	8	1	1	0	Success	None
7	65	M	10	1	1	0	Success	None
8	59	F	10	2	1	0	Success	None
9	85	M	5	1	1	0	Success	None

overcome this problem, several devices and techniques have been developed using the push-type papillotome, such as the Sohma[3] and Soehendra sphincterotomes[2]. These refinements allow proper orientation of the wire for the CBD and needle knife with guided techniques using a nasobiliary drain[17], cannula, or endoprosthesis[5]. However, effective push-type sphincterotomes or needle knives are, as yet, not as readily available as the standard pull-type sphincterotome.

Recently devised methods to resolve these problems rely on large balloon papillary dilation after minor EST with newly developed papillotomes[18-21], such as the rotatable papillotome[9] and scissors papillotome[10]. These methods are user-friendly, but difficult to use in performing major EST.

Another problem is the difficulty of handling the side-viewing duodenoscope through the afferent loop in a retrograde maneuver; the result is a high rate of failed procedures and serious complications that include perforation of the small bowel[22-24]. The side-viewing duodenoscope has the advantages of allowing an en-face view of papilla and an elevator to adjust the direction of accessories. However, the apparatus is not useful in B-Ⅱ anastomosis and increases the risk of perforation while passing the tortuous jejunum[14] and in cases of previous jejunal enteroanastomosis (Braun's anastomosis)[25].

This method contrasts with existing methods in three ways. First, a triple lumen needle knife was used instead of a push-type sphincterotome or rotatable papillotome. The obvious advantage of this knife is the simultaneous

use of the needle knife and guide wire through the same device, which provides an indication of where and how deeply to cut, as well as avoiding blind or inappropriate cutting under direct vision of the cutting device and the presence of a clear guide. This enables a large sphincterotomy that can still be safely performed even when done by a less experienced endoscopist. Actually, sphincterotomy in all patients reached the upper margin of the papillary roof and most CBD stones were removed without papillary balloon dilatation or mechanical lithotripsy, although most were small. These findings clearly showed different results compared with other studies[9,10]. In the current study, papillary large balloon dilation was performed in one patient as a rescue method due to a large CBD stone and distal CBD stricture, but mechanical lithotripsy was not necessary. Additionally, bleeding, pancreatitis and perforation after sphincterotomy did not occur in all patients.

Secondly, conventional guide wire was used instead of nasobiliary drain, a cannula, or endoprosthesis. A guide wire already inserted in the bile duct can reduce the time of removal and subsequent reintroduction of the endoscope or insertion of a plastic stent. Also, this technique is cost-effective compared with endoprosthesis-guided sphincterotomy using a plastic stent, as a guide wire permits directed movement in the bile duct and control of the depth of incision, similar to the role of a plastic stent in endoprosthesis-guided sphincterotomy.

Thirdly, a forward-viewing endoscope was used instead of the side-viewing version. The forward-viewing endoscope in patients with Billroth Ⅱ gastrectomy makes selective bile duct cannulation easier, as the endoscope and cannula are in line with the CBD[13,26]. Therefore, the lack of an elevator in forward-viewing endoscopes is only a slight disadvantage, and not a major factor in determining the success rate of cannulation and subsequence procedure. Also, a forward-viewing endoscope makes it easier to introduce the afferent loop and find the correct route, as well as making it easier to control for negotiating the acute angles of the anastomoses than side-viewing endoscope. These advantages were related with low complication rates in another study[14], and no complications, such as perforation, were noted in the current study.

Despite the several advantages of the current method, some problems remain. If the endoscopic approach to major papilla is difficult, biliary cannulation and sphincterotomy are also difficult. Therefore, for effective application of this technique, the endoscopy tip must be approached near the major papilla.

In patients with B-Ⅱ gastrectomy, we do not yet know which type of sphincterotomy is superior. The several techniques and accessories each have their own drawbacks. Currently, treatment strategy guided by personal preference or level of experience with specific techniques may be necessary. Our results support the use of the guide wire and triple lumen needle knife technique previously described as one option in patients with B-Ⅱ gastrectomy. The technique is especially attractive when less experienced endoscopists perform sphincterotomy in patients with B-Ⅱ gastrectomy.

In conclusion, guide wire using sphincterotomy by triple lumen needle knife through a forward-viewing endoscopy seems to be a safe, easy, and effective method for removing CBD stones in patients with B-Ⅱ gastrectomy. Further studies may be needed to compare the safety and efficacy of the technique in order to confirm our findings.

COMMENTS

Background

Endoscopic sphincterotomy is more difficult in an altered anatomy; the authors suggested a new technique by guide wire and triple lumen needle knife.

Research frontiers

Recently introduced methods have their limitations due to their difficultly in being controlled. Guide wire technique involving sphincterotomy by triple lumen needle knife through a forward-viewing endoscopy can be used more easily.

Innovations and breakthroughs

The authors demonstrated the effectiveness and safety of removing common bile duct (CBD) stones in patients with Billroth Ⅱ (B-Ⅱ) gastrectomy, although those that were removed were small in size.

Applications

Guide wire using sphincterotomy by triple lumen needle knife through a forward-viewing endoscopy are a feasible and effective intervention for removing CBD stones in patients with B-Ⅱ gastrectomy.

Peer review

This is a clinical study to evaluate the efficiency of a new technique for removing common bile duct stones in patients with gastrectomy. This is an important and novel topic in clinics.

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BRIEF ARTICLE

Improvement of type 2 diabetes mellitus after gastric cancer surgery: Short-term outcome analysis after gastrectomy

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Abstract

AIM: To evaluate the effect of gastrectomy on diabetes control in patients with type 2 diabetes mellitus and early gastric cancer.

METHODS: Data from 64 patients with early gastric cancer and type 2 diabetes mellitus were prospectively collected. All patients underwent curative gastrectomy (36 subtotal gastrectomy with gastroduodenostomy, 16 subtotal gastrectomy with gastrojejunostomy, 12 total gastrectomy) and their physical and laboratory data were evaluated before and 3, 6 and 12 mo after surgery.

RESULTS: Fasting blood glucose (FBS), HbA1c, insulin, C-peptide, and homeostasis model assessment-estimated insulin resistance were significantly improved 3 mo after surgery, regardless of operation type, and the significant improvement in all measured values, except HbA1c, was sustained up to 12 mo postoperatively. Approximately 3.1% of patients stopped diabetes medication and had HbA1c < 6.0% and FBS < 126 mg/dL. 54.7% of patients decreased their medication, and had reduced FBS or HbA1c. In multivariate analysis, good diabetic control was not associated with operation type, but was associated with diabetes duration.

CONCLUSION: Diabetes improved in more than 50% of patients during the first year after gastric cancer surgery. The degree of diabetes control was related to diabetes duration.

Key words: Type 2 diabetes mellitus; Gastrectomy; Gastric cancer; Short-term outcome; Glucose control

Core tip: Diabetes mellitus is one of the most important health problems and has an impact on the quality of life of gastric cancer patients as well as ordinary individuals. In this study, we evaluated the impact of conventional gastric cancer surgery on type 2 diabetes. Gastric cancer surgery led to a significant improvement in type 2 diabetes during the first year after surgery, and the degree of diabetes control was related to diabetes duration.

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INTRODUCTION

Gastric cancer is a leading cause of cancer death worldwide and is one of the most common cancers in Korea[1,2]. During the last several decades, there has been notable progress in the field of gastric cancer diagnosis and treatment, as indicated by the increasing proportion of early gastric cancers and improved survival rate[3,4]. Therefore, postoperative quality of life as well as the appropriate surgical treatment for a cure has become very important.

However, the increase in older patients due to an aging population and the increased incidence of lifestyle-related diseases including diabetes, hypertension, cardiovascular disease, and hypercholesterolemia make postoperative healthcare more difficult and complicated. Diabetes mellitus (DM) is one of the most difficult health problems worldwide as it is a multi-factorial chronic disease. In Korea, the prevalence of diabetes has increased dramatically from less than 1.5% in the 1970s to approximately 10% in the 2000s, and it is currently the 5th most common cause of death[5,6]. Although the prevalence of diabetes in gastric cancer patients has not been reported, it may be similar to that of the general population. After gastric cancer surgery, many surgeons focus on improving the nutritional status of patients rather than controlling diabetes as the main problem after gastric cancer surgery is weight loss. In addition, the beneficial effects of weight loss often lead to improvement in hyperglycemia, hypercholesterolemia, and hypertension.

Recently, metabolic surgery has become an appealing treatment option for patients with type 2 DM. The effects of metabolic surgery and the mechanism of action have been reported in several studies[7-9]. Although the purpose of metabolic surgery and gastric cancer surgery is completely different, there is a connection between the two procedures clinically and technically. In line with this thinking, the organized evaluation of the impact of conventional gastric cancer surgery on diabetes appears to be necessary. Such an evaluation will allow surgeons to select a favorable reconstruction type after gastrectomy in gastric cancer patients with diabetes. Therefore, in this study, we investigated the short-term effect of three types of routine gastric cancer surgery on type 2 DM.

MATERIALS AND METHODS

Patients

We analyzed the data from 64 early gastric cancer patients with type 2 DM who underwent curative gastrectomy for primary gastric cancer between 2009 and 2010. All of the patients had been diagnosed with type 2 DM after 40 years of age and were taking medication before the diagnosis of early gastric cancer. All the data were collected prospectively. Patients with the following conditions were excluded: (1) other malignancies; (2) pre- and postoperative chemotherapy or chemoradiotherapy; (3) other endocrine disorders such as thyroid or adrenal disease; (4) moderate to severe cardiovascular, pulmonary or renal disease; and (5) active infection. This study was reviewed and approved by the Institutional Review Board of Severance Hospital, Yonsei University College of Medicine, and written informed consent was obtained from all patients prior to surgery.

Surgical procedures

Subtotal or total gastrectomy was performed according to the tumor location. Billroth I or Ⅱ reconstruction was carried out after subtotal gastrectomy and Roux-en-Y esophagojejunostomy after total gastrectomy. In Billroth I reconstruction, the duodenum was transected 1 cm distal to the pyloric ring and gastroduodenostomy was performed using a circular stapler. In Billroth Ⅱ reconstruction, the length from the ligament of Treitz was approximately 20 cm and gastrojejunostomy was performed using a linear stapler. After total gastrectomy, the length of the esophagojejunostomy to jejunojejunostomy was approximately 45 cm, and the length of the ligament of Treitz to jejunojejunostomy was 20-25 cm. Esophagojejunostomy was performed using a circular stapler. Generally, D1+β or D2 lymph node dissection was performed according to the guidelines of the Japanese Gastric Cancer Association[10].

Evaluation of clinical variables and biochemical data during follow-up

All of the clinical and laboratory data were collected and recorded prospectively at each point of the routine follow-up. Blood samples were obtained after an overnight fast. Patients visited the hospital at 3-, 6- and 12-mo time points during the first year after gastrectomy for a physical examination, laboratory tests, imaging, and/or endoscopy. The variables for evaluating the status of glucose control included body weight, body mass index, biochemical data [serum glucose, HbA1c, insulin, C-peptide, homeostasis model assessment-estimated insulin resistance (HOMA-IR), low-density lipoprotein (LDL)-cholesterol, high-density lipoprotein (HDL)-cholesterol, triglyceride], and medication status, and were recorded preoperatively and 3, 6 and 12 mo after surgery. All of the patients completed the study.

The degree of diabetes control was divided into three groups: (1) Remission: No medication and FBS < 126 mg/dL and HbA1c < 6.0%; (2) Improved: Reduced medication and one of the following: FBS or HbA1c reduction; and (3) Stationary: No change of medication, or patients excluded from the improved and remission categories.

Statistical analysis

Statistical analysis was carried out using SPSS® version

Table 1 Patient demographics *n* (%)

Variables	*n* = 64
Age (yr)	62.7 ± 8.6
Range	45-77
Sex	
Male	43 (67.2)
Female	21 (32.8)
Body mass index (kg/m^2)	24.7 ± 3.4
Range	18.6-38.1
Smoking history	
No	36 (56.2)
Yes	28 (43.8)
Alcohol history	
No	38 (59.4)
Yes	26 (40.6)
Family history of DM	
No	52 (81.2)
Yes	12 (18.8)
Operation type	
STG B Ⅰ	36 (56.2)
STG B Ⅱ	16 (25.0)
TG	12 (18.8)
Surgical approach	
Open surgery	28 (43.7)
Laparoscopic surgery	36 (56.3)
Duration of DM (yr)	6.6 ± 6.4
Range	0.5-25
DM medication	
Oral hyperglycemic agents	60 (93.7)
Insulin only	1 (1.6)
Both	3 (4.7)

Data are expressed as absolute numbers (percentage) or mean ± SD. DM: Diabetes mellitus; STG B Ⅰ: Subtotal gastrectomy with Billroth I anastomosis; STG B Ⅱ: Subtotal gastrectomy with Billroth Ⅱ anastomosis; TG: Total gastrectomy.

15.0 for Windows® (SPSS, Chicago, IL, United States). Categorical variables were compared using the chi-square or Fisher exact test, and continuous data were compared by the Mann-Whitney *U* test. The Kruskal-Wallis test was used to compare biochemical data among the three surgical groups at the same evaluation time. The paired *t* test was used to compare preoperative and postoperative 12-mo biochemical data. Data are presented as mean ± SD. Binary logistic regression analysis was used to identify the independent variables associated with the degree of diabetic control. *P* values < 0.05 were considered statistically significant.

RESULTS

Patient demographics

The preoperative patient demographics are shown in Table 1. The mean body mass index (BMI) was 24.7 ± 3.4 kg/m^2. After subtotal gastrectomy, gastroduodenostomy (STG B Ⅰ) was performed in 36 patients and gastrojejunostomy (STG B Ⅱ) in 16 patients. Twelve patients underwent total gastrectomy with Roux-en-Y esophagojejunostomy (TG). Of the patients in this study, 18.8% had a family history of diabetes in first degree relatives, 93.7% were taking oral hyperglycemic agents, and 6.3% were taking insulin with or without oral agents.

Changes in biochemical data after surgery

All of the patients completed 12-mo follow-up. BMI, FBS, HbA1c, insulin, C-peptide, HOMA-IR, triglyceride, LDL-cholesterol, and HDL-cholesterol were determined preoperatively and 3, 6 and 12 mo after surgery (Table 2).

In the same operation type, data on preoperative day and postoperative 12 mo were compared. In addition, at the same follow-up points, variables of each operation type were compared. Figure 1 shows the changes in mean value of the biochemical data. BMI rapidly decreased during the first 3 mo after surgery and was maintained up to 12 mo (Figure 1A). In all operation types, the 12-mo postoperative BMI value significantly decreased to approximately 90% of the preoperative value. At the same follow-up point, BMI level showed no significant difference according to operation type. Despite this, the degree of weight loss tended to be greater after total gastrectomy than after subtotal gastrectomy.

The FBS levels at 3, 6 and 12 mo after surgery were lower than preoperative levels, and the difference in FBS levels at the preoperative time point and 12 mo after STG B I ($P = 0.001$) was statistically significant. As shown in Figure 1B, FBS levels decreased markedly up to 3 or 6 mo after surgery and then slowly declined or increased again up to 12 mo. There was no difference in the FBS level according to operation type at the same time points.

HbA1c levels improved 3 mo after each type of surgery, but increased 12 mo after subtotal gastrectomy and were maintained after total gastrectomy (Figure 1C). Therefore, there were no significant differences between preoperative and postoperative 12-mo HbA1c levels following the three types of surgery. This may be associated with the stabilization of BMI and FBS levels between 3 and 12 mo after surgery, which would result from an increase in food intake. Insulin levels rapidly decreased 3 mo after all types of surgery and then slowly decreased up to 12 mo (Figure 1D).

There were significant differences in insulin and C-peptide levels (Figure 1E) between 3 and 12 mo after surgery, but there were no differences according to operation type at the same follow-up points. HOMA-IR levels consistently improved at 3, 6 and 12 mo after all types of surgery (Figure 1F) and the levels at 12 mo after surgery were significantly lower than the preoperative levels. The HOMA-IR level at 6 mo follow-up after total gastrectomy was significantly lower than that after subtotal gastrectomy.

The lipid profile which included triglyceride, LDL, and HDL did not show significant differences between preoperative and postoperative 12-mo levels, with the exception of HDL level in the STG B I group (Figure 1G-I).

Diabetes control after surgery

Patients were divided into three groups based on their diabetes status: stationary, improved and remission (Table 3). Among the 64 patients, 35 patients (54.7%) improved and 2 patients (3.1%) went into remission. In the STG B I group, 58.3% of patients had improved 12 mo after

Table 2 Changes in biochemical data after surgery according to the follow up period and operation type

	Operation type	Preoperative	PO 3 mo	PO 6 mo	PO 12 mo	[1]*P* pre-12 mo
BMI (kg/m^2)	STG BⅠ	24.3 ± 2.9	22.5 ± 2.6	22.3 ± 2.8	21.9 ± 2.7	< 0.001
	STG BⅡ	24.7 ± 3.1	22.6 ± 2.9	22.9 ± 2.1	22.1 ± 1.9	0.002
	TG	25.7 ± 5.2	23.8 ± 5.7	22.5 ± 4.6	22.9 ± 4.4	0.004
	[2]*P*	0.793	0.965	0.616	0.780	
BMI	STG BⅠ	100%	92.9% ± 5.2%	92.2% ± 5.3%	92.0% ± 7.0%	< 0.001
	STG BⅡ	100%	91.6% ± 6.7%	93.3% ± 6.9%	90.3% ± 11.0%	0.004
	TG	100%	90.9% ± 6.4%	87.8% ± 5.7%	87.1% ± 8.1%	0.003
	[2]*P*	1.000	0.661	0.050	0.333	
Glucose (mg/dL)	STG BⅠ	147.3 ± 44.1	125.0 ± 30.3	122.3 ± 38.4	114.4 ± 21.7	0.001
	STG BⅡ	155.7 ± 40.0	136.4 ± 56.6	122.5 ± 31.9	126.4 ± 39.1	0.061
	TG	145.9 ± 37.5	114.3 ± 22.2	117.0 ± 28.0	115.4 ± 27.3	0.234
	[2]*P*	0.682	0.489	0.931	0.773	
HbA1c	STG BⅠ	7.2% ± 1.1%	6.8% ± 0.6%	7.0% ± 0.7%	7.1% ± 0.9%	0.834
	STG BⅡ	7.3% ± 1.3%	6.9% ± 1.4%	7.1% ± 1.3%	7.1% ± 1.6%	0.626
	TG	7.1% ± 0.8%	6.5% ± 0.7%	6.5% ± 0.5%	6.5% ± 0.6%	0.119
	[2]*P*	0.981	0.227	0.201	0.201	
Insulin (μIU/mL)	STG BⅠ	21.3 ± 20.7	8.6 ± 11.2	8.1 ± 11.3	5.1 ± 4.7	< 0.001
	STG BⅡ	22.5 ± 29.0	9.6 ± 8.8	8.9 ± 3.5	7.7 ± 7.1	0.041
	TG	18.1 ± 10.1	7.5 ± 5.7	4.0 ± 2.1	3.7 ± 1.9	0.02
	[2]*P*	0.679	0.652	0.050	0.350	
C-peptide (ng/mL)	STG BⅠ	3.4 ± 2.3	2.1 ± 1.6	2.3 ± 1.2	1.5 ± 0.8	< 0.001
	STG BⅡ	3.3 ± 1.8	2.0 ± 1.4	2.4 ± 0.9	2.1 ± 1.5	0.028
	TG	2.8 ± 1.3	2.1 ± 1.2	1.5 ± 1.3	1.6 ± 0.6	0.02
	[2]*P*	0.877	0.778	0.597	0.523	
HOMA-IR	STG BⅠ	8.7 ± 10.1	2.9 ± 4.7	2.6 ± 7.9	1.3 ± 0.9	0.001
	STG BⅡ	9.3 ± 13.5	3.3 ± 3.4	2.9 ± 2.0	2.5 ± 2.5	0.045
	TG	7.1 ± 4.1	2.1 ± 1.6	0.8 ± 0.2	1.1 ± 0.6	0.015
	[2]*P*	0.660	0.484	0.036	0.176	
TG (mg/dL)	STG BⅠ	126.1 ± 80.8	100.1 ± 51.6	115.9 ± 62.6	101.4 ± 41.7	0.341
	STG BⅡ	144.8 ± 102.6	118.5 ± 75.9	124.3 ± 53.7	105.2 ± 44.6	0.114
	TG	143.5 ± 88.9	130.5 ± 71.4	72.3 ± 36.6	96.6 ± 37.3	0.082
	[2]*P*	0.831	0.503	0.130	0.937	
LDL (mg/dL)	STG BⅠ	96.8 ± 34.7	89.0 ± 27.9	81.3 ± 23.5	90.8 ± 29.2	0.522
	STG BⅡ	97.4 ± 28.7	97.9 ± 33.5	94.4 ± 29.7	101.6 ± 37.5	0.515
	TG	113.6 ± 28.9	99.8 ± 28.0	63.5 ± 32.0	98.5 ± 32.0	0.023
	[2]*P*	0.299	0.556	0.192	0.594	
HDL (mg/dL)	STG BⅠ	43.9 ± 10.6	48.3 ± 11.4	49.4 ± 15.1	52.0 ± 15.1	0.002
	STG BⅡ	43.2 ± 9.2	42.6 ± 8.2	46.2 ± 10.6	46.5 ± 8.6	0.173
	TG	42.4 ± 10.5	45.0 ± 7.3	48.7 ± 3.2	46.3 ± 8.1	0.073
	[2]*P*	0.953	0.161	0.757	0.552	

[1]Paired *t*-test, mean ± SD; [2]Kruskal-Wallis test was used to evaluate the difference by operation type at the same follow up period. Significant values are indicated in bold face. BMI: Body mass index, BMI (%) refers to percentage of BMI at each follow-up compared to preoperative BMI. STG BⅠ: Subtotal gastrectomy with Billroth Ⅰ anastomosis; STG BⅡ: Subtotal gastrectomy with Billroth Ⅱ anastomosis; TG: Total gastrectomy; HOMA-IR: Homeostasis model assessment-estimated insulin resistance; LDL: Low-density lipoprotein; HDL: High-density lipoprotein.

surgery and 16.7% of patients stopped their medication. However, no patients went into remission. In the STG B Ⅱ group, one (6.2%) of 16 patients went into remission and 9 (56.2%) were improved 12 mo after surgery. In the TG group, 5 (41.7%) patients were improved and one (8.3%) was in remission 12 mo after surgery. Three (25%) patients stopped medication.

Factors for diabetes control 12 mo after surgery

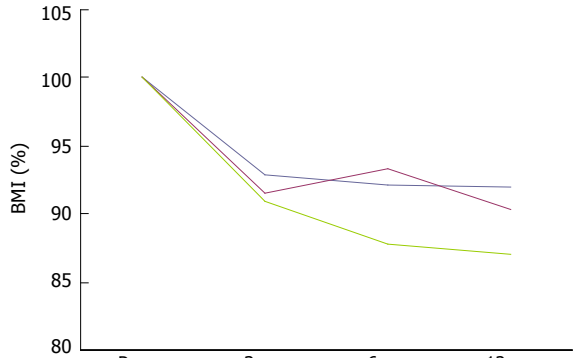
We compared the improved and in remission patients to those who were stationary to identify predictive factors for diabetes control. Age, sex, change in BMI, smoking, alcohol history, familial history (1st degree relatives) of type 2 DM, operation type, preoperative fasting blood glucose, HbA1c, insulin, C-peptide, HOMA-IR, triglyceride, LDL, and HDL were not associated with the degree of diabetes control 12 mo after gastrectomy (Table 4). Postoperative BMI changes, smoking history, and the duration of type 2 DM were predictive factors for diabetes control after surgery. BMI levels 3-, 6- and 12-mo after surgery were lower, the incidence of non-smokers was higher, and the duration of DM was shorter in patients satisfying improved or remission criteria than those in the stationary group.

In multivariate analysis, the duration of DM was the only significant factor associated with postoperative diabetic control (Table 5).

DISCUSSION

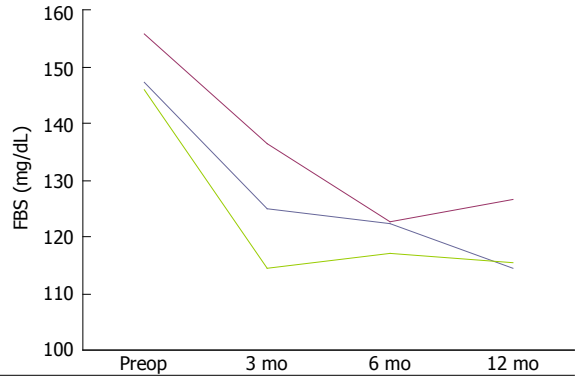
In recent studies which evaluated diabetes resolution after gastric cancer surgery, diabetes remitted in 15.1%-19.7% of gastric cancer patients[11,12]. However, because these studies involved a retrospective review of medical re-

A



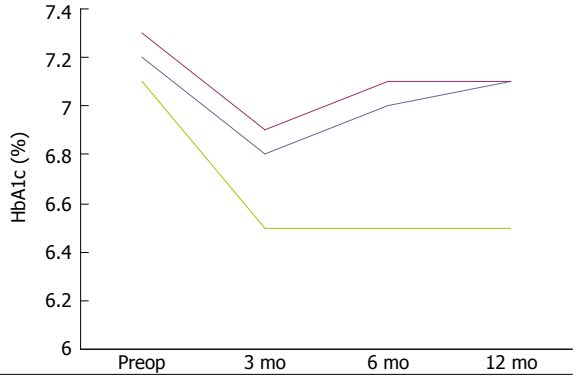
	Preop	3 mo	6 mo	12 mo
STG B I	100	92.9	92.2	92.0
STG B II	100	91.6	93.3	90.3
TG	100	90.9	87.8	87.1

B



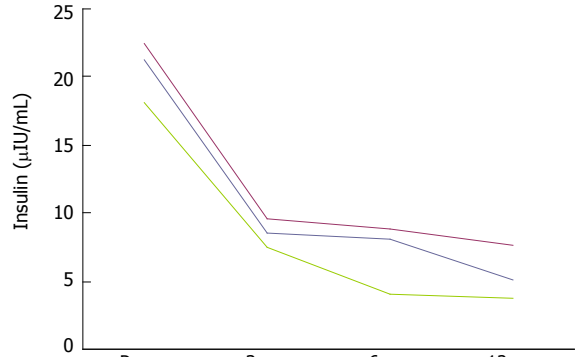
	Preop	3 mo	6 mo	12 mo
STG B I	147.3	125.0	122.3	114.4
STG B II	155.7	136.4	122.5	126.4
TG	145.9	114.3	117.0	115.4

C



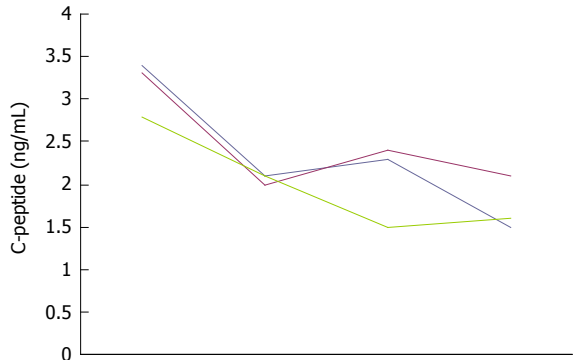
	Preop	3 mo	6 mo	12 mo
STG B I	7.2	6.8	7.0	7.1
STG B II	7.3	6.9	7.1	7.1
TG	7.1	6.5	6.5	6.5

D



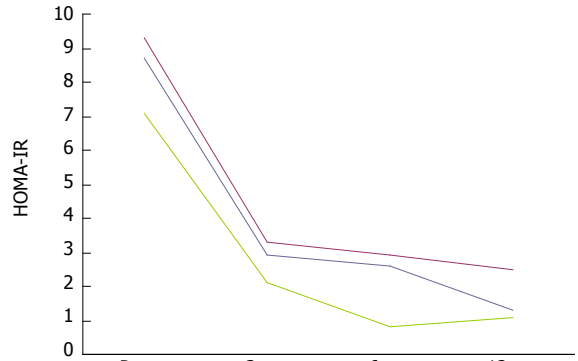
	Preop	3 mo	6 mo	12 mo
STG B I	21.3	8.6	8.1	5.1
STG B II	22.5	9.6	8.9	7.7
TG	18.1	7.5	4.0	3.7

E



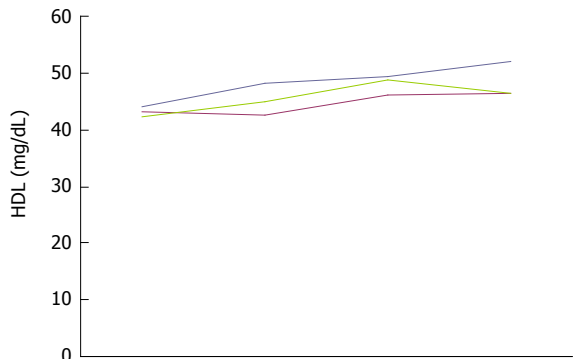
	Preop	3 mo	6 mo	12 mo
STG B I	3.4	2.1	2.3	1.5
STG B II	3.3	2.0	2.4	2.1
TG	2.8	2.1	1.5	1.6

F



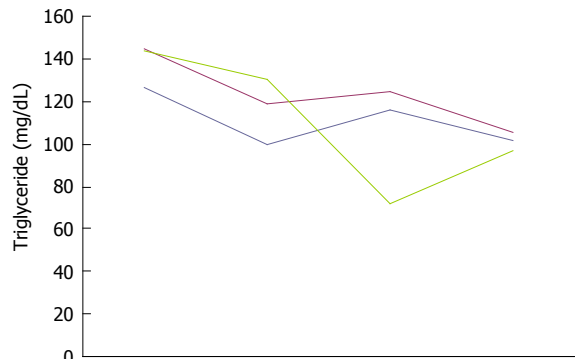
	Preop	3 mo	6 mo	12 mo
STG B I	8.7	2.9	2.6	1.3
STG B II	9.3	3.3	2.9	2.5
TG	7.1	2.1	0.8	1.1

G



	Preop	3 mo	6 mo	12 mo
STG B I	43.9	48.3	49.4	52
STG B II	43.2	42.6	46.2	46.5
TG	42.4	45.0	48.7	46.3

H



	Preop	3 mo	6 mo	12 mo
STG B I	126.1	100.1	115.9	101.4
STG B II	144.8	118.5	124.3	105.2
TG	143.5	130.5	72.3	96.6

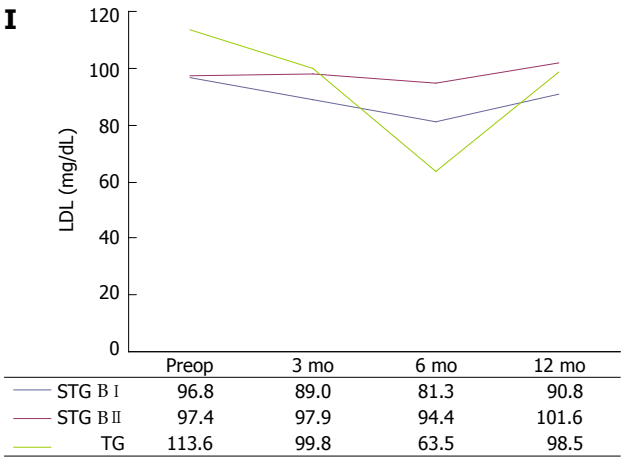


Figure 1 Changes in body mass index and serum biochemical data after gastric cancer surgery according to the follow-up periods and operation type. A: Body mass index; B: Fasting blood glucose level; C: HbA1c; D: Insulin; E: C-peptide; F: Homeostasis model assessment-estimated insulin resistance; G: Triglyceride; H: Low-density lipoprotein-cholesterol; I: High-density lipoprotein-cholesterol.

Table 3 Degree of diabetes mellitus control after surgery *n* (%)

	STG B I (*n* = 36)			STG B II (*n* = 16)			TG (*n* = 12)		
	3 mo	6 mo	12 mo	3 mo	6 mo	12 mo	3 mo	6 mo	12 mo
Stationary	16 (44.4)	16 (44.4)	15 (41.7)	8 (50)	8 (50)	6 (37.5)	6 (50)	7 (58.3)	6 (50)
Improved	19 (52.8)	20 (62.5)	21 (58.3)	8 (50)	8 (50)	9 (56.2)	6 (50)	4 (33.3)	5 (41.7)
Remission	1 (2.8)	0	0	0	0	1 (6.2)	0	1 (8.3)	1 (8.3)
Medication stopped	6 (16.7)	6 (16.7)	6 (16.7)	2 (12.5)	2 (12.5)	4 (25)	3 (25)	3 (25)	3 (25)

Stationary: No change in medication, or patients except improved and remission criteria; Improved: Reduced medication and a reduction in fasting blood glucose (FBS) or HbA1c; Remission: No medication and FBS < 126 mg/dL and HbA1c < 6.0%. STG B I: Subtotal gastrectomy with Billroth I anastomosis; STG B II: Subtotal gastrectomy with Billroth II anastomosis; TG: Total gastrectomy.

cords or interviewing, the available laboratory and physical parameters were limited. Although data analysis of the present study was performed retrospectively, all of our data were collected prospectively including laboratory data, body weight change, medical and familial history, and medication status. The low rate of diabetic remission in our study (3.1%) may be due to the strict evaluation of parameters reflecting diabetic control status at an exact time point, 12 mo after surgery.

The BMI of patients decreased by approximately 10% in the first 3 mo after surgery and was maintained or slightly decreased until the 12-mo evaluation. The FBS level showed rapid improvement at 3 mo and then slowed or was maintained up to 12 mo. HbA1c levels decreased at 3 mo and then increased or maintained between 3 and 12 mo after surgery. These patterns were similar in all three types of surgery and may be associated with the increased calorie intake and general recovery that occurs 3 mo after gastrectomy. These results suggest that weight loss is an important factor for diabetes improvement after gastric cancer surgery. Because one of the main treatments for type 2 diabetes is reduced calorie intake and gastrectomy is a type of restrictive surgery, our results are not unexpected[13].

However, serum insulin, C-peptide, and HOMA-IR continuously improved to at least 12 mo after surgery, even if the rate of improvement slowed 3 mo postoperatively. As shown in Table 2 and Figure 1, insulin, C-peptide, and HOMA-IR levels were significantly lower at the 12-mo evaluation compared to preoperative levels in all operation types. This suggests that insulin resistance continued to improve after surgery up to 12 mo, although body weight, FBS, and HbA1c did not. Gastric cancer surgery, including gastric resection with or without bypass procedures of a short segment of the proximal small bowel, appears to have a beneficial metabolic influence on diabetes control. However, considering that the pattern of biochemical data was similar in the STG B I and STG B II group, incomplete bypass of a short segment of proximal small bowel (B II) did not seem to provide significant additional benefits in terms of glucose metabolism.

The need for and amount of diabetes medication, FBS levels, and HbA1c levels are convenient tools for evaluating the impact of gastric cancer surgery on glucose control in clinical practice. We divided patients into three groups (stationary, improved, and remission) based on the severity of diabetes after surgery. Thirty three (51.6%) of 64 patients had improved glycemic control 3 mo after surgery and this increased to 54.7% 12 mo after surgery. One patient went into remission 3 mo after STG B I, however, this patient did not satisfy remission criteria at the 12 mo evaluation time. Finally, only 2 patients were in remission 12 mo after surgery: one (6.2%) in the

Table 4 Factors for diabetic control at postoperative 12 mo *n* (%)

	Stationary (*n* = 27)	Improved or remission (*n* = 37)	[1]*P* (univariate)
Age (yr)	64.3 ± 7.2	62.2 ± 8.4	0.582[2]
Sex			0.789
Male	19 (44.2)	24 (55.8)	
Female	8 (38.1)	13 (61.9)	
BMI, preop (kg/m^2)	24.5 ± 4.0	24.9 ± 3.0	0.434[2]
BMI			
3 mo	94.4% ± 5.0%	91.2% ± 5.3%	0.018[2]
6 mo	93.7% ± 6.8%	90.4% ± 5.7%	0.036[2]
12 mo	93.8% ± 9.1%	88.9% ± 7.8%	0.041[2]
Smoking			0.043
Yes	16 (57.1)	12 (42.9)	
No	11 (30.6)	25 (69.4)	
Alcohol history			0.132
No	13 (34.2)	25 (65.8)	
Yes	14 (53.8)	12 (46.2)	
DM duration (yr)			0.013
> 10	10 (76.9)	3 (23.1)	
5-10	8 (40.0)	12 (60.0)	
< 5	9 (29.0)	22 (71.0)	
Family history of DM			1.000
No	22 (42.3)	30 (57.7)	
Yes	5 (41.7)	7 (58.3)	
Operation type			0.799
STG B Ⅰ	15 (41.7)	21 (58.3)	
STG B Ⅱ	6 (37.5)	10 (62.5)	
TG	6 (50.0)	6 (50.0)	
Preop FBS	152.1 ± 43.6	145.6 ± 40.3	0.624[2]
Preop HbA1c	6.9 ± 0.8	7.4 ± 1.2	0.313[2]
Preop Insulin	21.7 ± 26.3	19.0 ± 17.8	0.804[2]
Preop C-peptide	3.1 ± 2.2	3.3 ± 1.9	0.509[2]
Preop HOMA-IR	9.2 ± 12.8	7.3 ± 8.0	0.835[2]

[1]χ^2; [2]Mann-Whitney *U* test, mean ± SD. Significant values are indicated in bold face. BMI: Body mass index, BMI (%) refers to the percentage of BMI at postoperative follow-up. DM: Diabetes mellitus; STG B Ⅰ: Subtotal gastrectomy with Billroth Ⅰ anastomosis; STG B Ⅱ: Subtotal gastrectomy with Billroth Ⅱ anastomosis; TG: Total gastrectomy; FBS: Fasting blood glucose; HOMA-IR: Homeostasis model assessment-estimated insulin resistance.

Table 5 Multivariate analysis of predictive factors for diabetic control at postoperative 12 mo

Variables	Odds ratio	95%CI	[1]*P*
Sex			
Male			
Female	0.168	0.018-1.529	0.113
Smoking			
Yes			
No	12.636	0.946-124.216	0.068
BMI (%)			
3 mo	0.869	0.690-1.094	0.232
6 mo	0.839	0.626-1.125	0.240
12 mo	1.055	0.893-1.246	0.526
DM duration (yr)			
> 10			
5-10	27.505	2.174-347.988	0.010
< 5	10.583	0.808-138.670	0.072
Operation type			
STG B Ⅰ			
STG B Ⅱ	0.547	0.086-3.493	0.523
TG	0.088	0.006-1.365	0.088

[1]Binary logistic regression. STG B Ⅰ: Subtotal gastrectomy with Billroth Ⅰ anastomosis; STG B Ⅱ: Subtotal gastrectomy with Billroth Ⅱ anastomosis; TG: Total gastrectomy.

STG B Ⅱ group and the other (8.3%) in the TG group. It seems to be difficult to adequately control diabetes with gastric cancer surgery to stop medication.

As shown in Table 5, the predictive factor for diabetes control 12 mo after surgery was the duration of diabetes. In univariate analysis, the rate of BMI change, smoking history, and diabetes duration were associated with diabetes control 12 mo after surgery. In multivariate analysis, diabetes was controlled in gastric patients with a shorter duration of diabetes. This result was similar to previous reports of type 2 DM patients who took oral hypoglycemic agents and for those who received bariatric surgery[14,15]. It is possible that islet cell function is less impaired in patients with a shorter duration of diabetes than in patients with a longer duration. Therefore, diabetes control would be more effective in gastric cancer patients with a short history of diabetes. The operation type which reflects the extent of gastric resection and the presence of bypass of a short segment of proximal jejunum, were not associated with diabetes control 12 mo after surgery. Although we failed to identify a difference in the efficacy of diabetes control based on the type of gastric cancer surgery, we did find that gastric cancer surgery positively affected diabetes. Although only 3.1% of the 64 patients went into remission, 57.8% showed improved glycemic control after surgery. Considering that approximately 10% of the general population suffers from diabetes and the incidence of diabetes in gastric cancer patients is similar to that of the general population, an improvement in diabetes after gastric cancer surgery would reduce health-care costs and improve the quality of life of these individuals.

Because more than half of the patients in this study were not obese, the impact of gastrectomy described should not be interpreted as if resulting from bariatric surgery. This study was initially planned to help surgeons select the most effective gastric cancer surgery for gastric cancer patients with diabetes. In other retrospective studies, diabetic resolution rates after TG ranged from 27.3%-50%, which were much higher than those after STG B Ⅰ and B Ⅱ. However, because the pattern of clinical and laboratory data were similarly changed in all three groups, we could not identify a difference between the STG B Ⅰ and STG B Ⅱ groups or between the STG and TG groups. Considering that the gap in HbA1c, insulin, C-peptide, and HOMA-IR levels between STG and TG widened at postoperative 6 mo, TG seemed to have more potential for better and more persistent glucose control than STG. However, they showed no significant difference at postoperative 12 mo and we cannot clarify whether the extent of gastrectomy or bypass length was more important in this study. We did not include patients who had STG with Roux-en-Y reconstruction which can provide a longer and complete bypass length of proximal jejunum than STG B Ⅰ and B Ⅱ. As the extent of gastrectomy mainly depends on the tumor location and extent, and the length of bypass of proximal small bowel is very short in conventional gastric cancer surgery, the

modification of bypass length of proximal small bowel would offer a better outcome for diabetic control. Therefore, a study that includes a larger number of patients and other types of surgery will be necessary to identify any differences between the types of gastric cancer surgery. In addition, we did not determine postprandial glucose and insulin level, thus we calculated only the insulin sensitivity index using a fasting-based formula. Therefore, further studies are needed to investigate the effect of gastric cancer surgery on gastric cancer patients with type 2 DM using an insulin sensitivity index derived from the oral glucose tolerance test.

In conclusion, gastric cancer surgery led to weight loss and a significant improvement in type 2 DM during the first year after surgery. The degree of diabetes control was related to diabetes duration in each patient. However, the impact of operation type in conventional gastric cancer surgery, such as the extent of gastric resection and current reconstruction methods, on diabetes remains to be determined.

COMMENTS

Background

Due to the increased incidence of early gastric cancers and improved survival, postoperative quality of life has become very important after gastric cancer surgery. Diabetes mellitus (DM) is an important health problem worldwide and in gastric cancer patients with DM.

Research frontiers

After gastric cancer surgery, many surgeons focus on improving the nutritional status of patients rather than controlling diabetes as the main problem after gastric cancer surgery is weight loss. Therefore, the effect of gastric cancer surgery on diabetic control in gastric cancer patients with type 2 DM has not yet been fully evaluated. In this study, the authors demonstrated the short-term effect of three types of routine gastric cancer surgery on type 2 DM.

Innovations and breakthroughs

In this study, the organized serial evaluation of the status of diabetic control was prospectively performed according to surgical extent and reconstruction type in conventional gastric cancer surgery.

Applications

This study will allow surgeons to select a favorable reconstruction type after gastrectomy in gastric cancer patients with diabetes.

Peer review

The study showed that diabetes mellitus was improved in more than 50% of patients during the first year after gastric cancer surgery and the degree of diabetes control was related to diabetes duration and not with the surgical type. However, the effect of gastric cancer surgery type on diabetic control should be further evaluated. These results are very original and support the possibilities that gastric surgery may be an alternative in the treatment of type 2 diabetes mellitus.

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BRIEF ARTICLE

Two surgical procedures for esophagogastric variceal bleeding in patients with portal hypertension

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Abstract

AIM: To determine the clinical value of a splenorenal shunt plus pericardial devascularization (PCVD) in portal hypertension (PHT) patients with variceal bleeding.

METHODS: From January 2008 to November 2012, 290 patients with cirrhotic portal hypertension were treated surgically in our department for the prevention of gastroesophageal variceal bleeding: 207 patients received a routine PCVD procedure (PCVD group), and 83 patients received a PCVD plus a splenorenal shunt procedure (combined group). Changes in hemodynamic parameters, rebleeding, encephalopathy, portal vein thrombosis, and mortality were analyzed.

RESULTS: The free portal pressure decreased to 21.43 ± 4.35 mmHg in the combined group compared with 24.61 ± 5.42 mmHg in the PCVD group ($P < 0.05$). The changes in hemodynamic parameters were more significant in the combined group ($P < 0.05$). The long-term rebleeding rate was 7.22% in the combined group, which was lower than that in the PCVD group (14.93%), ($P < 0.05$).

CONCLUSION: Devascularization plus splenorenal shunt is an effective and safe strategy to control esophagogastric variceal bleeding in PHT. It should be recommended as a first-line treatment for preventing bleeding in PHT patients when surgical interventions are considered.

Key words: Comparative study; Portal hypertension; Splenorenal shunt; Devascularization; Esophagogastric variceal bleeding

Core tip: A comparison of two surgical techniques for esophagogastric variceal bleeding in patients with cirrhotic portal hypertension was performed. Pericardial devascularization and shunt are the main surgical strategies for the prevention of esophagogastric variceal bleeding in patients with portal hypertension (PHT). In this study, we found that devascularization plus splenorenal shunt was an effective and safe strategy for controlling esophagogastric variceal bleeding in PHT patients. This surgical technique should be recommended as a first-line treatment for the prevention of bleeding in PHT patients when surgical interventions are considered.

Yang L, Yuan LJ, Dong R, Yin JK, Wang Q, Li T, Li JB, Du XL, Lu JG. Two surgical procedures for esophagogastric variceal bleeding in patients with portal hypertension. *World J Gastroenterol* 2013; 19(48): 9418-9424 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i48/9418.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i48.9418

INTRODUCTION

In China, portal hypertension (PHT) is a major health threat in patients with hepatitis-related cirrhosis. PHT usually leads to multiple complications including splenomegaly, ascites, hepatorenal syndrome, encephalopathy, and even variceal hemorrhage. Of these complications, the main complication associated with mortality risk is variceal hemorrhage, along with a high rate of recurrence[1]. Variceal hemorrhage develops in half of patients with cirrhosis, and bleeding occurs in approximately 20%. Patients with large varices have a 30% risk of bleeding over 2 years[2]. The risk of rebleeding without intervention is 65% over 2 years[3,4]. Although 70%-80% patients can be treated effectively with a non-cardioselective β-blocker and band ligation, 20%-30% of them will fail in such treatment and require further therapy[2]. In recent years, with the advent of alternative treatments, particularly the widespread use of endoscopy and transjugular intrahepatic portosystemic shunt (TIPS), the use of surgery in the acute management of active variceal bleeding has decreased[5].

Although the results of endoscopic procedures are satisfactory, the rate of hemostasis failure is almost 10%-20%, and mortality is approximately 60% if a second unsuccessful endoscopic treatment is performed without further intervention[6,7]. TIPS is currently one of the most commonly used therapies to decrease the free portal pressure (FPP) and stop bleeding. Although the rate of encephalopathy is higher (30%) with a high mortality rate[8,9], it results in better control of rebleeding, but no change in mortality[2]. TIPS is also a useful method for patients who are immediate candidates for liver transplantation, which is the only treatment to significantly prolong long-term survival in patients with cirrhosis.

However, in China, the majority of patients with refractory variceal bleeding do not have the opportunity of liver transplantation due to the high cost and a shortage of donor livers. Moreover, there are many patients with Child-Pugh class A disease who may not require transplantation for many years and TIPS is not recommended. Thus, in these cases, surgical intervention is necessary, and may be the only effective treatment to control rebleeding. Devascularization and shunts are two widely accepted surgical techniques for the management of portal hypertension. Although pericardial devascularization (PCVD) and the shunt procedure have their advantages, they also have disadvantages, such as a significant rebleeding rate following PCVD and a high encephalopathy rate following shunt procedures[10-12].

Hence, over the last two decades, we have performed a new combined operation (splenorenal shunt plus devascularization) to manage variceal esophageal bleeding resulting from portal hypertension secondary to cirrhosis. The aim of this operation is to combine the advantages of devascularization and the shunt, and to reduce the disadvantages of both techniques. In addition, we aimed to identify a more suitable treatment for those patients who have no other conditions or are unsuitable for liver transplantation or TIPS.

MATERIALS AND METHODS

Patients and exclusion criteria

From January 2008 to November 2012, 290 patients with PHT secondary to cirrhosis were hospitalized in our department. The patients were divided into two groups who received either the combined operation of PCVD and splenorenal shunt or PCVD only for esophageal and gastric varices. Exclusion criteria for the combined group were as follows: (1) thrombosis of the splenic vein; (2) the splenic vein was not suitable for a shunt; (3) extensive bleeding in the upper digestive tract associated with poor liver condition; (4) FPP lower than 30 mmHg after splenectomy; (5) patients with Child-Pugh class B or less, and poor condition; (6) regional portal hypertension; and (7) emergency surgery. Otherwise, patients received the combined operation. However, the final decision was often made during surgery.

Portal hypertension was due to liver cirrhosis in all patients, and these patients underwent routine preoperative clinical, biochemical and radiological evaluations, computed tomography scanning, and endoscopy. Prior to surgery, all patients were grouped according to Child-Pugh classification.

PCVD

PCVD was carried out using the modified Hassab procedure[13] first described by Qiu[10]. Briefly, we made a left transabdominal incision, and after the abdomen was opened, we measured the FPP *via* catheterization of the right gastroepiploic vein, and performed a splenectomy and PCVD. We used sequential ligation to devascularize the upper two-third vessels of both the lesser and greater curvatures of the stomach, including the left gastroepiploic vein, short gastric vein, and left gastric vein. The retrogastric venous collaterals running from the upper border of the pancreas to the gastroesophageal junction were meticulously divided and ligated. The lower 5 cm of the esophagus was devascularized *via* the transhiatal approach by sequential ligation.

PCVD plus a splenorenal shunt

In the combined group, after the PCVD was performed, a modified proximal splenocaval shunt was carried out. The tail and body of the pancreas, and the splenic vein and tributaries were carefully dissociated and placed to the right *via* the transverse mesocolon. The splenic vein was dissociated to a length of 2-3 cm and the infrarenal inferior vena cava was freed to a length of 4-5 cm for the preparation of a splenocaval anastomosis, and the splenocaval shunt was then performed. The diameter of the anastomotic stomas ranged from 6 to 8 mm, and the tail of the pancreas was fixed to the connective tissue surrounding the inferior vena cava to reduce the tension

Table 1 Clinical characteristics

Characteristics	PCVD group (n = 207)	Combined group (n = 83)
Mean age (yr)	45.79	43.72
SD	11.35	8.03
Range	17-77	30-63
Gender		
Male	130	55
Female	77	28
Etiology		
HBV-related	142	58
HCV-related	28	10
HBV and HCV	3	1
Other causes	14	4
Alcohol-related	20	10
Child-Pugh		
Grade A	134	73
Grade B	67	10
Grade C	6	0
Grade of varices		
Grade Ⅰ-Ⅱ	41	15
Grade Ⅲ-Ⅳ	143	56
Bleeding site		
Esophageal	55	25
Fundus varices	10	6

PCVD: Pericardial devascularization; HBV: Hepatitis B virus; HCV: Hepatitis C virus.

of the anastomotic stoma. During both operations, the FPP was measured *via* the right gastroepiploic vein after opening the abdomen, ligation of the splenic artery, removal of the spleen, and PCVD, respectively. The FPP was also measured in the combined group after the shunt procedure.

All patients underwent color Doppler ultrasound before and after the operation to measure the portal and splenic vein diameters, maximum velocity, flow direction, and to determine the presence of thrombosis in the portal system. A follow-up visit was scheduled after the patients were discharged from hospital. The postoperative mortality (defined as death in the perioperative period), the rate of complications, the incidence of rebleeding, the rate of encephalopathy, and survival were recorded.

Statistical analysis

The data were analyzed by SPSS 19.0 statistical software. All results are presented as mean ± SD. The Mann-Whitney U test and the χ^2 test were used appropriately. The Kaplan-Meier method (log rank test) was used to analyze long-term complications appropriately. $P < 0.05$ was considered statistically significant. This study was exempt from IRB review after institutional IRB review.

RESULTS

Clinical characteristics

Patient age, sex ratio and Child-Pugh classification were statistically similar between the two groups ($P > 0.05$). Of 290 patients, 207 underwent PCVD, and the remaining 83 patients underwent the combined operation. No emergency surgery was carried out in either of the two groups (Table 1).

Table 2 Intra- and post-operative clinical characteristics n (%)

Clinical characteristics	PCVD group	Combined group	P value
Intraoperative			
Operative time (min)	246 ± 71	307 ± 68	< 0.01
Blood loss (mL)	936 ± 1627	744 ± 832	< 0.01
Blood transfusion (mL)	843 ± 1237	760 ± 583	0.010
Postoperative			
Fever	56	10	< 0.01
Ascites	941 ± 833	759 ± 695	0.24
Rebleeding	12 (5.80%)	2 (2.41%)	0.04
Long-term complications			
Congestive gastropathy	35 (17.41%)	2 (2.41%)	< 0.01
Encephalopathy	3 (1.45%)	2 (2.41%)	0.58
Portal vein thrombosis	16 (7.96%)	3 (3.61%)	0.04
Rebleeding	30 (14.93%)	6 (7.22%)	< 0.05

PCVD: Pericardial devascularization.

Hemorrhage during the operation and operation time

During the operation, the average blood loss in the PCVD group was 936 ± 1627 mL compared with 744 ± 832 mL in the combined group ($P < 0.05$). Moreover, the operation time was significantly shorter in the PCVD group compared with the combined group (246 ± 71 min *vs* 307 ± 68 min, $P < 0.05$) (Table 2).

Complications in the perioperative period

In the perioperative period, the main complications were ascites and postoperative fever. After surgery, there were no significant differences in the mean amount of postoperative ascites, which was 941 ± 833 mL in the PCVD group and 759 ± 695 mL in the combined group (Table 2). However, the incidence of postoperative fever in the PCVD group (27.86%, 56 of 201 patients) was significantly higher than that in the combined group (12.04%, 10 of 83 patients) ($P < 0.05$) (Table 2).

Changes in free portal pressure

In both groups, the postoperative free portal pressure (FPP) was significantly lower than that preoperatively ($P < 0.01$). However, there was no difference in the first measured FPP at abdominal opening, 29.23 ± 4.58 mmHg in the PCVD group *vs* 29.81 ± 3.83 mmHg in the combined group. However, after PCVD and shunt surgery, a significant decrease in the combined group (21.43 ± 4.35 mmHg) was observed compared to the PCVD group (24.61 ± 5.42 mmHg) ($P < 0.01$) (Table 3).

Changes in hemodynamic parameters

Hemodynamic parameters of the portal vein (PV) were measured preoperatively and postoperatively (Table 4). There were no significant differences in the inner diameter, blood flow velocity and venous flow preoperatively, however, significant changes were found after surgery. In the PCVD group and the combined group, the postoperative inner diameter, blood flow velocity and venous flow of the PV were significantly decreased ($P < 0.01$),

Table 3 Changes of free portal pressure in the two groups (mmHg)

	PCVD group	Combined group	*Z*	*P* value
Abdominal opening	29.23 ± 4.58	29.81 ± 3.83	-0.36	0.72
Splenectomy	22.32 ± 5.33	24.60 ± 5.01	-2.91	< 0.05
PCVD	24.61 ± 5.42	22.06 ± 4.03	-3.08	< 0.05
Shunt		21.43 ± 4.35		

PCVD: Pericardial devascularization.

and the *D* values were also significantly different ($P < 0.01$), respectively. Similar results for the splenic vein (SV) and the superior mesenteric vein (SMV) are also shown in Table 4.

Rebleeding rate

The postoperative rebleeding rates in the PCVD and combined groups were 5.80% (12/207) and 2.41% (2/83), respectively. In the PCVD group, 10 of 12 patients had bleeding before the operation. Compared with the PCVD group, 2 patients had preoperative bleeding in the combined group (Table 2). The data in Table 2 also show the long-term results of rebleeding. In the 284 survived patients (6 died in the perioperative period), the overall incidence of rebleeding in the PCVD group and combined group was 14.93% (30/201) and 7.22% (6/83), respectively ($P < 0.05$). Twenty-one of 30 patients in the PCVD group had postoperative rebleeding, and 23 patients had preoperative bleeding, while 6 patients in the combined group had either preoperative bleeding or postoperative rebleeding.

Long-term results of complications

The incidence of congestive gastropathy in the PCVD group was 17.41% (35/201), which was significantly higher than that in the combined group, with only 2 patients (2.41%) affected ($P < 0.05$). The main long-term liver disease-related complications were encephalopathy and thrombosis. The incidence of encephalopathy was 1.45% (3/201) and 2.41% (2/83) in the PCVD group and combined group, respectively ($P = 0.58$). In each group, one patient died of progressive liver failure at 10 mo postoperatively due to severe encephalopathy. Portal vein thrombosis was found in 16 (7.96%) patients in the PCVD group and 3 (3.61%) patients in the combined group ($P = 0.04$), (Table 2).

Postoperative mortality and survival

As a result of the small number of deaths in both groups, we analyzed the cause of death and did not use statistical methods to evaluate mortality and survival. In contrast to the combined group with no death during the perioperative period, 6 of the 207 patients in the PCVD group died during the perioperative period. Two of these patients died due to rebleeding, 3 due to hepatic failure, and one due to multiple system organ failure (MSOF) caused by gastric fistula. The 3-year survival rate was 95.52% (192/201) in the PCVD group, 2 died due to rebleeding, 3 due to hepatic failure, 2 due to primary hepatic cancer, 1 due to cerebral hemorrhage, and 1 due to other reasons. The 3-year survival rate in the combined group was 96.39% (80/83), 1 died due to hepatic failure, 1 due to primary hepatic cancer, and 1 due to other reasons.

DISCUSSION

The main aims of the treatments used in PHT patients are to control variceal bleeding and to prevent rebleeding. In addition, it is necessary to maintain enough portal hepatopetal perfusion and protect the limited liver function. In China, PCVD and distal splenorenal shunt are favored and widely accepted by surgeons, and have prevailed until now. Although these two favorable procedures are based on two different hemodynamic theories, they achieve reliable effects by controlling variceal bleeding. However, a high rebleeding rate caused by recurrent varices or a high rate of residual varices and changes in the gastric mucosa following PCVD have been observed[14-16]. In addition, it was reported that the shunt can preserve hepatopetal perfusion to support liver function and improve the microcirculation of gastric mucosa[17-19], however, the incidence of hepatic encephalopathy needs to be reduced.

Thus, in order to combine the advantages of these two operations, we integrated these two different surgical procedures into one operation. Although there are limited reports on this combined operation, a comprehensive analysis is needed to prove its rationality. In this study, we retrospectively analyzed clinical data to determine the clinical value of the combined procedure in patients with cirrhotic portal hypertension and variceal bleeding. A total of 290 patients with portal hypertension of cirrhotic origin were enrolled in this study and received either PCVD only or the combined operation, respectively.

The primary end point of this study was variceal rebleeding. According to reports in the literature, the rebleeding rate of patients who underwent devascularization was 7.1%-37%[20-22], and following distal splenorenal shunt the rebleeding rate was approximately 5%-15%[2,22-25]. In China, the rebleeding rate after prolonged follow-up in patients who underwent the combined operation was 5%-10%, in contrast to 10%-30% in patients who underwent PCVD[26-29]. Furthermore, after the PCVD procedure, the postoperative venous pressure in the gastric wall increased and exacerbated pathologic changes and congestive conditions in the gastric mucosa, therefore increasing the risk of congestive gastropathy. It has been reported that the rate of postoperative rebleeding caused by congestive gastropathy is 20%-83%[21,30,31]. However, the rates of rebleeding and the incidence of congestive gastropathy in our study were lower than those reported in the literature. In addition, the rebleeding rate in our combined group was significantly lower than that following TIPS which was reported to be 10.5%[2].

Table 4 Comparison of hemodynamics in the two groups pre- and post-operatively

		Inner diameter (cm)				Blood flow velocity (cm/s)			
		Pre-op	Post-op	*D* value	*P* value	Pre-op	Post-op	*D* value	*P* value
PV	PCVD	1.42 ± 0.21	1.24 ± 0.26	0.15 ± 0.17	< 0.01	15.28 ± 4.69	13.27 ± 4.76	2.01 ± 3.01	< 0.01
	Combined	1.39 ± 0.26	1.01 ± 0.30	0.38 ± 0.25	< 0.01	16.52 ± 4.67	11.33 ± 3.78	5.19 ± 3.42	< 0.01
	P	0.57	< 0.01	< 0.01		0.06	< 0.05	< 0.01	
SV	PCVD	1.21 ± 0.24	1.05 ± 0.21	0.18 ± 0.13	< 0.01	17.65 ± 5.53	14.10 ± 5.58	3.55 ± 0.92	< 0.01
	Combined	1.25 ± 0.22	0.81 ± 0.22	0.43 ± 0.20	< 0.01	18.76 ± 5.76	13.10 ± 5.38	5.65 ± 3.00	< 0.01
	P	0.25	< 0.01	< 0.01		0.17	0.21	< 0.01	
SMV	PCVD	0.95 ± 0.70	0.81 ± 0.21	0.14 ± 0.17	< 0.01	13.17 ± 4.61	11.00 ± 4.76	2.13 ± 2.33	< 0.01
	Combined	0.92 ± 0.19	0.66 ± 0.23	0.26 ± 0.27	< 0.01	14.69 ± 5.23	10.40 ± 4.46	4.29 ± 3.07	< 0.01
	P	0.3	< 0.01	< 0.01		< 0.05	0.33	< 0.01	

PV: Portal vein; SV: Splenic vein; SMV: Superior mesenteric vein; PCVD: Pericardial devascularization.

The lower rebleeding rate and incidence of gastropathy in the combined group resulted from the following mechanisms. First, it has been demonstrated that extensive PCVD of at least 6-8 cm under the mediastinal esophagus and the dissociation of the uppermost gastric vessels[32] are necessary for the disappearance of esophageal varices[33]. Second, congestive gastropathy can be attenuated by an effective shunt, which can reduce the blood flow to the gastroesophageal mucosa and improve mucosal microcirculation in the stomach. Third, portal hypertension is relieved to some extent after splenocaval shunt surgery, and this may delay the reformation of gastroesophageal varices.

The changes in FPP and hemodynamics in the present study had significant clinical implications. In both groups, the postoperative FPP decreased significantly, however, in the PCVD group, it was still higher than normal. The postoperative FPP in patients who underwent the combined procedure decreased to a normal level. Furthermore, we also found that in both groups the PV, SV, and SMV parameters after the treatments were lower than preoperative levels. However, these changes were more significant in the combined group. These findings suggested that: (1) the combined operation efficiently reduced hypertensive congestion in the portal system; (2) a splenorenal shunt reduced the FPP more and markedly decreased hypertensive congestion in the portal system, and the combined procedure maintained PV patency and prevented a significant decrease in the pressure and blood flow of the PV; (3) the combined operation not only decreased PVF, but also resulted in good control of rebleeding. However, PCVD alone did not achieve control of rebleeding; (4) the postoperative FPP was normal in the combined group, and may have contributed to the complementary action of the hypotensive effect of the shunt and the hypertensive effect of PCVD; and (5) a significant decrease in the postoperative inner diameter and blood flow velocity in the combined group indicated that the PCVD plus shunt resulted in better hemodynamics.

These changes in hemodynamics may also maintain blood flow to the liver and prevent hepatic failure. Therefore, in our combined procedure we restricted the anastomotic stoma to maintain the hemodynamics in the appropriate range. Our historical experience showed that if we restricted the stoma more than 1.0 cm the FPP would decrease faster following PCVD, and the stoma would diminish due to an excessive drop in the FPP. In our department, we restrict the anastomotic stoma to 6-8 mm. This procedure also reduces the rate of encephalopathy. Based on previous experience, all types of shunts have been shown to have high encephalopathy rates due to a sharp drop in the PV and a reduction in portal pressure. In our study, the rate of encephalopathy in the two groups was similar, indicating that PCVD plus shunt did not increase the risk of encephalopathy.

During both procedures, splenectomy was performed in all patients due to splenomegaly associated with hypersplenism. However, after surgery the platelet count was elevated, which resulted in a high-coagulation status and injury to the inner mucosa of vessels, and blood flow velocity was reduced. As a result, thrombosis occurred in the portocaval stoma and portal vein. The rate of thrombosis is an important prognostic factor in patients with portal hypertension and cirrhosis. In our future studies, more effective efforts will be made to prevent thrombosis.

According to the results from our study, we can conclude that splenorenal shunt plus devascularization is an effective choice in patients with esophagogastric variceal bleeding due to PHT. The clinical characteristics in the combined group were better than those in the PCVD group. Although the surgical risk in the combined group was equal to the PCVD group, the combined procedure resulted in a lower rate of complications. Furthermore, the combined procedure maintained liver function, which is beneficial in patients who may have the opportunity of future liver transplantation.

In the present study, we compared the outcomes following treatment with PCVD and the combined operation. We hope that a comparative study of this combined procedure and other treatments in cirrhotic PHT patients can be carried out in the future.

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COMMENTS

Background

In China, portal hypertension (PHT) is a major threatening event due to hepatitis-related cirrhosis. In recent years, with the advent of alternative treatments, the role of surgery in the acute management of active variceal bleeding caused by PHT has decreased. However, devascularization and shunts are still two widely accepted surgeries for the management of portal hypertension. In this study, the authors investigated the clinical value of a splenorenal shunt plus pericardial devascularization (PCVD) in PHT patients with variceal bleeding.

Research frontiers

Liver transplantation is the major treatment for portal hypertension and upper gastrointestinal bleeding; however, due to the shortage of liver source and high cost, it is not acceptable extensively. It is important to find an effective method to control the complication of portal hypertension and prolong the survival time of the patients.

Innovations and breakthroughs

The authors performed a combined operation (devascularization plus splenorenal shunt) over the past two decades to manage variceal esophageal bleeding which results from the portal hypertension secondary to cirrhosis. They evaluated the clinical value of this combined surgery, and found that the devascularization plus splenorenal shunt is an effective and safe strategy to control esophagogastric variceal bleeding in PHT patients. It is superior to the traditional surgeries.

Applications

The devascularization plus splenorenal shunt is an effective and safe strategy to control esophagogastric variceal bleeding in PHT patients. It could be recommended as a first-line treatment for preventing bleeding in PHT patients when surgical interventions are considered.

Terminology

In the combined group, after the splenectomy and PCVD, a modified proximal splenocaval shunt was performed. The tail and body of the pancreas, and the splenic vein and tributaries were carefully dissociated and were turned right via the transverse mesocolon. The splenic vein was dissociated to a length of 2-3 cm and the infrarenal inferior vena cava was freed to a length of 4-5 cm for the preparation of a splenocaval anastomosis, and then the splenocaval shunt was performed. The diameter of the anastomotic stomas ranged from 6 to 8 mm, and the tail of the pancreas was fixed to the connective tissue surrounding the inferior vena cava to reduce the tension of the anastomotic stoma.

Peer review

This is a very interesting topic and has puzzled the surgeons for many decades. It still remains controversial in China. The authors investigated the surgical outcomes of patients with PHT who underwent PCVD alone or splenorenal shunt plus PCVD. They conclude that the devascularization plus splenorenal shunt is an effective and safe strategy to control esophagogastric variceal bleeding in PHT patients. Strengths of the study are the large number of cases, good follow-up and excellent annotation with clinical data.

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BRIEF ARTICLE

Overexpression of kallikrein gene 10 is a biomarker for predicting poor prognosis in gastric cancer

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Abstract

AIM: To analyze the expression of kallikrein gene 10 (*KLK10*) in gastric cancer and to determine whether *KLK10* has independent prognostic value in gastric cancer.

METHODS: We studied *KLK10* expression in 80 histologically confirmed gastric cancer samples using real-time quantitative reverse transcription-PCR and hK10 expression using immunohistochemistry. Correlations with clinicopathological variables (lymph node metastasis, depth of invasion and histology) and with outcomes (disease-free survival and overall survival) during a median follow-up period of 31 mo were assessed. Gastric cancer tissues were then classified as *KLK10* positive or negative.

RESULTS: *KLK10* was found to be highly expressed in 57/80 (70%) of gastric cancer samples, while its expression was very low in normal gastric tissues. Positive relationships between *KLK10* expression and lymph node metastasis (P = 0.048), depth of invasion (P = 0.034) and histology (P = 0.015) were observed. Univariate survival analysis revealed that gastric cancer patients with positive *KLK10* expression had an increased risk for relapse/metastasis and death (P = 0.005 and 0.002, respectively). Cox multivariate analysis indicated that *KLK10* was an independent prognostic indicator of disease-free survival and overall survival in patients with gastric cancer.

CONCLUSION: *KLK10* expression is an independent biomarker of unfavorable prognosis in patients with gastric cancer.

Key words: Kallikrein gene 10; Gastric cancer; Survival analysis; Prognostic biomarkers

Core tip: The study examined the clinicopathologic and prognostic significance of kallikrein gene 10 (*KLK10*) expression in gastric cancer. Based on collective findings, we hypothesize that *KLK10* expression in gastric cancer tissues may have prognostic/predictive value in patients with gastric cancer. *KLK10* expression is an independent biomarker for predicting unfavorable prognosis in patients with gastric cancer.

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INTRODUCTION

Gastric cancer is the fourth most common cancer, and the second leading cause of cancer death worldwide[1]. Mortality due to gastric cancer has risen in China over the past 20 years, especially in rural areas and in aging populations[2,3]. Although the increased use of screening for early disease diagnosis and the widespread administration of systemic adjuvant therapies have led to a decline in mortality rates, the incidence and mortality of gastric cancer are still second only to lung cancer[4,5].

The kallikrein gene family of secreted serine proteases, consisting of 15 genes, is localized in tandem on chromosome 19q13.4 and shows significant homologies at both the nucleotide and the protein levels[6,7]. Kallikrein-related peptidase 10 is a member of the kallikrein family and has been shown in numerous reports to be upregulated in ovarian cancer[8,9]. The human kallikrein (*KLK*) gene 10 encodes human kallikrein gene 10 (KLK10) protein. Recent studies have shown that human KLKs are involved in human carcinogenesis and that several KLKs are promising biomarkers of prostate, ovarian, testicular and breast cancer[10,11]. For instance, prostate-specific antigen (PSA/hK3) which is encoded by the *KLK3* gene is used as a cancer-specific marker for male population screening, early diagnosis and monitoring of prostate cancer[12]. In addition, quantification of KLK5 expression is critical for both the discovery of early cellular and molecular alterations in breast cancer, as well as the identification of novel diagnostic and prognostic biomarkers[13]. Many other KLKs are also expected to act as tumor biomarkers[14-17]. More recent evidence also implicates the KLKs in many cancer-related processes, including cell growth regulation, angiogenesis, invasion and metastasis[7]. Several authors have reported that *KLK10* mRNA was highly expressed in ovarian cancer tissue and that hK10 may be a useful serum biomarker for the diagnosis and management of ovarian cancer[18]. However, few studies have focused on *KLK10* expression in human gastric cancer.

In the present study, we examined the clinicopathologic and prognostic significance of *KLK10* expression in gastric cancer. Based on collective findings, we hypothesize that *KLK10* expression in gastric cancer tissues may have prognostic/predictive value in patients with this malignancy.

MATERIALS AND METHODS

Study population

Tumor specimens from 80 consecutive patients undergoing surgical treatment for primary gastric cancer at the Department of General Surgery, Tianjin First Central Hospital (Tianjin, China) were analyzed in this study. Patient age ranged from 35 to 74 years, with a median of 51 years (Table 1). All tumor specimens and matched control samples taken from normal tissues at the incision edge were snap-frozen in liquid nitrogen and stored at -80 ℃ for subsequent RNA extraction. Investigations were carried out in accordance with the ethical standards of the 1975 Helsinki Declaration, as revised in Tokyo 2004. The patients had not received hormonal therapy or chemotherapy prior to surgery. After surgery, all patients were treated with oxaliplatin-based chemotherapy regimens based on a platinum compound, alone or in combination with other drugs; grade 1 and stage I patients received no further treatment. Follow-up information (median follow-up period of 31 mo) was available for 80 patients (Table 1). Two time-to-event outcomes after surgery were recorded: disease-free survival (DFS) and overall survival (OS). DFS in each case was defined as the time interval between the date of primary cancer removal and the date of the first documented evidence of relapse. OS was defined as the time interval between the date of surgery and the date of death, or the date of last follow-up for those who were alive at the end of the study.

Clinical and pathological information documented at the time of surgery included clinical stage, histology, depth of invasion and lymph node metastasis (Table 2). All pathological factors were established as described by the 2010 National Comprehensive Cancer Network Guideline.

Table 1 Data of the study population

Variable	No. of patients	mean ± SE	Range
Age (yr)	80	51 ± 0.81	35-74
Lymph nodes[1]	80	30 ± 3.11	0-63
Follow-up (mo)[2]	80	31 ± 1.98	7-52

[1]Number of lymph nodes removed during surgery; [2]Follow-up time after surgery.

Table 2 Associations between kallikrein gene 10 status and other variables in 80 patients with gastric cancer

Variable	Total	*KLK10*-negative	*KLK10*-positive	*P* value[1]
Sex				
Male	56	14	42	
Female	24	9	15	0.258
Age (yr)				
< 60	31	10	21	
⩾ 60	49	13	36	0.581
Depth of invasion[2]				
T1	27	12	15	
T2-T3	23	7	16	
T4	30	4	26	0.034
Lymph node metastasis[2]				
N1	21	4	17	
N2	35	15	20	
N3	24	4	20	0.048
Differentiation[2]				
Well	18	10	8	
Moderate	37	7	30	
Poor	25	6	19	0.015

[1]χ^2 test; [2]TNM stage system of American Joint Committee on Cancer of 2012. *KLK10*: Kallikrein gene 10.

Ethics

The study protocol was approved by the Ethics Com-

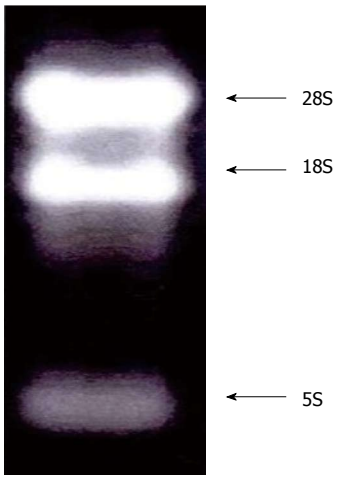

Figure 1 Confirmation of the integrity of total RNA.

mittee of the hospital and written informed consent was obtained from each patient.

Immunohistochemistry

Immunohistochemical studies of hK10 were carried out using the avidin-biotin-peroxidase method (LSAB2 kit, Dako, Kyoto, Japan) on formalin-fixed, paraffin-embedded surgical specimens from patients with gastric cancer. All sections were counterstained with hematoxylin. Primary goat polyclonal antibodies against hK10 (Santa Cruz, United States) were used at dilutions of 1:700.

All sections were independently examined by two researchers (Xin Jiao, Mi-Mi Zhai). The expression of hK10 was scored as positive when the carcinoma cell cytoplasm was stained brown. We examined hK10 protein expression in tumor tissues and corresponding normal tissues from 80 gastric cancer cases.

Total RNA extraction and reverse transcription

Tumor tissues of 100 mg were minced on dry ice using a scalpel and immediately transferred to 2 mL polypropylene tubes. Total RNA was isolated from these samples using TRI-reagent (Ambion Inc., Austin, TX, United States) following the manufacturer's instructions. Total RNA concentration and quality were determined spectrophotometrically at 260 and 280 nm, and RNA integrity was evaluated using agarose gel electrophoresis. Reverse transcription of the mRNA molecules into first-strand cDNA was carried out using 1 μg of total RNA from each tissue specimen, M-MuLV Reverse Transcriptase RNase H (Finnzymes Oy, Espoo, Finland) and an oligo(dT) oligonucleotide as a reverse transcription primer, according to the manufacturer's instructions. Confirmation of the integrity of total RNA is shown in Figure 1.

Real-time quantitative reverse transcription-PCR

Based on the mRNA sequences from the NCBI Sequence database, gene specific primers were designed and synthesized for the target *KLK10* gene (NCBI Reference Sequence: NM_002776) and HPRT1 (hypoxanthine phosphoribosyltransferase-1) endogenous reference gene (NCBI Reference Sequence: NM_000194.2) using the Primer Express software (Applied Biosystems, CA, United States). A *KLK10* fragment was amplified using the primers: forward, 5'-CTCTGGCGAAGCTGCTG-3' and reverse, 5'-ATAGGCTTCGGGGTCCAA-3', whereas the primers for HPRT1 were: forward, 5'-TGGAAAGGGTGTTTATTCCTCAT-3' and reverse, 5'-ATGTAATCCAGCAGGTCAGCAA-3'.

Real-time PCR assays of *KLK10* mRNA expression levels were performed using a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, United States), and the reagents TaqMan Fast Universal PCR Master Mix (29) (Applied Biosystems, United States) according to the manufacturer's instructions. With an initial polymerase activation step at 95 ℃ for 10 min, the amplification conditions of the 40 cycles consisted of denaturation at 95 ℃ for 15 s, annealing at 59 ℃ for 30 s, and elongation at 72 ℃ for 30 s. The products were then subjected to a temperature gradient from 55 ℃ to 95 ℃ at 0.1 ℃/s with continuous fluorescence monitoring to produce a melting curve of the products. *KLK10* mRNA expression was calculated from the standard curve, and quantitative normalization of cDNA in each sample was performed using the expression of GAPDH mRNA as an internal control[19]. We classified the 80 cases into two groups using the mean expression level of *KLK10* mRNA in tumor tissues (0.03): *i.e.,* a positive-expression group ($\geq$ 0.03, n = 57) and a negative-expression group ($<$ 0.03, n = 23).

Statistical analysis

Statistical analysis were performed using SPSS for Windows version 19.0 (SPSS, Chicago, IL, United States). Associations between clinicopathological parameters, such as depth of invasion, lymph node metastasis, histology and *KLK10* expression were analyzed by the Chi-square test or Fisher's exact test, where appropriate. Survival analysis were performed by constructing Kaplan-Meier DFS and OS curves and differences between curves were evaluated by the log-rank test (Mantel, 1966), and by estimating the relative risks for relapse and death using the Cox proportional hazards regression model (Cox, 1972). Cox analysis was conducted at both univariate and multivariate levels. Only the patients with known status of all variables were included in the multivariate regression models, which incorporated *KLK10* and all other variables, for which the patients were characterized.

RESULTS

Relationship between KLK10 expression and other parameters

Of the 80 patients included in this study, 57 (70 ℃) were positive for *KLK10* expression in gastric cancer tissues. In normal gastric tissues, the level of *KLK10* was undetectable or low. Table 2 shows the distribution of *KLK10*

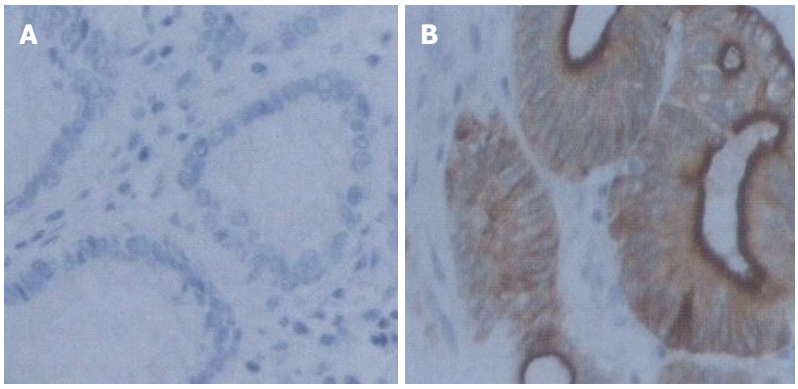

Figure 2 Immunohistochemical analysis of hK10 protein expression in normal gastric tissues and gastric tumor tissues. A: hK10 protein expression in normal gastric tissues; B: hK10 protein expression in gastric tumor tissues.

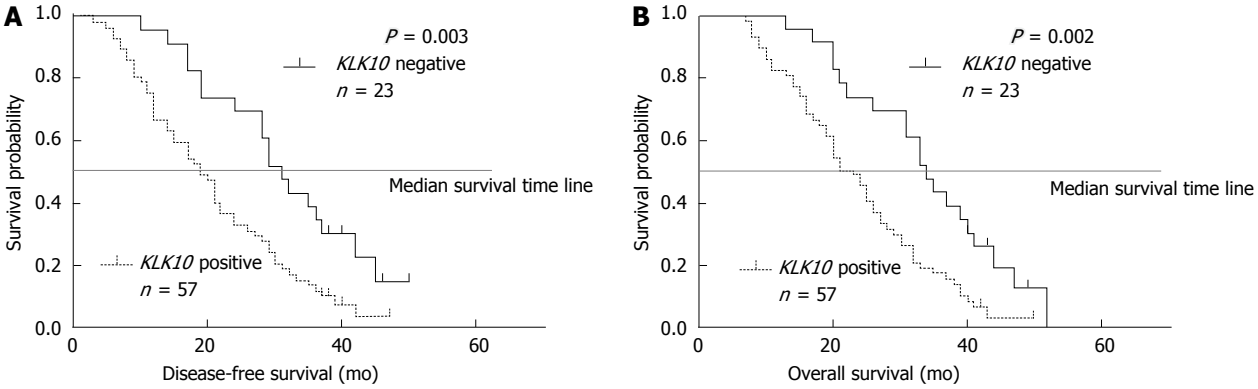

Figure 3 Kaplan-Meier survival analysis of disease-free survival (A) and overall survival (B) in gastric cancer patients who were either kallikrein gene 10 positive or kallikrein gene 10 negative. *KLK10*: Kallikrein gene 10.

expression (positive or negative) in gastric cancer tissues in relation to age, sex, lymph node metastasis, depth of invasion and histology. Patients with *KLK10*-positive gastric cancer more frequently had more lymph node metastasis ($P = 0.048$), greater depth of invasion ($P = 0.034$) and poorer histology ($P = 0.015$). No significant associations between *KLK10* expression and age ($P = 0.581$) and sex ($P = 0.258$) were found.

Immunohistochemistry

In 55 of the 57 patients who were positive for *KLK10* mRNA expression, specific expression of hK10 protein was only found in cancer tissues, but not in the corresponding normal tissues (Figure 2). In 23 cases with negative expression of *KLK10* mRNA, 20 exhibited negative or weak expression of hK10 in cancer tissues. In contrast, 2 cases with high *KLK10* mRNA expression exhibited negative or poor hK10 protein expression in cancer tissues.

Clinicopathologic significance of KLK10 mRNA expression in gastric cancer

The clinicopathologic factors analyzed in relation to *KLK10* mRNA expression in tumor tissues are shown in Table 2. The level of lymphatic invasion was significantly higher ($P = 0.048$) in the positive-expression group than in the negative-expression group. The depth of gastric wall invasion was greater ($P = 0.034$) in the positive-expression group than in the negative-expression group. The histotype also correlated with these groups ($P = 0.015$). In contrast, no significant difference was observed regarding age and sex. The 3-year actuarial OS rates in patients with gastric cancer and positive *KLK10* mRNA expression and in patients with negative *KLK10* mRNA expression were 20% and 42%, respectively (Figure 3). The survival difference between these two groups was statistically significant ($P = 0.002$; log-rank test).

Univariate and multivariate survival analysis

The degree of association between each clinicopathological variable and DFS and OS is shown in Table 3. In univariate analysis, patients with *KLK10*-positive gastric cancer had a significantly increased risk of relapse (decreased DFS) and death (decreased OS) (hazards ratios of 0.46 and 0.43; $P = 0.005$ and 0.002, respectively). Nevertheless, positive *KLK10* expression was weakly associated with an increased risk of death (decreased OS) (hazards ratio of 0.55; $P = 0.03$) in multivariate analysis compared with univariate analysis, while no significant association was found between the positive *KLK10* expression and relapse (decreased DFS) in patients with gastric cancer in multivariate analysis ($P = 0.06$).

Depth of invasion and histotype were the strongest independent indicators of poor prognosis ($P < 0.05$, except for depth of invasion for OS in multivariate analysis). As expected, Kaplan-Meier survival curves (Figure 3) indicated that patients with *KLK10*-positive gastric cancer had shorter DFS ($P = 0.003$) and OS ($P = 0.002$) com-

Table 3 Univariate and multivariate analysis of disease-free survival and overall survival

Survival variable	Disease-free survival			Overall survival		
	95%CI[2]	*P* value	HR[1]	95%CI[2]	*P* value	HR[1]
Univariate analysis						
KLK10						
Negative			1.00			1.00
Positive	0.25-0.74	0.002	0.46	0.26-0.79	0.005	0.43
Age	0.97-1.01	0.46	0.99	0.97-1.01	0.49	0.99
Sex	0.55-1.53	0.75	0.90	0.54-1.51	0.69	0.92
Depth of invasion	1.55-2.81	< 0.001	2.13	1.57-2.89	< 0.001	2.08
Lymph node metastasis	0.97-1.97	0.07	1.42	0.99-2.02	0.051	1.38
Histology	6.42-22.46	< 0.001	10.25	5.73-18.32	< 0.001	12.01
Multivariate analysis						
KLK10						
Negative		1.00			1.00	
Positive	0.31-0.96	0.03	0.58	0.33-1.02	0.06	0.55
Age	0.95-1.00	0.02	0.97	0.95-0.99	0.04	0.97
Sex	0.77-2.22	0.31	1.33	0.77-2.28	0.30	1.31
Depth of invasion	0.99-1.90	0.06	1.42	1.02-1.98	0.03	1.37
Lymph node metastasis	0.84-1.59	0.35	1.21	0.87-1.67	0.24	1.16
Histology	6.55-25.41	0.03	11.11	5.87-21.02	< 0.001	12.90

[1]Hazard ratio (HR) estimated from Cox proportional hazard regression model; [2]Confidence interval of the estimated HR. *KLK10*: Kallikrein gene 10.

pared with *KLK10*-negative patients.

DISCUSSION

Gastric cancer is a common malignant tumor of the gastrointestinal tract. The optimal management of patients with gastric cancer involves a multidisciplinary approach: diagnosis, surgery, and chemotherapy, including the use of biological markers. Several authors have reported that *KLK10* mRNA is highly expressed in human cancer tissues[20-23]. In the current study, we found a significant relationship between *KLK10* mRNA expression and lymph node metastasis, depth of invasion and histology in patients with gastric cancer, as shown in Table 1. These findings indicate that the overexpression of *KLK10* was significantly associated with both an increased incidence of lymphatic invasion and poor histology in patients with gastric cancer. These results suggest that enhanced expression of *KLK10* may play an important role in various pathologic processes of gastric cancer. The results obtained in this study are in agreement with previous studies which examined the association between *KLK10* expression status and the clinicopathological features of patients with gastric cancer[24].

Some members of the KLK family have been identified as potential biological markers of prognosis, including KLK5, KLK14 and KLK7[25]. For example, KLK5 expression is an indicator of poor prognosis in ovarian cancer[26]. Furthermore, stratifying patients based on the presence or absence of such markers may result in a different prognosis in individuals. For example, both KLK8 mRNA and KLK6 mRNA are highly expressed in human breast cancer tissues, however, it is unknown whether a breast cancer patient with high expression of KLK8 has a good/poor prognosis compared to a breast cancer patient with high expression of KLK6.

In this study, we identified *KLK10* as a new biomarker of poor prognosis in gastric cancer. Patients with *KLK10*-positive tumors were more likely to have poor histology and advanced stage disease. Our findings demonstrate that *KLK10* expression can reduce DFS in patients with gastric cancer in univariate, but not in multivariate analysis (Table 2). In addition, when assessing *KLK10* expression to predict survival outcomes, we found an increased risk of death in patients with *KLK10*-positive tumors in both univariate and multivariate analysis (Table 2). This indicates that *KLK10*-positivity may be an independent prognostic factor in patients with gastric cancer. That is, *KLK10* may induce gastric cancer cell growth and proliferation. However, the function of the *KLK10* signaling pathway is unclear. Some reports indicate that serine proteinases (the KLK gene family of secreted serine proteases includes 15 genes) participate in tumor growth and invasion by cleaving and activating proteinase-activated receptors (PARs: PAR-1 and PAR-2)[27-29]. A recent study demonstrated that KLK4 is aberrantly expressed in colon cancer and capable of inducing PAR-1 signaling in cancer cells[30]. KLK4 is a tumorigenic factor. Another report showed that KLK14 induced significant extracellular signal-regulated kinases 1 and 2 (ERK1/2) phosphorylation and HT29 cell proliferation, presumably by activating PAR-2. A PAR-2 cleavage and activation-blocking antibody markedly reduced KLK14-induced ERK1/2 signaling[31]. Our lack of knowledge of *KLK10* function and regulation in gastric cancer tissues does not allow us to formulate reasonable hypothesis to explain these observations. More studies with a larger group of patients are necessary to substantiate these findings.

The current study indicates that *KLK10* mRNA was significantly overexpressed in gastric cancer tissues and high *KLK10* expression levels were associated with lymphatic invasion, tumor invasion and poor patient prog-

nosis. hK3 has been well documented to be an excellent tumor marker for prostate cancer. Moreover, hK10 is a promising serum biomarker for ovarian cancer. Therefore, studies are now underway to investigate whether hK10 may also be a useful biomarker for gastric cancer using serum samples from the patients treated at our hospital.

COMMENTS

Background

Gastric carcinoma is one of the most common tumors worldwide. The expression of kallikreins is involved in cancer cell formation. Abnormal expression of kallikrein gene 10 *(KLK10)* is associated with carcinogenesis, and it is a promising serum biomarker for cancers.

Research frontiers

Several authors have reported that *KLK10* mRNA is highly expressed in ovarian cancer tissue and that hK10 could be a useful serum biomarker for the diagnosis and management of ovarian cancer. However, there is little information on *KLK10* expression in human gastric cancer.

Innovations and breakthroughs

This study assessed the clinicopathologic and prognostic significance of *KLK10* expression in gastric cancer. Furthermore, based on the collective findings, this study investigated whether *KLK10* expression in gastric cancer tissues may have prognostic/predictive value in patients with gastric cancer.

Applications

By exploring the relation between the expression of *KLK10* and clinicopathology in gastric cancer, this study may provide a strategy for predicting the prognosis of gastric cancer patients.

Peer review

It is a well written paper. The authors investigated the clinicopathologic and prognostic significance of *KLK10* expression in gastric cancer, which helps understand the pathogenesis and predict the prognosis of gastric cancer. The experimental procedure is quite well performed.

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BRIEF ARTICLE

Cystatin C is a biomarker for predicting acute kidney injury in patients with acute-on-chronic liver failure

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Abstract

AIM: To investigate serum cystatin C level as an early biomarker for predicting acute kidney injury (AKI) in patients with acute-on-chronic liver failure (ACLF).

METHODS: Fifty-six consecutive patients with hepatitis B virus-related ACLF who had normal serum creatinine (Cr) level (< 1.2 mg/dL in men, or < 1.1 mg/dL in women) were enrolled in the Liver Failure Treatment and Research Center of Beijing 302 Hospital between August 2011 and October 2012. Thirty patients with chronic hepatitis B (CHB) and 30 healthy controls in the same study period were also included. Measurement of serum cystatin C (CysC) was performed by a particle-enhanced immunonephelometry assay using the BN Prospec nephelometer system. The ACLF patients were followed during their hospitalization period.

RESULTS: In the ACLF group, serum level of CysC was 1.1 ± 0.4 mg/L, which was significantly higher (P < 0.01) than those in the healthy controls (0.6 ± 0.3 mg/L) and CHB patients (0.7 ± 0.2 mg/L). During the hospitalization period, eight ACLF patients developed AKI. Logistic regression analysis indicated that CysC level was an independent risk factor for AKI development (odds ratio = 1.8; 95%CI: 1.4-2.3, P = 0.021). The cutoff value of serum CysC for prediction of AKI in ACLF patients was 1.21 mg/L. The baseline CysC-based estimated glomerular filtration rate ($eGFR_{CysC}$) was significantly lower than the creatinine-based eGFR ($eGFR_{CG}$ and $eGFR_{MDRD}$) in ACLF patients with AKI, suggesting that baseline $eGFR_{CysC}$ represented early renal function in ACLF patients while the Cr levels were still within the normal ranges.

CONCLUSION: Serum CysC provides early prediction of renal dysfunction in ACLF patients with a normal serum Cr level.

Key words: Acute-on-chronic liver failure; Cystatin C; Creatinine; Acute kidney injury; Prediction

Core tip: Severe renal dysfunction often occurs in patients with acute-on-chronic liver failure (ACLF) due to circulatory abnormalities and inflammation. New biomarkers with higher reliability and specificity for monitoring renal function are required. Fifty-six patients with ACLF and normal serum creatinine (Cr) were enrolled. Our results showed that patients who developed acute kidney injury during hospitalization had significantly higher basal serum cystatin C (CysC) levels.

CysC-based estimated glomerular filtration rate more accurately represented renal function in ACLF patients. CysC can be used as an early biomarker for detection of renal dysfunction in patients with ACLF before any increase in serum Cr is detected.

Wan ZH, Wang JJ, You SL, Liu HL, Zhu B, Zang H, Li C, Chen J, Xin SJ. Cystatin C is a biomarker for predicting acute kidney injury in patients with acute-on-chronic liver failure. *World J Gastroenterol* 2013; 19(48): 9432-9438 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i48/9432.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i48.9432

INTRODUCTION

Acute-on-chronic liver failure (ACLF) encompasses patients with previously well-compensated liver disease in whom acute decompensation of liver function occurs because of a precipitating event[1]. In China, hepatitis B virus (HBV)-infected ACLF patients account for > 80% of ACLF patients, due to a high incidence of chronic HBV infection[2,3]. The progressive nature of ACLF affects many organ systems. Kidney dysfunction is a common complication of advanced liver disease and associated with a high mortality[4-6].

Acute tubular necrosis and hepatorenal syndrome (HRS) may account for the majority of cases of severe renal dysfunction in patients with ACLF due to underlying circulatory abnormalities and inflammation[4,7]. Recently, the Acute Kidney Injury Network (AKIN) proposed a new term for acute renal dysfunction, namely, acute kidney injury (AKI), which can represent the entire spectrum of acute renal dysfunction[8,9]. The definition of AKI is based on changes in serum creatinine (Cr). Unfortunately, Cr is an unreliable indicator during acute changes in kidney function because it is highly dependent on extrarenal factors during the estimation[10]. Serum Cr concentrations may not change until approximately 50% of kidney function has already been lost[11]. In addition, elevated serum bilirubin in ACLF patients can interfere with the measurement of serum Cr using the Jaffe method[12]. Therefore, a Cr-based estimation of the glomerular filtration rate (GFR) may overestimate renal function in patients with ACLF. Thus, new biomarkers with higher reliability and specificity for estimation of renal function are required.

Serum cystatin C (CysC) is currently being investigated for the prediction of AKI in patients with cardiac surgery[13], advanced liver diseases[14], and patients undergoing liver transplantation[15]. CysC is a ubiquitous protein that is freely filtered by the kidney and then metabolized by the tubules. Unlike Cr level, CysC level is independent of muscle mass, age or sex, and is not influenced by inflammatory conditions or malignancy[16,17]. CysC significantly outperforms both Cr and endogenous creatinine clearance rate and detects impairment of GFR earlier than Cr does[18]. Several reports have suggested that increased CysC levels are more sensitive for prediction of HRS development in patients with cirrhosis[14,19,20], but the data using CysC levels in ACLF patients are lacking.

The purposes of this study were to investigate whether serum CysC levels are increased in ACLF patients by comparing with chronic hepatitis B (CHB) patients and healthy controls, and to further determine whether CysC can be used as an early biomarker for predicting AKI in ACLF patients.

MATERIALS AND METHODS

Ethics

The protocol was approved by the Ethical Committee of Beijing 302 Hospital. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. Written informed consent was obtained from each patient before entering the study protocol.

Patients and controls

Fifty-six consecutive patients with HBV-related ACLF and normal serum creatinine level (male: < 1.2 mg/dL, female: < 1.1 mg/dL) were admitted to the Liver Failure Treatment and Research Center of Beijing 302 Hospital between August 2011 and October 2012. ACLF was diagnosed based on a recent increase in jaundice (serum total bilirubin > 171.0 μmol/L) and decreasing plasma prothrombin activity (< 40%)[21]. Thirty patients with CHB were enrolled during the same study period. CHB was diagnosed according to the criteria recommended by the Chinese Society of Infectious Diseases, and the Chinese Society of Hepatology[22]. Serum samples from 30 age- and sex-matched healthy volunteers were used to determine the normal values of the indicators. The ACLF patients were followed during their hospitalization. Patients with intrinsic renal disease, spontaneous bacterial peritonitis, sepsis or gastrointestinal bleeding at enrollment were excluded from the study.

Laboratory and clinical parameters

Consecutive serum samples from ACLF patients were collected upon admission and throughout hospitalization (every 3 d). The serum samples of healthy controls were collected when they came to the Health Examination Centre. The serum samples of CHB patients were collected on admission to our center. All samples were stored within 2 h at -20 ℃ until analysis. Biochemical tests, including blood urea nitrogen (BUN), sodium, albumin, and bilirubin, were routinely performed. Serum Cr levels were determined using the modified Jaffe method (Beckman, Hamburg, Germany). Serum CysC measurements were performed by the particle-enhanced immunonephelometry assay using the BN Prospec Nephelometer system (Dade Behring, Newark, DE, United States). The model for end-stage liver disease (MELD) score was

Table 1 Clinical characteristics of the study population at admission

Parameter	ACLF (*n* = 56)	CHB (*n* = 30)	Control (*n* = 30)
Male/female	40/16	21/9	14/6
Age (yr)	44 ± 11	40 ± 10	39 ± 8
Alanine aminotransferase (IU/L)	145 ± 189	71 ± 61	21 (10-31)
Total bilirubin (mg/dL)	20.2 ± 5.6	2.2 ± 1.3	0.7 ± 0.2
Albumin (g/L)	30.4 ± 4.8	33.5 ± 5.2	35.6 ± 4.9
BUN (mmol/L)	4.4 ± 2.1	4.1 ± 1.8	4.8 ± 1.5
Plasma sodium (mEq/L)	134.8 ± 4.5	135.6 ± 5.7	137.4 ± 3.8
Cr (mg/dL)	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
CysC (mg/L)	1.1 ± 0.4	0.7 ± 0.2	0.6 ± 0.3
International normalized ratio	1.9 ± 0.4	1.1 ± 0.2	0.9 ± 0.1
HBV DNA log10 (IU/mL)	4.75 ± 2.11	6.21 ± 2.78	
Ishak score	ND	4 (3-5)[1]	
MELD score	24 ± 3	8 ± 4	

[1]Histopathological data from 20 chronic hepatitis B (CHB) patients. ND: Not determined; BUN: Blood urea nitrogen; Cr: Creatinine; CysC: Serum cystatin C; HBV: Hepatitis B virus; MELD: Model for end-stage liver disease.

calculated as: 3.8ln (total bilirubin in mg/dL) + 11.2ln (INR) + 9.6ln (Cr in mg/dL) + 6.4. In addition, two methods of Cr-based estimated GFR (eGFR) were used: (1) the formula of Cockcroft and Gault[23] (eGFR$_{CG}$); and (2) the modification of the diet in renal disease (MDRD) equation (eGFR$_{MDRD}$)[24]. CysC-based GFR estimation was calculated using the Hoek formula (eGFR$_{CysC-1}$)[25] and the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (eGFR$_{CysC-2}$)[26].

AKI was diagnosed as follows[9]: an abrupt reduction in kidney function as manifested by an absolute increase in serum Cr by ⩾ 0.3 mg/dL, equivalent to a percentage increase in serum Cr by ⩾ 50% (⩾ 1.5 folds from baseline) without any evidence of pre-existing kidney disease.

Statistical analysis

The results are expressed as mean ± SD or the number of patients. Data processing was carried out using SPSS for Windows version 17.0 (SPSS, Chicago, IL, United States). Continuous variables were determined using Student's *t* test. Parameters with non-normal distribution were compared using the Mann-Whitney *U* test. Categorical data were compared by the χ^2 test. Spearman's correlation analysis was used to assess relationships between two parameters. Receiver operating characteristic curves (ROCs) were formed to detect sensitivity and specificity of CysC, Cr, BUN and serum sodium for predicting the development of AIK, using Medcalc 12.7.7 software. Multivariate analysis with logistic regression was used to determine independent factors. $P < 0.05$ was considered statistically significant.

RESULTS

Clinical characteristics of enrolled patients

A total of 86 patients with chronic HBV infection, including 56 with ACLF and 30 with CHB, and 30 healthy volunteers were enrolled in this study. The basal clinical characteristics of patients at admission are summarized in Table 1. Patients with ACLF comprised 40 men (71.4%) and 16 women (28.6%) with a mean age of 44 ± 11 years. The serum level of sodium in ACLF patients was 134.8 ± 4.5 mEq/L and hyponatremia (serum sodium level < 130 mEq/L) was documented in six patients (10.7%). The average level of Cr was 0.9 ± 0.1 mg/dL in ACLF patients. As shown in Table 1, the baseline serum level of CysC was 1.1 ± 0.4 mg/L in ACLF patients, which was significantly higher ($P < 0.01$) than those in CHB patients (0.7 ± 0.2 mg/L) and healthy controls (0.6 ± 0.3 mg/L). A moderate increase ($P > 0.05$) in CysC was found in CHB patients in comparison with healthy controls. Meanwhile, in ACLF patients, the serum CysC level showed a significant positive correlation with the MELD score ($r = 0.746$, $P < 0.001$) (Figure 1A). However, the serum CysC level was not correlated with the serum Cr level ($r = 0.193$, $P = 0.155$) (Figure 1B).

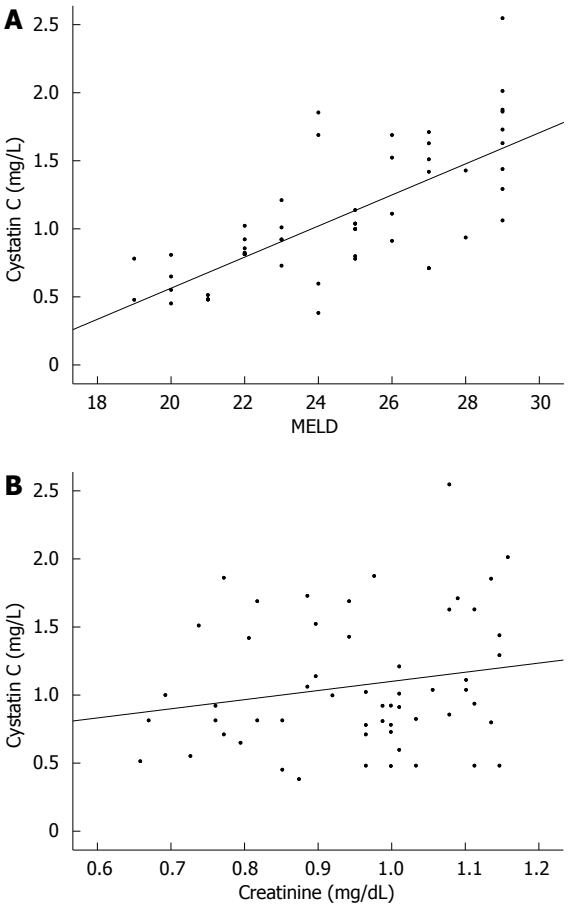

Figure 1 Scatter plots. A: Serum cystatin C (CysC) level *vs* model for end-stage liver disease (MELD) score; B: Serum CysC level *vs* serum creatinine (Cr) level.

Development of AKI

Patients with ACLF were followed during their hospitalization. The average hospitalization duration was 36 ± 10 d. During this period, eight (14.3%) of 56 patients developed AKI. The baseline clinical and laboratory charac-

Table 2 Comparison of baseline clinical characteristics in patients with or without acute kidney injury during hospitalization

Parameter	Without AKI (n = 48)	With AKI (n = 8)	P (univariate)	P (multivariate)	OR (95%CI)
Age (yr)	41 ± 9	55 ± 7	< 0.001		
Alanine aminotransferase (IU/L)	156 ± 158	62 ± 40	0.202		
Albumin (g/L)	30.1 ± 5.1	30.2 ± 2.1	0.965		
BUN (mmol/L)	4.2 ± 2.0	5.7 ± 1.1	0.065		
Sodium (mEq/L)	135.3 ± 4.2	132.1 ± 3.8	0.064		
Total bilirubin (mg/dL)	19.8 ± 5.6	21.3 ± 4.9	0.506		
Cr (mg/dL)	0.9 ± 0.1	1.0 ± 0.2	0.792		
International normalized ratio	1.9 ± 0.4	2.0 ± 0.3	0.367		
MELD score	24 ± 3	26 ± 2	0.094		
CysC (mg/L)	0.9 ± 0.3	1.8 ± 0.4	< 0.001	0.021	1.8 (1.4–2.3)

AKI: Acute kidney injury; BUN: Blood urea nitrogen; Cr: Creatinine; CysC: Serum cystatin C; MELD: Model for end-stage liver disease.

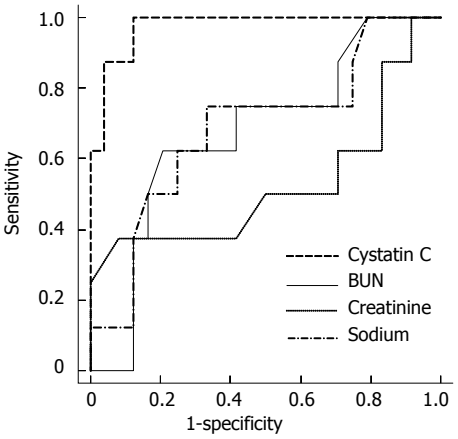

Figure 2 Receiver operating characteristic curve. Receiver operating characteristic curve analysis was performed to compare the efficacy of serum cystatin C, creatinine, blood urea nitrogen (BUN) and serum sodium level in predicting acute kidney injury.

teristics of patients with or without AKI are summarized in Table 2. Univariate analysis showed that patients who developed AKI were older (55 ± 7 years *vs* 41 ± 9 years, $P < 0.001$) and had a higher level of CysC (1.8 ± 0.4 mg/L *vs* 0.9 ± 0.3 mg/L, $P < 0.001$). The indicators (age, sodium, MELD score and CysC) which had $P < 0.1$ for patients with or without AKI were included in multivariate regression analysis. The results revealed that CysC level was the only independent predictive factor for the development of AKI in ACLF patients [odds ratio (OR) = 1.8; 95%CI: 1.4-2.3, $P = 0.021$].

ROC curve analysis was performed to compare the efficacy of CysC, Cr, BUN and serum sodium levels in predicting development of AKI during hospitalization (Figure 2). As shown in Table 3, the area under the curve (AUC) for CysC, Cr, BUN and serum sodium levels was 0.975, 0.526, 0.674 and 0.687, respectively. The results indicated that the AUC for CysC level had a better predictive value for development of AKI in ACLF patients ($P < 0.01$, DeLong's method for ROC curve comparison) in comparison with Cr, BUN and serum sodium. With an optimal cutoff value of 1.21 mg/L, the sensitivity and specificity of CysC for predicting the development of AKI were 100% and 87.5%, respectively ($P < 0.0001$).

Comparison of methods for estimating GFR using Cr- or CysC-based formulae

The methods for measuring eGFR included: Cr-based eGFR ($eGFR_{CG}$ and $eGFR_{MDRD}$), and CysC-based eGFRs ($eGFR_{CysC-1}$ and $eGFR_{CysC-2}$). These methods were compared between patients with or without AKI development (Figure 3). The four baseline eGFRs were not significantly different in patients without AKI (Figure 3A). In patients with AKI, baseline $eGFR_{CysC-1}$ when using the Hoek formula was 40.8 ± 9.7 mL/min, while the $eGFR_{CysC-2}$ from the Chronic Kidney Disease Epidemiology Collaboration equation was 40.3 ± 10.5 mL/min, which indicated a similar eGFR value using the two formulae. These two baseline $eGFR_{CysC}$ were significantly lower than $eGFR_{CG}$ (80.8 ± 19.6 mL/min, $P < 0.01$) and $eGFR_{MDRD}$ (79.8 ± 14.3 mL/min, $P < 0.01$) in patients with AKI (Figure 3A). The baseline $eGFR_{CysC-1}$ and $eGFR_{CysC-2}$ were significantly decreased in patients with AKI compared with those without AKI ($P < 0.001$). The baseline $eGFR_{CG}$ and $eGFR_{MDRD}$ were similar between patients with or without AKI. When AKI was diagnosed in ACLF patients, the $eGFR_{CG}$, $eGFR_{MDRD}$, $eGFR_{CysC-1}$ and $eGFR_{CysC-2}$ were 29 ± 3.3, 30.5 ± 5.1, 34.2 ± 5.7 and 33.3 ± 5.2 mL/min, respectively, suggesting no significant differences in the four eGFRs (Figure 3B). The results indicated that either Cr or CysC-based eGFRs reflected severe renal dysfunction when AKI occurred in ACLF patients. However, baseline $eGFR_{CysC}$ represented renal function of ACLF patients early during mild-to-moderate renal dysfunction, while the Cr levels were still within the normal ranges.

DISCUSSION

Patients with ACLF have immunological defects that are comparable to those in patients with sepsis. The clinical picture of both ACLF and septic shock is strikingly similar, and characterized by progressive vasodilatory shock and multiple organ failure[27]. Inflammation and oxidative stress also induce production of NO, which mediates the circulatory and renal disturbances of liver failure[28]. Recent reports from the European Association for the Study of the Liver (EASL) showed that the kidney failure

Table 3 Area under the curve for receiver operating characteristics and cutoff values for predicting acute kidney injury in acute-on-chronic liver failure patients

Parameter	Cutoff value	AUC (95%CI)	Sensitivity	Specificity	*P* value
CysC (mg/L)	1.21	0.974 (0.846-1.000)	100%	87.5%	< 0.0001
Cr (mg/dL)	1.1	0.526 (0.343-0.704)	37.5%	91.7%	0.828
BUN (mmol/L)	4.9	0.674 (0.487-0.829)	62.5%	79.2%	0.124
Serum sodium (mEq/L)	131	0.687 (0.500-0.839)	50%	87.5%	0.113

BUN: Blood urea nitrogen; Cr: Creatinine; CysC: Serum cystatin C; AUC: Area under the curve.

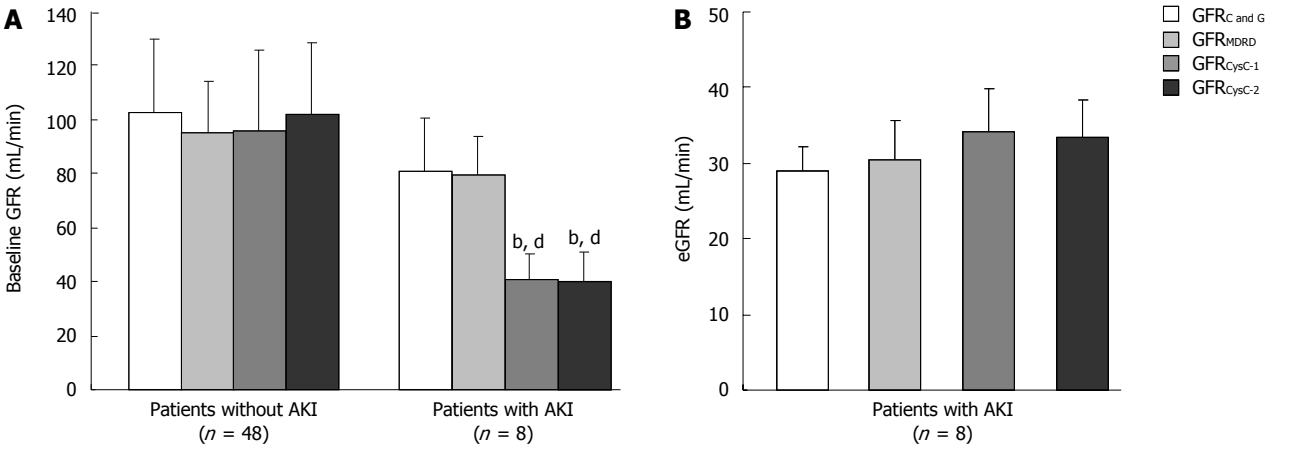

Figure 3 Performance of four equations for measuring estimated glomerular filtration rate in patients with acute-on-chronic liver failure. A: Baseline estimated glomerular filtration rate (eGFR) between patients with or without acute kidney injury (AKI); B: Comparison of four eGFR values in patients with AKI. $eGFR_{CG}$: The Cockcroft and Gault formula; $eGFR_{MDRD}$: The modification of the diet in renal disease equation; $eGFR_{CysC-1}$: Cystatin C-based Hoek estimate; $eGFR_{CysC-2}$: Chronic Kidney Disease Epidemiology Collaboration cystatin C equation. $^{b}P < 0.01$ *vs* GFR_{CysC} in patients without AKI; $^{d}P < 0.01$ *vs* e-GFR_{CG}, and e-GFR_{MDRD} in patients with AKI.

was clearly a risk factor for mortality in ACLF patients[6].

The progression of renal dysfunction in the presence of liver failure may be insidious and rapid or it may present as a mild or severe disturbance. Serum Cr is an easily measurable and widely used marker of renal function. However, Cr is an insensitive marker of kidney injury, and is usually maintained within the normal range until renal function is severely impaired, as in patients with cirrhosis and liver failure[29]. Several studies have reported that CysC is more useful for the assessment of renal function in patients with cirrhosis[14,19,20]. Assessment of CysC levels could be valuable in the early detection of renal dysfunction because they increase faster, as the GFR decreases, than do Cr levels[30]. However, data concerning CysC levels in ACLF patients are unavailable. All of the patients in the ACLF group had normal serum Cr level, with an average of 0.9 ± 0.1 mg/dL. Meanwhile, the average level of serum CysC was significantly higher in ACLF patients in comparison with healthy controls and CHB patients. Our results suggest that mild-to-moderate renal dysfunction may occur in ACLF patients who have a normal Cr level.

Patients with ACLF may have renal dysfunction other than HRS, due to the underlying circulatory abnormalities and sepsis[4,27,28]. Recently, the term AKI was proposed by AKIN, which may accurately represent the entire spectrum of acute renal dysfunction in ACLF patients. In our study, eight out of 56 ACLF patients (14.3%) developed AKI during hospitalization. Our results showed that CysC levels were significantly higher in patients who developed AKI during hospitalization. Our findings indicated that CysC could be used for the early detection of renal dysfunction in patients with ACLF before any increase in serum Cr levels is detected. Our multivariate analysis showed that age, Cr, sodium, and MELD score were not useful for predicting the development of AKI. The only independent predictive factor for AKI was CysC (OR = 1.8), which suggested that CysC represented renal function status more accurately in ACLF patients than did Cr. The results also indicated that CysC level might have accurately and rapidly reflected abnormalities in the renal handling of sodium and solute-free water before reduction in serum sodium level was detected. The diagnostic cutoff value of CysC for AKI prediction was 1.21 mg/L, which was different from that in cirrhosis patients reported previously[19,20].

It has been suggested that a Cr-based assessment of eGFR will overestimate the renal function in nonazotemic patients with cirrhosis and with moderate renal dysfunction (GFR < 60 mL/min)[31-33]. Two Cr-based and two CysC-based eGFR values were calculated in ACLF patients. The baseline Cr-based eGFR was similar in patients with or without AKI; however, baseline CysC-based eGFR was significantly lower in patients with AKI.

The results indicated that CysC-based eGFR was better in assessing kidney dysfunction in ACLF patients with a normal Cr level. In ACLF patients with an established diagnosis of AKI, both Cr-based and CysC-based eGFR were decreased to the same level, suggesting that these eGFR calculations were accurate in the case of severe renal dysfunction. Direct measurement of GFR using exogenous markers [Tc-99m diethylene-triamine-penta-acetic acid (DTPA) or inulin clearance] remains the standard for assessment of renal function[34,35]. Unfortunately, direct GFR assessment was not performed in our study because of the disease severity in ACLF patients. Demirtas *et al*[33] showed a correlation between CysC and 99mTc-DTPA clearance (r = -0.522, P = 0.006). They suggested that CysC assay, which has good analytical performance, could measure eGFR in patients with cirrhosis. That previous study, as well as our present study, suggests that CysC assay could replace Cr measurement for GFR assessment in patients with cirrhosis or ACLF.

In conclusion, CysC can be used as an early biomarker for the detection of renal dysfunction in patients with ACLF before any increase in the serum Cr levels can be detected. CysC-based eGFR calculation is more early represented renal function in ACLF patients during the period of mild-to-moderate renal dysfunction. The number size of patients for AKI development was small in this retrospective study. A prospective, large cohort study is ongoing in our research center to resolve this issue.

COMMENTS

Background

Kidney dysfunction is a common complication of advanced liver disease and associated with a high mortality. Serum creatinine (Cr) is an easily measurable and widely used marker of renal function. Unfortunately, Cr is an unreliable indicator during acute changes in kidney function because it highly depends on extrarenal factors such as muscle mass, gender, age and protein intake during the estimation. New biomarkers with higher reliability and specificity for estimation of renal function are required. Several reports have suggested that increased cystatin C levels are more sensitive for prediction of hepatorenal syndrome (HRS) development in patients with cirrhosis. However, data concerning serum cystatin C (CysC) levels in acute-on-chronic liver failure (ACLF) patients are unavailable.

Research frontiers

CysC is a ubiquitous protein that is freely filtered by the kidney and then metabolized by the tubules. Unlike Cr level, CysC level is independent of muscle mass, age or sex, and is not influenced by inflammatory conditions or malignancy. CysC detects impairment of glomerular filtration rate (GFR) earlier than Cr. The research hotspot is to investigate whether CysC can be used as an early biomarker for the detection of renal dysfunction in patients with ACLF before any increase in the serum Cr levels is detected.

Innovations and breakthroughs

Serum CysC is currently being investigated in the prediction of acute kidney injury (AKI) following cardiac surgery, advanced liver diseases, and undergoing liver transplantation, but the data using CysC levels in ACLF patients are lacking. Previous studies have reported that CysC was useful for the assessment of HRS in patients with cirrhosis. However, acute tubular necrosis and HRS may account for the majority of cases of severe renal dysfunction in patients with ACLF due to underlying circulatory abnormalities and inflammation. AKI which can represent the entire spectrum of acute renal dysfunction in ACLF patients was introduced in this paper.

Applications

CysC can be used as an early biomarker for the detection of renal dysfunction in patients with ACLF before any increase in the serum Cr levels. CysC-based eGFR more early represented renal function of ACLF patients during the period of mild-to-moderate renal dysfunction.

Terminology

AKI: a abrupt reduction in kidney function manifested by an absolute increase in serum creatinine by 0.3 mg/dL or more, equivalent to a percentage increase in serum creatinine by 50% or more (≥ 1.5 folds from baseline) without any evidence of preexisting kidney disease.

Peer review

The authors presented the finding that, in case of acute-on-chronic liver failure, the predictive performance of serum CysC and eGFR calculated from CysC is superior to that of serum creatinine and the other parameters. The prospective observation is excellent. The data collected in this study contribute to our understanding of common rule in the ACLF.

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BRIEF ARTICLE

Oxidative stress induces gastric submucosal arteriolar dysfunction in the elderly

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Abstract

AIM: To evaluate human gastric submucosal vascular dysfunction and its mechanism during the aging process.

METHODS: Twenty male patients undergoing subtotal gastrectomy were enrolled in this study. Young and elderly patient groups aged 25-40 years and 60-85 years, respectively, were included. Inclusion criteria were: no clinical evidence of cardiovascular, renal or diabetic diseases. Conventional clinical examinations were carried out. After surgery, gastric submucosal arteries were immediately dissected free of fat and connective tissue. Vascular responses to acetylcholine (ACh) and sodium nitroprusside (SNP) were measured by isolated vascular perfusion. Morphological changes in the gastric mucosal vessels were observed by hematoxylin and eosin (HE) staining and Verhoeff van Gieson (EVG) staining. The expression of xanthine oxidase (XO) and manganese-superoxide dismutase (Mn-SOD) was assessed by Western blotting analysis. The malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) content and the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were determined according to commercial kits.

RESULTS: The overall structure of vessel walls was shown by HE and EVG staining, respectively. Disruption of the internal elastic lamina or neointimal layers was not observed in vessels from young or elderly patients; however, cell layer number in the vessel wall increased significantly in the elderly group. Compared with submucosal arteries in young patients, the amount of vascular collagen fibers, lumen diameter and media cross-sectional area were significantly increased in elderly patients. Ach- and SNP-induced vasodilatation in elderly arterioles was significantly decreased compared with that of gastric submucosal arterioles from young patients. Compared with the young group, the expression of XO and the contents of MDA and H_2O_2 in gastric submucosal arterioles were increased in the elderly group. In addition, the expression of Mn-SOD and the activities of SOD and GSH-Px in the elderly group decreased significantly compared with those in the young group.

CONCLUSION: Gastric vascular dysfunction and senescence may be associated with increased oxidative stress and decreased antioxidative defense in the aging process.

Key words: Aging; Vascular dysfunction; Gastric blood flow; Oxidative stress; Human

Core tip: Aging is usually accompanied by a high risk of gastric disease. It is currently thought that adequate mucosal blood flow plays an important role in maintaining mucosal integrity. This study showed that oxidative stress induces gastric submucosal vascular structure dysfunction during the aging process. Vascular aging of gastric mucosa may lead to blood supply insufficiency, and thus increase the incidence of gastric diseases.

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INTRODUCTION

Although it is difficult to define the term "aging" in medical fields, it usually means the progressive accumulation of irreversible degenerative changes leading to loss of homeostasis. It is thought that there is also a modest decline in the structure and function of several digestive organs.

The most common stomach diseases in elderly individuals are atrophic gastritis and peptic ulcer disease[1]. The former is significantly associated with *Helicobacter pylori* infection and reduced acid secretion[2]. Hyposecretion of gastric acid reduces the absorption of vitamin B12, iron and calcium, and these deficits can lead to megaloblastic or iron-deficiency anemia and a higher frequency of osteoporosis[3]. Peptic ulcers in older patients are often caused by the use or overuse of nonsteroidal anti-inflammatory drugs[4].

It is currently thought that an adequate mucosal blood flow plays an important role in maintaining mucosal integrity. The blood supplies oxygen, nutrients and gastrointestinal hormones to support the correct structure, function and turnover of gastric mucosa. Blood flow is also important in the production and secretion of mucus and helps to maintain the mucosal barrier. In addition, the blood circulating in the surface mucosa removes waste materials and back-diffusing hydrogen ions and maintains the secretion of bicarbonate ions, protecting the mucosa by maintaining the neutral status of regional mucosa[5]. Numerous experimental studies have demonstrated the importance of mucosal blood flow in the defense of gastric mucosa against injury[6-13].

The structure and function of gastric blood vessels are important for determining blood flow, which plays an important role in maintaining mucosal integrity. There is considerable evidence showing that vascular aging is associated with an increased production of reactive oxygen species (ROS)[14]. There is a balance between the generation and elimination of ROS in vessels[15]. If the balance is destroyed, excess ROS will be produced, resulting in cellular dysfunction and vascular aging[16,17]. In the present study, human gastric submucosal arterioles from subtotal gastrectomy specimens were used to determine the relationship between gastric vascular dysfunction and oxidative stress, and to further explain the reason for the higher risk of gastric diseases in the elderly.

MATERIALS AND METHODS

Drugs and reagents

Acetylcholine (ACh) and sodium nitroprusside (SNP) were purchased from Sigma (St. Louis, MO, United States). The kits for assessing lipid oxidation injury including superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) were purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu Province, China). Polyclonal antibodies for xanthine oxidase (XO), manganese-superoxide dismutase (Mn-SOD) and the alkaline phosphorylase tagged goat anti-rabbit IgG antibody were purchased from Santa Cruz Biotechnology (CA, United States). The 5-bromo-4-chloro-3-indolyl phosphate/nitrotetrazolium blue chloride (BCIP/NBT) kit was purchased from Promega (Madison, WI, United States).

Harvesting of samples

The study was approved by the Ethics Committee of Xuzhou Medical College. Twenty male patients undergoing subtotal gastrectomy for gastric cancer in the Affiliated Hospital of Xuzhou Medical College were enrolled in this study. Patients with diabetes, hypertension or other cardiovascular diseases were excluded. All enrolled patients underwent conventional clinical examinations, including fasting blood glucose, total cholesterol, triglycerides and blood pressure, which were all found within normal ranges for clinic.

The patients were divided into two groups according to age: 10 patients aged 25-40 years were included in the young group and 10 patients aged 60-85 years were included in the elderly group. Gastric tissues, which were confirmed by pathology to be normal tissue, were obtained a distance from the cancer tissue and were undamaged due to surgical instruments. Gastric submucosal arterioles were immediately dissected free of fat and connective tissue in order to measure vascular function and changes in biochemistry and molecular biology.

Conventional clinical examinations in enrolled patients

Body mass index was calculated using body weight in kilograms divided by the square of the height in meters (kg/m^2). Fasting plasma total cholesterol, triglyceride concentration, systolic blood pressure, diastolic blood pressure and fasting plasma glucose concentration were measured. Arterial blood pressure was measured over the brachial artery during supine rest using a semiautomatic device (Dynamap XL, Johnson and Johnson). Fasting plasma metabolic parameters and oxidized low-density

lipoprotein were determined by standard assays. Plasma samples were also analyzed for oxidized low-density lipoprotein[18].

Histological staining

After surgery, sections of arteries were placed in phosphate buffered formaldehyde (4%) overnight, then stored in ethanol and embedded in paraffin. Cross sections (4 μm) were stained with hematoxylin and eosin (HE) staining and Verhoeff van Gieson (EVG) staining.

Arteriolar response to acetylcholine and sodium nitroprusside

Similar to our previous study[19], gastric submucosal arterioles, approximately 200 μm in maximal diameter and approximately 10 mm in length, were isolated and cannulated in a water-jacketed (37 ℃) perfusion device, intravascular pressure was maintained at 80 mmHg, and the changes in vascular diameter were recorded using a video monitor system. Dilations due to ACh (10^{-7} to 10^{-5} mol/L) and SNP (10^{-7} to 10^{-5} mol/L) were assessed in the arterioles from the young and elderly groups. Vasodilatation responses were expressed as the percentage of basal diameter at 80 mmHg.

ROS determination

Gastric arteries were isolated and crushed with liquid nitrogen and a homogenate was prepared. The homogenate was centrifuged, and the supernatant was used for biochemical analyses. The protein concentration in the supernatant was determined by the bicinchoninic acid assay (BCA assay, Nanjing Jiancheng Bioengineering Institute).

The MDA concentration in the homogenate was determined using a commercially available kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) based on thiobarbituric acid (TBA) reactivity. Briefly, after mixing trichloroacetic acid with the homogenate and centrifuging, a supernatant was obtained, and TBA was added. The developed red color of the resulting reaction was measured at 532 nm with a spectrophotometer. Other procedures were carried out following the manufacturer's protocols.

The content of H_2O_2 in gastric submucosal arteries was assessed using a commercially available kit (Nanjing Jiancheng Bioengineering Institute). H_2O_2 bound with molybdenic acid to form a complex, which was measured at 405 nm and the content of H_2O_2 was then calculated.

Antioxidant activity assay

SOD activity in gastric submucosal arteries was assessed using a commercially available kit (Nanjing Jiancheng Bioengineering Institute) based on the auto-oxidation of hydroxylamine. The developed blue color was measured at 550 nm.

GSH-Px activity was determined by the velocity method using a GSH-Px kit (Nanjing Jiancheng Bioengineering Institute). The reaction was initiated by the addition of H_2O_2. A series of enzymatic reactions was activated by GSH-Px in the homogenate which subsequently led to the conversion of GSH (reduced glutathione) to oxidized glutathione (GSSG). The change in absorbance during the conversion of GSH to GSSG was recorded spectrophotometrically at 412 nm.

Western blotting analysis

Gastric submucosal arteries from young and elderly patients were isolated and pooled in liquid nitrogen, respectively. Samples were solubilized in lysis buffer containing 1% protease inhibitor cocktail (Sigma) in ice for 30 min, followed by sonication for 1 min which was carried out twice at a 5-min interval. The supernatants were collected after centrifugation at 10000 × *g* for 15 min at 4 ℃. Protein concentrations were determined using the BCA protein assay kit. Samples (50 μg protein) were separated on 10% SDS-PAGE gels and transferred to a polyvinylidene fluoride membrane. The blots were incubated with 5% bovine serum albumin in TBST (10 mmol/L Tris, pH 7.5; 150 mmol/L NaCl, 0.05% Tween-20) at room temperature for 2 h, then incubated with primary antibodies (anti-Mn-SOD, polyclonal antibody 1:500, anti-XO polyclonal antibody 1:500) at 4 ℃ overnight. After washing with TBST, the blots were incubated with secondary antibody for 2 h and were determined using a BCIP/NBT assay kit. β-actin was used to normalize loading variations.

Statistical analysis

Data were expressed as mean ± SE. Comparisons between two groups were made using the Student's *t* test. Statistical analyses were performed using SPSS for Windows version 13.0. $P < 0.05$ was considered statistically significant.

RESULTS

Conventional clinical examinations in enrolled patients

Patients with diabetes, hypertension or other cardiovascular diseases which could affect the structure and function of vessels were excluded from the study. The results of conventional clinical examinations in the young and elderly patients are shown in Table 1. Fasting plasma total cholesterol, triglyceride concentrations, fasting plasma glucose concentrations, systolic blood pressure, diastolic blood pressure and mean arterial pressure were higher in the elderly group compared with the young group ($P < 0.05$). However, these values were within normal ranges for clinic. There were no differences in body mass index between the two groups.

Changes in morphology of gastric submucosal arteries

The overall structure of vessel walls was examined by HE and EVG staining, respectively (Figure 1). Disruption of the internal elastic lamina or neointimal layers was not observed in young or aged vessels; however, the cell layer number in the vessel walls increased significantly in the elderly group (Figure 1A and B). Compared with submucosal arteries in young patients, the amount of vascular collagen fibers, lumen diameter and media cross-sectional

Table 1 Clinical characteristics of young and elderly patients with isolated gastric submucosal arteries

	Young group	Elderly group
No. of patients	10	10
Age, yr	34.2 ± 4	71.6 ± 7[a]
Body mass index, kg/m^2	21.5 ± 2.6	20.5 ± 3
Fasting blood glucose, mmol/L	4.2 ± 0.7	5.4 ± 0.6[a]
Total cholesterol, mmol/L	3.87 ± 0.3	4.89 ± 0.17[a]
Triglycerides, mmol/L	1.03 ± 0.01	1.23 ± 0.11[a]
Systolic blood pressure, mmHg	112.2 ± 9.6	126.8 ± 8.6[a]
Diastolic blood pressure, mmHg	67.7 ± 3.7	77.9 ± 5.5[a]
Mean arterial pressure, mmHg	82.53 ± 2.98	94.2 ± 5.05[a]

Data are mean ± SE. [a]$P < 0.05$ *vs* young group.

area were significantly increased in the elderly group (Figure 1C and D).

Ach- and SNP-induced arteriolar response

ACh (10^{-7}, 10^{-6}, and 10^{-5} mol/L)- and SNP (10^{-7}, 10^{-6}, and 10^{-5} mol/L)-induced dilations were compared in gastric submucosal arterioles. Basal diameter and passive diameter at 80 mmHg of intravascular pressure in the arterioles from young and elderly patients were 142.8 ± 3.6 and 144.8 ± 2.8 μm, and 192.8 ± 4.1 and 189.0 ± 2.27 μm, respectively. As shown in Figure 2, dilation due to ACh, which stimulates NO synthesis and release from endothelium, was greatly reduced in the vessels from the elderly group compared with the young group ($P < 0.05$). Endothelium-independent vasodilatation was determined using SNP (a NO donor). Dilation due to SNP was also reduced in the vessels from the elderly group ($P < 0.05$), but the magnitude of this reduction was less.

Changes in MDA, H_2O_2, SOD and GSH-Px in gastric submucosal arteries

To determine whether oxidative stress participates in the aging process, we measured the contents of MDA and H_2O_2 and the activities of GSH-Px and SOD in gastric submucosal arteries, these are key markers of oxidative stress. Compared with the young group, there was a significant increase in MDA and H_2O_2 content ($P < 0.05$), and a decrease in SOD and GSH-Px activities ($P < 0.05$) in the elderly group (Figure 3).

Changes in XO and SOD protein expression in gastric submucosal arteries

Gastric submucosal arteries were isolated and the expression of XO and Mn-SOD was assessed. Figure 4 shows a representative Western blotting of XO and Mn-SOD in arteries. The XO protein was significantly increased (Figure 4A), whereas the expression of Mn-SOD (Figure 4B) was significantly reduced in the elderly group compared with the young group ($P < 0.05$).

DISCUSSION

Vascular aging is a key factor in accelerating the aging process and increasing the incidence of disease in humans. In this study, we demonstrated a marked deterioration in the structure and function of gastric blood vessels during aging. The underlying mechanisms may be associated with increased oxidative stress and decreased antioxidative defense, which induce an imbalance in oxidative stress during the aging process, thus accelerating vascular dysfunction and senescence in the elderly.

Aging is considered the leading cause of morbidity and mortality worldwide, and the proportion of elderly people is steadily growing[20]. With increasing age, the vasculature undergoes functional and structural impairment. Vascular changes during aging are manifested in various ways in many experimental animals; the most obvious changes noted are thickening of less compliant vessel walls[21]. In the present study, we evaluated the walls of gastric submucosal arteries from elderly patients, which were much thicker than the arteries from young patients. In addition, the collagen content was increased in aged gastric submucosal arteries (Figure 1). Aging can radically transform the endothelial layers lining the vessel wall in response to shear and stretch stress[22], and prompt them to thicken, as observed in many vascular models[23,24]. The changes in gastric submucosal arteries may be due to the recruitment of vascular smooth muscle cells for increased synthesis of interstitial, extra-cellular matrix proteins[25,26].

As expected, endothelium-dependent and -independent dilation were significantly attenuated in aged gastric submucosal arterioles compared with arterioles from young patients (Figure 2). Endothelial cell dysfunction has been observed in the elderly[27], and the production of endothelium-derived vasodilator substances is decreased[28,29]. In the present study, ACh-induced dilation of the gastric submucosal arteries from elderly patients was reduced, which was related to endothelial dysfunction during the aging process. Endothelium-independent dilation was also detected. SNP (a NO donor) induced dilation in the arterioles of elderly patients was reduced compared with that in arterioles from young patients. This change may be due to the thick vessel walls and increased collagen content. The changes in gastric submucosal vascular structure and function may lead to gastric blood supply insufficiency in the elderly.

Adequate mucosal blood flow plays an important role in maintaining mucosal integrity. Decreased gastric blood flow causes acute gastric mucosal lesions in animals and humans[9]. Gastric blood flow is important in the development of gastric ulceration and healing. Furthermore, the speed of ulcer healing is affected by the speed of blood flow at the ulcer edge[30]. Our group has recently shown that aspirin-induced injury of gastric mucous membrane was inhibited by increasing gastric mucosal blood flow[31]. The changes in gastric submucosal vascular structure and function which induced blood supply insufficiency may be one of the most important reasons for the higher incidence of gastric diseases in elderly subjects.

The mechanism involved in these changes in vascular structure and function in the elderly was also investigated

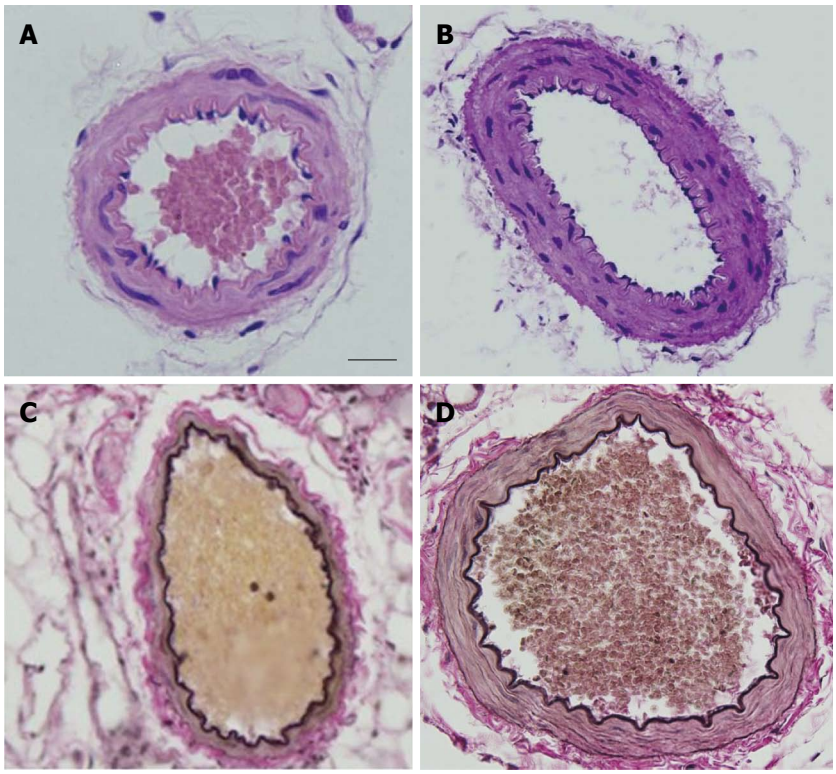

Figure 1 Changes in vascular structure of gastric submucosal arteries isolated from young and elderly patients shown by hematoxylin and eosin and Verhoeff van Gieson staining. A: Young, hematoxylin and eosin (HE); B: Elderly, HE; C: Young, Verhoeff van Gieson (EVG); D: Elderly, EVG. Images were obtained at × 400, Bar 50 μm.

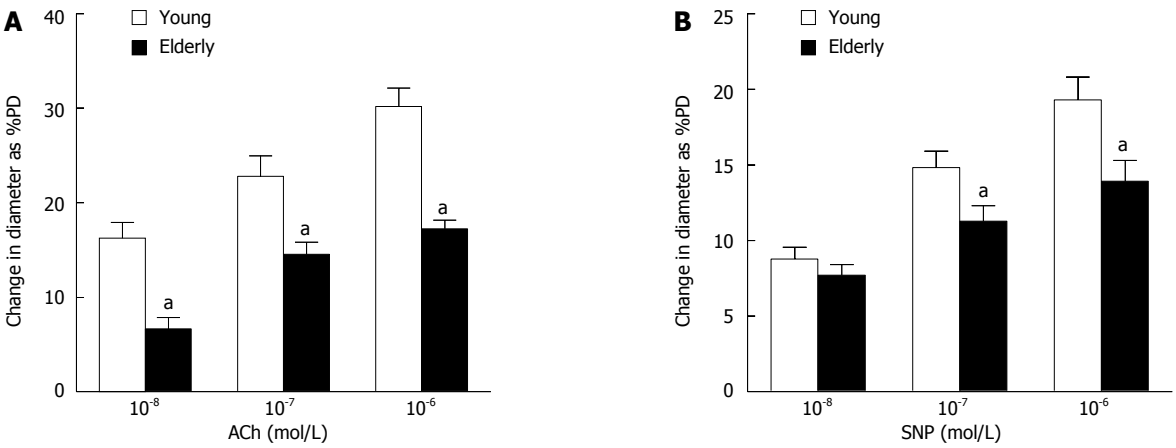

Figure 2 Effects of age on relaxant response to acetylcholine (A) and sodium nitroprusside (B) in gastric arterioles from young and elderly patients. Data are mean ± SE, $n = 6$. $^{a}P < 0.05$ *vs* young group. Ach: Acetylcholine; SNP: Sodium nitroprusside.

in the present study. MDA is a marker of free radical species-related injury, H_2O_2 is the main reactive oxygen species produced, and these are by-products of mitochondrial respiration. In our experiments, MDA and H_2O_2 were significantly increased in aged arteries compared to arteries from young patients. Age-related increases in oxidative stress may result in changes in vascular structure and function in aged gastric submucosal arteries.

Oxidative stress is associated with increased production of oxidizing species or a significant decrease in antioxidant defense capability[32]. XO is an important potential source of superoxide generation, it catalyzes the conversion reactions of hypoxanthine to xanthine and xanthine to uric acid, the last reaction in purine catabolism, with the byproduct of toxic superoxide radical[33]. XO protein levels in gastric submucosal arteries were significantly higher in elderly patients than in young patients. Increased expression of oxidases is the main source of reactive oxygen species in humans[34].

SOD, a major antioxidant enzyme, contributes to the destruction of free superoxide radicals and other reactive oxygen species, and blocks free radical-induced damage

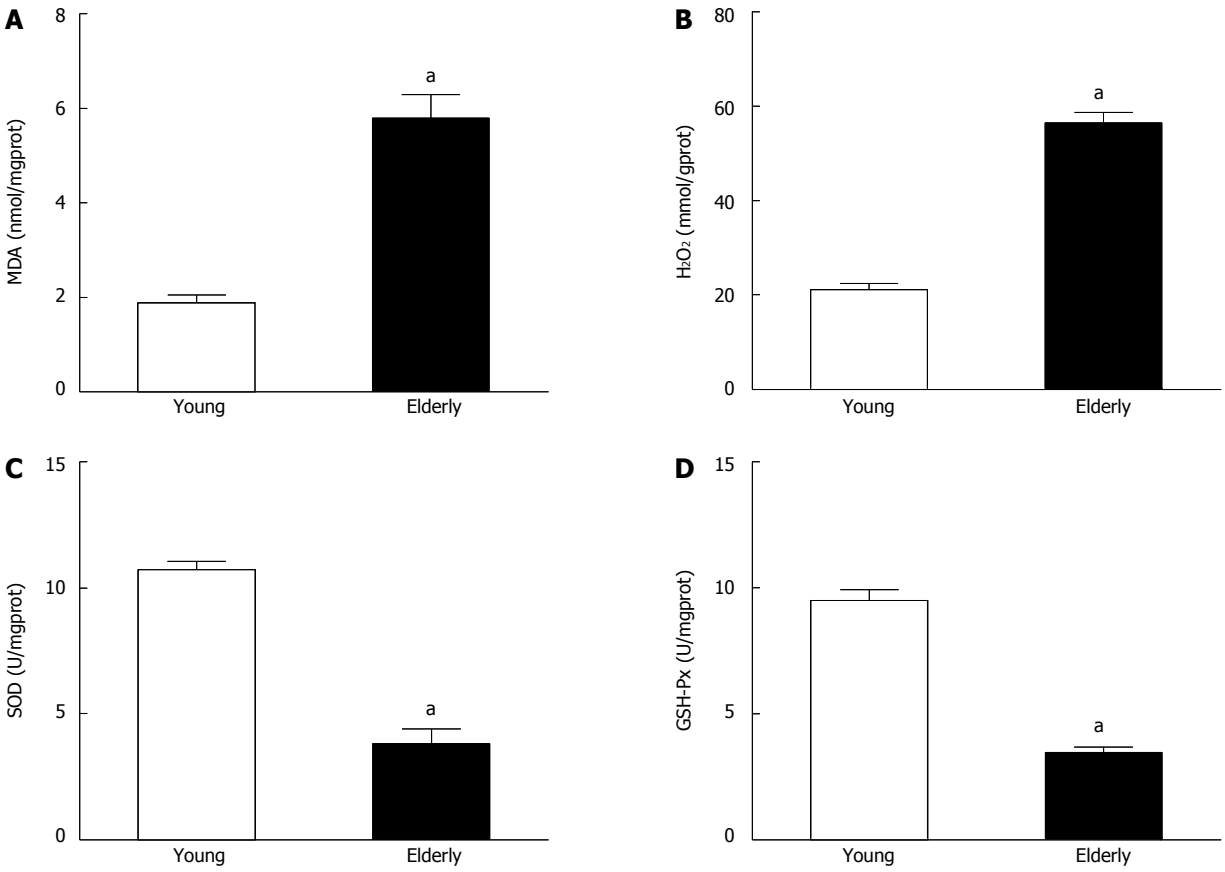

Figure 3 Changes in malondialdehyde (A) and hydrogen peroxide (B) and the activities of superoxide dismutase (C) and glutathione peroxidase (D) in gastric submucosal arteries isolated from young and elderly patients. Data are mean ± SE, $n = 10$. $^{a}P < 0.05$ *vs* young group. MDA: Malondialdehyde; H_2O_2: Hydrogen peroxide; SOD: Superoxide dismutase; GSH-Px: Glutathione peroxidase.

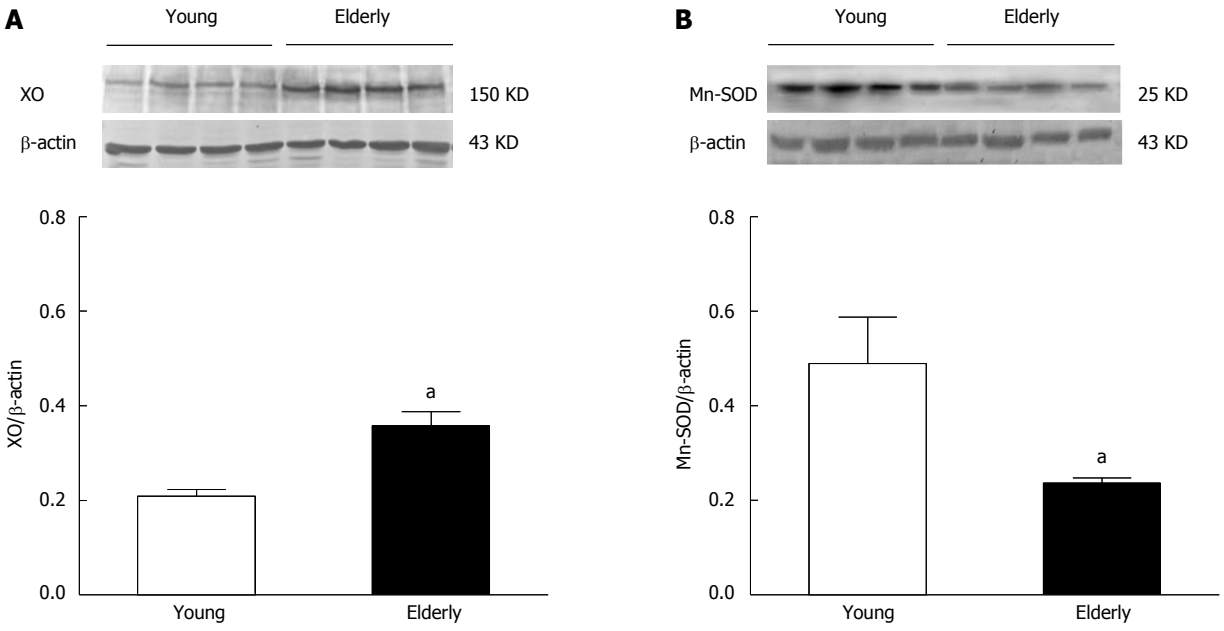

Figure 4 Expression of xanthine oxidase (A) and manganese-superoxide dismutase (B) protein in gastric mucosa substratum arteries isolated from young and elderly patients. $^{a}P < 0.05$ *vs* young group. β-actin was used to normalize loading variations (each bar represents the mean ± SE of 4 independent experiments). XO: Xanthine oxidase; Mn-SOD: Manganese-superoxide dismutase.

in the body[35,36]. There are three isoforms of SOD, cytosolic SOD or copper zinc SOD (CuZn-SOD or SOD-1), mitochondrial SOD or manganese SOD (Mn-SOD or SOD-2), and extracellular CuZn-SOD (EC-SOD or

SOD-3). Mn-SOD, which is found in mitochondria, plays an important role in the maintenance of vascular function. Previous animal studies found that the antioxidant enzyme content was decreased, while the expression of oxidative enzymes was significantly increased with aging[37]. The Mn-SOD protein level (Figure 4B) and SOD activity (Figure 3C) were significantly decreased in this study, which was consistent with previously reported findings.

GSH-Px is a free radical scavenging enzyme similar to SOD, which converts H_2O_2 to water independently[38]. In the present study, GSH-Px and total SOD were significantly lower in the elderly group compared with the young group. These results suggest that elderly patients have a higher risk of oxidative stress than younger patients and consequently greater vulnerability for chronic disease in old age. The decreased expression and activity of antioxidant enzymes will accelerate oxidative stress damage in aging vessels.

In summary, we demonstrated that oxidative stress and a decreased antioxidative defense induce vascular aging and enhance vascular dysfunction. Vascular aging of gastric mucosa may lead to blood supply insufficiency and an increased incidence of gastric diseases. This research has provided theoretical evidence suggesting that a decrease in oxidative stress during the aging process and improvement in the function of gastric submucosal vessels may be beneficial in the treatment of gastric disease in the elderly.

COMMENTS

Background

Aging is usually accompanied by a higher risk of gastric disease. Current opinion suggests that adequate mucosal blood flow plays an important role in maintaining mucosal integrity. The structure and function of gastric submucosal arteries are important for regulating gastric blood flow.

Research frontiers

Mucosal blood flow plays an important role in maintaining mucosal structure, function and turnover of gastric mucosa. Numerous experimental studies have demonstrated the importance of mucosal blood flow in the defense of gastric mucosa against injury. However, few studies have directly studied the structural and functional changes in gastric submucosal vessels.

Innovations and breakthroughs

This study showed that oxidative stress and a decreased antioxidative defense induce gastric vascular aging and enhance vascular dysfunction in the elderly. The structure and function of gastric submucosal arteries are important for regulating gastric blood flow. Vascular aging of gastric mucosa leads to blood supply insufficiency and an increase in the incidence of gastric disease.

Applications

This research provides theoretical evidence to suggest that a decrease in oxidative stress during the aging process and improvement in the function of gastric submucosal arteries may be beneficial in the treatment of gastric diseases in the elderly.

Terminology

Vascular aging involves vascular structure changes and dysfunction during the aging process. The aging of submucosal arteries leads to changes in mucosal blood flow which plays an important role in maintaining mucosal integrity. Inadequate blood flow will increase the incidence of gastric diseases.

Peer review

The authors demonstrated that aging induces vascular dysfunction through increasing oxidative stress in isolated human gastric submucosal arterioles. This study has provided theoretical evidence that a decrease in oxidative stress during the aging process and improvement in the function of gastric submucosal arterioles may be beneficial in the treatment of gastric disease in the elderly.

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BRIEF ARTICLE

Nedaplatin concurrent with three-dimensional conformal radiotherapy for treatment of locally advanced esophageal carcinoma

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Abstract

AIM: To evaluate the efficacy and toxicity of nedaplatin (NDP) concurrent with radiotherapy in the treatment of locally advanced esophageal carcinoma.

METHODS: Sixty-eight patients with locally advanced esophageal carcinoma were randomized into either a NDP group (n = 34) or a cisplatin (DDP) group (n = 34). The NDP group received NDP 80-100 mg/m^2 *iv* on day 1 + leucovorin (CF) 100 mg/m^2 *iv* on days 1-5 + 5-fluorouracil (5-FU) 500 mg/m^2 *iv* on days 1-5. The DDP group received DDP 30 mg/m^2 *iv* on days 1-3 + CF 100 mg/m^2 on days 1-5 + 5-FU 500 mg/m^2 *iv* on days 1-5. The treatment was repeated every 4 wk in both groups. Concurrent radiotherapy [60-66 Gy/(30-33 f)/(6-7 wk)] was given during chemotherapy.

RESULTS: There was no significant difference in the short-term response rate between the NDP group and DDP group (90.9% *vs* 81.3%, P = 0.528). Although the 1- and 2-year survival rates were higher in the NDP group than in the DDP group (75.8% *vs* 68.8%, 57.6% *vs* 50.0%), the difference in the overall survival rate was not statistically significant between the two groups (P = 0.540). The incidences of nausea, vomiting and nephrotoxicity were significantly lower in the NDP group than in the DDP group (17.6% *vs* 50.0%, P = 0.031; 11.8% *vs* 47.1%, P = 0.016; 8.8% *vs* 38.2%, P = 0.039). There was no significant difference in the incidence of myelosuppression, radiation-induced esophagitis or radiation-induced pneumonia between the two groups.

CONCLUSION: NDP-based concurrent chemoradiotherapy is effective and well-tolerated in patients with locally advanced esophageal carcinoma. NDP-based regimen has comparable efficacy to DDP-based regimen but is associated with lower incidences of gastrointestinal and renal toxicity.

Key words: Esophageal carcinoma; Chemoradiotherapy; Nedaplatin; Cisplatin

Core tip: This paper describes patients with locally advanced esophageal carcinoma who underwent nedaplatin (NDP) concurrent with radiotherapy. The survival and local control as well as the side effects during follow-up were analyzed by comparing with cisplatin (DDP). We found that NDP-based concurrent chemoradiotherapy is effective and well-tolerated. Compared with DDP, NDP-based concurrent chemoradiotherapy exhibits favorable efficacy with lower toxicity.

Shen ZT, Wu XH, Li B, Shen JS, Wang Z, Li J, Zhu XX. Nedaplatin concurrent with three-dimensional conformal radiotherapy

for treatment of locally advanced esophageal carcinoma. *World J Gastroenterol* 2013; 19(48): 9447-9452 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i48/9447.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i48.9447

INTRODUCTION

Radiotherapy is one of the main treatments for esophageal carcinoma, especially for patients with locally advanced esophageal cancer who have no indications for surgery. However, the 5-year survival rate for patients with non-early esophageal carcinoma after radiotherapy alone is only 8% to 17%[1]. Approximately 70%-80% of cases of radiotherapy failure are due to uncontrolled or recurrent localized disease. Chemotherapy given concurrently with radiotherapy can improve the efficacy of radiotherapy in esophageal carcinoma. Concurrent chemoradiotherapy has been recommended as the standard treatment for locally advanced esophageal carcinoma in some countries, and conventional fractionated radiotherapy plus cisplatin (DDP) and 5-fluorouracil (5-FU) has been advocated as a standard regimen for this malignancy[2,3]. However, the risk of gastrointestinal and renal toxicity associated with DDP-based PF regimen (DDP + 5-FU) limits its use. In the present study, we designed a randomized controlled phase Ⅱ trial to compare the efficacy, acute adverse reactions and late toxicity of three-dimensional conformal radiotherapy plus nedaplatin (NDP) and 5-FU versus plus the PF regimen in the treatment of locally advanced esophageal carcinoma, with an aim to find a regimen that has fewer adverse reactions and better efficacy than the PF regimen.

MATERIALS AND METHODS

Test drug

NDP injection (trade name, Jiebaishu, 10 mL) was provided by Simcere Pharmaceutical (Nanjing, China).

Subjects

Sixty-eight patients who were pathologically proven to have locally advanced esophageal squamous cell carcinoma by gastroesophagoscopy from March 2007 to September 2009 in Department of Radiation Oncology of the Nanjing General Hospital of Nanjing Military Region and had evaluable tumor lesions were included in the study. There were 38 males and 30 females, and their median age was 54 years (range, 26 to 72 years). According to the 1997 Union for International Cancer Control esophageal cancer staging system, 29 patients had stage Ⅱ disease and 39 had stage Ⅲ disease. The patients were randomly divided into either a NDP group (n = 34) or a DDP group (n = 34) to receive NDP + leucovorin (CF) + 5-FU and DDP + CF + 5-FU, respectively. In the NDP group, 14 patients had stage Ⅱ disease and 20 had stage Ⅲ disease. In the DDP group, 15 patients had stage Ⅱ disease and 19 had stage Ⅲ disease. The average age of patients in the NDP and DDP groups was 55 and 53 years old, and the median age was 54 and 53 years old, respectively. Clinical data for patients in both groups are shown in Table 1.

Table 1 Clinical data for patients in the nedaplatin group and the cisplatin group

	NDP group	DDP group	χ^2	*P* value
Case	34	34		
Gender				
Male	18	20	0.239	0.625
Female	16	14		
Age				
Range	27-72	26-70		
Median	54	53		
Clinical stage (Union for International Cancer Control)				
Ⅱa	4	6	0.478	0.787
Ⅱb	10	9		
Ⅲ	20	19		
Tumor length				
< 5 cm	14	17	0.534	0.465
⩾ 5 cm	20	17		
Cervical	5	3		
Location in the esophagus				
Upper	12	15	1.130	0.770
Middle	14	12		
Lower	3	4		
Medullary	20	22		
Fungoid	6	5		
Pathology				
Ulcer type	5	5	0.386	0.943
Sclerotic type	3	2		
General status (Eastern Cooperative Oncology Group score)				
0-1	24	21	0.591	0.442
2	10	13		

NDP: Nedaplatin; DDP: Cisplatin.

Inclusion criteria are (1) previously untreated, histologically or pathologically proven locally advanced esophageal carcinoma, with at least one measurable lesion (⩾ 2 cm); (2) Eastern Cooperative Oncology Group performance status score ⩽ 2; (3) expected survival for three months or more; (4) age between 26 and 72 years; (5) basically normal heart, lung, liver, kidney functions; (6) no previous thoracic radiotherapy or chemotherapy, and no significant chemotherapy contraindications; (7) no other malignancy; or (8) willing to provide signed informed consent.

Exclusion criteria included (1) participation in other drug trial or receiving anti-tumor therapy within 4 wk; (2) other serious complications that made the patient not to fit to the study; (3) pregnant or lactating women; and (5) allergy to the test drug.

Withdrawal criteria included (1) serious adverse reactions during treatment, such as life-threatening bleeding due to thrombocytopenia, life-threatening infections for leukopenia, and grade Ⅲ or more liver and kidney adverse reactions; (2) not being able to complete the treatment; (3) not willing to continue the trial; or (4) disease progression during treatment.

Treatments

The NDP group received NDP 80-100 mg/m^2 *iv* on day 1 + CF 100 mg/m^2 *iv* on days 1-5 + 5-FU 500 mg/m^2 *iv*

Table 2 Short-term response in the two groups *n* (%)

Group	*n*	CR	PR	SD	PD	RR	χ^2	*P* value
NDP group	33	6 (18.2)	24 (72.7)	3 (9.1)	0 (0)	90.9%	1.276	0.528
DDP group	32	5 (15.6)	21 (65.6)	6 (18.8)	0 (0)	81.3%		

RR = (PR+CR)/*n*. NDP: Nedaplatin; DDP: Cisplatin; CR: Complete response; PR: Partial response; SD: Stable disease; PD: Progressive disease; RR: Response rate.

on days 1-5. The DDP group received DDP 30 mg/m^2 *iv* on days 1-3 + CF 100 mg/m^2 on days 1-5 + 5-FU 500 mg/m^2 *iv* on days 1-5. The treatment was repeated every 4 wk in both groups. Before chemotherapy, prophylactic antiemetic therapy with 5-HT3 receptor antagonist was given. Granulocyte colony-stimulating factor (G-CSF) was administered when grade 3/4 neutropenia occurred. When anemia and grade 3/4 thrombocytopenia occurred, erythropoietin (EPO) and recombinant human interleukin-11 (IL-11) or therapeutic plateletpheresis were given, and the dose of main chemotherapy drugs was reduced by 25% in the next cycle or the interval between two cycles was extended. Concurrent radiotherapy was given during chemotherapy in both groups.

Three-dimensional conformal radiotherapy (3D-CRT) was adopted, with high-energy X-ray beams (6 MV) produced by a linear accelerator. Gross tumor volume (GTV) boundaries were determined by esophageal X-ray, barium meal, CT, and esophagoscopy. The upper and lower boundaries for clinical target volume (CTV) were defined as upper and lower boundaries for GTV plus 3 cm. The lateral boundaries for CTV were defined as the lateral boundaries for tumors plus 0.8 cm. Planning target volume (PTV) was defined as CTV plus 0.5 cm.

Efficacy was evaluated using the 2000 RECIST criteria based on physical examination and imaging data (X-ray, barium meal, chest CT). Imaging data were assessed independently by two professional radiologists. Patients were rated as complete response (CR), partial response (PR), stable disease (SD) or progressive disease (PD). The response rate (RR) was defined as (number of cases with CR + number of cases with PR)/total number of cases (*n*). Acute radiation injury was assessed using the Radiation Therapy Oncology Group (RTOG) acute radiation morbidity criteria (grades 0 to 4). Chemotherapy-associated adverse reactions were assessed using the U.S. National Cancer Institute common toxicity criteria (NCI-CTC), version 3.0 (grades 0-4).

Statistical analysis

Statistical analyses were performed using SPSS 13.0 software. Rates or percentages between two groups were compared using the chi-square test and Fisher exact test. Survival was analyzed using the Kaplan-Meier method. Survival curves were compared using the Log-rank significance test. Survival was defined as the period from the date of diagnosis to death. Two-tailed *P*-values <0.05 were considered statistically significant.

RESULTS

Short-term response

In the NDP group, 33 of 34 patients completed two or more cycles of treatment and were evaluable for efficacy and toxicity. In the DDP group, 32 patients completed two or more cycles of chemotherapy and can be evaluated for efficacy and toxicity, and the remaining two cases discontinued the treatment after one cycle of chemotherapy (one for intolerable side effects and the other for poor incompliance) but can be evaluated for toxicity. Short-term responses in the two groups are shown in Table 2.

Survival rate and causes of death

The 1-year overall survival rate was 75.8% (25/33) for the NDP group and 68.8% (22/32) for the DDP group, and the 2-year overall survival rate was 57.6% (19/33) and 50.0% (16/32), respectively. Although the overall survival rate was higher in the NDP group than in the DDP group, the difference was not statistically significant ($\chi^2 = 0.375$, $P = 0.504$).

During the follow-up period, 19 patients survived and 14 died in the NDP group. Of 14 dead patients, 9 died of distant metastasis, 3 of local control failure, and 2 of distant metastasis plus local control failure. Of 16 dead patients in the DDP group, 4 died of local control failure, 7 of local control failure plus distant metastasis, and 5 of distant metastasis. These findings suggest that distant metastasis was the main cause of death in both groups. The percentage of patients who died of distant metastasis showed no significant difference between the NDP group and DDP group (78.6% *vs* 75.0%, $\chi^2 = 0.053$, $P = 0.818$) (Figure 1).

Toxicity

Toxicity could be evaluated in all cases. In the NDP group, grades I -IV decreased hemoglobin developed in 20 patients (58.8%), grades I -IV leukopenia in 21 patients (61.8%), and grades I -IV thrombocytopenia in 19 patients (55.9%); the corresponding figures in the DDP group were 18 (52.9%), 19 (55.9%) and 14 cases (41.2%). The incidences of decreased hemoglobin, leukopenia and thrombocytopenia showed no significant differences between the two groups ($P = 0.990$, 0.805, 0.540). Although the incidence of hepatic dysfunction did not differ significantly between the two groups ($P = 0.565$), the incidence of renal toxicity was significantly higher in the DDP group (38.2% *vs* 8.8%, $P = 0.039$). The incidences

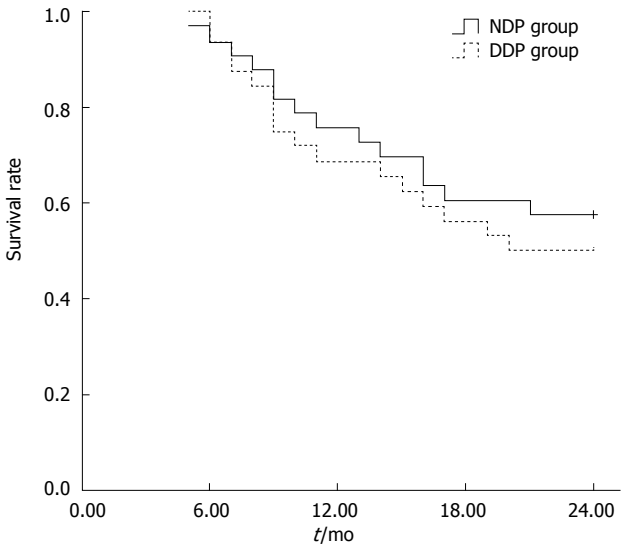

Figure 1 Survival curves for the two groups. NDP: Nedaplatin; DDP: Cisplatin.

of nausea and vomiting were significantly lower in the NDP group than in the DDP group (17.6% *vs* 50.0%, 11.8% *vs* 47.1%, $P = 0.031$, 0.016) (Table 3).

Late grade 4 esophageal toxicity was noted in one patient in the DDP group, but no patient developed late grade 3 or more esophageal toxicity in the NDP group. The incidences of late esophageal and lung toxicities showed no significant difference between the two groups ($P > 0.05$ for both). Serious late radiation toxicities such as radiation-induced myelitis and pericarditis were not observed (Table 4).

DISCUSSION

Esophageal carcinoma is one of the most common malignancies in China. Surgical excision is the standard treatment for esophageal carcinoma. Because most patients with esophageal carcinoma are diagnosed at the advanced stage, most of them have missed the chance of radical surgery. For patients without indications for surgery or those with localized disease after surgical resection, radiotherapy is another possible cure. However, both surgery alone and radiotherapy alone can not significantly improve the five-year survival rate in patients with esophageal carcinoma. To overcome this problem, worldwide scholars have tried a variety of comprehensive treatment from the 1970s to improve the therapeutic effect against this malignancy[4-8]. Chemotherapy combined with radiotherapy has yielded encouraging results. Particularly, the RTOG8501 trial conducted by Cooper *et al*[5] has provided conniving evidence to support the effectiveness of concurrent chemoradiotherapy in the management of esophageal carcinoma.

Compared to radiotherapy alone, concurrent chemoradiotherapy will further increase the incidence of side effects. Main side effects include radiation-induced esophagitis, pneumonia, bone marrow suppression, nausea, and vomiting. Seung *et al*[9] reported that the incidences of grade 2 and 3 esophagitis were 89% and 39%, respectively. Severe radiation-induced esophagitis is difficult to manage and often affects the implementation of treatment regimens or extends the total treatment time, thereby affecting therapeutic effects. Many studies have shown that the most commonly used PF regimen plus concurrent radiotherapy is associated with a high incidence of esophagitis. DDP is the main factor causing toxicity and is intolerable in some patients. Therefore, researchers have been seeking more efficient drugs or regimens with lower toxicity.

NDP (cis-diam-mincgly-colatoplatinum; formula, C2H8N2O3Pt; molecular mass, 303.18 kD) is a second-generation anti-cancer platinum derivative developed by Japanese pharmaceutical company Shionogi and approved for marketing in Japan in June 1995. Clinical studies have demonstrated that NDP is effective in esophageal carcinoma, head and neck cancer, lung cancer, cervical cancer, ovarian cancer, bladder cancer, testicular cancer and other solid tumors. It can be used alone or in combination with other chemotherapeutic drugs or radiotherapy to improve efficacy and reduce side effects. The mechanism of action of NDP is the same as that of DDP; they bind to DNA by forming platinum-nucleoside complexes and inhibit DNA replication[10]. The solubility of NDP is about 10 times that of DDP, and there exists certain cross-resistance between DDP and NDP[11]. NDP does not require hydration, has low renal and gastrointestinal toxicity, and shows a good synergistic effect when being used with other chemotherapy drugs. There is no complete cross-resistance between CDDP and NDP[12]. Although NDP has a high therapeutic index, its side effects are low. The dose-limiting toxicity of NDP is myelosuppression-induced thrombocytopenia, and its renal and gastrointestinal toxicity is low[13]. In recent years, many foreign clinical studies have demonstrated that the response rate of NDP-based regimens is above 50% in patients with advanced esophageal carcinoma, which is higher than or similar to those of conventional DDP-based regimens, but adverse reactions could be expected and well tolerated[14]. A similar study has also been reported in China[15]. Watanabe *et al*[16] reported the use of NDP and 5-FU with concurrent radiotherapy for advanced esophageal carcinoma. Kato *et al*[14] reported that NDP and 5-FU combined with radiotherapy achieved an overall response rate of 77%, a 1-year survival rate of 30.7%, a 2-year survival rate of 10.2%, and the median survival time of 10.1 mo in patients with unresectable advanced esophageal squamous cell carcinoma.

The present study showed that the short-term response rate and the 1- and 2-year survival rates were higher in the NDP group than in the DDP group (90.9% *vs* 81.3%, 75.8% *vs* 68.8%, 57.6% *vs* 50.0%), although the differences were not statistically significant. These findings suggest that NDP-based regimen has a trend to improve the short- and long-term response rates in locally advanced esophageal carcinoma, and that the efficacy of NDP-based concurrent chemoradiotherapy regimen is

Table 3 Acute adverse events in the two groups

Acute adverse reactions	NDP group (n = 34)						DDP group (n = 34)						χ^2	P value
	0	I	II	III	IV	Incidence	0	I	II	III	IV	Incidence		
Hemoglobin	14	9	5	5	1	58.80%	16	8	5	4	1	52.90%	0.303	0.990
Leukopenia	13	8	7	6	0	61.80%	15	8	6	4	1	55.90%	1.62	0.805
Platelet	15	7	6	5	1	55.90%	20	4	7	3	0	41.20%	3.109	0.540
Bilirubin	29	4	1	0	0	14.70%	30	3	1	0	0	11.80%	0.16	0.923
Transaminase	25	8	1	0	0	26.50%	27	7	0	0	0	20.60%	1.144	0.565
Urea nitrogen	30	4	0	0	0	11.80%	29	3	2	0	0	14.70%	2.16	0.340
Creatinine	31	2	1	0	0	8.80%	21	9	3	1	0	38.20%	8.378	0.039
Nausea	28	4	1	1	0	17.60%	17	7	6	4	0	50.00%	8.878	0.031
Vomiting	30	2	1	1	0	11.80%	18	6	4	6	0	47.10%	10.371	0.016
Esophagitis	8	18	7	1	0	76.50%	4	19	9	1	1	88.20%	2.61	0.625
Pneumonia	18	14	2	0	0	47.10%	12	17	4	1	0	64.70%	3.157	0.368

NDP: Nedaplatin; DDP: Cisplatin.

Table 4 Late adverse events in the two groups n (%)

Late adverse event	NDP group	DDP group	χ^2	P value
Late esophageal injury				
0	18 (52.9)	13 (38.2)		
I	10 (29.4)	12 (35.3)		
II	4 (11.8)	5 (14.7)	2.299	0.681
III	2 (5.9)	3 (8.8)		
IV	0 (0)	1 (2.9)		
Late lung injury				
0	24 (70.6)	20 (58.8)		
I	7 (20.6)	8 (23.5)		
II	2 (5.9)	4 (11.8)	1.43	0.698
III	1 (2.9)	2 (5.9)		
IV	0 (0)	0 (0)		

NDP: Nedaplatin; DDP: Cisplatin.

not lower, or slightly higher than that of traditional CDDP-based concurrent chemoradiotherapy regimen. With regard to adverse effects, the incidences of nausea and vomiting were significantly lower in the NDP group than in the DDP group (17.6% *vs* 50.0%, 11.8% *vs* 47.1%, $P < 0.05$ for both). The majority of cases of nausea and vomiting in the NDP group were grades Ⅰ-Ⅱ and could be easily managed using antiemetic therapy with 5-HT3 receptor antagonist, while the incidences of grades Ⅱ-Ⅲ nausea and vomiting were relatively high in the DDP group. The incidence of renal toxicity, mainly grades Ⅰ-Ⅱ, was significantly lower in the NDP group than in the DDP group (8.8% *vs* 38.2%, $P < 0.05$). There was no significant difference in the incidence of liver toxicity between the two groups ($P > 0.05$). The incidence of leukopenia, mainly grades Ⅰ-Ⅱ, was slightly higher in the NDP group than in the DDP group, but the difference was not statistically significant ($P > 0.05$). The incidence of thrombocytopenia (grades Ⅰ-Ⅱ: 38.2%; grades Ⅲ-Ⅳ: 17.6%) was also slightly higher in the NDP group. Thrombocytopenia occurred mainly 7 to 10 d after treatment and resolved in all cases 14 d after treatment. These results indicate that the incidence of gastrointestinal reactions such as nausea and vomiting was significantly lower in the NDP group. The liver and kidney toxicity was mild. The main dose-limiting toxicity was myelosuppression, especially thrombocytopenia, which can be managed by symptomatic and supportive treatment or dosage adjustment.

In conclusion, NDP is an effective drug for treatment of esophageal carcinoma. NDP combined with 5-FU is superior to DDP plus 5-FU in terms of reducing the incidences of gastrointestinal and renal toxicity and improving clinical tolerance. Since the sample size is small in the present study, further large-sample trials are required to evaluate the long-term efficacy and toxicity of NDP-based regimens.

COMMENTS

Background

Radiotherapy given concurrently with chemotherapy can improve the efficacy of radiotherapy in esophageal carcinoma. Concurrent chemoradiotherapy has been recommended as the standard treatment for locally advanced esophageal carcinoma, and conventional fractionated radiotherapy plus cisplatin (DDP) and 5-fluorouracil (5-FU) has been advocated as a standard regimen for this malignancy. However, the risk of gastrointestinal and renal toxicity associated with DDP-based PF regimen (DDP + 5-FU) limits its use.

Research frontiers

In the present study, the authors designed a randomized controlled phase Ⅱ trial to compare the efficacy, acute adverse reactions and late toxicity of three-dimensional conformal radiotherapy plus nedaplatin (NDP) and 5-FU *vs* plus the PF regimen in the treatment of locally advanced esophageal carcinoma, with an aim to find a regimen that has fewer adverse reactions and better efficacy than the PF regimen.

Innovations and breakthroughs

The survival and local control as well as the side effects during follow-up were analyzed by comparing with cisplatin. The authors found NDP-based concurrent chemoradiotherapy is effective and well-tolerated. Compared with DDP, NDP-based concurrent chemoradiotherapy exhibits favorable efficacy with lower toxicity.

Applications

The study results suggest that NDP-based concurrent chemoradiotherapy is a potential therapeutic regimen that could be used in locally advanced esophageal carcinoma.

Terminology

Cisplatin, a common chemotherapeutic drug, has been one of doctors' first lines of defense against tumors, especially those of the lung, ovary, testes and locally advanced esophageal carcinoma. Nedaplatin is a new platinum derivative, selected from a series of platinum analogues based on its pronounced preclini-

cal antitumor activity against various solid tumors with lower nephrotoxicity and gastrointestinal reactions.

Peer review

This is a good clinical study in which the authors evaluated the efficacy and safety of three-dimensional conformal radiotherapy plus NDP and 5-FU versus plus the PF regimen in the treatment of locally advanced esophageal carcinoma. The results suggest that NDP-based concurrent chemoradiotherapy exhibits favorable efficacy with lower toxicity.

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META-ANALYSIS

Endoscopic sphincterotomy plus large-balloon dilation *vs* endoscopic sphincterotomy for choledocholithiasis: A meta-analysis

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Abstract

AIM: To perform a meta-analysis of large-balloon dilation (LBD) plus endoscopic sphincterotomy (EST) *vs* EST alone for removal of bile duct stones.

METHODS: Databases including PubMed, EMBASE, the Cochrane Library, the Science Citation Index, and important meeting abstracts were searched and evaluated by two reviewers independently. The main outcome measures included: complete stone removal, stone removal in the first session, use of mechanical lithotripsy, procedure time, and procedure-related complications. A fixed-effects model weighted by the Mantel-Haenszel method was used for pooling the odds ratio (OR) when heterogeneity was not significant among the studies. When a Q test or I^2 statistic indicated substantial heterogeneity, a random-effects model weighted by the DerSimonian-Laird method was used.

RESULTS: Six randomized controlled trials involving 835 patients were analyzed. There was no significant heterogeneity for most results; we analyzed these using a fixed-effects model. Meta-analysis showed EST plus LBD caused fewer overall complications than EST alone (OR = 0.53, 95%CI: 0.33-0.85, P = 0.008); subcategory analysis indicated a significantly lower risk of perforation in the EST plus LBD group (Peto OR = 0.14, 95%CI: 0.20-0.98, P = 0.05). Use of mechanical lithotripsy in the EST plus LBD group decreased significantly (OR = 0.26, 95%CI: 0.08-0.82, P = 0.02), especially in patients with a stone size larger than 15 mm (OR = 0.15, 95%CI: 0.03-0.68, P = 0.01). There were no significant differences between the two groups regarding complete stone removal, stone removal in the first session, post-endoscopic retrograde cholangiopancreatography pancreatitis, bleeding, infection of biliary tract, and procedure time.

CONCLUSION: EST plus LBD is an effective approach for the removal of large bile duct stones, causing fewer complications than EST alone.

Key words: Balloon dilation; Cholangiopancreatography; Endoscopic retrograde; Choledocholithiasis; Endoscopic sphincterotomy; Meta-analysis

Core tip: This meta-analysis demonstrates that endoscopic sphincterotomy (EST) plus large-balloon dilation (LBD) is an effective approach for the removal of large bile duct stones. Specifically, when compared with the outcomes of EST alone, the combined technique is associated with fewer complications. Furthermore, use of mechanical lithotripsy in the EST plus LBD group decreased significantly, especially in patients with a stone size larger than 15 mm. However, more well-designed trials are required to clarify whether this combined technique is preferable.

Yang XM, Hu B. Endoscopic sphincterotomy plus large-balloon dilation *vs* endoscopic sphincterotomy for choledocholithiasis: A meta-analysis. *World J Gastroenterol* 2013; 19(48): 9453-9460

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INTRODUCTION

During endoscopic retrograde cholangiopancreatography (ERCP), endoscopic sphincterotomy (EST) or endoscopic papillary balloon dilation (EPBD) is the standard method of enlarging the papillary orifice before stone retrieval. However, the extent of orifice dilation with conventional EST or EPBD is limited[1-3], and the use of other methods such as mechanical lithotripsy, intraductal shock-wave lithotripsy, extracorporeal shock-wave lithotripsy or, if those fail, biliary stent placement with repeated ERCP or even surgery may be required in patients with difficult (usually large) stones[1]. These methods are not widely available, and a larger opening of the orifice by large-balloon dilation (LBD) seems to be necessary. Ersoz *et al*[4] first reported the use of LBD after sphincterotomy for large common bile duct stones and achieved a high stone clearance rate of up to 89%-95% without mechanical lithotripsy. Since then, a number of case series have also suggested that the combination technique facilitated large stone extraction and reduced dependence on mechanical lithotripsy, contributing to higher stone clearance in a single endoscopic session with an acceptable risk of complications[5-9]. However, the comparison of EST plus LBD and EST alone for removal of choledocholithiasis has given inconsistent results.

To the best of our knowledge, the only systematic review on the topic has been published by Liu *et al*[10]. This included non-randomized controlled trials (non-RCTs); two eligible abstracts[11,12] which were regarded as non-randomized in the review were in fact randomized; this was validated by contacting the authors. More recently, a well-arranged trial has been published and some conflicting results have emerged[13]. Therefore, we believe that an updated meta-analysis is required.

MATERIALS AND METHODS

Search strategy

A literature search was performed to identify all relevant studies that compared EST plus LBD and EST alone for removal of bile duct stones. The PubMed, EMBASE, Cochrane Library databases, and the Science Citation Index were searched systematically for all articles published up to May 2013, without language restriction, using the following terms in their titles, abstracts, or keyword lists: "balloon dilation," "sphincteroplasty," "sphincterotomy," "bile duct stone," and "choledocholithiasis." The references in retrieved articles were also screened manually. The abstracts of the United European Gastroenterology Week and Digestive Disease Week, from 2004 to 2012, were also searched systematically. An attempt to contact the first author was made when information was not extractable from potentially eligible published abstracts.

Study selection

Papers selected from this initial search were then screened for eligibility using the following criteria: (1) RCTs that evaluated a comparison of EST plus LBD (larger than 12 mm in balloon size) and EST alone in the removal of large common bile duct stones (larger than 10 mm in diameter); and (2) Outcomes of interest included complete stone removal, use of mechanical lithotripsy and complications. If reports came from the same study center, we only included data from the publication with the largest population. Comments, reviews, case reports, and guideline articles were excluded.

Data extraction

Data from eligible studies were extracted independently by two reviewers (Yang XM and Hu B) using standard forms, and consensus was reached on all items. Data were extracted on: first author, year of publication, country of origin, study setting, number, age and sex of patients, stone size, balloon diameter, complete stone removal, stone removal in the first session, use of mechanical lithotripsy, procedure time, and procedure-related complications.

Assessment of study quality

Two independent reviewers (Yang XM and Hu B) assessed the quality score of primary trials according to the Jadad scale[14]. Total scores ranged from 0 to 5. The Cochrane Collaboration's tool for assessing risk of bias was also used to address potential bias (Table 1). We defined studies with a Jadad score of 3 or more points and a low risk of bias as high quality in this meta-analysis. Disagreements were discussed by the reviewers and resolved through consensus.

Statistical analysis

For summary statistics in meta-analysis, the odds ratio (OR) is recommended for dichotomous data, and the weighted mean difference is recommended for continuous data. Complete stone removal, stone removal in the first session, use of mechanical lithotripsy and overall complications were summarized as OR with 95%CI. Peto OR with 95%CI was used for separate complications, including post-ERCP pancreatitis, bleeding, infection of biliary tract (including cholangitis and cholecystitis), and perforation, since it could generate the least biased pooled results of studies with zero event in both groups[15]. *P* values of less than 0.05 were considered significant.

Heterogeneity was assessed by visual inspection of a Forest plot, the Cochran Q test, and the I^2 statistic. Heterogeneity was considered significant by the Cochran Q test when $P < 0.1$ or $I^2 > 50\%$[16,17]. A fixed-effects model weighted by the Mantel-Haenszel method was used for pooling the OR when heterogeneity was not significant among the studies[18]. When a Q test or I^2 statistic indicated substantial heterogeneity, a random-effects model

Table 1 Characteristics of the included randomized controlled trials (according to the Cochrane Collaboration's tool for assessing risk of bias)

Ref.	Sequence generation	Allocation concealment	Blinding of participants	Incomplete outcome	Selective outcome	Other sources of bias
Heo *et al*[22] 2007	Computer random number generator	Sealed envelope	Outcome assessment blinded	No missing outcome data	All prespecified outcomes reported	No
Hong *et al*[11] 2009	Unclear	Not reported	Unclear	No missing outcome data	All prespecified outcomes reported	No
Kim *et al*[23] 2009	The order of the procedure	Not reported	Unclear	No missing outcome data	All prespecified outcomes reported	No
Kim *et al*[12] 2009	Unclear	Not reported	Unclear	No missing outcome data	All prespecified outcomes reported	No
Stefanidis *et al*[24] 2011	Random number table	Sealed envelope	Outcome assessment blinded	No missing outcome data	All prespecified outcomes reported	No
Teoh *et al*[13] 2013	Computer random number generator	Sealed envelope	Outcome assessment blinded	No missing outcome data	All prespecified outcomes reported	No

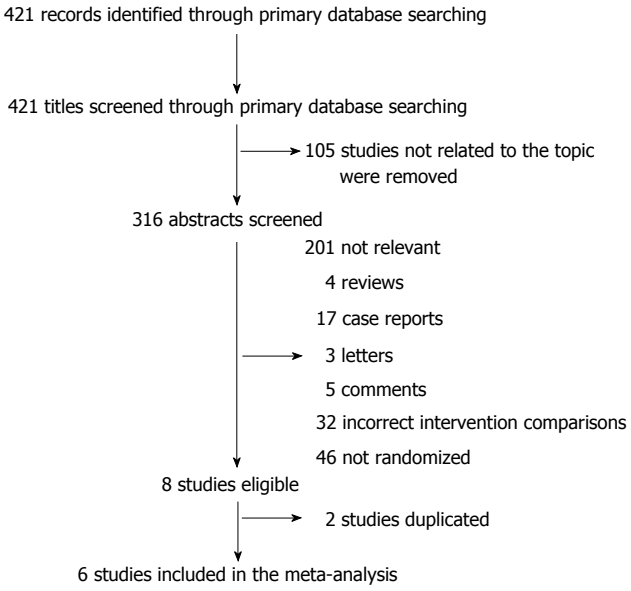

Figure 1 Flow chart of included and excluded trials.

weighted by the DerSimonian-Laird method was used[19]. We performed a sensitivity analysis by removing each study in turn from the overall data to evaluate the influence of a single study on the pooled analysis and by restricting the meta-analysis to high-quality studies. We also assessed the potential for publication bias through visual inspection of funnel plot asymmetry and evaluated the statistical significance of differences according to the methods of Begg *et al*[20] and Egger *et al*[21]. Statistical analyses were performed using Review Manager software (version 5.1 for Windows, Cochrane Collaboration, Oxford, United Kingdom).

RESULTS

Identification of eligible studies

The literature search yielded 316 abstracts for review, and 308 were excluded for the reasons shown in Figure 1. The results of two studies were conflated because they were from the same trial. Thus, six studies[11-13,22-24] were included, four of which were available as full texts and were high quality studies. The combined studies enrolled 835 patients who had been randomly allocated to the EST plus LBD group or the EST alone group. The characteristics of the included trials are listed in Tables 1 and 2, and the outcome data are shown in Table 3.

Efficacy

Six studies reported complete stone removal. Heterogeneity among these studies was not significant (P = 0.28, I^2 = 22%, Figure 2A). Thus, we used the fixed-effects model and found that there was no significant difference in complete stone removal between EST plus LBD and EST alone (OR = 1.41, 95%CI: 0.63-3.17, P = 0.40, Figure 2A). Sensitivity analysis by removing each study in turn from the overall data or by restricting the meta-analysis to high-quality studies showed that the result was robust. Four RCTs[12,13,22,23] reported stone removal in the first session, and there was no significant difference in stone clearance between the two methods (OR = 1.02, 95%CI: 0.65-1.61, P = 0.92). A comparison of EST plus LBD and EST alone in patients with stones larger than 15 mm was carried out, and five studies[11,13,22-24] with 377 patients were included. Meta-analysis showed that there was no significant difference in the complete stone removal rate according to the fixed-effects model (OR = 0.99, 95%CI: 0.35-2.81, P = 0.98, Figure 2B).

Use of mechanical lithotripsy

Six studies reported the use of mechanical lithotripsy during the stone removal process. The trials were heterogeneous (P < 0.001, I^2 = 87%), and a random-effects model analysis was performed. The results indicated a significantly reduced dependence on mechanical lithotripsy in the EST plus LBD group (OR = 0.26, 95%CI: 0.08-0.82, P = 0.02). We conducted a sensitivity analysis by excluding the study by Stefanidis *et al*[24], as no mechanical lithotripsy was used in the LBD group in this trial, and the result did not change (OR = 0.42, 95%CI: 0.18-0.98, P = 0.05). However, after removing the two eligible abstracts[11,12], there was no significant difference in the use of mechanical lithotripsy between EST plus

Table 2 Characteristics of the included randomized controlled trials

Ref.	Format	Country	Center involved	EST plus LBD, EST				Balloon diameter (mm)	Jadad score
				Number (*n*)	Male, female	Mean age (yr)	Stone size (mm)		
Heo *et al*[22] 2007	Full text	Korea	1	100	48, 52	64	16.0 ± 0.7[1]	12-20	4
				100	50, 50	63	15.0 ± 0.7[1]		
Hong *et al*[11] 2009	Abstract	Korea	1	70	Not reported	Not reported	> 15	15 or 20	1
				65			> 15		
Kim *et al*[23] 2009	Full text	Korea	1	27	12, 15	70	15-38.3	15, 16.5 or 18	3
				28	14, 14	70	15-48		
Kim *et al*[12] 2009	Abstract	Korea	1	104	53, 51	70	> 10	12-20	1
				100	49, 51	69	> 10		
Stefanidis *et al*[24] 2011	Full text	Greece	1	45	24, 21	69	12-20	15, 18 or 20	4
				45	22, 23	68	12-20		
Teoh *et al*[13] 2013	Full text	Hong Kong	2	73	32, 41	72	≥ 13	13-15	4
				78	40, 38	73	≥ 13		

[1]Values are mean ± SD. EST: Endoscopic sphincterotomy; LBD: Large-balloon dilation.

Table 3 Outcome data derived from the included randomized controlled trials *n* (%)

Ref.	Intervention	Complete stone removal	Stone removal in the first session	Mechanical lithotripsy	Overall complications	Pancreatitis	Bleeding	Infection of biliary tract	Perforation
Heo *et al*[22] 2007	Small EST plus LBD	97/100 (97)	83/100 (83)	8/100 (8)	5/100 (5)	4/100 (4)	0/100 (0)	1/100 (1)	0/100 (0)
	Full EST	98/100 (98)	87/100 (87)	9/100 (9)	7/100 (7)	4/100 (4)	2/100 (2)	1/100 (1)	0/100 (0)
Hong *et al*[11] 2009	Small EST plus LBD	70/70 (100)	Not reported	13/70 (19)	8/70 (11)	4/70 (6)	4/70 (6)	0/70 (0)	0/70 (0)
	Conventional EST	65/65 (100)		47/65 (72)	19/65 (29)	9/65 (14)	10/65 (15)	0/65 (0)	0/65 (0)
Kim *et al*[23] 2009	Small EST plus LBD	27/27 (100)	23/27 (85)	9/27 (33)	0/27 (0)	0/27 (0)	0/27 (0)	0/27 (0)	0/27 (0)
	Conventional EST	28/28 (100)	23/28 (82)	9/28 (32)	0/28 (0)	0/28 (0)	0/28 (0)	0/28 (0)	0/28 (0)
Kim *et al*[12] 2009	Small EST plus LBD	100/104 (96)	89/104 (86)	8/104 (8)	11/104 (11)	10/104 (10)	1/104 (1)	0/104 (0)	0/104 (0)
	Conventional EST	92/100 (92)	82/100 (82)	17/100 (17)	10/100 (10)	9/100 (9)	0/100 (0)	0/100 (0)	1/100 (1)
Stefanidis *et al*[24] 2011	Full EST plus LBD	44/45 (98)	Not reported	0/45 (0)	2/45 (4)	1/45 (2)	1/45 (2)	0/45 (0)	0/45 (0)
	Full EST plus ML	41/45 (91)		45/45 (100)	9/45 (20)	1/45 (2)	1/45 (2)	6/45 (13)	1/45 (2)
Teoh *et al*[13] 2013	Small EST plus LBD	71/73 (97)	65/73 (89)	21/73 (29)	5/73 (7)	2/73 (3)	1/73 (1)	2/73 (3)	0/73 (0)
	Full EST	78/78 (100)	69/78 (88)	36/78 (46)	8/78 (10)	3/78 (4)	0/78 (0)	3/78 (4)	2/78 (3)

EST: Endoscopic sphincterotomy; LBD: Large-balloon dilation.

LBD and EST alone (OR = 0.26, 95%CI: 0.05-1.48, *P* = 0.13). A subgroup analysis in patients with a stone size larger than 15 mm demonstrated that the use of mechanical lithotripsy in the EST plus LBD group decreased significantly (OR = 0.15, 95%CI: 0.03-0.68, *P* = 0.01, Figure 3A).

Safety

Six RCTs evaluated the safety in both groups (Table 4). The statistical results showed that EST plus LBD caused fewer overall complications than EST alone (OR = 0.53, 95%CI: 0.33-0.85, *P* = 0.008), and the result did not change by restricting the meta-analysis to the four high-quality studies (OR = 0.48, 95%CI: 0.24-0.99, *P* = 0.05). Subcategory analysis indicated that patients undergoing EST plus LBD had a lower risk of perforation (OR = 0.14, 95%CI: 0.20-0.98, *P* = 0.05). No significant difference was found in terms of post-ERCP pancreatitis (OR = 0.77, 95%CI: 0.43-1.39, *P* = 0.39), bleeding (OR = 0.50, 95%CI: 0.20-1.23, *P* = 0.13), and infection of the biliary tract (OR = 0.34, 95%CI: 0.11-1.02, *P* = 0.05).

Procedure time

Only two studies reported the total procedure time[13,23]. Meta-analysis showed no difference in ERCP duration between EST plus LBD and EST alone (OR = 1.55, 95%CI: -2.34-5.44, *P* = 0.44, Figure 3B).

Publication bias

The funnel plot did not show an asymmetrical pattern

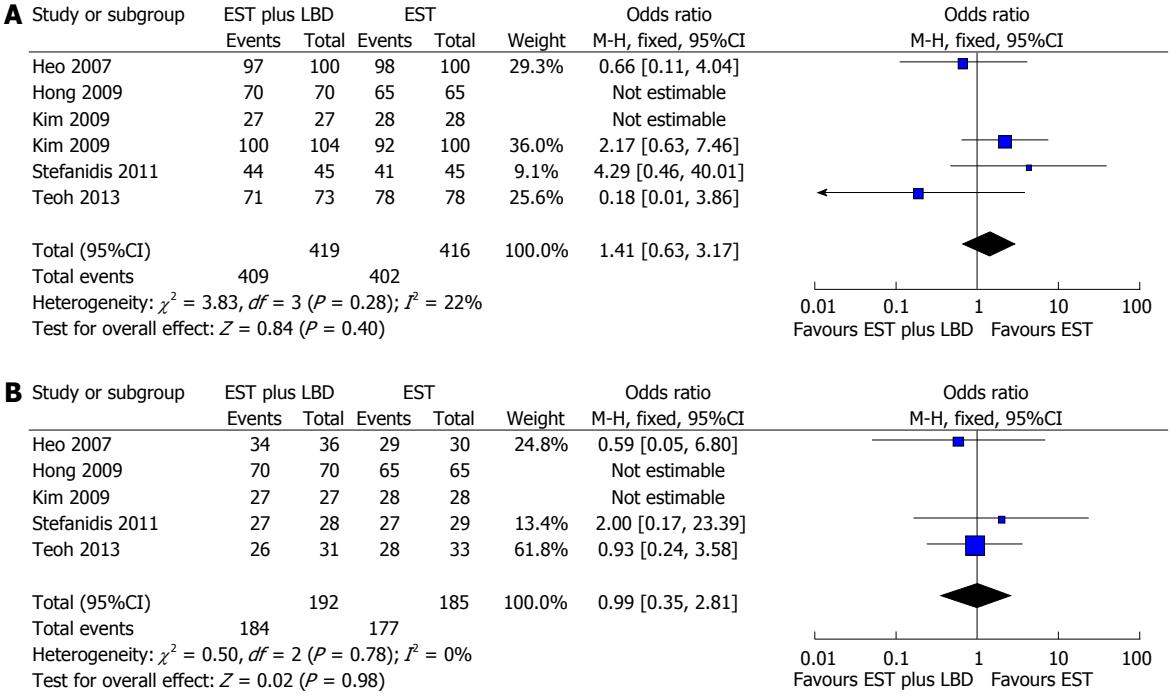

Figure 2 Forest plot demonstrating no significant difference in complete stone removal between endoscopic sphincterotomy plus large-balloon dilation and endoscopic sphincterotomy alone and in patients with stone size larger than 15 mm. A: Endoscopic sphincterotomy (EST) plus large-balloon dilation (LBD) and EST alone; B: EST plus LBD and EST alone and in patients with stone size larger than 15 mm.

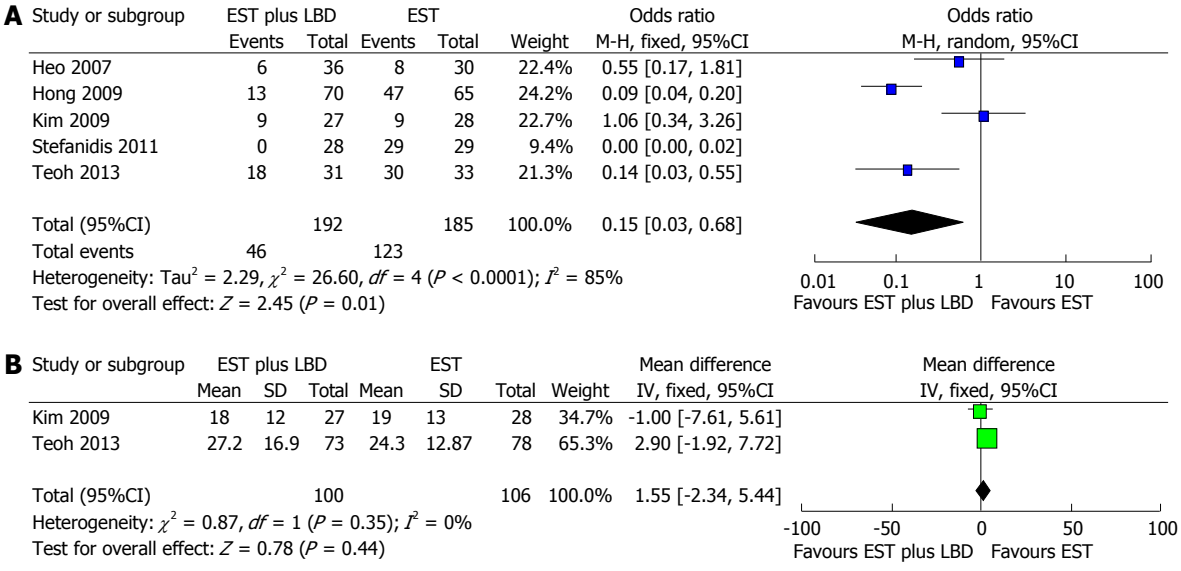

Figure 3 Forest plot demonstrating. A: The use of mechanical lithotripsy in the endoscopic sphincterotomy (EST) plus large-balloon dilation (LBD) group decreased significantly in patients with stone size larger than 15 mm; B: No significant difference in procedure time between EST plus LBD and EST alone.

(Figure 4). In addition, neither the Begg test nor the Egger test revealed significant publication bias (*P* = 0.148 and *P* = 0.426, respectively).

DISCUSSION

We performed this meta-analysis mainly to investigate whether EST plus LBD was feasible and safe for the removal of large stones. Theoretically, a large enough opening to the papilla may facilitate the extraction of large bile duct calculi. Our meta-analysis suggested that EST plus LBD achieved an equivalent success rate in stone clearance to that of EST alone. The use of mechanical lithotripsy in the EST plus LBD group decreased significantly, especially in patients with a stone size larger than 15 mm. Mechanical lithotripsy is a challenging technique and may create many stone fragments that are then difficult to clear[25], thus it is worth reducing dependence on mechanical lithotripsy.

Recent data has suggested that LBD does not cause

Table 4 Analyses of procedure-related complications *n* (%)

Complications	All 6 studies					Four full-text studies (high-quality)				
	Incidence EST plus LBD, EST	OR/Peto OR (95%CI)	*P* value	Heterogeneity		Incidence EST plus LBD, EST	OR/Peto OR (95%CI)	*P* value	Heterogeneity	
				I^2	*P* value				I^2	*P* value
Overall	31/419 (7.4) 53/416 (12.7)	0.53 (0.33-0.85)	0.008	28%	0.24	12/245 (4.9) 24/251 (9.6)	0.48 (0.24-0.99)	0.05	0%	0.37
Pancreatitis	21/419 (5.0) 26/416 (6.3)	0.77 (0.43-1.39)	0.39	0%	0.74	7/245 (2.9) 8/251 (3.2)	0.89 (0.32-2.49)	0.83	0%	0.95
Bleeding	7/419 (1.7) 13/416 (3.1)	0.50 (0.20-1.23)	0.13	22%	0.27	2/245 (0.8) 3/251 (1.2)	0.68 (0.12-3.93)	0.66	31%	0.24
Infection of biliary tract	3/419 (0.7) 10/416 (2.4)	0.34 (0.11-1.02)	0.05	28%	0.25	3/245 (1.2) 10/251 (4.0)	0.34 (0.11-1.02)	0.05	28%	0.25
Perforation	0/419 (0.0) 4/416 (1.0)	0.14 (0.02-0.98)	0.05	0%	1.00	0/245 (0.0) 3/251 (1.2)	0.14 (0.01-1.35)	0.09	0%	0.98

EST: Endoscopic sphincterotomy; LBD: Large-balloon dilation.

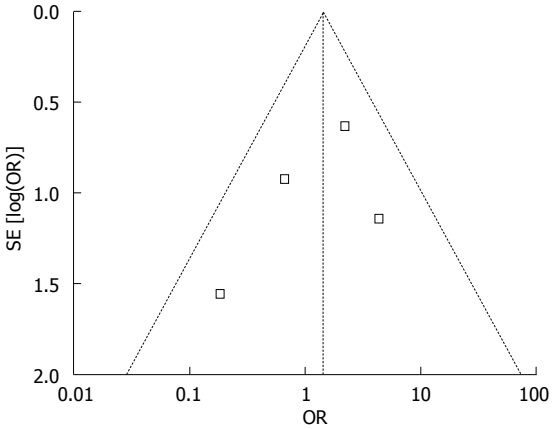

Figure 4 Funnel plot did not show publication bias.

serious complications such as severe pancreatitis and bile duct perforation if performed strictly under established guidelines[5,6,22]. Similarly, the current meta-analysis demonstrated that the incidence of overall complications was significantly lower in the EST plus LBD group. When standard EST is performed to remove large stones, a full or large incision may be made, possibly leading to bleeding or perforation. Our review showed that perforation occurred in four patients in the EST alone group, and in none in the EST plus LBD group. Furthermore, bleeding was rarer when balloon dilation was performed (1.7% *vs* 3.1%) after limited sphincterotomy, although no significant difference was observed. We presume that this may be due to the small incision made before LBD.

Many concerns have been raised about post-ERCP pancreatitis with increasing balloon size, especially for those over 15 mm. However, our meta-analysis showed that LBD did not increase pancreatitis. Theoretically, the initial sphincterotomy may orientate the direction of subsequent dilation, leading to a resultant tear away from the pancreatic orifice, which might decrease the risk of pancreatitis. Post-ERCP pancreatitis may also be associated with other factors such as cannulation time and stone removal time. Only two studies reported the total procedure time[13,23], and meta-analysis showed no difference in ERCP duration between the two groups. We cannot estimate the effect of procedure duration on the risk of pancreatitis.

Only the study by Teoh *et al*[13] compared the direct cost of the procedures between the two groups. A significant reduction in overall cost was noted in the EST plus LBD group [USD $5025 (interquartile range, $4140-$5235) *vs* $6005 (interquartile range, $4462-$5441), $P = 0.034$]. Whether this combined technique is less expensive requires clarification by conducting further trials.

Our findings are similar to those of the previous meta-analysis by Liu *et al*[10]. This previous meta-analysis included three RCTs[22,23,26], and summarized the results of RCTs and non-RCTs separately. One trial included in the previous meta-analysis which performed dilation using a small (8 mm) balloon[26] was excluded in our review. A well-arranged trial was excluded in the previous meta-analysis because mechanical lithotripsy was used in all the patients in the EST group, but in none of the patients in the EST plus LBD group[24], which did not accurately reflect the use of mechanical lithotripsy. We conducted a sensitivity analysis by excluding this study, and the result did not change. By contacting the authors, we found two eligible abstracts[11,12] regarded as non-randomized in the previous meta-analysis, which were in fact randomized. Furthermore, our meta-analysis included a recently published well-designed trial by Teoh *et al*[13]. The previous meta-analysis showed a significant reduction in the use of mechanical lithotripsy and overall complications for non-RCTs, but not for RCTs. However, our meta-analysis showed that EST plus LBD caused fewer overall complications than EST alone, and the result did not change by restricting the meta-analysis to high-quality studies. In addition, our meta-analysis showed that the use of mechanical lithotripsy in the EST plus LBD group decreased significantly, especially in patients with a stone size larger than 15 mm.

This meta-analysis also has some limitations. Firstly, it included two low-quality trials. It has been well documented that in RCTs and meta-analyses, low-quality studies are vulnerable to bias and may lead to exaggerated results. However, subgroup analysis of high-quality studies was also significant, which strengthened the results.

Secondly, only a few studies were included, which might decrease the robustness of the analysis and mask publication bias. Our meta-analysis showed that the significant reduction in perforations in the EST plus LBD group was marginal ($P = 0.05$), this was probably attributable to the small number of subjects with perforation ($n = 4$, all in the EST alone group).

In conclusion, large-balloon dilation following limited sphincterotomy appears to be an effective approach for large stone extraction. This method may cause fewer complications and reduce dependence on mechanical lithotripsy. However, it warrants more well-designed studies to clarify whether this combined technique is outweighed.

COMMENTS

Background

Endoscopic sphincterotomy (EST) or endoscopic papillary balloon dilation (EPBD) is the standard method for stone retrieval. However, the extent of orifice dilation with conventional EST or EPBD is limited, and the use of other methods, such as mechanical lithotripsy, may be required in patients with large stones. A larger opening of the orifice by large-balloon dilation (LBD) may facilitate stone removal. For the past few years, LBD following limited EST appears to be an alternative to EST alone for removing large bile duct stones. However, which one is predominant remains controversial.

Research frontiers

The current meta-analysis was carried out to comparatively assess LBD plus EST and EST alone for removal of large bile duct stones. The main outcome measurements included complete stone removal, stone removal in first session, use of mechanical lithotripsy, procedure time, and procedure-related complications.

Innovations and breakthroughs

The current meta-analysis demonstrated that EST plus LBD is an effective approach for the removal of large bile duct stones, causing fewer complications than EST alone. Furthermore, this combined technique may decrease dependence on mechanical lithotripsy during stone extraction.

Applications

The results from this meta-analysis suggest that LBD following limited EST is an effective alternative to EST alone for removing large bile duct stones, warranting routine clinical use.

Peer review

This is an interesting and well performed meta-analysis addressing the efficacy of EST plus LBD *vs* EST alone for removal of bile duct stones. The research design is solid, and its results have clinical relevancy as they demonstrate that EST plus LBD decreases complications and the usage of mechanical lithotripsy.

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META-ANALYSIS

TNF-α-308 polymorphism and risk of digestive system cancers: A meta-analysis

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Abstract

AIM: To evaluate the association between the tumour necrosis factor alpha-308 (*TNF-α-308*) gene polymorphism and the risk of digestive system cancers.

METHODS: All eligible case-control studies published up to December 2012 were identified by searching PubMed, Web of Science, Embase and China National Knowledge Internet without language restrictions. The risk of digestive system cancers associated with the *TNF-α-308* polymorphism was estimated for each study using odds ratio (OR) together with its 95%CI, respectively. Cochrane Collaboration RevMan 5.1 was used to perform the analysis. A χ^2-test-based Q statistic test and an I^2 test were performed to assess the between-study heterogeneity. When the Q test was significant ($P < 0.05$) or $I^2 > 50\%$, the random effects model was used, otherwise the fixed effects model was used.

RESULTS: Fifty-eight studies from fifty-five publications with a total of 9986 cancer patients and 15511 healthy controls were included. Overall, a significant association was found between the *TNF-α-308* polymorphism and the risk of digestive system cancers [dominant model: OR = 1.23, 95%CI: 1.09-1.39, (G/A) *vs* (G/G): OR = 1.15, 95%CI: 1.02-1.28, (A/A) *vs* (G/G): OR = 1.44, 95%CI: 1.19-1.73, recessive model: OR = 1.38, 95%CI: 1.15-1.66]. Furthermore, when the analysis was stratified by ethnicity, similar results were observed in both the Asian and Caucasian populations, except for the dominant model and heterozygote comparisons in the Asian population [dominant model: OR = 1.24, 95%CI: 0.99-1.56, (G/A) *vs* (G/G): OR = 1.09, 95%CI: 0.96-1.24]. When the cancer type subgroups were examined, similar results were detected in gastric and hepatocellular carcinomas; however, no significant association was observed among other digestive system cancers.

CONCLUSION: The *TNF-α-308* gene polymorphism may be significantly associated with the risk of gastric and hepatocellular carcinomas, but not colorectal, pancreatic, or oesophageal cancer, in the Asian population.

Key words: Tumour necrosis factor alpha; rs1800629; Polymorphism; Digestive system cancer; Meta-analysis; Association

Core tip: Genetic polymorphisms contribute to the risk of human malignant tumours. Many studies have reported the relationship between the tumour necrosis factor alpha-308 (*TNF-α-308*) gene polymorphism and risk of digestive system cancers. However, the results of these studies are inconsistent and contradictory. In this meta-analysis, our results suggest that the *TNF-α-308* polymorphism is significantly associated with the risk of gastric and hepatocellular carcinomas in the Asian

population (dominant model: 95%CI: 1.02-1.34, $P < 0.05$ and 95%CI: 1.20-2.54, $P < 0.05$, respectively). This finding indicates that certain polymorphisms and mutations at TNF-α-308 may increase susceptibility to digestive system cancers.

Guo XF, Wang J, Yu SJ, Song J, Ji MY, Cao Z, Zhang JX, Wang J, Dong WG. *TNF-α-308* polymorphism and risk of digestive system cancers: A meta-analysis. *World J Gastroenterol* 2013; 19(48): 9461-9471 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i48/9461.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i48.9461

INTRODUCTION

Digestive system cancers are the most common malignant tumours worldwide, with 3.4 million new cases each year, and their mortality rates have increased gradually over the past decade[1,2]. Molecular epidemiology has confirmed that carcinogenesis is a complex, multifactorial and multistep event, in which the interaction of environmental triggers and genetic susceptibility may play an important role. However, the exact mechanism of carcinogenesis is still not fully understood.

Tumour necrosis factor-alpha (TNF-α), which is mainly produced by macrophages, is a multifunctional cytokine that plays an important role in the pathogenesis of inflammatory, autoimmune, and malignant diseases[3]. The *TNF-α* gene is located in the major histocompatibility complex class Ⅲ region on the short arm of chromosome six. Several polymorphisms in the promoter region of the *TNF-α* gene have been identified and are implicated in the regulation of *TNF-α* transcription[4-5]. The *TNF-α-308* polymorphism (rs1800629) is the most extensively studied polymorphism in digestive system cancers[6-9]. However, the results of the studies on TNF-α-308 have been inconclusive or inconsistent. Therefore, we conducted a meta-analysis to evaluate the association between the *TNF-α-308* polymorphism and susceptibility to digestive system cancers.

MATERIALS AND METHODS

Search strategy

A literature search was conducted using PubMed, Web of Science, Embase and CNKI for studies that were published up to December 2012 without language restrictions. The relevant studies were identified using the following terms: ["tumour necrosis factor alpha or TNF alpha or TNF-α"] AND ["genetic polymorphism or polymorphisms or variant"] AND ["digestive system cancer or gastric cancer or colorectal cancer or hepatocellular carcinoma or pancreatic cancer or oesophageal cancer"]. The search was restricted to humans. Additional studies were identified by a manual search of references of original or review articles on this topic. If more than one cancer type was reported in one study, the data for each type was extracted separately. If data or data subsets were published in more than one article, only the publication with the largest sample size was included.

Inclusion and exclusion criteria

Studies were included if they met the following criteria: (1) studies that evaluated the association between the *TNF-α-308* polymorphism and digestive system cancer risk; (2) studies with a case-control study design; and (3) studies with detailed genotype frequencies for cases and controls or text that allowed for the calculation of these values. The major exclusion criteria were: (1) case-only studies, case reports, or review articles; (2) studies without raw data for the *TNF-α-308G/A* genotype; and (3) studies that compared the *TNF-α-308G/A* variants in precancerous lesions and other cancers.

Data extraction and quality assessment

Two investigators (Guo XF and Wang J) independently extracted the data and reached a consensus on each item. If the two investigators generated different results, they would check the data again and have a discussion to come to an agreement. If they could not reach an agreement, an expert (Dong WG) was invited to the discussion. The data extracted from the selected articles included the first author's name, year of publication, country of origin, ethnicity, cancer type, genotyping methods, and number of cases and controls. The ethnicities were categorised as Asian or Caucasian. The cancer types were categorised as gastric, colorectal, hepatocellular, pancreatic, or oesophageal.

Statistical analysis

The meta-analysis was performed using the Cochrane Collaboration RevMan 5.1 software (Copenhagen, 2008). The association between the risk of digestive system cancers and the *TNF-α-308* polymorphism was estimated for each study using the odds ratio (OR) and 95%CI. A χ^2 test-based calculation of the Q statistic was performed to assess the between-study heterogeneity[10]. We also quantified the effect of heterogeneity with an I^2 test. When the Q test was significant ($P < 0.05$) or $I^2 > 50\%$, indicating heterogeneity across studies, the random effects model was used[11]; otherwise, the fixed effects model was used[12]. Before estimating the relationship between the *TNF-α-308* polymorphism and digestive system cancer risk, we tested whether the genotype frequencies of the controls were in Hardy-Weinberg equilibrium (HWE) using a χ^2 test. We first estimated this relationship with the dominant model [G/A (GA) + A/A (AA) *vs* G/G (GG)] and the recessive model (AA *vs* GA + GG) and then with the co-dominant model (GA *vs* GG and AA *vs* GG). To evaluate the ethnicity-specific and cancer type-specific effects, we performed stratification analyses with respect to ethnicity and cancer type. Sensitivity analysis was performed to evaluate the stability of the results. Funnel plots were used to evaluate publication bias.

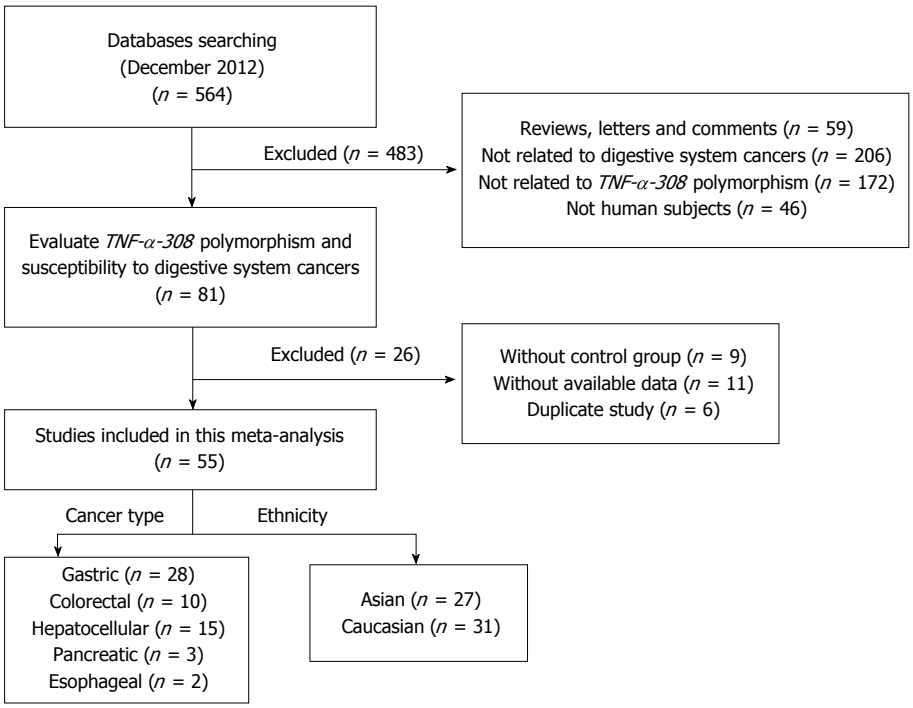

Figure 1 Flow chart showing study selection procedure. TNF-α: Tumour necrosis factor-alpha.

RESULTS

Study characteristics

The search strategy retrieved 564 potentially relevant studies. According to the inclusion criteria, 55 studies with full-text were included in this meta-analysis and 509 studies were excluded. A flow chart of the study selection is shown in Figure 1. Because the studies of El-Omar *et al*[9], Guo *et al*[13] and Jang *et al*[14] each included separate analyses of two cancer types, we treated them separately in this meta-analysis[9,13,14]. Therefore, as shown in Table 1, there were 58 case-control studies from 55 publications on the *TNF-α-308* polymorphism with a total of 9986 cancer cases and 15511 controls. Two ethnicities were addressed: 27 studies focused on Asian populations, and 31 studies focused on Caucasian populations. Five cancer types were addressed: 28 studies focused on gastric cancer[6-9,13-36], 10 studies on colorectal cancer[14,37-45], 15 studies on hepatocellular carcinoma[46-60], 3 studies on pancreatic cancer[61-63], and 2 studies on oesophageal cancer[9,13]. The genotype distribution in the controls was consistent with HWE for all of the selected studies, except for four studies on gastric cancer[7,13,33-34], one study on colorectal cancer[43], six studies on hepatocellular carcinoma[47,49,51-52,55-56], and one study on esophageal cancer[13].

Quantitative data synthesis

Overall, there was a significant difference in the *TNF-α-308G/A* genotype distribution between the digestive system cancer patients and the controls (dominant model: OR = 1.23, 95%CI: 1.09-1.39, $P < 0.00001$; GA *vs* GG: OR = 1.15, 95%CI: 1.02-1.28, $P < 0.0001$; AA *vs* GG: OR = 1.44, 95%CI: 1.19-1.73, $P = 0.23$; recessive model: OR = 1.38, 95%CI: 1.15-1.66, $P = 0.50$) (Table 2, Figure 2). In the analysis of the ethnic subgroups, similar results were observed in the Caucasian population; but in the Asian population, we found that there was no significant association between the *TNF-α-308* polymorphism and the risk of digestive system cancers in the dominant model and heterozygote comparisons (GA + AA *vs* GG: OR = 1.24, 95%CI: 0.99-1.56, GA *vs* GG: OR = 1.09, 95%CI: 0.96-1.24) (Table 2, Figure 2). When stratified by cancer type, similar results were detected for gastric and hepatocellular carcinomas; however, no significant association was observed among the other digestive system cancer types (Table 2, Figure 3). Furthermore, we found that there was significant heterogeneity for the dominant model and heterozygote comparisons both overall and in the stratified analyses: $I^2 = 64\%$ and 52% in the overall population, $I^2 = 66\%$ and 45% ($P = 0.008$) in the Asian population, $I^2 = 64\%$ and 58% in the Caucasian population, $I^2 = 76\%$ and 70% in colorectal cancer, and $I^2 = 73\%$ and 66% in hepatocellular carcinoma. In addition, there was evidence of heterogeneity in gastric cancer (dominant model: $P = 0.009$). Thus, the random effects model was employed in the OR calculations. Then, sensitivity analyses were conducted to determine whether modification of the inclusion criteria of the meta-analysis affected the final results. We examined the influence of these studies on the pooled OR by repeating the meta-analysis while excluding the study that was not in HWE. The estimated pooled OR did not show a significant change (Table 2), indicating that our results are statistically robust. The shapes of the funnel plots did not reveal any evidence of asymmetry, suggesting that there was no publication bias among the studies (Figure 4).

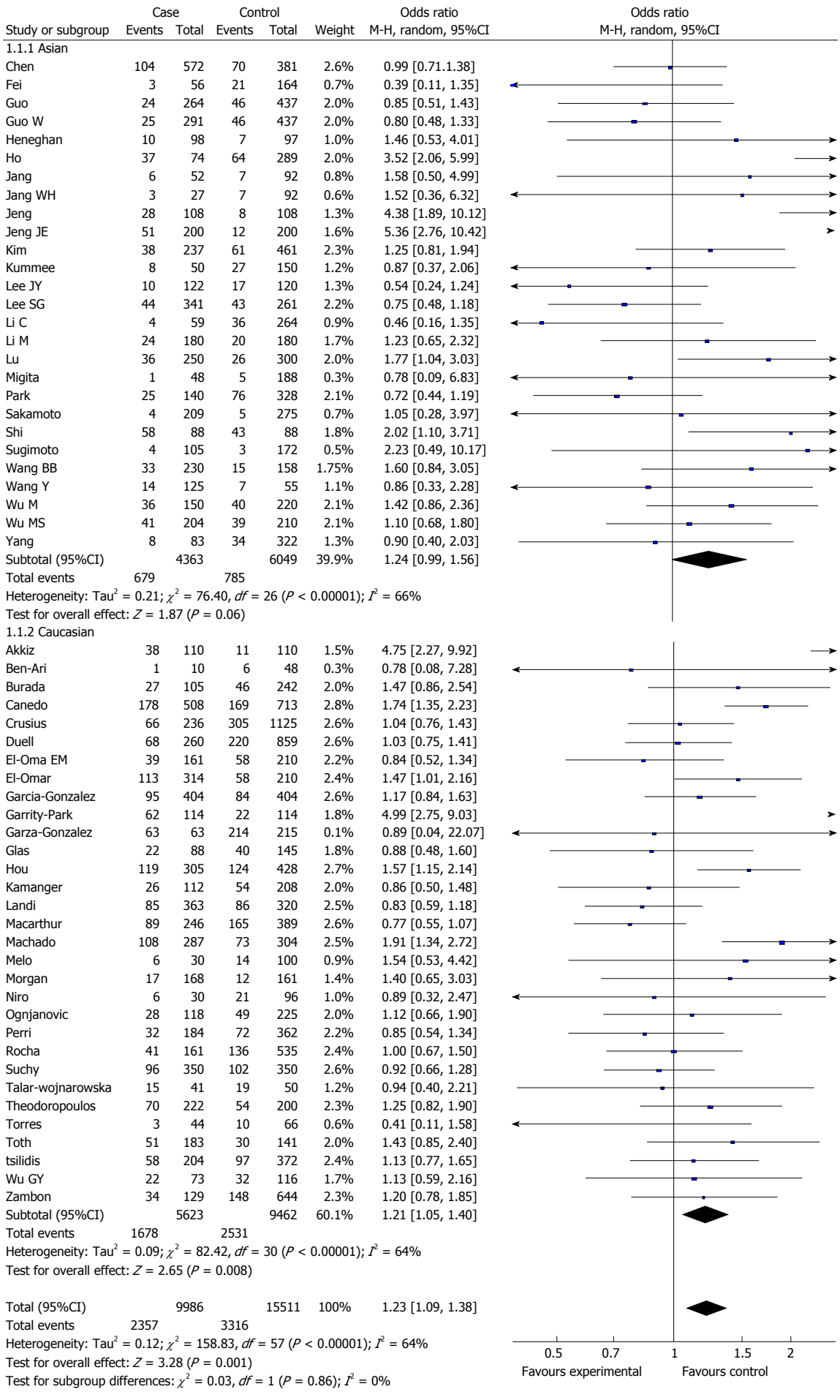

Figure 2 Subgroup analysis of tumour necrosis factor α-308 polymorphism by ethnicity (dominant model).

Study or subgroup	Case Events	Case Total	Control Events	Control Total	Weight	Odds ratio M-H, random, 95%CI
1.2.1 Gastric cancer						
Burada	27	105	46	242	2.0%	1.47 [0.86, 2.54]
Canedo	178	508	169	713	2.8%	1.74 [1.35, 2.23]
Crusius	66	236	305	1125	2.6%	1.04 [0.76, 1.43]
El-Omar	113	314	58	210	2.4%	1.47 [1.01, 2.16]
Fei	3	56	21	164	0.7%	0.39 [0.11, 1.35]
Garcia-Gonzalez	95	404	84	404	2.6%	1.17 [0.84, 1.63]
Garza-Gonzalez	63	63	214	215	0.1%	0.89 [0.04, 22.07]
Glas	22	88	40	145	1.8%	0.88 [0.48, 1.60]
Guo	24	264	46	437	2.0%	0.85 [0.51, 1.43]
Hou	119	305	124	428	2.7%	1.57 [1.15, 2.14]
Jang	6	52	7	92	0.8%	1.58 [0.50, 4.99]
Kamanger	26	112	54	208	2.0%	0.86 [0.50, 1.48]
Kim	38	237	61	461	2.3%	1.25 [0.81, 1.94]
Lee JY	10	122	17	120	1.3%	0.54 [0.24, 1.24]
Lee SG	44	341	43	261	2.2%	0.75 [0.48, 1.18]
Li C	4	59	36	264	0.9%	0.46 [0.16, 1.35]
Lu	36	250	26	300	2.0%	1.77 [1.04, 3.03]
Machado	108	287	73	304	2.5%	1.91 [1.34, 2.72]
Melo	6	30	14	100	0.9%	1.54 [0.53, 4.42]
Morgan	17	168	12	161	1.4%	1.40 [0.65, 3.03]
Perri	32	184	72	362	2.2%	0.85 [0.54, 1.34]
Rocha	41	161	136	535	2.4%	1.00 [0.67, 1.50]
Sugimoto	4	105	3	172	0.5%	2.23 [0.49, 10.17]
Torres	3	44	10	66	0.6%	0.41 [0.11, 1.58]
Wu M	36	150	40	220	2.1%	1.42 [0.86, 2.36]
Wu MS	41	204	39	210	2.1%	1.10 [0.68, 1.80]
Yang	8	83	34	322	1.3%	0.90 [0.40, 2.03]
Zambon	34	129	148	644	2.3%	1.20 [0.78, 1.85]
Subtotal (95%CI)		5061		8885	49.6%	1.17 [1.02, 1.34]
Total events	1204		1932			

Heterogeneity: Tau^2 = 0.05; χ^2 = 47.60, *df* = 27 (*P* = 0.009); I^2 = 43%
Test for overall effect: *Z* = 2.32 (*P* = 0.02)

1.2.2 Colorectal cancer						
Garrity-Park	62	114	22	114	1.8%	4.99 [2.75, 9.03]
Jang WH	3	27	7	92	0.6%	1.52 [0.36, 6.32]
Landi	85	363	86	320	2.5%	0.83 [0.59, 1.18]
Li M	24	180	20	180	1.7%	1.23 [0.65, 2.32]
Macarthur	89	246	165	389	2.6%	0.77 [0.55, 1.07]
Park	25	140	76	328	2.1%	0.72 [0.44, 1.19]
Suchy	96	350	102	350	2.6%	0.92 [0.66, 1.28]
Theodoropoulos	70	222	54	200	2.3%	0.25 [0.82, 1.90]
Toth	51	183	30	141	2.0%	1.43 [0.85, 2.40]
Tsilidis	58	204	97	372	2.4%	1.13 [0.77, 1.65]
Subtotal (95%CI)		2029		2486	20.7%	1.17 [0.87, 1.57]
Total events	563		659			

Heterogeneity: Tau^2 = 0.16; χ^2 = 37.06, *df* = 9 (*P* < 0.00001); I^2 = 76%
Test for overall effect: *Z* = 1.02 (*P* = 0.31)

1.2.3 Hepatocellular carcinoma						
Akkiz	38	110	11	110	1.5%	4.75 [2.27, 9.92]
Ben-Ari	1	10	6	48	0.3%	0.78 [0.08, 7.28]
Chen	104	572	70	381	2.6%	0.99 [0.71, 1.38]
Heneghan	10	98	7	97	1.0%	1.46 [0.53, 4.01]
Ho	37	74	64	289	2.0%	3.52 [2.06, 5.99]
Jeng	28	108	8	108	1.3%	4.38 [1.89, 10.12]
Jeng JE	51	200	12	200	1.6%	5.36 [2.76, 10.42]
Kummee	8	50	27	150	1.2%	0.87 [0.37, 2.06]
Migita	1	48	5	188	0.3%	0.78 [0.09, 6.83]
Niro	6	30	21	96	1.0%	0.89 [0.32, 2.47]
Ognjanovic	28	118	49	225	2.0%	1.12 [0.66, 1.90]
Sakamoto	4	209	5	275	0.7%	1.05 [0.28, 3.97]
Shi	58	88	43	88	1.8%	2.02 [1.10, 3.71]
Wang BB	33	230	15	158	1.7%	1.60 [0.84, 3.05]
Wang Y	14	125	7	55	1.1%	0.86 [0.33, 2.28]
Subtotal (95%CI)		2070		2468	19.9%	1.74 [1.20, 2.54]
Total events	421		350			

Heterogeneity: Tau^2 = 0.35; χ^2 = 51.15, *df* = 14 (*P* < 0.00001); I^2 = 73%
Test for overall effect: *Z* = 2.89 (*P* = 0.004)

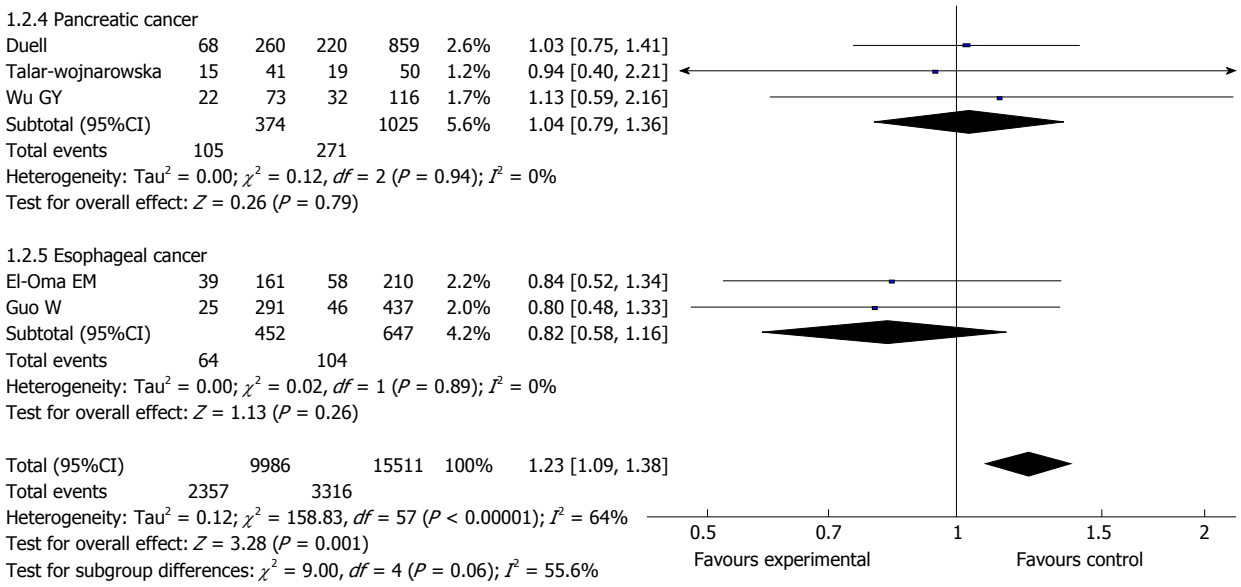

Figure 3 Subgroup analysis of tumor necrosis factor α-308 polymorphism by cancer type (dominant model).

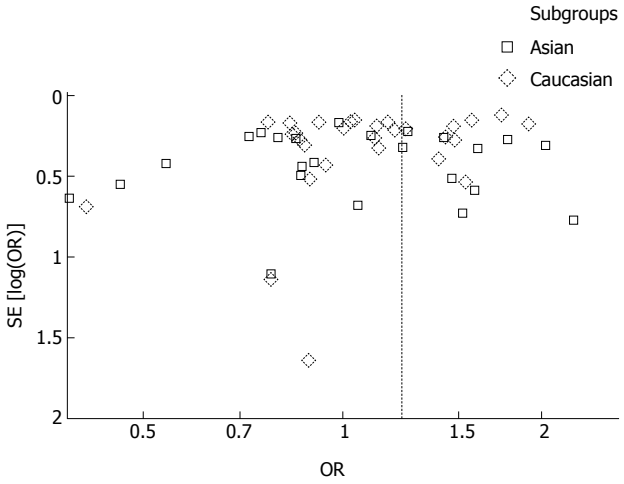

Figure 4 Funnel plots analysis to detect publication bias. Each point represents an independent study for the indicated association.

DISCUSSION

TNF, an important pro-inflammatory cytokine, plays an important role in the regulation of cell differentiation, proliferation and death as well as in inflammation and the innate and adaptive immune response. TNF has also been implicated in a wide variety of human diseases. The presence of DNA sequence variations in the regulatory region might interfere with transcription of the *TNF* gene, influencing the circulating level of TNF and thus increasing susceptibility to human diseases, such as cancer[64]. The TNF enhancer polymorphism has been implicated in several diseases, and the *TNF-α-308* polymorphism has been described as the most important TNF polymorphism in human disease susceptibility. The significance of these polymorphisms reflects their possible influence on the transcription of the *TNF* gene. However, the results of studies in this area are inconsistent. Canedo *et al*[7] found that the *TNF-α-308G/A* polymorphism increases the risk of gastric carcinoma. However, some studies have reported that no statistically significant association exists between the *TNF-α-308G/A* polymorphism and cancer risk[14,20].

The current meta-analysis, which included 58 case-control studies and 25497 subjects, was conducted to explore the association of the *TNF-α-308* polymorphism with digestive system cancer risk. Overall, a significant association was identified between the *TNF-α-308* polymorphism and the risk of digestive system cancers. When the analysis was stratified by ethnicity, we found a statistically significant association between this polymorphism and the risk of these cancers in the Caucasian population. However, no significant association was observed in the dominant model and heterozygote comparisons in the Asian population, which could be due to ethnic differences. When the analysis was stratified by cancer type, we found a significant association between this polymorphism and gastric and hepatocellular carcinoma risk under all four genetic models, but no significant association was observed among colorectal, pancreatic or oesophageal cancer.

Heterogeneity is a potential problem when interpreting the results of meta-analyses. In this meta-analysis, heterogeneity was found in the dominant model and heterozygote comparisons in both the overall and subgroup analyses; thus, the random effects model was used. Sensitivity analyses were also conducted by excluding the study that was not in HWE. With this exclusion, the estimated pooled OR did not change significantly, strengthening our confidence in our results. This finding suggests that the population selection and the study that was not in HWE were not sources of heterogeneity. Alternatively, lifestyle, environment and other unknown factors may be sources of heterogeneity. Moreover, no publication bias was shown, suggesting that our results

Table 1 Characteristics of studies included in the meta-analysis

Ref.	Year	Country	Ethnicity	Cancer type	Genotyping method	Case				Control				*P*
						Total	GG	GA	AA	Total	GG	GA	AA	
Burada *et al*[6]	2012	Romania	Caucasian	Gastric	TaqMan	105	78	26	1	242	196	44	2	0.78
Canedo *et al*[7]	2008	Portugal	Caucasian	Gastric	TaqMan	508	330	178[1]		713	544	169[1]		NA
Crusius *et al*[8]	2008	Spain	Caucasian	Gastric	Real-time PCR	236	170	64	2	1125	820	274	31	0.17
El-Omar *et al*[9]	2003	United States	Caucasian	Gastric	TaqMan	314	201	87	26	210	152	52	6	0.55
Guo *et al*[13]	2005	China	Asian	Gastric	PCR-RFLP	264	240	20	4	437	391	40	6	< 0.01
Jang *et al*[14]	2001	South Korea	Asian	Gastric	PCR-RFLP	52	46	4	2	92	85	7	0	0.70
Fei *et al*[15]	2004	China	Asian	Gastric	PCR	56	53	3	0	164	143	20	1	0.74
Garcia-Gonzalez *et al*[16]	2007	Spain	Caucasian	Gastric	TaqMan	404	309	84	11	404	320	77	7	0.35
Garza-Gonzalez *et al*[17]	2005	Mexico	Caucasian	Gastric	PCR-RFLP	63	0	8	55	215	1	35	179	0.61
Glas *et al*[18]	2004	Germany	Caucasian	Gastric	PCR-RFLP	88	66	19	3	145	105	36	4	0.67
Hou *et al*[19]	2007	Poland	Caucasian	Gastric	TaqMan	305	186	98	21	428	304	109	15	0.19
Kamangar *et al*[20]	2006	Finland	Caucasian	Gastric	TaqMan	112	86	23	3	208	154	52	2	0.29
Kim *et al*[21]	2006	South Korea	Asian	Gastric	PCR-RFLP	237	199	34	4	461	400	59	2	0.91
Lee *et al*[22]	2004	South Korea	Asian	Gastric	PCR	341	297	43	1	261	218	42	1	0.49
Lee *et al*[23]	2005	South Korea	Asian	Gastric	PCR-RFLP	122	112	10	0	120	103	17	0	0.40
Li *et al*[24]	2005	China	Asian	Gastric	PCR-RFLP	59	55	4	0	264	228	34	2	0.56
Lu *et al*[25]	2005	China	Asian	Gastric	PCR-DHPLC	250	214	36	0	300	274	24	2	0.08
Machado *et al*[26]	2003	Portugal	Caucasian	Gastric	PCR-SSCP	287	179	105	3	304	231	69	4	0.65
Melo *et al*[27]	2009	Brazil	Caucasian	Gastric	PCR-RFLP	30	24	5	1	100	86	13	1	0.53
Morgan *et al*[28]	2006	Honduras	Caucasian	Gastric	TaqMan	168	151	17	0	161	149	12	0	0.62
Perri *et al*[29]	2005	Italy	Caucasian	Gastric	PCR-RFLP	184	152	30	2	362	290	65	7	0.15
Rocha *et al*[30]	2005	Brazil	Caucasian	Gastric	PCR-RFLP	161	120	37	4	535	399	123	13	0.34
Sugimoto *et al*[31]	2007	Japan	Asian	Gastric	PCR-RFLP	105	101	4	0	172	169	3	0	0.91
Torres *et al*[32]	2004	Colombia	Caucasian	Gastric	PCR	44	41	3	0	66	56	10	0	0.51
Wu *et al*[33]	2002	China	Asian	Gastric	Direct sequencing	150	114	27	9	220	180	27	13	< 0.01
Wu *et al*[34]	2004	China	Asian	Gastric	Direct sequencing	204	163	29	12	210	171	26	13	< 0.01
Yang *et al*[35]	2009	South Korea	Asian	Gastric	SNaPshot	83	75	8	0	322	288	34	0	0.32
Zambon *et al*[36]	2005	Italy	Caucasian	Gastric	TaqMan	129	95	31	3	644	496	138	10	0.91
Garrity-Park *et al*[37]	2008	Ireland	Caucasian	Colorectal	PCR, sequencing	114	52	49	13	114	92	20	2	0.46
Jang *et al*[14]	2001	South Korea	Asian	Colorectal	PCR-RFLP	27	24	3	0	92	85	7	0	0.70
Landi *et al*[38]	2003	Spain	Caucasian	Colorectal	TaqMan	363	278	80	5	320	234	76	10	0.22
Li M *et al*[39]	2011	China	Asian	Colorectal	PCR-RFLP	180	156	15	9	180	160	19	1	0.60
Macarthur *et al*[40]	2005	Scotland	Caucasian	Colorectal	TaqMan	246	157	74	15	389	224	145	20	0.58
Park *et al*[41]	1998	South Korea	Asian	Colorectal	PCR-RFLP	140	115	24	1	328	252	72	4	0.65
Suchy *et al*[42]	2008	Poland	Caucasian	Colorectal	PCR-RFLP	350	254	87	9	350	248	95	7	0.55
Theodoropoulos *et al*[43]	2006	Greece	Caucasian	Colorectal	PCR-RFLP	222	152	56	14	200	146	44	10	0.01
Toth *et al*[44]	2007	Hungary	Caucasian	Colorectal	PCR-SSP	183	132	48	3	141	111	30	0	0.16
Tsilidis *et al*[45]	2009	United States	Caucasian	Colorectal	TaqMan	204	146	55	3	372	275	90	7	0.91
Akkiz *et al*[46]	2009	Turkey	Caucasian	Hepatocellular	PCR-RFLP	110	72	35	3	110	99	11	0	0.58
Ben-Ari *et al*[47]	2003	United States	Caucasian	Hepatocellular	PCR-SSP	10	9	1[1]		48	42	6[1]		NA
Chen *et al*[48]	2005	China	Asian	Hepatocellular	TaqMan	572	468	95	9	381	311	67	3	0.77
Heneghan *et al*[49]	2003	China	Asian	Hepatocellular	ASO-PCR	98	88	10	0	97	90	6	1	0.03
Ho *et al*[50]	2004	China	Asian	Hepatocellular	PCR-RFLP	74	37	34	3	289	225	62	2	0.30
Jeng *et al*[51]	2007	China	Asian	Hepatocellular	PCR-SSO	108	80	28[1]		108	100	8[1]		NA
Jeng JE *et al*[52]	2009	China	Asian	Hepatocellular	PCR-SSO	200	149	51[1]		200	188	12[1]		NA
Kummee *et al*[53]	2007	Thailand	Asian	Hepatocellular	PCR-RFLP	50	42	8	0	150	123	26	1	0.77
Migita *et al*[54]	2005	Japan	Asian	Hepatocellular	PCR-SSP	48	47	1	0	188	183	5	0	0.85
Niro *et al*[55]	2005	Italy	Caucasian	Hepatocellular	Direct sequencing	30	24	6[1]		96	75	21[1]		NA
Ognjanovic *et al*[56]	2009	United States	Caucasian	Hepatocellular	TaqMan	118	90	28[1]		225	176	49[1]		NA
Sakamoto *et al*[57]	2008	Japan	Asian	Hepatocellular	PCR-RFLP	209	205	4	0	275	270	5	0	0.88
Shi *et al*[58]	2011	China	Asian	Hepatocellular	PCR-RFLP	88	30	43	15	88	45	35	8	0.75
Wang *et al*[59]	2003	Japan	Asian	Hepatocellular	Direct sequencing	125	111	13	1	55	48	6	1	0.16
Wang *et al*[60]	2010	China	Asian	Hepatocellular	PCR-SSO	230	197	30	3	158	143	15	0	0.53
Duell *et al*[61]	2006	United States	Caucasian	Pancreatic	PCR-RFLP	260	192	63	5	859	639	198	22	0.16
Talor-wojnarowska *et al*[62]	2009	Poland	Caucasian	Pancreatic	PCR-RFLP	41	26	12	3	50	31	17	2	0.86
Wu GY *et al*[63]	2010	Germany	Caucasian	Pancreatic	PCR-RFLP	73	51	20	2	116	84	30	2	0.72
El-Omar *et al*[9]	2003	United States	Caucasian	Esophageal	TaqMan	161	122	34	5	210	152	52	6	0.55
Guo *et al*[13]	2005	China	Asian	Esophageal	PCR-RFLP	291	266	21	4	437	391	40	6	< 0.01

[1]Numbers of GA+AA. P_{HWE} was calculated by goodness-of fit χ^2-test, and $P_{HWE} < 0.05$ was considered statistically significant. PCR-DHPLC: Polymerase chain reaction-based denaturing high-performance liquid chromatography; HWE: Hardy-Weinberg equilibrium; NA: Not available; GG: Guanine/Guanine; GA: Guanine/Adenine; AA: Adenine/Adenine; PCR-RFLP: Polymerase chain reaction-restriction fragment length polymorphism.

are accurate.

Some limitations of this meta-analysis should be addressed. First, the number of published studies, especially for oesophageal and pancreatic cancers, was not suf-

Table 2 Stratified analysis of the tumor necrosis factor alpha polymorphism and digestive system cancers risk

Group	GA + AA *vs* GG			GA *vs* GG			AA *vs* GG			AA *vs* GA + GG		
	n	OR (95%CI)	*P*[1]	*n*	OR (95%CI)	*P*[1]	*n*	OR (95%CI)	*P*[1]	*n*	OR (95%CI)	*P*[1]
Overall	58	1.23 (1.09, 1.38)[2]	< 0.00001	52	1.14 (1.01, 1.28)[2]	< 0.00001	44	1.43 (1.19, 1.73)	0.26	44	1.38 (1.15, 1.66)	0.55
Studies with HWE	46	1.18 (1.03, 1.34)[2]	< 0.00001	46	1.14 (1.00, 1.29)[2]	< 0.00001	38	1.54 (1.25, 1.90)	0.15	38	1.48 (1.20, 1.81)	0.40
Cancer type												
Gastric	28	1.23 (1.12, 1.34)[2]	0.009	27	1.15 (1.04, 1.27)	0.07	22	1.38 (1.06, 1.80)	0.63	22	1.33 (1.03, 1.72)	0.67
Colorectal	10	1.17 (0.87, 1.57)[2]	< 0.0001	10	1.10 (0.83, 1.45)[2]	0.0004	9	1.45 (0.76, 2.75)[2]	0.02	9	1.40 (0.99, 2.00)	0.07
Hepatocellular	15	1.74 (1.20, 2.54)[2]	< 0.00001	10	1.58 (1.05, 2.39)[2]	0.002	8	2.55 (1.38, 4.70)	0.49	8	2.15 (1.19, 3.90)	0.66
Pancreatic	3	1.04 (0.79, 1.36)	0.94	3	1.04 (0.79, 1.38)	0.88	3	0.99 (0.46, 2.14)	0.63	3	0.99 (0.46, 2.13)	0.60
Esophageal	2	0.82 (0.58, 1.16)	0.89	2	0.80 (0.55, 1.15)	0.89	2	1.01 (0.42, 2.43)	0.95	2	1.05 (0.44, 2.51)	0.92
Ethnicity												
Asian	27	1.24 (0.99, 1.56)[2]	< 0.00001	25	1.07 (0.94, 1.22)[2]	0.008	19	1.55 (1.11, 2.17)	0.43	19	1.47 (1.05, 2.06)	0.60
Caucasian	31	1.21 (1.05, 1.40)[2]	< 0.00001	27	1.17 (1.01, 1.35)[2]	< 0.0001	25	1.38 (1.10, 1.74)	0.18	25	1.34 (1.08, 1.67)	0.39

[1]Test for heterogeneity; [2]Random-effects model was used when the *P* for heterogeneity test was < 0.05. GG: Guanine/Guanine; GA: Guanine/Adenine; AA: Adenine/Adenine; HWE: Hardy-Weinberg equilibrium.

ficiently large for a comprehensive analysis, and some studies with small sample sizes may not have enough statistical power to prove authentic associations. Therefore, our analysis should be interpreted with caution, and more studies are needed. Second, our results were based on unadjusted estimates, and lack of information for the data analysis may cause serious confounding bias. Third, significant heterogeneity was found in some models, which may lead to failure to confirm marginal associations. In spite of these limitations, our meta-analysis had several advantages. First, a substantial number of cases and controls were pooled from different studies, which significantly increased the statistical power of the analysis. Second, the quality of the case-control studies included in the current meta-analysis was satisfactory and met our inclusion criteria. Third, we did not detect any publication bias, suggesting that the whole pooled result is unbiased.

In summary, this meta-analysis suggests that the *TNF-α-308* polymorphism increases susceptibility to digestive system cancers in the Caucasian population. The *TNF-α-308 AA* genotype is closely related to the risk of digestive system cancers in people of Asian descent. The *TNF-α-308* polymorphism may be significantly associated with the risk of gastric and hepatocellular carcinomas, but not colorectal, pancreatic, or oesophageal cancer. Future studies should use standardised unbiased genotyping methods, examine homogeneous cancer patients and well-matched controls, and include multiethnic groups.

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COMMENTS

Background

Digestive system cancers are the most common malignant tumors worldwide. Tumor necrosis factor alpha-308 (*TNF-α-308*) polymorphism (rs1800629) is the most extensively studied polymorphism in digestive system cancers. However, the results are different or even inconsistent.

Research frontiers

Molecular epidemiology has confirmed that carcinogenesis is a complex, multifactorial, and multistep event, and genetic mutation play an important role in the process. Many studies have reported the association between the *TNF-α-308* polymorphism and human malignant tumors, but no agreements have been reached till now.

Innovations and breakthroughs

This meta-analysis systemically assessed the association between *TNF-α-308* polymorphism and risk of digestive system cancers. Results show that *TNF-α-308* polymorphism may be significantly associated with the risk of gastric and hepatocellular carcinomas in Asians.

Applications

This study results indicate that *TNF-α-308* polymorphism may be used as a detectable biomarker for gastric and hepatocellular carcinoma patients.

Peer review

The authors present a meta-analysis study over the influence of a polymorphism of TNF-α on digestive system cancers. The manuscript is well written and interesting, especially because it is the first meta-analysis study on the subject.

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META-ANALYSIS

Association of interleukin-10 polymorphisms with risk of irritable bowel syndrome: A meta-analysis

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Abstract

AIM: To clarify the current understanding of the association between interleukin-10 (*IL-10*) polymorphisms and the risk of irritable bowel syndrome (IBS).

METHODS: We searched for studies in any language recorded in PubMed, Embase and Cochrane library before August 2013. The associations under allele contrast model, codominant model, dominant model, and recessive model were analyzed. The strengths of the association between *IL-10* polymorphisms and IBS risk were estimated using odds ratios (OR) with 95% confidence interval (CI). Fixed effects model was used to pool the result if the test of heterogeneity was not significant, otherwise the random-effect model was selected.

RESULTS: Eight case-control studies analyzing three single-nucleotide polymorphisms rs1800870 (-1082 A/G), rs1800871 (-819C/T), and rs1800872 (-592A/C) of the *IL-10* gene, which involved 928 cases and 1363 controls, were eligible for our analysis. The results showed that rs1800870 polymorphisms were associated with a decreased risk of IBS (GG+GA *vs* AA: OR = 0.80, 95%CI: 0.66-0.96), (AA+GA *vs* GG: OR = 0.68, 95%CI: 0.52-0.90). Subgroup analysis revealed such association only existed in Caucasian ethnicity (AA+GA *vs* GG, OR = 0.70, 95%CI: 0.55-0.89). The *rs1800872* polymorphisms were associated with an increased risk of IBS in Asian ethnicity (CC *vs* GG: OR = 1.29, 95%CI: 1.01-1.16). There were no associations between rs1800871 polymorphisms and the IBS risk.

CONCLUSION: The results suggest that IL-10 rs1800870 confers susceptibility to the risk of IBS in Caucasian ethnicity, and the rs1800872 may associate with IBS risk in Asians. However, no significant associations are found between rs1800871 and IBS risk.

Key words: Interleukin-10; Irritable bowel syndrome; Gene polymorphism; Case-control; Meta-analysis

Core tip: Interleukin-10 (*IL-10*) polymorphisms have been identified as a biomarker causally associated with occurrence of irritable bowel syndrome (IBS) and receives extensive interest. However, its relationship with IBS remains obscure. In this paper, after combing the data from 8 case-control studies with 928 cases and 1363 controls, the authors found that the IL-10 rs1800870 confers susceptibility to the risk of IBS in Caucasian ethnicity, and the rs1800872 may associate with IBS risk in Asians. However, no significant associations are found between rs1800871 and IBS risk.

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INTRODUCTION

Irritable bowel syndrome (IBS) is a type of functional gastrointestinal disorder that has a multi-factorial origin. The exact pathophysiology leading to the occurrence of IBS is largely unknown, although inflammatory reactions are believed to play an important role in its pathogenesis. In some animal studies, researchers have found that inflammatory responses can alter the function of gut smooth muscles, enteric nerves, and interstitial cells of Cajal[1-3]. Moreover, IBS patients show an increase in the number of inflammatory cells in the gut[4-6]. The clustering of IBS in families and the results from twin studies have also provided evidence for a role of hereditary factors in the propensity of developing IBS[7,8].

Cytokines are important modulators of immune responses and inflammatory reactions and play a central role in intestinal inflammation[9]. The production of cytokines can be affected by genetic polymorphisms within the coding and promoter regions of cytokine genes[10,11]. Therefore, a genetic predisposition for the high or low production of a particular cytokine may affect disease susceptibility and clinical outcome[12,13]. Interleukin 10 (IL-10), also known as a human cytokine synthesis inhibitory factor, is an anti-inflammatory cytokine capable of inhibiting the synthesis of proinflammatory cytokines, such as interferon-γ, IL-2, IL-3, and tissue necrosis factor-α, which are produced by macrophages and regulatory T-cells[14]. Several studies have shown that serum IL-10 levels are significantly lower in IBS patients than in normal controls, suggesting that altered IL-10 levels may be involved in the pathogenesis of IBS and may be an IBS biomarker[15-17].

Some reports[18-21] have also indicated a significant association between *IL-10* polymorphisms and IBS risk; however, other studies[13,22-24] have failed to find such associations. Generally, this disparity may be partly due to ethnic differences or to the limited numbers of subjects involved in the studies. Therefore, the relationship between IBS risk and *IL-10* polymorphisms is not confirmed and needs further study with a large, genetically homogenous sample. The current study is a comprehensive meta-analysis performed to further evaluate the associations between *IL-10* polymorphisms and the risk of IBS.

MATERIALS AND METHODS

Search strategy and study selection

All methods were based on the guidelines proposed by the Human Genome Epidemiology Network for systematic reviews of genetic association studies, and followed the PRISMA guidelines[25]. A systematic literature search was performed using PubMed, Embase, the Cochrane Library, Google Scholar databases, Chinese National Knowledge Infrastructure (CNKI), and conference abstracts to identify published studies evaluating genetic association between *IL-10* polymorphisms and IBS risk published prior to August 2013; letters and abstracts were included. The Medical Subject Headings and text words used for the search were "interleukin-10" or "IL-10", "polymorphism," and "irritable bowel syndrome" or "IBS". Search results were limited to human studies. All languages were searched, and the retrieved articles were translated, when necessary. The references of the identified publications were searched for additional studies, and the MEDLINE option for searching for related articles was used to examine all relevant articles.

Inclusion and exclusion criteria

Studies were included if they (1) examined the association between *IL-10* polymorphisms and IBS risk; (2) had a case-control design; and (3) contained sufficient information on genotype frequency. To achieve adequate statistical power, only single-nucleotide polymorphisms (SNPs) reported in > 2 publications were selected. For studies describing results from the same or overlapping groups of subjects or controls, but reported in > 1 publication, only the largest published data set was included.

Studies were excluded if they did not evaluate the association between *IL-10* polymorphisms and the risk of IBS or if the genotype and allele frequency was inadequately reported, and such data could not be obtained by contacting the authors. Studies reporting associations with SNPs described by fewer than 3 publications were also excluded. In the event of duplicate publications, the smaller data set was excluded.

Data extraction

Two investigators independently extracted data from the identified publications, including the first author's name, year of publication, source of publication, diagnostic criteria for IBS, method of genotyping, number of cases and controls, genotype frequency, and allele frequency. Discrepancies in data extraction were resolved by repeating the study review and discussing the results.

Statistical analysis

Associations found with the allele contrast, codominant, dominant, and recessive models were analyzed. The strengths of the associations between *IL-10* polymorphisms and risk of IBS were estimated using odds ratios (OR) with 95% confidence interval (CI). We assessed the heterogeneity among the studies using the Cochran's Q-test. We also calculated the inconsistency index I^2 to quantify heterogeneity[26]. A fixed effects model ($P > 0.05$) was used to pool the results if a heterogeneity test was not significant, otherwise a random-effects model was

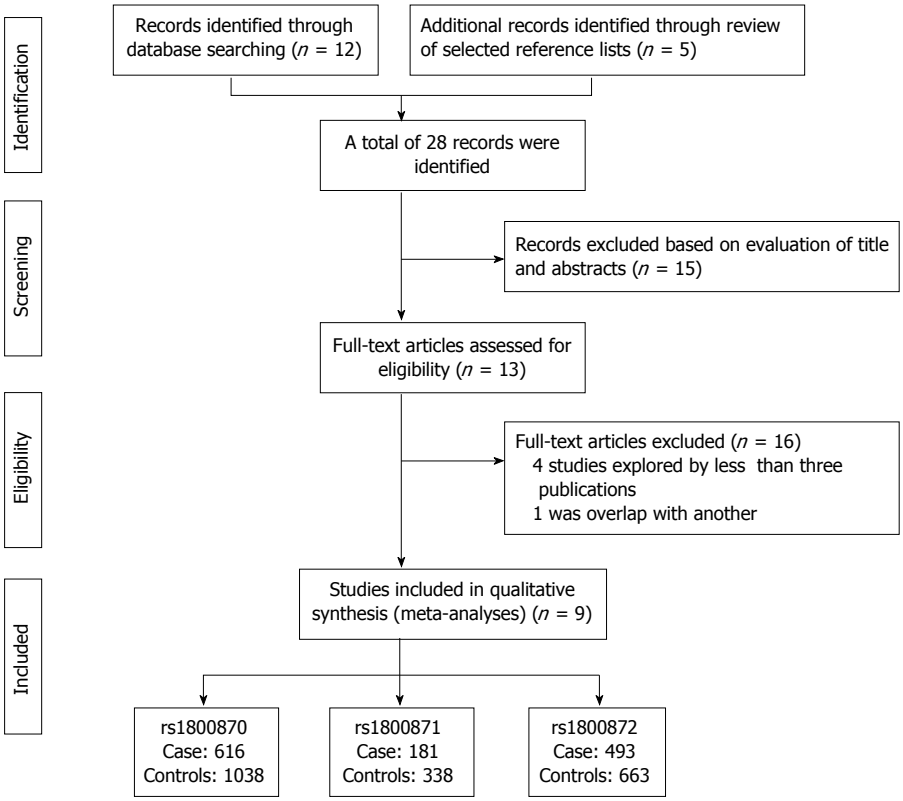


Figure 1 Flow chart of study selection.

used ($P < 0.05$)[27,28]. Subgroup analyses were performed to investigate narrower subsets of studies.

A Hardy-Weinberg equilibrium (HWE) was applied to the control population to evaluate data quality. The HWE analysis for genotype distribution among control populations was performed using a Chi-squared test. Further, a sensitivity analysis was performed to exclude studies that were not in HWE[29]. An asymmetric plot was used to suggest possible publication biases. Publication bias was examined using the Begg's and Egger's tests for each SNP publication[30,31]. All statistical tests were two-sided, and a P value < 0.05 was considered statistically significant. STATA, version 11.2 (Stata, College Station, TX, United States), was used for all statistical analyses.

RESULTS

Study selection process

The initial search yielded 28 studies; 15 were excluded because they were review articles, animal studies, or non-case-control design studies. After screening the full text of the remaining 13 studies, an additional 5 studies were excluded; of these, 4 explored SNPs reported by < 3 studies and one[19] did not provide sufficient genotype frequency data, even after contacting the original authors. Thus, a total of 8 case-control studies[18-24,32], involving 928 cases and 1363 controls, were included in the present meta-analysis. The studies analyzed 3 *IL-10* SNPs, rs1800870 (1082 A/G), rs1800871 (819 C/T), and rs1800872 (592 A/C). Figure 1 provides a summary of the selection process.

Characteristics of included studies

One study selected IBS patients using the Rome I criteria, 3 used the Rome II criteria, and the other 4 studies used the Rome III criteria. Patients from 4 studies[18-21] were Caucasians, and 4 studies involved Asians[22-24,32]. The characteristics of the 8 studies and the results of the HWE test for the distribution of the genotype in the control population are shown in Table 1.

IL-10 rs1800870 and IBS risk

Seven studies[13,18,20-24], involving 616 IBS subjects and 1038 controls, analyzed the association between the *IL-10 rs1800870* polymorphism and IBS risk. The distribution of the controls in 2 studies[20,21] deviated from the HWE. Overall, the GG+GA *vs* AA (OR = 0.80, 95%CI: 0.66-0.96, $P = 0.018$) and AA+GA *vs* GG (OR = 0.68, 95%CI: 0.52-0.90, $P = 0.007$) models presented a decreased risk of IBS. Little heterogeneity was found in the AA+GA *vs* GG model ($I^2 = 0.0\%$, $P = 0.542$) by the I^2 test and Q-test; however, there was significant heterogeneity in the GG+GA *vs* AA ($I^2 = 79.4\%$, $P = 0.000$) model. There were no significant associations between the GG *vs* AA ($P = 0.523$) and G *vs* A ($P = 0.892$) models and IBS risk. Egger's and Begg's tests suggested little publication bias in the 4 models (all, $P > 0.05$) (Table 2, Figure 2A). In the sensitivity analysis, after removing 2 studies[20,21] in which the controls deviated from the

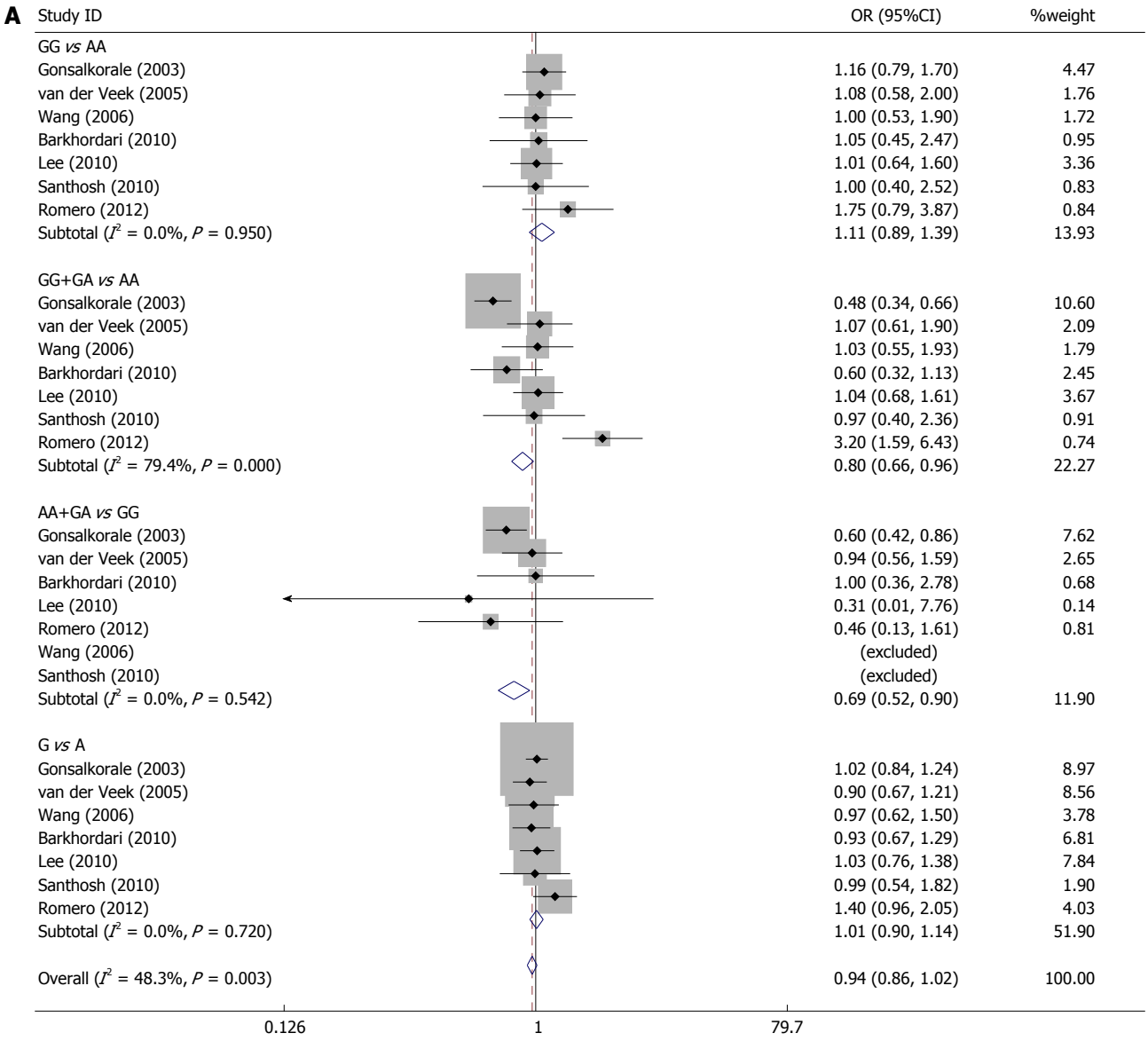
Table 1 Characteristic of individual studies in the meta-analysis

Ref.	Year	Country/ethnicity	SNP of *IL-10*	IBS/controls	Genotyping methods	Diagnostic criteria	HWE of control
Gonsalkorale *et al*[21]	2003	United Kingdom	rs1800870	230/450	PCR-SSP	RomeI	0.000
van der Veek *et al*[13]	2005	Netherlands	rs1800870	111/128	PCR-RFLP	Rome Ⅱ	0.707
Wang *et al*[24]	2006	China	rs1800870	43/41	PCR-RFLP	Rome Ⅱ	0.678
Barkhordari *et al*[18]	2010	Iran	rs1800870	70/140	PCR-SSP	Rome Ⅲ	0.041
Lee *et al*[22]	2010	South Korea	rs1800870	94/88	PCR-RFLP	Rome Ⅲ	0.707
Santhosh *et al*[23]	2010	Indian	rs1800870	23/20	PCR-SSP	Rome Ⅱ	0.717
Romero-Valdovinos *et al*[20]	2012	Mexico	rs1800870	45/173	PCR-RFLP	Rome Ⅲ	0.000
Wang *et al*[24]	2006	China	rs1800871	43/41	PCR-RFLP	Rome Ⅱ	0.619
Barkhordari *et al*[18]	2010	Iran	rs1800871	70/140	PCR-SSP	Rome Ⅲ	0.907
Santhosh *et al*[23]	2010	China	rs1800871	23/20	PCR-SSP	Rome Ⅱ	0.662
Romero-Valdovinos *et al*[20]	2012	Mexico	rs1800871	45/173	PCR-RFLP	Rome Ⅲ	0.920
Wang *et al*[24]	2006	China	rs1800872	43/41	ARMS-PCR	Rome Ⅱ	0.619
Barkhordari *et al*[18]	2010	Iran	rs1800872	70/140	PCR-RFLP	Rome Ⅲ	0.97
Jiang *et al*[32]	2010	China	rs1800872	312/325	PCR-SSP	Rome Ⅲ	0.255
Santhosh *et al*[23]	2010	Indian	rs1800872	23/20	PCR-SSP	Rome Ⅱ	0.438
Romero-Valdovinos *et al*[20]	2012	Mexico	rs1800872	45/173	PCR-RFLP	Rome Ⅲ	0.018

SNP: Single nucleotide polymorphism; HWE: Hardy-Weinberg equilibrium; IBS: Irritable bowel syndrome; PCR: Polymerase chain reaction; SSP: Sequence specific primer; RFLP: Restriction fragment length polymorphism; ARMS: Amplification refractory mutation system.

Table 2 Summary odds ratios of polymorphisms with irritable bowel syndrome risk

	P value	OR (95%CI)	I^2	*P* heterogeneity	Begg' test	Egger test
rs1800870						
GG *vs* AA	0.338	1.11 (0.89-1.39)	0.0%	0.950	1.000	0.694
Caucasian[13,18,20,21]	0.228	1.19 (0.90-1.58)	0.0%	0.776		
Asian[22-24]	0.963	1.01 (0.71-1.42)	0.0%	0.999		
GG+GA *vs* AA	0.018	0.80 (0.66-0.96)	79.4%	0.000	1.000	0.200
Caucasian	0.003	0.70 (0.55-0.89)	88.5%	0.000		
Asian	0.860	1.03 (0.74-1.43)	0.0%	0.990		
AA+GA *vs* GG	0.007	0.68 (0.52-0.90)	0.0%	0.542	1.000	0.899
Caucasian	0.008	0.69 (0.52-0.91)	0.0%	0.415		
Asian	0.478	0.31 (0.01-7.76)	-	-		
G *vs* A	0.815	1.01 (0.90-1.14)	0.0%	0.720	0.764	0.773
Caucasian	0.799	1.02 (0.89-1.16)	17.1%	0.306		
Asian	0.976	1.00 (0.80-1.260	0.0%	0.974		
rs1800871						
AA *vs* GG	0.698	0.90 (0.53-1.54)	0.0%	0.440	0.308	0.326
Caucasian[18,20]	0.205	0.56 (0.23-1.37)	22.6%	0.255		
Asian[23,24]	0.530	1.25 (0.62-2.54)	0.0%	0.969		
AA+GA *vs* GG	0.969	0.99 (0.61-1.60)	32.4%	0.218	0.308	0.146
Caucasian	0.182	0.58 (0.26-1.29)	0.0%	0.365		
Asian	0.252	1.45 (0.77-2.76)	0.0%	0.364		
GG+GA *vs* AA	0.651	0.92 (0.64-1.33)	0.0%	0.720	0.734	0.080
Caucasian	0.985	1.00 (0.68-1.49)	0.0%	0.906		
Asian	0.219	0.54 (0.20-1.45)	0.0%	0.967		
A *vs* G	0.496	1.09 (0.85-1.38)	0.0%	0.809	1.000	0.924
Caucasian	0.939	1.01 (0.75-1.37)	0.0%	0.631		
Asian	0.314	1.23 (0.83-1.82)	0.0%	0.671		
rs1800872						
CC *vs* AA	0.989	1.00 (0.77-1.19)	0.0%	0.456	0.806	0.506
Caucasian[18,20]	0.379	0.73 (0.37-1.47)	41.6%	0.191		
Asian[23,24,32]	0.730	1.05 (0.80-1.38)	0.0%	0.466		
CC+CA *vs* AA	0.028	1.29 (1.03-1.62)	0.0%	0.717	0.221	0.196
Caucasian	0.417	1.27 (0.71-2.26)	0.0%	0.573		
Asian	0.042	1.29 (1.01-1.66)	0.0%	0.410		
AA+CA *vs* CC	0.833	0.97 (0.72-1.31)	60.2%	0.040	0.806	0.384
Caucasian	0.578	1.13 (0.72-1.71)	3.2%	0.310		
Asian	0.382	0.82 (0.53-1.27)	76.0%	0.016		
C *vs* A	0.112	1.12 (0.97-1.28)	0.0%	0.863	0.086	0.266
Caucasian	0.614	1.12 (0.97-1.28)	0.0%	0.669		
Asian	0.114	1.14 (0.97-1.33)	0.0%	0.618		



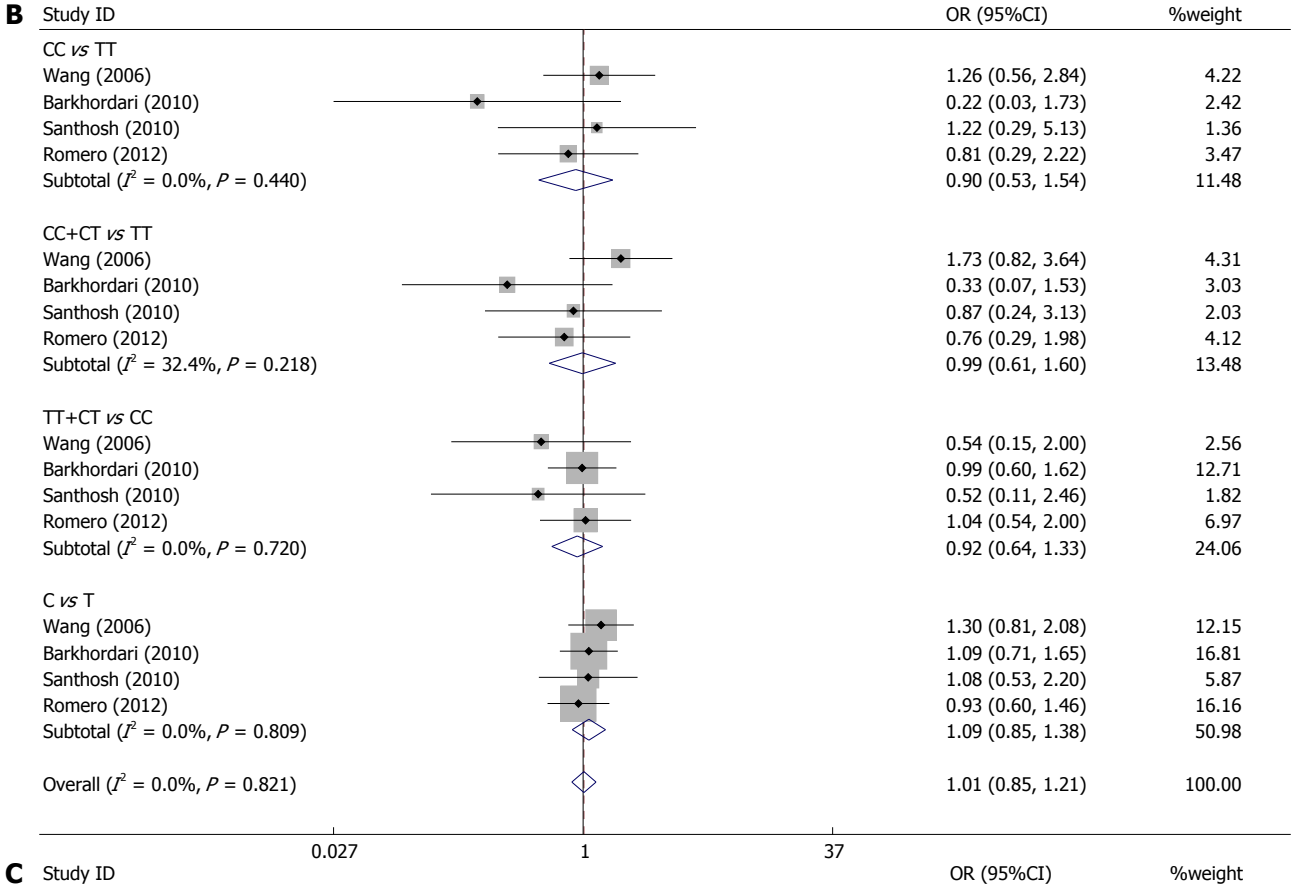
HWE, significant associations with the GG+GA *vs* AA model were no longer observed, and the heterogeneity became negligible (data not shown); the associations remained for the AA+GA *vs* GG (OR = 0.69, 95%CI: 0.52-0.91, P = 0.008) model. Subgroup analysis revealed associations between the *IL-10 rs1800870* polymorphisms and IBS risk in Caucasians[18,20,21] (P = 0.003), but not in Asians[22-24] (P = 0.860) (Table 2).

IL-10 rs1800871 and IBS risk

Four studies[18,20,23,24], involving 493 IBS subjects and 663 controls, analyzed the associations between the *IL-10 rs1800871* polymorphisms and IBS risk; the distribution of controls in the studies fulfilled the HWE. In the meta-analysis, no significant associations were observed for any of the 4 models: AA *vs* GG (P = 0.698), AA+AG *vs* GG (P = 0.969), GG+AG *vs* AA (P = 0.651), and A *vs* G (P = 0.496). Either significant heterogeneity or publication bias was found associated with each of the 4 models (all, P > 0.05). A sensitivity analysis, after excluding studies in turn, indicated that the null associations remained (data not shown). Further, subgroup analyses did not find any associations between the *IL-10 rs1800871* polymorphisms and IBS risk, regardless of ethnicity[18,20,23,24] (Table 2, Figure 2B).

IL-10 rs1800872 and IBS risk

Five studies[18,20,23,24,32], involving 181 IBS subjects and 338 controls, analyzed the associations between IL-10 rs1800872 polymorphisms and IBS risk; the distribution of the controls in the studies fulfilled the HWE. The meta-analysis demonstrated that the CC+CA *vs* AA model was associated with susceptibility to IBS (OR = 1.29, 95%CI: 1.03-1.62, P = 0.028). However, significant associations were not found between the other 3 models, CC *vs* AA (P = 0.989), AA+CA *vs* CC (P = 0.833), and C *vs* A (P = 0.112), and IBS risk. Either significant heterogeneity or publication bias was found in each of the 4 models (all, P > 0.05). A sensitivity analysis, after removing each study sequentially, demonstrated that the results remained similar to the initial results. Subsequent subgroup analyses showed associations between rs1800872 and IBS risk



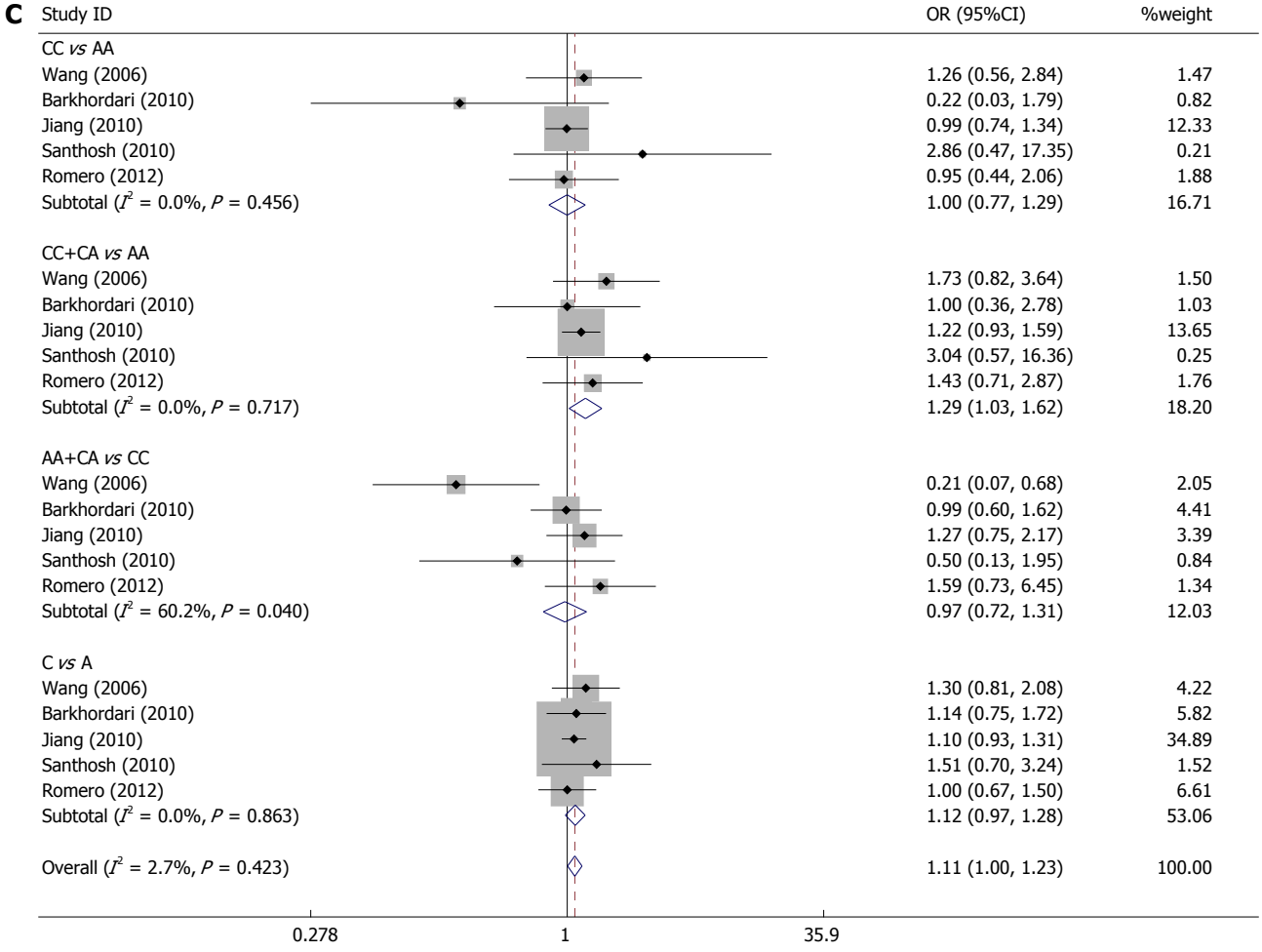


Figure 2 Meta-analysis. A: Interleukin-10 (*IL-10*) *rs1800870* polymorphisms and irritable bowel syndrome risk; B: *IL-10 rs1800871* polymorphisms and IBS risk; C: *IL-10 rs1800872* polymorphisms and IBS risk.

in Asians[23,24,32] (P = 0.042), but not in Caucasians[18,20] (P = 0.417) (Table 2, Figure 2C).

DISCUSSION

The gene encoding IL-10 is located on chromosome 1q31-1q32, and has 3 confirmed biallelic polymorphisms in the promoter region, *i.e.*, rs1800870, rs1800871, and rs1800872. A genetic predisposition for low IL-10 production is associated with the development of IBS[33-35], and previous studies have shown that IL-10 SNPs and some haplotypes are associated with an increased IBS risk[5,13,18,23]. Likewise, production of the anti-inflammatory cytokine IL-10 is also associated with SNPs at specific positions.

The A allele of rs1800870 has been reported to be associated with lower production of IL-10 and an accordingly stronger inflammatory response[36]. More recently, Bashashati *et al*[37] reported the results of a meta-analysis that showed that the A/G of rs1800870 conferred susceptibility to IBS. However, their study only included 5 studies, and most of the included subjects were Caucasians. When considering the disparity in genetic factors among different racial groups, it is difficult to conclude that rs1800870 was associated with IBS risk, without adjusting for ethnicity and sample size. In the present study, although the overall meta-analysis results showed the presence of significant associations between rs1800870 and IBS risk in the GG+GA *vs* AA (P = 0.018) and AA+GA *vs* GG (P = 0.007) models, a subgroup analysis revealed that such association existed only for Caucasians, and not for Asians. This disparity between the two ethnicities might be explained by variations in allelic frequencies between the ethnic groups. This was possible since the frequency of the rs1800870 A allele is associated with significantly higher production of IL-10 in Caucasians than in Asians. Moreover, another study showed that the frequency of the high producer *IL-10* genotype is much higher in the Irish population than in Africans or Singaporean Chinese[38]. In the present study, 4[13,18,20,21] of the 8 included studies analyzed Caucasian subjects, and the frequency of the *rs1800870 GG* genotype in controls was higher than that observed in Asian subjects. The present meta-analysis results confirmed the association of rs1800870 and IBS risk, but also demonstrated that the association varies according to ethnicity.

The role of rs1800871 in IL-10 production is incompletely understood. Although some studies have reported that rs1800871 is associated with several diseases, such as endometriosis[39] and periodontitis[40], the association between rs1800871 and IBS has remained controversial[18,20,23,24]. In our study, the overall results and the results of the subgroup analysis failed to show a significant association between rs1800871 and IBS risk, regardless of the examined ethnicity. This observation indicates similar distribution of rs1800871 among both IBS patients and controls, supporting the observation that the genetic make-up for IL-10 production levels does not differ between IBS patients and normal subjects[13,18,24]. Although a direct link between rs1800871 and IL-10 production levels has not been established, previous reports have suggested that rs1800871 polymorphisms are in linkage disequilibrium with rs1800871 polymorphisms; that the haplotypes for rs1800870, rs1800871 and rs1800872 are common in Caucasians; and that the GCC/GCC haplotypes are commonly associated with high IL-10 production, whereas the *ATA/ATA* genotype is associated with low IL-10 production[41]. Because only limited data are available in the included studies, further studies are required to perform a haplotype analysis to explore the associations between rs1800871 haplotypes and other SNPs with IBS.

With regard to rs1800872, studies have reported that the presence of rs1800872 confers susceptibility to some diseases such as leprosy[42] and hepatocellular carcinoma[43]. In studies investigating the association of IL-10 with IBS risk, Santhosh *et al*[23] reported that the C allele was much lower in individuals with IBS than among normal controls (41.3% *vs* 73.5%), in an Indian population. Wang *et al*[24] found similar results in a Chinese population (IBS *vs* controls, 9.3% *vs* 17.1%). However, a Mexican study[18] failed to show a significant difference between IBS patients and normal controls with respect to the C allele (72.51% *vs* 71.1%); similar results were reported in 2 other studies[19,20]. In the present meta-analysis, we found that CC+CA *vs* AA was associated with an increased IBS risk, and the sensitivity analysis further confirmed this association. However, the subgroup analysis revealed that only Asians demonstrated this association. Although 5 studies were included in the present analysis, the sample size was relatively small; hence, these results should be interpreted with caution.

The present comprehensive meta-analysis demonstrated an association between *rs1800871* and *rs1800872* polymorphisms and IBS risk, and that the *rs1800872* polymorphism conferred susceptibility to IBS. In addition, although Bashashati *et al*[37] reported a meta-analysis demonstrating that rs1800870 conferred susceptibility to IBS, we expanded this finding to show that this association existed in Caucasians but not in Asians, which had not been previously reported. The present study also involved a sensitivity analysis demonstrating that the distribution of controls deviated from the HWE, guaranteeing the reliability of the results. The Begg's and Egger's test results did not show a significant publication bias in the eligible as well as the non-English studies, which also attests to the robustness of the results.

Some limitations to this meta-analysis require careful consideration. First, because only limited data were available, we did not analyze the association between *IL-10* polymorphisms and different types of IBS, *e.g.*, diarrhea or constipation. Thus, the associations between different types of IBS require further investigation. Second, other factors such as genetic and environmental factors, which also may affect susceptibility to IBS, were not adjusted in the present studies. Hence, a well-designed study is warranted to account for potential confounders and to provide a more precise association. Third, also because

of the limited number of available studies, the subgroup analysis of rs1800872 involved comparatively few studies; thus, its association with IBS needs to be confirmed by a study involving a larger number of subjects.

In conclusion, the present meta-analysis suggests that the *rs1800870* polymorphism of IL-10 may represent an increased risk of IBS in Caucasians, but not in Asians. Similarly, *rs1800872* polymorphisms may represent an increased risk of IBS in Asians, but future studies are necessary to reinforce these findings. The present study failed to find an association between rs1800871 and IBS risk, regardless of ethnicity.

COMMENTS

Background

Cytokines are important modulators in the immune responses and inflammatory reaction, which play a central role in intestinal inflammation.

Research frontiers

Interleukin-10 (*IL-10*) polymorphisms have been identified as a biomarker causally associated with occurrence of irritable bowel syndrome (IBS) and receives extensive interest. However, its relationship with IBS remains obscure.

Innovations and breakthroughs

This is the first paper conducting a comprehensive meta-analysis to investigate the association of *IL-10* polymorphisms with IBS risk. The authors showed that IL-10 rs1800870 is associated with IBS risk in Caucasian ethnicity, and rs1800872 associate with IBS risk in Asians.

Applications

This study furthers the understanding of the association of *IL-10* polymorphisms with IBS risk.

Peer review

The study deals with the important topic related to the association between single nucleotide polymorphisms of genes coding for inflammation-linked factors with pathogenesis of chronic inflammatory diseases such as IBS.

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CASE REPORT

Endoscopic management of a rare granulation polyp in a colonic diverticulum

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Abstract

There are many case reports on colon diverticula that cause irritable bowel syndrome, constipation, bleeding, diverticulitis, stricture due to multiple recurrences of diverticulitis, and perforation. However, few articles have examined neoplasms that arise from a diverticulum, such as adenoma and adenocarcinoma, and there have been no reports of granulation polyps that arise from a colon diverticulum after recurrent diverticulitis. We observed a rare granulation polyp that arose from a diverticulum as a result of repeated episodes of local diverticulitis. Narrow band imaging magnified colonoscopy was very useful to diagnose the polyp as a granulation polyp because of the absence of a pit pattern on the surface of the polyp. We successfully resected the polyp using endoscopic mucosal resection. We inverted the diverticulum, and the resected stalk of the polyp was used to close the diverticulum with an over-the-scope clip. If a granulomatous polyp could arise from a diverticulum, differential diagnosis between a colon neoplasm and a granulomatous polyp would not only be difficult but also necessary for suitable endoscopic treatment.

Key words: Diverticulitis; Endoscopy; Granulation polyp; Mucosal resection; Neoplasm; Recurrence

Core tip: The study observed a rare granulation polyp that arose from a diverticulum as a result of repeated episodes of local diverticulitis. The authors successfully resected the polyp using endoscopic mucosal resection. The diverticulum was inverted, and the resected stalk of the polyp was used to close the diverticulum with an over-the-scope clip.

Mori H, Tsushimi T, Kobara H, Nishiyama N, Fujihara S, Matsunaga T, Ayagi M, Yachida T, Masaki T. Endoscopic management of a rare granulation polyp in a colonic diverticulum. *World J Gastroenterol* 2013; 19(48): 9481-9484 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i48/9481.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i48.9481

INTRODUCTION

A colon diverticulum is caused by increased intra-colonic pressure or by a weakened colonic wall. Most colon diverticula consist of acquired pseudodiverticula and have been observed in the sigmoid colon of patients in Western countries and in the ascending colon of patients in Japan[1]. The most reliable method to identify colon diverticula is a barium enema; however, once a diverticu-

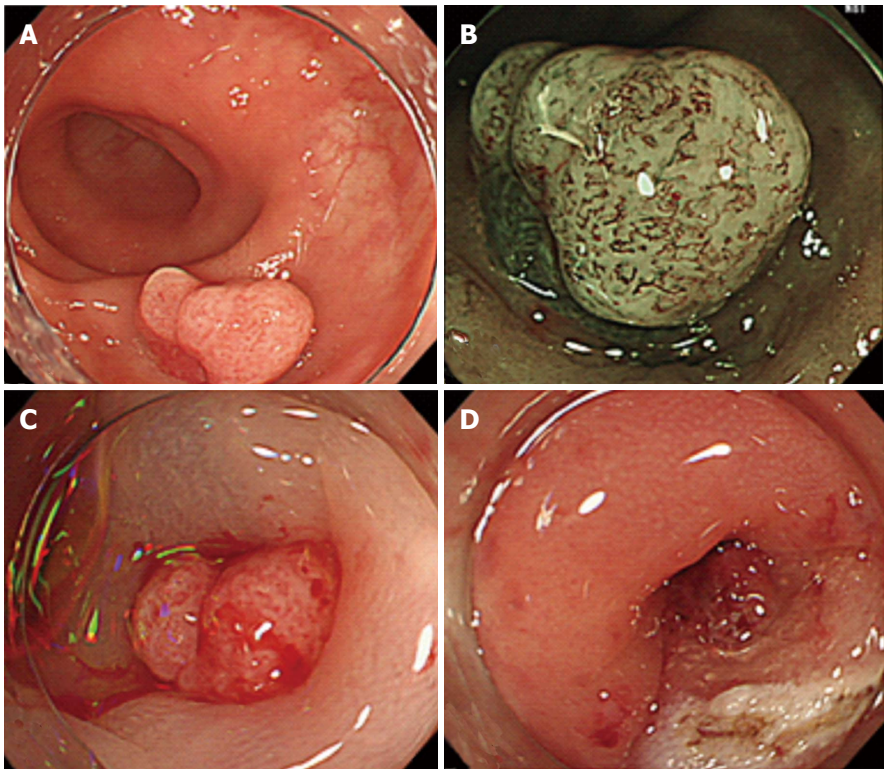

Figure 1 Endoscopic mucosal dissection of the sigmoid colon polyp. A: A sigmoid colon polyp approximately 25 mm in diameter; B: Narrow band imaging magnified colonoscopy was performed to investigate the polyp in greater detail. Several irregular microvessels were observed on the surface of the polyp, but there was no pit pattern on the surface; C: A local saline injection was administered, and we observed slight elevation of the polyp; D: After the endoscopic mucosal resection procedure and the removal of the polyp, the diverticulum was identified using the resected stalk of the polyp.

lum begins to bleed, colonoscopy is a useful modality to treat the bleeding vessels[2,3]. Although the incidence of colonic diverticular bleeding is increasing, treatments have not yet been well established. The risk factors contributing to recurrent hemorrhage after initial improvement in colonic diverticular bleeding are past histories of hypertension or renal deficiency. Follow-up colonoscopy after the initial improvement in colonic diverticular bleeding is needed in patients with hypertension or renal deficiency[4]. In addition, local peritonitis due to diverticulitis, and perforation are serious complications[5]. Although 85% of patients with colonic diverticulitis will recover with non-surgical treatment, some patients may have complications such as abscesses, fistulas, obstruction and panperitonitis[6,7]. On the other hand, few articles have examined neoplasms that arise from the diverticulum, such as adenoma and adenocarcinoma[8].

We describe a rare case of a granulomatous polyp which arose from a colon diverticulum.

CASE REPORT

A 62-year-old woman who suffered from repeated left lower abdominal pain and high fever (38 ℃) underwent a colonoscopy and was diagnosed with a sigmoid colon polyp that was approximately 25 mm in diameter (Figure 1A). Twice during the previous year, she had suffered from abdominal pain and a high fever, and her blood laboratory data were as follows: a white blood cell count of 12000/μL and a C-reactive protein level of 5.59 mg/mL. After undergoing colonoscopy, her symptoms disappeared. Additionally, narrow band imaging (NBI) magnified colonoscopy was performed to diagnose the polyp in greater detail. Several irregular microvessels were found on the surface of the polyp. However, the pit pattern which is usually observed in neoplasms, such as adenoma and adenocarcinoma, was absent from the surface of the polyp (Figure 1B). The surface was smooth, and we were unable to determine whether the polyp was a neoplasm or an inflammatory polyp. To confirm the qualitative histological diagnosis, we performed endoscopic mucosal resection (EMR) of the polyp. We obtained written informed consent from the patient to perform the EMR procedure for treatment of the polyp. During EMR, a local saline injection was administered, which slightly elevated the polyp (Figure 1C), and allowed resection of the polyp. After removing the polyp, we identified the diverticulum using the resected stalk of the polyp (Figure 1D). A closer view of the resected surface revealed that the cavity of the diverticulum was irregular, and exposed vessels were observed (Figure 2A). The resection of the polyp indicated that it arose from the diverticulum (Figure 2A). To prevent post-EMR bleeding and delayed perforation, we inverted the diverticulum and sutured the inverted diverticulum, including the resected stalk of the polyp, with an over-the-scope clip (OTSC) (Figure 2B). After the EMR procedure, computed tomography was performed to examine the soft tissue density around the OTSC and the increased fat density around the resected site (Figure 2C and D).

According to the clinical course of the patient, which included high fever and repeated left lower abdominal pain, we suspected that post-inflammation granulation tissue arose from the bottom of the diverticulum after repeated episodes of diverticulitis. Seven days after the procedure, the histology of the polyp revealed that it was

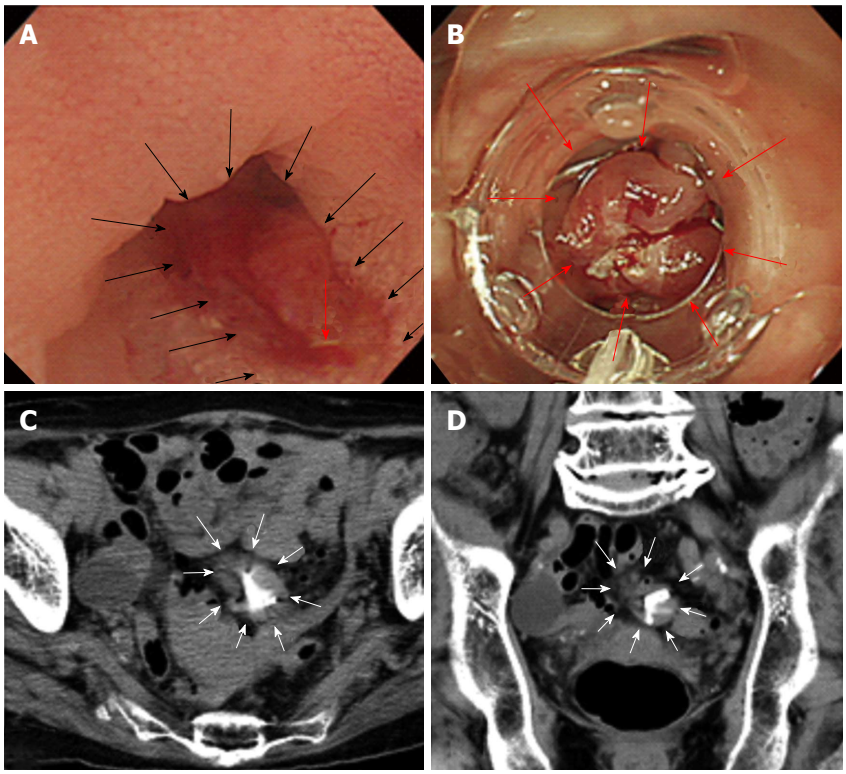

Figure 2 Closure of the diverticulum using the resected stalk of the polyp. A: In a closer view of the resected surface, the cavity of the diverticulum was irregular (the black arrows), and an exposed vessel was identified (red arrow); resection of the polyp indicated that it arose from the diverticulum; B: To prevent bleeding and delayed perforation following the endoscopic mucosal resection (EMR) procedure, we inverted the diverticulum and sutured the inverted diverticulum, including the resected stalk of the polyp, using an over-the-scope clip (red arrows); C, D: After the EMR procedure, computed tomography was performed to examine the soft tissue density around the over-the-scope clip and the increased fat density around the resected site (white arrows).

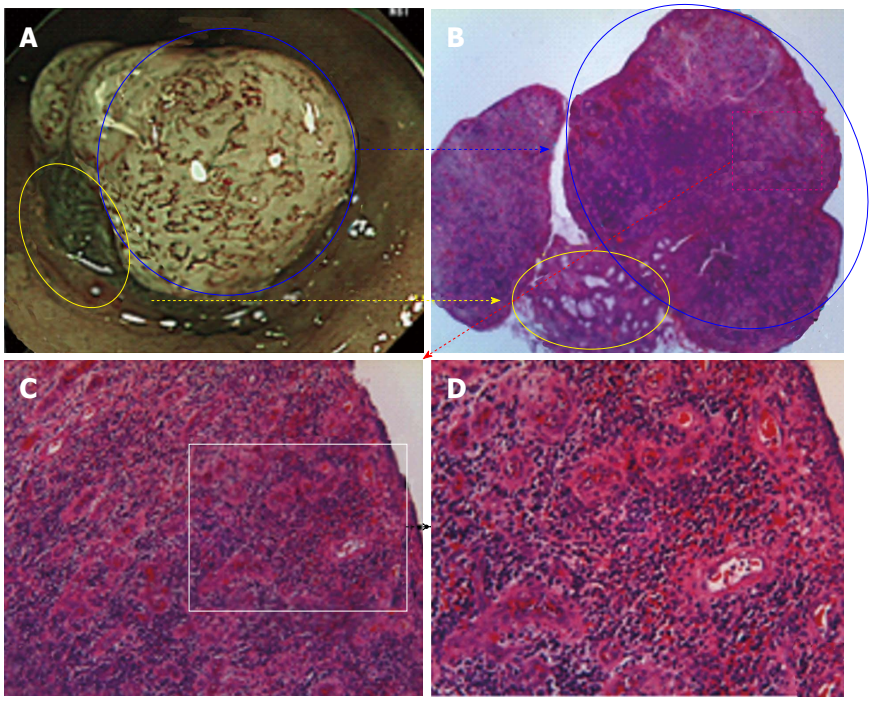

Figure 3 Histological findings of the resected polyp. A: A 20 magnified narrow band imaging image of the granulomatous polyp; B: A 20 magnified image with a hematoxylin and eosin (HE) stain, the yellow and blue circles in Panel A corresponding to those in Panel B; C: A 100 magnified image with a HE stain reveals significant infiltration of lymphocytes and plasma cells; D: A 200 magnified image with a HE stain reveals increased outgrowth of microvascular structures and infiltration of lymphocytes, neutrophils and plasma cells, which indicates granulation tissue. There were no atypical cells or structural atypia.

composed only of granulation tissue with no neoplasm (Figure 3). The patient was discharged from our hospital without any complications.

DISCUSSION

We report the unique case of a granulomatous polyp that arose from a single diverticulum after repeated episodes of local diverticulitis. A colonic diverticulum causes some serious complications such as bleeding, stricture due to multiple recurrences of diverticulitis, and perforation[5-7]. Several case reports have also described neoplasms that arose from a single diverticulum, which was successfully treated with EMR and completely closed with the assistance of laparoscopy[8]. However, there have been no previous reports of granulomatous polyps that arose from a single diverticulum after repeated episodes of local diverticulitis, which was treated with EMR and closed with an OTSC. When polyps are diagnosed as neoplastic, magnifying chromoendoscopy and NBI magnifying

image-enhanced endoscopy can be used to detect the pit pattern. A granulomatous polyp should be diagnosed when no pit pattern is observed because a pit pattern reveals the surface characteristics of a neoplasm[9]. The resection of a polyp arising from a diverticulum is associated with a risk of perforation or bleeding. A full-thickness resection using a pre-full thickness suture with an OTSC was reported to be the safest method to resect the full-thickness wall of the colon[10]. Similarly, after inverting the diverticulum and the polyp into the colon, a full-thickness suture using an OTSC and a full-thickness resection may be safely performed. In this case, we should have initially inverted the diverticulum and the polyp and safely resected the full-thickness of the colon wall after diagnosing the granulomatous polyp that arose from a diverticulum. When many granulomatous polyps arise from many diverticula and the neighboring granuloma fuses, a stricture of the colon may develop; therefore, a differential diagnosis between colon cancer and a granulomatous stricture would be difficult[11].

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CASE REPORT

Primary hepatic choriocarcinoma in a 49-year-old man: Report of a case

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Abstract

We report a case of hepatic choriocarcinoma in a man diagnosed at autopsy after a rapid downhill clinical course. The patient was a 49-year-old man who presented with acute right-sided abdominal pain. There were no masses palpable on physical examination. Radiographic findings showed large multi-nodular tumors mainly in the right lobe of the liver. Fludeoxyglucose-positron emission tomography scan showed uptake only in the liver, and no uptake in the testes. We initially planned to perform a liver resection for the presumed diagnosis of intra-hepatic cholangiocarcinoma. However, the tumors grew rapidly and ruptured. Multiple lung metastases rapidly developed resulting in respiratory failure, preventing liver resection or even biopsy. He died 60 d after initial presentation with no pathological diagnosis. Postmortem studies included histopathological and immunohistological examinations which diagnosed a primary choriocarcinoma of the liver. Primary hepatic choriocarcinoma is very rare but should be considered in the differential diagnosis of a liver tumor in a middle aged man. Establishing this diagnosis may enable treatment of the choriocarcinoma. Liver biopsy and evaluation of serum human chorionic gonadotropin are recommended in these patients.

Key words: Hepatic choriocarcinoma; Male; Human chorionic gonadotropin; Liver biopsy; Fludeoxyglucose-positron emission tomography

Core tip: Evaluation of serum human chorionic gonadotropin levels in addition to other liver tumor markers should be performed in middle-aged men with undiagnosed hepatic tumors, to rule-out the possibility of primary hepatic choriocarcinoma. Liver biopsy is important to diagnose this rare and highly malignant tumor.

Sekine R, Hyodo M, Kojima M, Meguro Y, Suzuki A, Yokoyama T, Lefor AT, Hirota N. Primary hepatic choriocarcinoma in a 49-year-old man: Report of a case. *World J Gastroenterol* 2013; 19(48): 9485-9489 Available from: URL: http://www.wjgnet.com/1007-9327/full/v19/i48/9485.htm DOI: http://dx.doi.org/10.3748/wjg.v19.i48.9485

INTRODUCTION

Choriocarcinoma is a rare, aggressive, malignant germ-cell neoplasm of trophoblastic cells, which are among the first cells to differentiate from the fertilized egg to enable

implantation. Choriocarcinoma is prone to rapid hematogenous metastases, and the first clinical manifestation is often metastatic lesions[1]. The characteristic laboratory finding in patients with choriocarcinoma is an elevated serum human chorionic gonadotropin (hCG) level. Choriocarcinoma is less common in men than women, and comprises only 1% of all germ-cell tumors, most often with the primary lesion in the testes[2]. There are only seven patients previously reported in the English literature with primary choriocarcinoma of the liver[3-5]. These patients have been reported from Asia, including Japan and China. We report here a 49-year-old Japanese male with primary choriocarcinoma of the liver diagnosed at autopsy, who presented initially with acute abdominal symptoms and a rapid downhill clinical course. Establishing the diagnosis early may enable treatment of choriocarcinoma. Consideration of this lesion in a patient with an undiagnosed liver mass is essential, necessitating evaluation of serum hCG level and urgent liver biopsy.

CASE REPORT

A 49-year-old male presented to the emergency room with acute right-sided abdominal pain and fever. He had a previous history of diabetes mellitus and hepatitis C. Physical examination was positive for abdominal tenderness. Contrast-enhanced computed tomography (CT) and magnetic resonance imaging (MRI) scans revealed a multi-nodular hepatic tumor more than 10 cm in diameter in the right lobe (Figure 1A). Laboratory data showed white blood count (WBC) and liver function tests within normal limits but an elevated C-reactive protein to 8.75 mg/L. Serum carcinoembrionic antigen (CEA) was elevated to 18.5 ng/mL but α-fetoprotein (AFP) and CA19-9 were within normal limits. We suspected a metastatic liver tumor or intra-hepatic cholangiocarcinoma. Endoscopy found no primary lesion in the gastrointestinal tract and fludeoxyglucose-positron emission tomography (FDG-PET) scan showed abnormal uptake only in the liver (Figure 1B). We planned to perform liver resection with a presumptive diagnosis of intra-hepatic cholangiocarcinoma but avoided performing a liver biopsy due to the risk of dissemination. Before he could undergo liver resection, the tumors grew rapidly and ruptured (Figure 2A). Multiple lung metastases rapidly developed, accompanied by severe respiratory failure (Figure 2B). Due to pulmonary, biopsy or resection of the liver were not possible and the patient died 60 d after initial presentation.

At autopsy, the liver weighed 4080 g with numerous hemorrhagic satellite nodules in the right lobe. There were multiple hemorrhagic lung nodules up to 3 cm in diameter, and microscopic metastases were identified in other viscera, including a para-aortic lymph node, the right adrenal gland, peritoneum, right renal capsule, and spleen. There was no malignant change or scar in the testes. Histological findings of the hepatic tumors showed choriocarcinoma with a biphasic pattern of mononuclear cytotrophoblasts and giant multi-nucleated syncytiotrophoblast cells (Figure 3A).

Immunohistochemistry was positive for an antibody to hCG subunits α (Figure 3B) and β (Figure 3C). Control tissue slides of placental chorionic villi stained with the same antibody showed staining limited to the syncytiotrophoblast layer. The syncytiotrophoblast cells in the liver were strongly positive for hCG, as were those in the other organs involved. Serum hCG was evaluated postmortem, significantly elevated at 53000 IU/mL.

DISCUSSION

Choriocarcinoma is an uncommon, aggressive trophoblastic malignant neoplasm that is prone to early hematogenous metastases. It typically presents as a primary tumor of the uterus or genital tract in gestational females. In males the primary lesion is usually in the testes, but represents only 1% of all testicular tumors[2]. Extragenital choriocarcinomas are less common, and often exist with other carcinomas, tending to occur in mid-line organs[1]. Pure extra-genital non-midline choriocarcinomas are the least common type. Only seven previous male patients with choriocarcinoma of the liver have been reported in the English language literature (Table 1)[3-5]. Hepatic choriocarcinoma has been recognized as a primary malignant tumor of the liver since 1992 when first reported by Fernández Alonso *et al*[3]. The other patients were reported from Asia (China and Japan), with a majority from China[4,5].

The patient in this report presented with acute abdominal symptoms and a multi-nodular tumor in the right lobe of the liver. Based on radiographic appearance, an elevated serum CEA and the absence of a lesion in the gastrointestinal tract, the leading diagnosis was intrahepatic cholangiocarcinoma. FDG-PET scan showed no other lesions, including the testes. Based on these findings, a liver resection was planned in this patient. FDG-PET scan has been reported previously in the diagnosis of choriocarcinoma[6,7]. Furthermore, FDG-PET scan is also useful to evaluate the efficacy of treatment of liver lesions such as surgery or chemotherapy[6].

In the differential diagnosis of malignant liver tumors in a patient presenting with an acute abdomen, sarcomatous changes from hepatocellular carcinoma or cholangiocarcinoma must be considered. Both of these tumors can have a rapid clinical course and generally have poor outcomes. Sarcomatous change in primary liver tumors has been reported from Asian countries as well as choriocarcinoma[8-10]. Sarcomatous changes are seen in about 2%-4% of patients with resected hepatocellular carcinoma. Patients with sarcomatous changes have a worse prognosis than that in patients with typical hepatic lesions. More than half of the patients with sarcomatous changes died within a year of resection[8].

The characteristic laboratory finding in choriocarcinoma is an elevated hCG level in the blood or urine. This patient had an elevated serum hCG level in a post-

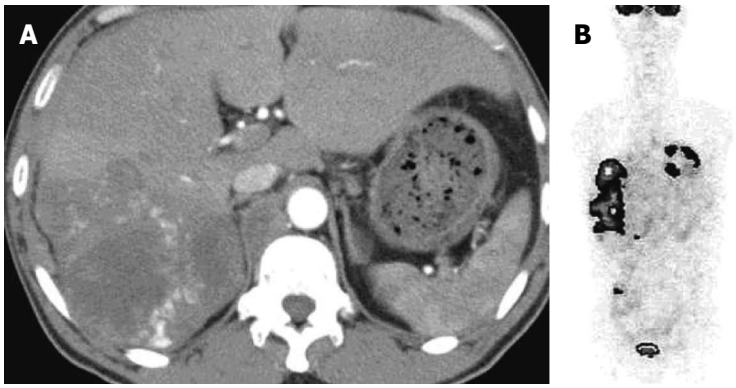

Figure 1 Enhanced computed tomography of the liver and fludeoxyglucose-positron emission tomography. A: Enhanced computed tomography showed a multi-nodular tumor in the right lobe of the liver; B: Fludeoxyglucose-positron emission tomography scan showed accumulation in the liver with no accumulation in the testes.

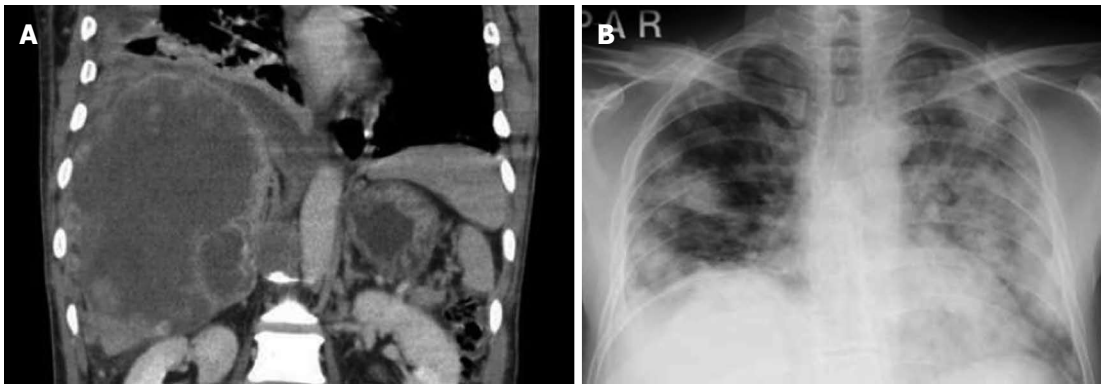

Figure 2 Computed tomography and chest X-ray following ruptured of the tumors with respiratory failure. A: Computed tomography scan following rupture of the tumors showed an enlarged tumor with ascites and a right pleural effusion; B: Chest X-ray showed multiple lung metastases clinically associated with severe respiratory failure.

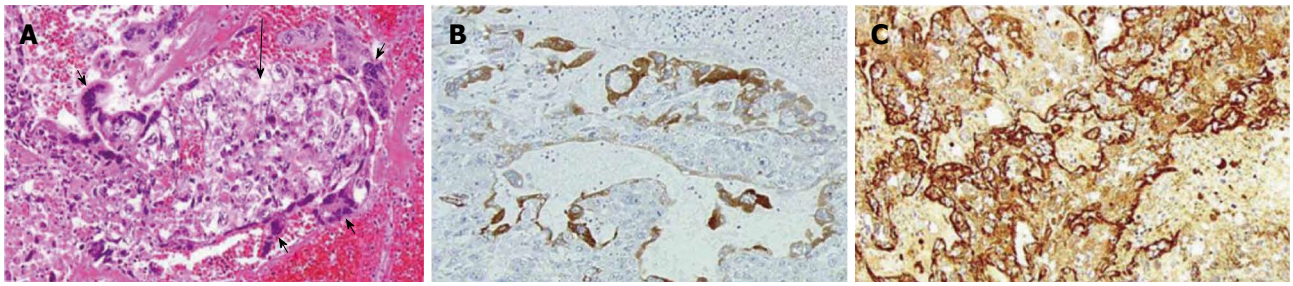

Figure 3 Photomicrographs of the liver tumor. A: Ovoid mononuclear cytotrophoblast cells (long arrow) encircled by large amorphous multinuclear syncytiotrophoblast cells (short arrows) among dark-red hemorrhagic tissue (hematoxylin and eosin, × 400); B: Immunohistochemical stain showing α-human chorionic gonadotropin (hCG) positive cells (× 400); C: Immunohistochemical stain showing β-hCG positive syncytiotrophoblast cells (× 400).

mortem blood sample. In male patients, there are few reasons to evaluate serum hCG levels except in patients with testicular tumors[11-13]. When we evaluate a middle aged patient with an aggressive liver tumor, we recommend checking serum hCG as tumor marker in addition to AFP, CEA and CA19-9.

The strategy for choriocarcinoma of the liver is not established because of its rarity and highly malignant behavior. We believe that urgent liver resection before manifestation of distant metastases and chemotherapy may be the best course for prolongation of survival. In a patient with gastric choriocarcinoma and multiple liver metastases, Waseda *et al*[14] reported pathological complete response using etoposide and cisplatinum, with a two year disease-free survival after surgical resection. Methotrexate and actinomycin D may also be important agents in the treatment of choriocarcinoma. The use of cyclophosphamide, etoposide and vincristine have also been reported. Cisplatinum and 5-FU were used in other reports. Shi *et al*[5] reported five patients with hepatic choriocarcinoma. Two of the five patients underwent liver resection with adjuvant chemotherapy, and three of the five patients

Table 1 Previous reports of men with primary hepatic choriocarcinoma

Ref.	Year	Age, yr	Time to death
Fernández Alonso *et al*[3]	1992	62	1 yr
Arai *et al*[4]	2001	65	45 d
Shi *et al*[5]	2010	39	6 mo
Shi *et al*[5]	2010	45	2 mo
Shi *et al*[5]	2010	48	3 mo
Shi *et al*[5]	2010	36	5 mo
Shi *et al*[5]	2010	40	8 mo
Present case	2011	49	2 mo

with distant metastases were treated with chemotherapy after needle biopsy. The two patients who underwent resection survived only six and eight months respectively, despite having received adjuvant chemotherapy. Of the three other patients reported, two were diagnosed with undifferentiated carcinoma and one with metastatic choriocarcinoma by needle biopsy. These patients underwent chemotherapy including 5-FU and platinum, but all died within five months.

Liver biopsy was not performed in the present patient because the diagnosis of intra-hepatic cholangiocarcinoma was suspected, and biopsy could result in an increased risk of tumor dissemination. His condition rapidly deteriorated due to respiratory failure, which precluded the safe conduct of any invasive procedures. The diagnostic accuracy of needle or aspiration biopsy is not adequate, and may lead to an incorrect diagnosis of poorly differentiated carcinoma because of the similarity to cytotrophoblasts. The accuracy of liver biopsy is still controversial in establishing the diagnosis of choriocarcinoma. However, in order to enable rapid treatment of such an aggressive tumor, liver biopsy should be performed without hesitation.

In a middle-aged male patient with an aggressive liver tumor, evaluation of serum hCG levels in addition to other liver tumor markers should be performed. Liver biopsy is important, especially in Chinese and Japanese patients, to detect this rare and highly malignant tumor.

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Written informed consent was obtained from the patient's younger brother for publication of this report and accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal.

COMMENTS

Case characteristics

Acute abdominal pain and fever in a 49-year-old man.

Clinical diagnosis

Primary hepatic choriocarcinoma.

Differential diagnosis

Sarcomatous changes from hepatocellular carcinoma or cholangiocarcinoma.

Loboratory diagnosis

Elevated hCG in blood or urine is definitive.

Imaging diagnosis

Multi-nodular liver tumor with uptake by FDG-PET.

Pathological diagnosis

Liver biopsy with immunohistochemistry is recommended.

Treatment

Urgent surgical resection and chemotherapy is recommended.

Term explanation

Hepatic choriocarcinoma in a middle-aged male is the least common.

Experiences and lessons

In a middle-aged male with an aggressive liver tumor, evaluation of serum hCG levels and liver biopsy should be performed.

Peer review

The study reported a case of a patient suffering primary hepatic choriocarcinoma, which is a kind of very rare, especially in men, and malignant trophoblastic cancer. The description of this case is very interesting for the detection and differentiation of this type of aggressive tumour among other primary liver cancers and the conclusions are enlightening for the clinical management of these patients.

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CASE REPORT

IgG4-related autoimmune pancreatitis overlapping with Mikulicz's disease and lymphadenitis: A case report

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Author contributions: Qu LM, Wen XY, Liu YH, Li YJ performed the majority of the experiments; Liu YH contributed the study design, data collection; Gao RP designed the study and wrote the manuscript; Brigstock DR co-ordinated the study and edited the manuscript; Qu LM and Liu YH contributed equally to the paper.
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Abstract

Autoimmune pancreatitis (AIP) is a form of chronic pancreatitis that is categorized as type 1 or type 2 according to the clinical profile. Type 1 AIP, which predominantly presents in a few Asian countries, is a hyper-IgG4-related disease. We report a case of IgG4-related AIP overlapping with Mikulicz's disease and lymphadenitis, which is rare and seldom reported in literature. A 63-year male from Northeast China was admitted for abdominal distension lasting for one year. He presented symmetric swelling of the parotid and submandibular glands with slight dysfunction of salivary secretion for 6 mo. He had a 2-year history of bilateral submandibular lymphadenopathy without pain. He underwent surgical excision of the right submandibular lymph node one year prior to admission. He denied any history of alcohol, tobacco, or illicit drug use. Serological examination revealed high fasting blood sugar level (8.8 mmol/L) and high level of IgG4 (15.2 g/L). Anti-SSA or anti-SSB were negative. Computed tomography of the abdomen showed a diffusely enlarged pancreas with loss of lobulation. Immunohistochemical stain for IgG4 demonstrated diffuse infiltration of IgG4-positive plasma cells in labial salivary gland and lymph node biopsy specimens. The patient received a dose of 30 mg/d of prednisone for three weeks. At this three-week follow-up, the patient reported no discomfort and his swollen salivary glands, neck lymph node and pancreas had returned to normal size. The patient received a maintenance dose of 10 mg/d of prednisone for 6 mo, after which his illness had not recurred.

Key words: IgG4-related disease; Type 1 autoimmune pancreatitis; Mikulicz's disease; Lymphadenitis

Core tip: We report a rare case of a 63-year-old Northeast Chinese man who suffered from IgG4-related disease (RD) which involved the salivary glands, lymph node and pancreas. The patient responded promptly to prednisone therapy. Further identification and characterization of such cases is required to elucidate the prevalence and clinical features of IgG4-RD in China.

Qu LM, Liu YH, Brigstock DR, Wen XY, Liu YF, Li YJ, Gao RP. IgG4-related autoimmune pancreatitis overlapping with Mikulicz's disease and lymphadenitis: A case report. *World J Gastroenterol* 2013; 19(48): 9490-9494 Available from: URL:

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INTRODUCTION

Autoimmune pancreatitis (AIP) is an uncommon form of chronic pancreatitis that was first described in Japan in 1995[1]. Two subtypes of AIP have been so far recognized[2,3]. Type 1 AIP is related to high levels of serum IgG4, dense periductal lymphoplasmacytic infiltration and obliterative venulitis, while type 2 AIP is an IgG4-independent pancreatic disease that is characterized by neutrophilic infiltration into the epithelium of the pancreatic duct[3,4]. Although 20%-40% of AIP cases are type 2 in the United States and Europe, most cases of AIP in Japan and Korea are type 1, and type 2 is quite rare[5]. The prevalence and clinical features of AIP in China has not been fully clarified so far.

Mikulicz's disease (MD) refers to bilateral and symmetrical swelling of the lacrimal, parotid, and submandibular glands. Based on histological similarities reported by Morgan *et al*[6] in 1953, MD was considered a subtype of Sjögren's syndrome (SS). However, several recent reports from Japan have revealed that MD is associated with elevated serum IgG4 levels and prominent infiltration of IgG4-positive plasmacytes[7,8]; these findings are distinct from those of SS and have resulted in the recognition of MD as a singular systemic IgG4-related plasmacytic disease[9].

In this report, we describe a case from Northeast China of IgG4-related autoimmune pancreatitis overlapping with Mikulicz's disease and lymphadenitis. This rare clinical condition has seldom been reported in literature.

CASE REPORT

A 63-year male from Northeast China was admitted for abdominal distension lasting for one year. He presented symmetric swelling of the parotid, and submandibular glands with slight dysfunction of salivary secretion for 6 mo. He had a 2-year history of bilateral submandibular lymphadenopathy without pain. He underwent surgical excision of the swollen lymph node in the right submandibular region one year prior to hospital admission. The patient denied any history of alcohol, tobacco, or illicit drug use. On admission, his blood pressure was 136/88 mmHg, pulse rate was 72/min, and body temperature was 36.7 ℃. On examination, he had bilateral swelling of the parotid and submandibular glands as well as left swelling of the submandibular lymph node. His mouth was dry. Abdominal examination revealed mild epigastric tenderness to deep palpation without rebound.

The laboratory test data on admission revealed an elevated neutrophil ratio of 76%, and an elevated fasting blood sugar level of 8.8 mmol/L. Serum amylase was 42 U/L and serum lipase was 65 U/L, both within normal limits. Serological testing for autoimmune function displayed high levels of IgG4 (15.2 g/L) and IgG (18.5 g/L), and negative values of anti-SSA and anti-SSB. A computed tomography (CT) scan of the abdomen revealed diffuse enlargement of the pancreas and loss of normal pancreatic lobulation, consistent with autoimmune pancreatitis (Figure 1A).

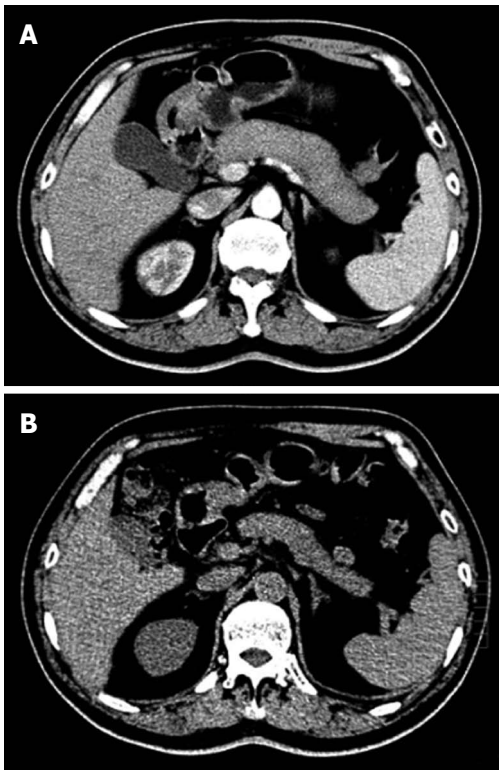

Figure 1 Typical imaging features of type 1 autoimmune pancreatitis. Computed tomography (CT) scan showing diffuse swelling of the pancreas with loss of lobulation (A), and a dramatic decrease in swelling of the pancreas after 3 wk of steroid treatment (B).

The patient underwent a minor labial salivary gland biopsy for a possible diagnosis of MD. Labial gland specimens stained with hematoxylin and eosin revealed significant infiltration of lymphocytoplasma cells in the patient, but no infiltration of these cells in a healthy individual (Figure 2A and B). Immunohistochemical staining showed numerous IgG4-positive plasmacytes in the labial gland of the patient, with a ratio of IgG4/IgG-positive plasmacytes of more than 50% (Figure 2C and D).

Since both autoimmune pancreatitis and MD meet the criteria for IgG4-related disease, we investigated the IgG4 status of the patient's swollen lymph nodes. Lymph node specimens collected from the patient by excision of the right submandibular lymph node one year prior to admission ago were examined for IgG4 and IgG using immunohistochemistry. As shown in Figure 3, there were diffuse infiltrations of IgG4-positive plasma cells in the patient's lymph node. The ratio of IgG4/IgG-positive cells was greater than 40% thus meeting the diagnostic criteria for IgG4-related lymphadenitis.

On the 8th d after admission, the patient was diagnosed with IgG4-related systemic disease. He received 30 mg/d of prednisone for three days without any side effects, and was then discharged with the same steroid

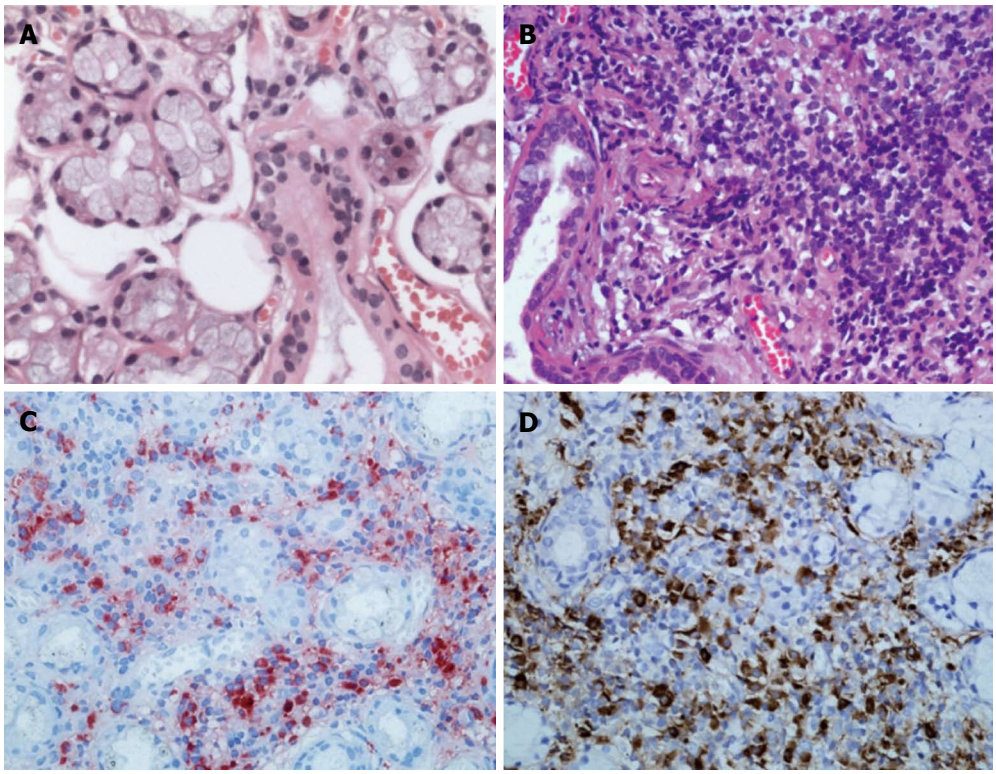

Figure 2 Histological findings of labial salivary gland specimens. A: Hematoxylin and eosin stain showing normal labial gland; B: Diffuse infiltration of lymphoplasma cells from the patient; C, D: Immunohistochemical staining for IgG4 (C) or IgG (D) in plasma cells from the patient, consistent with Mikulicz's disease. Original magnification, × 400.

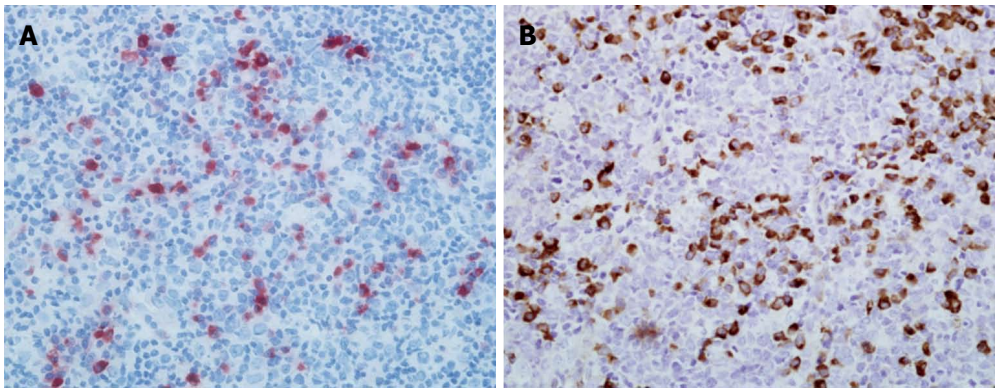

Figure 3 Histological findings of submandibular lymph node specimen. Immunohistochemical staining showing IgG4-positive plasma cells (A) and IgG-positive plasma cells (B) in lymph node sections of the patient. Original magnification, × 400.

dose for the following 3 wk. At three-week follow-up the patient exhibited no signs of either a dry mouth or abdominal distension. His swollen glands including parotid and submandibular glands as well as left submandibular lymph node were no longer palpable. The enlarged pancreas had returned to its normal size (Figure 1B) and elevated morning glucose levels were within the normal range. The patient then received a long-term maintenance dose of 10 mg/d of prednisone after steroid tapering. At six-month follow-up, his illness had not recurred.

DISCUSSION

Yoshida *et al*[1] first proposed the concept of AIP in 1995 based on observations of patients who had hyper-γ globulinemia, various autoantibodies, lymphocytic infiltration into pancreatic tissue, and good steroid responsiveness. In 2002 Hamano *et al*[10] reported high serum IgG4 concentrations in Japanese AIP patients and abundant IgG4-producing plasma cell infiltration in pancreatic tissue. Some cases of AIP in Europe or America appear to represent an "idiopathic duct-centric chronic pancreatitis", which are caused by neutrophilic granulocyte infiltration and are not related to IgG4[11]. In 2009, Sugumar *et al*[12] suggested that IgG4-related AIP should be named as type 1 and neutrophilic granulocyte lesions of AIP as type 2[12]. Over the last decade, international consensus diagnostic criteria for AIP were established to be applicable

worldwide and to distinguish between the two types of AIP. The diagnosis of AIP can be usually made based on the presence of at least one of the five cardinal features (*i.e.*, imaging, serology, other organ involvement, histology, and response to steroid therapy)[3]. However, sufficient biopsy specimens from the pancreas are difficult to obtain using standard procedures, except laparotomy. The clinical diagnostic criteria to establish type 1 AIP from Japan were the presence of pancreatic swelling together with high serum levels of IgG4 and/or prominent IgG4-producing plasma cell infiltration in pancreas tissue[11]. By contrast, in the case reported here, the patient presented a diffuse swelling of the pancreas with loss of lobulation, high serum IgG4 concentration, abundant IgG4-positive plasma cell infiltration into labial or lymph node tissues, and good steroid responsiveness, thus fully meeting multiple type 1 AIP diagnostic criteria[3,11]. Additionally, in this case report, three-week steroid treatment caused dramatic improvements in either exocrine insufficiency (abdominal distention) or endocrine insufficiency (elevated fasting blood sugar level) as well as dramatic reduction of the enlarged pancreas, suggesting that both exocrine and endocrine insufficiency might be reversible in IgG4-related type 1 AIP.

The first case of MD was reported by Mikulicz-Radecki in 1888, which was described as bilateral symmetrical enlargement of the salivary and lacrimal glands, and lymphocytic infiltration into lacrimal and salivary gland tissues[9]. Since 1953, when Morgan *et al*[6] found similarities in histology between MD and SS, MD was considered as a subtype of SS. However, this concept has been modified over the decade in light of compelling Japanese studies showing that MD is associated with elevated serum IgG4 levels and prominent infiltration of IgG4-positive plasmacytes into lacrimal and salivary glands[7,8]. Thus, a modern clinical concept of MD is that it is distinct from SS and is instead part of the spectrum of IgG4-RD[9]. Diagnostic criteria for IgG4-related MD were approved by the Japanese Sjögren's Syndrome Society in 2008. According to these critera, IgG4-related MD is defined in the presence of persistent (≥ 3 mo), symmetrical swelling of the lacrimal, parotid and submandibular glands involving at least two pairs, together with either high serum levels of IgG4 (≥ 1.35 g/L) and/or marked IgG4-positive plasmacyte infiltration (≥ 50% IgG4-positive/IgG-positive cells in five high power fields) into lacrimal and salivary gland tissues[9]. In this study, the patient presented six-month symmetric swelling of the parotid and submandibular glands, elevated serum IgG4 levels, and prominent IgG4-positive plasmacyte infiltration into the labial gland tissue, which fully met the diagnostic criteria for IgG4-related MD[9]. Furthermore, both the swollen salivary glands and the dry mouth of the patient promptly recovered in response to prednisone therapy, indicating that IgG4-related MD is a reversible disorder which differs from SS.

Recently, IgG4-RD has been defined as a novel clinical entity with multi-organ involvement and associated abundant infiltration of IgG4-positive cells[11,13]. In this case, the clinical and histopathological features of the patient met diagnostic criteria for IgG4-related type 1 AIP and MD respectively. Additionally, the patient presented abundant infiltration of IgG4-positive plasma cells in lymph node tissue (> 40% IgG4/IgG-positive cells) and good steroid responsiveness, which fully met the diagnostic criteria for IgG4-related lymphadenitis[13].

In summary, we report a rare case of a 63-year-old Northeast Chinese man who suffered from IgG4-RD which involved the salivary glands, lymph node and pancreas. The patient responded promptly to prednisone therapy. Further identification and characterization of such cases is required to elucidate the prevalence and clinical features of IgG4-RD in China and their relationship to similar cases in Japan.

COMMENTS

Case characteristics

The patient presented symmetric swelling of the parotid and submandibular glands as well as a diffusely enlarged pancreas with loss of lobulation.

Clinical diagnosis

The authors report a rare case of a 63-year-old Northeast Chinese man who suffered from IgG4-related disease which involved the salivary glands, lymph node and pancreas.

Differential diagnosis

Immunohistochemical staining for IgG4 and IgG is the major method for differential diagnosis between IgG4-related disease and other diseases.

Laboratory diagnosis

Serological testing for autoimmune function displayed high levels of serum IgG4 and IgG, and negative values of anti-SSA and anti-SSB in the patient.

Imaging diagnosis

A computed tomography scan of the abdomen revealed diffuse enlargement of the pancreas and loss of normal pancreatic lobulation.

Pathological diagnosis

Immunohistochemical staining showed numerous IgG4-positive plasmacytes in labial gland and lymph node of the patient, with a ratio of IgG4/IgG-positive plasmacytes of more than 40% in both tissues.

Treatment

The patient received a dose of 30 mg/d of prednisone for three week, and a long-term maintenance dose of 10 mg/d of prednisone.

Term explanation

Mikulicz's disease (MD) refers to bilateral and symmetrical swelling of the lacrimal, parotid, and submandibular glands. MD, as a singular systemic IgG4-related plasmacytic disease, was considered a subtype of Sjögren's syndrome. IgG4-related disease (IgG4-RD) has been defined as a novel clinical entity with multi-organ involvement and associated abundant infiltration of IgG4-positive plasmacytes.

Experiences and lessons

The authors report a rare case of a 63-year-old Northeast Chinese man who suffered from IgG4-RD which involved the salivary glands, lymph node and pancreas. This rare clinical condition has seldom been reported in literature.

Peer review

Type 1 autoimmune pancreatitis is related to high levels of serum IgG4, dense periductal lymphoplasmacytic infiltration and obliterative venulitis. In this manuscript, the authors reported a interesting case of IgG4-related autoimmune pancreatitis overlapping with Mikulicz's disease and lymphadenitis. This case is very rare and seldom reported in literature.

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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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